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PHARMACOLOGICAL ASPECTS OF HEART DISEASE

Proceedings of an International Symposium on Heart Metabolism in Health and Disease and the Third Annual Cardiology Symposium of the University of Manitoba, July 8–11, 1986, Winnipeg, Canada

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This book is dedicated to
Dr. Lionel H. Opie for his
distinguished contributions to
pharmacology of heart in
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CONTENTS

E. NEWER INOTROPIC AGENTS AND CALCIUM

Preface

The importance of heart and artery disease as a cause of death and disability is difficult to exaggerate: it causes over half of all deaths in the western world and now accounts for one-quarter of deaths in the entire world. This appalling incidence persists in spite of commendable progress in treatment and prevention, particularly in the last two or three decades. Deaths from coronary disease have decreased by a third in the past twenty years and stroke has decreased by a half in the same period. This remarkable improvement, saving thousands of lives per year, has come about due to changes in life style (low fat diet, control of high blood pressure, less smoking and more exercise) and progress in treatment (more effective drugs, coronary care units, pacemakers, and cardiac surgery). Progress in understanding the pathophysiologic and pharmacologic mechanisms operative in heart disease have been paramount in the development of more rational and more effective therapy.

Dramatic and spectacular surgical treatments have fired the public imagination. Bypass surgery is commonplace and results in complete or considerable relief of symptoms in the majority of patients operated upon. Heart transplants are increasingly frequent and now show a one year survival rate of 80 per cent and a four year survival of 70 per cent. One has lived for 17 years. Artificial hearts are being used as a final resort on an experimental basis. Nevertheless it must be recognized with realism and regret that all of these heroic measures are rather crude and only palliative. The underlying disease continues and eventually reappears in the heart or elsewhere in the body. They are procedures to be used only until superior methods of treatment or prevention are available.

Best hope for the ultimate conquest of these deadly afflictions lies in increased knowledge of the intricate structure and behavior of the cells which make up the heart and blood vessels. This, in turn, requires a comprehension of the nature and functions of the minute components of cells at the subcellular, molecular and atomic levels. To this end an appreciation of the myriad enzymes, hormones, and biochemical substances which govern the life of the cell both in health and disease is required. This complex interplay may be defined as the metabolism of the cell. While much is known about how and why heart cells contract and then relax in cyclic manner to produce the pumping action of the heart, very little is known regarding the exact relationship among metabolic, ultrastructural,

electrical and mechanical aspects of cardiac muscle. To increase knowledge and to enable better prevention and treatment it is necessary to integrate the diverse and somewhat scattered information to be found in physiology, biochemistry, pathology, pharmacology and clinical cardiology. In an effort to accomplish such an integration, the Department of Physiology (Section on Experimental Cardiology) of the University of Manitoba Medical School organized an international symposium on "Heart Metabolism in Health and Disease" which was held in the Winnipeg Convention Centre July 8-11, 1986. The Meeting comprised the 8th annual meeting of the International Society for Heart Research (American Section), a Satellite Symposium of the XXX International Physiological Congress (held in Vancouver) and the 3rd Annual Cardiology Symposium of the University of Manitoba. Several sessions were devoted to presentations by clinical cardiologists with research interests and constituted the program of the Cardiology Symposium of the University of Manitoba. Through the integration of basic science with clinical problems of patients affected by these diseases, this meeting may be one of the most significant ever held anywhere or anytime in the global pursuit of the control and eventual eradication of cardiovascular disease. It does not appear unrealistic to imagine that this could lead to future advances that will one day make bypass surgery, heart transplants and the artificial heart as obsolete as sanatoria and mutilating chest surgery have now become for tuberculosis.

For the benefit of those who were unable to attend this meeting, we have selected 31 articles in the field of pharmacology and therapy of heart disease. In this book these papers have been arranged under five sections namely Antiarrhythmic Agents, Therapy of Hypertension and Heart Failure, Calcium Antagonists and Beta Adrenergic Receptor Blockers, Cardiac Glycosides and Sodium Pump, and Newer Inotropic Agents and Calcium. It is hoped that the information contained in this book will result in improved treatment of the common cardiovascular problems encountered in the practice of cardiology.

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A. ANTIARRHYTHMIC AGENTS

1

APPROACHES TO THE SELECTION OF AN ANTIARRHYTHMIC DRUG

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There have been many advances in our understanding of the mechanism of cardiac arrhythmia, improvements in the techniques to document the occurrence of arrhythmia and new pharmacologic options for therapy. Although antiarrhythmic drugs remain the most frequently utilized approach to treat patients with arrhythmia, the selection of an effective and well tolerated agent continues to challenge the cardiologist. While the antiarrhythmic drugs may be classified (Table 1) and arrhythmias categorized based on mechanism (Table 2), this does not help in the selection of an effective and well tolerated agent for the individual patient.

Table 1. Classification of antiarrhythmic drugs.

Class 1 - Membrane stabilizers (local anesthetics)
- 1A. moderate slowing of conduction (moderate QRS widening)
  marked prolongation of repolarization (JT increased)
  quinidine, disopyramide, procainamide
- 1B. little slowing of conduction (QRS unchanged)
  shortening of repolarization (JT unchanged or decreased)
  lidocaine, tocainide, mexiletine
- 1C. marked slowing of conduction (marked QRS widening)
  no change in repolarization (JT unchanged)
  encainide, flecainide, propafenone

Class 2 - Beta blockers
propranolol, acebutolol, timolol, atenolol, metoprolol, nadolol, sotalol

Class 3 - Repolarization prolongators
marked JT prolongation
bretylium, sotalol, amiodarone*

Class 4 - Calcium channel blockers
verapamil, diltiazem

*also is membrane stabilization, beta blocker and calcium channel blocker

Table 2. Mechanisms of arrhythmia.

I. Abnormality of impulse generation
   A. Enhanced automaticity (ectopic focus)
   B. Triggered automaticity
      1. early afterpotentials
      2. delayed afterpotentials

II. Abnormality of impulse conduction
   A. Block and reentry
   B. Block and reflection

Therefore, the choice of an antiarrhythmic drug remains empiric. Since there are no guidelines for determining efficacy, the only way to document the drug's action on arrhythmia is by its administration. The effect of one agent will not predict the response to another one even if they are structurally and electrophysiologically similar. Waxman and coworkers (1) reported that the response to intravenous procainamide correlated with response to other similar agents, specifically disopyramide, quinidine, lidocaine or phenytoin. In this study, electrophysiologic testing was used to judge drug effect and 30 patients were rendered noninducible with procainamide, of whom 25 (83%) also responded to another agent. In contrast, 69 patients did not respond to procainamide and in only 9 (13%) was another agent effective. However, a number of other investigators have not found procainamide response to be predictive (2, 3). Tocainide and mexiletine are lidocaine congeners and are structurally similar. Nevertheless, response to one agent does not predict the response to the other. In a study by Hession and coworkers (4) involving 79 patients treated with both mexiletine and tocainide in random fasion the results of therapy were concordant in only 42 patients (53%). Winkle and coworkers (5) reported that the effect of intravenous lidocaine on arrhythmia has some predictive value for response to oral tocainide. In a group of 26 patients, 63% of those who responded to lidocaine also responded to tocainide, while in 83% of patients not responding to lidocaine, tocainide was ineffective. Hohnloser and coworkers (6) reported on 85 patients who were evaluated with both intravenous lidocaine and oral tocainide. Of the 48 patients in whom lidocaine was ineffective, 40 (83%) did not respond to tocainide. Lidocaine was effective in 37 patients of whom 20 (54%) also responded to tocainide. Overall response to lidocaine had only a 71% predictive accuracy. Despite the similarity

of these two drugs, lidocaine does not always predict the response to tocainide. Not unexpectedly, a negative response to lidocaine is more helpful than a positive response to this agent.

It may be concluded from these studies that each drug is unique and one does not predict the effect of another, even if structurally similar. Since drug selection is empiric and without adequate guidelines, the only reliable way to determine drug effect is by its administration. With the availability of many oral agents, the selection of an effective and well tolerated drug is complicated and time consuming. There is therefore a need for a systematic and rational approach to drug therapy.

At the present time there are two established methods for the evaluation of antiarrhythmic drugs. Noninvasive techniques involve 24 hour ambulatory monitoring and exercise testing, with efficacy based on the suppression of spontaneous arrhythmia (7, 8). The basis for the use of noninvasive methods is that certain types of ventricular premature beats, specifically repetitive forms, are markers for the presence of active reentrant circuits which predispose to a sustained ventricular tachyarrhythmia. In patients with a history of malignant ventricular arrhythmias, these repetitive forms, primarily runs of nonsustained ventricular tachycardia (VT), represent clinical markers for an unstable myocardium, an appropriate electrophysiologic substrate. The goal of antiarrhythmic therapy is the suppression of these forms, which reflects the elimination of the reentrant circuits and correlates with the inability of the myocardium to generate or sustain ventricular arrhythmia. The second approach is invasive and involves electrophysiologic testing (9, 10). The goal of therapy is the inability to reinduce an arrhythmia which was previously inducible. The use of electrophysiologic testing is based on the concept that the addition of appropriately timed extrastimuli will activate a reentrant circuit and induce the same sustained ventricular tachyarrhythmia that occurred spontaneously. In patients with infrequent repetitive arrhythmia, electrophysiologic testing is useful for inducing the arrhythmia when necessary. Drug efficacy is defined as the inability to reinduce the arrhythmia indicating that the reentrant circuit is altered and is no longer capable of generating or sustaining arrhythmia.

Regardless of the method employed for drug selection, drug administration is the only way to evaluate efficacy. In order to shorten and simplify this process, we have developed a systematic approach which can be applied to both invasive and noninvasive methods. There are 4 phases of study (7, 8).

Phase 0 or the control period involves the acquisition of baseline data regarding the frequency, type and reproducibility of ventricular arrhythmia. After admission to the hospital, previous antiarrhythmic drug therapy is discontinued. After an appropriate washout period, each patient undergoes 48 hours of ambulatory monitoring and an exercise test on a motorized treadmill adhering to a Bruce protocol.

Ventricular arrhythmia is categorized using the Lown grading system (7, 11).

Grade 0 - no ventricular premature beats (VPBs)
Grade 1A - infrequent (<30/hour) VPBs and <1/min
Grade 1B - infrequent VPBs and occasionally >1/min
Grade 2 - frequent (>30/hour) VPBs
Grade 3 - multiformed VPBs
Grade 4A - repetitive VPBs - couplets
Grade 4B - repetitive VPBs - runs of VT (3 or more successive VPBs)
Grade 5 - early R on T VPBs

On the basis of this grading system, an arrhythmia equation is developed. This categorizes the data derived from 24 hours of monitoring data and results in the development of a simple equation (Table 3).

Table 3. Arrhythmia equation.

0^4 $1A^3$ $1B^5$ 2^{12} $3^{14}{}_3$ $4A^{12}{}_8$ $4B^{11}{}_{6,9(190)}$

Grade	Interpretation
0^4	4 hours of no VPBs
$1A^3$	3 hours of infrequent VPBs, always <1/min
$1B^5$	5 hours of infrequent VPBs, occasionally >1/min
2^{12}	frequent VPBs for 12 hours
$3^{14}{}_3$	14 hours during which multiform VPBs occurred, 3 different forms
$4A^{12}{}_8$	couplets present during 12 hours, as many as 8/hour
$4B^{11}{}_{6,9(190)}$	runs of VT occurred during 11 hours with up to 6 runs/hour, 9 beats in length and with rates up to 190

Once these baseline studies are completed, a decision about the approach to drug evaluation is made. If spontaneous arrhythmia is of high density and is reproducible, noninvasive methods are used. An adequate density of arrhythmia includes: 1) grade 2 VPBs for at least 50% of the hours during each 24 hour period; 2) at least 3 hours of repetitive forms during each 24 hour monitoring period; 3) the provocation of at least 2 VPBs/minute during exercise test; 4) during exercise the occurrence of repetitive forms.

If this density of arrhythmia is absent or if arrhythmia is not reproducibly present on both days, the patient undergoes invasive electrophysiologic testing.

Regardless of the approach used, drug evaluation is performed in 2 ways. Phase 1 or acute drug testing is a rapid screening of drug effect (12) (Figure 1).

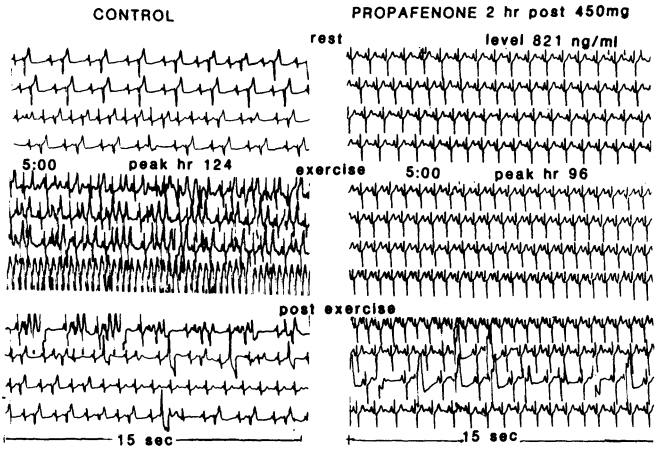

Figure 1. Example of acute drug test. During control the patient has frequent ventricular premature beats while with exercise ventricular tachycardia (VT) is induced. Two hours after propafenone, arrhythmia is abolished.

After 30 minutes of observation at rest and a brief period of exercise on a bicycle ergometer, a large single oral dose of drug is administered (Table 4).

Table 4. Doses of drugs used.

Drug	Dose (mg) Phase 1	Phase 2
atenolol	--	100 daily
disopyramide	300	100-150 TID or QID
encainide	--	25-50 TID or QID
ethmozine	--	200-400 TID
flecainide	--	100-200 BID
indecainide	--	50-100 TID
lorcainide	--	100-200 BID or TID
metoprolol	100	25-50 BID or TID
mexiletine	400	200-400 TID
procainamide	1500	500-1000 TID or QID
propafenone	450	150-300 TID
propranolol	80	20-40 TID or QID
quinidine	600	200-400 QID
tocainide	800	400-800 TID

Continuous monitoring is performed for 3 hours. Each hour bicycle exercise is repeated, an ECG for intervals recorded, and blood obtained for drug level. The drugs tested acutely are those which are rapidly absorbed, achieve peak levels within 2 hours, have no important active metabolites and are rapidly cleared so that on the following day another drug can be tested. The dose administered is approximately 1/2 of the usual daily maintenance dose. Although blood levels are not at steady state, a "therapeutic" level is achieved within 2 hours, permitting rapid evaluation of drug effect. If the patient is undergoing electrophysiologic testing, a study is repeated 2 hours after a dose, at a time of peak blood level.

After the completion of a number of acute drug tests, the agent that appears to be the most effective is selected for short term maintenance or phase 2. Drugs which have active metabolites or which are slowly absorbed and require several days before therapeutic levels are achieved cannot be tested acutely, but are only used during phase 2 study. Multiple doses of the selected drug are administered for 72 to 96 hours. At the end of this time 24 hour monitoring and exercise testing (Figure 2) or electrophysiologic testing are repeated (Figure 3).

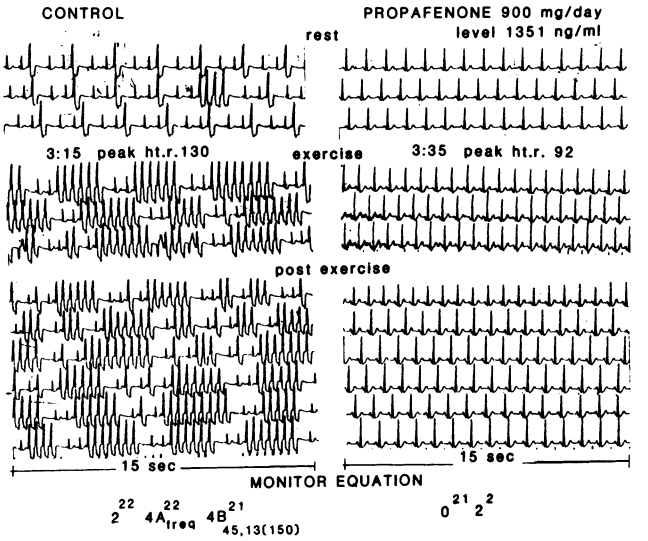

Figure 2. Example of phase 2 drug study. Prior to drug therapy the patient has frequent runs of ventricular tachycardia (VT) with exercise while monitoring demonstrates frequent VPBs and couplets for 22 hours (2^{22}, $4A^{22}$) and runs of VT for 21 hours with as many as 45/hour, 13 beats in length and at rates of 150. During propafenone therapy VT and couplets are eliminated and there are only 2 hours of frequent VPBs (2^{2}) on monitoring.

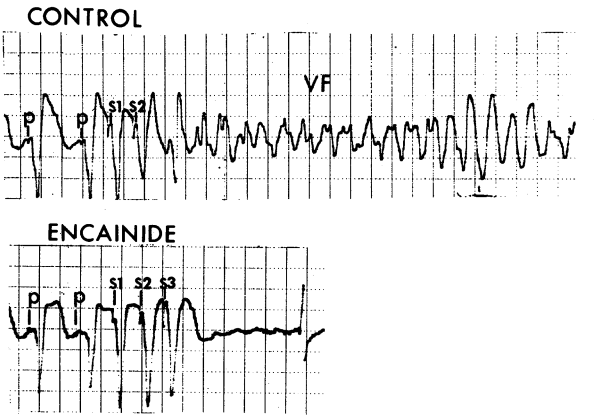

Figure 3. Electrophysiologic drug study. During control, 2 extrastimuli (S_1, S_2) added during a paced rhythm (P) induce ventricular fibrillation (VF). With encainide therapy, no arrhythmia is provoked with 3 extrastimuli (S_1, S_2, S_3).

The purpose of phase 2 is to correlate the results with the acute drug test, confirm drug efficacy and identify any side effects which may preclude the long term use of the agent. If the drug is effective and well tolerated, the patient is discharged and followed long term or phase 3.

Of critical importance are the criteria for defining drug efficacy. When noninvasive techniques are employed, a drug is considered effective if the following are achieved with both monitoring and exercise testing:

1) total elimination of runs of VT
2) >90% reduction in couplets
3) >50% reduction in VPBs

When electrophysiologic testing is used, the criterion for efficacy is the inability to induce 3 or more repetitive responses when using up to 3 extrastimuli during ventricular pacing at a rate of 100 and 120 beats per minute.

One of the advantages of such a systematic approach is the identification of several effective or partially effective agents. This is important for not infrequently combinations of antiarrhythmic drugs are preferable or necessary. Although for many patients a single antiarrhythmic drug is effective, the dose necessary may cause intolerable side effects. Adding another effective agent permits a reduction of drug dose while maintaining efficacy (13). Not infrequently no agent when administered singly is completely effective for suppressing arrhythmia. We have observed that a combination of two partially effective agents will often result in synergistic activity enhancing antiarrhythmic efficacy (14, 15).

An additional benefit of a systematic evaluation is the exposure of potentially serious side effects. While each drug has its unique profile of toxic reactions, aggravation of arrhythmia occurs with all drugs and is a frequent side effect which is unpredictable and not easily identified. This serious toxic reaction has been reported to occur in 11% of noninvasive drug tests (16) and in 16% of electrophysiologic studies (17) (Figure 4).

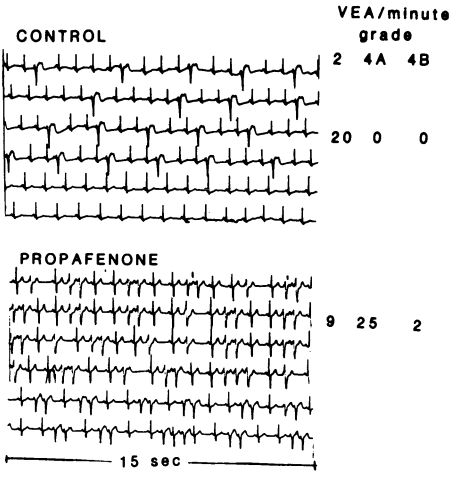

Figure 4. Aggravation of arrhythmia by antiarrhythmic drugs. During control the patient had ventricular premature beats (grade 2) with a frequency of 20/minute. During propafenone therapy the patient now has 25 couplets (grade 4A) per minute and 2 runs of ventricular tachycardia per minute (grade 4B). VEA = ventricular ectopic activity.

There are no ECG changes predictive of this complication and blood levels are usually in the defined therapeutic range. Aggravation with one drug does not predict this complication when another antiarrhythmic drug of the same class is administered. While arrhythmia aggravation is more common in patients with a history of a sustained ventricular tachyarrhythmia, especially in the presence of significant left ventricular dysfunction, there are no other clinical parameters which are helpful for identifying patients at risk (18). The unpredictable nature of this complication mandates cautious use of all antiarrhythmic agents.

An important question is whether antiarrhythmic drugs will prevent a recurrence of serious arrhythmia and prolong life. Although there are no placebo controlled studies in patients with a history of serious arrhythmia, there are reports of improved outcome when a drug program is tailored for the individual patient, with selection guided by arrhythmia suppression. In a report by Graboys and coworkers (15), 123 patients with malignant arrhythmia underwent a noninvasively guided evaluation of drug effect. In 98 patients an effective agent was identified and continued long term. The annual sudden death rate was 2.3%. In contrast, 25 patients continued to have runs of VT despite the administration of the most effective agent. In this group the annual sudden death rate was 44%. Stein and coworkers (19) reported that in a group of 107 patients who responded to mexiletine the annual sudden death rate was 3.6% during a follow-up of 23 months. In a study of long term tocainide therapy reported by Hohnloser and coworkers (6), 73 patients responding to the drug were discharged and the annual sudden death rate was 4.4%. Similar results have been reported when drug selection is guided by electrophysiologic techniques. Ruskin and coworkers (9) reported on 31 patients with sudden death of whom 25 were inducible. An effective drug was identified in 19 and none had a recurrence after 15 months. In contrast, 3 of 6 patients who were still inducible had a recurrence. Similar results were reported by Swerdlow and coworkers (20). In our experience with 45 patients who were inducible in control, 36 were rendered noninducible during drug therapy and the annual recurrence was 2.8% (10). Of 9 patients still inducible, 5 (56%) had a recurrence.

In conclusion, both noninvasive and invasive techniques are important and reliable methods for evaluating antiarrhythmic drugs. Since there are no guidelines for selecting an effective agent for an individual patient, a systematic approach to drug evaluation is necessary. Drug therapy must be tailored to each patient, based on the suppression of spontaneous arrhythmia evaluated noninvasively or the failure to induce arrhythmia with electrophysiologic techniques. If an effective and well tolerated drug is identified for long term therapy, arrhythmia recurrence can be prevented in the vast majority of patients.

References

1. Waxman, H.L., Buxton, A.E., Sadowski, C.M., Josephson, M.E. The response to procainamide during electrophysiologic study for sustained ventricular tachycardia predicts the response to other medication. Circulation 67:30-37, 1983.
2. Oseran, D., Gang, E., Dexer, T., Mandel, W. The efficacy of oral type 1 agents in the treatment of ventricular tachycardia cannot be determined by response to intravenous procainamide. Circulation 70(SupplII):II-447, 1984.
3. Fogoras, R.N., Fiedler, S. Serial drug testing for ventricular arrhythmia. Inability of early failure with procainamide to predict failure with all standard drugs. Circulation 70(SupplIII): II-447, 1984.
4. Hession, M., Blum, R., Podrid, P.J., Lampert, S., Stein, J., Lown, B. Mexiletine and tocainide: Does response to one predict response to the other? J Am Coll Cardiol 7:338-343, 1986.
5. Winkle, R.A., Mason, J.W., Harrison, D.C. Tocainide for drug resistant ventricular arrhythmias: Efficacy, side effects and lidocaine responsiveness for predicting tocainide success. Am Heart J 100:1031-1036, 1980.
6. Hohnloser, S.H., Lange, H.W., Raeder, E.A., Podrid, P.J., Lown, B. Short and long term therapy with tocainide for malignant ventricular tachyarrhythmias. Circulation 73:143-147, 1986.
7. Lown, B., Podrid, P.J., DeSilva, R.A., Graboys, T.B. Sudden cardiac death: Management of the patient at risk. Curr Prob Cardiol 4(12): 1-62, 1980.
8. Podrid, P.J., Lown, B., Graboys, T.B., Lampert, S. Use of short term drug testing as part of a systematic approach for evaluating antiarrhythmic drugs. Circulation 73(SupplII):II81-91, 1986.
9. Ruskin, J.N., DiMarco, J., Garan, H. Out of hospital cardiac arrest. Electrophysiologic observations and selection of long term antiarrhythmic therapy. N Engl J Med 303:607-613, 1980.
10. Podrid, P.J., Schoeneberger, A., Lown, B., Lampert, S., Matos, J., Porterfield, J., Raeder, E., Corrigan, E. Use of nonsustained ventricular tachycardia as a guide to antiarrhythmic drug therapy in patients with malignant ventricular arrhythmia. Am Heart J 105:181-188, 1983.
11. Lown, B., Wolf, M. Approaches to sudden death from coronary heart disease. Circulation 44:130-142, 1971.
12. Gaughan, C.E., Lown, B., Lanigan, J., Voukydis, J., Besser, W. Acute oral testing for determining antiarrhythmic drug efficacy. I. Quinidine. Am J Cardiol 38:677-684, 1976.
13. Duff, H.J., Roden, D., Primm, R.K., Oates, J.A., Woosley, R.L. Mexiletine in the treatment of resistant ventricular arrhythmias. Enhancement of efficacy and reduction of dose related side effects by combination with quinidine. Circulation 67:1124-1128, 1983.
14. Hirsowitz, G., Podrid, P.J., Lampert, S., Stein, J., Lown, B. The role of beta blocking agents as adjunct therapy to membrane stabilizing drugs in malignant ventricular arrhythmia. Am Heart J 111:625-631, 1986.
15. Graboys, T.B., Lown, B., Podrid, P.J., DeSilva, R.A. Long term survival of patients with malignant ventricular arrhythmia treated with antiarrhythmic drugs. Am J Cardiol 50:437-443, 1982.

16. Velebit, V., Podrid, P.J., Cohen, B., Lown, B., Graboys, T.B. Aggravation and provocation of ventricular arrhythmia by anti-arrhythmic drugs. Circulation 65:886-894, 1982.
17. Poser, R.F., Podrid, P.J., Lombardi, F., Lown, B. Aggravation of arrhythmia induced with antiarrhythmic drugs during electrophysiologic testing. Am Heart J 110:9-16, 1985.
18. Slater, W., Podrid, P.J., Lampert, S., Lown, B. Are there clinical predictors for arrhythmia aggravation. J Am Coll Cardiol 7 (Suppl A):158A, 1986.
19. Stein, J., Podrid, P.J., Lampert, S., Hirsowitz, G., Lown, B. Long term mexiletine for ventricular arrhythmia. Am Heart J 107: 1091-1098, 1984.
20. Swerdlow, C.D., Winkle, R.A., Mason, J.W. Determinants of survival in patients with ventricular tachyarrhythmia. N Engl J Med 308: 1436-1441, 1983.

2

ANTIARRHYTHMIC ACTION OF CALCIUM ANTAGONISTS

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INTRODUCTION

The marked coronary dilating and negative inotropic effect of prenylamine /1/ as well as that of verapamil /2/ was described in the early sixties, however Fleckenstein et al. /3/ were the first to show that these agents are capable of depressing cardiac contractility in concentrations not yet affecting the action potential. Thus they inhibit the electromechanical coupling. The authors attributed this effect to a drug induced depression of the transmembrane inward Ca^{2+} current hence the name: calcium antagonists /Ca-s/. Later Grün and Fleckenstein /4/ have shown electromechanical uncoupling effect of CA-s in the smooth muscle too. The steadily growing interest has initiated a number of experimental and clinical studies showing that in addition to its scientific importance introduction of this new group of drugs into clinical therapy represents a major breakthrough.

The main sites of action and indications for clinical application are summarized in Fig. 1. In view of all these facts it is surprising to find that contrary to expectations the use of CA-s in antiarrhythmic therapy is rather limited and essentially confined to tachyarrhythmias of supraventricular origin.

Therefore aim of the present paper was twofold: first to clear the cause of this limitation and secondly to suggest new fields for clinical use against cardiac arrhythmias taking into account the very heterogenous chemical structure and pharmacological actions of these agents.

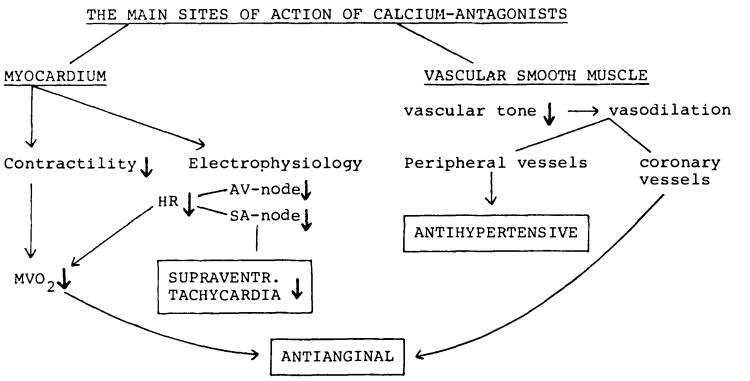

Fig. 1. The main sites of action and clinical indications of CA-s

RESULTS AND DISCUSSION

Because of the dissimilar structure and pharmacological profile of CA-s their mode of action seems to be rather complex - nevertheless they all possess a common action, namely the more or less expressed inhibition of the slow inward Ca^{2+} current.

Accordingly the myocardial action of CA-s is mainly focussed on the automatic cells characterized by the predominantly Ca^{2+} inward current dependent slow diastolic depolarisation. It should be mentioned here that the typical electrophysiological changes summarized in the next figure are characteristic of verapamil and to a lesser extent of diltiazem but not of nifedipine, which in the therapeutic dose range has no substantial electrophysiological and antiarrhythmic effects in man /5/. Therefore it was omitted from further comparative studies.

In addition to these "pure" classical CA-s which may block Na^{+} channel only in very high and toxic concentrations, there is a group of agents - such as prenylamine, its derivative: demethylprenylamine or fendiline, furthermore a new compound: substance "A", made available by courtesy of Dr. Korbonits - which besides their Ca^{2+} antagonistic action may also block the fast Na^{+} channel considerably. In the following the two latter agents called by us shortly as "mixed"

CA-s will be also included into our comparative studies as will be two standard Class I antiarrhythmic drugs: quinidine and mexiletine. The calcium antagonistic properties of substance "A" are demonstrated in Fig. 2. In Tyrode solution

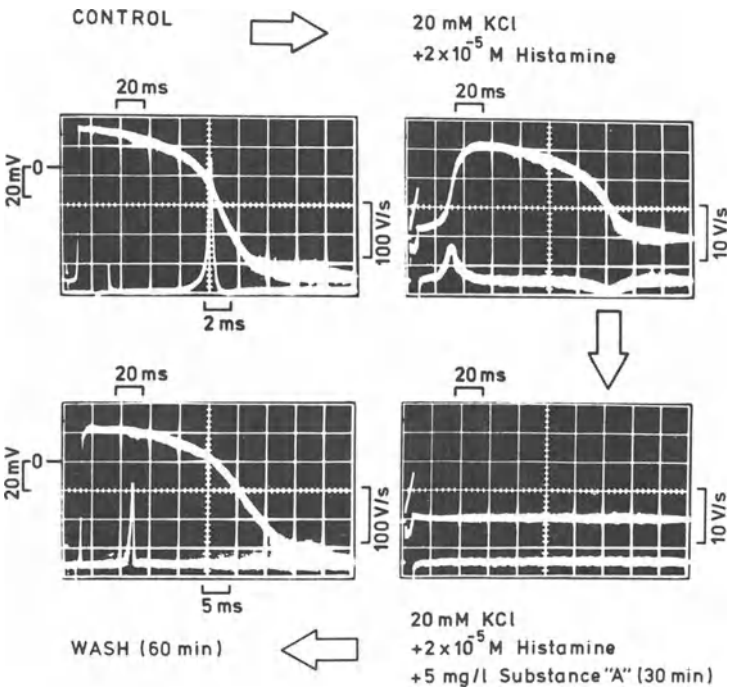

Fig. 2. Effect of substance "A" on the slow Ca^{2+} current dependent action potential arising in the presence of high /20 mM/ KCl solution, and histamine $2x10^{-5}$ M. Left upper panel: control, intracellular recording from right ventricular myocardium of the rabbit /first curve = normal AP; second curve = rate of depolarisation; dV/dt_{max}/. Right upper panel: slow potential; right lower panel: substance "A" abolished slow potential by inhibition of inward Ca^{2+} current; left lower panel: wash and recovery.

containing high 20 mM KCl inactivating fast Na^{+} channel but leaving slow inward Ca^{2+} current intact, density of this latter is increased by the adenylate cyclase activator

histamine /$2x10^{-5}$ M/. The arising slow potential is extinguished in the presence 5 mg/l substance "A". The sodium channel blocking effect the same substance is shown in Fig. 3, where depression of the rate of rise is specially conspicuous.

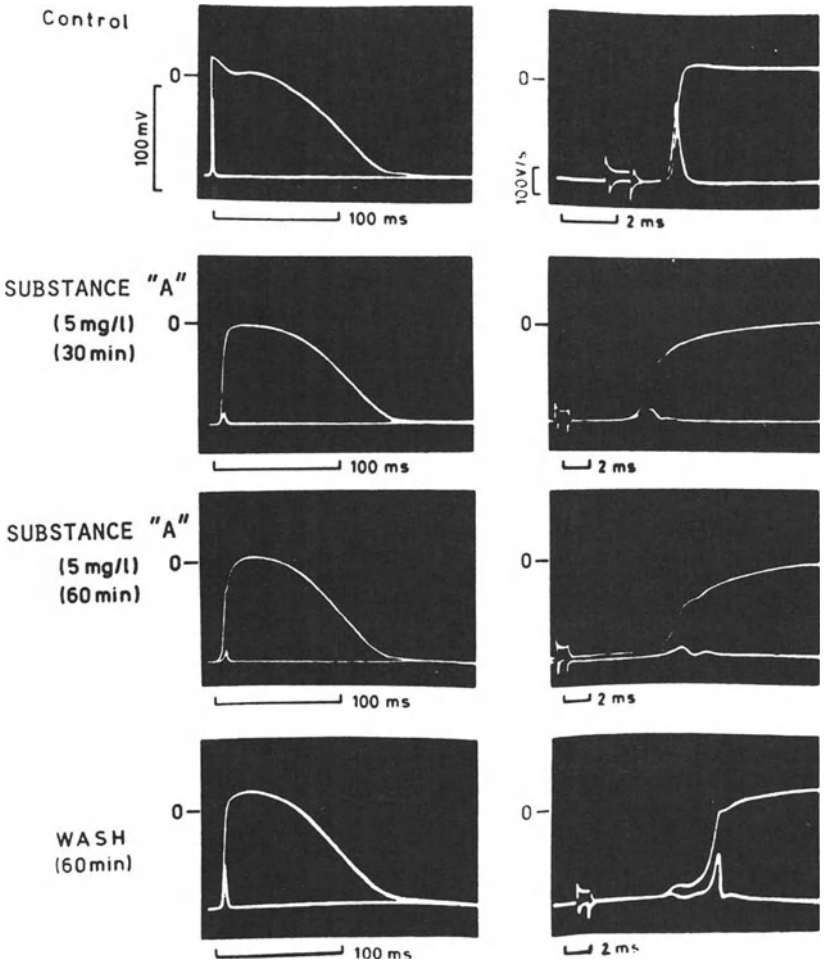

Fig. 3. Effect of substance "A" on the transmembrane action potential recorded from the right ventricular distal Purkinje fiber strand. Panels on the left = intracellular records photographed at slow sweep speed /upper curve = AP; lower curve = dV/dt_{max}/; panels on the right = the same with fast sweep speed.

The cardiac electrophysiologic effects of CA-s are summarized in Table 1. Accordingly CA-s reduce the amplitude of the action potential and the rate of spontaneous firing in the sinoatrial /SA/ pacemaker. Their bradycardiac effect is a

Table 1. Cardiac electrophysiologic effects of /verapamil type/ Ca^{2+}-antagonists

Site of action	Electrophysiologic changes
Sinus node	AP-amplitude ↓ Rate of firing ↓ Recovery time ↑ SA conduction time ↑
Atrium	normal < conduction ∅ latent pacemakers ↓ diseased or dilated < conduction ↓ AP-amplitude ↓ rate of rise of depol. ↓
A-V node	conduction time ↑ refractoriness ↑
Accessory pathways	antegrade < conduction ∅ refractoriness ∅ or ↓ retrograde < conduction ∅ refractoriness ∅ or ↑
His Purkinje system and ventricles	A-H interval ↑ H-V interval ∅ or slightly ↑ nonischemic conduction ∅ ischemic conduction time ↓ in reentrant pathway

AP = action potential, SA = sinoauricular,
A-H = atrial-His, H-V = His-ventricular

dose dependent direct action on the sinus node which cannot be abolished by atropine. It may be however sometimes compen-

sated by a reflex sympathetic tachycardia evoked by the drug induced peripheral vasodilatation.

The CA-s also depress the amplitude of the junctional action potentials and thus slow down A-V conduction. His-bundle recordings in patients and in experimental animals have shown that these drugs may prolong A-H time, an indicator of intranodal conduction without substantially affecting P-A, H-V and QRS intervals.

Accessory pathways are not substantially affected.

In view of the aforementioned facts it was of interest to compare the depressant action of "pure" and "mixed" CA-s on SA, AV and Purkinje fiber automaticity. Therefore right atrial, atrioventricular and Purkinje fiber right ventricular preparations from the same rabbit heart were suspended in oxygenated Tyrode solution and subjected to increasing concentrations of fendiline, substance A and verapamil respectively. As it may be seen in Table 2, in contrast with the "mixed" CA-s markedly depressing both higher and especially the lower /Purkinje/ automatic centers, the "pure" CA: verapamil, a potent inhibitor of the spontaneous SA and junctional firing did not affect substantially Purkinje firing rate. Thus probably no great protection against heterotopic discharges can be expected from verapamil type CA-s.

These findings are clearly reflected by the clinical use and therapeutic value of verapamil type CA-s as shown in Table 3. For comparison effects quinidine and lidocaine are also shown.

The marked inhibition of AV node is the mechanism by which verapamil may be useful against paroxysmal supraventricular tachycardias or junctional tachycardias both arising mainly on the basis AV nodal reentry. This mechanism is also responsible for the protection of the ventricles in atrial flutter and atrial fibrillation, when the number of impulses conducted to the ventricles is reduced to more physiological values. Although as mentioned the drug has no effect on accessory pathways, in praeexcitation syndromes

Table 2. Negative chronotropic effect of calcium antagonists on automaticity of different pace-maker areas of the isolated rabbit heart

Drug concentr. /mg/l/	% Change in spontaneous rate		
	SA-node	AV-node	Purkinje fiber
Fendiline			
0.5	- 7.8^{NS} / 9/	-10.2^{NS} / 8/	-31.6^{NS} / 4/
1.5	- 8.9^{NS} / 9/	-13.7* / 6/	-87.0*** / 6/
3.0	-14.8** / 9/	-18.6* / 7/	-92.1*** / 6/
5.0	-29.8** / 9/	-24.6** / 9/	-95.8*** / 5/
Substance A			
1.0	-15.1^{NS} / 6/	-13.2^{NS} / 7/	-49.0** / 9/
2.0	-21.6** / 8/	-22.4^{NS} / 8/	-78.9** / 6/
4.0	-24.0** / 5/	-44.4*** /12/	-87.6*** /14/
Verapamil			
0.125	- 7.9^{NS} / 6/	-14.1^{NS} / 9/	+16.3^{NS} / 8/
0.25	-20.5** /10/	-26.7* /11/	-13.3^{NS} / 8/
0.5	-25.8*** /16/	-40.2*** /11/	-18.2^{NS} / 8/

Statistical significance of the difference from control values: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS = non significant
Number of preparations in brackets.

such as WPW it could block reentry in the A-V node and prevent junctional reciprocal tachycardia. It may be useful in the treatment of supraventricular tachycardias associated with myocardial infarction. In animal experiments it may reduce the amplitude of oscillatory afterpotentials appearing in the diastolic phase of the AP after intoxication with cardiac glycosides.

Certainly under pathological conditions, e.g. in local myocardial ischaemia due to sudden coronary occlusion transmembrane potentials could be so much depressed, that only Ca^{2+} current dependent slow response electrical activity is possible. This may activate latent pacemaker activity in the working myocardium and specially in the presence of increased sympathetic tone maintain slow conduction and give

Table 3. Clinical use and approximate therapeutic value of Verapamil in different forms of cardiac arrhythmias as compared with quinidine and lidocaine

Type of arrhythmia	Drugs and their approximate therapeutic value		
	Verapamil	Quinidine	Lidocaine
Supraventricular			
Atrial paroxysmal tachycardia	++++	+++	ϕ
Atrial extrasystoles /ES/	++++	++++	ϕ
Atrial flutter and fibrillation ↓of ventricular rate	++++	ϕ	ϕ
conversion to sinus rhythm	++	+++	ϕ
maintenance of sinus rhythm	++	+++	ϕ
A-V junctional			
ES and tachycardia	+++	+++	ϕ
Ventricular			
Ventricular ES	ϕ	+++	++++
Ventricular tachycardia /VT/	ϕ	++	++++
Digitalis intoxication			
Atrial ES and tachycardia	+++	++	ϕ
Ventricular ES and VT	ϕ	++	++++

rise to reentry. This is a valid indication for the use of Ca^{2+} antagonists in postinfarction arrhythmias however a much higher dose is required and here the great sensitivity of the AV node to verapamil is the dose limiting factor, since AV block should be avoided. This is clearly seen in Table 4, in which the potency of different CA-s and of quinidine and mexiletine relative to quinidine was estimated in the heart "in situ" of anesthetized dogs by comparing their ED_{25} values on heart rate /HR/, corrected sinus node recovery time /CSNRT/, atrial /ARP/ and ventricular refractory period /VRP/ and the refractory period of the AV-node /AVRP/. Verapamil proved to be about 270-times more active on this latter than quinidine, whereas corresponding values for HR were = 196, for CNSRC : 22, for ARP : 46 and

for VRP : 40.

Table 4. Efficacy of different calcium antagonists and of some standard antiarrhythmic drugs on different electrophysiological parameters in the heart "in situ" of anesthetized dogs estimated by comparison of the ED_{25} values and related to the effects of quinidine

Substance	↓HR	↑CNSR	↑ARP	↑VRP	↑AVRP
	Rel.activity	Rel.activity	Rel-activity	Rel.activity	Rel.activity
Quinidine /n=5/	1.00	1.00	1.00	1.00	1.00
Mexiletine /n=5/	1.17	1.20	0.91	1.44	1.89
Fendiline /n=5/	2.90	1.80	1.96	2.63	4.82
Substance A /n=5/	2.93	2.45	1.66	1.96	6.08
Diltiazem /n=5/	25.86	21.14	14.03	33,75	66.38
Verapamil /n=5/	196.21	21.93	46.28	40.38	269.69

HR = heart rate; CNSR = corrected sinus node recovery time; ARP = atrial refractory period; VRP = ventricular refractory period; AVRP = atrioventricular refractory period

A similar though less expressed tendency concerning prolongation of the AVRP in seen with the other "pure" CA: diltiazem, however this drug has a relatively marked effect on VRP too and so its use in postischemic ventricular arrhythmias seems to be promising. The same holds for the "mixed" CA-s as fendiline and substance "A".

Indeed CA-s possessing definite electrophysiological actions as verapamil or diltiazem were able to reduce the number of extrasystoles appearing during 5 min occlusion and subsequent release of the left anterior descending coronary artery /LAD/ in the presence of a critical constriction of the left circumflex coronary artery /LXC/

in anesthetized thoracotomized dogs /Table 5/. The method

Table 5. Effect of calcium antagonists on the number of extrasystoles and the incidence of ventricular fibrillation following acute LAD occlusion and reperfusion in the presence of a critical constriction of the left circumflex /LCX/ artery in anesthetized thoracotomized dogs

	LCX critical constriction			
	LAD occlusion /A/	Reperfusion /A'/	LAD occlusion /B/	Reperfusion /B'/
			Diltiazem 0.3 mg/kg i.v.	
ES/5 min	34.0±4.8	31.0±3.2	11.7±3.2 -66.8% A-B***	16.5±4.5 -46.7% A'-B'NS
VF	2/15 /13%/	3/13 /23%/	0/10 /0%/	0/10 /0%/
			Verapamil 0.15 mg/kg i.v.	
ES/5 min	28.8±2.8	21.3±4.6	4.45±1.8 -84.5% A-B***	8.8±2.4 -58.7% A'-B'**
VF	3/26 /11%/	4/23 /17%/	1/19 /5%/	2/18 /11%/
			Nifedipine 0.015 mg/kg i.v.	
ES/5 min	36.5±3.1	38.4±2.8	36.2±4.6 A-BNS	28.4±3.2 -26% A'-B'*
VF	1/11 /9%/	3/10 /30%/	0/7 /0%/	1/7 /16%/

Values are: Mean ± S.E.M., % difference from the untreated control occlusion and reperfusion values. Difference significant at level: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS = non significant. ES = extrasystole; VF = ventricular fibrillation; LAD = left anterior descending coronary artery

imitating the multivessel disease of coronary patients has been described by us earlier /6/. Both agents also considerably reduced ischemic ST-segment elevation in the epi- and endocardial electrogram but nifedipine possessing a similar antiischemic action was unable affect early post-occlusion arrhythmias although it moderated reperfusion arrhythmias. All three drugs seemed to protect more or less

against ventricular fibrillation /VF/ due to coronary occlusion and reperfusion respectively.

Analysis of late postocclusion arrhythmias appearing 24 hrs after delayed two stage ligation of the LAD has shown that ES could be influenced only by substance "A" but not by verapamil or fendiline. Under such conditions neither drug affected HR /Table 6/.

Table 6. Effect of calcium antagonists 24 hours after two stage ligation of the LAD on the number of ES appearing within 10 minutes after i.v. administration of the drug

Substance	Dose mg/kg /i.v./	n	Changes in the first 10 minutes following drug administration	
			% occurence of ES	HR
Control		5	68 ± 5	172 ± 10
Substance A	2.0	5	40 ± 4*	162 ± 12
			-41 %	-6 %
Control		5	50 ± 4	149 ± 15
Verapamil	0.15	5	46 ± 6	148 ± 15
				-1 %
Control		5	53 ± 4	139 ± 17
Fendiline	3.0	5	55 ± 6	136 ± 16
				-2 %

Values are = Mean ± S.E.M.
n = number of experiments; HR = heart rate; ES = extrasystoles
Statistically significant difference from control value at level: *$p < 0.05$

In view of the steadily increasing importance attributed to reperfusion arrhythmias in the clinical practice - protection against this latter by CA-s was studied in the isolated Tyrode perfused Langendorff rabbit heart subjected to 25 min global ischemia followed by reperfusion /Table 7/. The drugs tested /verapamil, substance "A" and fendiline/

Table 7. Protective effect of Substance A, Verapamil and Fendiline against reperfusion arrhythmias in the isolated rabbit heart subjected to 25 min global ischemia followed by reperfusion

Drug concentration /mg/l/	VF + VT	ES	No arrhythmia
Control	7/10 /70 %/	1/10	2/10 / 20 %/
Substance A			
0.25	$3/10^{NS}$	2/10	$5/10^{NS}$
0.50	2/10* /20 %/	2/10	$6/10^{NS}$
1.0	0/10** / 0 %/	0/10	10/10*** /100 %/
2.0	0/10** / 0 %/	0/10	10/10*** /100 %/
Verapamil			
0.125	1/10** /10 %/	6/10	$3/10^{NS}$
0.25	1/10** /10 %/	4/10	$5/10^{NS}$
0.50	0/10** / 0 %/	3/10	7/10* / 70 %/
Fendiline			
0.50	$4/10^{NS}$	3/10	$3/10^{NS}$
1.0	2/10* /20 %/	4/10	$4/10^{NS}$
2.0	2/10* /20 %/	2/10	$6/10^{NS}$

Each dose was tested on 10 hearts
Significant difference from control was calculated according to χ^2 method, at level = *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; NS = non significant; VF=ventricular fibrillation; VT=ventricular tachycardia; ES=extrasystoles

were dissolved in the reperfusion fluid. The occurence of reperfusion induced VT and VF was considerably reduced by all three drugs, except for the lowest concentration range; the occurence of ES was not affected or has even somewhat increased whereas reperfusion arrhythmias were completely absent after 1.0 and 2.0 mg/l concentrations of substance "A". Thus on the basis of the above discussed data it seems as if indication of CA-s in the antiarrhythmic therapy could be extended to early and late postischemic and reperfusion arrhythmias. The well known antiischemic effect of these

agents may certainly play here a role, however as negative results with nifedipine have shown: electrophysiologic effects are of importance too.

What concerns the possible therapeutic use of these agents their evaluation cannot be based on the effective dose required but rather on the relationship of this latter to the dose just evoking side effects. Until now only the electrophysiological limiting factor: namely the AV depression has been mentioned, but the unfavourable hemodynamic actions

Table 8. Tentative comparing of calcium antagonists on the basis of their favourable /antiischemic, antiarrhythmic/ versus unfavourable /hypotensive, negative inotropic/ actions estimated by means of the relevant ED_{25} values in mg/kg

Parameters and tendency of changes	Verapamil	Fendiline	Substance A
Hemodynamics: /anesthetized dogs/			
↓BP	0.20	0.74	2.25
↓HR	0.75	5.00	7.20
↓dP/dt max	0.07	0.49	6.00
Atrial FFT ↑ /anesthetized cats/	*	2.8	1.8
Vasopressin angina /anesthetized rats/	1.0	1.2	0.6
$\frac{BP}{Antianginal}$ ED_{25}	0.02	0.62	3.75
$\frac{BP}{Atrial\ FFT}$ ED_{25}	*	0.26	1.25
$\frac{dP/dt\ max}{Antianginal}$ ED_{25}	0.007	0.41	10.00
$\frac{dP/dt\ max}{Atrial\ FFT}$ ED_{25}	*	0.175	3.33

Note: BP = blood pressure; HR = heart rate; dP/dt max = left ventricular contractility; FFT = fibrillo-flutter threshold, *can not be determined

of CA-s: as the blood pressure fall and the negative inotropic action could also limit elevation of the dose to values necessary to reach adequate therapeutic effect.

Therefore in the following an attempt was made to compare "pure" and "mixed" CA-s on the basis of their favourable: antiarrhythmic and antiischemic versus unfavourable, hypotensive and negative inotropic actions all measured by the relevant ED_{25} values /Table 8/.

Although the calculated values are rough estimations based on data determined in different species - they clearly show the tendency that the "mixed" CA-s could have more therapeutic importance than assumed on the basis of their low efficacy as compared with that of the "pure" CA-s. To prove this further and more adequate and accurate evidence is needed, however our data concerning efficacy of "mixed" CA-s in model arrhythmias seem to be convincing enough to encourage further investigations in this direction.

SUMMARY

Clinical use of calcium antagonists /CA-s/ is essentially confined to tachyarrhythmias of supraventricular origin, an indication based on their action depressing the sinoatrial and particularly the atrioventricular /AV/ node. Therapy of ventricular arrhythmias involving slow channels is limited because of the marked AV blocking, negative inotropic and hypotensive effect of these drugs. Our present experiments in which the "pure" CA: verapamil compared with the mixed /Ca^{2+} and N^{+}-channel blocker / antagonists as fendiline or substance "A" have shown that the "mixed" CA-s are effective in early and late reperfusion arrhythmias. Their use proved to be favourable on the relationship between of hypotensive and fibrillation threshold increasing effect and on that between negative inotropic and fibrillation threshold increasing effect estimated by the ED_{25} values. Since a similar shift concerning relationship between hypotensive and antianginal ED_{25} values was observed, mixed CA-s could be advantageously used against arrhythmias associated with

myocardial ischaemia.

REFERENCES

1. Lindner, E. Arzneim. Forsch. 10: 569-573, 1960.
2. Haas, H. and Härtfelder, G. Arzneim. Forsch. 12: 549-558, 1962.
3. Fleckenstein, A., Kammermeier, H., Döring, H. and Freund, H.J. Z. Kreislaufforsch. 56: 716-744, 839-853, 1967.
4. Grün, G. and Fleckenstein, A. Arzneim. Forsch. 22: 334-344, 1972.
5. Henry, P.D. Am. J. Cardiol. 46: 1047-1058, 1980.
6. Szekeres, L., Udvary, É. and Végh, Á. Br. J. Pharmacol. 86: 341-350, 1985.

3

QUINIDINE REDUCES OUTWARD CURRENT IN SINGLE CANINE CARDIAC PURKINJE CELLS

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INTRODUCTION

The commonly used antiarrhythmic drug quinidine is a member of the local anesthetic class. In cardiac muscle it reduces sodium (Na) current (1), thereby slowing the action potential (AP) upstroke (2), slowing conduction velocity, and reducing excitability. Quinidine also affects AP repolarization. Typical changes are a prolongation of the AP and a negative shift in the plateau voltage (3), although other studies show either AP shortening (4) or both lengthening and shortening (5). A number of ionic currents contribute to the plateau of the AP, including the inward calcium (Ca) current, slowly inactivating (or "window") Na current, and three potassium (K) currents: the inward rectifier current, the delayed rectifier, and the transient outward current (I_{to}). The complex response of the AP to quinidine suggests that it may affect more than one of these repolarizing currents. Effects of drugs on several membrane channels is not rare, but the drug interaction must be to some shared molecular components of the diverse channels. This report addresses the question of quinidine's effect on I_{to} in canine cardiac Purkinje cells.

The importance of these effects of quinidine on repolarization can be found in two associated areas. Clinically, quinidine can produce a characteristic ventricular tachycardia called "Torsade de Pointes", probably the result of prolongation of repolarization and induction of early afterdepolarizations (6). Secondly, quinidine may produce a reduction in contraction strength. It is well-known that cardiac contraction strength depends on the duration of the AP or the depolarization (5,7,8). Consequently, the effect of quinidine on the AP may be a part of the mechanism of

its negative inotropic effect. These two effects of quinidine on repolarization may be the basis of its cardiac toxicity. Elucidation of the mechanism of these effects could influence the clinical use of quinidine and contribute to development of equally effective, safer antiarrhythmic drugs.

The strategy used in these studies was to study single cardiac Purkinje cells. They are known to have a large I_{to} (9), and the new patch pipette method permits good voltage control and better regulation of the intracellular and extracellular ionic environment. We avoided Na and Ca currents by holding the cell membrane potential at a voltage where all of the Na current was inactivated and by using Ca-free solutions. Delayed rectifier K current was not seen in these cells, but I_{to} could be easily elicited by depolarizing steps. The results support the conclusion that quinidine is a potent blocker of the I_{to} channel, probably by interacting with the open state of the channel.

METHODS

Single Purkinje cells were isolated from fibers harvested from canine hearts by the method of Sheets et al (10). The yield varied from 40-80%, but viable cells were chosen for study on the basis of clearly visable cross-striations and the absence of blebs. These are large cells, 121±26 µm long and 28±6 µm in diameter (n=32), and they have no transverse tubules (11). After separation, they were stored in Tyrode solution containing (in mM) NaCl 142, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 5, and HEPES-NaOH buffer 5 (pH = 7.4). The Ca-free solution contained no added Ca, $MgCl_2$ 5, and EGTA 0.1. Experiments were done at 20±1 °C. Patch pipettes were made according to the method of Hamill et al (12) from borosilicate glass. They were fire polished to a tip size of 4-7 µm, so that the resistance when filled with isotonic salt solution was about 0.5-1.5 MΩ. The pipettes were usually filled with (in mM) K-aspartate 110, KCl 20, KH_2PO_4 2, glucose 10, HEPES 10, Mg-ATP 5, BAPTA 5, and Na-creatine phosphate 5. Voltage clamp was made with a Dagan 8900 clamp without the capacitive correction circuit (feedback resistance 20 MΩ), with command signals and data acquisition at 1-2K Hz by an IBM-XT using a 12 bit A-D and D-A converter (DT 2818, Data Translation, Inc.). The holding potential was set near -40 mV and voltage steps were made usually for 700 msec at one minute intervals. Measured voltages were adjusted for the junction potential of -8 mV inside the

pipette. Exponential curve-fitting was in a Masscomp 550 using the DISCRETE algorithm. Quinidine sulfate was added from a 10 mM stock solution.

RESULTS

Effects of quinidine in Tyrode solution

The need for isolation of I_{to} can be demonstrated by examination of the effects of quinidine in normal Tyrode solution (Fig. 1). Sodium current was inactivated by holding the membrane potential at -42 mV. Steps in the depolarizing direction demonstrated an inward Ca current overlapping with a large outward I_{to} current. Quinidine reduced both currents, but the activation phase and the maximal level of I_{to} could not be accurately measured in the presence of the Ca current.

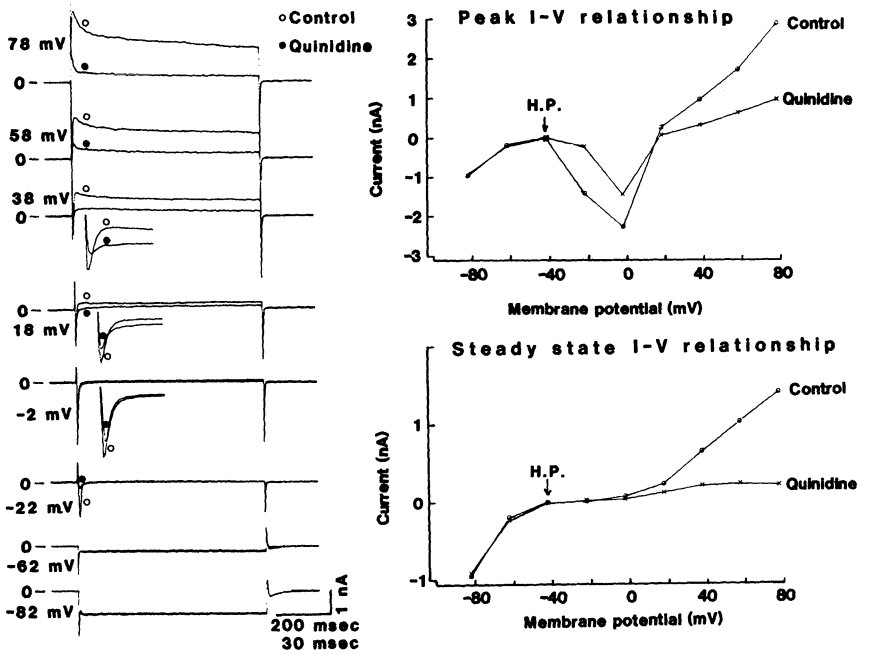

Fig. 1. Quinidine effect in normal solution. The left panel depicts currents recorded from one cell. Hyperpolarizing and depolarizing steps were applied from a holding potential of -42 mV. The right panel shows the current-voltage (I-V) relationships in the presence and absence of 20 μM quinidine. I_{to} activates at potentials positive to -18 mV. The Ca current activates at potentials between -22 and +38 mV. Both currents were reduced by addition of quinidine to bath solution. The inward rectifier K current activates negative to -82 mV. Bath solution was normal Tyrode solution with 1.8 mM Ca.

I_{to} under Ca-free conditions

In order to avoid the distortion produced by Ca current, we performed the rest of the studies in solutions that were effectively free of Ca. Although there has been a suggestion that I_{to} is a Ca-activated current (13), it is apparent in Fig. 2 that I_{to} is still present when Ca is omitted from the extracellular solution. More detailed study of the role of Ca in controlling I_{to} will be reported separately (Nakayama & Fozzard, unpublished observations). Fig. 2 also illustrates the lack of an outward tail of current upon clamping back to -42 mV after 700 msec or back to -22 mV after 4 sec. Under conditions of these experiments we were unable to see any evidence for the delayed rectifier K current.

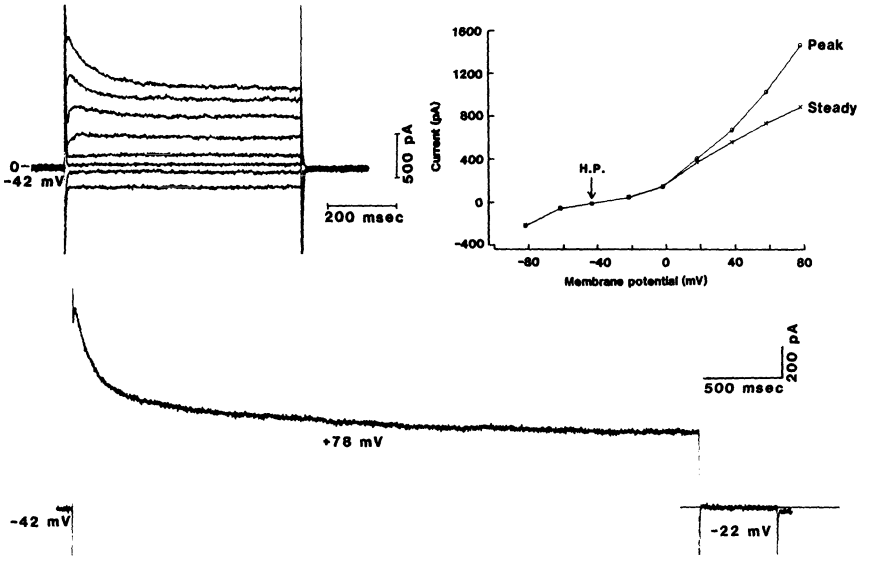

Fig. 2. The family of currents in the upper left panel was recorded under Ca-free conditions. The I-V relationship is shown in the right panel. Time-dependent I_{to} and time-independent currents were recorded from a holding potential of -42 mV. No tail currents were observed upon clamp-back to -42 mV (upper panel) or to -22 mV (lower panel). Any Na current through the Ca channels that might have occurred because of the absence of Ca was blocked by 5 mM Mg.

Recovery of I_{to} from inactivation

A characteristic of I_{to} is its slow recovery from inactivation (9,14). Under the conditions of these experiments recovery was exponential, with time constants of less than 1 sec (Fig. 3).

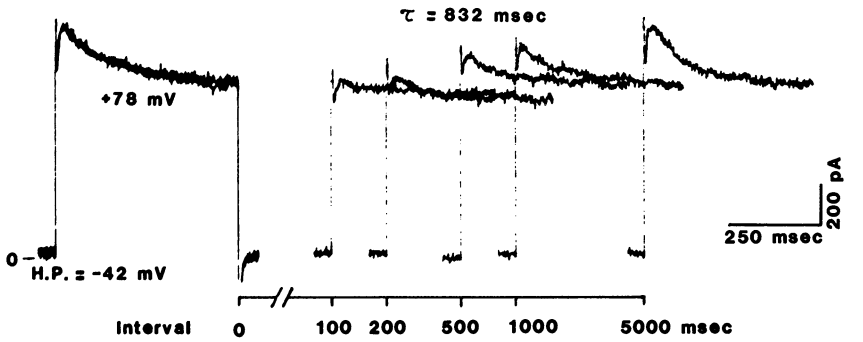

Fig. 3. Currents elicited by pairs of identical steps to +78 mV from a holding potential of -42 mV. The pairs of steps were separated by varying intervals, and a pair was separated from another pair by 60 sec. The first current trace has superimposed on it the 60 sec step. The second to sixth currents were separated by the interval shown on the lower scale. Recovery followed a single exponential time course with a time constant of 832 msec.

Quinidine effect in Ca-free solutions

Quinidine in micromolar doses produced a dramatic fall in peak amplitude of I_{to} (Fig. 4). Quantitation of this effect was sometimes difficult because the rapid decline of I_{to} made separation of the peak current from the capacity transient uncertain.

There was a dramatic fall in the steady state current at 700 msec. From these studies it is not possible to say if this is a steady state component of I_{to} that is blocked by quinidine or an effect of the drug on the leakage K current. This question was investigated by comparing the effect of quinidine to that of 4-aminopyridine (4AP), which is known to block I_{to} (15). In six experiments 1 mM 4AP reduced the steady state current by 24%. By analogy it appeared that there was a steady state I_{to} that was blocked by quinidine, but that there may also be some block of the leakage K current by quinidine.

The effect of quinidine on I_{to} was dose dependent, with a half effect achieved at a concentration of 2.3×10^{-5}M (Fig. 5).

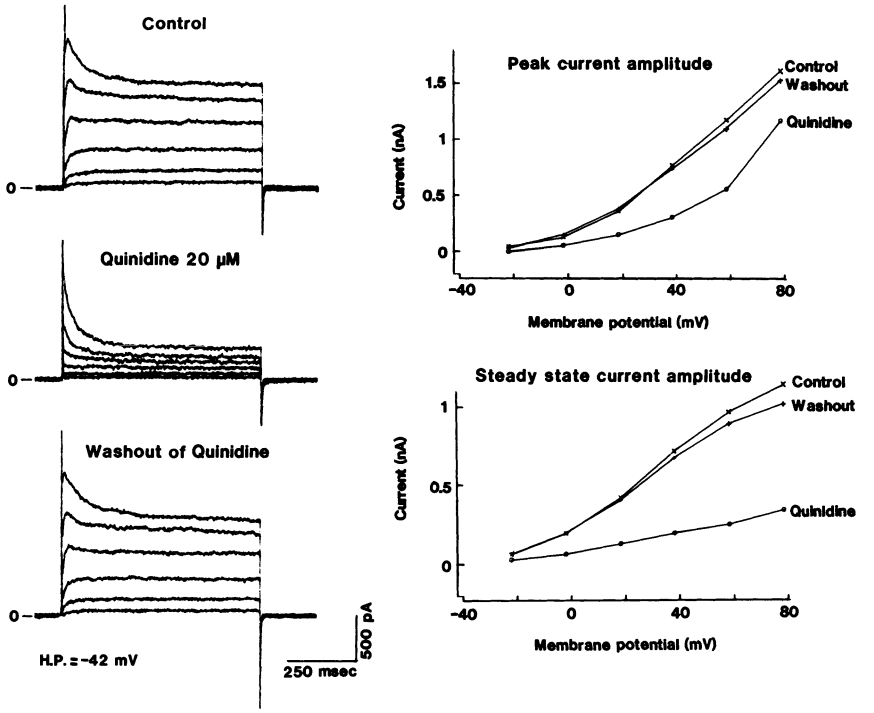

Fig. 4. The left three current traces were recorded from the same cell. Depolarizing steps, incremented by 20 mV, were applied from a holding potential of -42 mV in the presence and absence of 20 µM quinidine. The right panels show the I-V relationships for both peak and late (700 msec) currents. Both the peak and the steady state currents were reduced by quinidine.

Effect of quinidine on I_{to} kinetics

Some information about the nature of the quinidine block can be gained by examining the drug's effect on current kinetics. Recording of the quinidine effect at higher time resolution demonstrated that the peak of I_{to} was advanced, as well as reduced (Fig. 6). This suggests that the block by quinidine occurs during the period when the channel is open. On the other hand, inactivation was not much affected. Previous studies have indicated that I_{to} is not inactivated as a single exponential, but is better fit with two exponentials (14). Also illustrated in Fig. 6 are inactivation curves before and after quinidine, with exponential fits.

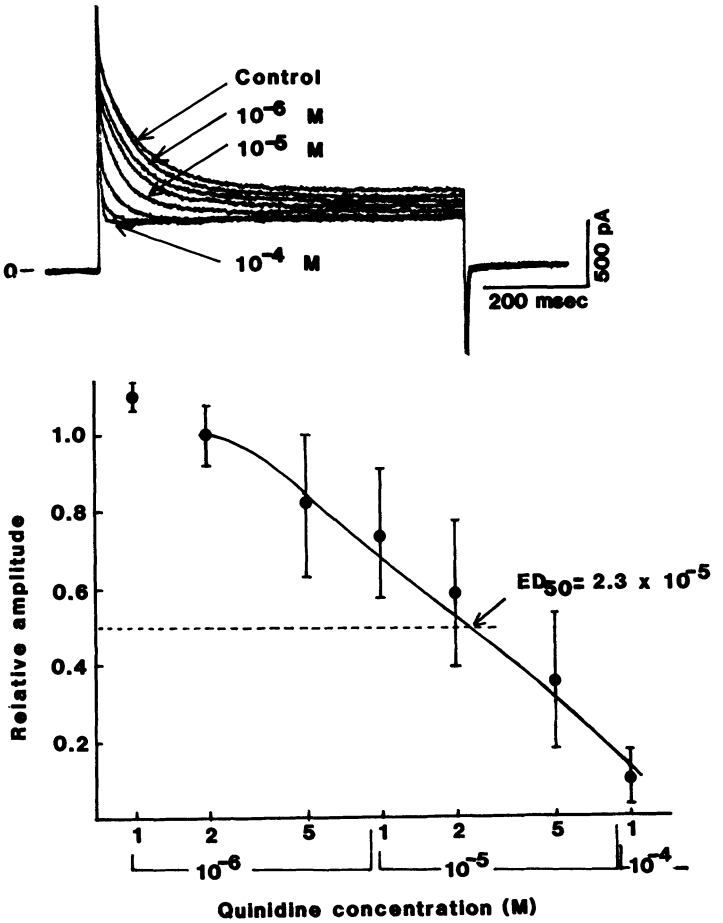

Fig. 5. The upper panel shows superimposed currents from a cell that was repeatedly depolarized to +78 mV from a holding potential of -42 mV during exposure to different concentrations of quinidine. The dose-response curve for peak amplitudes, which were determined from exponential fits, is shown in the lower panel. The half maximal effect was estimated to be 23 μM.

Effect of Na removal on I_{to}

Because the reversal potential of the putative I_{to} channel was influenced by the Na gradient (14), we examined I_{to} in solutions free of both Na and Ca (Fig. 7). Characteristics of I_{to} were unchanged by Na removal, and block of both the peak current and the steady state current was not affected. It was difficult to determine if the magnitude of I_{to} was altered by Na removal, because the experiments were performed in separate cells. However, if there was a magnitude effect, it must have been small.

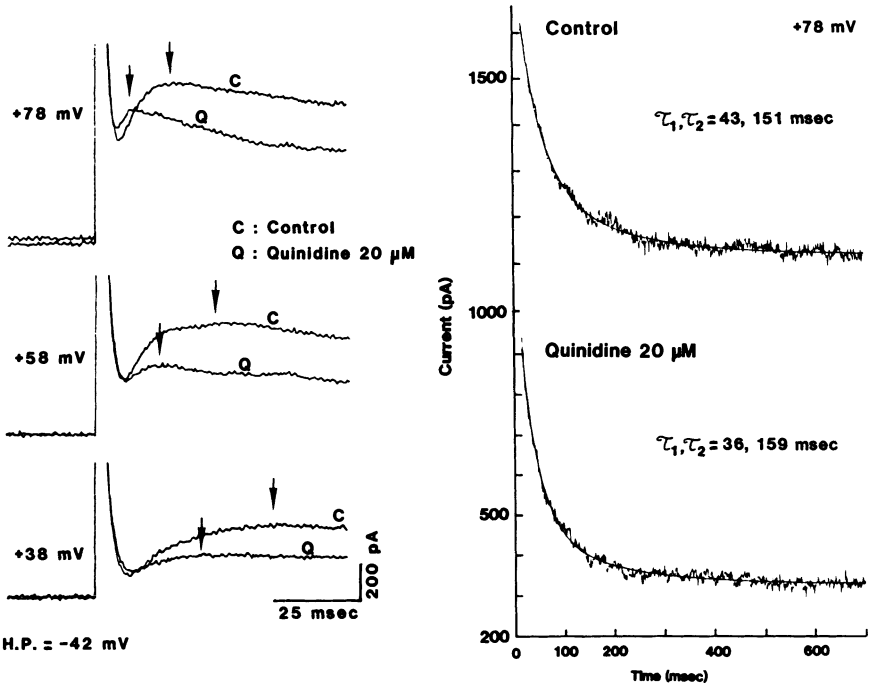

Fig. 6. Effect of quinidine on I_{to} kinetics. The left panel shows the activation of I_{to} at 15±1°C. After application of quinidine, both the peak (indicated by the arrow) and the time of the peak were decreased. The right panel shows the inactivation phase of I_{to} at 20±1°C. In most cases the decay of the current could be fit with two exponentials.

DISCUSSION

Characteristics of I_{to}

The transient outward current in heart muscle was first described and characterized in Purkinje fibers (9,15,16,17). Initially, it was thought that the ionic carrier of I_{to} in the heart was chloride (Cl). While it remains possible that part of I_{to} is carried by Cl (16), the predominant carrier appears to be K. As with any large current in multicellular preparations, it is necessary to be concerned about the possibility of poor voltage control and of accumulation or depletion of ions in the limited extracellular spaces (18,19). This study is the first made in single Purkinje cells, where voltage control is good and where extracellular accumulation or depletion is minimal. The principal characteristics of I_{to} in the single cells are not different from those already described for

multicellular preparations. In particular, it is important to note that under these relatively favorable conditions, there was still two time constants to I_{to} inactivation.

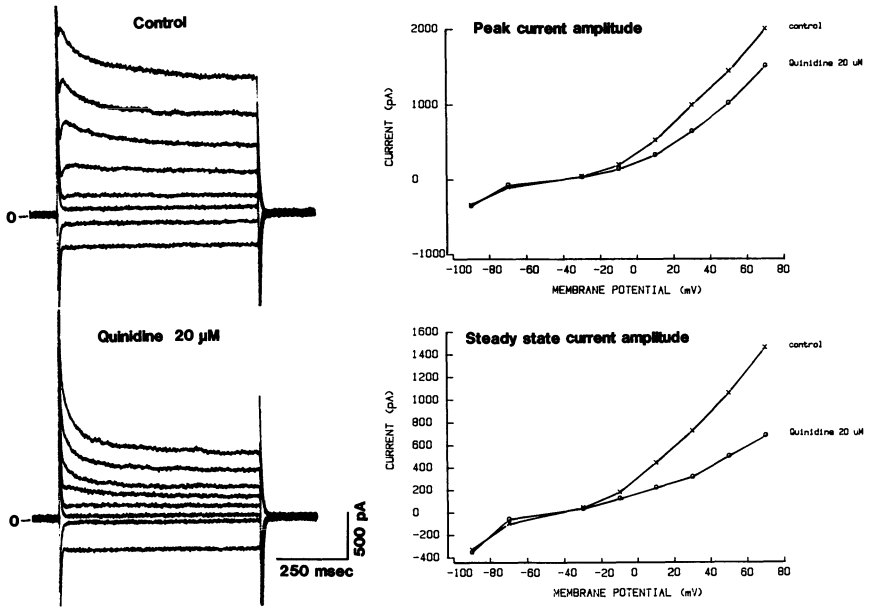

Fig. 7. The left panel illustrates currents from the same cell bathed in a Na- and Ca-free solution. Voltage steps were applied from a holding potential of -42 mV. The right panel shows the I-V relationships for the peak and steady state currents in the presence and absence of quinidine 20 µM.

Other studies of I_{to} in single cardiac cells are those of Nakayama and Irisawa (14) in rabbit AV node cells, Josephson et al(20) in rat ventricular cells, and Giles and van Ginneken (21) in rabbit atrial cells. Nakayama and Irisawa (14) correlated I_{to} with a transiently activated single channel that was predominantly selective for K, but probably also was responsive to changes in the Na gradient. A similar transient outward current has been identified and studied in neuronal cells, where it is called i_A (22,23).

Removal of Ca from the superfusing solution improved the recording of I_{to} by avoiding the overlap with Ca current. This was particularly

important for examination of the activation phase of I_{to}, but also it was important to remove any effect of long-lasting or tonic Ca currents. I_{to} itself was clearly not dependent on Ca for its activation, since extracellular Ca was kept low with EGTA and intracellular Ca was buffered with BAPTA. Removal of Na from the superfusing solution was without significant effect on I_{to}.

Quinidine block of I_{to}

These studies demonstrate a dramatic block of I_{to} by quinidine in micromolar doses, similar to those that affect Na currents. Furthermore, the effect appears to be open channel block, demonstrated by the shortening of time-to-peak current, as well as peak magnitude, without significant effect on the time course of inactivation. This sort of interaction has been proposed for quinidine interaction with Na channels (24), so it may indicate that the site of action of quinidine may be within the channel itself and may not be a specific binding site. Certainly the speed of action of quinidine on I_{to} is sufficiently fast that it could influence normal action potentials.

The half effective dose of quinidine for block of I_{to} was 23 μM. This is the range that produces reduction in the Na currents (1), delayed rectifier K current (25), and Ca currents (Salata & Wasserstrom, unpublished observations).

Quinidine had an effect on steady state outward current in the depolarized voltage range that was equivalent to its effect on the transient component of outward current. Normally it is assumed that all of I_{to} is inactivated, as implied in its name. However, the channel responsible for this current could have a tonic opening probability, generating a steady current. We compared the block of steady state current by quinidine to that of 4AP. Kenyon & Gibbons (15) had already shown that 4AP blocked some of the steady state outward current. We found no clear difference in blockade by the two drugs, but this is not sufficient evidence for a steady state I_{to}.

I_{to} block and the action of quinidine on the action potential

I_{to} is an outward current that favors repolarization after the action potential peak. Its block by 4AP results in a positive shift in the plateau of the AP with little change in its duration (16). Consequently, one would expect that a similar block by quinidine would not lengthen the AP. If, however, there is a tonic component to I_{to}, then it would shorten the AP.

With the evidence in hand, it appears safe to conclude that the shape change of the AP produced by quinidine is not caused by blockade of I_{to}. A more plausible explanation is blockade of the delayed rectifier K current (25). We were not able to see this current under our experimental conditions. The reason for its absence its not clear, but perhaps it is related to the absence of Ca intracellularly or to the washout of some important intracellular promoter of that current.

SUMMARY

I_{to} was studied in single canine cardiac Purkinje cells by the patch clamp method in the whole cell configuration. I_{to} could be easily seen during depolarizing steps from a holding potential of -42 mV. The activation phase of I_{to} could be determined best when studies were made without extracellular or intracellular Ca, confirming that the current does not require Ca for its activation. Inactivation was multiexponential.

Quinidine blocked the peak value of I_{to} with a half-maximal effect at 23 μM. Time-to-peak of I_{to} was earlier in the presence of quinidine, as expected if the drug blocks when the channel is open. There was no effect on the two time constants of inactivation. Quinidine blocked the steady state outward current. 4AP also blocks steady state outward current, so there may be a tonic component to I_{to}.

Since quinidine blocks several channels at about the same concentration, and the block occurs in the open channel state, there may be an internal reactive site that is structurally similar in several ionic channels. This property of affecting several channels makes interpretation of quinidine-induced AP changes difficult.

ACKNOWLEDGEMENTS

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REFERENCES

1. Lee,K.S., Hume,J.R., Giles,W., and Brown,A.M. Nature 291: 235-327, 1981.
2. Weidmann,S. J. Physiol. (Lond.) 129: 568-582, 1955.
3. Vaughn Williams,E.M. Brit. J. Pharmacol. 13: 276-278, 1958.
4. Carmeliet,E., and Saikawa,T. Circ. Res. 50: 257-272, 1982.
5. Nawrath,H.J. Pharmacol. Exp. Ther. 216: 176-182, 1981.
6. Roden,D.M., and Hoffman,B.F. Circ. Res. 56: 857-867, 1985.
7. Wood,E.H., Heppner,R.L., and Weidmann,S. Circ. Res. 24: 409-445, 1969.
8. Gibbons,W.R., and Fozzard,H.A. Circ. Res. 28: 446-460, 1971.
9. Fozzard,H.A., and Hiraoka,M. J. Physiol. (Lond.) 234: 569-586, 1973.
10. Sheets,M.F., January,C.T., and Fozzard,H.A. Circ. Res. 53: 544-548, 1983.
11. Eisenberg,B.R., and Cohen,I.S. Proc. Roy. Soc. Lond. B 217: 191-213, 1983.
12. Hamill,O.P., Marty,A., Neher,E., Sakmann,B., and Sigworth,F.J. Pfluegers Arch. 391: 85-100, 1981.
13. Siegelbaum,S.A., and Tsien,R.W. J. Physiol. (Lond.) 299: 485-506, 1980.
14. Nakayama,T., and Irisawa,H. Circ. Res. 57: 65-73, 1985.
15. Kenyon,J.L., and Gibbons,W.R. J. Gen. Physiol. 73: 139-157, 1979.
16. Kenyon,J.L., and Gibbons,W.R. J. Gen. Physiol. 73: 117-138, 1979.
17. Dudel,J., Peper,K., Rudel,R., and Trautwein,W. Pfluegers Arch. 295: 197-212, 1967.
18. Baumgarten,C.M., and Isenberg,G. Pfluegers Arch. 368: 19-31, 1977.
19. Levis,R.A., Mathias,R.T., and Eisenberg,R.S. Biophys. J. 44: 225-248, 1983.
20. Josephson,I.R., Sanchez-Chapula,J., and Brown,A.M. Circ. Res. 54: 157-162, 1984.
21. Giles,W.R., and van Ginneken,A.C.G. J. Physiol. (Lond.) 368: 243-264, 1985.
22. Connor,J.A., and Stevens,C.F. J. Physiol. (Lond.) 213: 21-30, 1971.
23. Hagiwara,S. Membrane potential-dependent ion channels in cell membrane. Raven Press, New York, 1983.
24. Hondeghem,L.M., and Katzung,B.G. Biochim. Biophys. Acta 472: 373-398, 1977.
25. Colatsky,T.J. Circ. Res. 50: 17-27, 1982.

4

ANTIARRHYTHMIC DRUG THERAPY FOR PATIENTS WITH VENTRICULAR TACHYARRHYTHMIAS IN THE SETTING OF LEFT VENTRICULAR DYSFUNCTION:

1. THE ROLE OF PROPAFENONE
2. THE ROLE OF ADJUNCT BETA-BLOCKADE

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INTRODUCTION:

The isolated occurrence of either congestive heart failure or ventricular tachycardia (VT) carries a poor prognosis. Frequently, congestive heart failure and complex ventricular arrhythmia coexist in patients (1-5). The combined occurrence of congestive heart failure and complex ventricular arrhythmia has a worse prognosis than either alone, with an annual reported mortality of between 50 and 60 percent (2). Previous attempts at extending survival in this group of patients has primarily focused on treatment of heart failure (1,2). Despite control of heart failure, mortality remained high as patients frequently succumbed to sudden death. However, previous investigators have not pursued aggressive arrhythmia management in this group of patients.

Antiarrhythmic drug therapy can prevent recurrent arrhythmias (6). However, antiarrhythmic drugs have many side effects, including the possibility of worsening left ventricular function (7). Many antiarrhythmic drugs have been evaluated for their effect on left ventricular function. Although both quinidine and procainamide can reduce ventricular contractility, they rarely cause clinical congestive heart failure (8). Disopyramide, on the other hand, can reduce ventricular contractility and frequently may cause congestive heart failure especially in patients with pre-existing left ventricular dysfunction (7). This may relate to changes in systemic vascular resistance. Studies of the antiarrhythmic

drugs amiodarone and mexiletine have shown no appreciable effect on left ventricular function (9,10).

Propafenone is a promising new antiarrhythmic agent. In addition to a membrane stabilizing action it also is a weak beta blocking agent and to a lesser degree is a calcium channel antagonist (11,12). Propafenone has efficacy in suppressing both supraventricular and ventricular arrhythmias (13-16). Recent studies have yielded conflicting data as to its effect on left ventricular function (15-17). The efficacy and safety of propafenone in patients with malignant ventricular arrhythmias and congestive heart failure have not been established.

Beta-blocker therapy has been successfully employed in the treatment on some patients with ventricular arrhythmia (18-21). Beta-blockers are the only drugs which have been shown to reduce mortality after myocardial infarction, in part by reducing sudden cardiac deaths (18). The successful use of beta-blockers as solitary or combination therapy for life-threatening ventricular arrhythmias has been reported (19-21). The identification of patients who are appropriate candidates for beta-blocker therapy has not been resolved. While beta-blockers have the potential to worsen left ventricular function, they can be carefully titrated to avoid hemodynamic compromise, in fact a recent study demonstrated that beta-blockers may improve prognosis in patients with impaired left ventricular function (22).

We report our experience in two groups of patients with life-threatening ventricular tachyarrhythmias and impaired left ventricular function. One group was treated with propafenone and the second treated with a partially effective class I antiarrhythmic drug combined with beta-blockade.

METHODS

Definitions:

Standard definitions were utilized for ventricular tachycardia, whether spontaneous, or induced by programmed stimulation (23) Spontaneous sustained VT was defined as lasting longer than 30 seconds and requiring an intervention for termination. Spontaneous nonsustained VT was defined as greater

than or equal to 3 consecutive ventricular ectopic depolarizations. Inducible sustained ventricular tachycardia was defined as 50 or greater repetitive ventricular responses to extrastimulation or requiring intervention (pacing, cardioversion, or defibrillation) for reversion to a stable rhythm. Inducible nonsustained VT was defined as six or greater, but less than 50 repetitive ventricular responses to extrastimulation and terminating spontaneously.

Base line evaluation:

Upon referral, heart failure was managed in a traditional fashion, and when controlled, all drugs, including antiarrhythmic agents and digoxin, were discontinued for 48 hours. Patients then had base line studies including 24-hour ambulatory electrocardiographic monitoring, left ventricular ejection fraction measurement and invasive cardiac electrophysiologic study. The testing procedures have been previously described (20).

Antiarrhythmic Drug Program:

All patients were treated with antiarrhythmic drugs. Antiarrhythmic drug therapy was guided utilizing electrophysiologic testing, ambulatory electrocardiographic monitoring, exercise testing or a combination of these modalities.

Heart Failure Management:

All patients in New York Heart Association class four were initially given digoxin, diuretics and vasodilators. All patients in functional class three were given digoxin and diuretic therapy. The patients in functional class two were given a variety of therapies. Serum potassium and magnesium levels were maintained at greater than 4.0 milliequivalents/liter and 2.0 milligrams/deciliter respectively by, if necessary, vigorous electrolyte replacement or the use of potassium-sparing diuretics.

Follow-up Evaluation:

Repeat noninvasive evaluation was done at least every three to six months.

RESULTS:

Propafenone:

Fifteen patients with ventricular tachyarrhythmias and symptoms of congestive heart failure with ejection fractions of 40 percent or less were given a trial of propafenone. All fifteen patients had failed trials of conventional antiarrhythmic drugs (one to four drugs). The patient's clinical characteristics are summarized in table 1.

Propafenone was given orally as a one-time loading dose of from 300 to 450 milligrams. Subsequently, propafenone was given at an interim maintenance dosage of 300 milligrams orally every eight hours. One subject (patient number 5) had worsening of arrhythmia (recurrent cardiac arrest) within three hours after the loading dose and propafenone was discontinued. At least five oral doses of propafenone were given prior to arrhythmia evaluation in the remaining 14 patients. Blood levels were not obtained.

Antiarrhythmic Effects:

Seven patients underwent invasive arrhythmia assessment before and during propafenone therapy. Ventricular tachycardia was induced in all seven patients during base line cardiac electrophysiologic study. Ventricular tachycardia could not be induced in three of the seven patients during solitary propafenone therapy. One additional patient was noninducible while receiving concomitant beta-blockade. Three patients remained inducible of which one was then discontinued from the propafenone protocol.

After excluding both the patient who had worsening of his arrhythmia during loading and one of the patients with persistently inducible VT, the remaining 13 patients were placed on maintenance drug therapy. These 13 patients underwent ambulatory ECG monitoring both before and during propafenone therapy (Table 2). Base line ambulatory ECG monitoring revealed frequent ventricular premature depolarizations (VPDs), ventricular couplets and VT in the patients prior to propafenone therapy. During propafenone therapy there was a significant reduction in the frequency of ventricular arrhythmia.

Effects on Ventricular Function:

Table 1.
Basic Patient Characteristics:

Patient	Age(yr)	Sex	Disease	EF(%)	FC	Rhythm	Symptoms
1	55	M	CAD	20	4	VF	CA
2	44	M	CAD	26	2	VF	CA
3	62	F	CAD	31	3	VF	CA
4	65	M	CAD	34	2	VF	CA
5	44	M	CAD	19	3	VTs	CA
6	54	M	CAD	37	2	VTs	S
7	57	M	CAD	28	3	VTs	PS
8	61	M	CAD	34	2	VTs	PS
9	55	M	CAD	40	2	VTs	PS
10	60	M	IDCM	22	3	VTns	PALP
11	89	M	CAD	22	2	VTns	PALP
12	64	F	CAD	38	3	VTns	PALP
13	66	M	IDCM	39	2	VTns	PALP
14	78	M	CAD	35	3	VTns	PALP
15	66	M	CAD	34	2	VTns	PALP

Abbreviations: FC = New York Heart Association functional class; M = Male; F = Female; CAD = Coronary Artery Disease; IDCM = Idiopathic Dilated Cardiomyopathy; VF = Ventricular Fibrillation; VTs = Sustained Ventricular Tachycardia; VTns = Nonsustained Ventricular Tachycardia; CA = Cardiac Arrest; S = Syncope; PS = Presyncope; PALP = Palpitations.

Table 2.
Ambulatory Monitoring:

	VPDs/Hour		Couplets/24 hours		VT runs/24 hours	
Patient	Base line	PPF	Base line	PPF	Base line	PPF
1	18	17	7	0	2	0
2	40	1	0	0	8	0
3	100	21	2	3	0	0
4	4	1	1	0	1	0
6	1067	31	5200	18	28	1
7	1813	28	226	0	3188	1
9	3	0	0	0	0	0
10	1200	1	4800	0	240	0
11	28	10	12	1	3	0
12	900	30	376	1	126	0
13	360	58	694	23	228	0
14	34	1	9	0	3	0
15	2459	122	24991	337	592	30
p Value	<0.05		<0.05		<0.05	

Abbreviations: VPDs = Ventricular Premature Depolarizations; VT = Ventricular Tachycardia; PPF = Propafenone Therapy

Of the 13 patients on maintenance therapy, eight had their left ventricular ejection fractions determined by nuclear ventriculography both before and during propafenone therapy. Initial measurements were from 22 to 39 (mean 30) percent. Subsequent determinations during propafenone therapy were from 22 to 48 (mean 30) percent. In two patients, base line nuclear ventriculograms were performed, but repeat nuclear ventriculograms were not obtained because the drug was stopped due to side effects.

Side Effects:

In four of the 13 patients on maintenance therapy, significant side effects occurred requiring the discontinuation of propafenone. Three patients had increasing dyspnea necessitating discontinuation of propafenone therapy including two who had clinical worsening of left heart failure. The other patient had a history of severe pulmonary disease and had a marked increase in dyspnea suggesting pulmonary disease. In only one of these patients was a follow up ventricular function study available during propafenone therapy. In the fourth patient persistent dizziness during propafenone therapy required discontinuation.

Follow-up:

Seven patients continuing on propafenone therapy have been followed for between three to 46 (mean 23) months. One patient suffered a recurrence of sustained VT shortly after his discharge. He was subsequently well controlled with an increased dose of propafenone. Two patients were discontinued from therapy for nonmedical reasons after uneventful follow-up for 12 and 18 months. Adjustment of propafenone dosage and heart failure therapy have been made as needed. The daily propafenone dose has ranged from 450 to 1050 (mean 750) milligrams. Out of the eight patients who failed or discontinued propafenone therapy, six remain alive while two have died on other treatment.

Adjunct Beta Blockade:

From November 1982 through April, 1984, fifteen consecutive patients with coronary artery disease and left ventricular

dysfunction were referred for evaluation of recurrent ventricular fibrillation or recurrent sustained or symptomatic ventricular tachycardia. Nine of these patients had inducible sustained monomorphic VT during base line programmed stimulation. Using serial electrophysiologic testing, solitary class I drug therapy was shown to be completely effective in three patients, but not in six. A completely effective drug response was defined as limiting repetitive responses to less than six. The six patients with a less than completely effective solitary drug response are the subject of this report. A class I drug with partial efficacy was identified for each patient. This was defined by conversion from sustained to nonsustained ventricular tachycardia (one patient), at least a ten percent slowing in the rate of the inducible sustained VT (three patients), or inducible sustained VT that required greater provocation (more stimuli) than during the base line study (two patients). After at least two single class I antiarrhythmic drugs had been assessed in each patient, the best partially effective class I drug was maintained and metoprolol was added. Metoprolol, (in divided doses of 50 to 250 milligrams per day) was titrated to limit exercise peak heart rate to less than 120 beats per minute and to prevent symptoms of congestive heart failure. While on combination therapy, each patient underwent repeat electrophysiologic study proving noninducibility.

Prior to antiarrhythmic drug therapy, each patient had a measured left ventricular ejection fraction of between 18 and 40 (mean 27.8) percent. Following combination drug therapy, the ejection fraction measured from 21 to 37 (mean 27.2) percent (p = not significant). Five of the six patients remain alive during a follow-up ranging from 26 to 40 (mean 31) months. One patient died 11 months after initiation of combination therapy following discontinuation of his beta-blocker therapy.

DISCUSSION:

Ventricular fibrillation and sustained or hemodynamically compromising VT are life-threatening complications in patients with cardiac diseases. Ventricular tachycardia can usually be

provoked by electrophysiologic study in patients who have had the rhythm clinically (5). Frequently, despite antiarrhythmic drug therapy, VT remains inducible (24-27). Patients who have partial suppression of inducible ventricular arrhythmia, have more subsequent clinical events than those with complete suppression (28). In the present report a series of class I drugs were tested and the dose of most effective drug advanced to its therapeutic limit. Because of patient selection in this study, the best class I drug was only partially effective in all six patients reported. A beta-blocking drug was added in an attempt to find a drug combination that would produce complete efficacy. The addition of a beta-blocker to the best partially effective class I antiarrhythmic drug yielded complete efficacy in all six patients.

Previous investigators have had variable success in managing cardiac arrhythmias with combination antiarrhythmic drug therapy (21,26,27,29,30). Combination therapies using class I drugs usually resulted in induction of slower VT in most of the patients (26). Previous reports using combination therapy of class I drugs and beta-blockers have suggested some degree of efficacy for a variety of cardiac arrhythmias (21,27,29,30). One of the reasons why these previous reports evaluating combination antiarrhythmic drug therapy may have had variable results is the different patient populations and methods used in the various studies (21,26,27,29,30).

Solitary beta-blocker therapy for VT has been effective when used in the management of patients without significant underlying cardiac disease (20). Beta-blockers alone have often not been effective when used in patients with ventricular tachycardia and significant cardiac dysfunction (31). We did not evaluate solitary beta-blocker therapy in these patients, and cannot be certain the beneficial results were not due to metoprolol alone.

Various mechanisms have been postulated to explain the efficacy of beta-blockers in the management of cardiac arrhythmias. Beta-blockers have been shown to have direct membrane stabilizing effects on cardiac tissue (32). Because

the plasma levels of beta blocking drugs that are needed to produce an antiarrhythmic effect in humans are much lower than the drug concentration needed to produce direct effects on cell membranes, most of the antiarrhythmic effects of beta-blockers are thought due to beta receptor blockade (32). One possible mechanism is suggested by the fact that sympathetic stimulation enhances afterdepolarizations which in turn may trigger automatic type arrhythmias (33). Because beta-blockers have been shown to suppress afterdepolarizations, it is possible that they exert an antiarrhythmic effect through this mechanism. A second possible mechanism is suggested by the observation that beta-blockers are effective in treating reentry arrhythmias. Although sympathetic stimulation does not significantly affect conduction in ventricular muscle, it does shorten the effective refractory period (34). Dispersion of refractoriness may by caused by sympathetic stimulation to an area with an inhomogenous distribution of sympathetic nerves (34). If this should occur, a functional reentrant pathway might be established and reentry continued resulting in ventricular tachycardia (33). In this context, beta-blockers might work by decreasing inhomogeneous conduction. Support for this second mechanism is found in the present study. First, the ventricular arrhythmias were considered to be reentry type because they were inducible during programmed stimulation. Second, the addition of beta-blockers to a class I drug abolished the inducible ventricular tachyarrhythmias. Third, beta-blockers were used in low doses limiting the possibility of any membrane effects. The effective combination of class I drugs and beta-blockers suggests a possible synergism in membrane effects and beta-receptor blockade.

Our results also indicate that propafenone is an effective agent for the management of many patients with life-threatening ventricular tachyarrhythmias and congestive heart failure. Studies in the 13 patients placed on maintenance therapy revealed control of arrhythmias using propafenone. Although side effects necessitated discontinuation of propafenone in 4 patients, in only 2 of these was definite worsening of

congestive heart failure noted. The 7 patients who continued on propafenone have survived over a mean follow-up of 23 months. Therefore, propafenone was effective in 7 of 15 patients (47%) with life-threatening ventricular arrhythmias and congestive heart failure.

Recent studies have yielded conflicting data as to the effect of propafenone on left ventricular function, especially in patients with congestive heart failure (14-16). Despite measured changes in left-ventricular function, only two of the 92 patients in the previous studies required discontinuation of propafenone therapy due to worsening of heart failure. In the present study, seven of 15 patients with clinical congestive heart failure maintained propafenone therapy. There was no significant change in the mean left ventricular ejection fraction from base line to treatment with propafenone as measured by nuclear ventriculography in eight patients. Only two of ten patients had worsening of clinical congestive heart failure requiring discontinuation of propafenone.

Propafenone is an effective antiarrhythmic drug for the management of many patients with life-threatening ventricular arrhythmias. In patients with left ventricular dysfunction, the drug appears to be useful, but must be used with caution.

SUMMARY:

This report details our experience using two separate approaches in the treatment of patients with life threatening ventricular tachyarrhythmia in the setting of congestive heart failure. First, propafenone, a new antiarrhythmic drug was tested on 15 patients. Second, beta-blockers were tested in combination with class I agents in 6 patients.

We administered propafenone to 15 patients with ventricular tachyarrhythmias and left ventricular ejection fractions less than or equal to 40%. Propafenone significantly reduced ventricular arrhythmia evaluated by both ambulatory monitoring and programmed stimulation. In 8 patients studied before and during therapy, there was no significant change in left ventricular ejection fraction as determined by nuclear ventriculography. Propafenone was discontinued in 4 patients due

to side effects. Seven patients receiving continuing propafenone therapy remain alive with only 1 patient suffering a recurrence of his arrhythmia. Therefore, propafenone is an effective drug for the management of ventricular tachyarrhythmias and may be used for patients with impaired left ventricular function.

In the 2nd study, 6 patients who had failed solitary class I antiarrhythmic drug therapy were given beta-blockers in combination with the best partially effective class I antiarrhythmic drug to determine if this type of combination therapy could enhance efficacy in abolishing inducible ventricular tachyarrhythmias. All 6 patients had underlying coronary artery disease and reduced ejection fractions (range 18-40, mean 28%). Sustained VT was reproducibly demonstrated in all 6 patients during baseline invasive electrophyiologic study. Multiple class I antiarrhythmic drugs were then evaluated in each patient but were, at best, only partially effective. Beta-blockers were added to the best partially effective class I antiarrhythmic drug. Subsequent electrophysiologic study demonstrated abolition of inducible VT in each patient. Five of the 6 patients remain alive during a follow-up of 26-40 (mean 31) months. It is concluded that beta-blockade can be a useful adjunct in the management of patients with life-threatening ventricular tachyarrhythmias when used in combination with a class I antiarrhythmic drug that has shown partial efficacy.

REFERENCES:

1. Franciosa JA, Wilen M, Ziesche S, Cohn JN. Am J Cardiol 1983;51:831-836.
2. Wilson JR, Schwartz JS, St John Sutton M, Ferraro N, Horowitz LN, Reichek N, Josephson ME. J Am Coll Cardiol 1983;2:403-410.
3. Poll DS, Marchlinski FE, Buxton AE, Doherty JU, Waxman HL, Josephson ME. Circulation 1984;70:451-456.
4. Swerdlow CD, Winkle RA, Mason JW. N Engl J Med 1983;308:1436-1442.
5. Roy D, Waxman HL, Kienzle MG, Buxton AE, Marchlinski FE, Josephson ME. Am J Cardiol 1983;52:969-974.
6. Graboys TB, Lown B, Podrid PJ, DeSilva R. Am J Cardiol 1982;50:437-443.
7. Podrid P, Schoeneberger A, Lown B. N Engl J Med 1980;302:614.
8. Smitherman TC, Gottlich CM, Narahara KA, Osborn RC, Platt M, Rude RE, Lepscomb K. Chest 1979;76:552-556.
9. Trobaugh GB, Kudenchuk PJ, Greene Hl, Tutt RC, Kingston E, Gorham Jr, Gross BW, Graham EL, Sears GK, Werner JA. Am J Cardiol 1984;54:1263-1266.
10. Stein J, Podrid P, Lown B. Am J Cardiol 1984;54:575-578.
11. McLeod AA, Stiles GL, and Shand DG. J Pharmacol and Exp Therapeutics 1984;228:461-466.
12. Dukes ID, Vaughan Williams EM. European Heart Journal 1984;5:115-125.
13. Walleffe A, Mary-Rabine L, de Rijbel R, Soyeur D, Legrand V, Kulbertus HE. European Heart Journal 1981;2:345-352.
14. Salerno DM, Granrud G, Sharkey P, Asinger R, Hodges M. Am J Cardiol 1984;53:77-83.
15. Shen EN, Sung RJ, Morady F, Schwartz AB, Scheinman MM, Dicarlo L, Shapiro W. J Am Coll Cardiol 1984;3:1291-1297.
16. Podrid PJ, Lown B. J Am Coll Cardiol 1984;4:117-125.
17. Baker BJ, Dinh HA, Kroskey D, de Soyza NDB, Murphy ML, Franciosa JA. Am J Cardiol 1984;54:20D-22D.
18. The Norwegian Multicenter Study Group. N Engl J Med 1981;304:801-807.
19. Coltart DJ, Gibson DG, Shand DG. British Med J 1971;1:490-491.
20. Brodsky MA, Sato DA, Allen BJ, Chesnie BN, Henry WL. Chest 1986;89:790-794.
21. Hirsowitz G, Podrid P, Lampert S, Stein J, Lown B: J Am Coll Cardiol 1978;3:618.
22. Swedberg K, Waagstein F, Hjalmarson A, Wallentin: Lancet 1979; 1:1374-1375.
23. Wellens HJJ, Brugada P, Stevenson WG: Circulation 1985;72:1-7.
24. Swiryn S, Bauernfiend RA, Strasberg B, Palileo E, Iverson N, Levy PS, Rosen KM. Am Heart J 1982;104:43-50.
25. Horowitz LN, Josephson ME, Farshidi A, Spellman SR, Michelson EL, Greenspan AM. Circulation 1978;58:986-997.

26. Duffy CE, Swiryn S, Bauernfeind RA, Strasberg B, Pallileo E, Rosen KM. Am Heart J 1983;106:450-458.
27. Ross DL, Sze DY, Keefe DL, Swerdlow CD, Echt DS, Griffin JC,Winkle RA, Mason JW. Circulation 1982;66: 1205-1210.
28. DiMarco J, Garan H, Ruskin J: Circulation 1980; 62:261.
29. Dreifus LS, Lim HR, Watanabe Y, McKnight E, Frank Mn. JAMA 1968;204:736-737.
30. Fors WJ, Vanderark CR, Reynolds EW. Am J Cardiol 1971;27:190-194.
31. Mason JW, Winkle RA. Circulation 1978;58:971-985.
32. Wit AL, Hoffman BF, Rosen MR. (Parts A,B,C). Am Heart J 1975;90:521-523, 665-675, 795-803.
33. Arnsdorf MF. J Pharmacol and Exp Therapeutics 1977;201:689-700.
34. Han J, Garcia DP, Moe GK. Circulation Res 1964;14:516-524.

5

PROTECTIVE EFFECT OF NIFEDIPINE UPON OUABAIN-INDUCED ARRHYTHMIAS.

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INTRODUCTION

Calcium channel blockers represent a group of compounds which have the common property of inhibiting transmembrane calcium influx via the voltage gated channels (1). Recent reports suggest the existence of a kinetic interaction between calcium channel blockers and cardiac glycosides which leads to the elevation of plasma digoxin level (2-4). This phenomena is partly attributed to the reduced renal clearance of digitalis (5).
Nifedipine, a prototype of the dyhydropyrdine group of compounds, has been demonstrated to increase the plasma digoxin level by 25-45% in the healthy individuals (2). Sixty to eighty percent elevation of plasma digoxin level was seen when digoxin was combined with verapamil in the treatment of atrial fibrillation. However none of these regimen seems to change the electrophysiological correlates (6-8). In fact some of the observations suggest that the incidence of cardiac toxicity is lower in patients receiving combined therapy of verapamil and digoxin than in those receiving digitalis alone (9).

Generally the electrical toxicity of digitalis manifests as either an abnormality of the conduction or an abnormal impulse initiation. In toxic concentrations, cardiac glycosides can provoke oscillatory potentials that are coupled to and triggered by preceding action potentials (10). If they are large enough to reach the threshold, the oscillatory potentials might initiate a premature response or self-sustained rhythm (11). Such

phenomenon have been observed in purkinje fiber (12,13), specialized atrial fibers (14) and ventricular muscle (15,16). The oscillatory potentials are increased in magnitude by an increase in $[Ca^{2+}]o$ (13) and diminished by a reduction in $[Na^{+}]o$ (17,18) and can be abolished altogether by verapamil, tetrodotoxin (19) or Mn^{2+} (13). These studies would indicate that in some way both Na^{+} and Ca^{2+} inward currents are involved in generating the oscillatory potentials. Tsien et al (20) have, however, suggested that a rise in $[Na^{+}]i$, due to an inhibition of Na^{+}, K^{+}-ATPase, may cause an increase in the intracellular Ca^{2+} through Na^{+} - Ca^{2+} exchange and consequently an increase in the inward oscillatory current (18). It is conceivable that Ca^{2+} influx in the presence of toxic concentration of digitalis may raise the cytoplasmic Ca^{2+} to a level that causes Ca^{2+} overload in organelles such as mitochondria (21) and sarcoplasmic reticulum (22). A direct correlation between Ca^{2+} overload, oscillatory potentials and early ischemic cardiac arrhythmias has recently been shown (23). All the above studies suggest that calcium plays a vital role in digitalis induced cardiac arrhythmias. Since the calcium channel blockers inhibit the transmembrane calcium influx (1) and may thus prevent intracellular calcium overload it might reduce the incidence of cardiac arrhythmias caused by digitalis.

Our previous studies in both the isolated guinea pig heart and in the intact guinea pig model indicated that verapamil has a significant protective effect against ouabain induced mechanical and electrical toxicity (24,25). We carried out the present study to investigate the possible protective effect of another widely used calcium channel blocker, nifedipine, upon ouabain induce cardiac arrhythmias in the intact guinea pig model.

MATERIALS AND METHODS

Experimental Model

Guinea pigs of either sex, weighing between 300-350 gm, were anesthetized with ureathane 500 mg/kg and alpha-chloralose 60 mg/kg intraperitonealy. Trachea cannulated and the chest was cut open under artificial respiration. Lead II EKG, left ventricular pressure and the maximum rate of its rise (+dp/dt) were recorded using Stratham P23Gb transducers and Hewlett Packard recorder (No. 1308A). Drugs were infused at a slow rate via the external jugular vein.

Animals were divided into 4 groups and were examined to investigate the protective effect of nifedipine upon ouabain-induced arrhythmias.

Group I

When the preparation was stable, ouabain was infused at the rate of 1.4 ug/min in 23 ul of saline. Ventricular ectopics appeared in 18-20 min. When the arrhythmias appeared to be fatal, (consistent and repeated 5-6 ventricular ectopics, ventricular tachycardia or ventricular fibrillation) the infusion of ouabain was stopped. Mortality rate in this group was more than 95%.

Group II

In this group, animals were administered with nifedipine in slow infusion at a rate of 0.9 ug/min. Infusion was continued until the total dose of nifedipine reached 100 ug/kg. The animals were monitored for changes in EKG, left ventricular pressure, dp/dt and heart rate.

Group III

To this group of animals 35 ug/kg nifedipine was infused (rate - 0.9 ug/min) in combination with the toxic dose of ouabain (0.14 mg/kg). One-third of the above nifedipine dose (11.66 ug/kg) was infused before starting the ouabain administration and then both the drugs were infused simultaneously. Infusion was stopped after the

predetermined doses were administered and the animals were monitored for the development of arrhythmias.

Group IV

This group of animals was divided into several subgroups and each subgroup was given varying doses of nifedipine 15-50 ug/kg combined with ouabain which was continuously infused until the fatal cardiac arrhythmias appeared. The nifedipine infusion was started prior to the ouabain infusion as in Group III, and stopped after the predetermined doses were administered.

Drug Solutions

Ouabainoctahydrate was purchased from Sigma Pharmaceutical Co. The solution was prepared in saline. Nifedipine (10 mg capsules) was obtained from Miles Pharmaceuticals. The capsules were dissolved in 70% alcohol and further diluted in saline. Final solution of nifedipine had 0.1% ethanol. Nifedipine solution was always prepared in the dark and was stored in a brown bottle at 4^{o} C.

Statistical Analysis

The data are expressed as the mean $\pm$ S.E. The student's t-test or analysis of variance was used for statistical analysis taking $P < 0.05$ as the level of significance.

RESULTS

Genesis of ouabain-induced cardiac arrhythmias in guinea pig

In the control group of animals (Fig. 1), when ouabain was infused at a rate of 1.4 ug/min, the maximum inotropic response, as demonstrated by an increase in the dp/dt, occurred in about 8-10 min of continuous infusion. The dose of ouabain required for the initiation of the maximum increase in dp/dt was 0.044 $\pm$ 0.002 mg/kg.

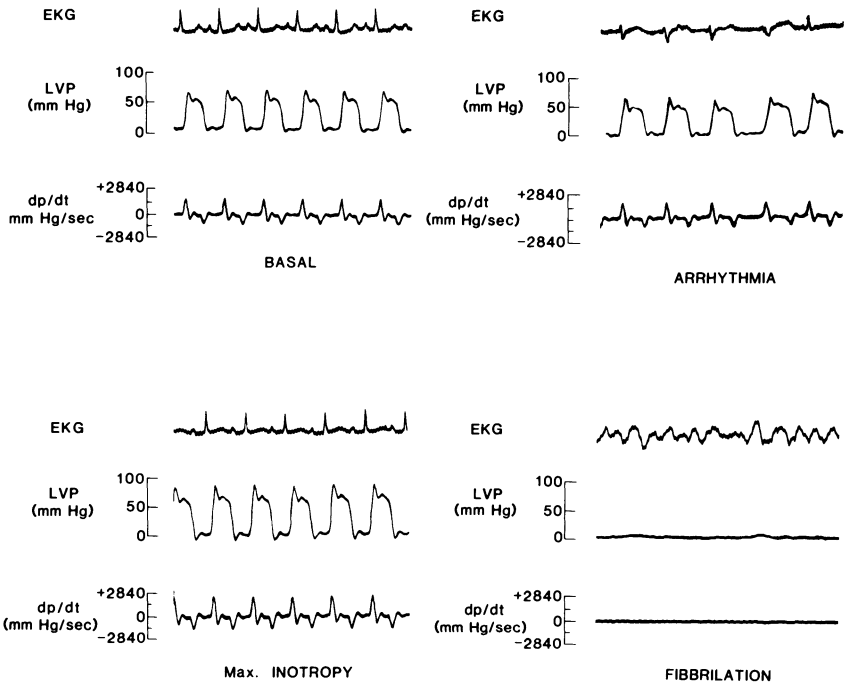

Fig. 1. Simultaneous recordings of EKG, left ventricular pressure (LVP) and the rate of its rise (+dp/dt) during ouabain infusion to the control animals (Group I). Typical recordings from 8-10 experiments.

Arrhythmias appeared with the ouabain dose of 0.1 ± 0.007 mg/kg in about 20-23 min. These cardiac arrhythmias were mainly of ventricular origin, e.g. ventricular extrasystoles, which progressed into ventricular fibrilla-

tion and the animals died with a ouabain dose of 0.14 ± 0.007 mg/kg in about 30 min.

Table 1. Alterations in the heart rate and PR interval during the infusion of either nifedipine and ouabain alone or with combined infusion.

EXPERIMENTAL CONDITION	HEART RATE (BEATS/MIN)	PR INTERVAL (SEC)
BASAL VALUE	259 ± 5	0.058 ± 0.001
NIFEDIPINE (30-35 UG/KG)	250 ± 17	0.058 ± 0.003
MAX. INOTROPY (OUABAIN)	262 ± 7	0.061 ± 0.002
MAX. INOTROPY (OUABAIN + 30 UG/KG NIF)	248 ± 8*	0.061 ± 0.001
IMMEDIATELY BEFORE ARRYTHMIAS (OUABAIN)	240 ± 10	0.09 ± 0.008*
IMMEDIATELY BEFORE ARRHYTHMIAS (OUABAIN + 30 UG/KG NIF)	222 ± 4*	0.081 ± 0.005*

Each value represents mean ± S.E. of 8-10 experiments.
*Indicates statistical significance ($P < 0.05$) as compared to the basal value.

Analysis of the heart rate and the PR interval demonstrated that these parameters did not change significantly during ouabain-induced maximum inotropy (Table 1). However, just before the initiation of arrhythmias, ouabain caused a significant reduction in the heart rate and prolonged PR interval.

Effects of nifedipine on ouabain-induced arrhythmias

Infusion of nifedipine alone (0.9 ug/min) caused a slowing of the heart rate at a dose of 30 ug/kg which continued to drop with further infusion of nifedipine. However, a significant drop in heart rate was observed only after the dose of nifedipine reached 80 ug/kg. Although, there was a slight increase in the dp/dt with the infusion of nifedipine up to 80 ug/kg and reduction between 80-100 ug/kg, these changes were not significantly different.

When nifedipine (35 ug/kg) was added to the infusion of a toxic dose of ouabain (0.14 mg/kg), a dose which will normally produce terminal arrhythmias in 25-30 min (Fig. 1), no arrhythmias were observed even after several hours of monitoring. Figure 2 illustrates the recordings up to two hours after the combined infusion of ouabain with nifedipine.

In another setting, a dose-response effect of nifedipine on the dose of ouabain required to induce arrhythmias was investigated. In this setting, when different doses of nifedipine were combined with a continuous infusion of ouabain, nifedipine caused a dose-dependent increase in both the arrhythmogenic as well as the lethal dose of ouabain. In Fig. 3, 35 ug/kg of nifedipine was combined (0.9 ug/min) with a continuous infusion (1.4 ug/min) of ouabain and the animals wre monitored until the terminal arrhythmias appeared.

As shown in Fig. 4, nifedipine in the doses between 30-37.5 ug/kg caused a significant elevation in the doses of ouabain necessary to induce both arrhythmias and death. Further increase in the dose of nifedipine did not elevate the arrhythmogenic dose of ouabain, although it further delayed the terminal arrhythmias to cause death of the animals.

In both the experimental groups where nifedipine was combined with ouabain (Groups III and IV) neither the

inotropic response (Fig. 5) nor the dose of ouabain (Fig. 4) for the inotropic effect was altered except with the higher doses of nifedipine. A dose of 50 ug/kg nifedipine reduced this ouabain-induced inotropic response to 33%. Ouabain alone increased the dp/dt by 53% in the control group (Fig. 5).

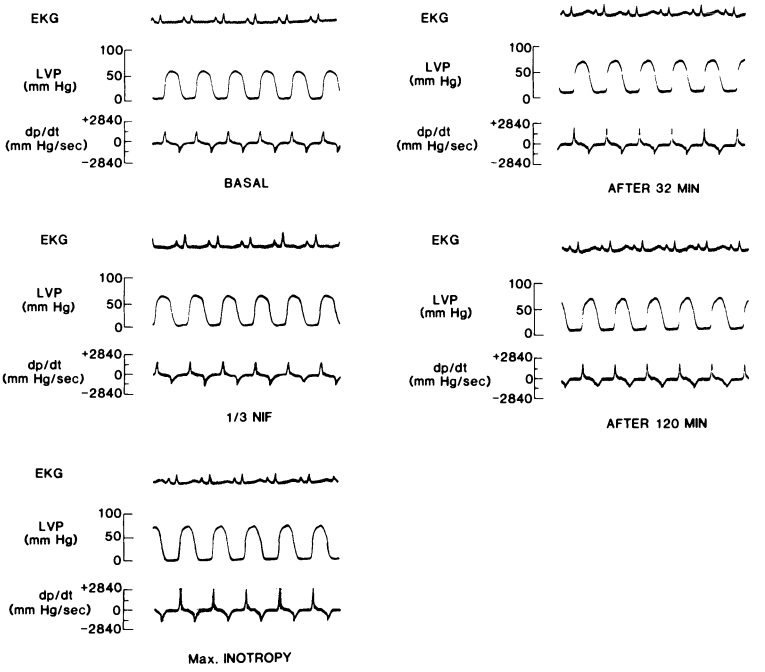

Fig. 2. Effects of combined infusion of ouabain (0.14 ug/kg) and nifedipine (35 ug/kg) on EKG, LVP and dp/dt. Typical records of 8-10 experiments.

Ouabain produced a significant drop in the heart rate immediately before the onset of arrhythmias (Table 1). With the addition of nifedipine to the system, as in Groups III and IV, drop in heart rate was seen earlier, at the stage of maximum inotropy. In these groups, PR intervals were prolonged with the infusion of ouabain and it was 0.08 $\pm$ .005 immediately before arrhythmias.

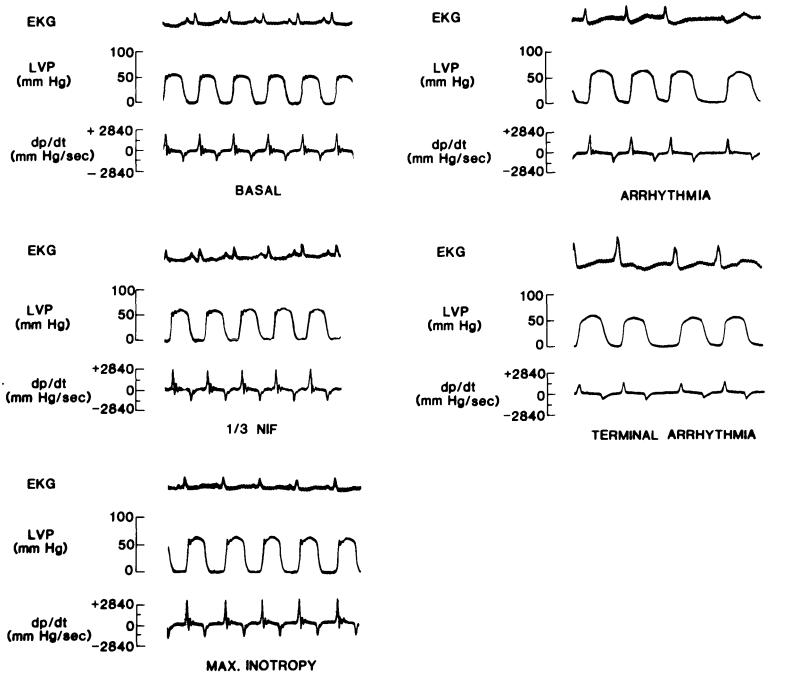

Fig. 3. EKG, LVP and dp/dt during continuous infusion of ouabain combined with 35 ug/kg nifedipine. Typical recordings of 8-10 experiments.

At this stage the PR interval in the ouabain control group was 0.09 ± 0.005. However both of these results are not significantly different from each other.

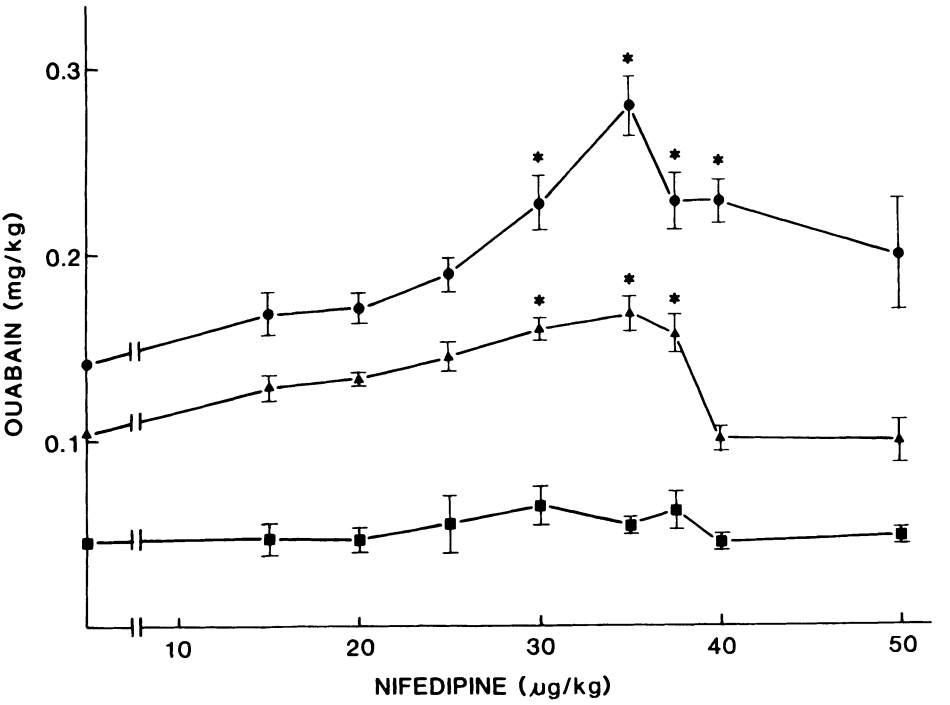

Fig. 4. Influence of nifedipine on the doses of ouabain required to produce maximum inotrophy (■---■), arrhythmias (▲---▲), and death (●---●). Each value represents mean ± S.E. of 8-10 experiments.

DISCUSSION

The above data demonstrate that nifedipine exerts a protective effect against ouabain-induced cardiac arrhythmias, without significantly influencing the ouabain-induced inotropic response. These effects of nifedipine were demonstrable with 30-35 ug/kg of nifedipine, doses well within the therapeutic range in humans. Nifedipine

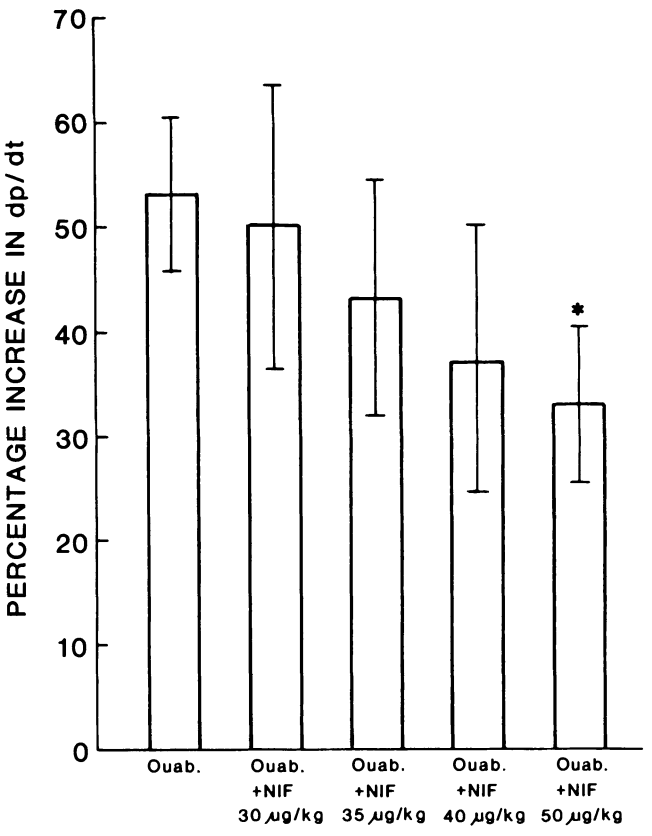

Fig. 5. Dose related effects of nifedipine upon ouabain induced increase in dp/dt *Indicates statistcial significance ($P<0.05$) when compared with dp/dt in presence of ouabain along.

in doses of 40 ug/kg or higher were less protective in the ouabain initiation of arrhythmias, although these doses were still effective in improving the survival rate of the animals.

The well accepted mechanism of digitalis is the inhibition of Na^+, K^+-ATPase, recently reviewed by Schwartz (26). The pump inhibition causes an increase in the $[Na^+]i$ and consequently $[Ca^{2+}]i$ through Na^+-Ca^{2+} exchange (27-29). An increased Na^+-Ca^{2+} exchange in the cultured myocardial cells was recently shown to be related to the arrhythmogenic action of digitalis (30). That Ca^{2+} plays a significant role in the genesis of digitalis-induced arrhythmias, is also supported by many electrophysiological studies. Ferrier and Moe (13) suggested that the transient inward current is a reflection of Ca^{2+} influx, which contributes significantly to the genesis of ouabain-induced arrhythmias. Kass et al hypothesized that, oscillatory release of Ca^{2+} from an intracellular source may alter the conduction of sarcolemmal membrane to other ions such as Na^+, which might cause rhythmic disturbance (31). It has now been shown that there is a direct correlation between $[Ca^{2+}]i$ overload, oscillatory potentials and early ischemic cardiac arrhythmias (23). Similarly in canine cardiac purkinje fibers, Weir et al (22) have demonstrated that the diastolic oscillations of membrane potential, induced by cardiotonic steroids, are always associated with the aequorin Ca^{2+} signals and suggested that Ca^{2+} is exclusively involved in the ouabain-induced electrical toxicity. Accordingly, calcium antagonists should prove to be able to reduce or abolish digitalis-induced rhythmic disturbances.

Calcium channel blockers inhibit calcium influx through the voltage gated channels (1), an important source of Ca^{2+} in the beat to beat regulation of the heart (32,33). Presence of nifedipine during digitalis administration may prevent the elevation of $[Ca^{2+}]i$ to a calcium

overload like state and help maintain the electrical and the mechanical activity of the heart for a longer period of time. We, infact, have earlier demonstrated that both verapamil and nifedipine not only reduce ouabain-induced mechanical toxicity in the guinea pig heart (24), but also preserve the functional and structural integrity of the mitochondria in the toxic hearts (34).

In the present study, nifedipine substantially increased the dose of ouabain to produce cardiac toxicity without any significant change in the dose of ouabain necessary to produce inotropic response or the magnitude of the inotropic response, except in higher doses. This suggests that the $[Ca^{2+}]i$ necessary for the inotropic response of ouabain was not significantly reduced, when its administration was combined with nifedipine.

The protective effect of nifedipine upon ouabain-induced cardiac arrhythmias has also been observed in cats (35). In our experimental model nifedipine reduced the heart rate and it was facilitated by the administration of ouabain. Studies in patients and in dogs with rapid infusion of nifedipine resulted in an increase in the heart rate due to the reflex elevation of catecholamines (36,37). The reduction in the heart rate in our model may be due to the species difference. Slowing of the heart rate was also observed by Olea and Quevedo in cats (35).

Unlike verapamil and diltiazem, nifedipine does not delay the atrioventricular conduction (38). The same observation was made in our experimental model even with the combination of nifedipine and ouabain. Infact nifedipine seems to improve the AV conduction although it is not statistically significant. Facilitation of AV conduction by nifedipine has been demonstrated in the isolated AV node preparation of cats (39).

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REFERENCES

1. Fleckenstein, A. Ann. Rev. Pharmacol. Toxicol. 17: 149-166, 1977.
2. Belz, G.G., Doering, W., Munkes, R., Mathews, J. Clin. Pharmacol. Therap. 33: 410-417, 1983.
3. Klein, H.O., Lang, R., Weiss, E., Segni, E.D., Libhaber, C., Gueirero, J., Kaplinsky, E. Circ. 65: 998-103, 1982.
4. Pedersen, K.E. Acta. Medica. Scand. Suppl. 681: 31-36, 1982.
5. Pedersen, K.E., Pedersen, A.D., Hivide, S., Klitgaard, N.A., Kudsk, F.N. Clin. Pharmacol. Therap. 30: 311-316, 1981.
6. Heng, M.K., Singh, B.N., Roche, M.B., Norris, R.N., Mercer, C.J. Am. Heart J. 90: 487-498, 1975.
7. Pandis, P.I., Morganvoth, J. and Baessler, C. Am. J. Cardiol. 52: 1197-1201, 1983.
8 Schwartz, J.B. Am. J. Cardiol. 55: 31E-34E, 1985.
9. Babich, M., Hamann, S.R., McAllister, R.G., Pratapreddy, C. Pharmacology 29: 224-232, 1984.
10. Ferrier, G.R. Prog. Cardiovas. Dis. 19: 459-474, 1977.
11. Cranefield, P.F. Circ. Res. 41: 415-423, 1977.
12. Rosen, M.R., Gelband, H. and Hoffman, B.F. Circ. 47: 65-71, 1973.
13. Ferrier, G.R. and Moe, G.K. Circ. Res. 33: 508-515, 1973.
14. Hashimoto, K. and Moe, G.K. Circ. Res. 32: 618-624, 1973.
15. Ferrier, G.R., Saunders, J.H. and Mendez, C. Circ. Res. 32: 600-609, 1973.
16. Ferrier, G.R. J. Pharmacol. Exp. Ther. 215: 332--341, 1980.
17. Lin, C.I. and Vassalle, M. Am. J. Physiol. 234: H477-H486, 1978.
18. Tsien, T.W. and Carpenter, D.O. Fed. Proc. 37: 2127-2131, 1978.
19. Rosen, M.R. and Danilo, P. Circ. Res. 46: 117-124, 1986.
20. Tsien, R.W., Kass, R.S. and Weingart, R. Ann. N.Y. Acad. Sci. 307: 483-490, 1978.
21. Tower, D.B. Exp. Brain Research 6: 273-283, 1968.
22. Weir, W.G. and Hess, P.J. Gen. Physiol. 83: 395-415, 1984.
23. Clusin, W.T., Buckbinder, M. and Harrison, D.C. Lancet I: 272-274, 1983.
24. Khatter, J.C., Agbanyo, M., Hoeschen, R.J.,

Navaratnam, S. and Bains, R. J. Pharmacol. Exp. Ther. (In press), 1986.
25. Khatter, J.C., Agbanyo, M., Nero, B., Hoeschen, R.J., and Navaratnam, S. J. Mol. Cell Cardiol. (In Press) 1986.
26. Schwartz, A., Whitmer, K., Grupp, G., Grupp, I., Adams, R.T. and Lee, S.W. Ann. N.Y. Acad. Sci. 402: 253- 271, 1982.
27. Reuter, H. Circ. Res. 34: 599-605, 1974.
28. Mullins, L.J. Am. J. Physiol. 236: C103-C110, 1979.
29. Lee, C.O., Abek, P., Pecker, M., Sonn, J.K., Vassalle, M. J. Mol. Cell Cardiol. 17: 1043-1053, 1985.
30. Goshima, K. and Wakabayashi, S. J. Mol. Cell Cardiol. 13: 489-509, 1981.
31. Kass, R.S., Lederer, W.J., Tsien, R.W., and Weingart, R. J. Physiol. 281: 187-208, 1978.
32. Reuter, H. and Scholz, H. J. Physiol. (London) 264: 17-47, 1977.
33. Reuter, H. Ann. Rev. Physiol. 41: 413-424, 1979.
34. Agbanyo, M., Khatter, J.C. and Hoeschen, R.J. Biochem. Pharmacol. (submitted for publication) 1986.
35. Olea, J.P. and Quevedo, M. Arzneimittel Forschung 34(11): 1271-1274, 1984.
36. Kawai, C., Konishi, T., Matsuyama, E., Okazaki, H. Circulation 63: 1035-1042, 1981.
37. Millard, R.W., Grupp, G., Grupp, I.C., Disalvo, J., DePover, A., Schwartz, A. Circ. Res. 52 (suppl I): 29-39, 1983.
38. Rowland, E., Evans, T., Krikler, D. Br. Heart J. 42: 124-127, 1979.
39. Taira, N. and Narimatsu, A., Naunyn-Schmiedeberg's Arch. Pharmacol. 290: 107-112, 1975.

6

EFFECT OF ISOPRENALINE OR STRESS ON ADRENALINE-INDUCED ARRHYTHMIAS IN ALBINO RATS

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INTRODUCTION

Rona et al. (1) described for the first time that isoprenaline (ISP) produces infarct-like myocardial lesions in experimental animals. Since then the ISP model has been widely used to understand the pathophysiology of myocardial ischemia. During the subsequent years it has been reported that rat-myocardium develops relative insensitivity to the necrosis-inducing effects of ISP after a prior exposure to myocardial insults (2,3,4,5). Selye et al. (2) showed that ligation of the left coronary artery provided resistance against ISP-induced myocardial necrosis in rat. This resistance was reported to be maximal after 48 hr after coronary ligation and then gradually declined, the myocardium regaining sensitivity 3 wk after coronary ligation (3). Balazs et al. (4) found that myocardial resistance to ISP challenge occurred in rats pretreated with ISP for 2 consecutive days, and that the sensitivity had returned 11 wk after the initial treatment. The development of resistance was found to be dependent on the initial production of myocardial lesions, and not on the size of the necrosis (5). In a subsequent study, it was reported that it took at least 5 days for the myocardial resistance to develop after a single dose of ISP, and the normal myocardial sensitivity after multiple doses of ISP did not return even after 19-20 wk of the initial insult (6). In another study it has been reported that rats which were pretreated with ISP have more hypoxic tolerance 14 days after pretreatment with ISP than after 48 hr (7).

During the recent years there has been a large number of retrospective and prospective studies to determine the effects

of physical exercise on the development of coronary heart disease. The literature on this subject has been recently reviewed by Leon (8). The general consensus of opinion was that the relative risk of total coronary heart disease for men engaged in sedentary work was 2 to 2.5 times that of men engaged in physical work.

It, therefore, appears that exposure of heart to certain forms of non-fatal challenge, like ISP, myocardial ischemia or physical exercise, induces resistance to the heart to withstand further insults. One of the dreaded complications of coronary heart disease is cardiac arrhythmias, and exaggerated adrenergic activity plays a dominant role in the initiation and perpetuation of such an event. The present studies were undertaken to ascertain the effect of stress on the propensity of the rat heart to adrenaline (AD)-induced arrhythmias. Two forms of stress were selected - ISP challenge, and physical exercise. Based on the reports of Joseph et al. (6), the doses and modes of administration of ISP were so selected as to certainly produce myocardial necrosis. Swimming was used as a form of physical exercise, and the rats were subjected to swim-exercise-stress for a similar period of 3 wk. Our earlier studies showed that cholinergic system has an important role in the modulation of cardiac arrhythmias (9,10,11,12). The role of cholinergic system in the modulation of stress-induced modifications of AD-arrhythmias was, therefore, also studied.

MATERIALS AND METHODS

Experiments were conducted on adult Wistar strain albino rats of either sex weighing between 125 - 150 g. All the experimental animals were kept in identical environmental conditions including diet and access to water for 4 wk before using them, and throughout the experimental period. The rats were subjected to any of the following 2 types of stress for 21 days.

Isoprenaline-stress: Isoprenaline HCl (ISP) was administered sc once daily for 21 days in the dose of either 50 (ISP-50) or 100 (ISP-100) μg/kg.

Exercise-stress: The rats were subjected to swimming in a water tank (0.75 m diameter, 0.5 m deep) having a water temperature of 30 C for 30 min/day for 21 days.

In both the groups concurrent controls were kept. In the ISP group, the control rats received equivalent volume of distilled water sc every day for 21 days.

Adrenaline-induced arrhythmias: The rats were anesthetised with pentobarbitone sod. 35 mg/kg ip on the 22nd day, and Lead II EKG was monitored. Adrenaline bitartrate (AD) iv in graded doses was used as an arrhythmogen. The appearance of a minimum of 10% ventricular extrasystole was considered as a positive response. The minimum arrhythmogenic dose was determined in each rat. In a separate group of ISP-100 rats, atropine sulphate 1 mg/kg ip was administered on the 22nd day, 30 min before the administration of AD. The arrhythmogenic dose of AD was determined in each of the following experimental groups:

1a. ISP-50, b. ISP-100, c. ISP-100 atropine-pretreated.

2. Exercise-stress.

3. Concurrent Controls for each experimental group.

Ventricular Acetylcholine (ACh): In the following groups of rats, after ISP-100 treatment for 21 days, ACh from both the ventricles was extracted (13) on the 22nd day and biologically assayed (14):

1. ISP-100. 2. Concurrent control.

RESULTS

Effect of Isoprenaline pretreatment on Adrenaline-arrhythmias.

None of the rats treated with 50 or 100 µg/kg of ISP sc daily for 21 days died during the 22 days of observation period. The effects of ISP pretreatment on AD-induced arrhythmias have been summarised in Table 1. In the Control rats, significant arrhythmias were not observed up to 3 µg/kg of AD, while the mean arrhythmogenic dose of AD was found to be about 8 µg/kg. In contrast to this, ISP pretreatment markedly increased the arrhythmogenic dose of AD in a dose-

dependent manner. In the ISP-50 group, the maximal ED0 of AD was about 8 μg/kg, and the minimal ED100 was 21.00 ± 1.9 μg/kg. In the ISP-100 group, the maximal ED0 of AD was 12.5 ug/kg, and ED100 was 54.06 ± 7.47 μg/kg.

Table 1

Effect of Isoprenaline (ISP) pretreatment daily for 21 days on Adrenaline-induced arrhythmias on the 22nd day in albino rats.

Group	n	Arrhythmogenic dose of Adrenaline in ug/kg. (Mean ± SEM)	P value
Control	6	8.20 ± 0.49	
ISP (50 μg/kg) pretreated	6	21.00 ± 1.90	<0.001
Control	16	8.06 ± 0.40	
ISP (100 μg/kg) pretreated	16	54.06 ± 7.47	<0.001

Effect of Atropine pretreatment on Adrenaline arrhythmias.

In the second set of experiments, the role of cholinergic system in AD-arrhythmias was studied. Atropine sulphate 1 mg/kg sc was used to block the muscarinic receptors. The effects of atropine pretreatment on AD-arrhythmias have been summarised in Table 2.

Atropine pretreatment significantly reduced the arrhythmogenic dose of AD from the Control value of 8.51 ± 0.52 μg/kg to 4.71 ± 0.52 μg/kg. In the ISP-100 pretreated rats, however, atropine treatment markedly reduced the arrhythmogenic dose of AD from 49.20 ± 9.12 to 8.57 ± 1.34 μg/kg.

Dose-response relationship of AD in different groups of rats has been shown in Fig 1. It was found that ISP pretreatment markedly shifted to the right the dose-response curve of AD. Atropine pretreatment shifted the dose-response curves of both control as well as ISP-100 rats to the left, the shift was, however, marked in the ISP-100 rats. But the arrhythmogenic dose of AD in Atropine-ISP-100 group was higher than in the Control Atropine group.

Table 2

Effect of Atropine (ATR; 1mg/kg ip single dose, 30 min) pretreatment on Adrenaline (AD)-induced arrhythmias in different groups of albino rats.

Group	Arrhythmogenic dose of Adrenaline in ug/kg (Mean ± SEM)			P value
	n Control	n	ATR pretreated	
Control	8 8.51±0.52	7	4.71 ± 0.52	<0.001
ISP-100[a]	8 49.20±9.12	7	8.57 ± 1.34	<0.001 <0.025[b]

a: Isoprenaline (100 µg/kg sc daily for 21 days) pretreated.
b: P value in relation to Control Atropine pretreated group.

Effect of Exercise-stress on the Adrenaline-arrhythmias

The effects of 21 days swimming exercise on the arrhythmogenic effect of AD has been summarised in Table 3. The arrhythmogenic dose of AD in the exercise rats was significantly higher on the 22nd day as compared to that in the Controls, the values being 8.00 ± 0.58 and 19.75 ± 3.77 µg/kg in the Control and exercised rats respectively.

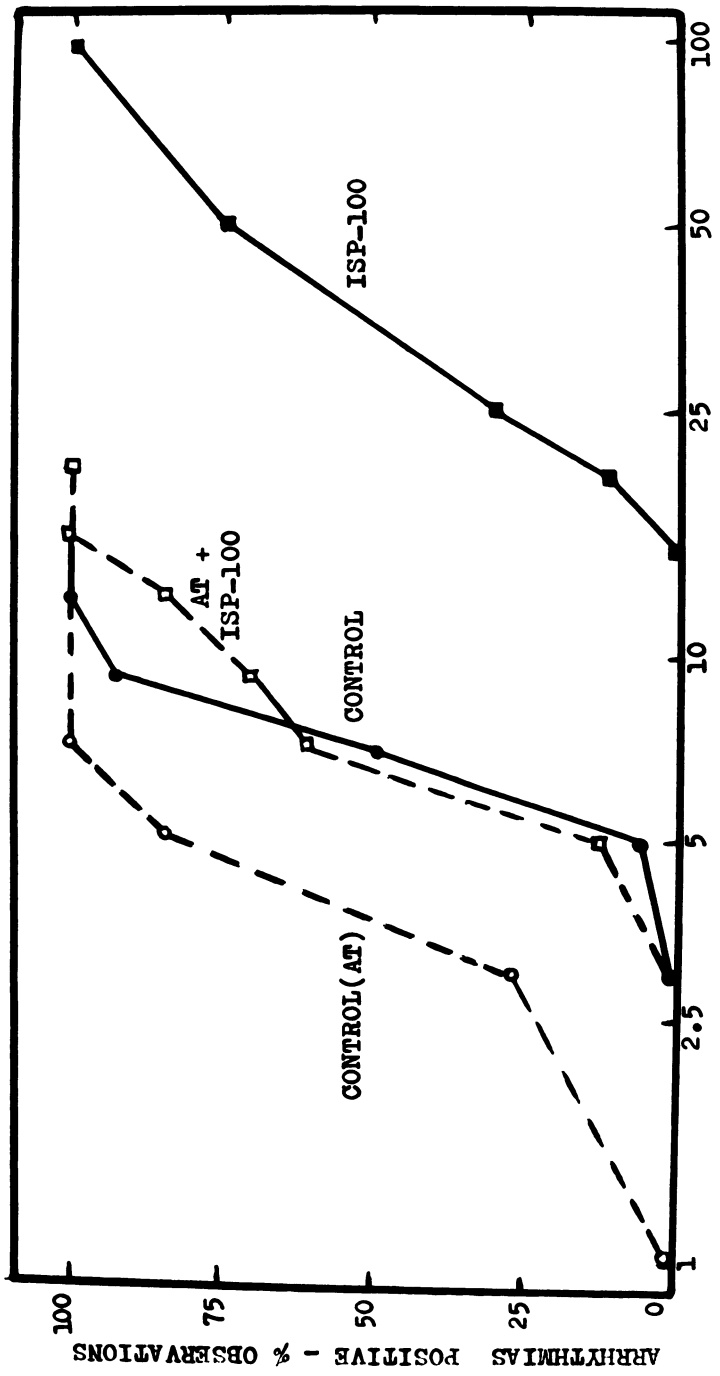

Fig. 1. Effect of pretreatment with Atropine (1 mg/kg ip, AT) and/or Isoprenaline (100 ug/kg sc, for 21 days, ISP-100) on the dose-response (arrhythmias) curves of Adrenaline.

Effect of Isoprenaline pretreatment on Ventricular Acetylcholine.

In the earlier sets of experiments it was found that ISP pretreatment for 21 days markedly decreased the arrhythmogenic effect of AD. This ISP-effect was also found to be markedly inhibited by atropine pretreatment. It was, therefore, considered worthwhile to study the effect of ISP treatment on the myocardial acetylcholine (ACh) content. The data have been given in Table 4.

Table 3

Effect of Swimming (30 min/day for 21 days) on Adrenaline-induced arrhythmias in albino rats.

Group	n	Arrhythmogenic dose of Adrenaline in ug/kg (Mean ± SEM)	P value
Control	6	8.00 ± 0.58	
Swimming	6	19.75 ± 3.77	<0.025

Table 4

Effect of Isoprenaline (100 µg/kg sc daily for 21 day, ISP-100) pretreatment on ventricular Acetylcholine (ACh) content in albino rats.

Group	n	Ventricular ACh content in µg/g (Mean ± SEM)	P value
Control	15	0.508 ± 0.012	
ISP-100	15	0.636 ± 0.013	<0.001

The ventricular ACh content was found to be significantly

higher in the rats pretreated with ISP 100 μg/kg daily for 21 days as compared to that seen in the Control rats, the values being 0.508 ± 0.012 and 0.636 ± 0.013 μg/g in the Control and ISP-100 rats respectively.

DISCUSSION

It has been reported by various workers that rat myocardium loses its sensitivity to the necrosis-inducing effects of ISP after an initial insult of either ISP, coronary artery ligation or strenuous muscular exercise (2,4,6). In humans, there is a general consensus of opinion that indulgence in a certain amount of physical exercise reduces the chances of developing coronary heart disease (8). Cardiac arrhythmias are associated with a number of cardiac diseases, and sometimes these arrhythmias are the cause of a terminal event, specially so after coronary heart disease. Exaggerated adrenergic discharge is known to be an important cause of initiation as well as perpetuation of cardiac arrhythmias associated with several cardiac conditions, viz. coronary heart disease, thyrotoxicosis, acute stress, during anesthesia, etc. The present studies were conducted to investigate whether prior exposure of heart to two different forms of stress, viz. ISP challenge, and physical exercise, for a period of 3 wk can lead to the development of myocardial resistance to catecholamine-induced arrhythmias. It was found that 3 wk exposure to ISP markedly decreased the arrhythmogenic effect of AD in a dose-dependent manner. In ISP-100 rats the increase in the ED100 of AD was by 6 times. Similarly, in the 3 wk exercised rats the arrhythmogenic activity of AD was markedly inhibited. In amount of AD needed to produce arrhythmias in exercised rats was 2.5 times that needed in Control sedentary rats. These results, therefore, show that exposure of rats to ISP challenge or to physical exercise protects the heart against AD-arrhythmias.

The mechanism of ISP-induced myocardial resistance has been studied by a number of workers. It has been shown that prolonged in vivo infusion of catecholamines results in beta-

receptor down regulation, and desensitization of cardiac muscle to exogenous catecholamines (15,16). An internalization of beta-receptors into sequestered cytosolic vesicles has also been suggested (17). In studies on human neutrophils, catecholamine-induced desensitization has been found to be associated with a reduction in the number of beta-receptors and a 'relative uncoupling' of remaining receptors (18). Hayes et al. (19) studying the cardiovascular responses to ISP, dopamine and tyramine after prolonged infusion of ISP found that there was a down regulation of beta-receptors, unmasking of alpha-mediated cardiovascular responses and depletion of myocardial stores of norepinephrine. However, an enzymatic adaptation has also been suggested to be the cause of development of myocardial resistance. Joseph et al. (6) have hypothesized that the ISP-induced myocardial resistance may not be due to alteration in the intensity of pharmacologic events, but due to an adaptation to ISP-induced hypoxia due to a shift of LDH-H to LDH-M subunits in the heart enabling it to adapt to anerobic metabolism.

Our earlier studies had shown that facilitation of in vivo cholinergic activity by the use of an anti-cholinesterase agent in dogs inhibited the propensity of heart to arrhythmias in normothermic as well as hypothermic state (9,10). Recently, the interaction of cholinergic and adrenergic system in cardiac arrhythmias was studied by subepicardial infusion of interacting agents and development of unifocal ventricular arrhythmias in dogs (12). In this self control study, it was concluded that cholinergic innervation to the ventricular myocardium and Purkinge fibers inhibits the arrhythmogenic activity of the adrenergic system, and the blockade of the cholinergic system potentiates arrhythmogenicity. It was, therefore, hypothesized that the myocardial resistance to AD-arrhythmias after an initial exposure to ISP could be due to an endogenous facilitation of cholinergic system. In case the hypothesis is correct, the ISP-induced resistance would be neutralised by an anti-muscarinic agent. It was found that ISP-induced myocardial resistance to

AD-arrhythmias was markedly inhibited by atropine treatment. In addition, as expected, arrhythmogenicity of AD in Control rats was also inhibited to some extent. The facilitation of cholinergic system in ISP-treated rats is further substantiated by the fact that the myocardial ACh content was higher in ISP-treated rats than that of the Controls. These data,therefore, show that facilitation of cardiac cholinergic system plays a significant role in the development of myocardial resistance in ISP-challenged rats.

Role of physical exercise in the prevention of coronary heart disease has been a subject of extensive study during the last more than a decade. The American Heart Association's Subcommittee on Exercise/Cardiac Rehabilitation (20) has summarised that 'Exercise training can increase cardiovascular functional capacity decreasing myocardial oxygen demand for any given levels of physical activity in normal persons as well as most cardiac patients.' In addition, 'evidence suggests that regular moderate or vigorous occupational or leisure-time physical activity may protect against coronary heart disease, and may improve the likelihood of survival from a heart attack.' The physiologic sequelae of chronic dynamic exercise has been reviewed by Hammond and Froelicher (21). The changes associated with chronic dynamic exercise include resting bradycardia, decrease in intrinsic heart rate, 'relative' bradycardia at matched absolute work loads, unaltered plasma and myocardial levels of catecholamines with a reduced circulating catecholamines at matched absolute submaximal work loads, but a similar ISP dose-response relationship in sedentary versus highly trained atheletes. No cardiac adrenergic or cholinergic receptor changes was found in chronic exercise rats (22). It appears that exercise leads to an increase in parasympathetic tone (21). In rats, physical training may lead to an increase in myocardial concentration of ACh (23). In the present studies, exercise has been found to reduce markedly the arrhythmogenic activity of AD. The role of cholinergic system in this modulation needs investigation similar to that seen in the case of ISP.

The present studies show that exposure of heart to a continued low-grade stress whether by physical exercise or chemically mediated overactivity/non-fatal necrosis leads to a development of myocardial resistance to withstand further insults. It is known that in an acute stress there is an overstimulation of the sympathetics which disproportionately increases myocardial oxygen consumption (24), and may be cardiac arrhythmias. The decrease in the catecholamine-induced arrhythmias may be life-saving in a chronic stress-conditioned heart. The studies further suggest that facilitation of cholinergic activity may be one of the mechanisms of development of myocardial resistance in a stress-conditioned heart.

SUMMARY

Wistar strain albino rats of either sex weighing 125-150 g were either pretreated with isoprenaline 50(ISP-50) or 100(ISP-100) ug/kg/day sc, or subjected to swimming for 30 min/day for 21 days. On the 22nd day,the rats were anesthetised with pentobarbitone sod., and EKG was monitored. The minimal arrhythmogenic ED100 of adrenaline(AD) was determined in each rat. ISP pretreatment markedly inhibited the arrhythmogenic activity of AD in a dose-dependent manner. Arrhythmogenic activity of AD was also markedly reduced in swim-exercised rats. The effect of ISP-100 pretreatment on AD-arrhythmias was markedly reduced by atropine sulphate. The ventricular acetylcholine content of ISP-100 rats was found to be significantly higher than in the Control rats. The results show that ISP treatment or exercise for 3 wk protected the heart against AD-induced arrhythmias. This protection seemed to be due to facilitated cholinergic activity.

REFERENCES

1. Rona, G., Chappel, C.I., Balazs, T. and Gaudry, R. Arch. Pathol. 67: 443-445, 1959.
2. Selye, H., Veilleux, R. and Grasso, S. Proc. Soc. Exp. Biol. Med. 104: 343-345, 1960.
3. Dusek, J., Rona, G. and Kahn, D.S. J.Pathol. 105: 279-282, 1971.

4. Balazs, T., Ohtake, S. and Noble, J.F. Toxicol. Appl. Pharmacol. 21: 200-213, 1972.
5. Balazs, T. Toxicol. 2: 247-255, 1974.
6. Joseph, X., Bloom, S., Pledger, G. and Balazs, T. Toxicol. Appl. Pharmacol. 69: 199-205, 1983.
7. Bhatia, B., Datta, K. and Nijhawan, R. In: Advances in Myocardiology II (Eds. M. Tajuddin, B. Bhatia, H.H. Siddiqui and G. Rona), University Park Press, Baltimore, 1980, pp. 153-163.
8. Leon, A.S. Med.Clinics North Am. 69: 3-20, 1985.
9. Das, P.K. and Bhattacharya, S.K. Brit. J. Pharmacol. 44: 397-403, 1972.
10. Das, P.K. and Sinha, P.S. Brit. J. Pharmacol. 44: 391-397,1972.
11. Das, P.K. and Bhattacharya, S.K. In: Advances in Myocardiology II (Eds. M. Tajuddin, B. Bhatia, H.H. Siddiqui and G. Rona), University Park Press, Baltimore, 1980,p. 85-115.
12. Das, P.K., Bhattacharya, T.K. and Gambhir, S.S. In: Advances in Myocardiology VI(Eds. N.S. Dhalla and D.J. Hearse), Plenum Publishing Corp., 1985,pp. 349-365.
13. Anand, B.K. Am. J. Physiol. 168: 218-225, 1952.
14. Richter, D. and Crossland, J. Am. J. Physiol. 159: 247-251, 1949.
15. Chang, H.Y., Kelin, R.M. and Kunos, G. J. Pharmacol. Exp. Ther. 221: 784-789, 1982.
16. Kenakin, T.P. and Ferris, R.M. J. Cardiovasc. Pharmacol. 5: 90-97, 1983.
17. Stadel, J.F., Strulovici, B., Nombi, P., Lavin, T.N., Briggs, M., Caron, M.G. and Lefkowitz. R.J. J. Biol. Chem. 258: 3032-3038, 1983.
18. Davies, A.O. and Lefkowitz, R.J. J. Clin. Invest. 71: 565-571, 1983.
19. Hayes, J.S., Pollock, G.D. and Fuller, R.W. J. Pharmacol. Exp. Ther. 231: 633-639, 1984.
20. American Heart Association Subcommittee on Exercise/Cardiac Rehabilitation. Circulation. 64: 1302-1304, 1981.
21. Hammond, H.K. and Froelicher, V.F. Med. Clinics North Am. 69: 21-39, 1985.
22. Williams, R.S. Cardiovasc. Res. 14: 177-182, 1980.
23. Ekstrom, J. Q. J. Exp. Physiol. 59: 73-80,1974.
24. Raab, W., Stark, E., Macmilan, W.H. and Gigee, W.R. Am. J. Cardiol. 8: 203-211, 1961.

B. THERAPY OF HYPERTENSION AND HEART FAILURE

7

CLINICAL TRIALS IN HYPERTENSION AND CONGESTIVE HEART FAILURE

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In cardiovascular disease, many treatments aim to prevent future disease complications and/or premature death. This applies both to asymptomatic conditions such as hypertension and to symptomatic illnesses such as congestive heart failure (CHF). The main purpose of this review is to consider the results of randomized clinical trials in these conditions, and to examine the overall effects of drug treatment on all-cause mortality. For the hypertension trials, stroke and coronary heart disease (CHD) incidence will also be reviewed.

METHODOLOGY

In this overview the results of individual studies have been combined. Such "pooling" of data from the hypertension trials has been conducted in order to provide a more stable estimate of treatment effect by reducing random errors and to increase the statistical power to detect moderate benefits. Most trials in hypertension were not of sufficient size or duration to detect even a 20-25% treatment effect, either for all-cause mortality and/or cause-specific mortality and morbidity. Treatment benefits of 10-15% have important public health consequences given the high prevalence of hypertension in the population. With one recent exception (1) all CHF trials have been small and of short duration. A systematic overview of the CHF trial findings was conducted in order to determine trends and develop hypotheses for testing in future large-scale prevention trials.

For any overview to be reliable, data should be available from all trials, published and unpublished. This can sometimes be a problem since trials with indifferent or unfavorable findings are more likely to be terminated early, completed but never

written up, or unsuccessfully submitted for publication. For this review, every attempt was made to ensure that all randomized trials were included. It is unlikely that any large hypertension trial has been missed. The focus of this presentation is on trials in less severe hypertension involving predominantly primary prevention. More comprehensive reports have been published elsewhere (2,3). The authors have previously presented two overviews of CHF trials (4,5) which include complete references.

The overview is limited to randomized controlled trials, and the analyses adhere to the intention-to-treat principle. The procedure for pooling developed by Peto and colleagues (6) was employed. The definitions of cause-specific mortality and morbidity (particularly for non-fatal myocardial infarction) were not the same across all the trials and, thus, these pooled data ought to be interpreted with some caution.

RESULTS

Trials in less severe hypertension

Seven placebo or "no treatment" control trials and two "referred care" control studies have been reviewed (Table 1). A large proportion of the control subjects in the former trials were placed on active treatment during the average follow-up of 5 1/2 years and, thus, the distinction between the two types of trials is not sharp. However, the true benefit of treatment is expected to be underestimated in studies employing referred care controls.

The reviewed trials represent over 43,000 subjects. Their mean diastolic blood pressure (DBP) at entry was 98.5 mg Hg. The treatment regimen was similar in the trials. The predominant drugs used were thiazide or thiazide-like diuretics. The step-up regimens included rauwolfia drugs, alpha-methyldopa, hydralazine and/or beta-blockers. The MRC trial (7) evaluated two active interventions, a thiazide diuretic and propranolol.

At the completion of the follow-up, DBP was 5.5 mm Hg lower in the treatment group than in the controls. The corresponding difference for systolic blood pressure was 10.6 mm Hg (data available from six trials). The blood pressure reduction was associated with a lower all-cause mortality among actively treated subjects in six of the nine trials. In only one of these,

Table 1. Randomized treatment trials in less severe hypertension: Design and blood pressure results

Trial	Sample Size	Follow-up Years	Diastolic Blood Pressure Baseline	Net Change*
VA (1970)	380	3.8	104	-19
USPHS (1977)	389	6.5-9.0	99	-10
VA-NHLBI (1978)	1,012	1.5	93	-7
Helgeland (1980)	785	5.5	97	-10
ANBP (1980)	3,427	4.0	100	-6
EWPHE (1985)	840	4.7	101	-10
MRC (1985)	17,354	5.5	98	-6
HDFP (1979)	10,940	5.0	101	-5
MRFIT**(1980)	8,012	7.0	96	-4
TOTAL	43,139	5.6	98.5	-5.5

* Difference of mean DBP between treatment and control groups
** Hypertensives only

however, the Hypertension Detection and Follow-Up Trial, (8) did the difference reach statistical significance. The pooled results show an 11% lower mortality in the treated group compared to the controls, a statistically significant difference with a 95% confidence interval of -19% to -2%.

For the outcome measure of stroke, treatment was remarkably effective. In several individual trials, statistically significant reductions were observed. The pooled results indicate a 38% reduction in stroke mortality (95% CI: -53% to -19%) and a 43% benefit for nonfatal stroke (95% CI: -54% to -29%). For neither CHD mortality nor incidence of nonfatal myocardial infarction (MI) was there a statistically significant benefit associated with active treatment. Combining the results yielded a 8% lower CHD mortality rate in subjects from the treatment groups (95% CI: -21% to +6%) and a 6% lower rate for MI incidence (95%

CI: -22% to +14%).

Data on the incidence of CHF are only available from four trials in less severe hypertension. In order to provide more complete picture of the effect of antihypertensive therapy on the CHF incidence, the findings from three trials in severe hypertension are also considered and included in Table 2. In severe hypertension, CHF is a common complication -- 16 events among 146 subjects. Treatment had a dramatic benefit and almost abolished the risk of CHF among the study participants. The incidence of CHF was intermediate in subjects with less severe hypertension and signs of end organ damage -- 17 events in 413 subjects. None of the actively treated subjects developed CHF during the trial. In the group with less severe hypertension without end organ damage, the incidence of CHF was very low, approximately 2 per 1,000.

Trials in CHF

Nineteen trials of vasodilators have been reviewed. They include (1) direct-acting nitrates, hydralazine and minoxidil, (2) neurohumoral antagonists like prazosin, and (3) angiotensin - converting enzyme (ACE) inhibitors such as captopril and enalapril. None of the vasodilator trials showed a significant reduction in mortality, nor did the combined data for all vasodilators produce any conclusive findings: 170 deaths/742 controls (22.9%) vs. 205 deaths/829 actively treated (24.7%). However, the combined data from the trials of ACE inhibitors, 24/292 controls (8.2%) vs. 11/292 treated patients (3.8%), and the direct-acting vasodilators, 132/364 controls (36.3%) vs. 88/277 treated (31.8%), appear promising. The recently published VA trial (1), by far the largest trial in CHF completed so far, reported a favorable trend for the combination of hydralazine and isosorbide dinitrate. Caution is advised in drawing conclusions from the pooled data regarding treatment efficacy. For example, non-favorable results in a completed comparison of 300 patients randomized to digitalis, captopril and placebo or from other studies could easily alter the picture.

The available information on trials of inotropic agents is incomplete. Because of the incompleteness and the very small

Table 2. Incidence of CHF in placebo-controlled trials of hypertension

Trial*	Events/Number of Patients Treatment	Control
Severe Hypertension		
Hamilton et al. (1964)	1/30	4/31
Wolfe & Lindeman (1966)	0/45	8/42
VA (1967)	0/70	4/73
SUBTOTAL	1/145	16/146
Moderate Hypertension with End Organ Damage		
VA (1970)	0/186	11/194
HSCS (1974)	0/233	6/219
SUBTOTAL	0/419	17/413
Moderate Hypertension without End Organ Damage		
Helgeland (1980)	0/406	1/379
ANBP (1980)	3/1,721	3/1,706
SUBTOTAL	3/2,127	4/2,085

*For references see (2,3)

number of deaths in the available trials, it is difficult to draw any firm conclusion (5). It is notable that there is not even a suggestion of favorable effect of inotropic agents on mortality.

DISCUSSION

The results of the nine long-term trials in subjects with less severe hypertension are consistent with the findings of the trials in severe hypertension. The major benefit seems to be a reduction in pressure-related complications such as stroke and CHF. Most of

the reduction in all-cause mortality can be attributed to this effect. This conclusion would suggest that the findings reported here, which mostly relate to diuretic-based therapy, apply to other antihypertensive agents as well. If the benefits of various antihypertensives are similar, then other treatment-related factors such as frequency of adverse effects, impact on quality of life (9), and cost will become even more important in the decision as to which drug or drugs to prescribe.

The small, statistically non-significant effects of treatment on CHD mortality and MI incidence are disappointing. As judged from prospective epidemiological studies, one would have expected a greater benefit from a mean reduction in DBP of 5.5 mm Hg. Whether the explanation lies in certain characteristics of the drugs evaluated in the nine trials (10) or the duration of the trials can only be conjectural. Unfortunately, large trials comparing the various classes of antihypertensive agents are difficult to conduct. Other issues raised by the trials relate to retrospective subgroups analyses (3). For example, are there differences in response to specific drugs between whites and blacks, smokers and non-smokers, men and women, subjects with and without ECG abnormalities, as some trials have suggested?

Currently there is no conclusive scientific evidence that any drug therapy improves survival in CHF. The combined data from trials of certain vasodilators, including ACE inhibitors, appear promising. Large, well-designed, long-term trials are required to establish their effect on mortality in CHF. To date, the limited published data provide no suggestion of a beneficial effect of inotropic agents on survival.

SUMMARY

In cardiovascular disease many treatments aim to prevent future disease complications and/or premature death. This applies both to asymptomatic conditions such as hypertension (HT) and to symptomatic illnesses such as congestive heart failure (CHF). Several large, long-term HT trials have clearly documented a reduction in "hypertensive" events (CHF, stroke, etc.) with active treatment. The pooled data from 9 trials in mild-to-moderate HT (N approximately 43,000) showed an 11% reduction in all-cause

mortality. Coronary heart disease mortality and non-fatal myocardial infarction rates were lower in active treatment groups, but not significantly so. Post-hoc subgroup analyses have raised questions regarding the effects in subsets of hypertensives. In contrast, the randomized trials in CHF have been small and of short duration. Pooling of trials evaluating inotropic agents showed no favorable effect at all on mortality. Vasodilator therapy might convey some benefit; this is suggested by the promising pooled results of trials of angiotensin converting enzyme inhibitors, and suggestive evidence from the combination of isosorbide dinitrate and hydralazine in the V-HEFT study. However, larger and long-term trials are needed before we know with certainty whether this class of drugs prolongs life in CHF patients.

REFERENCES

1. Cohn, J.N., Archibald, D.G., Ziesche, S., Franciosa, J.A., Harston, W.E., Tristani, F.E., Dunkman, W.B., Jacobs, W., Francis, G.S., Flohr, K.H., Goldman, S., Cobb, F.R., Shah, P.M., Saunders, R., Fletcher, R.D., Loeb, H.S., Hughes, V.C. and Baker, B. N. Engl. J. Med. 314:1547-1552, 1986.
2. Cutler, J.A. and Furberg, C.D. Prev. Med. 14:499-519, 1985.
3. MacMahon, S.W., Cutler, J.A., Furberg, C.D., and Payne, G.H. Progr. Cardiovasc. Dis. 1986 (in press).
4. Furberg, C.D. and Yusuf, S. Am. J. Cardiol. 55:1110-1113, 1985.
5. Furberg, C.D. and Yusuf, S. Adv. Cardiol. 34, 1986.
6. Yusuf, S., Peto, R., Lewis, J., Collins, R. and Sleight, P. Progr. Cardiovasc. Dis. 27:335-371, 1985.
7. Medical Research Council Working Party. Br. Med. J. 291: 97-104, 1985.
8. Hypertension Detection and Follow-up Program Cooperative Group. JAMA 242:2562-2571, 1979.
9. Croog, S.H., Levine, S., Testa, M.A., Brown, B., Bulpitt, C.J., Jenkins, C.D., Klerman, G.L. and Williams, G.H. N. Engl. J. Med. 314:1657-1664, 1986.
10. Multiple Risk Factor Intervention Trial Research Group. Am. J. Cardiol. 55:1-15, 1985.

8

INTERACTION OF CALCIUM ENTRY BLOCKERS AND ADRENERGIC SYSTEM IN HYPERTENSION AND HEART FAILURE

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Intracellular calcium plays a pivotal role in the maintenance of smooth muscle tone (1). It was not surprising therefore that calcium entry blockers have met a widespread success in the treatment of hypertension, a disease characterized by peripheral arteriolar vasoconstriction (2). However, it was soon found that the antihypertensive action of calcium entry blockers was due not only to the peripheral vasodilation (3-7) but also to other effects including natriuresis (8-10) and alteration of autonomic nervous system (11,12). Despite this multifaceted antihypertensive effect, the ability to reduce peripheral vascular resistance remained the most widely recognized mediator of blood pressure control by this class of drugs. Not only peripheral vascular resistance was found to be reduced during therapy with various CEB, but also forearm blood flow increased significantly after acute and chronic nifedipine treatment (13). It is the mechanism by which this decrease in vascular smooth muscle tone is achieved that needs further explanation.

In addition to its direct regulatory effect on actin-myosin interaction, intracellular calcium influences smooth muscle contraction indirectly by altering neural, humoral and other ionic factors (1,10). Because of this diversity of effects of calcium, it is not surprising that the vasodilator effect of CEB has been ascribed to their ability to block calcium entry by competing with calcium at the transmembrane transport system (14); a competitive displacement of Ca^{++} from its transmembrane carrier system has been described with resultant interruption of excitation-contraction coupling. In this respect, the mechanism of the direct vasodilator effect of CEB is different from that of other known direct vasodilators. Nitrates, for example, were found to minimize the availability of free intracellular calcium ions by stimulating biochemical mechanisms of cellular calcium sequestration or by enlarging the capacity for calcium storage (14).

In addition to their direct regulatory role, CEB were reported to influence the response of receptor-mediated stimuli, particularly those acting on the α-adrenergic receptors (15-18). Moreover, it was suggested from the studies of

Pedrinelli and Tarazi (19,20) and others (17,18,21-26) that the influence of calcium on α_1 and α_2 adrenergic stimulation differed according to the experimental conditions. Thus, whereas calcium antagonists exhibited preferential antagonism of α_2-adrenergic mediated pressor responses in vivo, a considerable body of in vitro evidence appeared to support an equivalent degree of external calcium mobilization during both α_1 and α_2 stimulation. In this respect, experiments by Pedrinelli and Tarazi (19) have demonstrated that calcium entry blockade by either nitrendipine or verapamil interfered effectively with pressor responses to infused norepinephrine when the α_2-adrenergic sites were left exposed (pretreatment with prazosin and propranolol); however, these responses were not significantly antagonized in the group pretreated with propranolol and yohimbine, in which presumably only the α_1-adrenergic receptors were exposed. It was suggested, therefore, that calcium entry blockade, in the pithed Sprague-Dawley rat, antagonizes preferentially in vivo α_2-mediated adrenergic vasoconstriction. These conclusions were supported by the findings that nitrendipine was less potent against the pressor responses evoked by neural stimulation (predominantly α_1 mediated) than against responses to exogenous norepinephrine (predominantly α_2 mediated) (17,27-30).

Contrary to these findings in the "intact" pithed Sprague-Dawley rat, calcium entry blockade was shown to interfere also with contractile responses to α_1 stimulation in some isolated vascular tissues (24-26), and in autoperfused hindquarters of pithed Sprague-Dawley rats (20) as well as in vitro perfused rat hindquarters (31). In this context, studies by Pedrinelli and Tarazi (20) have shown that the calcium entry blocker, nitrendipine, did not produce any differential effect on the responses of the rat autoperfused hindquarter vascular bed to both Cirazoline (a selective α_1-adrenergic agonist) and B-HT 920 (a selective α_2 agonist), (Fig. 1,2). Thus, external calcium appeared important for the development of adrenergic-mediated vasoconstriction irrespective of the particular subset of alpha adrenoreceptors involved. Similar findings were also reported by Llenas and Gassingham (30) using another calcium antagonist, cinnarizine.

To reconcile these discrepancies between findings in the whole animal and in an isolated vascular region, several suggestions were presented (20) including a) a selective activity of α_1 and α_2 stimuli on resistance and capacitance vessels, b) the adrenergic control of the hindlimb vascular region in the rat is mainly a function of α_1 adrenoreceptor activation, and c) a low receptor reserve

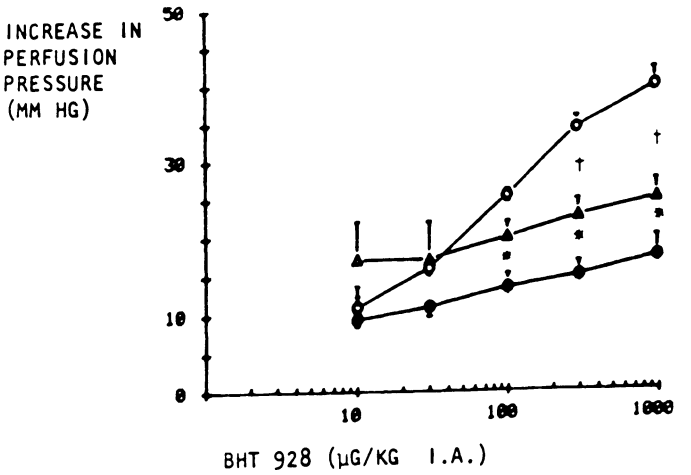

FIGURE 1: Autoperfused hindquarters of pithed, reserpinized rats. Perfusion pressure log dose-response curve to B-HT 920 during vehicle (o) or nitrendipine (▲, 3.0 ± 0.2 μg/kg/min; ●, 31.3 ± 2.3 μg/kg/min) infusion, $P < .05$ or less nitrendipine (∿30 μg/kg/min) vs vehicle. Means ± S.E.M. n = 5-7 animals per group. With permission from Pedrinelli and Tarazi (20).

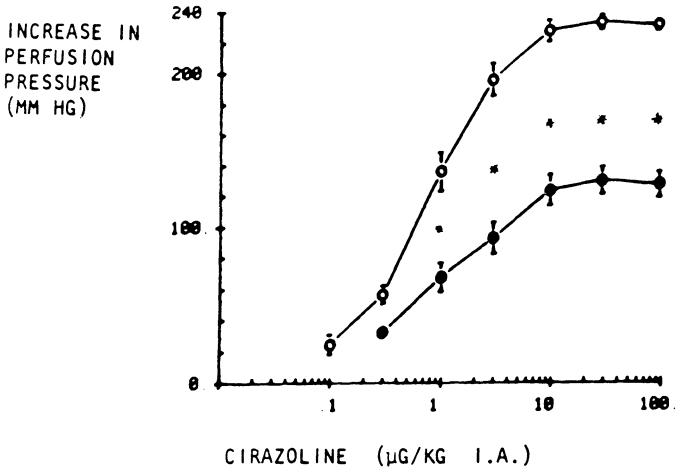

FIGURE 2: Autoperfused hindquarters of pithed, reserpinized rats. Perfusion pressure log dose-response curves to cirazoline during vehicle (o) and nitrendipine (•) (33.9 ± 1.1 μg/kg/min) infusion. Means ± S.E.M.; n = 4-5 animals per group. $P < .05$ or less nitrendipine vs vehicle. With permission from Pedrinelli and Tarazi (20).

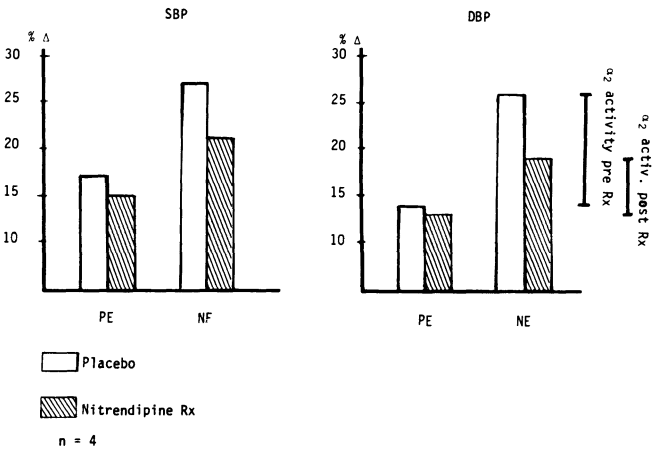

FIGURE 3: Effect of nitrendipine on blood pressure response to exogenous sympathomimetics.

at the hindquarter level, mainly a muscular vascular bed in which local metabolic regulation prevails over adrenergic tone (32) as compared to the overall vascular system.

Such studies of differential receptor responses are difficult to perform in man. However, a simplified pharmacological approach was used in our laboratory. Results revealed a reduction in α_2 responsiveness to agonists in the presence of nitrendipine (Fig. 3). Our approach consisted of comparing the blood pressure change induced by sequential injection of phenylephrine and norepinephrine in the same subject (hypertensive patients) during placebo treatment then after 2 weeks of nitrendipine therapy. Since phenylephrine is mostly an α_1 stimulant while norepinephrine stimulates both α_1 and α_2-adrenergic receptors, we asssumed that the difference in blood pressure change by the two drugs will represent the effect of α_2 receptor stimulation. Under such conditions, and in a small number of patients, we found that nitrendipine interfered with α_2 responsiveness. These data did not allow us to assess the effect of the compound on α_1 adrenoceptors; however, the response to physiologic stimuli such as Valsalva maneuver and cold pressor test was unchanged by nitrendipine. This is in agreement with the findings of McLeay et al. (12) that the vasopressor responses to tilt, cold pressor test and hand grip were not altered by nifedipine therapy compared to the pretreatment phase. The sparing of α_1 receptors in the intact organism explains the normal vascular response to head-up posture in patients treated with CEB; orthostatic hypotension is not one of the recognized side effects of treatment with calcium entry blockade.

Reflex adrenergic stimulation has been indeed well documented during treatment with both nifedipine and nitrendipine; hyperkinetic circulation documented by symptoms and hemodynamic indices (5,33,34) occurred in many of our treated patients; the hemodynamic change was dose-dependent in at least one subject (Table 1). The increase in heart rate accompanying this hyperkinetic circulation could be explained by several factors at the level of the central nervous system, sinoatrial node, or sinoaortic baroreflex. There was no experimental evidence that CEB modulate directly central nervous mechanisms or sinoatrial node function. On the other hand, McLeay et al. (12) have reported resetting and increased sensitivity of the sinoaortic baroreflex after chronic nifedipine therapy; they have shown a shift to the left as well as a significant increase in the slope of the baroreflex regression line (response to IV phenylephrine). These changes were attributed to the relaxing effect of the

TABLE 1

NITRENDIPINE THERAPY FOR HYPERTENSION:
HYPERKINETIC CIRCULATION IN ONE PATIENT

	Control	2 wk	1 mo
MAP (mmHg)	140	114	117
HR (bpm)	68	89	90
CO (l/min/m^2)	2.01	2.76	3.99
TPR (U. m^2)	70	41	29
MTT (sec)	10	8.8	6.1
EF (%)	45	61	59
Weight (kg)	123.3	123.8	123.6
TBV (ml/cm)	31.6	31.6	35.1
CPVI (ml/m^2)	335	405	405
CV (%)	44.5	44.5	45.8

MAP = mean arterial pressure; HR = heart rate; CO = cardiac output; TPR = total peripheral resistance; MTT = mean transit time; EF = ejection fraction; TBV = total blood volume; CPVI = cardiopulmonary volume index; CV = cell volume; wk = week; mo = month.

calcium entry blocker on the smooth muscles in the carotid sinus and aortic arch, thus favoring the functional characteristics of the pressure receptors located in these areas (12,36). Thus, peripheral vasodilation will be counteracted by reflex sympathetic stimulation and vagal withdrawal. This nonadrenergic (vagal withdrawal) component explains experimental findings in the conscious dog; the increase in heart rate in animals receiving nifedipine, diltiazem or Verapamil was only partly inhibited by beta blockade (12), indicating that a nonadrenergic mechanism was contributing to the response (37). Irrespective of its mechanisms, this increased baroreceptor sensitivity is of clinical significance; presumably, it will result in improved buffering of transient increases in blood pressure.

It is important to mention that several studies were not in agreement with the above findings; in contrast, heart rate changes during CEB therapy were reported to be not significant (4,12). Obviously, changes in heart rate differ in the individual patient depending on the duration of treatment, the age of the patient and the functional state of baroreceptors. Moreover, Tarazi et al. (7) have shown that there is a relative balance between the venodilator and arteriolar dilator effects of nitrendipine relative to the dose used. Thus, small doses of nitrendipine produced no increase in cardiac output despite the reduction in systemic resistance and peripheral arteriolar dilation, a pattern similar to that produced by antihypertensive agents with combined arteriolar and venodilator effect (38). On the other hand, large doses of nitrendipine (3 mg/kg) produced the hemodynamic pattern expected for potent and predominantly arteriolar vasodilators including fall in blood pressure and peripheral resistance and marked reflex increase in heart rate and cardiac output (38,39).

In summary, in vivo preferential α_2 postsynaptic antagonism by calcium entry blockers plays a role in their antihypertensive effects without interfering with neurogenic vasopressor responses or baroreceptor mediated reflexes. Thus, orthostatic hypotension does not complicate treatment with CEB. Moreover, tachycardia and absence of cardiac depresssion after calcium blockers may be attributed to baroreceptor-mediated sympathetic discharge counteracting the possible negative chronotropic and inotropic effects of the drugs (11). It is not sure, however, if these negative effects of CEB operate in vivo since Kazda et al. (40) and others (11,41) have reported that oral therapeutic doses of nifedipine, diltiazem or lidoflazine produce free plasma concentrations which are at least 10 times below those required to depresss myocardial contractility in vitro. Of importance, in this respect, are the observations during antianginal therapy, that

combination of calcium blockers with beta blockers did not unmask negative inotropic effects in vivo (42a-c) as measured by LV dP/dt and ejection fraction.

Calcium entry blockers were used indeed for treatment of CHF. However, observations applicable to a normal functioning heart are not necessarily true in the case of the failing heart because of altered compensatory mechanisms; it is well known, for example, that baroreceptor reflexes are blunted in heart failure (43). Thus, Fifer et al. (44) demonstrated that in the absence of reflex sympathetic stimulation, sublingual administration of nifedipine decreased peak positive left ventricular dP/dt in heart failure patients despite the reduction in blood pressure and the increase in LV end diastolic pressure. On the other hand, equihypotensive doses of nitroprusside did not produce any depression of this index. The lack of reflex sympathetic stimulation in this study was manifested by the absence of significant changes in heart rate or catecholamine levels and was attributed to the well known blunted baroreceptor reflexes in heart failure patients (43). Similarly, Elkayam et al. (45) showed that oral nifedipine given to patients with chronic severe congestive heart failure failed to increase stroke volume despite marked reduction in systemic vascular resistance. Thus, the intrinsic negative inotropic activity of the CEB was unmasked when reflex sympathetic stimulation did not occur in response to the decrease in systemic arterial pressure. However, the effect of CEB on myocardial performance in the failing heart remains controversial. In fact, beneficial effects of nifedipine and other CEB on cardiac function have been demonstrated in some studies in heart failure patients. Thus, Prida et al. (46) showed that stroke volume increased and pulmonary wedge pressure decreased during nifedipine treatment for CHF. Also, Miller et al. (47) reported persistent decrease in pulmonary wedge pressure and left ventricular volume during nifedipine therapy; the occurrence of these changes in the absence of evidence of venodilation was attributed to a beneficial effect of nifedipine on LV relaxation. Such beneficial effects of CEB in heart failure have been supported by several other studies (48-50). One particular subset of patients in whom nifedipine seemed particularly beneficial were the hypertensives with congestive heart failure (51). Finally, another beneficial effect of CEB could be related to their role in the coronary circulation; increased resting coronary venous flow was shown by Tweddel and Hutton (52) in heart failure patients treated with felodipine, and by Kobayashi and Tarazi (53) in hypertensive rats chronically treated with nitrendipine. Still appropriate to the heart failure population is the reported observation that Ca-antagonistic compounds counteract glycoside-5

induced coronary vasoconstriction (14,54). This beneficial effect was not described for other coronary vasodilators which do not significantly interfere with excitation-contraction coupling (14). Such combination therapy is therefore of therapeutic significance and is particularly suitable where the beneficial positive-inotropic glycoside effect on the myocardium is needed whereas the undesired coronary vascoconstriction is abolished (14).

In conclusion, the interaction of calcium entry blockers and adrenergic nervous system occurs at different levels of the system, and influence the hemodynamic and clinical response in both hypertension and congestive heart failure. Preferential antagonism of α_2-adrenergic constriction in vivo adds to the direct vasodilating effect of calcium entry blockade. The effects on the heart represent, however, the complex balance between the changes in peripheral and in coronary circulation and the direct negative inotropic effect of the drug.

REFERENCES

1. Rasmussen, H. Ann. Intern. Med. 98 (Pt. 2): 809-816, 1983.
2. Tarazi, R.C., Ibrahim, M.M., Dustan, H.P. and Ferrario, C.M. Circ. Res. (Suppl. I) 34: 213-221, 1974.
3. Aoki, K., Kondo, S., Mochizuki, A., Yoshida T., Kato, S., and Takikawa, K. Am. Heart J. 96: 218-226, 1978.
4. Olivari, M.T., Bartorelli, C., Polese, A., Fiorentini, C., Maruzzi, P., and Guazzi, M. Circulation 59: 1056-1062, 1979.
5. Pederson, O.L. Acta Pharmacol. Toxicol. 49 (Suppl. II): 1, 1981.
6. Fouad, F.M., Aboul-Khair, M., Tarazi, R.C. J. Cardiovasc. Pharmacol. 4: S383-S386, 1982.
7. Tarazi, R.C., Aboul-Khair, M., and Kobayashi, K. In: Nitrendipine (Eds. A. Scriabine, S. Vanov, and K. Deck), Urban and Schwarzenberg, Baltimore-Munich, 1983, pp. 251-270.
8. Leonetti, G., Cuspidi, C., Smapieri, L., Terzoli, L., Zanchetti, A. J. Cardiovasc. Pharmacol. 4: S319-S324, 1982.
9. Garthoff, B., Knorr,A., Thomas, G., Kazda, S. Biochem, Pharmacol. 18: 3015-3016, 1982.
10. Luft, F.C., Aronoff, G.R., Sloan, R.S., Fineberg, N.S., and Weinberger, M.H. Hypertension 7: 438-442, 1985.
11. Henry, P.D. In: Calcium Blockers , Mechanisms of Action and Clinical Applications (Eds. S.F. Flaim and R. Zelis), Urban & Schwarzenberg, Baltimore-Munich, 1982, pp 135
12. McLeay, R.A.B., Stallard, T.J.,Watson, R.D.S., and Littler, W.A. Circulation 67: 1084, 1983.
13. Pederson, L.O., Mikkelsen, E. Eur. J. Clin. Pharmacol. 14: 375, 1978.
14. Fleckenstein, A. Calcium Antagonism in Heart and Smooth Muscle, John Wiley & Sons, New York, 1983, pp. 209-285.
15. Casteels, R. Chest 78 (Suppl.): 150, 1978.
16. Van Breemen, C., Aaronson, P., Loutzenhiser, R., Meisheri, K. Chest 78 (Suppl.): 157, 1978.
17. Van Meel, J.C.A., De Jonge, A., Kalkman, H.O., Willfert, B., Timmermans, P.B.M.W.M., Van Zwieten, P.A. Eur. J. Pharmacol. 69: 205-208, 1981a.
18. Van Zwieten, P.A.,Van Meel, J.C.A. and Timmermans, P.B.M.W.M. Hypertension 5 (Suppl. II): 8-17, 1983.
19. Pedrinelli, R. and Tarazi, R.C. Circulation 69: 1171-1176, 1984.
20. Pedrinelli, R. and Tarazi, R.C. J. Pharm. & Exp. Therap. 233: 643-649, 1985.
21. Cauvin, C., Loutzenhiser, R., Hwang, O. and Van Breeman, C. Eur. J. Pharmacol. 84: 233-235, 1982.
22. Awad, R., Payne, R. and Deth, R.C. J. Pharmacol. & Exp Therap. 227: 60-67 1983.
23. Janssens, W. and Verhaeghe, R. J. Physiol. (London) 347: 525-532, 1984.
24. DeMey, J. and Vanhoutte, P.M. Circ. Res. 48: 875-884, 1981.
25. Muller-Schweinitzer, E. Naunyn-Schiedeberg's Arch. Pharmacol. 324: 64-69, 1983.
26. Beckering, J.J., Thoolen, M.J.M.C., De Jonge, A., Willfert, B., Timmermans, P.B.M.W.M., and Van Zwieten, P.A. J. Pharmacol. Exp. Therap. 229: 515-521, 1984.
27. Langer, S.Z., Shepperson, N.B. Br. J. Pharmacol. 74: 942, 1981.
28. Van Meel, J.C.A., De Zoeten, K., Timmermans, P.B.M.W., Van Zwieten, P.A. J. Auton. Pharmacol. 2: 13, 1982.

29. Timmermans, P.B.M.W., De Jonge, A., Van Meel, J.C.A., Mathy, M.J., and Van Zwieten, P.A. J. Cardiovasc. Pharmacol. 5: 1, 1983.
30. Llenas S. and Massingham, R. Eur. J. Pharmacol. 87: 53, 1983.
31. Kobinger, W., Lillie, C. and Pichler, L. Circ. Res. (Suppl. 1) 46: 21-25, 1980.
32. Folkow, B. Circulation 21: 760-768, 1960.
33. Guazzi, M.D., Fiorentini, C., Olivari, M.T., Bartorelli, A., Necchi, G. and Polese, A. Circulation 61, 913-919, 1980.
34. Fouad, F.A., Pedrinelli, R., Bravo, E.L., Abi-Samra, F., Textor, S.C. and Tarazi, R.C. Clin. Pharmacol. & Therap. 35: 768-775, 1984.
35. Brown, A.M. Circ. Res. 46: 1-10, 1980.
36. Watson, R.D.S., Stallard, T.J. and Littler, W.A. Clin. Sci. 57: 241, 1979.
37. Millard, E.W., Lathrop, D.A., Grupp, G., Ashraf, M., Grupp, I.L. and Schwartz, A. Am. J. Cardiol. 49: 499-505, 1982.
38. Tarazi, R.C. Cardiovasc. Med. 3: 1125-1131, 1978.
39. Tarazi, R.C. and Fouad, F.M. J. Hypertension 3 (Suppl. 2): S27-32, 1985.
40. Kazda, S., Garthoff, B., Meyer, H., Schlossmann, K., Stoepel, K., Towart, R., Vater, W. and Wehinger, E. Arzneim, Fosch. 30: 2144-2162, 1980.
41. Triggle, D.J. In: New Perspectives on Calcium Antagonists (Ed. G.B. Weiss), American Physiological Society, Bethesda, 1981, pp. 1-18.
42a. Dargie, H., Rowland E, E. and Krickler, D. Br. Heart J. 468: 8-16, 1981.
42b. Leon M.B., Rosing, D.R., Bonow, R.O., Lipson, L.C. and Epstein, S.E. Am. J. Cardiol. 48: 131-139, 1981.
42c. Bela Subramanian, V., Bowles, M.J., Davies, A.B. and Raftery, E.B. Am. J. Cardiol. 49: 125-132, 1982.
43. Goldstein, R.E., Beiser, G.D., Stampfer, M. and Epstein, S.E. Circ. Res. 36: 571-578, 1975.
44. Fifer, M.A., Colucci, W.S., Lorell, B.H., Jaski, B.E. and Barry, W.H. J. Am. Coll. Cardiol. 5: 731-737, 1985.
45. Elkayam, U., Weber, L., Torkan, B., Berman, D. and Rahimtoola, S.H. Am. J. Cardiol. 52: 1041-1045, 1983.
46. Prida, X.E., Kubo, S.H., Laragh, J.H. and Cody, R.J. Am. J. Med. 75: 795-800, 1983.
47. Miller, A.B., Conetta, D.A. and Bass, T.A. Am. J. Cardiol. 55: 1359-1362, 1980.
48. Olivari, M.T., Levine, T.B. and Cohn, J.N. J. Cardiovasc. Pharmacol. 6 (Suppl. 7): S1002-S1005, 1984.
49. Kubu, S.H., Fox, S.C., Prida, X.E. and Cody, R.J. Am. Heart J. 10: 1032-1034, 1985.
50. Lahiri, A., Tovey, J., Kohli, R.S., Robinson, C.W., Caruana, M.P., Harlow, B.J. and Raftery, E.B. Postgraduate Med. Journal 60 (Suppl 4): 35-38, 1984.
51. Given, B.D., Lee, T.H., Stone, P.H. and Dzau, V.J. Arch. Intern. Med. 145: 281-285, 1985.
52. Tweddel, A.C. and Hutton, I. Eur. Heart J. 7: 54-60, 1986.
53. Kobayashi, K. and Tarazi, R.C. Hypertension 5 (Suppl II): II-45 - II-51, 1983.
54. Cantelli, I., Pavesi, P.C., Parchi, C., Naccarella, F. and Bracchetti, D. Am. Heart J. 106: 308-315, 1983.

9

PRAZOSIN THERAPY IN CHRONIC CONGESTIVE HEART FAILURE

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INTRODUCTION

Management of congestive heart failure is based on recognition of the specific underlying cardiac disease, understanding of the pathophysiology involved, exclusion of extracardiac factors perpetuating cardiovascular dysfunction, and thorough knowledge of medical and surgical therapy. Although most patients respond favorably to conventional treatment consisting of digitalis and diuretics, relatively refractory heart failure may develop in advanced myocardial heart disease (the specific, idiopathic, and ischemic cardiomyopathies) unamenable to operative intervention. The most important recent advance in the medical therapy for such patients with acute and chronic pump dysfunction has been the application of systemic vasodilator drugs to reduce excessive left ventricular afterload, thereby improving lowered cardiac output and decreasing elevated pulmonary venous pressure.

The cardiac output delivered from the intact heart is governed by integration of four principal determinants: 1) preload (ventricular end-diastolic volume), 2) contractility (variable force of ventricular contraction independent of loading), 3) impedance (instantaneous aortic pressure-flow ratio), and 4) heart rate (1). The two principal factors regulating afterload (ejection tension) of the left ventricle are its radius and systolic pressure, respectively related to chamber volume (preload) and aortic impedance (controlled by arterial compliance and by total peripheral vascular resistance of the systemic arterioles) (1). Since both preload and impedance are increased in heart failure, intramyocardial wall tension (afterload), a principal determinant of myocardial energetics, is also elevated in the failing heart, thereby raising cardiac oxygen requirements (1).

The cardiac output of the normal heart is principally governed by systemic venous return (directly related to venous tone), with the left ventricle operating on the steep ascending limb of its function curve (Fig. 1), aortic impedance being of little importance (2). In contrast, in the presence of left ventricular dysfunction, arterial resistance is elevated and cardiac output becomes very dependent on outflow impedance (Fig. 1), venous return having minimal influence with the ventricle operating at the apex of its depressed function curve (2). The profoundly heightened sympathetic activity in response to lowered cardiac output is excessively operative as an inherent reflex compensatory mechanism attempting to maintain cardiocirculatory integrity in the heart failure state (2). Because of this, systemic vascular resistance is greater than necessary to sustain arterial pressure, with the result that cardiac output is further decreased, thus evoking a deleterious cycle in which pump function is progressively depressed (2). In addition, systemic venoconstriction is above that required to enhance venous return to the failing ventricle on its flattened function curve characteristic of decreased contractility, thus exacerbating pulmonary edema and excessively increasing myocardial oxygen demand (2). Importantly, systemic vasodilator therapeutic reduction of both impedance and preload interrupts this harmful sequence by partially counteracting adrenergic overstimulation, thereby augmenting cardiac output, diminishing pulmonary congestion and improving myocardial energetics.

RESULTS AND DISCUSSION

Vasodilator Spectrum.

The vasodilators produce disparate modifications of cardiac function, depending on their differing alterations of preload versus impedance (1). Non-intravenous nitrates principally cause venodilation (decrease left ventricular filling pressure) (3). In contrast, intravenous nitroprusside (4) and oral prazosin (5) produce relatively balanced venous and arterial dilation (decrease left ventricular filling pressure and increase cardiac output), provided left ventricular end-diastolic pressure is maintained at its upper limits of normal (6). The vasodilator spectrum of hemodynamic profiles is completed by oral hydralazine which predominantly effects arteriolar dilation (increases cardiac output).

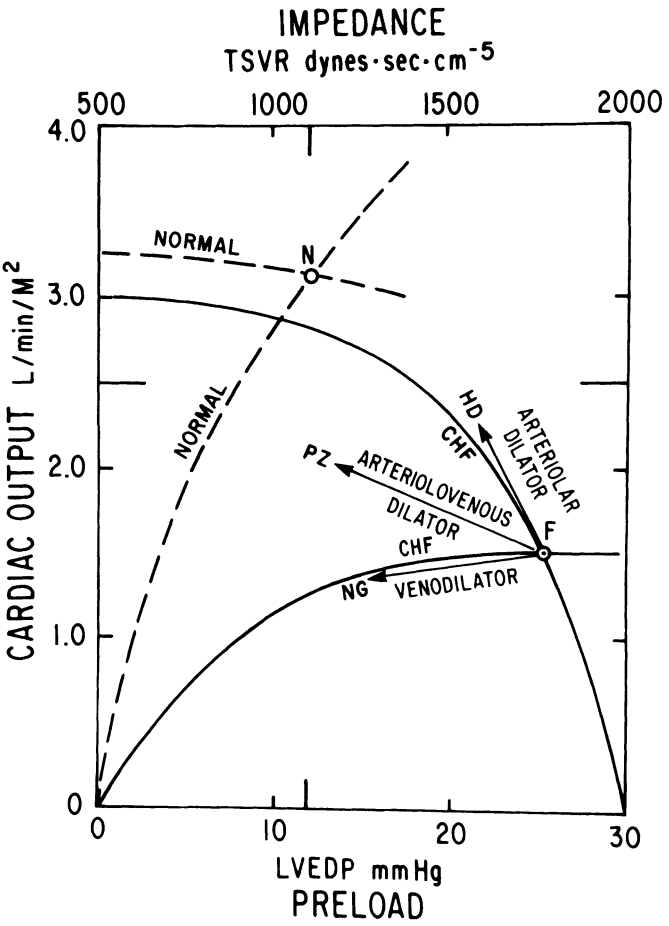

Fig. 1. Relationship of left ventricular preload (LVEDP) and aortic impedance (TSVR) to cardiac output (CO) in a normal and a failing left ventricle. In the normal heart (point N), CO is principally regulated by changes in preload (left-to-right ascending broken line relating LVEDP to CO); alterations in impedance are of minor importance (right-to-left horizontal broken line relating TSVR to CO). In contrast, in the failing heart (point F) with congestive heart failure (CHF), CO is principally regulated by changes in impedance (right-to-left ascending unbroken line relating TSVR to CO); alterations in preload are of minor importance (depressed left-to-right horizontal line relating LVEDP to CO). In the failing heart, the pure arteriolodilator, hydralazine, raises lowered CO

markedly with mild decline of elevated LVEDP (vertical arrow from point F); the balanced arteriolovenous dilator, prazosin, raises lowered CO and decreases elevated LVEDP (diagonal arrow from point F); and the pure venodilator, sublingual nitroglycerin, decreases elevated LVEDP markedly with little or no improvement of lowered CO (horizontal arrow from point F). (Reprinted with permission from reference 2.)

Ambulatory Prazosin Therapy.

Concerning the long-term management of advanced congestive heart failure, orally administered prazosin affords chronic efficacy for several months with addition of this vasodilator to the standard regimen of digitalis and diuretics (7). Thus, balanced peripheral arterial and venous relaxation (nitroprusside-like effect) of a single dose of prazosin (8) enhances lowered cardiac output (improves fatigue) by decreasing total systemic vascular resistance (impedance), to a similar extent and duration (six to eight hours) as the agent reduces excessive left ventricular filling pressure (preload) (relieves pulmonary congestion and dyspnea) by decreasing venous tone (Fig. 2). Concordantly, myocardial energetics are improved by decline of elevated left ventricular afterload (a preload-impedance product). Prolonged administration of prazosin (2 to 7 mg three or four times daily; average, 20 mg/day) provides increased treadmill exercise tolerance, improves New York Heart Association symptomatic classification of dyspnea and fatigue, and causes elevation of reduced scintigraphic and echocardiographic left ventricular ejection fractions (7). These prolonged salutary effects have been demonstrated by serial testing to be sustained for more than 12 months of continued ambulatory prazosin therapy in patients with chronic coronary heart disease (9).

Subacute Attenuation. Rapid attenuation of the favorable acute hemodynamic effects of prazosin has been observed in some congestive heart failure patients. To delineate the importance of this subacute phenomenon, we performed serial cardiocirculatory measurements during constant dose (5 mg four times daily) continuous oral therapy (10). The acute vaso-relaxant actions were blunted during the brief subacute period in three of the nine patients tested with congestive heart failure; however, within a few days of maintained prazosin administration, there was spontaneous complete restoration of the marked initial vasodilator efficacy (Fig. 3). Thus, while subacute hemodynamic prazosin attenuation occurs in a minority of heart failure patients, it is a temporary phenomenon (related to peripheral vascular alpha receptor blockade) without impact on the success of sustained ambulatory vasodilator therapy with this agent (10).

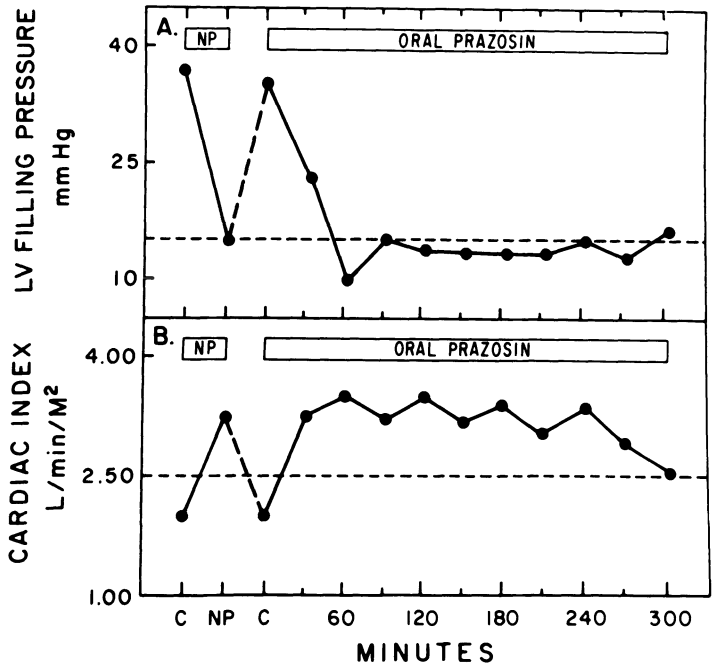

Fig. 2. Comparative effects of nitroprusside (NP) and prazosin on left ventricular (LV) filling pressure (A) and concomitantly on cardiac index (B) in a single patient serving as his own control, with severe congestive heart failure due to chronic coronary heart disease. The actions of NP were initially observed, then the infusion discontinued (diagonal broken lines), and a 4 mg oral dose of prazosin next given. (Reprinted with permission from Mason, D.T., Awan, N.A., Lee, G. and DeMaria, A.N. In: Advances in Heart Disease, Vol. 3 (Ed. D. T. Mason), Grune & Stratton, Inc., New York, 1980, pp. 403-446.)

Chronic Tolerance. With prazosin treatment of congestive heart failure for more than three months, late tolerance of the drug may occur in up to one-third of patients (9). This chronic phenomenon (different from subacute attenuation) may take place with long-term use of any of the vasodilator agents (10), and appears to be related to accompanying salt and water retention including the vascular wall itself (11), necessitating increased diuretic requirements of furosemide and especially the aldosterone antagonist, spironolactone. Prazosin late tolerance can be circumvented, with full responsiveness of the drug reestablished (Fig. 4),

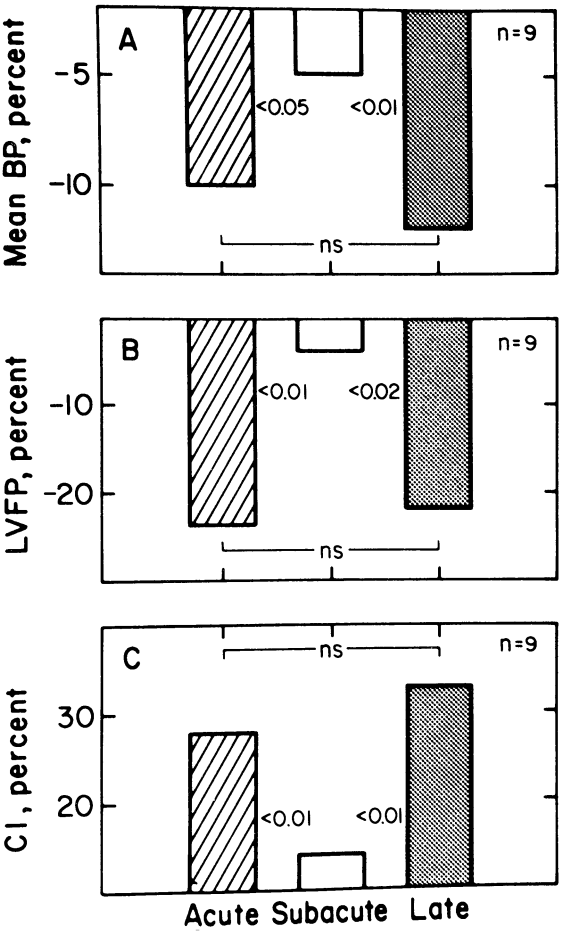

Fig. 3. Sequential hemodynamic cardiocirculatory actions of prazosin (PZ) during continuous ambulatory vasodilator therapy (5 mg four times daily) of severe chronic congestive heart failure (CHF). The initial (Acute) modest lowering of systemic blood pressure (BP, panel A), substantial decline of left ventricular filling pressure (LVFP, panel B) and considerable elevation of cardiac index (CI, panel C) were attenuated at 4 days (Subacute) but were spontaneously restored within 30 days (Late) during uninterrupted oral PZ therapy of CHF. (Reprinted with permission from reference 10.)

by discontinuation of the therapy for a few weeks or by temporary substitution of an alternate vasodilator, such as high-dose hydralazine (75 to 100 mg three or four times daily) plus oral nitrates (isosorbide dinitrate 20 to 40 mg four times daily) (9).

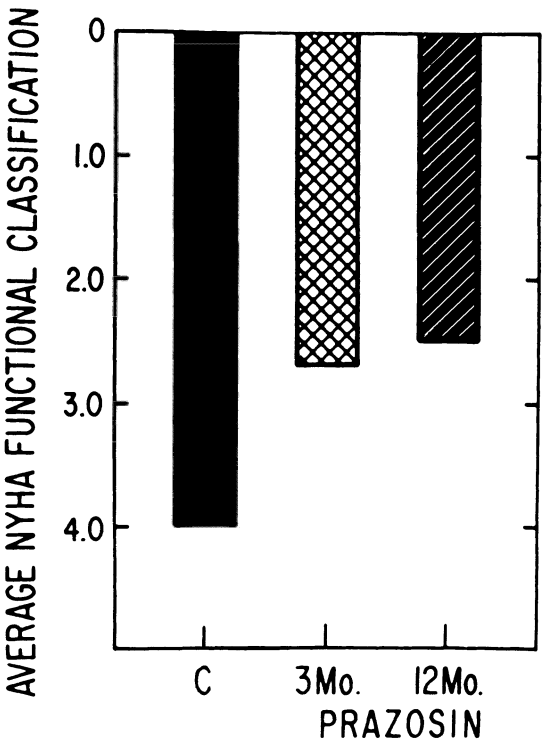

Fig. 4. Effects of chronic prazosin therapy on the New York Heart Association (NYHA) functional classification in the six of sixteen chronic heart failure patients developing PZ-vasodilator late tolerance to chronic PZ dosage of 16 mg daily. C = pre-chronic PZ therapy; 3 mo. = 3 months of chronic PZ therapy; 12 mo. = 12 months of chronic PZ therapy. (Reprinted with permission from reference 9.)

Captopril.

Since the renin-angiotensin-aldosterone system is activated in chronic congestive heart failure, the therapeutic efficacy of the oral angiotensin-converting enzyme inhibitor, captopril, has been evaluated in patients with normotensive cardiac dysfunction. Captopril causes nearly balanced systemic venous and arterial relaxation, resulting in marked decline in elevated left ventricular preload and moderate elevation of depressed cardiac output (12). Furthermore, the beneficial cardiocirculatory actions of chronically administered oral captopril (12.5 to 25 mg three times daily) provide prolonged improvement of ventricular function and clinical status in refractory congestive heart failure (13). In addition, long-term therapy

with the agent tends to decrease rather than increase diuretic requirements due to prevention of secondary hyperaldosteronism.

Conclusions.

The physiologic approach to medical treatment of chronic congestive heart failure is based on improving the four principal determinants of cardiac function (preload, afterload, contractility, and heart rate) to allow the depressed contractile force of the failing pump to deliver normal cardiac output without excessive filling pressure. Management centers on correcting three important aspects of the congestive heart failure state: 1) impaired myocardial contractility, 2) excessive cardiac workload, and 3) body salt and water retention. In the ambulatory management of chronic refractory congestive heart failure, it is now our policy (14) to initially augment the therapeutic regimen with captopril when digitalis and diuretics alone are insufficient and then add conventional vasodilators (prazosin, hydralazine and nitrates) and finally the new oral cardiotonics (beta-adrenergic agonists and milrinone) (Fig. 5). In chronic congestive heart failure intractable to these outpatient measures, relatively sustained benefit may be provided by short periods of in-hospital intravenous therapy by dobutamine or dopamine with nitroprusside or by amrinone.

SUMMARY

In the long-term management of advanced congestive heart failure (CHF), orally administered prazosin (PZ) affords chronic efficacy for several months with addition of this vasodilator to the standard regimen of digitalis and diuretics. Thus, balanced peripheral arterial and venous relaxation of a single PZ dose enhances decreased cardiac output by decreasing total systemic vascular resistance to a similar extent and duration (6-8 hours) as the agent reduces excessive left ventricular (LV) filling pressure by decreasing venous tone. Concordantly, myocardial energetics are improved by decline of elevated LV afterload. Prolonged PZ administration (2-7 mg, 3-4 times daily; average 20 mg/day) provides increased treadmill exercise tolerance, improves NYHA symptomatic class, and causes elevation of reduced scintigraphic and echo LV ejection fraction. These prolonged salutary effects have been demonstrated by serial testing to be sustained for more than 12 months of continued ambulatory PZ therapy in chronic cardiomyopathy.

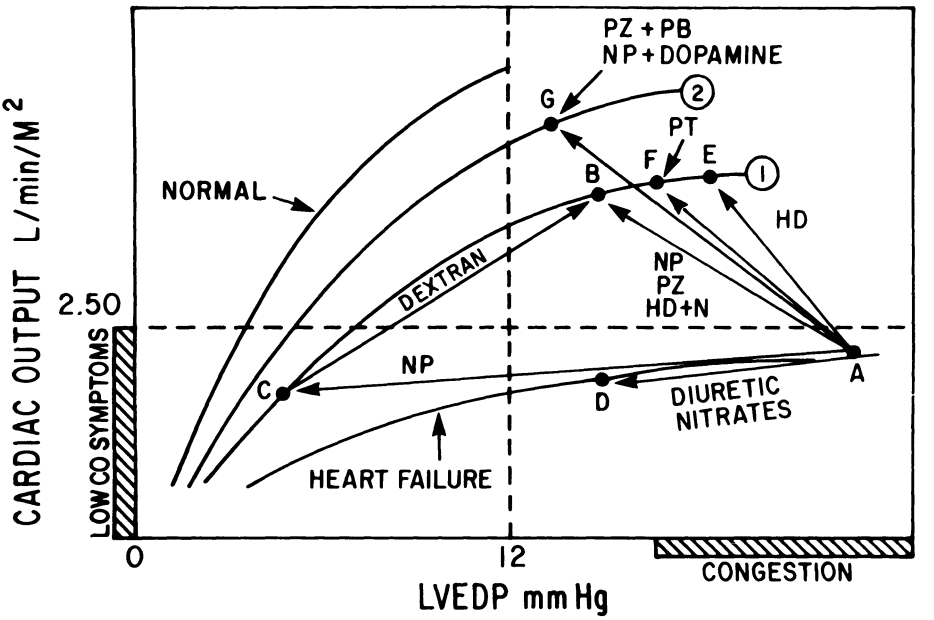

Fig. 5. Relationship between cardiac output (CO) and left ventricular end-diastolic pressure (LVEDP) in a normal subject (left curve) and a patient with congestive heart failure (CHF) (right curve). Point A indicates operation of the dysfunctioning left ventricle in CHF. Intermediate curve (1) shows improved relationships between CO and LVEDP after administration of hydralazine (point E); nitroprusside, prazosin, or combined hydralazine and nitrates to above LVEDP of 12 mm Hg (point B); and nitroprusside to below LVEDP of 12 mm Hg (point C) with addition of dextran (point B). Enhanced CO and reduced LVEDP following administration of phentolamine are shown by point F. Note that improvements from point A on lowest ventricular function curve to point B on intermediate function curve (1) after administration of nitroprusside, prazosin, or hydralazine and nitrates and to point F after phentolamine are not the result of increased contractility; rather, they are due to enhanced relationship between CO and LVEDP as the result of reduction of impedance to left ventricular ejection produced by these vasodilators. Point D on CHF curve is LVEDP after diuretic or nitrate therapy. Intermediate curve (2) demonstrates improvement in CO and decrease of LVEDP achieved with combined intravenous nitroprusside and dopamine or dobutamine therapy, or with combined oral prazosin and pirbuterol administration (point G). Horizontal broken line indicates lower limit of normal for CO and vertical broken line indicates upper limit of normal of LVEDP. Congestion indicates pulmonary congestion. (Reprinted with permission from reference 2.)

With PZ treatment of CHF for more than 3 months, late tolerance of the drug may occur in up to one-third of patients. This phenomenon may take place with long-term use of any of the conventional vasodilator agents, and appears related to accompanying salt and water retention including the vascular wall itself, necessitating increased diuretic requirements and aldosterone antagonism. Prazosin late tolerance can be circumvented, with full responsiveness of the drug reestablished, by discontinuation of PZ therapy for a few weeks or by temporary substitution of an alternate vasodilator.

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References:

1. Mason, D.T. Am. J. Med. 65:106-125, 1978.
2. Mason, D.T., Awan, N.A., Lee, G. and DeMaria, A.N. Arch. Int. Med. 140:1577-1581, 1980.
3. Williams, D.O., Bommer, W.J., Miller, R.R. and Mason, D.T. Am. J. Cardiol. 39:84-90, 1977.
4. Miller, R.R., Vismara, L.A., Williams, D.O. and Mason, D.T. Circ. Res. 39:127, 1976.
5. Awan, N.A., Miller, R.R. and Mason, D.T. Circulation 57:152-159, 1978.
6. Miller, R.R., Williams, D.O., DeMaria, A.N. and Mason, D.T. In: Congestive Heart Failure (Ed. D. T. Mason), Yorke Medical Books, New York, 1976, pp. 343-364.
7. Awan, N.A., Miller, R.R., DeMaria, A.N. and Mason, D.T. Circulation 56:346-354, 1977.
8. Miller, R.R., Awan, N.A. and Mason, D.T. N. Engl. J. Med. 297:303-309, 1977.
9. Awan, N.A., DeMaria, A.N. and Mason, D.T. Am. Heart J. 101:541-547, 1981.
10. Awan, N.A. and Mason, D.T. Am. Heart J. 101:695-700, 1981.
11. Zelis, R., Mason, D.T. and Braunwald, E. J. Clin. Invest. 47:960-970, 1968.
12. Awan, N.A. and Mason, D.T. Am. Heart J. 101:22-31, 1981.
13. Chatterjee, K., Parmley, W.W., Cohn, J.N., Levine, B., Awan, N.A., Mason, D.T., Faxon, D.P., Creager, M., Gavras, H.P., Fouad, F.M., Tarazi, R.C., Hollenberg, N.K., Dzau, V., LeJemtel, T.H., Sonnenblick, E.H., Turini, G.A., Brunner, H.R. Am. Heart J 110:439-447, 1985.
14. Mason, D.T., Chan, M.D. and Lee, G. In: International Practice of Cardiology (Ed. T. O. Cheng), Pergamon Press, New York, 1986, in press.

10

INFLUENCES OF A LONG-TERM TREATMENT WITH ANTIHYPERTENSIVE DRUGS ON LEFT VENTRICULAR MYOSIN ISOENZYME PATTERN IN SPONTANEOUSLY HYPERTENSIVE RATS

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INTRODUCTION

Rat's left ventricular myosin is separated into three isoenzymes by pyrophosphate gel electrophoresis (1-3),i.e. VM-1,2,3, which have greater electrophoretic mobility and ATPase activity in order. Thyroid hormone's administration and endurance swimming training can induce the shift of ventricular myosin isoenzyme pattern towards VM-1. Aging, chronic pressure overload and thyreostatic treatment, on the other hand, can shift the pattern towards VM-3 (4-12). The shift of myosin isoenzyme pattern towards VM-3 in pressure overloaded myocardium is thought to be an adaptational process to keep force development efficient with low oxygen and energy utilization (13,14). In spontaneously hypertensive rats (SHR) the concentration of VM-3 isomyosin in left ventricle is higher than that of age-matched normotensive rats. The aim of the present study is to examine to what extent alters myosin isoenzyme pattern with some antihypertensive drugs.

MATERIALS AND METHODS

20-22 week-old SHR were divided into 5 groups: control without treatment, groups treated with bunitrolol, verapamil, hydralazine and captopril. Bunitrolol and verapamil were administered 30-40 mg/kg/day, hydralazine and captopril were administered 80-90 mg/kg/day and 50-60 mg/kg/day respectively for 8-10 weeks (per os). Systolic blood pressure and heart rate were measured at tail by rat tail manometer-tachometer system (Natsume KN-210-1). Polyacrylamide gel electrophoresis in the presence of pyrophosphate

was carried out as described elsewhere (1-3). The gel contained 3.8% acrylamide and 0.12% N,N'-methylene bisacrylamide. Electrophoresis buffer was 20 mM $Na_4P_2O_7$ (pH 8.8) in the presence of 10% glycerol. Native myosin of left ventricle was extracted with a solution consisting of 100 mM $Na_4P_2O_7$ (pH 8.8), 5 mM 1,4-dithiothritol, 5 ug/ml leupeptin. Electrophoresis was carried out for 30h at 2 C and a voltage gradient of 13.3 V/cm.

RESULTS

As is shown in Table 1, heart weight to body weight ratios of the groups treated with bunitrolol and captopril were significantly smaller than that of non-treated control. The ratio of the group treated with verapamil was also smaller than that of control, although not significant in statistics. In contrast, the ratio of the groups treated with hydralazine was not different from that of control.

Table 1 Comparisons of body weight and heart weight

	Body weight (g)	Heart weight (mg)	$\frac{\text{Heart weight}}{\text{Body weight}} \times 1000$
Control (n=6)	364.2 ± 14.3	1372.2 ± 110.3	3.77 ±0.18
Bunitrolol (n=7)	360.0 ± 34.4	1243.9 ± 166.1	3.45** ±0.17
Verapamil (n=8)	354.4 ± 35.5	1259.5 ± 138.6	3.56$^{\triangle}$ ±0.22
Hydralazine (n=2)	330.0* ± 0.0	1223.0 ± 35.4	3.71 ±0.11
Captopril (n=2)	335.0* ± 7.1	1032.0** ± 56.6	3.08*** ±0.10

means±SD. $^{\triangle}$: P<0.1, *: P<0.05, **: P<0.01, ***: P<0.005

Heart rates of all treated groups except hydralazine-treated group were not different from that of non-treated control, but hydralazine-treated group showed significantly higher heart rate than control (Fig. 1). Blood pressure of hydralazine-treated group was considerably lower than that of control, and this was also seen in captopril-treated group. Blood pressure of the group treated with verapamil was slightly but significantly lower than that of control, but

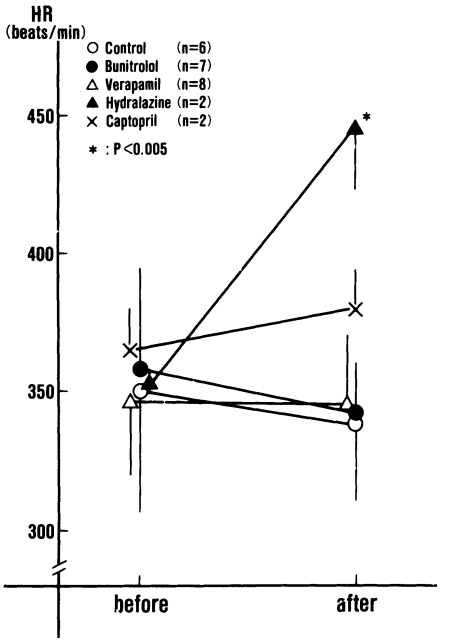

Fig.1 Influence of anti-hypertensive drugs on heart rate

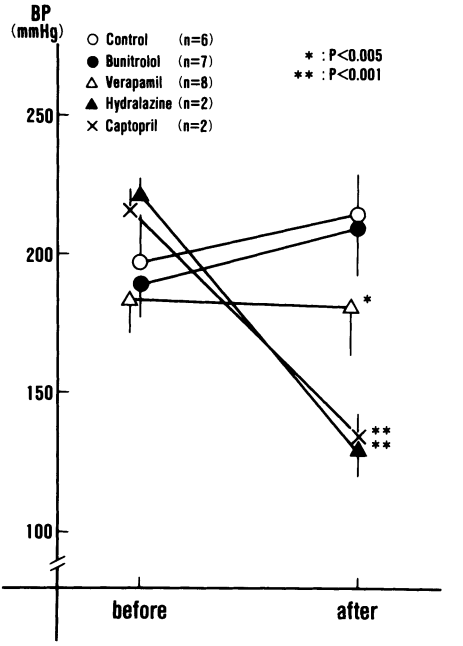

Fig.2 Influence of anti-hypertensive drugs on blood pressure

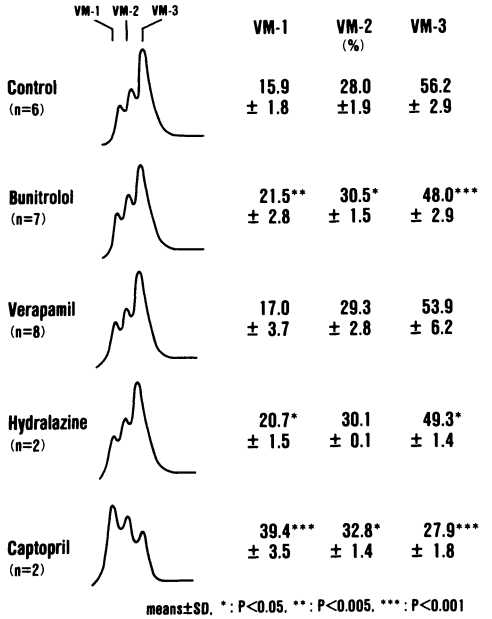

Fig.3 Left ventricular myosin isoenzyme patterns revealed by pyrophosphate gel electrophoresis

there was no significant difference in blood pressure between bunitrolol-treated group and control (Fig. 2).

Fig. 3 shows the comparison of left ventricular myosin isoenzyme pattern. In bunitrolol-treated and hydralazine-treated group myosin isoenzyme pattern was shifted towards VM-1 as compared to control and this was more remarkable in captopril-treated group. In verapamil-treated group myosin isoenzyme pattern was not significantly different from that of control, although slightly shifted towards VM-1.

DISCUSSION

In the present study four antihypertensive drugs were used. Bunitrolol has a beta-blocking effect with a small effect of alpha-blockade, verapamil is calcium channel blocker, hydralazine dilates peripheral arterial smooth muscle and stimulates indirectly adrenergic activity and captopril inhibits the angiotensin-converting enzyme. In bunitrolol-treated group blood pressure was not different from that of control after 8-10 weeks' treatment, but heart weight to body weight ratio was significantly smaller than that of control. In contrast, blood pressure in hydralazine-treated group was considerably reduced by treatment as compared to non-treated control, heart weight to body weight ratio was, however, not different from that of control. These results coincide with those of other authors (15-17), suggesting that adrenergic system may also be related with cardiac hypertrophy. Left ventricular myosin isoenzyme pattern of bunitrolol-treated group shifted towards VM-1, inspite of no significant change of blood pressure. Verapamil-treated group showed little alteration in myosin isoenzyme pattern, although blood pressure was reduced by treatment. This might be because the reduction of blood pressure was not sufficient to induce significant alterations in myosin isoenzyme pattern. Captopril-treated and hydralazine-treated groups showed remarkable reduction of blood pressure in the same degree. Left ventricular myosin isoenzyme pattern of captopril-treated group shifted

remarkably towards VM-1, but the pattern of hydralazine-treated group did not shift so remarkably towards VM-1 as that of captopril-treated group, although the shift was similar to that of bunitrolol-treated group. From these results it may be said that not only cardiac pressure overloads but also other factors, adrenergic system, for example, play a role in alteration of left ventricular myosin isoenzyme pattern in SHR.

SUMMARY

20-22 week-old SHR were treated with antihypertensive drugs and alterations in blood pressure, heart weight and left ventricular myosin isoenzyme pattern were examined. Rats were divided into 5 groups: control SHR without treatment, groups treated with bunitrolol, verapamil, hydralazine and captopril. Bunitrolol and verapamil were administered 30-40 mg/kg/day, hydralazine and captopril were administered 80-90 mg/kg/day and 50-60 mg/kg/day respectively for 8-10 weeks (per os). All treated groups except bunitrolol-treated group showed lower blood pressure than non-treated control SHR. Heart weight to body weight ratios of the groups treated with bunitrolol and captopril were smaller than that of control. Left ventricular myosin isoenzyme pattern revealed by pyrophosphate gel electrophoresis was shifted towards VM-1 in the groups treated with bunitrolol, hydralazine and captopril. It is concluded that not only cardiac pressure overloads but also other factors, adrenergic system, for example, may play a role in cardiac hypertrophy and alterations of left ventricular myosin isoenzyme pattern in SHR.

REFERENCES

1. Hoh,J.F.Y., McGrath,P.A. and Hale,P.T.J.Mol.Cell. Cardiol. 10: 1053-1076,1978.
2. d'Albis,A., Pantaloni,C. and Becher,J.-J.Eur.J.Biochem. 99: 261-272,1979.
3. Rupp,H. and Jacob,R. Can.J.Physiol.Pharmacol. 60: 1098-1103,1982.

4. Lompre,A.-M., Schwartz,K., d'Albis,A., Lacombe,G., Van Thiem,N. and Swyghedauw,B. Nature 282:105-107,1979.
5. Mercadier,J.J., Lompre,A.M., Wisnewsky,C., Samuel,J.L., Bercovici,J., Swynghedauw,B. and Schwartz,K. Circ.Res. 49: 525-532,1981.
6. Rupp,H. Basic.Res.Cardiol. 76: 79-88,1981.
7. Alpert,N.R., Mulieri,L.A. and Litten,R.Z. In: Cardiac adaptation to hemodynanic overload, training and stress (Eds. R.Jacob, R.W.Gülch and Kissling), Dr.D.Steinkopff Verlag, Darmstadt, 1982, pp.147-157.
8. Jacob,R., Rupp,H., Ebrecht,G., Holubarsch,Ch. and Kissling,G. Z.Kardiol. 71: 553-565,1982.
9. Martin,A.F., Pagani,E.D. and Solaro,R.J. Circ.Res. 50: 117-124,1982.
10. Rupp,H. Basic.Res.Cardiol. 77: 34-46,1982.
11. Takeda,N., Dominiak,P., Türck,D., Rupp,H. and Jacob,R. Basic.Res.Cardiol. 80: 88-99,1985.
12. Takeda,N., Dominiak,P., Türck,D., Rupp,H. and Jacob,R. Basic.Res.Cardiol. 80: 384-391,1985.
13. Jacob,R., Rupp,H., Ebrecht,G., Gülch,R.W. and Kissling, G. In: Cardiac structure and metabolism 4 (Eds. M.Nagano and I.Seki), Roppo Shuppansha, Tokyo, 1981,pp. 1-28.
14. Jacob,R., Kissling,G., Ebrecht,G., Holubarsch,Ch., Medugorac,I. and Rupp,H. In: Advances in Myocardiology 4 (Eds. E.Chazov, V.Saks and G.Rona), Plenum Publishing Corporation, New York, 1983, pp.55-77.
15. Sen,S., Tarazi,R.C., Khairallah,P.A. and Bumpus,F.M. Circ.Res. 35: 775-781,1974.
16. Sen,S., Tarazi,R.C. and Bumpus,F.M. Cardiovasc.Res. 11: 427-433,1977.
17. Pegram,B.L., Ishise,S. and Frohlich,E.D. Cardiovasc. Res. 16: 40-46,1982.

11

ENHANCED ACTION OF NIFEDIPINE UPON VASCULAR SMOOTH MUSCLE FROM HYPERTENSIVE RATS

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INTRODUCTION

Calcium channel blockers are compounds that seem to be more active in lowering arterial pressure in hypertensive persons than in normotensives (1,2). Actually, a lack of effect of nifedipine (NIF) upon blood pressure in normotensive humans has been reported (3). Experiments from Bühler et al (1) and from our own laboratory (2) show that the extent of the decrease in arterial pressure is directly related to the pretreatment level of arterial pressure. The reason for the enhanced action of these drugs in hypertension (4,5) is still unknown, and it is atractive not only because of their therapeutic advantages but also because of the possibility of modifying the mechanism causing the hypertensive state.

The experiments we are going to present were designed to answer the following questions:

1) Is NIF active on blood pressure levels of normotensive humans?.
2) Does the hypotensive action of NIF depend on pretreatment blood pressure?.
3) Is the hypotensive action related to the etiology of hypertension?.
4) Can we explain this effect by its vasodilator properties through the calcium influx blockage?.

MATERIALS AND METHODS

Fifteen normotensive volunteers ages between 19 and 33 years and eleven patients with essential hypertension were studied before and after a sublingual dose of NIF, 20 mg. Blood

pressure, heart rate, forearm blood flow and forearm vascular resistance were calculated as described elsewhere (2).

Male Wistar rats weighing 150-200 g were used for control (NR) and for the production of experimental hypertension. Normotensive (WKY) and hypertensive (SHR) rats were obtained from an offspring of the Okamoto-Aoki strain (5). Hypertensive rats by deoxycorticosterone acetate (DOCA) and renal hypertensive rats (2K-1C and 1K-1C) were obtained as described elsewhere (5,6). A local strain obtained by crossing SHR with NR was also used (L-SHR), in order to obtain a lower level of arterial pressure, similar to that of 2K-1C and 1K-1C. Systolic blood pressures of the different experimental groups appear in Table 1.

Table 1. Systolic blood pressure in the different groups.

NR	WKY	SHR	DOCA	L-SHR	2K-1C	1K-1C
126	141	203	164	168	167	176
± 2	± 6	±10	± 5	± 2	± 4	± 9
(10)	(6)	(4)	(6)	(40)	(8)	(6)

Mean ± SEM with number of animals between parentheses. Abbreviations as in Methods.

Concentration-response curves to NIF and nitroglycerin were performed in aortic rings studied under isometric conditions and precontracted by high K (35 mM), as described (5-9).

Calcium influx was studied by the lanthanum method described by Karaki and Weiss (10,11) with minor modifications.

Chemically skinned aortic smooth muscle was prepared following the procedure described by Ruegg and Paul (12).

RESULTS

Fig. 1 shows that after 30 min of the administration of NIF to normotensive humans, mean arterial blood pressure fell 8 ± 2 mmHg, heart rate increased 10 ± 2 beats/min, forearm blood flow increased 1.5 ± 0.2 ml/min . 100 ml and forearm vascular resistance decreased by 22 ± 9 %.

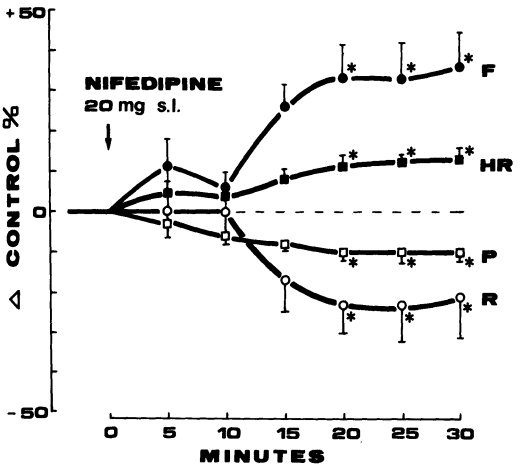

Fig. 1. Hemodynamic effects of NIF 20 mg sublingual in 15 normotensive volunteers. Forearm blood flow (F) and heart rate (HR) increased after NIF, while mean arterial pressure (P) and calculated forearm vascular resistance (R) decreased. *: $P < 0.05$ with respect to control values. Note that all measurements are expressed as % of change from control values. (Reprinted with permission from Ref. 2).

Fig. 2 shows the correlation between pretreatment diastolic blood pressure after 20 mg of NIF. The higher the pretreatment level of arterial pressure, the greater was the decrease in pressure after NIF. The correlation coefficient was -0.64.

Fig. 3 shows a typical experiment in which a single dose of NIF 10^{-13} M relaxes the high K-contracted ring from an SHR aorta, but fails to relax the WKY ring.

Fig. 4 depicts concentration-response curves to the relaxant effect of NIF in aortic smooth muscle from normotensive and hypertensive rats contracted by high K. Rings from any of the hypertensive rats relax much more than rings from normotensive rats at any of the concentrations employed. Within the different hypertensive groups, a greater shift to the left of the concentration response curve was evident in SHR. Active tension after exposure to high K was similar in all groups (inset).

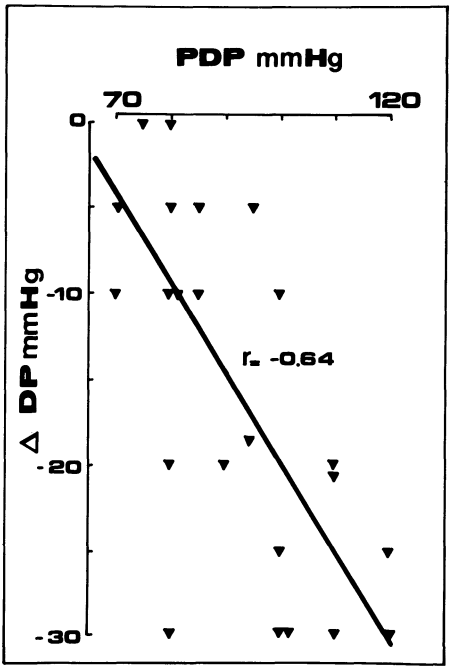

Fig. 2. Fall in diastolic blood pressure after 20 mg NIF sublingual (ΔDP) plotted as a function of pretreatment levels of diastolic blood pressure (PDP). Data from 15 normotensive and 11 hypertensive patients. Note that the higher the PDP, the greater was the decrease in diastolic blood pressure after NIF. (Reprinted with permission from Ref. 2).

Fig. 5 shows the EC50 (concentration necessary to relax by 50% the previous contracture) for NIF plotted as a function of the systolic blood pressure of normotensive and hypertensive rats. EC50 seems to be a function of the blood pressure of the group and independent of the method by which hypertension was produced. Note that groups with similar blood pressure, like L-SHR and 2K-1C, have also similar EC50.

Fig. 6 depicts concentration-relaxation curves to nitroglycerin in WKY, L-SHR, 2K-1C and 1K-1C. Both the contractile response to high K (inset) and the relaxant response to nitroglycerin were not different in the group studied.

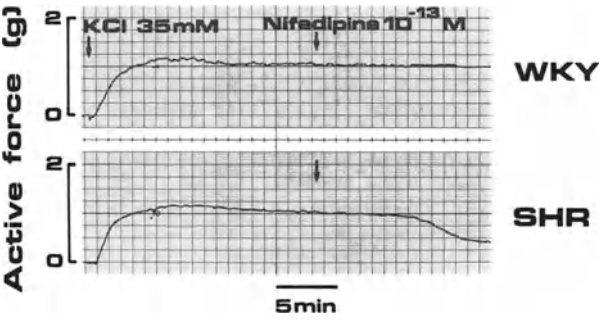

Fig. 3. Typical experiment showing the effect of a single dose of nifedipine (NIF) 10^{-13} M on KCl-contracted aortic rings from WKY and SHR rats. NIF had no effect on WKY, but produced significant relaxation in SHR (Reprinted with permission from ref. 5).

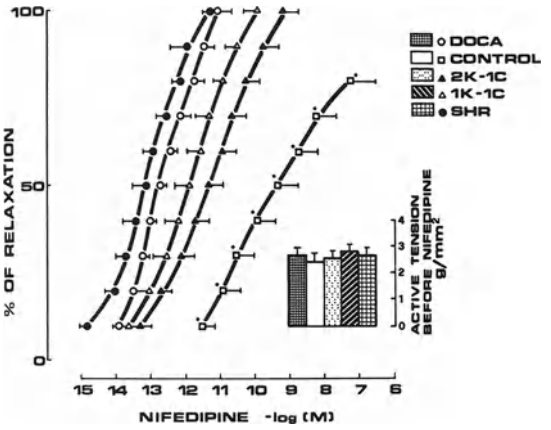

Fig. 4. Relaxant effect of nifedipine (NIF) on KCl-contracted aortas from DOCA, NR, 2K-1C and SHR rats. Note that a much lower concentration of NIF was necessary to relax any of the rings from hypertensive rats as compared with NR rats. Inset: active tension before exposure to NIF was similar in all groups. Horizontal bars represent 1 SEM above and below mean; $P < 0.05$ with respect to control (Reprinted with permission from ref. 5).

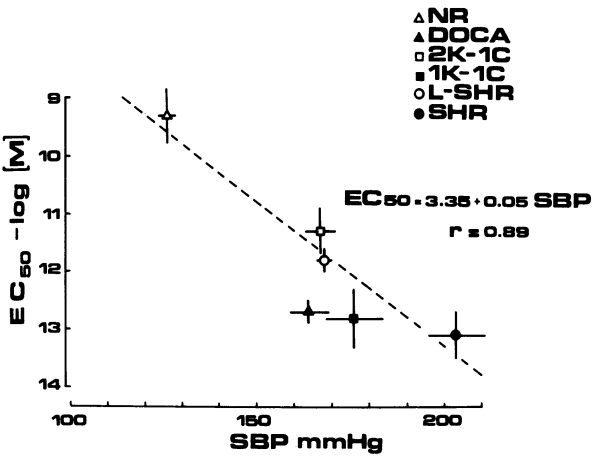

Fig. 5. Mean systolic blood pressure (SBP) of NR, DOCA, 2K-1C, 1K-1C, L-SHR and SHR rats plotted as a function of the EC50 for the relaxant effect of nifedipine (NIF) for each group. Note that the higher the SBP, the lower the EC50 for NIF. Bars represent 1 SEM above and below mean (Reprinted with permission from ref. 5).

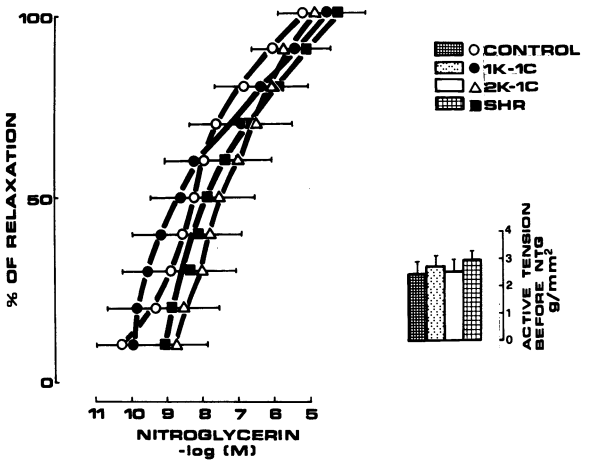

Fig. 6. Relaxant effect of nitroglycerin (NTG) in KCl-contracted aortic rings from NR, 1K-1C, 2K-1C and SHR rats. Note that the relaxant effect of NTG was similar in all groups. Inset: Active tension before exposure to NTG, showing no differences between groups. Horizontal bars represent 1 SEM above and below mean. (Reprinted with permission from ref. 5).

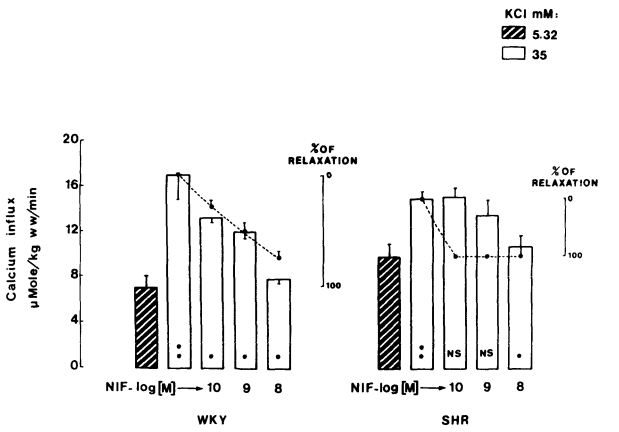

Fig. 7. Calcium influx blockage and relaxation measured in WKY and SHR. In both groups K-PSS augments significantly the calcium influx (first open bar with respect to cross-hatched bar). In WKY the effect of NIF from 10^{-10} to 10^{-8} M on calcium influx (open bars) is accompanied by mechanical relaxation (broken line). On the contrary, in SHR the mechanical effect is not accompanied by calcium influx blockage at concentrations of NIF of 10^{-10} and 10^{-9} M. NIF 10^{-8} M promotes calcium influx blockage with the muscle already relaxed. **P < 0.05 with respect to KCl 35 mM.

Fig. 7 shows the effects of NIF upon calcium fluxes (bars) and high K-induced contracture (broken lines). Basal influx (cross-hatched bars) is significantly increased by exposure to high K in both WKY and SHR (first open bar). In WKY, exposure to NIF 10^{-10}, 10^{-9} and 10^{-8} M produced Ca^{2+} influx blockage and graded relaxation of the contracture. In SHR relaxation was 100% with any of the concentrations of NIF, but Ca^{2+} influx blockage was significant only with the 10^{-10} M concentration. Then, in SHR, the mechanical effect of NIF was not related to its effect upon Ca^{2+} influx.

Fig. 8 shows the effect of NIF 10^{-10} M on chemically skinned arteries from WKY and SHR, contracted by exposure to pCa 6. NIF elicits significant relaxation in SHR but not in WKY.

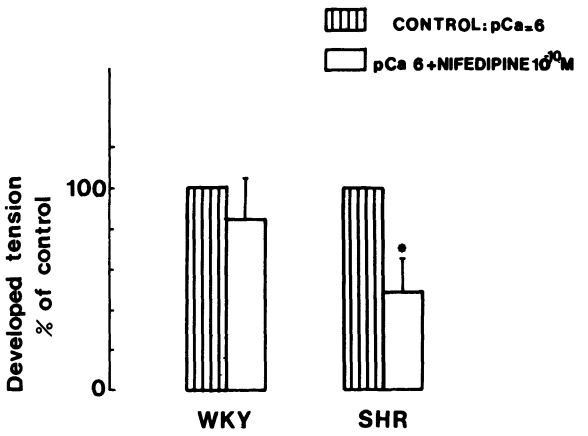

Fig. 8. Effect of NIF 10^{-10} M on chemically skinned aortic smooth muscle. A contraction was induced by exposure to pCa= 6 (cross hatched bar). NIF 10^{-10}M relaxes SHR arteries but not WKY arteries. *: $P < 0.05$ with respect to its own control.

DISCUSSION

The data presented here show modest but statistically significant effects of NIF upon forearm blood flow of normotensive human beings. This effect results in a slight decrease in arterial pressure. The hypotensive action seems to be a function of pretreatment levels of arterial pressure, the effect being greater when the pretreatment arterial pressure is higher. These data are consistent with previous observations (1,23).

The calcium channels are the binding sites for dihydropyridines and their mechanism of action to produce relaxation in vascular smooth muscle seems to be the blockage of calcium influx through the voltage-operated channels. A greater sensitivity of aortic smooth muscle from hypertensive rats was detected, whereas no differences in sensitivity to other vasodilator (nitroglycerin) were found.

A shift to the left in concentration-response curves was detected in hypertensive smooth muscle. The shift is signifi-

cant with any of the hypertensive models used (SHR, DOCA, 2K-1C and 1K-1C). The greatest shift, however, was detected between normotensive rats and SHR. This finding could be related to the mechanism involved in the pathogenesis of hypertension or could be a simple consequence of the higher level of arterial pressure in the last experimental group. Fig. 5 shows that the sensitivity to the relaxant effect of NIF seems to be correlated with the arterial pressure of the animal and independent of the way used to produce hypertension. L-SHR and 2K-1C rats, with similar blood pressure are having similar EC50 (concentration necessary to relax by 50 % the precontracted rings) in spite of the etiology of hypertension being different.

The term "calcium antagonist" was coined after Fleckenstein (13). However, the term "calcium entry blockers" was proposed lately based on the involved correlation between calcium influx blockage and relaxation (14,15). This correlation was also detected in our experiments with aortic rings from normotensive rats. If we assume this mechanism as valid, an enhanced relaxation should be accompanied by a greater calcium influx blockage. However, a lack of correlation between calcium influx blockage and relaxation was detected in hypertensive smooth muscle. A dose of nifedipine of 10^{-10} M elicited a complete relaxation in the SHR without significant effect in calcium influx blockage. Furthermore, increasing the concentration of the compound to 10^{-9} and 10^{-8}M,a calcium influx blockage under the completely relaxed state was detected. These data are consistent with a mechanism other than calcium influx blockage producing relaxation in hypertensive smooth muscle.

If the greater sensitivity of hypertensive smooth muscle to nifedipine is not followed by a greater calcium influx blockage (actually no calcium influx blockage was detected at concentrations producing 100 % of relaxation) other mechanisms should be considered. A greater calcium efflux promoted by the compound in the hypertensive smooth muscle could account for the enhanced relaxation. In normal smooth muscle a lack of effect of nifedipine upon calcium efflux was previously reported (16) and we were unable to detect changes in hypertensive

aortic smooth muscle (data not shown).

Our data are consistent then with the hypothesis that nifedipine produces smooth muscle relaxation in hypertensive smooth muscle through mechanisms other than calcium influx blockage, and consistent also with the possibility that this pharmacological intervention results in a diminished formation of the Ca^{2+}-calmodulin-MLCK complex. The diminished formation could be the result of a binding of the drug to calmodulin (17), a decrease in affinity of the Ca^{2+}-CaM complex for the light chain kinase, an increase in phosphatase activity (18) or a direct action of the drug upon the MLCK. In connection with this, the binding of dihydropyridines to calmodulin has been reported but at doses higher than 10^{-10} M (19-21). If the binding of dihydropyridines to calmodulin could play a role in the mechanism of relaxation, we would predict a greater effect if a deficit in calmodulin exists. A decrease in calmodulin concentration in smooth muscle from hypertensives was reported (22) and could explain the differences in pharmacological sensitivity to the compound if we accept an anticalmodulin effect of the drug. In any case, in the light of the present results, it seems reasonable to re-examine the mechanism responsible for the vasodilator effect of NIF in hypertension, and to consider the possibility that the term "calcium influx blocker" for NIF should be challenged at least in hypertension.

SUMMARY

The data presented by us in normotensive human beings show slight but statistically significant decrease in arterial pressure after 20 mg of NIF. The same dose produced an effect related to the pretreatment level of arterial pressure in hypertensives: the higher the pressure, the greater the effect.

Aortic smooth muscle from rats made hypertensive by different methods were compared in their sensitivity to the relaxant effect of nifedipine (NIF) with vascular smooth muscle from normotensive rats.

The relaxant effect of NIF was much more pronounced in

hypertensive than in normotensive rats, while the relaxation produced by nitroglycerin (NTG) was similar. The increased sensitivity seems to be related to the level of arterial pressure and independent of the etiology of the hypertension. Rats with spontaneous hypertension have EC50's not different to those of rats with renal hypertension when arterial pressure levels were similar.

Whereas the relaxant effect of NIF in normotensive rats seems to be correlated with its effect upon calcium channel blockage, this was not the case in hypertensive rats. The relaxant effect of NIF was dissociated from its effect upon calcium influx blockage in hypertensive rats. NIF 10^{-10} M relaxed the hypertensive smooth muscle by 100 % without producing calcium influx blockage. Chemically skinned aortic fibers from hypertensive rats were more sensitive to the relaxant effect of NIF.

Our data suggest that at least in hypertension NIF can produce smooth muscle relaxation by a mechanism other than calcium influx blockage.

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REFERENCES

1. Bühler, F.R., Hulthen, V.L., Kiowski,W. and Bolli,P. Clin. Sci. 63: 439s-442s, 1982.
2. Rinaldi, G.J. and Cingolani, H.E. Acta Physiol. Pharmacol. Latinoam. 36(2), 1986 (in press).
3. Lederballe-Pedersen,O., Christensen,N.J. and Ramsch,K.D. J. Cardiovasc. Pharmacol. 2: 357-366, 1980.
4. Kazda,S., Garthoff,B. and Knorr,A. J. Cardiovasc. Pharmacol. 7 (suppl. 6): 561-565, 1985.
5. Amado Cattaneo,E., Rinaldi,G.J., Mattiazzi,A.R. and Cingolani,H.E. J. Cardiovasc. Pharmacol., 1986 (in press).
6. Gende,O.A., Mattiazzi,A.R., Camilión,M.C., Pedroni,P., Taquini,C., Gómez Llambi, H. and Cingolani,H.E. Am. J. Physiol. 249: H814-H819, 1985.
7. Rinaldi,G.J. and Cingolani,H.E. Circulation 68: 1315-1320, 1983.
8. Lardani,H.E., Rinaldi,G.J. and Cingolani,H.E. Am. J. Cardiol. 50: 869-873, 1982.
9. Camilión de Hurtado,M.C., Rinaldi,G.J., Mattiazzi,A.R. and

Cingolani,H.E. Medicina (Buenos Aires) 44: 137-144, 1984.
10. Karaki,H. and Weiss,G.B. J. Pharmacol. Exp. Ther. 211(1): 86-92, 1979.
11. Karaki,H. and Weiss,G.B. J. Pharmacol. Exp. Ther. 213(3): 450-455, 1980.
12. Ruegg,J.C. and Paul,R.J. Circ. Res. 50: 394-399, 1982.
13. Fleckenstein,A. Toxicol. 17: 149-166, 1977.
14. Van Breemen,C., Hwang,O.K. and Meisheri,K.D. J. Pharmacol. Exp. Ther. 218: 459-463, 1981.
15. Godfraind,R., Miller,R.C. and Socrates Lima,J. Br. J. Pharmacol. 77: 597-604, 1982.
16. Van Breemen,C., Lukeman,S. and Cauvin,C. Am. J. Med. 77 (4A): 26-30, 1984.
17. Johnson,J.D. and Fugman,D.A. J. Pharmacol. Exp. Ther. 226: 330-334, 1983.
18. Bialojan,C., Ruegg,J.C. and Di Salvo,J. Proc. Soc. Exp. Biol. Med. 178: 36-45, 1985.
19. Bostrom, S.-L., Ljung,B., Mardh,S., Forsen,S. and Thulin,E. Nature 292: 777-778, 1981.
20. Epstein,P., Fiss,K., Hachisu,R. and Andrenyak,D.M. Biochem. Biophys. Res. Commun. 105: 1142-1149, 1982.
21. Lamers,J.M.J., Crosouw,K.J. and Verdouw,P.D. Biochem. Pharmacol. 34: 3837-3843, 1985.
22. Hermsmeyer,K. and Kuthe,C. J. Cardiovasc. Pharmacol. 6: S933-S936, 1984.
23. Hulthen,U.L, Bolli,P., Amann,F.W., Kiowski,W. and Buhler, F.R. Hypertension 4 (Suppl. II): II-26/II-31, 1982.

12

AJMALOON A UNIQUE HERBAL POLYPHARMACEUTICAL POSSESSING BOTH HYPOTENSIVE AND CADMIUM LOWERING PROPERTY.

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INTRODUCTION

The role of Cardiovascular diseases, in mortality statistics has long been firmly established. However, after improvement of treatment for essential hypertension the mortality due to cardiac & renal failure has decreased which were once the major causes for death due to hypertension. Although the principal causes of mortality are cerebral & myocardial infarction due to associated atherosclerosis (1). Epidemiological studies show essential hypertension is most commonly prevalent among the age group of 40-60 yrs in both sexes.

Regarding the pathogenesis of hypertension a number of theories has been proposed. A new concept of the role of trace elements is in consideration now-a-days. Trace elements which can be associated with the pathogenesis of hypertension are Cadmium, Lead, Mercury & Sodium as pressors and Zinc, Copper, Magnesium, Potassium and Selenium as depressors on the basis of animal experiments and epidemiological studies (2).

Elements particularly essential trace elements play both curative & preventive roles in combating diseases. The curative features displayed by iron in anemia, iodine in goitre and fluorine for dental caries are clinical examples. Elements such as cadmium, mercury, lead and thallium are known as inherently toxic as they impair health in minute concentrations (3).

Regarding the pathogenesis of hypertension, Cadmium has been given a special emphasis as a possible factor

for hypertension and arteriosclerotic diseases (4,5,6,7,8, 9,10). Cadmium in small amounts when injected parenterally or orally induces hypertension (10,11,12,13) in experimental animals. Talwar et al (14) recently reported that a biphasic response induced by Cadmium, in rats, i.e. an initial fall followed by sustained rise in blood pressure was antagonised by Zinc sulphate. However the rise in blood pressure in reserpinised rats due to cadmium injected intravenously was not statistically significant (15) which supported that cadmium induced hypertension was associated with rise in plasma noradrenaline level (16) and gave a ground for development of newer catecholamine depleting drugs for hypertension.

Recently it was shown that serum cadmium concentrations were significantly higher and serum zinc levels were lower in hypertensives (17). This finding is in support to an earlier observation that plasma cadmium/ zinc ratio was significantly greater in hypertension and there was a significant positive co-relation between plasma cadmium/zinc ratio and arterial blood pressure (18).

The present study was conducted with a view to vindicate the above observations and to develop a new effective and safe drug from Rauvolfia serpentina which will not possess the dangerous side effects of reserpine (19,20,21,22,23).

MATERIALS AND METHODS :

The drug used for clinical trial, code named as Ajmaloon is a herbal polypharmaceutical derived from the combination of alcoholic extract of Rauvolfia serpentina (Roots) as main active ingredient and other toxicity corrective herbs

Each tablet of Ajmaloon weighs 500 mg and had the following herbal ingredients :- (See next page) (Table 1)
Ajmaloon is developed by Hakim Abdul Hameed.

(Table 1)

BOTANICAL NAME	PARTS USED	PROPERTY	QUANTITY
1. Zea mays	Styles	Diuretic (24)	50 mg.
2. Cicer arietinum	Seed coat	Diuretic (24)	50 mg.
3. Juniperus communis	Berries (Oil)	Diuretic (24)	1 mg.
4. Rauvolfia serpentina	Root Alcoholic Ext.	Anti-hypertensive	6.2 mg.
5. Hordeum vulgare	Grains	Demulcent (24)	25 mg.

The drug was standardized phytochemically, using various physical & chemical parameters and estimation of total alkaloids for determining the various organic constituents. Elementological standardization of the drug was done for essential & toxic elements viz As, Na,K,Ca,Mg, Co, Ni, as they play some role in cardiovascular diseases. Biological standardization was also done by comparing Ajmaloon with reserpine by lethal activity in mice and hypotensive activity in dogs (25).

Toxicity

Acute toxicity studies of the drug in animals revealed no serious adverse effects except ptosis. No mortality occured during 24hrs to 4-6 weeks at dose level of 25.6 g/kg,about forty times the therapeutic dose in hypertensive patients. An institutional review board was formed consisting of physicians, pharmacists, jurists, consumer organizations representatives for approving the clinical trial or to recognize any ethical objection (26). It is evident that before a drug can be used to treat patients, elementary safety evaluation was done in healthy human volunteers starting with single dose and progressively increasing doses. The volunteers were screened clinically, biochemically & pathologically before and after treatment with the drug. Clinically slight fall in Blood pressure was observed although statistically insignificant, otherwise the pathological & biochemical changes were within normal limits.

Clinical trial

170 patients of essential hypertension between

the ages of 40-60 yrs visiting Hamdard Clinic at Asaf Ali Road of both the sexes were selected. 127 patients were treated with drug and 43 with Placebo. Placebo treated patients were having diastolic pressure of the range 90-105. A detailed investigational proforma including the patients personal, diet, job and history of residential area was recorded. Detailed clinical Electocardiographic, fundoscopic and relevent pathological, biochemical & elementological investigations were conducted. Fundoscopy was done as a routine to classify the grade of hypertension. Blood pressure was checked everyday and a proper record was maintained.

The dose of the drug ranged between 500 mg to 3 gm daily in divided doses according to the level of diastolic pressure as per table given below (Table 2).

(Table 2)

BLOOD PRESSURE PATIENTS	DOSES
Diastolic B.P. $>$ 120 -	6 tablets daily 2 TDS
" B.P. $<$ 120 $>$ 100	4 tablets daily 2 BD
" B.P. $<$ 100 $>$ 90	2 tablets daily 1 BD
" B.P. $<$ 90 $>$ 80	1 tablet daily.

All the patients were kept on salt restricted diet maximum upto 5 gms. Elemental analysis of 75 hypertensives & 60 normotensives were performed by flame atomic absorption spectrophotometer with (Perkin elmer 303) apparatus for zinc. Flameless AAS (Perkin Elmer 306) with graphite furnace HGA 400 was used for the analysis of Cadmium (27,28,29). Serum of hypertensives were analysed before and after treatment with Ajmaloon.

RESULTS

The effect of drug Ajmaloon in lowering hypertension has been found highly significant ($P < 0.001$). It was observed that medicine has very good effect in mild & moderate hypertensives within the Ist month, as the blood pressure was reduced appreciably. However, continuous use of the drug over a reasonable period of 6

months is able to control the blood pressure even in severe hypertensives (For details see Fig.1&2).

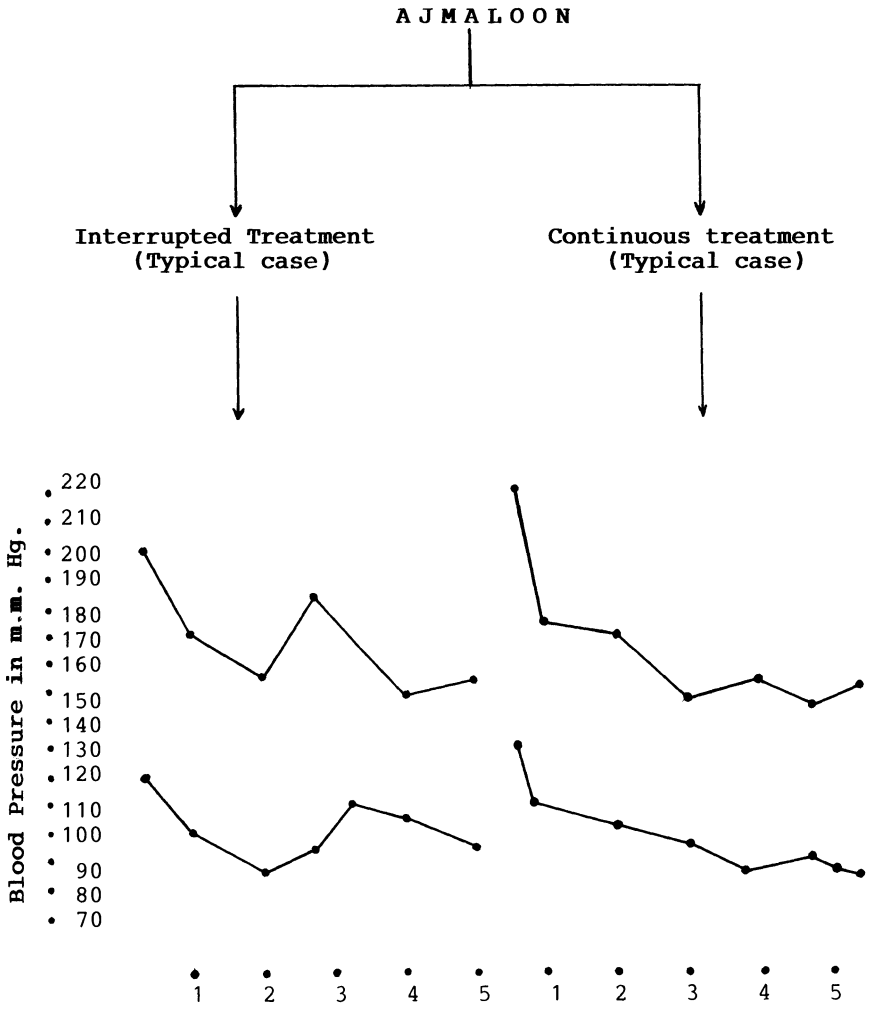

Fig.1 Effect of Ajmaloon in typical hypertensive patient.

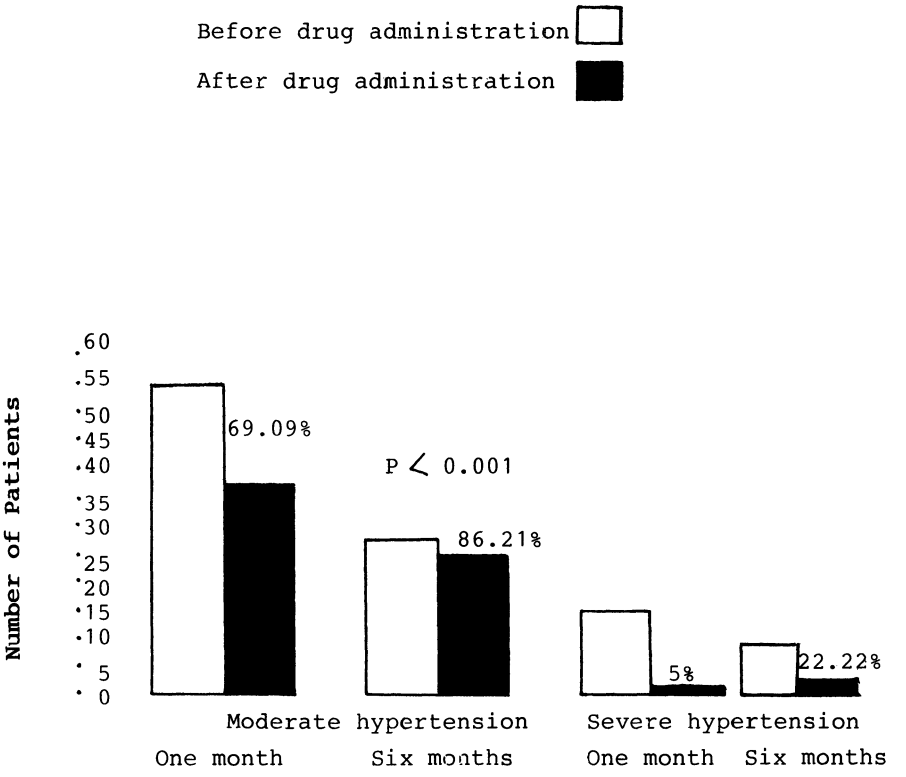

Fig.2 Benefit in diastolic blood pressure of hypertensives by administration of Ajmaloon.

Serum cadmium & zinc levels were analysed in 75 hypertensives. It was found that in hypertensive patients the cadmium level was 38.9% higher and zinc level was 25% lower when compared with 60 normotensives. Another observation on these serum inorganic elemental levels showed that drug Ajmaloon tries to normalise the serum cadmium level but the serum zinc levels were unchanged (see Fig.3). However long term stutides on a large number of patients are mandatory before a categorical statement can be made.

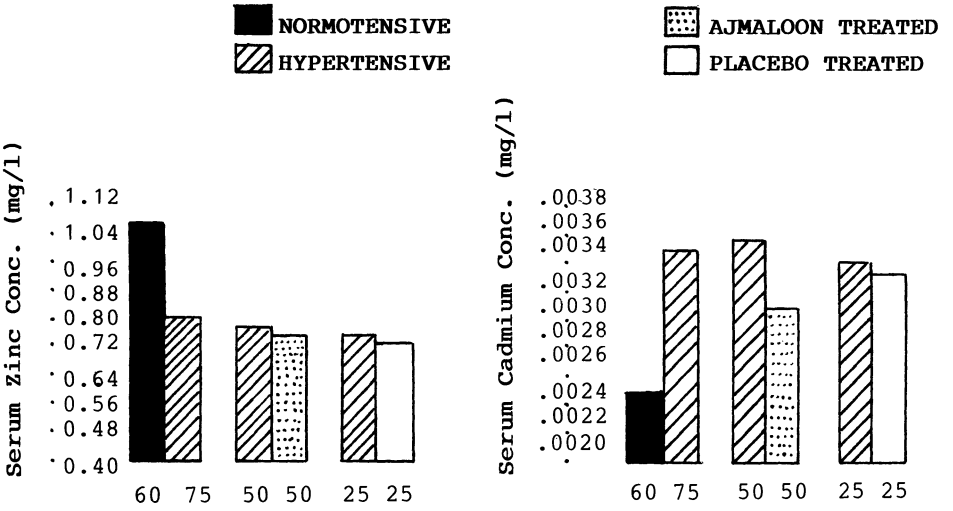

Fig.3 Comparison of Zinc and Cadmium level of normotensives and hypertensives before and after administration of Ajmaloon

DISCUSSION

A new concept of medical elementology i.e. the role of trace elements on human health makes it important for the physicians, to the consideration of trace elements deficiencies & excesses in the various causes of pathological disease in humans. Among the numerous trace elements currently 15 are thought to be essential for human beings - Arsenic, Chromium, Cobalt, Copper, Iodine, Fluorine, Iron, Manganese, Molybdenum, Nickel, Selenium, Silicon, Tin, Vanadium, Zinc (30).

Elements such as Cadmium, Mercury, Lead &Thallium are known as inherently toxic since they impair health even at minute concentration (30). Cadmium has received considerable attention in terms of its relationship to hypertension (31,32). Relationship of experimental hypertension due to cadmium intake and human disease have been reviewed in literature (33,34). Metals like zinc in the form of zinc chelate & zinc sulphate have been shown to reverse Cadmium induced hypertension (35,20) which is in support to the earlier observation of disappearance of cadmium from rat plasma following intravenous injection of zinc (36) and possible explanation was displacement of albumin cadmium by excess zinc. A further review suggests that Cadmium administration can give rise to severe symptoms of zinc deficiency (37,38) and recently an interaction between the macro-molecules of cadmium & zinc has been suggested (39).

This created an incentive for our clinical work on the role of cadmium & zinc in human hypertension. Our purpose is not to persuade a role of Cadmium as the data are too inconclusive, rather we are trying to convince you that this field warrants further work. At present Cadmium has no known biological effects in man except for its well recognized toxic effects which are associated with extensive and easily demonstrable exposure. Literature reveals that human beings who had significant long term low-level cadmium exposure had shown a considerable accu-

mulation of cadmium in the body. Chronic effects of cadmium exposure in humans have been well documented in last two decades. long term exposure to cadmium leads to the development of lung insufficiency, osteomalacia, anaemia & hypertension (40).

The severe form of chronic cadmium poisoning in the itai-itai disease which is characterized by osteomalacia and prevalent among elderly women in zinzu river area of Toyama, due to prolonged oral intake of cadmium, are also suffering from severe renal dysfunction (41) and enteropathy (42). Cadmium toxicity has become an occupational hazard in the industries manufacturing alkaline batteries with nickel and cadmium electrodes (43,44,45 46). Hypertensive individuals have been reported to have more renal cadmium than normotensive subjects. It has been also shown that known hypertensive patients excrete upto 40 times as much cadmium in their urine as do the normal controls (47).

In one of our recent studies we have shown that serum cadmium concentrations were significantly higher while serum zinc levels were significantly lower in hypertensive individuals (48,49) when compared to normal control group. Although the mechanism by which cadmium induces hypertension remains unclear, 4 potential pressure effects (50,51) have been observed : (a) Sodium retention (50,51) (b) increased peripheral resistance (52) (c) hyperreninemia (53) (d) increased cardiact output (52). Recently action of cadmium as an antinatriuretic and a pressure agent has been demonstrated (54). Experimental studies on animals also suggest that protein and carbohydrates have synergestic effect on cadmium induced hypertension while calcium has preventive role against this induction (55,56). In the present work it has been observed that serum cadmium levels are higher & serum zinc levels are lower in hypertensives in comparison to normal controls of the same age group. Cadmium & zinc interaction is due to competitive inhibition of zinc uptake process by cadmium as revealed

by intestinal kinetic studies on experimental animals (57,58). Cadmium is concentrated in the kidney where it is bound to specific protein, metallothionein. Zinc and other metals are also bound to metallothionein which has no known function, however, cadmium is bound more tightly than zinc and can displace it. The incorporation of cadmium increases the half life of the metallothionein molecule and cadmium zinc ratio (59). This exposure to cadmium may affect zinc homestasis through metallothionein. A number of allopathic drugs have been developed; however, in view of their toxicity the need for developing the new drugs has never waned.

Reserpine, an alkaloid extracted from Rauvolfia serpentina has been in use as an antihypertensive agent, for past many years for moderately severe hypertension but one of its serious side effects viz mental depression leading to suicidal tendencies by some patients is the main draw back of the use of the drug (19,20,21,22,23). In this present paper Ajmaloon, a herbal polypharmaceutical developed from the combination of Alcoholic extract of Rauvolfia serpentina (roots) as main ingredient and Zea mays (styles), Cicer arietinum (Seed Coat), Juniperus communis (Berries), Hordeum vulgare (Grains) as toxicity corrective herbs was tried in the hypertensive individuals and its efficacy was found to be statistically significant by double blind clinical trials.

The greatest drawback in the present antihypertensive drug profile is in the intermediate area between mild & powerful antihypertensive agents. One can confirm that Ajmaloon will emerge as a unique herbal polypharmaceutical for mild & moderate hypertension and is devoid of serious side effects of Reserpine i.e. mental depression.

The mechanism of action of Ajmaloon is still under study but efforts have been made to tune its beneficial effect as one of normalising the deranged serum elemental concentration. Ajmaloon tends to decrease the elevated serum cadmium levels in hypertensive individuals but

is unable to elevate the decreased level of serum zinc in the same individuals. However a long term study on large number of patients is necessary before a categorical statement can be made.

SUMMARY

Cadmium and zinc levels were estimated in 60 normotensive and 75 hypertensive individuals. It was observed that in individuals with moderate & severe hypertension having diastolic blood pressure above 110 mm Hg the Serum cadmium levels were 30 to 40% higher than normal values of 0.001 to 0.0033 mg/lit while the serum zinc level appeared to be 25% lower than normal controls of 0.84 to 1.43 mg/litre. Ajmaloon having Rauvolfia serpentina, Zea mays, Cicer arietinum, Juniperus communis and hordeum vulgare as its major ingredients, effectively lowered the raised blood pressure ($p < 0.001$) even in moderate hypertensives. Action starts in 24-48 hrs & reaches its peak in 5-7 days. It also tends to lower the raised serum cadmium levels.

Cadmium has been implicated unequivocally in pathophysiology of hypertension in both clinical and experimental situations. Ajmaloon thus occupies a unique position since it is an effective & safe hypotensive agent possessing also cadmium lowering activity.

ACKNOWLEDGEMENTS

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REFERENCES

1. Davidson, S. and Macleod, J. The Principles and practice of medicine, Churchill Livingstone, 1973.
2. Hathcock, J.N. Ed. Nutritional Toxicology Vol. 1 Academic Press, New York, London 1982.
3. Iyenger G.V. Science Today, Feb. 32,35, 1985.
4. Perry, H.M. Jr. and Schroeder, H.A. J. Lab. Clin. Med. 46: 636-640, 1955.
5. Schoeder, B.A. J. Chronic Dis. 18: 647-556, 1965.
6. Carroll, R.E. J. Am. Med. Assoc. 198: 267-269, 1965.
7. Perry. H.M. Jr. and Erlanger, M.W., Am. J. Physiol. 220: 808-811, 1971.
8. Perry, H.M. Jr., and Erlanger, M.W. and J. Lab. Clin. Med. 82: 808-811, 1971.
9. Perry, H.M. Jr., Erlanger, M.W. and Perry, E.F. Am. J. Physiol. 232: 114-121, 1977.
10. Perry. H.M. Jr., Erlanger, M.W. and Perry, E.F. Environ. Health Perspect. 28: 251-260, 1979.
11. Thind, G.S. and Fischer, G.M. Exe. Mol. Pathol. 22: 326-334, 1975.
12. Schroeder, H.A. and Vinton, W.H. Am. J. Physiol. 202: 515-518, 1962.
13. Schroeder, H.A., Nason, A.P. and Mitchener, M. Am. J. Physiol. 214: 796-800. 1968.
14. Talwar, S., Qadry, J.S., Pillai, K.K. and Arora, R.B. Ind. J. Pharmacol. 17 Suppl. 1: Abs. No.084, 1985.
15. Talwar S., Qadry, J.S., Pillai, K.K. and Arora R.B. 4th int. Cong. Orient. Med. Tokyo, Japan, Oct. 1985.
16. Revis, N.W., Major, T.C., Horton, C.Y., J. Am. Coll. Toxicol. 2 (2): 165, 1983.
17. Roonwal, G.S., Bhatia, B., Khan, S.U. and Arora, R.B. (1985) : Ind. J. Pharmacol. 17 Suppl. 1:111, 1985.
18. Thind, G.S. and Fisher G.M. Clinical Science and Molecular Medicine, 51: 483-486, 1975.
19. Quetsch, R.M., Achor, R.W.P., Litin, E.M. and Faucett, R.L. Circulation, 19: 366-375, 1959.

20. Nayler, W. J. Pharmac. Exp. Ther. 139: 222-229, 1962.
21. Emas, S., and Fyro, B. Acta Physiol Scand, 63: 358-369, 1965.
22. Gaunt, R., Chart, J.J. and Renzi, A.A.A.A. Rev Pharmac. 2: 109-128, 1963.
23. Goodwin, F.K., Ebert, M.H. and Bunney, W.E. Jr. R. - Shader, Raven Press, New York pp. 25-47, 1972.
24. Nadkarni, A.K. Indian materia medica, Bombay popular Prakashna, 1, 1976.
25. Khan, S.U., Kheterapal, K., Khanna, T., Zahid, M. and Arora, R.B. (Abstract). Accepted for second International Conference on elements in health and disease-1987.
26. Department of health, Education and Welfare, Food and Drug Administration U.S. Federal Register, Aug. 8, 1978 Part IV, 35186-35208.
27. Hundnick V., Gomiseek M.M. and Gomiseek S. Anal. Chim. Acta, 157: 143-150, 1984.
28. Hundnick V., Gomiseek M.M. & Gomiseek S. Anal. Chim, Acta, 157: 303-311, 1984.
29. Khan, S.U., Roy S. and Arora, R.B. In "Development of Unani drugs from herbal sources and the role of elements in their mechanism of action" Ed. R.B. Arora Hamdard National Foundation monograph, IHMMR, printing press, New Delhi, 1985.
30. Iyengar, G.V., In: Development of Unani drugs from herbal sources and the role of elements in their mechanism of action. (Ed. Arora R.B.) Hamdard National Foundation, Monograph, 1985. pp. 21-44.
31. Schroeder, H.A. and Ballasa, J.J. Am. J. Physiol. 209: 62-66, 1964.
32. Schroeder, H.A. and Buckman. Arch. Environ. Health 14: 693-697, 1967.
33. Schroeder, H.A., Circulation 35: 570-82, 1967.
34. Schroeder H.A. North Am. 58: 381-396, 1974.
35. Perry, H.M. Jr. Erlanger M.W. and Perry, E.F. 8th Annual Conference on Trace substances in 1974, Environmental Health. Univ. of Missouri, pp. 51-57.
36. Frazeir, J.M. Fundam, Appl. Toxicol. 1(6): 452-456, 1981.
37. Petering, H.G., Johnson, M.A. and Stemmer, K.L. (Abst) Proc. Fed. A. Soc. Exp. Biol. 28: 9-61, 1969.
38. Petering, H.G., Johnson,M.A. and Stemmer, K.L. Arch. Environ. Health 23: 93-101, 1971.
39. Verma M.P., Sharma, R.P. and Bourcier, P.R. Biol. Trace Hem, Res. 4: 35-43, 1982.
40. Lanwerys, R. In: The chemistry, Biochemistry and Biology of Cadmium." pp. 433-455, Webb, Med., Elservier.
41. Friberg, L., Piscator, M., Nordberg, G.F. and Kjellstrom, T. Second Ed. Chemical Rubber Co. Press, Cleveland 1974.

42. Murata, I., Hirono, T.O., Saeki, Y. and Nakagawa, S. Bull. Soc. Int. Chir. 1: 34, 1970.
43. Baader, E.W. Dtsch. Med. Wochenschr. 76: 487-494, 1951.
44. Hunter, Dr. Arh. Hig. Rada, 5: 221-224, 1954.
45. Friberg. L. Acta Med. Scand. 138 (Suppl.): 240, 1950.
46. Friberg, L., Proc. 9th Int. Congr. Ind. Med. pp. 641-44, 1948.
47. Perry, H.M. Jr. and Schroeder, H.A. J. Lab. clin. Med. 46: 936, 1955.
48. Roonwal, G.S., Bhatia, B., Khan, S.U. and Arora R.B. Asian Congress of Pharmacology, New Delhi, 1985.
49. Arora, R.B., Roy, S. and Khan, S.U., Acta Pharmacologica et. Toxicologica 58 (Suppl 11): 1986 In Print.
50. Doyle, J.J., Bernhoft, R.A. and Sandstead, H.H. J. Lab. Clin. Med. 86: 57-63, 1975.
51. Lener, J. and Musit, J. Experentia. 29: 902, 1971.
52. Perry, H.M. Jr., Erlanger, M.W., Yunice, A. and Perry, E.F.J. Lab. Clin. Med. 70: 963-72, 1967.
53. Perry, H.M. Jr. and Erlanger, M.W. J. Lab. Clin. Med. 82: 339-405, 1973.
54. Perry, H.M. Jr. and Erlanger, M.W. Cadmium as both an antinaturetic and pressure agent. In "Trace substance in Environ Health" XIV ed. Hemphill. D.D. University of Missouri, 280-286, 1980.
55. Revis, N.W., Zinsmeister and Bull, R. Proc. natl. Acad. Sci. USA 78: 6494-6498, 1981.
56. Prasad, R., Sharma, N., Paliwal, V.K., Annrel, I.S. and Nath, R. Proc. Nat. conf. on lead, zinc and cadmium at work place: Environment and Health care, New Delhi 2: 1-16, 1982.
57. Lyall, V., Mahmood, A. and Nath, R. Ind. Jour. Biochem Biophys. 16: 80-83, 1979.
58. Prasad, R., Paliwal, V.K., Sharma, N. and Nath, R. (abstract Toxicology Letters, 18: 8, 1983.
59. Cain, K. and Holt, D.G. Chem. Biol. Interact, 28: 91--106, 1979.

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PATHOPHYSIOLOGY AND TREATMENT OF DECREASED CARDIAC OUTPUT COMPLICATING PULMONARY HYPERTENSION

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A decrease in cardiac output due to increased right ventricular (RV) afterload may complicate several clinical conditions such as ARDS and pulmonary embolism (1,2).

A recent canine study investigated treatment of shock in a canine model of pulmonary embolism (3). Autologous blood clots were injected over approximately 25 minutes, and when mean BP had fallen to 70 mmHg (shock), dogs were treated according to prior randomization. Four groups of six dogs were studied. One group served as controls. Another group was treated with volume expansion, a third with isoproterenol and the final group with norepinephrine (NE).

As per experimental design, blood clot emboli decreased mean CO to <.8 l/min and decreased mean BP to 70 mmHg prior to onset of therapy. In controls and in dogs treated with volume or isoproterenol, hemodynamic state continued to deteriorate, and all dogs died within ten minutes. In contrast, all six dogs treated with NE demonstrated marked hemodynamic improvement and remained stable during one hour of continuous infusion.

Table 1 illustrates hemodynamic effects of pulmonary emboli and NE. Note the marked deterioration in RV function as afterload increased. NE increased BP, and CO increased from an unmeasurable value to 2.3 l/min. Corresponding to the increase in CO, RVEDP decreased. These changes signal an improvement in RV pump performance, due to a direct inotropic effect and/or increased contractility due to increased BP and improved RV perfusion (4).

Table 1. HEMODYNAMIC EFFECTS OF NORADRENALINE TREATMENT*

	BASELINE	TREATMENT	15 MIN	60 MIN
CO ($L \cdot min^{-1}$)	3.5±1.5	–	2.3±0.7	2.3±0.3
BP (mmHg)	140±22	71±2	112±25	106±16
RVEDP (mmHg)	0.7±0.8	10±1	5±5	5±3
PAP (mmHg)	13±3	62±11	55±7	50±6
PVR ($mmHg \cdot L^{-1} \cdot min$)	2.5±0.7	–	28±8	31±18

* Values are mean ±SD

Table 2. HEMODYNAMIC EFFECTS OF EMBOLIZATION, NORADRENALINE AND METHOXAMINE

	BASELINE	EMBOLIZATION	NORADRENALINE	TIME CONTROL	METHOXAMINE
CO L/min	4.8±1.4	1.0±0.3^{+}	2.0±0.5^{x}	1.2±0.4	1.1±0.3
BP mmHg	144±10	64±13^{x}	122±5^{x}	68±18	121±10^{x}
PAP mmHg	16.2±1.6	47.8±16.5^{+}	52.1±16.6	38.3±9.1	37.9±10.4
RVEDP mmHg	4.4±2.0	10.7±4.3*	8.0±3.1	8.4±3.2	12.8±4.0
PVR $mmHg \cdot L^{-1} \cdot min$	1.5±0.6	44±17^{+}	24±12^{+}	28±11	28±15
RVCPP mmHg	132±10	35±11^{x}	93±10^{x}	48±17	94±7^{+}

* $p < 0.05$ vs previous baseline
+ $p < 0.01$ vs previous baseline
x $p < 0.001$ vs previous baseline
RVCPP = Right ventricular coronary perfusion pressure

A recent study by Ducas et al (5) was designed to determine which of the above mechanisms best explained the improvement in RV performance. In an attempt to separate direct inotropic effects from indirect effects due to increased BP and improved RV perfusion, acute hemodynamic effects of NE and methoxamine were compared in the same dogs. RV afterload was increased via injection of small (80-120 u) glass beads to decrease BP to approximately 65 mmHg. In this model, this was the lowest BP where hemodynamic stability was maintained. Mean results are illustrated in Table 2. Note that embolization dramatically decreased CO, BP and RV coronary perfusion pressure. NE doubled BP and CO and almost tripled RV coronary perfusion pressure. Note that PVR, calculated as PVR = PAP - LVEDP/CO decreased with NE. In contrast, despite a similar improvement in BP and RV coronary perfusion pressure, CO did not change with methoxamine.

These results suggest that noradrenaline improved RV pump performance primarily via a direct inotropic effect.

While it is possible that the failure for RV function to improved with methoxamine was due to an increase in coronary vascular resistance, offsetting the increase in coronary perfusion pressure, previous work does not support this possibility (6).

The results of this study compliment those of Vlahakes et al (4). In that study, RV function in the setting of marked systemic hypotension was improved by infusion of an antagonist (phenylephrine). The authors attributed the increased RV function to the improvement in BP and RV coronary perfusion pressure reversing RV ischemia. However, in that study, BP was much lower during RV failure (48 mmHg) than in the study of Ducas et al in which an α agonist had no effect on RV function. Accordingly, the more pronounced level of hypotension prior to treatment probably explains the increase in CO which occurred with phenylephrine,

whereas direct inotropic effects were necessary to improve CO and RV function without frank shock.

While isoproterenol was ineffective in treatment of shock due to acute pulmonary emboli (3), can isoproterenol improve RV performance in the setting of pulmonary hypertension without frank circulation instability?

To test this hypothesis, a recent study compared acute cardiopulmonary effects of NE and isoproterenol in a canine model of increased RV afterload and decreased CO (7). In six anesthetized dogs, autologous blood clots were injected over approximately two hours to increase RV afterload and decrease CO 40%. Mean results are illustrated in Table 3. Note that while both drugs increased SV, only isoproterenol increased CO. Corresponding to the increase in flow, RV filling pressure and PVR decreased with isoproterenol.

Zapol et al reported beneficial hemodynamic effects of isoproterenol in patients with ARDS and pulmonary hypertension (1). In another study, Snider et al investigated effects of isoproterenol in patients with ARDS and mild pulmonary hypertension (8). Isoproterenol increased CO and caused a small decrease in PVR.

Accordingly, when a moderate decrease in CO complicates an increase in RV afterload, isoproterenol may be an excellent drug to increase CO and improve RV function.

In a variety of conditions vasodilators are used to decrease systemic and pulmonary vascular resistance and increase CO.

A recent study compared acute cardiopulmonary effects of nitroprusside (NP) and hydralazine in a canine model of pulmonary hypertension and decreased CO (9). Mean results are illustrated in Table 4. Note that while both drugs decreased ventricular filling pressures and systemic vascular resistance, only hydralazine decreased PVR and increased CO. Note that PAP and BP did not change with hydralazine, and that while the mean value for arterial 0_2

Table 3. HEMODYNAMIC EFFECTS OF INOTROPIC AGENTS IN PULMONARY HYPERTENSION

	CO L/min	SV .ml/beats	PAP mmHg	RVEDP mmHg	BP mmHg	PVR $mmHg \cdot L^{-1} \cdot min$
CONTROL 1	1.3±0.3*	8±2*	44±5‡	9±4	93±16*	34±10
ISOPROTERENOL	3.0±0.8	15±4	49±9	5±3	71±11	16±4*
CONTROL 2	1.2±0.4	7.5±3.5	40±6	9±4	84±18*	33±10
NORADRENALINE	1.4±0.5	12±5*	45±11	8±5	117±20	32±14
CONTROL 4	1.2±0.4	7.4±2.5	42±5	9±4	90±18	34±12

* = $p < 0.01$, + = $p < 0.025$, ‡ = $p < 0.05$
Comparing control 1 to isoproterenol
Comparing control 3 to noradrenaline

Table 4. HEMODYNAMIC EFFECTS OF INCREASED PVR AND VASOACTIVE DRUGS

	CO L/min	PAP mmHg	RVEDP mmHg	BP mmHg	SVR $mmHg \cdot l^{-1} \cdot min$	PVR $mmHg \cdot l^{-1} \cdot min$
BASELINE	3.0±1.3	14±2	3±2	139±21	53±20	3±3
CONTROL 1	1.6±0.7[B]	47±5[B]	6±3[A]	144±32	93±26[B]	31±17[B]
NITROPRUSSIDE	1.8±2.2	44±5	4±2[A,D]	97±18[B]	63±25[B,C]	30±30
CONTROL 2	1.5±0.6	43±5	5±3	134±26	94±8	29±15
HYDRALAZINE	2.7±1.5[A]	42±6	4±3[A,D]	133±31	55±19[B,C]	18±12[B]

Statistical comparisons: [A]$p < .05$ parameters vs control; [B]$p < .01$ parameters vs control; [C]$p < .05$ comparing differences from control; D not significant, comparing differences from control

tension decreased with nitroprusside, it increased with hydralazine. Similar results are reported when hydralazine is given to patients with primary and secondary pulmonary hypertension, with and without RV dysfunction (10, 11).

Accordingly, while hydralazine may be useful to decrease RV afterload and increase CO when a low output state complicates an acute increase in RV afterload, extreme care should be taken to ensure that excessive falls in BP and RV perfusion do not occur. Conceivably, NE, which probably does not increase PVR (see section on the flow resistive characteristics of the pulmonary vasculature), could be used in conjunction with a vasodilator if the latter is felt to be indicated.

THE FLOW RESISTIVE CHARACTERISTICS OF THE PULMONARY VASCULATURE

To this point, this review has focused on pathophysiology and treatment on RV dysfunction complicating acute changes in afterload. The genesis for RV dysfunction in this setting, occurs as a function of alterations in the pulmonary vascular bed, and the physiological mechanisms responsible for the alterations in pulmonary hemodynamics have only recently been investigated. Conventionally, PVR, calculated as mean PAP - LVEDP ÷ CO is assumed to reflect the flow resistive properties of the pulmonary vasculature. This approach forces one to make assumptions on the vascular effects of the drug on the basis of single pressure-flow (P-Q) measurements before and during therapy. When this data is used to define a "resistance," as derived from Poiseuille's Law, it is implied that the relationship between pressure and flow in the pulmonary circulation is linear, and that flow begins (Zone III) when the upstream pressure (PAP) exceeds the apparent downstream (LVEDP or LA) pressure (12, 13). Several studies employing isolated lobe or lung preparations have demonstrated that at physiologic rates of flow, the pulmonary P-Q relationship, which defines the incremental resistance, be described by a linear

relationship (12-15). However, the pressure intercept (effective outflow pressure), obtained by linear extrapolation of the P-Q line to zero flow, has been reported to exceed the apparent downstream pressure (14, 15).

Accordingly in the setting of pulmonary hypertension, such as following pulmonary emboli, elevation in the PAP may be related to an increase in mean outflow pressure, an increase in incremental resistance or a combination of these two factors. Similarly, vasoactive compounds may produce alterations in pulmonary hemodynamics, by altering these factors either separately or in combination.

A recent canine study investigated pulmonary vascular effects of pulmonary emboli and hydralazine (16). To define the vascular P-Q relationship, multiple PAP-Q coordinates were obtained by opening systemic A-V fistulae fitted with variable resistors.

As in the study by Lee et al (9), hydralazine approximately doubled CO, decreased calculated PVR and did not affect PAP. Effects of pulmonary emboli and hydralazine on pulmonary P-Q characteristics in four dogs are illustrated in Figure 1. Note that while emboli increased incremental resistance, the predominant explanation for the increase in PAP was the large increase in outflow pressure. Further, note that the predominant effect of hydralazine was not to alter incremental resistance, but to decrease outflow pressure (mean change, 24%). To test the hypothesis that the extrapolated pressure intercept reflects the effective downstream or outflow pressure, in four dogs, left atrial pressure was progressively raised, by inflating a left atrial balloon at constant CO, see Figure 1. Before embolization, increases in left atrial (LA) pressure over a given range (approximately 8 to 17 mmHG) caused similar changes in PAP, indicating LA pressure approximated the effective outflow pressure. In contrast, after embolization, changes in LA pressure over the same range had

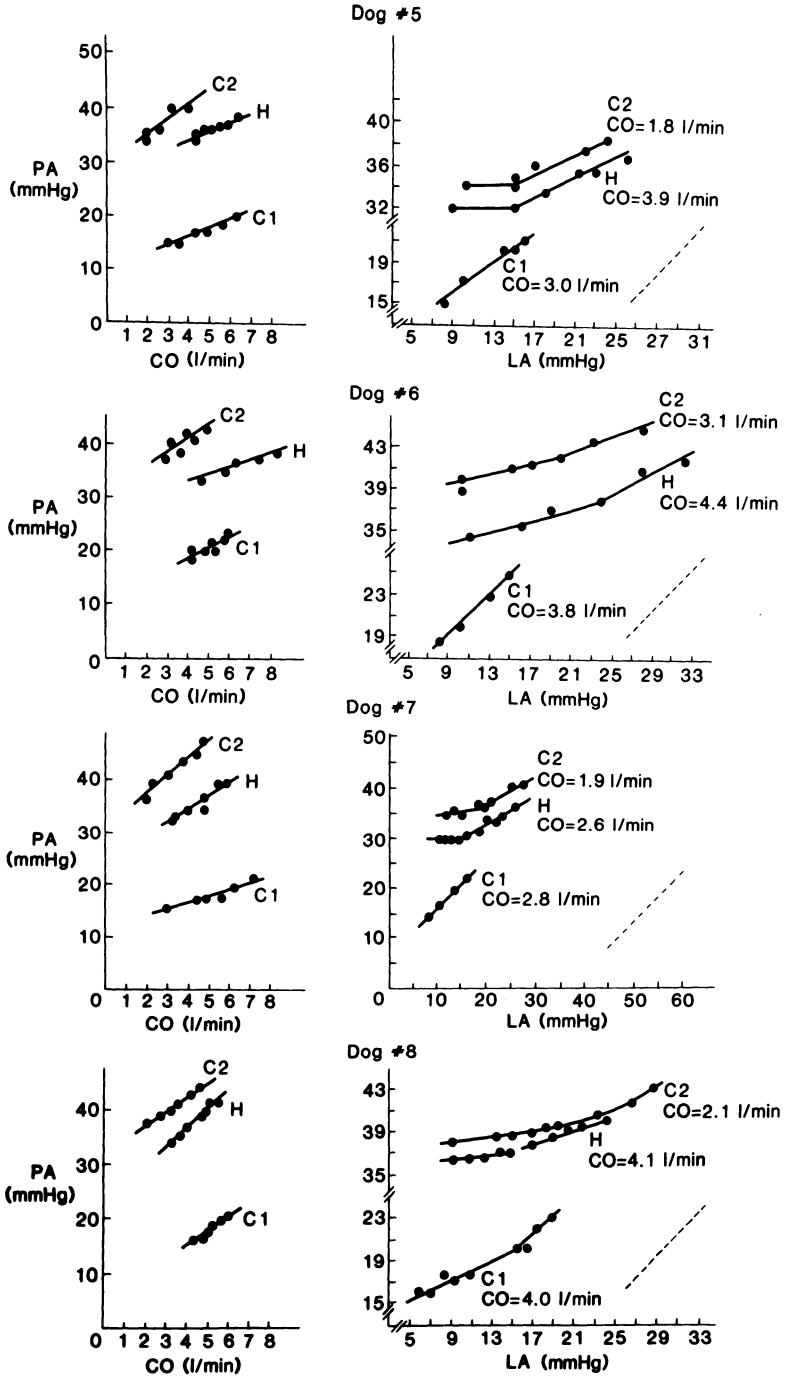
Dog #5
PA (mmHg)
CO (l/min)
C2
H
C1
C2 CO=1.8 l/min
H CO=3.9 l/min
C1 CO=3.0 l/min
LA (mmHg)
Dog #6
C2 CO=3.1 l/min
H CO=4.4 l/min
C1 CO=3.8 l/min
Dog #7
C2 CO=1.9 l/min
H CO=2.6 l/min
C1 CO=2.8 l/min
Dog #8
C2 CO=2.1 l/min
H CO=4.1 l/min
C1 CO=4.0 l/min

FIGURE 1 (Preceding page)

On the left, the coordinates for PA and CO have been plotted for four dogs. The lines drawn are from linear regression. On the right, for each dog, the relationship between PA pressure and LA pressure at constant cardiac output are plotted. The lines drawn are from a visual best fit. The dashed line indicates the slope of the line of identity. See text for further discussion.

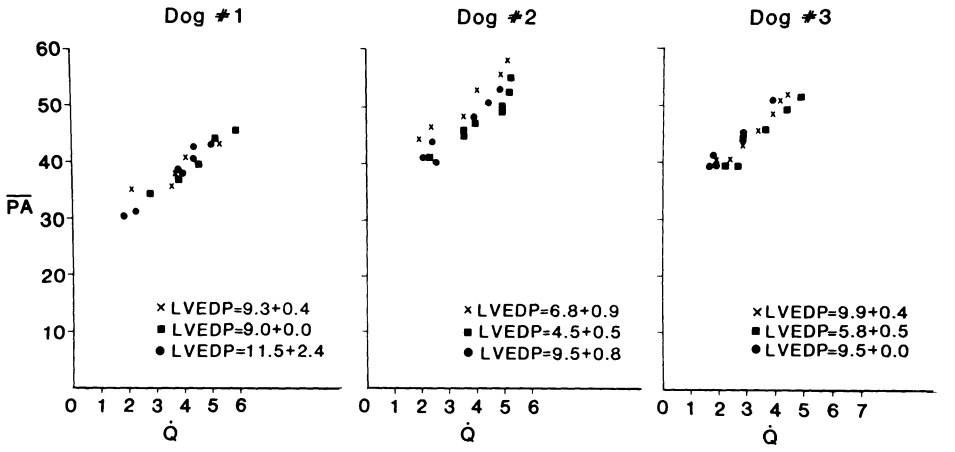

FIGURE 2

The PA-CO coordinates for three dogs post emboli are plotted prior to noradrenaline (NE) (x) during NE (●), and time control-TC after NE infusion (■). For each dog, analysis of covariance revealed no differences in slope or intercept.

absent to trivial effects on PAP. Accordingly, as signaled by the change in extrapolated pressure intercept, embolization resulted in a marked increase in the effective outflow pressure.

Another recent canine study investigated effects of pulmonary emboli and NE on P-Q characteristics (17). P-Q characteristics were defined as described above (16). Emboli increased mean incremental resistance from 1.9 to 5.5 mmHg/l/min and caused a marked upward shift in the extrapolated pressure intercept, from 8.1 to 28.3 mmHg. Both before and after emboli NE produced significant increases in CO and BP. Also, as seen in the studies of Molloy et al (3) and Ducas et al (5), NE decreased traditionally calculated PVR. However, as illustrated in Figure 2, NE did not alter incremental resistance or outflow pressure.

These findings further emphasize the utility of NE for treatment of low output states due to increased RV afterload. That is the beneficial inotropic and pressor effects which improve RV function, CO and BP are not associated with deleterious pulmonary vascular effects, so that the increase in PAP with NE is due to the corresponding change in flow.

SUMMARY

When shock complicates an acute increase in RV afterload, initial therapy should be directed toward restoration of an adequate BP (RV coronary perfusion pressure) and CO. Current results indicate that NE may be an excellent agent for acute resuscitation and short-term maintenance of hemodynamic stability.

In the absence of shock, when a moderate decrease in CO complicates pulmonary embolism, isoproterenol, hydralazine or other vasodilators, may be employed to improve flow. However, these agents may decrease BP so careful monitoring is required.

Recent canine studies indicate that an increase in vascular outflow pressure is the predominant mechanism explaining the increase in PAP and apparent increase in PVR complicating pulmonary embolism. Accordingly, in addition to decreasing vascular resistance, therapy could be directed toward decreasing outflow pressure.

REFERENCES

1. Zapol, W. M. and Snider, M. T. N Engl J Med 296: 476-480, 1977.
2. Alpert, J. S., Smith, R., Carlson, J., Oskene, I. S., Dexter, L. and Saline, J. E. JAMA 236: 1477-1480, 1976.
3. Molloy, W. D., Lee, K. Y., Girling, L., Schick, U and Prewitt, R. M. Am Rev Respir Dis 130: 870-874, 1984.
4. Vlahakes, G. J., Turley, K. and Hoffman, J. I. E. Circ 63: 87-95, 1981.
5. Ducas, J., Deutscher, R. and Prewitt, R. M. Am Rev Respir Dis (suppl) 131(4): A149, 1985.
6. West, J. W. and Aviado, D. M. Am J Med Sci 231: 599-600, 1956.
7. Molloy, D. W., Lee, K. Y., Jones, D., Penner, B. and Prewitt, R. M. Chest 88: 432-435, 1985.
8. Snider, M. T., Rie, M. A. and Lauer, J. et al. Am Rev Respir Dis (suppl) 121: 191, 1980.
9. Lee, K. Y., Molloy, D. W., Slykerman, L. and Prewitt, R. M. Circ 68(6): 1299-1303, 1983.
10. Rubin, L. J. and Peter, R. H. N Engl J Med 302: 69, 1980.
11. Rubin, L. J. and Peter, R. H. Am J Cardiol 47: 116, 1981.
12. Graham, R., Skoog, C., Macedo, W., Carter, J., Oppenheimer, L., Rabson, J. and Goldberg, H. S. J Appl Physiol 54(5): 1277, 1983.
13. Mitzner, W. Clin Chest Med 4(2): 127, 1983.
14. Graham, R., Skoog, C., Oppenheimer, L., Rabson, J. and Goldberg, H. S. Circ Res 50: 566, 1982.
15. Mitzner, W., Sylvester, J. T. J Appl Physiol 51(5): 1065, 1981.
16. Ducas, J., Girling, L., Schick, U. and Prewitt, R. M. Circ 73(5): 1050-1057, 1986.
17. Duval, D., Ducas, J., Molloy, W. D., Girling, L. and Prewitt, R. M. Am Rev Respir Dis 129(4): A63, 1984.

C. CALCIUM ANTAGONISTS AND BETA ADRENERGIC RECEPTOR BLOCKERS

14

Ca ANTAGONISTS IN CARDIOPULMONARY CHANGES INDUCED BY HIGH ALTITUDE HYPOXIA

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INTRODUCTION

Calcium antagonists have been increasingly used in the last decade both as valuable cardiovascular drugs and as tools to investigate the pharmacology of slow channels of many excitable cells. Although very different in chemical structure, all these compounds reduce rather specifically the Ca influx through the voltage dependent Ca channels in cardiac and vascular smooth muscle cells, which results in negative inotropic, chronotropic and dromotropic as well as vasodilating properties of these drugs. Thus, calcium antagonists are successfully used for the treatment of coronary heart disease, arrhytmias and systemic hypertension (1, 2).

Much less is known about the effect of these substances on pulmonary circulation. It has been shown that acute administration of calcium channel antagonists such as verapamil and nifedipine inhibits the pulmonary pressure response to alveolar hypoxia in isolated lungs (3, 4), intact animals (5) and human subjects (6). This suggests that membrane depolarization and Ca influx through voltage sensitive Ca channels are components of an as yet unexplained mechanism of hypoxic pulmonary vasoconstriction. The results with longlasting administration of calcium antagonists in chronically hypoxic subjects are, however, still controversial. Whereas some observations suggest that repeated administration of verapamil and diltiazem prevented right ventricular

hypertrophy in hypoxic animals (7, 8), other experiments failed to show any protective effect (9).

The present survey summarizes some of our results concerning the effect of preventive and therapeutic treatment of cardiopulmonary changes induced in rats by high altitude hypoxia, simulated in a barochamber. Since recent evidence suggests significant age-related differences in the cardiovascular sensitivity to interventions affecting transsarcolemal Ca fluxes (10, 11, 12), particular attention was paid to the effect of calcium antagonists on the developing cardiopulmonary system.

RESULTS AND DISCUSSION

Preventive and therapeutic pharmacological treatment in adult rats

Exposure of experimental animals to chronic high altitude hypoxia may both stimulate favourable cardiopulmonary acclimatization and impose stress, the magnitude of which depends upon the intensity and duration of the hypoxic stimulus. Our previous results have shown that intermittent high altitude hypoxia (IHA, barochamber, stepwise up to 7000 m, 8 h/day, 5 days a week) can induce pulmonary hypertension and right ventricular hypertrophy in a relatively short time. Furthermore, at the beginning of the precess of acclimatization focal myocardial necroses can be observed, localized predominantly in the right ventricular myocardium (13,14). It is interesting to note that such primarily affected myocardial tissue is significantly more resistant to acute anoxia in vitro or to isoprenaline-induced necrotic lesions (15, 16).

Calcium antagonists were used in order to estimate the possibilities of pharmacological reduction of IHA induced cardiopulmonary changes. In the first part of our study verapamil was administered from the beginning of hypoxia before each of the 24 exposures (17). Preventive administration of the calcium antagonist (8 $mg.kg^{-1}$) did not affect right ventricular systemic pressure and right ventricular

weight in the sea level group (Fig. 1), nor did it affect the left ventricular weight and hematocrit values even in rats exposed to IHA. This latter finding agrees with the observation of Davidson et al. (7) and suggests that the hypoxic stimulus was the same in verapamil treated and non-treated animals. Administration of verapamil to IHA exposed animals significantly reduced - but did not normalize - the degree of pulmonary hypertension and right ventricular hypertrophy (Fig. 1) and partially prevented the development of hypertensive changes in the pulmonary vasculature (Fig. 2). Furthermore, verapamil reduced significantly the incidence of necrotic lesions in the myocardium (Table 1) and diminished the positive sign of acclimatization, i.e. cardiac resistance to acute anoxia, as judged from the decreased recovery of isotonic contractions of isolated right ventricle.

Table 1. The effect of verapamil on myocardial lesions (IHA - 8 hr/day, 24 exposures)

	n	Without pathological changes	Focal myofibrosis	Disseminated myofibrosis
Controls	10	100 %	0	0
Hypoxia	10	0	10 %	90 %
Hypoxia + verapamil	10	80 %	20 %	0

These results are consistent with the hypothesis that calcium antagonists inhibit the pulmonary pressure response to alveolar hypoxia in vitro (3, 4). This hypothesis was recently supported by the fact that a structural analogue of nifedipine, BAY K 8644, which promotes Ca influx through voltage dependent Ca channels, potentiates hypoxic vasoconstriction in isolated lungs (18). The degree of protective effect depends probably on the relation between the dose, and intensity and duration of hypoxic exposure. The reduction of right ventricular hypertrophy in chronically hypoxic, verapamil or diltiazem treated animals was also observed by Davidson et al. (7) and Reeves et al. (19); the hemodynamic

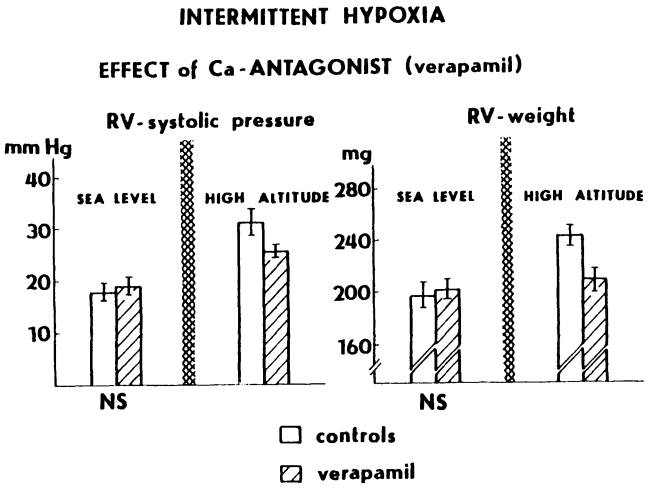

Fig. 1
Effect of preventive administration of verapamil on the right ventricular systolic pressure and right ventricular weight in "sea level" and "high altitude" exposed adult animals. (Reprinted from ref. 22 with permission.)

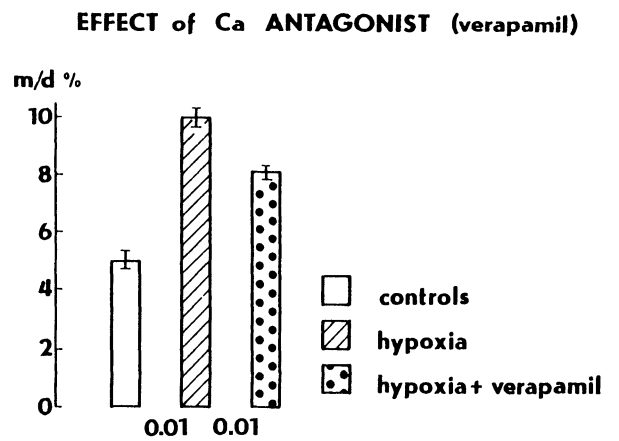

Fig. 2
Average percentage medial thickness (m/d %) of the distal pulmonary arteries in "sea level" and verapamil treated and non-treated"high altitude" adult animals.
(Data from ref. 17.)

as well as morphometric changes were, however, not investigated. Similarly, Kentera et al. (8) have shown that verapamil even in drinking water significantly reduced the degree of hypoxic pulmonary hypertension and right ventricular hypertrophy in rats.

The mechanism of IHA-induced myocardial changes is complex: in addition to the effect of hypoxia and stress, connected with the stay of animals in a hypobaric chamber (right and left ventricle) the influence of increased work load (right ventricle) cannot be excluded. According to Fleckenstein (20) an intracellular Ca overload followed by high energy phosphate defficiency and Ca impairment of mitochondria proved to be the decisive pathogenetic factor in the etiology of myocardial fibre necrosis, produced under various circumstances. Protective effect of verapamil on the hypoxic heart muscle can be accounted for in terms of its sparing effect on the rate of depletion of the endogenous ATP and CP reserves (21). In the case of right ventricular myocardium the protective effect of verapamil may at least partly be explained by a decreased work load due to the significantly reduced hypoxic pulmonary hypertension. Our results thus support the hypothesis that the transmembrane influx of extracellular calcium is an important component of both the mechanism of hypoxic pulmonary vasoconstriction and the IHA induced myocardial lesions.

In the second experimental situation, calcium antagonists verapamil (8 $mg.kg^{-1}$) and nifedipine (8 $mg.kg^{-1}$) were used therapeutically in pulmonary hypertension and right ventricular hypertrophy already developed (22). Untreated animals were exposed to IHA, 8 hr/day, for 24 exposures. The rats were then exposed to another 20 exposures for 4 hr/day and simultaneously treated with the calcium antagonist. The degree of pulmonary hypertension as well as right ventricular hypertrophy was, however, not influenced by the administration of different drugs (Fig. 3).

It may therefore be concluded that, whereas preventive administration of calcium antagonists significantly reduced

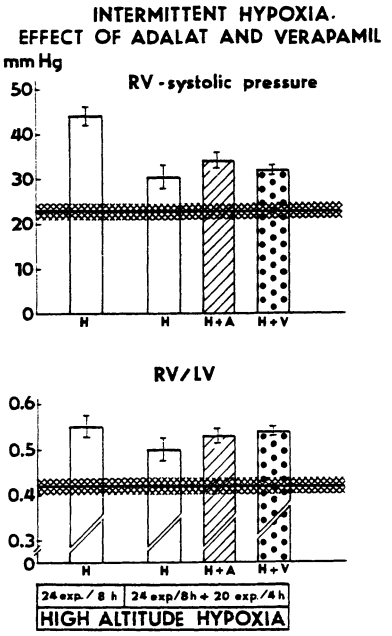

Fig. 3
Effect of therapeutic administration of verapamil and adalat on the right ventricular systolic pressure and right to left ventricular ratio.
H = hypoxia exposed non treated animals; H+A = hypoxia exposed and adalat treated animals; H+V = hypoxia exposed and verapamil treated animals.
(Reprinted from ref. 22 with permission.)

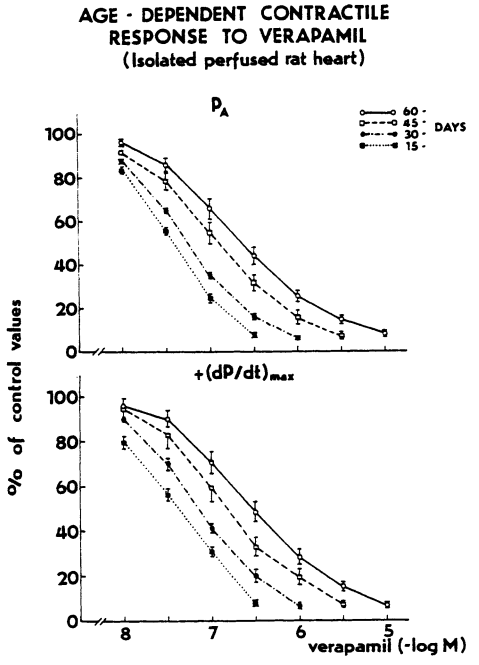

Fig. 4
Effect of verapamil on left ventricular pressure amplitude (P_A) and the maximum rate of pressure development (+(dP/dt)max) in isolated perfused hearts of rats of different age; means ± SEM.

the development of IHA-induced cardiopulmonary changes, therapeutic treatment was without any effect. On the basis of our observation it is difficult to decide what is responsible for such a striking difference in the action of calcium antagonists. For explanation of this finding, possible differences in calcium dependent smooth muscle tone in the pulmonary circulation under different hypoxic load should be taken into consideration. Nevertheless, our experimental data are in good agreement with the heretofore unconvincing results of chronic administration of calcium antagonists in patients with hypoxic pulmonary hypertension.

Calcium antagonists and ontogenetic development

To follow the ontogenetic differences in cardiac sensitivity to verapamil, mortality rate and the intensity of negative inotropic response of the isolated perfused heart were studied in rats during their postnatal development.

Mortality of verapamil treated animals was age-dependent (Table 2). Whereas the first death was observed in 90-day-old rats when a huge dose of 100 $mg.kg^{-1}$ of verapamil had been injected, with decreasing age the mortality rate significantly increased. In newborn rats 25 % mortality was still observed after 2.5 $mg.kg^{-1}$ of verapamil.

Table 2. Mortality rate after verapamil treatment (Reprinted from ref. 12 with permission)

Verapamil ($mg.kg^{-1}$)	n	Mortality rate (%) Age (days)			
		3	15	30	90
0.5	10	0	0	0	0
1.0	10	0	0	0	0
2.5	8	25	0	0	0
5.0	10	60	0	0	0
7.5	10	90	80	0	0
10.0	8	100	75	0	0
25.0	10	100	100	0	0
50.0	10	100	100	90	0
100.0	8	100	100	100	12.5

Similarly, significant age-related differences in contractile response can be observed. The sensitivity to verapamil increases with decreasing age of rats (from the 60th to 15th day of postnatal life) as judged from the values of the left ventricular pressure amplitude and maximum rate of pressure development (+(dP/dt) max) (Fig. 4). The values of ID_{50} (concentration of the drug that reduced +(dP/dt) max to 50 % of control, Table 3) demonstrate significant differences even between 45-day-old and 60-day-old animals.

Table 3. Concentrations of verapamil that reduced +(dP/dt) max to 50 % of control - ID_{50}; isolated perfused rat heart

Age (days)	n	+(dP/dt) max
15	6	$4.36 \pm 0.42 \times 10^{-8}$
30	8	$7.25 \pm 0.61 \times 10^{-8}$
45	8	$1.72 \pm 0.36 \times 10^{-7}$
60	9	$3.46 \pm 0.70 \times 10^{-7}$

Comparable age-related negative inotropic effect was described in the dog (verapamil, ref. 23) and rabbit heart (verapamil, nifedipine, diltiazem, ref. 10). Recent evidence both from our laboratory (12) and others (11) obtained on isolated immerse preparations demonstrates that these age-related differences can be attributed to direct myocardial effects and are independent of differences in innervation, myocardial blood flow, heart rate, vascular responses or pharmacokinetics. For explanation of these findings structural and functional developmental changes of systems responsible for myocardial calcium handling have to be considered. It has been shown that sarcoplasmic reticulum of immature heart is not fully developed (24) and Ca-induced release of Ca from sarcoplasmic reticulum is thus much less expressed (25). The immature heart depends, therefore, to a great extent upon transsarcolemal calcium influx. However, as follows from our results, the changes in the cardiac sensitivity to verapamil were observed even at the time (the 45th day) when structural and functional development of sarcoplasmic reticulum was already completed (26, 27). This

suggests that alternative or additional age-related differences in the mechanism of negative inotropic action of verapamil cannot be excluded.

The ontogenetic difference in the sensitivity to calcium antagonists is the self-evident limitting factor for the use of these drugs in young subjects. In order to estimate the effect of calcium antagonists on the development of IHA-induced cardiopulmonary changes in young rats, the dose was determined according to the above mortality rate. The animals were exposed to the simulated high altitude from the 5th day of postnatal life, stepwise up to 7000 m, 8 hr/day, 5 days a week. Up to the 11th day they received verapamil in a dose of 25 $mg.kg^{-1}$, then 5.0 $mg.kg^{-1}$ and from the 22nd day 7.5 $mg.kg^{-1}$, before each of 24 IHA exposures.

It was found that, similarly as in adults, IHA-induced significant pulmonary hypertension and right ventricular hypertrophy (Fig. 5). The administration of verapamil did not affect right ventricular systolic pressure and right ventricular weight in the sea level group; in contrast to adults, preventive administration of verapamil did not influence right ventricular pressure even in IHA exposed animals; right ventricular systolic pressure was reduced only insignificantly. On the other hand, verapamil markedly increased heart rate, both in sea level and IHA exposed animals (Fig. 6); such increase was not observed in adult animals.

The effect of calcium antagonists on the development of IHA-induced pulmonary hypertension and right ventricular hypertrophy was significantly less expressed as compared with adults. Unfortunately, there are not comparable data in the literature, since calcium antagonists have not been used as long-term vasodilators in neonates. Redding et al. (28) were the only authors to study the acute hemodynamic effects of nifedipine in unanesthetized piglets to test the hypothesis that nifedipine attenuates pulmonary vasoconstriction associated with alveolar hypoxia in newborn animals. They found that nifedipine lowers total pulmonary resistance

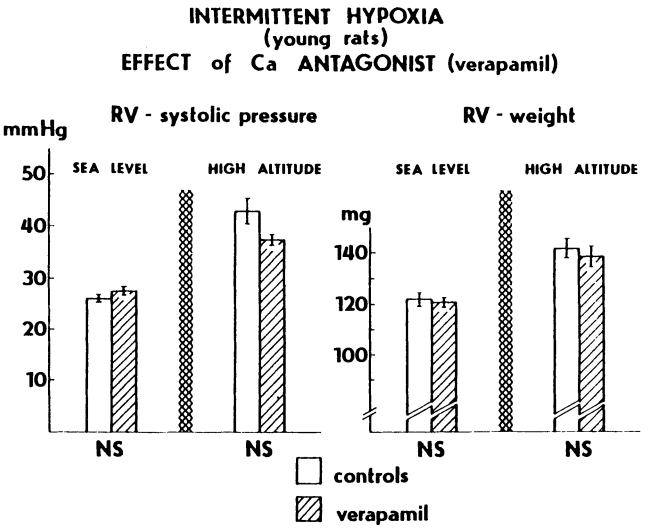

Fig. 5
Effect of preventive administration of verapamil on the right ventricular systolic pressure and right ventricular weight in "sea level" and "high altitude" exposed young animals.

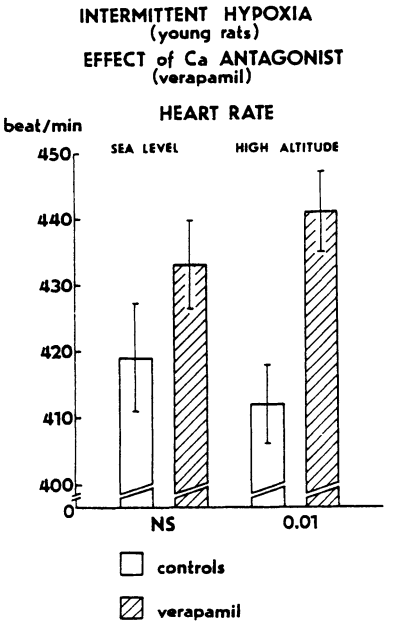

Fig. 6
Effect of verapamil on heart rate in "sea level" and "high altitude" exposed young animals.

during hypoxia in association with an increase in cardiac output, but not with a reduction in pulmonary artery pressure in this age group. The rise in heart rate, observed in our experiments, indicates that similar mechanism may operate also after repeated administration of calcium antagonists in chronically hypoxic rats.

It may be assumed that significant ontogenetic differences exist in cardiovascular sensitivity to verapamil both in "in vivo" and "in vitro" conditions and after acute or repeated administration of this substance. We thus stress the possible negative consequences of clinical use of calcium antagonists in the youngest age group.

SUMMARY

Calcium antagonists were used in order to estimate the possibilities of the pharmacological reduction of intermittent high altitude (IHA)-induced cardiopulmonary changes in young and adult rats. Wistar male rats were acclimatized for 8 hr/day, stepwise up to 7000 m in a barochamber. Preventive administration of verapamil (before each of 24 IHA exposures) to adult animals significantly reduced the development of pulmonary hypertension, right ventricular hypertrophy, hypertensive vascular changes and cardiac necroses. Therapeutic treatment with verapamil and/or nifedipine (started when IHA-induced changes were already developed) was, however, without any effect. The sensitivity to verapamil (mortality rate, negative inotropic response) is age-dependent: it decreases with increasing age of animals. The protective effect of verapamil on IHA-induced changes was in rats acclimatized from the 5th day of postnatal life significantly less expressed as compared with adults. On the other hand, verapamil markedly increased heart rate both in sea level and hypoxic animals; such increase was not observed in adults. It may be assumed, that significant ontogenetic differences exist in cardiovascular sensitivity to verapamil.

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REFERENCES

1. Schramm, M. and Towart, R. Life Sci. 37: 1843-1860, 1985.
2. Fleckenstein, A., Frey, M., Zorn, J. and Fleckenstein-Grün, G. Am. J. Cardiol. 56: 3H-14H, 1985.
3. McMurtry, I. F., Davidson, A. B., Reeves, J. T. and Grover, R. F. Circulation Res. 38: 99-104, 1976.
4. Suggett, A. J., Mohammed, F. H. and Barer, G. R. Clin. Exp. Pharmacol. Physiol. 7: 263-274, 1980.
5. Tucker, A., McMurtry, I. F., Grover, R. F. and Reeves, J. T. Proc. Soc. Exp. Biol. Med. 151: 611-614, 1976.
6. Simonneau, G., Escourrou, P., Duroux, P. and Lockhart, A. N. Engl. J. Med. 304: 1582-1585, 1981.
7. Davidson,A., McMurtry, I. F. and Reeves, J. T. Am. Heart J. 95: 810, 1978.
8. Kentera, D., Sušič, D. and Zdravkovič, M. Respiration 37: 192-196, 1979.
9. Sheldon, J. W. S., Cameron, I. R. Bull. Europ. Physiopath. Resp. 16: 101P, 1980.
10. Boucek, R. J., Shelton, M., Artman, M., Mushlin, P. S., Starnes, V. A. and Olson, R. D. Pediatric Res. 18: 948-952, 1984.
11. Artman, M., Graham, T. P., Boucek, R. J. J. Cardiovasc. Pharmacol. 7: 850-855, 1985.
12. Škovránek, J., Oštádal, B., Pelouch, V. and Procházka, J. Paediatric Cardiol. 1986 (in press).
13. Widimský, J., Urbanová, D., Ressl, J., Oštádal, B., Pelouch, V. and Procházka, J. Cardiovasc. Res. 7: 798-808, 1973.
14. Oštádal, B. and Widimský, J. Intermittent hypoxia and cardiopulmonary system, Academia, Prague, 1985.
15. Poupa, O., Krofta, K., Procházka, J. and Turek, Z. Fed. Proc. 25: 1243-1246, 1966.
16. McGrath, J. J., Oštádal, B., Procházka, J., Wachtlová, M., Rychterová, V. J. Appl. Physiol. 39: 205-208, 1975.
17. Oštádal, B., Ressl, J., Urbanová, D., Procházka, J., Pelouch, V. and Widimský, J. Respiration 42: 221-227, 1981.
18. McMurtry, I. F. Am. J. Physiol. 249 (Heart Circ. Physiol. 18): H741-H746, 1985.
19. Reeves, W. C., Wood M. S., Whitesell, L., Schidt, N. Am. J. Cardiol. 56: 470-472, 1985.
20. Fleckenstein, A. Calcium antagonism in heart and smooth muscle. J. Wiley and Sons, New York, 1983.
21. Nayler, W. G. In: The use of calcium antagonists in heart disease, Knoll AG, Ludwigshafen, 1978, pp. 55-72.
22. Oštádal, B., Procházka, J., Pelouch, V., Urbanová, D., Widimský, J. and Staněk, V. Prog. Resp. Res. 20: 17-25, 1985.

23. Gibson, R., Driscoll, D., Gillette, P., Hartley, C. and Entman, M. L. Dev. Pharmacol. Therap. 2: 104-116, 1981.
24. Schiebler, T. H. and Wolff, H. H. Zellforsch. 69: 29-40, 1966.
25. Fabiato, A. and Fabiato, F. Ann. N. Y. Acad. Sci. 307: 491-522, 1978.
26. Sulakhe, P. V. and Dhalla, N. S. Life Sci. 9: 1363-1370, 1970.
27. Page, E., Earley, J. and Power, B. Circulation Res. 35 (Suppl. II): 12-16, 1974.
28. Redding, G. J., Tuck, R. and Escourrou, P. Am. Rev. Resp. Dis. 129: 785-789, 1984.

15

EFFECT OF CRYSTALLOID CARDIOPLEGIA AND DILTIAZEM ON THE CARDIAC FUNCTION AND SOME BIOCHEMICAL PARAMETERS DURING HYPOTHERMIC CARDIAC ARREST USING CARDIOPULMONARY BYPASS

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INTRODUCTION

Cardiac surgery requires a still and relaxed heart on which to operate. Functional recovery of the heart after operation depends upon the functional survival of the myocardium during cardiac arrest. Many technical modifications have been made to protect the myocardium from ischemic injury. Currently cold crystalloid cardioplegia with various modifications are being used for cardiac arrest (1-6). The period of ischemia is often quite damaging to the heart and can severely impair the cardiac function even after restoration of coronary flow (7-8).

Ischemia has been reported to increase the tissue Ca^{++} (9-10). A massive increase in intracellular Ca^{++} has been demonstrated upon reperfusion of ischemic myocardium (11-13). Impairment of functional integrity of sarcolemma early during ischemic injury is accompanied by a marked inward Ca^{++} influx (14). A high level of intracellular Ca^{++} is known to produce cell death by activating a number of energy consuming reactions notably those of cardiac contractile proteins and of mitochondria (15). A decline in intracellular ATP during ischemia would produce contracture and would also impair the mechanism that pumps out Ca^{++}, thus accentuating the problem. A calcium channel blocker would, therefore, be helpful in reducing the intracellular Ca^{++} and its deleterious effects on the myocardium during ischemic cardiac arrest.

Many other factors may be involved in the left ventricular failure and cardiac contracture. Sarcoplasmic reticular system plays an important role in the muscle contraction and relaxation. The ability of sarcoplasmic reticulum for Ca^{++} binding and uptake might be affected

due to ischemia or ischemia related release of chemicals and enzymes. A failure of relaxation would produce a decrease in contractility, and contracture. Sarcolemmal Na^+-K^+-ATPase has been implicated in the pathogenesis of heart failure (16-19). Myocardial infarction is accompanied by an elevated serum MBCK (20,21).

The present investigation was undertaken to assess (i) the functional recovery of the heart after ischemic cardiac arrest with cold crystalloid cardioplegia with and without diltiazem (Ca^{++} channel blocker); (ii) the changes in the serum MBCK, sarcolemmal and sarcoplasmic reticular ATPase; and Ca^{++} binding and uptake by sarcoplasmic reticulum after ischemic cardiac arrest by cold crystalloid cardioplegia with and without diltiazem; (iii) if the functional recovery are related to the changes in biochemical parameters measured.

METHODS

Hemodynamic Measurements

Healthy, adult mongrel dogs of either sex weighing between 20 and 32 kg were anesthetized with pentothal soidum (25 mg/kg intravenously). A satisfactory plane of anesthesia was maintained with halothane via a closed-cuff endotracheal tube. A 7 French gauge Cournard (Cordis GF) catheter was positioned at the aortic arch through the femoral artery to record aortic pressure. The same catheter was pushed into the left ventricle to record left ventricular pressure. A catheter was positioned into the right atrium through external jugular vein to record the right atrial pressure. A Swan-Ganz balloon-tiped flow directed catheter was positioned into the pulmonary artery to record the pulmonary arterial wedge pressure and to determine the cardiac output by thermodilution technique. The first derivative of the left ventricular pressure was recorded with a differentiating device coupled to left ventricular pressure at a frequency response of 100 Hz. Aortic, right atrial, left ventricular, and the dp/dt of the left ventricular pressure and lead II ECG were recorded simultaneously on a Beckman R411 dynograph recorder. The pressures were recorded with an Ailtech microdot pressure transducer. The ratio of (dp/dt)/IIP was used as one index of myocardial contractility because it is not affected by preload and by a small change in the heart rate (22,23).

The dp/dt is affected not only by contractility but also by preload, afterload and heart rate (23). The left ventricular work index (LVWI), total systemic vascular resistance (TSVR) and cardiac index (CI) were calculated by previously described methods (17,24). For cardiac index the body surface area of the dog was determined according to the method of Ettinger and Suter (25).

Extra-corporeal circulation

The dogs were then placed on total cardiopulmonary bypass using Harvey oxygenator gassed with 95% O_2 and 5% CO_2. The system was primed with a solution of the following composition: Ringer lactate: 35 ml/kg; heparin sodium: 125 U/kg; potassium chloride: 1 mEq/kg; sodium bicarbonate: 1 mEq/kg; 20% solution of mannitol: 2 ml/kg; 5% dextrose in distilled water: 5 ml/kg. After initiation of complete cardiopulmonary bypass with left ventricle decompressed, the systemic temperature was lowered to 22 to 24 ^{o}C. The ascending aorta was then clamped to render the heart ischemic. Immediately following clamping 200 ml of cold cardioplegic solution of the following composition: Normosol R (pH 7.4), 500 ml; potassium chloride, 20 mEq: procaine HCl 2%, 50 ml; insulin, 20 units; dextrose 50%, 34 ml; sodium bicarbonate 24mEq at $4^{o}C$ was injected in the aortic root using pressure of 120 to 140 mmHg. Reinjection of 100 ml of cold cardioplegia in the aortic root were repeated at 15, 30, 45 and 60 minutes of cross clamping of aorta. Pericardial sac was filled with cold normal saline and the myocardial temperature was maintained at 16 to $18^{o}C$. Total-body perfusion was maintained at a minimal flow rate of 100 ml/kg/min. At the end of one hour, the corss clamp was removed to restore perfusion and rewarming started. Difibrillation was started when the cardiac muscle temperature rose close to $34^{o}C$. After one half hour of reperfusion and rewarming, cardiopulmonary bypass was discontinued. The hemodynamic stability was achieved through transfusion of the left over blood and perfusion fluid in the extracorporeal system (mean right atrial pressure maintained at 7-10 mmHg. At the end of one hour off bypass all the hemodynamic measurements were made.

CK and MBCK Measurement

The method for measurement of CK and MBCK were the same as described earlier by Prasad et al (26). Two milliliters of blood was collected in a test tube containing 0.1 ml EGTA (20 moles solution)

and was centrifuged to collect plasma. N-acetyl cystine was added in the amount of 0.1 ml of 1 mM solution per ml of plasma as a reducing agent. Creatine kinase-isoenzymes were separated using column chromatography method of Mercer (27). The ion exchanger was prepared for column packing by mixing, twenty-five gram of DEAE Sephadex A50-100 in one liter of buffer #1. This buffer contained Tris-hydrochloride (0.05M/l, pH 8.0) and sodium chloride (100 mM/l).

The mini-column consisted of a 12.5 cm Pasteur pipette. A 3 mm glass bead was placed at the top of the pipette taper. The column was filled with Sephadex to a height of 6 cm above the glass bead. The columns were washed with 2 mls of buffer #1. A plasma sample of 0.5 ml containing CK enzyme was applied to the top of the column and the effluent collected was discarded. The column was then washed with 5 ml of buffer #1 and the effluent collected in a test tube kept in ice bath, each containing 0.5 ml of EGTA (0.5 M). This fraction is MM fraction of CK. The columns were then washed with buffer #2 which contains 0.05 M Tris buffer pH 8.0 containing 50 mM EGTA and 150 mM NaCl. The eluate collected is MB fraction of CK. The CK and MBCK determination was made using Worthington statzyme CPK-N-1 kit (Worthington Diagnostic System Inc., Freehold, N.J. 07728).

Sarcolemmal Na^+-K^+-ATPase Measurement

The method of measurement of sarcolemmal Na^+-K^+-ATPase was similar to that reported earlier by Prasad et al (18). Samples from left ventricle were taken and frozen quickly for estimation of Mg^{++} dependent Na^+-K^+-ATPase activity. The ATPase activity in the presence of 10^{-3} M/L ouabain in the assay medium was substracted from that in the absence of ouabain in the assay medium to obtain the Mg^{++} dependent Na^+-K^+-ATPase, ouabain sensitive portion of ATPase activity.

Sarcoplasmic Ca^{++}-ATPase, Ca^{++}-binding and uptake

Sarcoplasmic Ca^{++}-ATPase activity was determined by the method of Sulakhe et al, (28). Ca^{++}-binding and Ca^{++}-uptake by sarcoplasmic reticulum were determined using millipore filtration technique described by Narayanan (29).

The animals were divided into three experimental groups:

Group I: Sham Control

Eight dogs were included in this group. The chest was opened and the dogs were placed on cardiopulmonary bypass for 3 hours. The aorta

was not cross clamped.

Group II: Cold Crystalloid Cardioplegia

Nine dogs were included in this group. Two hundred mls of cold crystalloid cardioplegia was injected in the aortic root immediately after cross clamping of aorta. Reinjection of 100 ml of cold cardioplegia was repeated at 15, 30, 45 and 60 minutes. External cooling of the heart was done by keeping the heart immersed in cold saline. Myocardial temperature was maintained at 16 to 18^{o}C during aortic cross clamping.

Group III: Cold Crystalloid Cardioplegia Containing Diltiazem

Nine dogs were in this group. Diltiazem in the dose of 150 mg/kg was used. Half of the dose was given in bolus form intravenously 20 minutes before clamping the aorta. The other half dose was added to the cold crystalloid cardioplegia (600 ml). The rest of the procedures were the same as with cold crystalloid cardioplegia group.

Groups II and III were put on total cardiopulmonary bypass for 30 minutes and then the aorta was cross clamped for one hour. The cross clamp was removed and the dog was on bypass for 30 minutes. Thereafter the dogs were taken off the bypass for one hour. The hemodynamic measurements were made before opening the chest (pre.op), before going on pump (pre-pump) and at the end of 1 hr off pump. The blood samples for CK, and MBCK were collected before opening the chest (S_1), before going on bypass (S_2), 5 minutes after declamping the aorta (S_3), 30 minutes off bypass (S_4) and 60 minutes off bypass (S_5).

Statistical analysis was made using the Student Paired "t" test and the probability (p) was deemed significant when less than 0.05.

RESULTS

Cardiac Function

Pre-operative hemodynamic values for sham bypass, and cold crystalloid with and without diltiazem are summarized in Table I. Index of myocardial contractility (dp/dt/IIP), cardiac index, LVWI were lower in the sham bypass group than those in the other two groups. However, the values for LVEDP were higher in sham bypass as compared to those in other two groups. The hemodynamic parameters for the groups of cold crystalloid with or without diltiazem were similar. Because of the variability in certain hemodynamic parameters, the results for

TABLE 1

THE PRE-OPERATIVE AND PRE-PUMP HEMODYNAMIC PARAMETERS IN THE GROUPS OF SHAM BYPASS, COLD CRYSTALLOID CARDIOPLEGIA WITH AND WITHOUT DILTIAZEM.

	Sham Bypass	Cold Crystalloid Cardioplegia	Cold Crystalloid Cardioplegia & Diltiazem
PRE-OPERATIVE VALUES			
(dp/dt)/IIP (sec^{-2})	105 ± 9.2	174 ± 33.6	212 ± 34.2
CI ($L/min/m^2$	2.72 ± 0.19	4.8 ± 0.69	4.53 ± 0.72
TSVR ($dynes \cdot sec \cdot cm^{-5}$)	2071 ± 246	1902 ± 311	2067 ± 384
LVWI ($kg\text{-}m/min/m^2$)	6.29 ± 0.65	8.68 ± 0.88	9.32 ± 2.0
LVEDP (mm Hg)	20.0 ± 3.06	12.89 ± 2.11	7.86 ± 2.26
MAP (mm Hg)	130.8 ± 8.6	107.1 ± 4.2	110.8 ± 6.74
PRE-PUMP VALUES			
(dp/dt)/IIP (sec^{-2})	80.67 ± 12.64	203.0 ± 42.0	185 ± 30
CI ($L/min/m^2$)	2.69 ± 0.31	4.77 ± 0.53	4.51 ± 0.51
TSVR ($dynes \cdot sec \cdot cm^{-5}$)	1863 ± 540	1707 ± 172.8	1659 ± 263
LVWI ($kg\text{-}m/min/m^2$)	4.17 ± 0.43	7.87 ± 0.99	9.50 ± 1.57
LVEDP (mm Hg)	15.67 ± 3.8	11.4 ± 2.5	10.5 ± 3.4
MAP (mm Hg)	93.8 ± 6.7	113.5 ± 7.68	114.6 ± 10.0

The values are mean ± standard error. (dp/dt)/IIP - index of left ventricular contractility; CI - cardiac index; TSVR - total systemic vascular resistance; LVWI - left ventricular work index; LVEDP - left ventricular end-diastolic pressure; MAP - mean aortic pressure.

post-pump have been expressed as percentage of the pre-pump and pre-operative value.

The reperfusion hemodynamic parameters were compared with those of pre-pump and pre-operative period. The results are summarized in Table II and III. When the reperfusion hemodynamic parameters were compared with those of pre-operative values, there were no significant changes in index of myocardial contractility, LVEDP and TSVR for the sham bypass (SB) group. However, there was a marked decrease in the post perfusion CI, LVWI and MAP. When the reperfusion parameters were compared with those of pre-pump values, a marked decrease in the LVWI and MAP was observed in the sham group. The other parameters were unaffected in this group. It appears that sham bypass in general produced a marked decrease in the MAP and LVWI.

TABLE II

HEMODYNAMICS AFTER ONE AND A HALF HOURS OF REPERFUSION FOLLOWING ONE HOUR OF ISCHEMIC CARDIAC ARREST. THE RESULTS ARE EXPRESSED AS % OF PRE-OPERATION VALUES

	Sham Bypass	Cold Crystalloid Cardioplegia	Cold Crystalloid Cardioplegia & Diltiazem
(dp/dt)/IIP	112.7 ± 50.9	82.17 ± 16.2*	130.7 ± 23.7+
CI	70.6 ± 7.7	46.74 ± 2.16*	56.3 ± 6.0+*
TSVR	83.0 ± 25.3	80.0 ± 7.3	92.8 ± 13.8
LVWI	32.2 ± 6.4	38.8 ± 6.5	43.7 ± 4.18
MAP	48.0 ± 12.3	55.9 ± 9.5	56.4 ± 8.4
LVEDP	108.3 ± 25.6	156.0 ± 39.2	187.0 ± 79.0

The values are mean ± standard error. (dp/dt)/IIP - index of myocardial contractility; CI - cardiac index; TSVR - total systemic vascular resistance; MAP - mean aortic pressure; LVEDP - left ventricular end-diastolic pressure.

* $P<0.05$; Sham Bypass vs Cold Crystalloid with and without diltiazem.
\+ $P<0.05$; Cold Crystalloid vs Cold Crystalloid with diltiazem.

Total systemic vascular resistance, LVWI, MAP and LVEDP of cold crystalloid cardioplegic (CCC) group were similar to those of SB group when these values were compared to pre-operative values. However, there was a significant decrease in the cardiac index and a tendency for a decrease in the index of myocardial contracility. When the reperfusion hemodynamic parameters were expressed as percentage of prepump values, there was a marked decrease in the CI, TSVR and MAP as compared to the SB group. The value of the index of myocardial contractility, LVWI and LVEDP in CCC group were similar to those in SB group.

TABLE III

HEMODYNAMICS AFTER ONE AND A HALF HOURS OF REPERFUSION FOLLOWING ONE HOUR OF ISCHEMIC CARDIAC ARREST. THE RESULTS ARE EXPRESSED AS % OF PRE-PUMP VALUES.

	Sham Bypass	Cold Crystalloid Cardioplegia	Cold Crystalloid Cardioplegia & Diltiazem
(dp/dt)/IIP	126.0 ± 34.4	103.0 ± 15.8*	145.3 ± 38.0+
CI	78.5 ± 14.9	51.1 ± 4.56*	51.9 ± 4.8*
TSVR	138.6 ± 24.5	69.3 ± 7.9*	103.7 ± 28.7+
LVWI	52.9 ± 15.1	47.6 ± 11.3	41.0 ± 9.2
MAP	67.8 ± 15.4	45.9 ± 6.1*	53.2 ± 12.3
LVEDP	141.7 ± 46.4	97.5 ± 20.3	114.4 ± 43.8

The values are mean ± standard error. (dp/dt)/IIP - index of myocardial contractility; CI - cardiac index; TSVR - total systemic vascular resistance; MAP - mean aortic pressres; LVEDP - left ventricular end-diastolic pressure.

* $P<0.05$; Sham Bypass vs Cold Crystalloid with and without diltiazem.
\+ $P<0.05$; Cold Crystalloid vs Cold Crystalloid with diltiazem.

It was observed that all the parameters in the group of cold crystalloid with diltiazem (CCCD) were similar to the SB group except that CI was significantly lower in the CCCD group. It was also

observed that the index of myocardial contractility and cardiac index were slightly greater in the CCCD group than in the CCC group. The values for index of myocardial contractility, TSVR, LVWI, MAP and LVEDP in CCCD group were similar to those is SB group. However, the CI in CCCD group was still significantly lower than that in the SB group. There was a tendency for an increase in the index of myocardial contractility with CCCD group as compared to CCC group.

The hemodynamic data suggest that cold crystalloid cardioplegia with diltiazem may be slightly better than cold crystalloid cardioplegia alone because hemodynamic parameters were better preserved with the former intervention.

Serum CK and MBCK

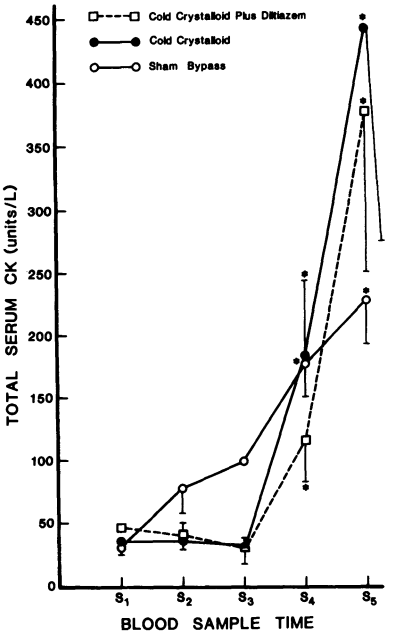

FIGURE 1. Total serum CK activity in the three experimental groups of dogs (sham bypass, cold crystalloid, cold crystalloid plus diltiazem). S_1, pre-operative period; S_2, pre-pump period; S_3, 5 minutes after declamping of aorta following 1 hour of ischemic cardiac arrest; S_4, 30 min. of bypass; S_5, 60 min. off bypass. Note the marked increase in CK values after ischemic arrest and reperfusion.

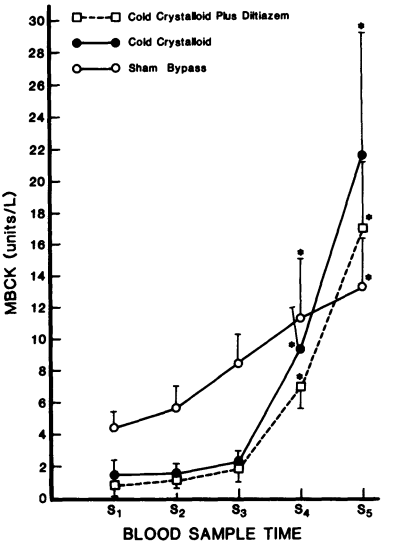

FIGURE 2. Serum MBCK activity in the three experimental groups of dogs (sham bypass, cold crystalloid, cold crystalloid plus diltiazem). The blood sample times are the same as described in Fig. 1. Note the marked increase in the serum MBCK following ischemic cardiac arrest.

The results of the serial measurements of the serum CK in the three groups of animals are summarized in Fig. 1. Total CK increased progressively throughout the experiment in the sham bypass group. However, this enzyme increased after 30 minutes of reperfusion in the cold crystaloid cardioplegic groups with and without diltiazem and these increases were progressive till the end of the protocol. There were no significant differences in the CK values of the three groups at the end of 1 1/2 hours of reperfusion after one hour of ischemic cardiac arrest.

Although the values of serum MBCK was highest in the cold crystalloid group and lowest in the sham bypass group at the end of 1 1/2 hours of reperfusion, these values were not significantly different from each other.

Ca^{++} accumulation, binding and Ca^{++} ATPase

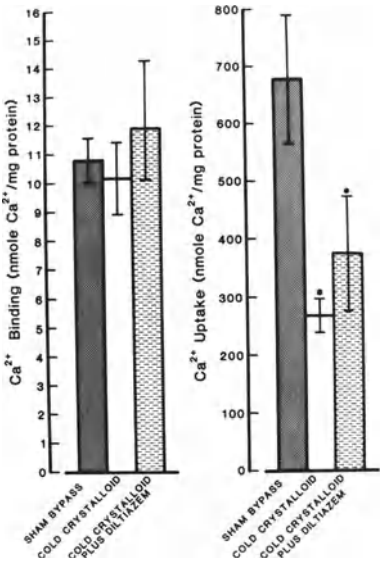

FIGURE 3. Effects of various interventions on the Ca^{++} binding and Ca^{++} uptake by sarcoplasmic reticulm. The results are shown as mean + S.E. performed in duplicate•$P<0.05$ compared to sham bypass.

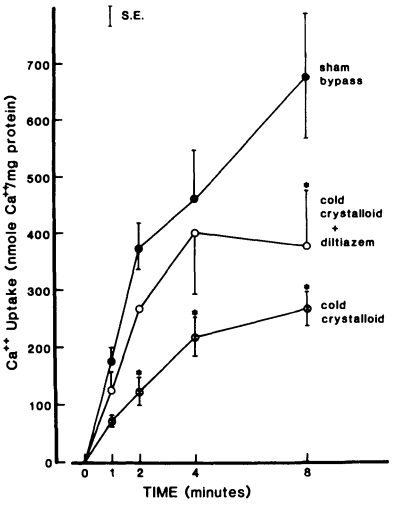

FIGURE 4. The time course of Ca^{++} uptake by cardiac sarcoplasmic reticulum in three experimental groups. The results are expressed as mean + S.E. $P<0.05$ sham bypass vs the other two groups at various time intervals.

Figure 3 shows Ca^{++} binding and uptake by cardiac sarcoplasmic reticulum from dogs with sham bypass; and cold crystalloid cardioplegia with and without diltiazem. There were no significant differences in the Ca^{++} binding by sarcoplasmic reticulum from the three groups. The rate of binding was similar in all the groups. Binding was highest at 1 minute. The Ca^{++} accumulation by sarcoplasmic reticulum from dogs with cold crystalloid cardioplegia was considerably less (39.6%) than that from dogs with sham bypass. Membrane from group with cold crystalloid plus diltiazem showed lower (55%) Ca^{++} accumulation than from groups with sham bypass. However there were no significant differences in the Ca^{++} accumulation by the sarcoplasmic reticulum from the groups with cold drystalloid cardioplegia and cold crystalloid cardioplegia with diltiazem. The rate of Ca^{++} accumulation by sham bypass group was greater than that by crystalloid cardioplegia with or without dilitazem at all levels of incubation (Fig. 4).

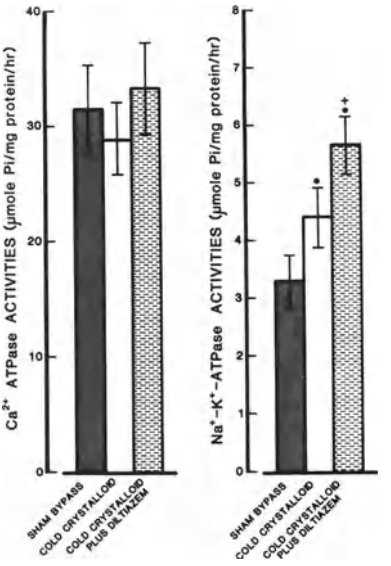

FIGURE 5. Effect of sham bypass, cold crystalloid and cold crystalloid with diltiazem on the Ca^{++} ATPase activity of cardiac sarcoplasmic reticulum (left hand side) and on the sarcolemmal Na^{+}-K^{+}-ATPase (right hand side). The results are expressed as mean $\pm$ S.E. $^{*}P<0.05$, sham bypass vs cold crystalloid with or without diltiazem. $^{+}P<0.05$ cold crystalloid vs cold crystalloid plus diltiazem.

Since the Mg^{++} dependent, Ca^{++} stimulated ATPase of sarcoplasmic reticulum energizes the active transport of Ca^{++} across the membrane, the Ca^{++} ATPase activities of the three groups were also determined. Ca^{++} stimulated ATPase of the membrane fraction from these groups (sham bypass, cold crystalloid cardioplegia, and cold crystalloid cardioplegia plus diltiazem) were not significantly different from each other (Fig. 5).

Sarcolemmal Na^{+}-K^{+}-ATPase

An increase in the sarcolemmal Mg^{++}-dependent-Na^{+}-K^{+}-ATPase has been shown to be associated with a decrease in the myocardial contractility and vice-versa. It was therefore, decided to determine the cardiac sarcolemmal Na^{+}-K^{+}-ATPase in these three groups of animal. The basal Mg^{++}-ATPase activities were 19.30 $\pm$ 3.1 (S.E.), 15.3 $\pm$ 2.69 (S.E.) and 14.29 $\pm$ 4.52 (S.E.) in groups of sham bypass, cold crystalloid cardioplegia and cold crystalloid cardioplegia plus diltiazem respectively. These values were not significantly different from each other. The Na^{+}-K^{+}-ATPase activity of sarcolemmal fraction from group of cold crystalloid cardioplegia with and without dilitazem were higher than that of sham bypass group (Fig. 5). The Na^{+}-K^{+}-ATPase activity of diltiazem was greater than that of cold crystalloid group.

DISCUSSION

The results of the present study show that the pre-operative and pre-pump hemodynamic values in the three groups were very similar except that index of cardiac contractility (dp/dt/IIP) and CI were lower in the bypass group. Because of this variability in some of the hemodynamic parameters, the post-bypass reperfusion hemodynamic values have been expressed as percentage of either pre-operative or pre-pump values. Since the sham bypass alone had deleterious effects on some of the hemodynamic parameter (CI, LVWI and MAP) as shown in Tables II and III, the post-bypass reperfusion hemodynamic values with different interventions have been compared with those of sham bypass group.

The effect of various interventions on the hemodynamic recovery following 1 1/2 hour of reperfusion after one hour of ischemic cardiac arrest suggested that cold crystalloid cardioplegia with diltiazem had better protective effect on the myocardial contractility and cardiac index than cold crystalloid cardioplegia alone. The protective effect

of calcium channel blockers on the recovery of left ventricular function (dp/dt and CI) after ischemic cardiac arrest have also been shown by other investigators (30,31). These investigators used dp/dt as index of myocardial contractility. Since this parameter is affected not only by contractility but also by preload, afterload and heart rate, this parameter may not be the true index of myocardial contractility. The index of myocardial contractility (dp/dt/IIP) used in the present study is not affected by pre-load and hence is a better index of contractility than dp/dt. This index of contractility was better preserved with cold crystalloid plus diltiazem than with cold crystalloid alone.

Calcium binding by, and Ca^{++}-ATPase of sarcoplasmic reticulum was not affected by the reperfusion for 1 1/2 hours following one hour of ischemic cardiac arrest with the two cardioplegic solutions (cold crystalloid cardioplegia with and without diltiazem). However, the Ca^{++} uptake by sarcoplasmic reticulum is markedly reduced in the group of cold crystalloid cardioplegia. Cold crystalloid with diltiazem produced a partial restoration of Ca^{++} uptake by the sarcoplasmic reticulum towards the control value (sham bypass).

The role of sarcoplasmic reticulum in process of cardiac relaxation is well established (32). Although it is known that availability of intracellular Ca^{++} is essential for cardiac contraction, the source of Ca^{++} is not well established. The cell membrane depolarization probably serves as a trigger not only for the displacement of Ca^{++} across sarcolemma but also for the displacement of Ca^{++} from intracellular binding sites including the sarcoplasmic reticulum and release of these ions into the myoplasm. Conditions which result in an increased amount of Ca^{++} being accumulated in sarcoplasmic reticulum probably result in additional Ca^{++} being available for release during subsequent depolarization.

The present results show that the decrease in the contractility index and cardiac function which accompanies cold crystalloid cardioplegia is associated with a decrease in the Ca^{++} uptake by the sarcoplasmic reticulum. Also the improvement in the index of cardiac contractility with cold crystalloid plus diltiazem was associated with a tendency for an increase in the Ca^{++} uptake by the sarcoplasmic reticulum. No results are available on the Ca^{++} uptake and binding by

sarcoplasmic reticulum and Ca^{++}-ATPase of sarcoplasmic reticulum in a bypass setting similar to that in the present studies. Both increases, decreases and no changes in these parameters in failing and hypertrophied heart have been reported (33-36). Myocardial ischemia induced in dogs by coronary artery ligation has resulted in a decrease in the Ca^{++} uptake by sarcoplasmic reticulum (37). A decrease in the Ca^{++} uptake by sarcoplasmic reticulum in the failing heart without any change in the Ca^{++}-ATPase of sarcoplasmic reticulum may be because of the uncoupling of these two events.

Sarcolemmal Na^{+}-K^{+}-ATPase increased with both the cold crystalloid without diltiazem and with diltiazem. These findings are consistent with those in failing heart (16-19). Since there was an improvement in the index of myocardial contractility with diltiazem, one would have expected a decrease in the sarcolemmal Na^{+}-K^{+}-ATPase. However, the results show that there was a significant increase in sarcolemmal Na^{+}-K^{+}-ATPase with cold crystalloid plus diltiazem when these values were compared to those of cold crystalloid group.

The serum creatine kinase in dog pre-operatively was found to be similar to those reported by Knob and Seidl (38) and Ahmed et al, (39). In the present investigation six of 26 dogs did not show MBCK in the serum pre-operatively. The remaining 20 dogs had a very small amount of MBCK (1.92 to 7.72 units/l) in the serum pre-operatively. Our results are contrary to that of Knob and Seidl (38) and Klein et al (40) who could not detect any MBCK in the blood of healthy dogs. Although there was a progressive increase in the total CK and MBCK in all the three groups, the increases were more with cold crystalloid without and with diltiazem. Furthermore the increases in these enzymes were less with crystalloid plus diltiazem than with cold crystalloid alone, although the differences were not significant between these two groups.

These results suggest that cold crystalloid with diltiazem cardioplegia is better than cold crystalloid cardioplegia in protecting the cardiac function, and certain biochemical changes during ischemic cardiac arrest using cardiopulmonary bypass.

REFERENCES

1. Engelman, R.M., Levitsky, S., O'Donoghue, M.J. and Auvil, J. Circulation 58: (Suppl. I) 107 -113, 1978.

2. Harlan, B.J., Ross, D., Macmanus, I., Knight, R., Luber, J. and Starr, H. Circulation 58: (Suppl.I) 114-124, 1978.
3. Kay, H.R., Levine, F.H., Fallon, J.T., Grotte, G.J., Butchart, E.G., Rao, S., McEnany, M.T. and Buckley, M.J. J. Thorac. Cardiovasc. Surg. 76: 590-603, 1978.
4. Sink, J.D., Pellom, G.L., Currie, W.D., Chitwood, W.R., Hill, R.C. and Wechsler, H.S. Circulation 60: (suppl. I) 158-163, 1979.
5. Conti, V.R., Bertranow, E.G., Blackstone, E.H., Kirklin, J.W. and Digerness, S.B. J. Thorac. Cardiovasc. Surg. 76: 577-589, 1978.
6. Folletta, D.M., Mulder, D.G., Maloney, J.V., Jr. and Buckberg, C.D. J. Thorac. Cardiovasc. Surg. 76: 604-619, 1978.
7. Engelman, R.M., Chandra, R. and Bauman, F.G. Surgery 80: 266-276, 1976.
8. Henry, P.D., Snuchleib, R., Davis, J., Weiss, E.S. and Sobel, B.E. Am. J. Physiol. 233: H677-H684, 1977.
9. Snen, A.C. and Jennings, R.B. Am. J. Pathol. 67: 441-452, 1972.
10. Nayler, W.G., Yebez, C., Grau, A. and Slade, A. Cariovasc. Res. 10: 650-662, 1976.
11. Jennings, R.B., Ganote, C.E. and Reimer, K.A. Am. J. Pathol. 81: 179-198, 1975.
12. Lakkatta, E.G., Nayler, W.G. and Poole-Wilson, P.A. Europ. J. Cardiol. 10: 77-87, 1977.
13. Prasad, K., Khatter, J.C. and Bharadwaj, B. J. Cardiovasc. Surg. 22: 153-162, 1981.
14. Whalen, D.A., Jr, Hamilton, D.G., Ganote, C.E. and Jennings, R.B. Am. J. Pathol. 74: 381-398, 1974.
15. Katz, A.M. and Reuter, H. Am. J. Cardiol. 44:188-190, 1979.
16. Khatter, J.C. and Prasad, K. Cardiovasc. Res. 10: 637-641, 1976.
17. Prasad, K., Khatter, J.C. and Bharadwaj, B. Cardiovasc. Res. 13: 95-104, 1979.
18. Prasad, K., O'Neil, C.L. and Bharadwaj, B. Am. Heart. J. 108: 1487-1494, 1984.
19. Prasad, K., O'Neil, C.L. and Bharadwaj, B. Cardiovasc. Res. 19: 406-410, 1985.
20. Anumada, G., Roberts, R. and Sobel, B.E. Progr. Cardiovasc. Dis. 18: 405-420, 1976.
21. Snell, W.E., Lavell, J.F. and Covel, J.W. J. Clin. Invest. 52: 2579-2590, 1973.
22. Siegel, J.H., Sonnenblick, E.H., Judge, R.D. and Wilson, W.S. Cardiologia. 45: 189-221, 1964.
23. Yang, S.S., Bentivoglio, L.G., Maranhao, V. and Goldberg, H. Philadelphia FA Davis Company, p 157, 1972.
24 Prasad, K. and Khatter, J.C., Bharadwaj, B. Jap. Heart J. 18: 823-840, 1977.
25. Ettinger, J.S. and Suter, P.F. Philadelphia W.B. Saunders Co., pp 233-235, 1970.
26. Prasad, K., Kim, R.B. and Debnath, D. Cand. J. Cardiol. 2: 34-41, 1986.
27. Mercer, D.W. Clin. Chem. 20: 36-40, 1974.
28. Sulakhe, P.V., Drummond, G.I. and NG, D.C. J. Biol. Chem. 248: 4158-4162, 1973.
29. Narayanan, N. J. Mol. Cell. Cardiol. 15: 7-15, 1983.
30. Vouhe, P.R., Helias, J. and Grondin, C.M. Annals. Thorac. Surg. 30: 342-348, 1980.
31. Magovern, G.J., Dixon, C.M. and Burkholder, J.A. J. Thorac. Cardiovasc. Surg. 82: 239-244, 1981.

32. Nayler, W.G. In modern trends in Cardiology - 3 eds. M.F. Oliver. Butterworths, London 154-181, 1983.
33. Harigaya, S. and Schwartz, A. Circ. Res. 35: 781-794, 1969.
34. Ito, Y. and Chidsey, C.A. J. Mol. Cell. Cardiol. 6: 237-247, 1974.
35. Mead, R.J., Peterson, M.B. and Welty, J.D. Circ. Res. 29: 14-20, 1971.
36. Sordahl, L.A., McCollum, W.B., Wood, W.G. and Schawartz, A. Am. J. Physiol. 244: 497-502, 1973.
37. Lee, K.S., Ladinsky, H. and Stuckey, J.H. Circ. Res. 21: 439-444, 1967.
38. Knob, M. and Seidl, I. Clin. Chim. Acta. 106: 287-293, 1980.
39. Ahmed, S.A., Williamson, J.R., Roberts, R., Clark, R.E. and Sobel, B.E. Circulation 54: 187-193, 1976.
40. Klein, M.S., Shell, W.E. and Sobel, B.E. Cardiovasc. Res. 7: 412-418, 1973.

16

EFFECTS OF VERAPAMIL ON METABOLISM AND FUNCTION OF HEARTS FROM NORMAL AND HYPERTHYROID RATS.

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INTRODUCTION

That thyroid hormones sensitize the heart and other organs to the metabolic effects of catecholamines is generally accepted. A possible important factor in mediating this hypersensitivety is the intracellular concentration of calcium ions. Hartley and McNeill (1) studied the effect of calcium on cardiac metabolism and myocardial contraction in isolated, perfused hearts from hyperthyroid rats a decade ago. They reported that pretreatment of rats with triiodothyronine did not enhance the elevation in cardiac phosphorylase a activity produced by progressively increasing doses of calcium ions in the fluid perfusing the isolated rat hearts.

Although a positive role for thyroxine to increase the phosphorylase activating effect of calcium was not established by the experiments of Hartley and McNeill (1), we considered it worthwhile to initiate a study to determine the effect of calcium deprivation on cardiac metabolism in hearts from hyperthyroid rats. A major impetus for this investigation was our finding that the glycogen content in animals pretreated with thyroxine is significantly lower than normal. Therefore, of special interest was the action of a slow-channel calcium blocking drug, verapamil, on glycogen metabolism and contractile force in hearts from normal and thyrotoxic rats in the presence and absence of isoproterenol.

METHODS

Hearts from normal and hyperthyroid rats weighing 200-250g were used in these experiments. Hyperthyroidism was produced by injecting thyroxine intramuscularly, 250 μg/rat, for five days. Rats were sacrificed by decapitation, the hearts removed immediately and placed on a Langendorff perfusion apparatus. Measurements of heart rate and force of contraction were made with a force-displacement transducer attached to the apex of the heart. Signals from

the transducer were recorded on a Hewlett-Packard oscillograph. In those experiments in which isoproterenol was given, the drug was infused at a rate of 0.5 ml/minute for two minutes. The total dose of the amine was 0.05, 0.1 or 1.0μg. Control hearts were infused with an equal volume of 0.9% NaCl. Effluent fluid from the heart was collected at appropriate intervals for measurements of lactate production.

At the end of each experiment the heart was frozen with Wollenberger tongs precooled in liquid nitrogen. The hearts were stored in liquid nitrogen until assayed for tissue glycogen content (2,3) and glucose-6-phosphate (4).

RESULTS

As would be predicted, administration of isoproterenol to perfused hearts from euthyroid rats caused a dose-dependent increase in lactate production (Fig. 1). It is apparent from results given in Fig. 1 that lactate production by hearts from hyperthyroid animals, even in the absence of isoproterenol, is significantly higher than that from hearts of normal rats. In response to increasing doses of the catecholamine, the output of lactate from hearts of animals pretreated with thyroxine is enormously increased over normal (Fig.1).

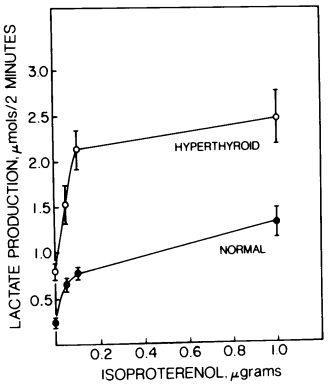

Fig. 1. Lactate production by isolated, perfused hearts from normal and hyperthyroid rats after administration of isoproternol.

Lactate production in hearts stimulated by 1μg isoproterenol in the presence and absence of verapamil is shown in Fig. 2. Again it is evident that the lactate produced by hearts from hyperthyroid rats under all circumstances is significantly greater than that by hearts from control animals. Inclusion of verapamil in the perfusion fluid had no significant effect on lactate production either in hearts from euthroid or thyroxine-treated rats. Stimulation of the production of lactate by isoproterenol was also unaffected by verapamil in hearts from the two groups of animals. (Fig.2).

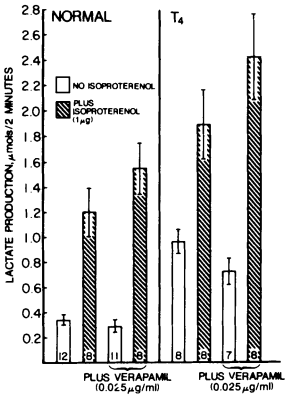

Fig. 2. Effect of isoproternol on lactate production by isolated, perfused hearts from normal and hyperthyroid rats in the presence and absence of verapamil.

As mentioned previously, glycogen content in hearts from rats pretreated with thyroxine is approximately 55% of that measured in hearts from normal animals (Fig.3). Only after a large dose of isoproterenol (1μg) were glycogen levels decreased in hearts from euthyroid rats. In hearts from hyperthyroid rats, where the glycogen content was initially very low, even the highest dose of isoproterenol failed to cause a further reduction in the amount of glycogen in these hearts (Fig.3).

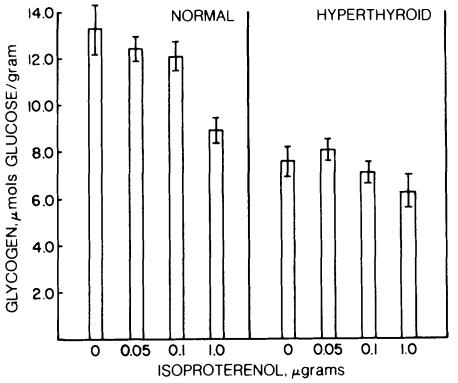

Fig. 3. Glycogen content in isolated, perfused hearts from normal and hyperthyroid rats after administration of isoproterenol.

The decrease in cardiac glycogen produced by the large dose of isoproterenol given to hearts from control rats was still present with verapamil in the perfusion medium (Fig. 4). Similarly, verapamil had no effect on glycogen content in the absence or presence of isoproterenol (1μg) in hearts from hyperthyroid animals (Fig. 4).

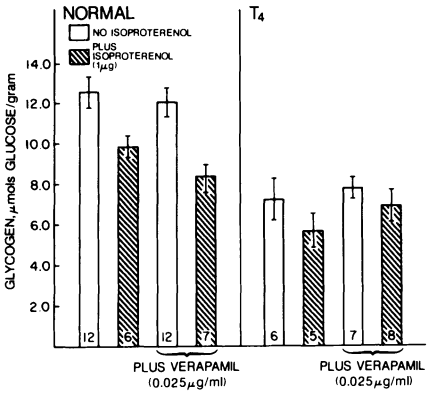

Fig. 4. Effect of isoproterenol on glycogen content of isolated, perfused hearts from normal and hyperthyroid rats in the presence and absence of verapamil.

Hyperthyroidism per se caused an elevation in the concentration of cardiac glucose-6-phosphate, which was not further increased by infusion of isoproterenol (Fig. 5). Administration of the amine to hearts from normal rats produced a significant rise in glucose-6-phosphate (Fig.5), which was not attenuated by verapamil.

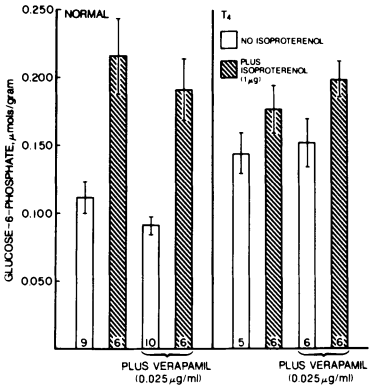

Fig. 5. Effect of isoproterenol on glucose-6-phosphate content of isolated, perfused hearts from normal and hyperthyroid rats in the presence and absence of verapamil.

Isometric force of contraction was measured in response to isoproterenol, verapamil and isoproterenol after verapamil. The results of these measurements are given in Fig. 6. Parenthetically, it should be noted that the force of contraction of the heart in the absence of any drug was of equal magnitude in hearts from euthyroid and hyperthyroid rats. This observation is in agreement with the earlier work reported by Hartley and McNeill (1). As can be seen, in Fig. 6 verapamil caused an equal depression of contractile force in hearts from both groups of rats.

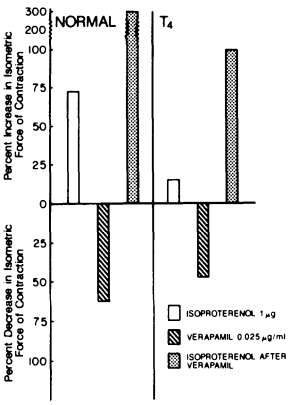

Fig. 6. Isometric force of contraction in response to isoproterenol and/or verapamil in hearts from normal and hyperthyroid rats.

A suprising finding was the difference in isometric force of contraction seen in hearts from normal versus hyperthyroid animals when isoproterenol was given alone or to hearts depressed by verapamil. In the former case, isoproterenol caused a marked increase in force of contraction with or without verapamil in the perfusion fluid, while in hearts from thyroxine-treated rats, the increase in contractile force was considerably less (Fig. 6). Apparently, perfused hearts from hyperthyroid animals are less able to respond to catecholamine-induced stimulation and verapamil is more effective than in hearts from normal rats in limiting the inotropic effect of isoproterenol.

DISCUSSION

Alterations in cardiac glycogen have been shown to be characteristic of several endocrinopathies. For example in experimental diabetes, the concentration of glycogen in the heart is abnormally high (3,5,6). Results reported in this paper showed that in hyperthyroid rats, myocardial glycogen content was approximately half that found in hearts of normal animals. Since calcium ions are intimately involved in regulating carbohydrate metabolism, it is logical to investigate the effect of slow channel calcium antagonists (calcium entry blockers) on thyroxine-induced changes in cardiac metabolism. Early work by

Nayler et al. (7) demonstrated that in intact dogs thyroid hormone does indeed enhance the ability of sarcoplasmic reticulum to accumulate calcium. One can assume that in order for the sarcoplasmic reticulum to take up more calcium, increased calcium ions must be present in the cytoplasm, presumably through the action of thyroxine. By decreasing the entry of calcium into the cell with verapamil, the number of calcium ions available to be taken up by the sarcoplasmic reticulum and subsequently released during depolarization would be substantially reduced. Such an interruption in the supply of calcium, would inhibit the conversion of phosphorylase b to a by phosphorylase b kinase, a calcium-dependent enzyme. A reduction in glycogenolysis after verapamil should then be expected to raise the depleted concentration of cardiac glycogen in hearts of hyperthyroid rats. This was the rationale for conducting these experiments.

Because lactate production was increased in hearts from euthyroid and hyperthyroid rats by infusion of doses of isoproterenol which did not diminish myocardial glycogen, we feel that the outpouring of lactate in these experiments was due to an increase in glycolysis rather than enhanced glycogenolysis. When the largest dose of isoproterenol (1 μg) was used, glycogen content in normal hearts was reduced, but the presence of verapamil in the perfusion fluid did not prevent this catecholamine-induced decrease in cardiac glycogen. The failure of verapamil to counteract the glycogenolytic action of isoproterenol is not without precedence, since Watanobe and Besch(8) found that an anolog of verapamil, D600, did not inhibit stimulation of adenylate cyclase activity or production of cyclic AMP caused by injection of epinephrine into perfused guinea pig hearts.

In hearts from hyperthyroid animals in which cardiac glycogen was decreased as a result of chronic treatment with thyroxine, perfusion with medium containing verapamil did not restore glycogen levels to normal. It may be that calcium entry must be inhibited for a longer period of time in order for the restoration of normal concentrations of heart glycogen to occur. Of interest is the observation that infusion of a large amount of isoproterenol (1 μg) into hearts from thyroxine-treated rats caused no further reduction in these glycogen-depleted hearts. This would imply that the concentration of cardiac glycogen can be decreased to a certain critical level by thyroxine, beyond which it does not fall even when the heart is exposed to high concentrations of known glycogenolytic drugs.

Unlike hearts in situ in hyperthyroid animals, isolated, perfused rat hearts from thyroxine-treated animals did not exhibit any greater basal contractile force than isolated, perfused hearts from normal animals. While hearts from euthyroid and hyperthyroid rats were equally vulnerable to the negative inotropic effect of verapamil, the positive inotropic response to isoproterenol alone and in the presence of the calcium-blocking drug was quite different in the two groups of hearts. With hearts from hyperthyroid rats the increase in contractile force produced by the catecholamine in the presence or absence of verapamil was significantly less than that generated when isoproterenol was infused into normal hearts with or without verapamil in the perfusion fluid. A reasonable explanation for the decreased contractile response to isoproterenol in hearts from thyroxine-treated rats is their lower-than-normal content of glycogen. In other words, glycogen stores were sufficient to permit normal, basal contractile force, but insufficient to allow the heart to respond fully to the positive inotropic action of isoproterenol.

SUMMARY

Lactate production by the isolated, perfused rat heart is markedly increased by the hyperthyroid state. Isoproterenol stimulates the output of lactate to a greater extent in hearts from hyperthyroid rats as compared to that produced by hearts from euthyroid animals. Verapamil does not inhibit formation of lactate induced by isoproterenol either in hearts from normal or hyperthyroid rats.

In hyperthyroid rats, cardiac glycogen content is significantly lower than normal and is not further reduced by a dose of isoproterenol (1 μg) which effectively decreases the amount of glycogen in hearts from euthyroid animals.

Verapamil fails to affect the stimulatory action of isoproterenol on lactate output or glucose-6-phosphate production in hearts from normal or hyperthyroid rats.

In hearts from either group of animals, verapamil causes an equal reduction of isometric force of contraction (IFC). The inotropic response to isoproterenol either in the presence or absence of verapamil is significantly less in hearts from thyroxine-treated animals than in hearts from normal rats.

REFERENCES

1. Hartley, E.J. and McNeill, J.H. Can. J. Physiol. Pharmacol. 54:590-595, 1976.
2. Sølling, H. and Esmann, U.A. Analyt. Biochem 68:664-668, 1975.
3. Haugaard, N., Hess, M.E., Locke, C.L., Torbati, A. and Wildey, G. Biochem. Pharmacol. 33:1503-1508, 1984.
4. Lamprecht, W. and Trautschold, L. In: Methods of Enzymatic Analysis (Bergmeyer, H.U., Editor) Academic Press, New York, 1974, Vol. 4, pp2101-2109.
5. Cruickshank, M.B. J. Physiol. 47:1-14, 1913.
6. Shipp, J.C., Opie, L.H. and Challoner, D. Nature, London, 189:1018-1019, 1961.
7. Nayler, W.G., Merrillees, N.C.R., Chipperfield, D. and Kurtz, J.B. Cardiovas. Res. 5:469-482, 1971.
8. Watanabe, A.M. and Besch, H.R., Jr. J. Pharmacol. Exp. Ther. 191:241-251, 1974.

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REGULATION OF BETA-ADRENERGIC PATHWAYS IN THE HEART

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Beta-adrenergic pathways are affected by several physiologic and pathologic processes involving the heart [1-29] (Table 1).

Table 1: Effect of hypertrophy and other pathologic conditions on cardiac β-adrenoceptors and isoproterenol stimulate adenylate cyclase.

	β-Adrenoceptor		
	Bmax	K_D	Adenylate Cyclase
Hypertrophy			
Isoproterenol[1]	Decreased	Unchanged	Decreased
Norepinephrine[2]	Increased	Unchanged	Increased
Aortic constriction[3]	Increased	Unchanged	----
[4]	Increased	Increased	Unchanged
Thyroxine[5-8]	Increased	Increased	Increased
SHRs[9-14]	Decreased	Increased	Decreased
Salt-DOCA[15]	Decreased	Increased	
Renal Hypertension[12,16]	Decreased	Increased	Decreased
[17]	Unchanged	Increased	----
[18]	Increased	Increased	Decreased
Dahl[19]	Increased	Unchanged	Increased
Exercise[20]	Decreased	Unchanged	
[21]	Unchanged	Unchanged	
Ischemia[23,24]	Increased	Increased	Decreased
[25]	Increased	Unchanged	----
Diabetes[26]	Decreased	Unchanged	
Heart Failure			
Humans[27]	Decreased	Unchanged	Decreased
Guinea-pigs[28]	Increased	Unchanged	----
Dogs[29]	Increased	Increased	----

In general, changes in β-receptor numbers/affinities are accompanied by concordant changes in inotropic responsiveness to β-agonists. There is a clinical correlate of these observations since a decline in functional β-receptors is a regular feature of the failing heart[27] and contributes to the inotropic impairment in this syndrome. In addition, this decline limits the effectiveness of inotropic drugs acting through β-receptor stimulation. There is, thus, considerable incentive for studying the control of β-receptor number and their functional coupling to adenylate cyclase in the heart. The following is a brief selective overview of the potential mechanisms involved.

Since β-receptors are glycoproteins embedded in the cell membrane bilayer,[30] their apparent number and degree of coupling to adenylate cyclase may be modulated by altering the physical state of the membrane in their microenvironment. There are indeed several reports of agonist binding modulation by fatty acids[31] or phospholipid-active compounds.[32] In addition, phospholipid methylation, which increases the fluidity of the membrane and is altered in cardiac hypertrophy,[33] enhances the number of membrane-bound β-receptors in the heart.[34]

Changes in their rate of synthesis is an obvious site for regulating the number of cellular receptors. Relatively little is known, however, about the contribution of altered synthetic rate to the reported changes in membrane-bound β-receptors during physiologic and pathologic conditions. Pitha and colleagues[35,36] have used the irreversible alkylating beta-blocker bromoacetylalprenololmenthane (BAAM) to study the rate of β-receptor reappearance (which reflects de novo synthesis). After a single BAAM injection, complete recovery of cardiac β-receptors occurs within 7 days. Aging significantly prolongs the time required for complete recovery indicating an age-dependency of β-receptor synthesis in the heart.[36] Comparable data for pathologic conditions affecting the myocardium are not available. We have recently examined whether the decline in cardiac β-receptors in spontaneously hypertensive rats (SHRs) is due to defective receptor synthesis. After a single intraperitoneal injection of BAAM, β-receptors on cardiac myocytes decreased by about 50% in both SHRs and WKYs (Figure 1). The rate of reappearance was actually faster in SHRs with complete recovery by the sixth postinjection day compared to 7-8 days in WKYs (Figure 2). A similar difference was noted between the two experimental groups in the recovery of the ability of isoproterenol to stimulate

cyclic AMP but this recovery lagged behind the reappearance of β-receptors in both hypertensive and normotensive animals (Figure 3).

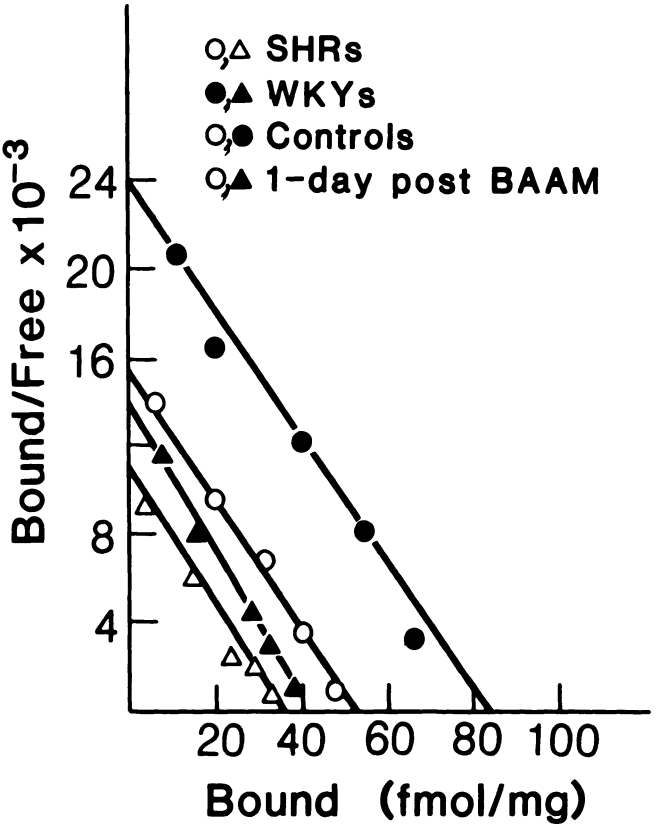

Figure 1: Scatchard plot of [^{3}H]CGP-12177 binding to cardiac myocytes from SHRs (o,Δ). Myocyte suspension of control (o,o) or BAAM-injected (Δ,Δ) animals were incubated with varying concentrations (1-20 nM) [^{3}H]CGP=12177 for 16 hours at 4°C prior to filtration and counting.

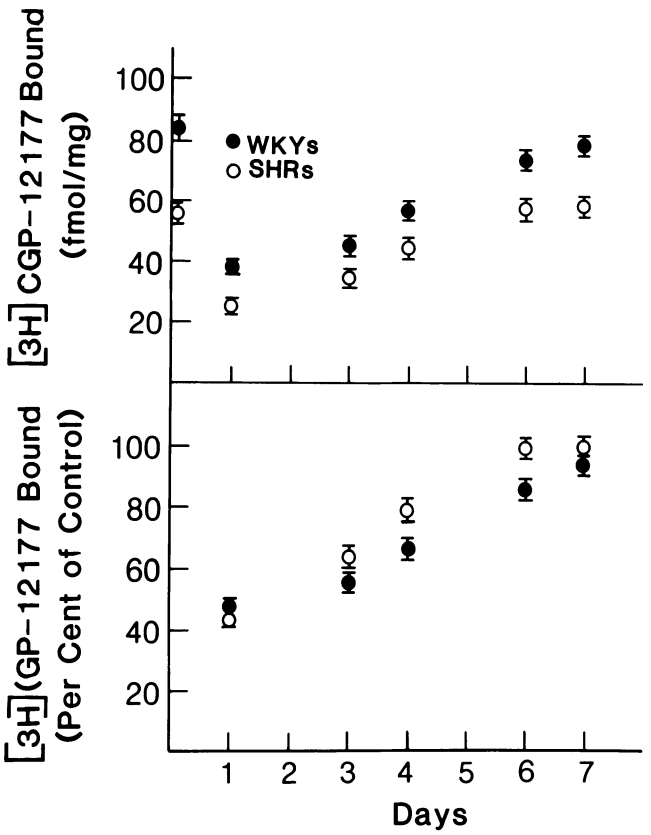

Figure 2: Time course of cardiac beta-receptor recovery following BAAM. SHRs (o) and WKYs (o) were injected with 35 mg/kg I.P. BAAM and sacrificed at varying intervals thereafter. Cardiac beta-receptors were assayed in myocyte suspensions using 6 nM [^{3}H]CGP-12177. Results are given as mean ± SE for 4-7 animals in each group. In the lower panel cardiac beta-receptors are expressed as percent of controls (vehicle only injected animals).

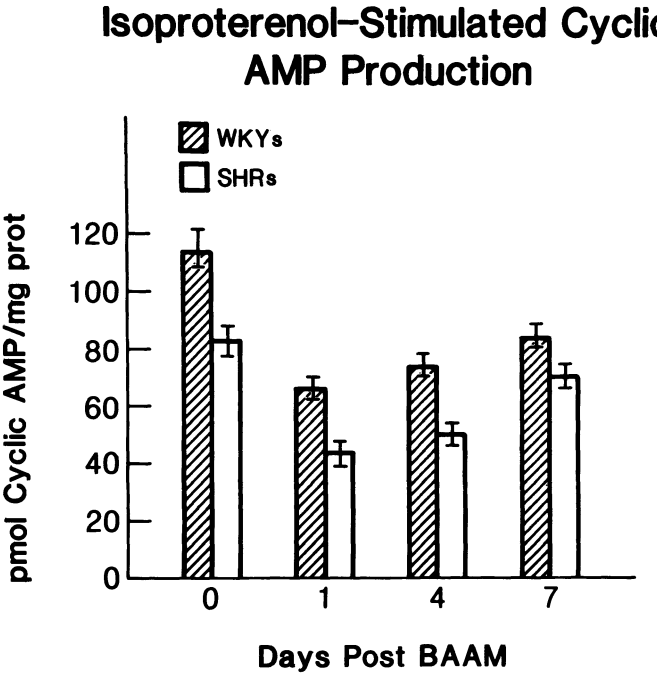

Figure 3: Isoproterenol-induced cyclic AMP production by cardiac myocytes of SHRs (o) and WKYs (o). Myocyte suspensions were incubated at 37°C for 10 minutes in the presence of 0.1 mM isobutylmethylxanthine and the indicated concentrations of (-) isoproterenol. Results represent mean ± SE for four separate experiments.

It would appear from these results that reappearance of membrane-bound beta-receptors precedes the recovery of functional adenylate cyclase (or coupling of the receptors to the adenylate cyclase). Similar observations have recently been made in other cell systems.[37] BAAM may provide us with a tool to study the mechanisms for establishing effective coupling between beta-receptors and components of the adenylate cyclase complex. Although differences in establishing such coupling are not evident in the myocardium SHRs and WKYs, it is conceivable that, in other models of hypertrophy, enhanced rates of beta-receptor synthesis may not be translated into equivalent numbers of functional receptor-adenylate cyclase units.

Once synthesized and inserted into the plasma membrane, β-receptors still participate in intracellular traffic as a consequence of their interactions with agonists. There is accumulating evidence that receptors shuttle between the plasma membrane and an intracellular site. Although this reversible cycling may occur in the absence of agonist binding, for β-receptors it is usually associated with the process of homologous desensitization i.e. decreased magnitude of agonist-mediated physiologic responses following exposure of the receptors to their agonists. This desensitization is associated with a loss of membrane-bound β-receptors and/or adenylate cyclase activity. Two mechanisms have been proposed for "desensitization" to agonist effects:[38-41] (a) "down" regulation which results in an apparent loss of detectable receptors, and (b) "uncoupling" of the β-receptor from the adenylate cyclase manifested as unchanged number of β-receptors associated with diminished cyclic AMP generation. The two mechanisms are not mutually exclusive and, in some cells, uncoupling of the β-receptors temporally precedes their "loss" following prolonged exposure to isoproterenol.

Relatively little is known about the process of desensitization in the normal and diseased myocardium. Marsh et al[42] and Bobik et al[43] have observed that, in cultured chick heart cells, decreased inotropic responsiveness after a short preincubation with this agonist was due to uncoupling of the β-receptor from the adenylate cyclase. This contrasts with the decline in cardiac β-receptors noted after in vivo isoproterenol administration[1].

Using isolated cardiac myocytes, we have recently noted a rapid decline in the numbers of cardiac β-receptors after in vitro incubation of cardiac

myocytes with (-) isoproterenol[44]. This decline was reversible upon agonist removal (85% of the "lost" β-receptors reappear within 20 minutes). The recovery of the β-receptors is energy-dependent and requires intact microtubule assembly. The mechanism of the isoproterenol-induced "down" regulation was studied following in vivo administration of a single (-) isoproterenol dose in rats.[45] A decline in the number of β-receptors on cardiac membranes was associated with a parallel increase in cytosolic binding sites. This redistribution of β-receptors was prevented by the in vivo administration of colchicine and vinblastine, but not lumicolchicine. None of these compounds affected the numbers of β-receptors when added directly to the assay medium. Receptor redistribution was a consequence of the isoproterenol-β-receptor interaction since it was prevented by alprenolol pretreatment. In contrast, no redistribution of adenylate cyclase was induced by isoproterenol. Similar results have recently been reported for cultured heart cells.[46]

These results suggest that isoproterenol-induced desensitization involves the translocation of cardiac β-receptors to the cytosol where they may be further processed. This translocation appears to depend on intact microtubule assembly. The subcellular structures into which internalized cardiac β-receptors are associated have not been unequivocally identified yet. In other cell systems, "down" regulation is associated with sequestration of the β-receptors in a vesicular fraction devoid of plasma membrane markers and occupying a low-density position on sucrose gradients. Whether this fraction indeed represents an intracellular structure in intact cells or a specialization of the plasma membrane has not been conclusively proven.

A novel mechanism of heterologous β-receptor desensitization has recently been studied, i.e., phorbol ester- and diacylglycerol-mediated.[47] Phorbol esters have been the focus of intense investigation because of their growth-promoting properties. It has become apparent, however, that they have additional important effects including the modification of hormone-receptor interactions, such as the transferrin, insulin, epidermal growth factor and β-adrenergic receptors. It is thought that phorbol esters exert their effects by binding to cellular receptors which copurify with and may be identical to the Ca^{2+}/phospholipid protein kinase (protein kinase C). According to current concepts, phorbol esters substitute for

unsaturated diacylglycerols, the endogenous activator of protein kinase C, in lowering the Ca^{2+} requirements of the enzyme. Both phorbol esters and unsaturated diacylglycerols can be used, therefore, as probe of the biological role of protein kinase C.

We have recently described the presence of specific phorbol ester receptors on rat cardiac myocytes.[48] In addition, protein kinase C is present in the myocardium and may play an important role in the regulation of cardiac function.

We have recently shown[47] that biologically active phorbols and the synthetic diacylglycerol 1-oleyl-2-acetyl diglycerol (OADG) promote desensitization of the β-receptors which is microtubule-dependent and involves β-receptor internalization.

Incubation of enzymatically dissociated rat cardiac myocytes in vitro with OADG, phorbol dibutyrate or TPA (but not the inactive 4α-phorbol or 4α-phorbol-12,13-dibutyrate) resulted in a time-and concentration-dependent loss of β-receptors detectable with [^{3}H]CGP-12177. There was a small additive effect when preincubation with either phorbol dibutyrate or OADG was carried out in the presence of isoproterenol, suggesting separate sites of action.

Both OADG- and phorbol dibutyrate-mediated loss of cardiac β-receptors was largely prevented by colchicine but not the inactive analog, trimethylcolchicinic acid. This points to a requirement for intact microtubule assembly. The mechanism of the β-receptor loss was then investigated by lysing control and phorbol dibutyrate- or OADG-pretreated cells and separating the membrane and cytosolic fractions. Following a one-hour 158,000 xg centrifugation of the cytosol, a vesicular fraction devoid of plasma membrane markers was isolated. Both phorbol dibutyrate and OADG induced a redistribution of the β-receptor from the cell membrane to the vesicular fraction without a significant change in the total β-receptor numbers. These results are consistent with internalization of the β-receptor as a result of phorbol dibutyrate-or OADG-pretreatment.

Since diacylglycerols which activate protein kinase C derive from receptor-linked phosphoinositide hydrolysis,[48] we examined the effects of carbachol, a muscarinic agonist.[49] Carbachol induces a time- and concentration-dependent decline of β-receptors on isolated cardiac myocytes. Scatchard plot of the binding data reveals that carbachol induces a

decline in receptor affinity as well as the total receptor numbers. Gallamine, which blocks binding to the M_2-receptor does not affect carbachol-induced desensitization. Similarly, oxotremorine, which binds preferentially to the M_2 receptor, does not influence β-receptor numbers or affinities. These results suggest that the effect of carbachol on the β-receptors is mediated through the M_1 receptor which has been shown to promote phosphoinositide hydrolysis and diacylglycerol generation[45]. It is likely, therefore, that the M_1 receptor-linked desensitization of the β-receptors is mediated through the activation of protein kinase C.

It is interesting that both homologous and heterologous desensitization are associated, least initially, with a redistribution of β-receptors between the plasma membrane and the vesicular fraction. Several processes leading to cardiac hypertrophy and the clinical syndrome of heart failure have the potential of activating the desensitization pathways. For example, activation of the sympathetic nervous system, reflected in higher plasma norepinephrine levels, accompanies the induction of some hypertrophy models (such as the spontaneously hypertensive rat) as well as the development to heart failure.[50,51] It is notable that these models are consistently characterized by a decline in membrane-bound β-receptors. It has not been determined, however, whether this decline is part of a redistribution of the β-receptor such as seen during short-term exposure to β-agonists. It is conceivable that post-receptor defects may follow β-receptor desensitization and further alter the functional effectiveness of β-receptor-cyclase-protein kinase pathways. We have recently examined this issue in the spontaneously hypertensive rat. The cellular distribution of the β-receptors was examined by two methods: a) acid elution of membrane-bound receptors, and b) differential centrifugation to separate the plasma membrane from the vesicular fraction. Both approaches gave comparable results: about 30% of all β-receptors of normotensive animals were recovered in the vesicular fraction. In contrast, about 45% of total receptors of SHRs were in the vesicular fraction. The total number of cellular β-receptors did not differ significantly in the two experimental groups. This pattern is consistent with chronic desensitization secondary to sympathetic nervous system activation. However, these changes in β-receptors were associated with mirror-image changes in cardiac α-receptors i.e. increase in membrane-bound and a corresponding decline in

α-receptors recovered in the vesicular fraction. It is not possible, on the basis of available evidence, to ascribe these changes to increased norepinephrine levels. It may be necessary to invoke a different mechanism for the redistribution of α-receptors. Since both α- and β-adrenoceptors subserve inotropism (albeit through different pathways), the increase in α-receptors may be viewed as a compensatory response. The mechanism(s) mediating this response, however, remain unclear. Kunos and colleagues[52,53] have suggested that, under certain experimental conditions, α- and β-receptors interconvert through pathways which may involve products of arachidonate metabolism. Whether these observations are relevant to the reciprocol changes in α- and β-adrenoceptors in the myocardium of SHRs is not known. It is clear, however, that such reciprocal changes are not a universal feature of cardiac hypertrophy since renovascular hypertension is associated with lower α- and β-adrenoceptors in cardiac membranes.[15] Hyperthyroidism, on the other hand, is characterized by reciprocal changes in membrane-bound adrenoceptors.[5,7] It is evident that the functional implications of changes in β-adrenoceptors will have to be evaulated within the context of changes in other receptor systems as well as other biochemical adaptations to the process of cardiac hypertrophy. Furthermore, pathologic conditions involving the heart may introduce additional mechanisms for modifying β-adrenoceptor function. There is evidence, for example, that alloimmune I gG may impair ligand binding to β-receptors.[54] Immune mechanisms seem to play an important role in Chagas' cardiomyopathy.[55] Similarly, a high percentage of patients with idiopathic cardiomyopathy have serum factor(s) inhibiting [^{3}H]dihydroalprenolol binding to cardiac myocytes.[56] Further studies are needed to elucidate the precise role of humoral and cellular immunity in promoting β-receptor changes in heart disease.

REFERENCES

1. Tse J, Powell JR, Baste CA, Priest RE, Kuo JF. Endocrinology 105: 246-255, 1979.
2. Raum WJ, Laks MM, Garner D, Ikuhara MH, Swerdloff RS. Am J Physiol 246: H31-H36, 1984.
3. Limas CJ. Biochim Biophys Acta 588: 174-178. 1979.

4. Vatner DE, Homcy CJ, Pit SP, Manders WT, Vatner SF. J Clin Invest 73 1473-1482, 1984.
5. Williams LT, Lefkowitz RJ, Watanabe AM, Hathaway DR, Besch HR Jr. J Biol Chem 252: 2787-2789, 1977.
6. Ciaraldi T, Marinetti GV. Biochem Biophys Res Commun 74: 984-990, 1977.
7. Fox AW, Juberg EN, May JM, Johnson RD, Abel PW, Minneman KP. J Pharmacol Exp Ther 235: 715-723, 1980.
8. Tse J, Wrenn RW, Kuo JF. Endocrinology 107: 6-16, 1980.
9. Limas C, Limas CJ. Biochem Biophys Res Commun 83: 710-716, 1978.
10. Limas CJ, Limas C. Hypertension 6 (Suppl I): 31-39, 1984.
11. Woodcock EA, Funder JW, Johnston CI. Clin Exp Pharmacol 5: 545-550, 1978.
12. Kumano K, Upsher ME, Khairallah PA. Hypertension 5 (Suppl I): 175-183, 1983.
13. Robberecht P, Winand J, Chatelain P, Poloczek P, Camus JC, DeNeef P, Christophe J. Biochem Pharmacol 27: 1967-1971, 1978.
14. Bhalla RC, Sharma RB, Ashley T. Biochem Biophys Res Commun 82: 273-280, 1978.
15. Woodcock GA, Johnston CI: Circ Res 46: 876, 1984.
16. Saragoca MA, Tarazi RC. Hypertension 3: 380-385, 1981.
17. Gende OA, Mattiazzi A, Camilion MC, Pedroni P, Taquini C, Gomez Llambi H, Cingolani HE. Am J Physiol 249: H814-J819, 1985.
18. Giachetti A, Clark TL, Berti F. J Cardiovasc Pharmacol 1: 467-471, 1979.
19. Limas C, Limas CJ. Hypertension 7: 760-766, 1985.
20. Sylvestre-Gervais L, Nadeau A, Nguen MH, Tancrede G, Rosseau-Migneron S. Cardiovasc Res 16: 530-534, 1982.
21. Williams RS. Cardiovasc Res 14: 177-182, 1980.
22. Cervoni P, Herzlinger H, Lai FM, Panikella P. Br J Pharmacol 74: 517, 1981.
23. Mukherjee A, Wong TM, Buja LM, Lefkowitz RJ, Willerson JT. J Clin Invest 64: 1423-1428, 1979.
24. Devos C, Robberecht P, Nokin P, Waelbroeck M, Clinet M, Camus JC, Beaufort P, Schoenfeld P, Christophe J. Naunyn Schmiedebergs Arch Pathol 331: 71-75, 1985.
25. Maisel AS, Motulsky HJ, Insel PA. Science 230: 183-186, 1985.
26. Heyliger CE, Pierce GN, Singal PK, Beamish RE, Dhalla NS. Basic Res Cardiol 77: 610-618, 1982.
27. Bristow MR, Ginsburg R, Minobe W, Cubicciota RS, Sagman WC, Lurie K. New Eng J Med 307: 205-211, 1982.
28. Karliner JS, Barnes P, Brown M, Dollery C. Eur J Pharmacol 67: 115-118, 1980.
29. Vatner DE, Vatner SF, Fuji AM, Homcy CJ. J Clin Invest 76: 2259-2264, 1985.
30. Shorr RGL, McCaslin DR, Strohsacker MW, Alianell G, Rebar R, Stadel JM, Crooke ST. Biochemistry 24: 6869-6875, 1985.
31. Thang NX, Borsodi A, Wollemann M. Biochemi Pharmacol 29: 2791-2797, 1980.
32. Lefkowitz RJ, Haber E, O'Hara D. Proc Natl Acad Sci USA 69: 2828-2832, 1972.
33. Limas CJ. Biochem Biophys Acta 632: 254-259, 1980.
34. Limas CJ. Circ Res 47: 536-541, 1980.
35. Pitha J, Hughes BA, Kusiak JW, Dax EM, Baker SP. Proc Natl Acad Sci USA 79: 4424-4427, 1982.
36. Baker SP, Pitha J. J Pharmacol Exp Ther 220: 241-251, 1982.

37. Insel PA, Mahan LC. Fed Proc 43: 876, 1984.
38. Kebabian JW, Katz M, Romero JA, Axelrod J. Proc Natl Acad Sci USA 72: 3735, 1975.
39. Su YF, Harden TK, Perkins JP. J Biol Chem 255: 7410-7419, 1980.
40. Homburger V, Lucas M, Cantau B, Barabe J, Penit J, Bockaert J. J Biol Chem 255: 10436-10444, 1980.
41. Stadel JM, Strulovici B, Nambi P, Lavin TN, Briggs MM, Caron MG, Lefkowitz RJ. J Biol Chem 258: 3032-3038, 1983.
42. Marsh JD, Barry WH, Neer EJ, Alexander RW, Smith TW. Circ Res 47: 493-501, 1980.
43. Bobik A, Campbell JH, Carson V, Campbell GR. J Cardiovasc Pharmacol 3: 541-545, 1981.
44. Limas CJ, Limas C. Cir Res 55: 524-531, 1984.
45. Limas CJ, Limas C. Biochim Biophys Acta 735: 181-184, 1983.
46. Karliner JS, Simpson PC, Hombo N, Woloszyn W. Cardiovasc Res 90: 221-228, 1986.
47. Limas CJ, Limas C. Circ Res 57: 443-449, 1985.
48. Berridge MJ. Bichem J 220: 345-360, 1984.
49. Limas CJ, Limas C. Biochem Biophys Res Commun 128: 699-704, 1985.
50. Thomas JA, Marks BH. Am J Cardiol 41: 233-243, 1978.
51. Cohn JN, Levine TB, Francis GS, Goldsmith S. Am Heart J 102: 509-516, 1981.
52. Kunos G, Kunos I, Hirata F, Ishac EJN. J Allergy Clin Immunol 76: 346-352, 1985.
53. Kunos G. Br J Pharmacol 59: 177-189, 1977.
54. Sterin-Borda L, Cremaschi G, Pascual J, Genaro A, Borda E. Clin Exp Immunol 58: 223-228, 1984.
55. Borda E, Pascual J, Cossio PM, Bega M, Arana R, Sterin-Borda L. Clin Exp Immunol 57: 676-686, 1984.
56. Goldenberg IF, Limas CJ. J Am Coll Cardiol 7: 94A, 1986.

18

INVOLVEMENT OF β_2-ADRENERGIC RECEPTORS IN THE POTENTIATION OF THE CHRONOTROPIC ACTION OF ISOPRENALINE EVOKED IN ROCKER-CULTURED NEONATAL RAT HEART CELLS BY PYRUVATE AND L(+)-LACTATE

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INTRODUCTION

Some time ago we reported (1) that cells from the ventricles of newborn rats, cultured as monolayers on the bottom of flasks that were continuously agitated on a rocking tray for insuring an adequate supply of oxygen (2) had a lower lactate content than did their counterparts in stationary (immobile) culture. The lowered lactate level was associated with subsensitivity to ß-adrenergic stimulation in terms of chronotropic responsiveness. Prior exposure of the rocked cells to L(+)-lactate (1-10 mM) or pyruvate (1 mM) for a minimum of 45-60 min led to a potentiation of the chronotropic response to the ß-adrenergic agonist isoprenaline by several orders of magnitude (3), causing the cells at 3 mM L(+)-lactate and 1 mM pyruvate to become even supersensitive to this catecholamine. The potentiation was abolished by low concentrations of propranolol, indicating involvement of ß-adrenergic receptors in this phenomenon. In the following we present data indicating that the receptors involved in the potentiation are of the β_2 subtype and we present evidence suggesting that treatment with pyruvate and lactate makes these receptors, which otherwise appear to be inaccessible to isoprenaline, available for activation by this ß agonist.

METHODS AND MATERIALS

Cell culture and counting of beats. As in the earlier experiments (1) trypsin-dissociated cells from the heart ventricles of 1 to 2 day-old Wistar rats were cultured at 37 °C for 8 days (unless stated otherwise) as monolayers on the 17 cm^2 bottom of 45 ml Müller flasks containing 3 ml of Halle SM 20-I medium equilibrated with air and supplemented with 10 per cent heat-inactivated calf serum,

0.1 mU insulin/ml, and 2 μM fluorodeoxyuridine, the latter to prevent proliferation of non-muscle cells. The flasks were attached in an incubator to a metal tray on which they were continuously and gently rocked back and forth at an angle of $\pm$ 30° twice a min. Medium was renewed at the end of the first, fourth, and seventh day. On the eigth day the cell monolayer was washed with serum-free culture medium (which contains 11 mM glucose) and incubated at 37 °C in 1 ml of this medium with continued rocking for 2 hours without or with added lactate or pyruvate. The flasks were then transferred to the heated stage of an inverted microscope where 10 small circular fields of the cell layer were observed at 37 $\pm$ 0.3 °C through the perforations of a metal template. The number of beats of a selected isolated myocardial cell or synchronously beating group of cells was counted for 15 sec, beginning 3 min after addition, with vigourous mixing, of 10 μl of serum-free medium (control beating rate) and thereafter of 10 μl of a solution in this medium (0.5 mM ascorbic acid present) of the agent or agents to be tested. Three to five culture flasks, corresponding to 30-50 fields, were used to determine the effect of a given agent. Control beating rates on the eigth day averaged 136.4 $\pm$ 6.6 per min (mean $\pm$ S.E., n = 130). Concentration-response curves were obtained by cumulative addition of the agents. Tachyphylaxis was not observed except at very high concentrations of agonists considered irrelevant to the experiment.

Results are expressed as means $\pm$ standard errors, the latter being pictured as vertical bars in the figures. EC_{50} values were determined from computer-fitted concentration-response curves by non-linear least square regression analysis.

Preparation of serum gamma globulin fraction. This protein fraction was precipitated at 5 °C in 1.8 ml blood serum samples by ammonium sulfate at 40 % saturation, washed repeatedly, dissolved in dialysis buffer (154 mM NaCl, 10 mM sodium phosphate, pH 7.2) and dialyzed for 36-48 hours at 5 °C against 5 x 1 l of this buffer. Aliquots were precipitated with antihuman gamma globulin. The supernatant was lyophylized and taken up in distilled water.

Treatment with trypsin. The washed cell monolayer was incubated at 37 °C for 3 min with 1 ml of a 0.1 % solution of crude trypsin in serum-free medium. The reaction was stopped by excess trypsin

inhibitor.

Materials. The following compounds were received as kind gifts: Lithium D(-)-lactate from Boehringer Mannheim GmbH, (±)-isoprenaline (isoproterenol) sulfate from VEB Berlin-Chemie, (-)-propranolol hydrochloride from VEB Isis-Chemie Zwickau, clenbuterol from Dr. Karl Thomae GmbH, prenalterol hydrochloride from Hässle Pharmaceuticals ICI 118,551 from Dr. J.D. Fitzgerald, Imperial Chemical Industries Ltd., acebutolol from Bayer AG, CGP 12227 from Prof. M. Staehelin, and phenoxybenzamine hydrochloride from Smith Kline and French Laboratories. Sodium L(+)-lactate and sodium pyruvate were purchased from Boehringer Mannheim GmbH, antihuman gamma globulin from Staatliches Institut für Immunpräparate und -medien Berlin, trypsin (crude preparation) from Leidholdt Biochemica, dithiothreitol from Calbiochem-Behring, and soja bean trypsin inhibitor from Serva Feinbiochemica. All other chemicals were commercial products of highest purity grade.

RESULTS

Sensitization by pyruvate and L(+)-lactate to the chronotropic action of isoprenaline. Figs. 1 and 2 are presented to recall (3) the increase in sensitivity to the chronotropic action of isoprenaline evoked in rocker-cultured neonatal rat heart myocytes by preincubation with pyruvate and with L(+)-lactate. The resulting concentration--response relationship is in both cases atypical in that it is not described by the usual sigmoid graph, but by long drawn-out curves extending over 8 log concentration units, due to a major leftward shift of the foot of the original concentration - response curves without change in the position of the maxima. Obviously the new curves are of composite nature, representing a complex stimulus--response relationship.

As demonstrated in Fig. 1 for the pyruvate-treated cultures, the curve obtained after pyruvate treatment can be resolved into 2 components by subtracting from it the control curve. The difference curve (inset) then represents the pyruvate-induced component of the chronotropic response to isoprenaline. The EC_{50} values and confidence intervals for the positive chronotropic action of (±)-isoprenaline under control conditions and for the pyruvate-induced

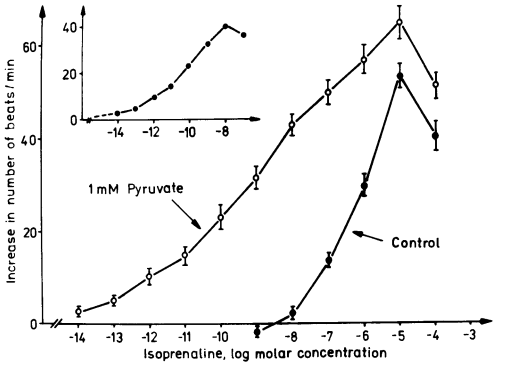

Fig. 1. Effect of pretreatment with 1 mM pyruvate on the chronotropic response of rocker-cultured neonatal rat ventricle cells to isoprenaline. Four-day cultures; mean beating rate: 168/min. Inset: Pyruvate--induced component of the chronotropic response, obtained by subtracting the control curve from the pyruvate curve and assuming that there was no increase in the rate of beat in the absence of added pyruvate at isoprenaline concentrations of 10^{-9} M and below. Nominations of coordinates as in main part of the figure.

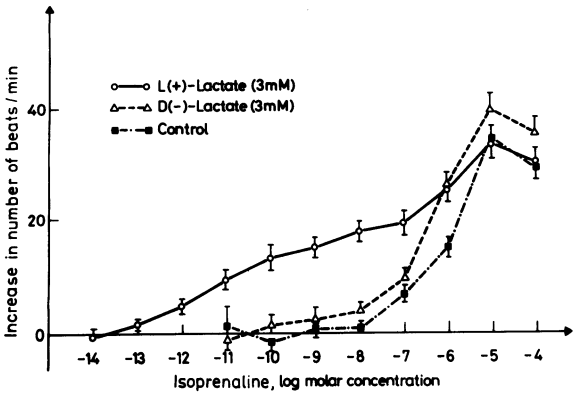

Fig. 2. Sensitization of the rocker-cultured heart muscle cells to isoprenaline by pretreatment with L(+)-lactate.

part of its action were determined to be $6.7\ (6.4\text{-}7.1) \times 10^{-7}$ M and $5.4\ (4.9\text{-}6.0) \times 10^{-11}$ M, respectively. Judging from these EC_{50} values pyruvate treatment may be credited with having increased the sensitivity of the rocker-cultured myocardial cells to isoprenaline more than 10.000-fold.

An increase in sensitivity to isoprenaline of about the same magnitude was caused by treatment with 3 mM L(+)-lactate as can be gathered from a comparison of Figs. 1 and 2. This effect was shared to only a very minor extent by D(-)-lactate (Fig. 2). Lithium chloride (3 mM) was without effect. Neither were the concentration--response curves in Figs. 1 and 2 altered by phenoxybenzamine (5 μM), added as an inhibitor of extraneuronal catecholamine uptake. The EC_{50} of (±)-isoprenaline for the L(+)-lactate-induced component of its chronotropic action

(not shown) was estimated to be 10^{-11} M.

Effect of ß-subtype selective antagonists on the potentiated chronotropic response to isoprenaline. In Fig. 3 the "No antagonist" curve represents the potentiated chronotropic response to isoprenaline, brought about by prior incubation of the heart cell cultures for 2 hours with 1 mM pyruvate. Comparison with Fig. 1 shows that the potentiating action of pyruvate represented by the lower, left-hand part of the "No antagonist" curve, is eliminated, without change in the response represented by the top part of the curve, by the $ß_2$-selective adrenergic antagonist ICI 118,551 (30 nM), yielding a new concentration-response curve for isoprenaline that nearly coincides with the control curve in Fig. 1. Conversely, the $ß_1$-selective antagonist acebutolol shifts, at 100 nM, the upper, right-hand part of the "No antagonist" curve by about 1 log unit to the right, but has no effect on the chronotropic action of subnanomolar concentrations of isoprenaline that became effective after treatment

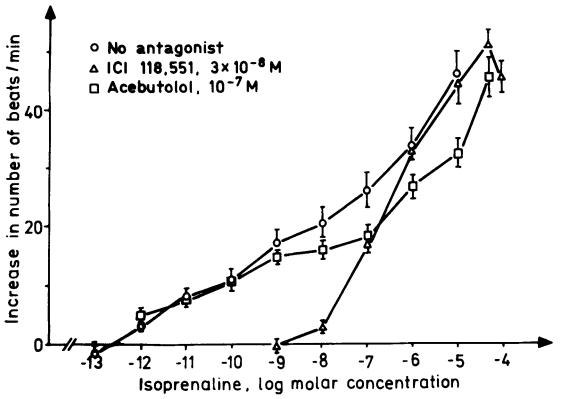

Fig. 3. Modification of the chronotropic response of the pyruvate-treated cultured heart cells by ß subtype-selective antagonists.

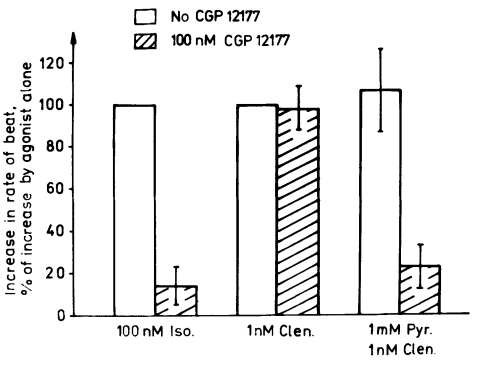

Fig. 4. Permissive role of pyruvate in the inhibition of the chronotropic action of clenbuterol by CGP 12177. Four-day cultures.

of the cultured heart cells with pyruvate. It appears from these data that the "ordinary", non-potentiated chronotropic response of rocker-cultured neonatal rat heart muscle cells to the β_2 β_1-adrenergic agonist isoprenaline is mediated chiefly by β_1-adrenergic receptors, while the potentiation of this response under the influence of pyruvate and, by implication, of L(+)-lactate is chiefly mediated by β_2 receptors.

Effect of pyruvate treatment on the action of CGP 12177. CGP 12177 is a hydrophilic partial ß-adrenergic antagonist that binds to cell surface receptors of intact cells (4). It blocked, at 10^{-7} M, the positive chronotropic response of the cultured heart myocytes to isoprenaline (10^{-7} M), which likewise is hydrophilic, but did not influence the positive chronotropic action of the lipophilic β_2-selective agonist clenbuterol, exerted at a concentration of the latter (10^{-9} M, Fig. 4) at which a β_1 component of its action can be assumed to be negligible. However, in cultures pretreated with 1 mM pyruvate CGP 12177 strongly opposed the chronotropic effect of clenbuterol (Fig. 4). These findings suggest that β_2-adrenergic receptors accessible to lipophilic, but not to hydrophilic ß-adrenergic ligands were made accessible to the latter during incubation of the heart cell cultures with pyruvate. Fig. 4 also provides an example of the fact that pyruvate, in contrast to its strong potentiating effect on isoprenaline chronotropism (Fig. 1), does not significantly enhance the potency of action of clenbuterol on the automaticity of the rocker-cultured heart cells. Lemoine et al. (5) had similarly found that the action of the β_2-selective, moderately lipophilic agonist fenoterol on the beat frequency of guinea pig right atria is not potentiated by pyruvate.

Effect of pyruvate and L(+)-lactate on the action of the serum gamma globulin fraction from asthmatic patients. Venter et al. (6) have identified autoantibodies to β_2-adrenergic receptors in the serum of patients with asthma and allergic rhinitis as a possible cause of adrenergic hyporesponsiveness in these diseases. The autoantibodies are contained in the gamma globulin fraction of blood serum. We have used them as an auxiliary tool in the subclassification of the chronotropic ß receptors in our heart cell cultures. A representative example taken from this work (7) is presented in

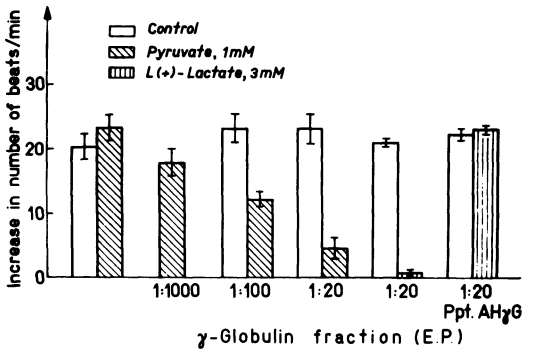

Fig. 5. Inhibition of the chronotropic action of clenbuterol (2 μM) on pyruvate- and lactate-treated neonatal rat heart cells in 4-day culture by the gamma globulin fraction of the serum of a patient (E.P.) with allergic asthma. Annulment of the inhibition by immunoprecipitation with antihuman gamma globulin (Ppt. AHγG).

Fig. 5. The gamma globulin fraction of the serum of E.P., a patient with allergic asthma, had no effect on the acceleration of beating of the cultured myocardial cells caused by the lipophilic β_2 agonist clenbuterol in the absence of added pyruvate and lactate, but inhibited the acceleration in a dose-dependent fashion following a 2-hour treatment with pyruvate or L(+)--lactate. The inhibitory effect was overcome by immunoprecipitation with antihuman gamma globulin. Results qualitatively identical to those depicted in Fig. 1 were obtained with serum of 6 other out of a total of 7 patients (including E.P.) with allergic asthma. Given the specificity and selectivity of the autoantibodies in question - there was no cross-reaction with β_1 receptors (7; see also 6) and no inhibitory effect on beating by the gamma globulin fraction from healthy individuals - these findings may be taken as further evidence for the β_2 character of the adrenergic receptors that under present culture conditions become subject to activation or inhibition by non-lipophilic ß-receptor ligands following treatment with pyruvate or lactate. We have called attention to the rocker-cultured heart cell model as a prospective functional test system for the detection of antibodies to the β_2-adrenergic receptor (8).

Permissive role of pyruvate in the inhibition of the chronotropic action of clenbuterol by dithiothreitol. A disulfide bond has been reported to be essential in maintaining the conformation of the catecholamine binding site in ß-adrenergic receptors (9, 10). Reduction of this bond by dithiothreitol (DTT) was found to inactivate the ß receptors. Fig. 6 depicts the results of experiments in which

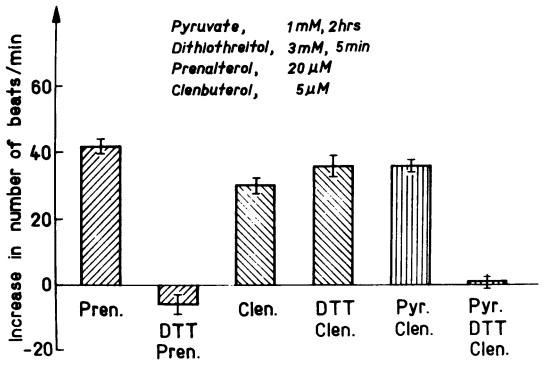

Fig. 6 Effect of dithiothreitol on the positive chronotropic response of the cultured myocardial cells to ß-adrenergic agonists. Permissive role of pyruvate.

we examined the effect of 1 mM DTT on the chronotropic response of our cultured heart muscle cells to the amphiphilic nonselective partial ß agonist prenalterol (found in one investigation (11), however, to be $ß_1$-selective) and to the strongly lipophilic $ß_2$-agonist clenbuterol. Only that part of the experiments is shown in which DTT was added prior to the agonists. It is seen that the chronotropic effect of prenalterol was eliminated by DTT, whereas that of clenbuterol (pyruvate absent) was unaffected, in apparent agreement with data assembled from the literature (12), according to which DTT at 1 millimol/l does not interfere with the binding of the nonselective ß-receptor ligand [^{125}I]-iodohydroxybenzylpindolol to $ß_2$ receptors. However, in cells pretreated with pyruvate the chronotropic response to clenbuterol was likewise eliminated by DTT (Fig 6). A plausible explanation to be offered for this finding in conjunction with the interpretation of the results obtained with CGP 12177 is that the critical disulfide bond in the clenbuterol-binding receptors was shielded from DTT in a hydrophobic domain and that pyruvate treatment opened the S-S bond site to the disulfide reducing reagent.

As was to be expected on the basis of the literature data, DTT was without effect when the agonists were added first.

Susceptibility to trypsinization. ß-Adrenergic cell surface receptors are easely susceptible to proteolytic degradation by trypsin (13). In the experiment represented in Fig 7 the cultured rat heart cells were treated with a 0.1 % solution of trypsin for 3 min, at which time the action of the enzyme was stopped by excess trypsin inhibitor. The cells were then challenged by isoprenaline and by clenbuterol. The figure shows that the chronotropic response to the

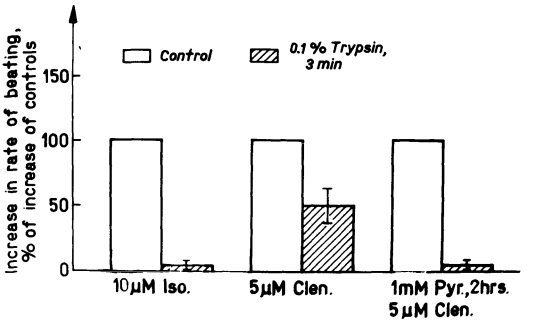

Fig. 7. Differential effect of trypsin on the chronotropic responses of the cultured myocardial cells to isoprenaline and clenbuterol and influence of pyruvate pretreatment.

hydrophilic nonselective ß agonist isoprenaline was nearly eliminated by the trypsinization, whereas the response to the lipophilic $ß_2$ agonist clenbuterol (pyruvate absent) was merely weakened. After preincubation with 1 mM pyruvate the response to clenbuterol had vanished too. These results may be interpreted to mean that in the absence of pyruvate the receptors activated by clenbuterol were less exposed to the added trypsin than were the receptors activated by isoprenaline, but that this was not anymore the case following treatment with pyruvate.

DISCUSSION

The starting point of the present investigation was our previous finding (3), documented once more in Figs. 1 and 2, that rocker--cultured neonatal rat cells, which have a low lactate content and are subsensitive to isoprenaline in terms of chronotropic responsiveness are resensitized and even become supersensitive to this catecholamine after incubation with 1 mM pyruvate or 3 mM lactate, as indicated by a decrease in the EC_{50} of (-) isoprenaline to 54 and 10 pM, respectively, for the pyruvate- and lactate-induced components of the action of the catecholamine. This sensitization, previously found to be abolished by propranolol (3), could in the present experiments be eliminated by the $ß_2$-selective antagonist ICI 118,551 at a concentration (30 nM), the negative logarithm of which exceeds significantly the pA_2 (14, 15) and pK_B (5) values of this antagonist for $ß_1$ receptors and which did not appreciably affect the positive chronotropic response to isoprenaline in the absence of added pyruvate and lactate. The latter response, on the other hand, was sensitive to a concentration of the $ß_1$-selective antagonist acebutolol

(100 nm) that left the potentiation of the chronotropic response to isoprenaline unaltered. These findings indicate a participation of $ß_2$-adrenergic receptors in the potentiated chronotropic response.

From the results of the experiments with CGP 12177, the serum gamma globulin fraction, DTT, and trypsin it can be gathered that these receptors or their ligand binding site are under present culture conditions shielded in a hydrophobic environment from hydrophilic low molecular weight ß adrenoreceptor ligands, autoantibodies to the $ß_2$ receptor, DTT, and, to some degree, from extracellular trypsin and that access to the receptors is provided or promoted for these agents by treatment with pyruvate and L(+)-lactate. It is not far-fetched to think that these are the $ß_2$ receptors that are involved in the pyruvate- and lactate-evoked sensitization of the rocker-cultured cardiomyocytes to the chronotropic action of isoprenaline.

The results discussed above leave it open, whether removal of an accessibility barrier to the chronotropic $ß_2$ receptors was both a necessary and sufficient condition for the potentiation of the chronotropic effect of isoprenaline or whether facilitation of a postreceptor step in the stimulus-response path was the decisive requirement. The latter alternative appears unlikely in view of the fact that the chronotropic effect of the $ß_2$-selective agonist clenbuterol, which was highly sensitive to ICI 118,551, but not to acebutolol (see also 16) and bisoprolol ($ß_1$-selective), was not influenced by treatment with L(+)-lactate and pyruvate and that clenbuterol was a full agonist in our system, eliciting maximal chronotropic responses equaling those elicited by isoprenaline in pyruvate- and lactate-treated cultures. Well compatible with the present results, however, is the supposition that the chronotropic $ß_2$ receptors were highly efficiently coupled to the catecholamine-regulated sarcolemmal ionic channel or channels subserving pacemaker function, but that coupling itself was not dependent on pyruvate and lactate.

It will have been noticed that the concentration-effect curve in the inset of Fig. 1, which represents the pyruvate-induced component of the chronotropic action of isoprenaline, is still anomalous, though simpler than the "pyruvate" curve from which it was derived by subtraction of the control curve, in that it extends over 6 log units. Similar curves were found to describe the chronotropic response of

cultured neonatal rat heart myocytes to clenbuterol (16) and to several other β_2-selective adrenergic agonists (17). The last-cited authors attributed the chronotropic action of these agents to stimulation of β_1 receptors, without, however, having looked at their action in the presence of β subtype-selective antagonists. The concentration--response curves just mentioned and the similarly shaped difference curve in Fig. 1 may harbor 2 components into which they might be separable by appropriate analysis. A possibility to be considered is that the foot part of the curves expresses activation of β_2 receptors while the top reflects β_1 receptor activation. Alternatively, one may be dealing with two affinity states of β_2 receptors or with β_2 receptors differing in tightness of coupling to the effector. A possible involvement of a non-adrenergic mechanism has also been envisaged (17).

The idea that β_2-adrenergic receptors have a place in the regulation of the heart rate has steadily been gaining ground ever since it was first proposed by Carlsson and coworkers (18) in 1972 and it has lately received renewed endorsement (19). Evidence for the existence of chronotropic β_2 receptors has also extended to cultured ventricle cells of the neonatal rat (16, 20). On the other hand, only β_1 receptors were detected by the radioligand binding technique in myocyte-enriched cultures of these cells (21, 22). This method, however, would not have discriminated β_2 receptors, unless they constituted more than 10 per cent of the total β receptor population (23). With [^{3}H]CGP 12177 bound to intact cells computer analysis of the competition binding curve obtained with ICI 188,551 indicated the presence of a minor population of β_2 receptors with high affinity for ICI 118,551 on the surfaces of pyruvate-treated neonatal rat ventricle cells in myocyte-enriched rocker culture (7). A minor fraction of the total receptor population on and in these cells would presumably suffice for the mediation of pronounced increases in beating frequency since cultures of neonatal rat heart cells (24), like intact cardiac tissue of older rats (25), dispose of a large reserve of chronotropic receptors for isoprenaline.

At the presently chosen density of plating (1.4×10^5 cells/cm^2) most of the contracting cells were connected to one another in clusters and reticula in which the cell with the highest intrinsic pacemaker activity can be presumed to have dictated or partly

determined the rate of beating of its followers (26). The possibility cannot a priori be dismissed that these "leaders" may have been cells of the Purkinje system, since automaticity is a property of Purkinje fibers and not normally of cells of the working ventricular myocardium (27). This possibility is especially relevant to the present issue at stake in that Purkinje fibers have been reported (28) to be relatively rich in β_2 receptors. Experiments conducted in the same way as the present ones, except that only the outer ventricle walls served as source tissue and that seeding density was reduced 10 and 20-fold, yielded a population of mostly solitary beating cells that on treatment with 3 mM L(+)-lactate displayed a potentiated chronotropic response to isoprenaline and a high sensitivity to the β_2-selective agonist clenbuterol (7). Since Purkinje cells constitute only a small minority of the cells making up the outer ventricle walls and since cell proliferation did not occur, there is justification to believe that the effects reported in this communication were a property, though not necessarily an exclusive one, of cells of the working myocardium. It would, of course, be of much interest to know, whether these effects have a pendant in cultures of Purkinje (29) and also of sinoatrial node cells (30).

High sensitivity of the heart to catecholamines may have serious consequences in acute myocardial ischemia and infarction and be a factor in the elicitation of the dangerous arrhythmias that are encountered in these situations (31). The lactate concentration of 3 mM that was found to sensitize the lactate-poor rocker-cultured heart myocytes to the chronotropic action of isoprenaline lies within the lactate level range observed in acutely ischemic myocardial tissue (32). An increase in ß-adrenergic receptors bound to [^{3}H]dihydroalprenolol has been reported to take place in the myocardium after 60 min of ischemia and was thought to reflect, among other possible changes, an unmasking of latent receptors or a shift of ß receptors from an intracellular site to the cell surface (33). A shift of this sort, associated with an increase in isoprenaline-stimulated adenylate cyclase activity, was actually seen to occur in cardiac muscle after 30-90 min of ischemia (34). Unmasking was also offered as one explanation for increases in the number of ß receptors in cultures of neonatal rat heart cells following 1 to

4-hour periods of impaired energy metabolism (35). The possibility of a participation of β_2-adrenergic receptors in the ischemia- and energy deficiency-induced changes was not a matter of concern in these investigations. That the sensitization of our lactate- and pyruvate-treated cultured heart cells occurred with a latency of the same or similar length as the just mentioned ß receptor changes in ischemia and energy deficiency may or may not be a mere coincidence. Extension of the present type of experiments to intact cardiac tissue may disclose, whether the results reported here are of more general significance.

SUMMARY

1. It was confirmed that neonatal rat heart cells in rocker culture, which previously were found to have a low lactate content and which are subsensitive to the chronotropic action of isoprenaline, are resensitized and even become supersensitive to this catecholamine following a 2-hour treatment with 1 mM pyruvate or 3 mM L(+)-lactate. The sensitization (examined in the case of pyruvate) was eliminated by the β_2-selective adrenergic antagonist ICI 118,551 at 30 nM, a concentration without significant effect on the chronotropic response to isoprenaline in the absence of added pyruvate and lactate. It was not affected by the β_1-selective antagonist acebutolol.

2. CGP 12177, a hydrophilic non-subtype-selective ß receptor ligand that binds to surface receptors, blocked, at 100 nM, the positive chronotropic response to the hydrophilic β_2,β_1-adrenergic agonist isoprenaline (100 nM), but did not influence, in the absence of added pyruvate and lactate, the positive chronotropic effect (selectively sensitive to ICI 118,551) of the lipophilic β_2-selective agonist clenbuterol (1 nM). However, in pyruvate-treated cultures CGP 12177 nearly abolished the chronotropic response to clenbuterol. Pretreatment with pyruvate or L(+)-lactate was also a prerequisite for the inhibition of the chronotropic action of clenbuterol by the serum gamma globulin fraction of patients with allergic asthma, an inhibition attributed to the presence of autoantibodies to the β_2-adrenergic receptor. A permissive role of pyruvate was likewise evident in the inhibition of the chronotropic action of

clenbuterol by dithiothreitol, an agent that reduces a critical disulfide bond at the ligand binding site of ß-adrenergic receptors, and by limited trypsinization.

3. These findings point to the presence on rocker-cultured neonatal rat heart cells of chronotropic $ß_2$-adrenergic receptors that are shielded from hydrophilic low molecular weight ß receptor ligands, autoantibodies to $ß_2$ receptors, dithiothreitol, and to some degree, from extracellular trypsin, but that become accessible to, or easier to be reached by these molecules following treatment with pyruvate or L(+)-lactate. It is suggested that these are the $ß_2$-adrenergic-receptors that are involved in the observed potentiation of the chronotropic action of isoprenaline.

REFERENCES

1. Wallukat, G. and Wollenberger, A. Acta Biol.Med.Germ. 39: K7-K13, 1980.
2. McLimans W.F., Blumenson L.E. and Tunnah, K.V. Biotechnol. Bioeng. 10: 741-763, 1968.
3. Wollenberger, A. and Wallukat, G. In: Advances in Studies on Heart Metabolism (Eds. C.M. Caldarera and P. Harris), CLUEB, Bologna 1982 pp. 133-137.
4. Staehelin M. and Simons P. Embo J 1: 187-190, 1982.
5 Lemoine, H., Ehle, B. and Kaumann, A.J Naunyn-Schmiedeberg's Arch. Pharmacol. 331: 40-51, 1985.
6. Venter G.C., Fraser C.M. and Harrison L.C. Science 207: 1361-1363.
7. Wallukat, G. and Wollenberger, A. Unpublished experiments.
8. Wallukat, G. and Wollenberger, A. Acta Histochem. __: in press, 1987.
9. Vauquelin, G., Bottari, S. Kanarek, L. and Strosberg, A.D. J.Biol.Chem. 254: 4462-4469, 1979.
10. Lucas M., Hanoune, J. and Bockaert, J. Mol.Pharmacol. 14: 227-236.
11. Williams, R.S. J Cardiovasc.Pharmacol. 5: 266-271.
12. Strauss, W.L., In: Receptor Biochemistry and Methodology I (Eds. J.C. Venter and L.C. Harrison), Alan R. Liss Inc., New York, 1984, pp. 85-97.
13. Strulovici B. and Lefkowitz, R.B. J Biol.Chem. 259: 4389-4395.
14. O'Donnel, S.R. and Wanstall, J.C. Life Sci. 27: 671-677, 1980.
15. Bilsky, A.J., Halliday, S.E., Fitzgerald, J.D. and Wale, J.L. J. Cardiovasc.Pharmacol. 5: 430-437.
16. Wollenberger, A. and Wallukat, G. Biomed.Biochim. Acta 42: 917-929, 1983.
17. Freyss-Beguin M., Griffaton, G., Lechat, P., Picken, D., Quennedey, M.C., Rouot, B. and Schwartz, J. Brit.J.Pharmacol. 78: 717-723, 1983

18. Carlsson E., Ablad E., Brändström, A., and Carlsson, B., Life Sci. 11, Pt.I: 963-958, 1972.
19. Brown, J.E., McLeod, A A. and Shand, D.G. Am.J.Cardiol. 57: 7F-10F, 1986.
20. Etaiche M.P , Athias, P., Variot, N. and Klepping, J. Can. J.Physiol.Pharmacol. 63: 1221-1227, 1985.
21. Lau, Y.H., Robinson, R B., Rosen, M.R. and Bilezikian, J.P. Circ.Res 45: 41-48, 1980
22. Karliner, J S. Simpson, P C., Taylor, J E., Honbo, N. and Woloszyn, W. Biochem.Biophys.Res Commun. 128: 376-382, 1985.
23. DeLean, A., Hancock, A.A. and Lefkowitz, R J Mol.Pharmacol. 21: 5-16, 1981
24. Kaumann, A.J. Naunyn-Schmiedeberg's Arch Pharmacol. 320: 119-129.
25. Buckner, C.K., Torphy, T. and Costa, D.J Eur J Pharmacol. 47: 259-271, 1978.
26. DeHaan, R L. and Sachs, H.G. Curr.Top.Dev.Biol. 7: 193-228, 1972.
27. Hoffmann, B.F. and Cranefield, P.F. Electrophysiology of the Heart. McGraw-Hill, New York, 1960.
28. Buchthal, S.D., Bilezikian, J P. and Danilo, Jr., P Circ 72: Supp. III: 330, 1985
29 Sheets, M.F., January, C.T. and Fozzard, H.A. Circ.Res. 53: 544-548, 1983
30. Marvin Jr., W J., Chittick, V.L., Rosenthal, J.K., Sandra A., Atkins, D.L. and Hermsmeyer, K. Circ.Res. 55: 253-260, 1984.
31. Lown, B., Verrier, R.L. and Rabinowitz, S.H. Am.J.Cardiol. 39: 890-902, 1976
32 Wollenberger, A and Krause, E.-G. Am.J.Cardiol. 22: 349-359, 1968.
33 Mukherjee, A., Wong, T.M., Buja, L M., Lefkowitz, R.J. and Willerson, J.T. J Clin.Invest. 64: 1423-1428, 1979.
34. Maisel, A.S., Motulsky, H.J. and Insel, P.A. Science 230: 183-186, 1985
35. Buja, L M., Muntz, K.H., Rosenbaum,T., Haghani, Z., Buja, D,K., Sen, A , Chien, K.R. and Willerson, J T. Circ.Res 57: 640-645, 1985.

19

CARDIAC EFFECTS OF ß-ADRENERGIC BLOCKERS IN COMBINATION WITH METABOLIC INTERVENTIONS

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INTRODUCTION

It is well known that catecholamines have pronounced hemodynamic and metabolic effects. As shown in Fig. 1, catecholamines interact with the ß-receptor of the myocardial cell and stimulate via a GTP-binding protein adenylate cyclase (1). The resulting increase in cAMP (2) activates protein kinases that are involved in different metabolic and functional processes. Apart from stimulating lipolysis mainly in organs other than the heart, there is an increase in glycogenolysis. Phosphorylation appears to be also involved in the initiation of functional effects. In this way, the Ca^{++} influx through the sarcolemma (3,4) becomes enhanced and thus the increased contractility is brought about. On the other hand, troponin I (5) and phospholamban are phosphorylated (6), the latter resulting in an increased uptake of Ca^{++} into the sarcoplasmic reticulum and in the acceleration of relaxation. Catecholamines also influence glycolysis and the pentose phosphate pathway (7,8) and, as a consequence of this, the de novo synthesis of adenine nucleotides (9) and ultimately RNA and protein synthesis (10).

Fig. 2 demonstrates the effect of isoproterenol on glycogenolysis, on functional parameters, on the activity of glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the oxidative pentose phosphate pathway, and on the de novo synthesis of cardiac adenine nucleotides. It is obvious that the increase in glycogenolysis as evidenced by the elevation in the cAMP and glucose-6-phosphate contents correlates with the increase in heart rate and the maximal rate of rise in left ventricular pressure (LV dP/dt_{max}). After 12 hours, both glycogenolysis and heart function have become normalized again. It is exactly at this time that the activity of glucose-6-phosphate dehydrogenase starts to increase reaching a maximum after 2 days, well after the peak in glycogenolysis. As far as the de novo synthesis of cardiac adenine nucleotides is concerned, there is an immediate stimulation which

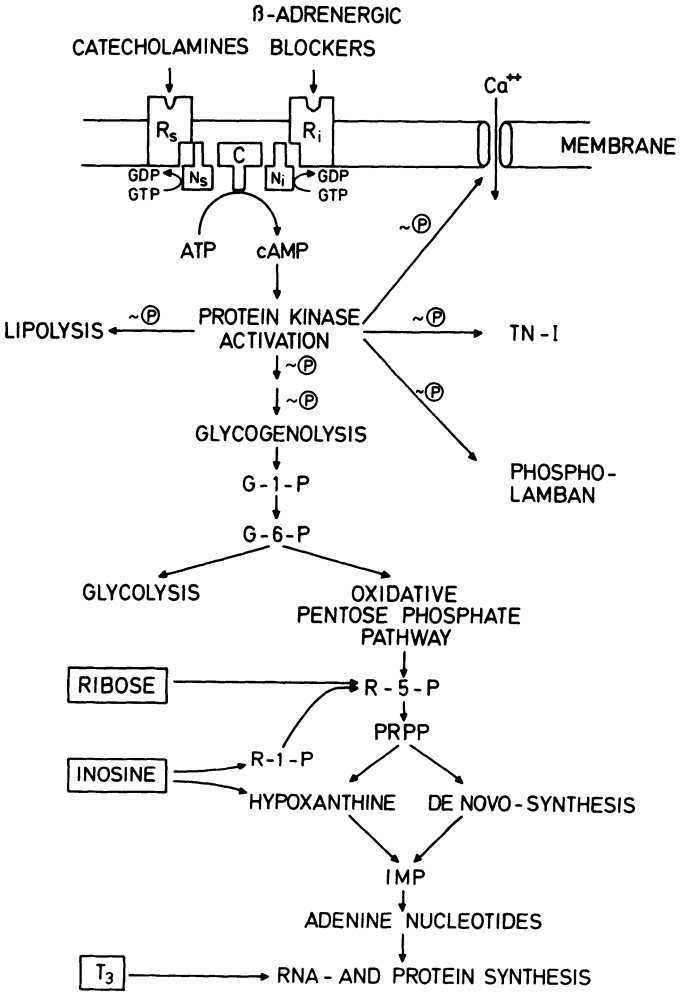

Fig. 1. Schematic presentation of the metabolic and functional effects of catecholamines, and the influence of ß-adrenergic blockers. The components of the hormone responsive adenylate cyclase system have been adapted from (1). G-1-P: glucose-1-phosphate; G-6-P: glucose-6-phosphate; R-5-P: ribose-5-phosphate; R-1-P: ribose-1-phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; T_3: triiodothyronine. For details see text.

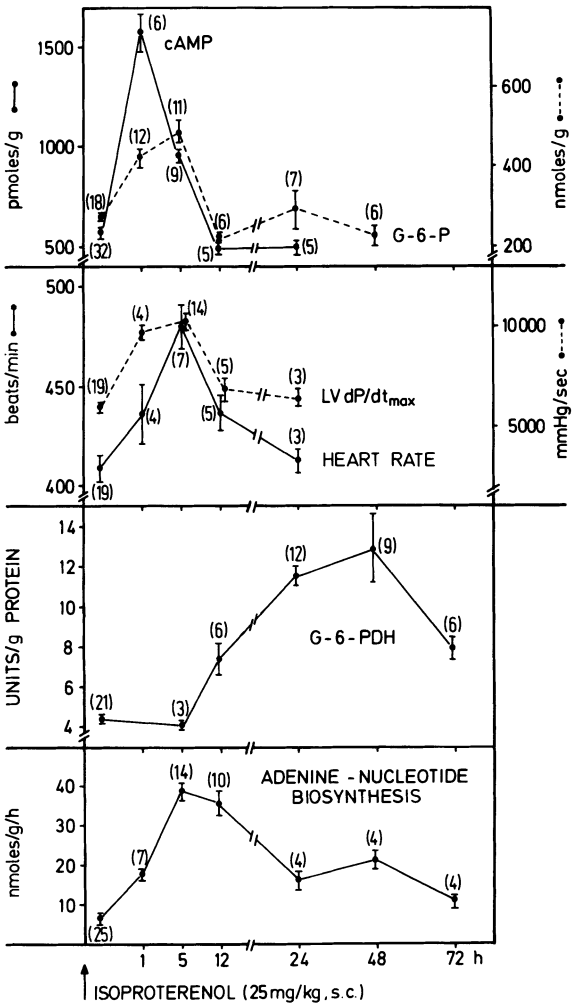

Fig. 2. Effects of isoproterenol on cardiac glycogenolysis, i.e. the contents in cAMP and in glucose-6-phosphate (upper panel), on hemodynamic parameters, i.e. heart rate and maximal rate of rise in left ventricular pressure (LV dP/dt_{max}) (second panel), on the activity of myocardial glucose-6-phosphate dehydrogenase, and on the rate of cardiac adenine nucleotide biosynthesis (bottom panel). Data are means $\pm$ SEM; numbers of experiments are given in parentheses.

coincides with the increase in glycogenolysis, thereafter the enhancement is maintained on a somewhat lower level. Thus, there are two distinct phases: In the first phase, glycogenolysis, heart function and adenine nucleotide de novo synthesis (= biosynthesis) are enhanced with no alteration in glucose-6-phosphate dehydrogenase activity. In the second phase, when glycogenolysis and left ventricular function have returned to the respective control levels, glucose-6-phosphate dehydrogenase activity is enhanced, and this is associated with the maintenance of the increased adenine nucleotide biosynthesis. All isoproterenol-elicited metabolic and functional alterations can be prevented entirely by ß-adrenergic blockers (8,9).

It is the purpose of this contribution to examine the hemodynamic and metabolic effects of ß-adrenergic blockers in combination with metabolic interventions. The rationale for this approach is that, if metabolic interventions can be found that are suitable for administration in patients, the conventional cardiac therapy must be continued. ß-Adrenergic blockers are presently widely used drugs, and there should be no interference of the possible metabolic intervention with the therapeutic agent used.

In our studies on rats in vivo we have selected three metabolic interventions that are directed at different points in cardiac metabolism (Fig. 1). Ribose bypasses the limiting step in the oxidative pentose phosphate pathway, elevates the available pool of 5-phosphoribosyl-1-pyrophosphate and stimulates adenine nucleotide de novo synthesis specifically in muscular organs such as the heart and skeletal muscle (11). We have shown previously that any experimentally induced decline in ATP can be attenuated or even entirely prevented by ribose (12,13). The effects of inosine on cardiac adenine nucleotide content are qualitatively similar to those of ribose, but the mechanism is quite different. Inosine is degraded to ribose-1-phosphate and hypoxanthine (14) which then uses up the available 5-phosphoribosyl-1-pyrophosphate to form IMP. The latter is subsequently converted to AMP, ADP and ATP. The last metabolic intervention, 3,3',5-triiodo-L-thyronine, interferes at a completely different site, the nuclear receptors, and stimulates RNA andprotein synthesis which leads to cardiac hypertrophy (15,16,17,18). It will be shown in this contribution that these three metabolic interventions have different interactions with the $ß_1$-selective adrenergic blocker metoprolol.

MATERIALS AND METHODS

Isoproterenol, D(-)ribose, inosine and 3,3',5-triiodo-L-thyronine were obtained from Sigma Chemie. Metoprolol-tartrate was a gift from Astra Chemicals. All other chemicals were purchased from Merck and were of analytical grade. The experiments were done on female Sprague-Dawley rats (220 - 250 g body weight) fed a diet of Altromin^{R} with tap water ad libitum.

For the measurements of left ventricular functional parameters, the ultraminiature catheter pressure transducer, 3 French (model PR 249, Millar Instruments, Inc.) was used. The catheter was attached to a Millar transducer control unit (model TC-100) which was connected to a HSE Electromamometer (Hugo Sachs Elektronik). The maximal rate of rise in left ventricular pressure (LV dP/dt_{max}) was obtained with an electronic differentiation system (Physio-Differentiator, Hugo Sachs Elektronik). The recording system was a Gould Brush 2600 recorder. Calibration of pressure was done with a pressure calibrator (type 367, Hugo Sachs Elektronik). The ultraminiature catheter pressure transducer was inserted into the right carotid artery in the closed-chest animals anesthetized with thiobutabarbital sodium (Inactin^{R} Byk, 80 mg/kg, i.p.). It was then quickly advanced into the left ventricle (19). When the measurements of left ventricular hemodynamic parameters had been completed, the ultraminiature catheter pressure transducer was withdrawn from the left ventricle and positioned in the aorta so that the diastolic aortic pressure could be obtained. The cardiac output index was determined using the thermodilution technique. A 100 µl bolus of 0.9% NaCl (18°C) was injected into the right jugular vein. A thermodilution microprobe, French 1.5, was positioned in the aorta for monitoring blood temperature. Cardiac output index was calculated from the thermodilution curve by "Cardiomax II" (Columbus Instruments) which is specifically designed for the measurements in small laboratory animals.

The available pool of 5-phosphoribosyl-1-pyrophosphate (PRPP) was assessed by measuring the incorporation of ^{14}C-adenine into cardiac adenine nucleotides after an in vivo exposure time of 15 min. Adenine consumes the available 5-phosphoribosyl-1-pyrophosphate for the formation of AMP, and this is then converted to ADP and ATP. Thus, the radioactivity of myocardial adenine nucleotides is an indirect measure of the available pool of 5-phosphoribosyl-1-pyrophosphate (20). The rates of myocardial

adenine nucleotide biosynthesis were determined by measuring the incorporation of 1-^{14}C-glycine into adenine nucleotides. The total radioactivity of adenine nucleotides was related to the mean specific activity of the tissue glycine precursor pool so that actual rates of adenine nucleotide biosynthesis were obtained (21). The incorporation of 1-^{14}C-glycine into cardiac proteins was measured as previously described (22).

RESULTS

In the first series of experiments, metoprolol was administered as continuous i.v. infusion for 24 hours in a dose of 2 mg/kg/h which is clearly cardiodepressive. As is shown in Fig. 3, heart rate, left ventricular systolic pressure (LVSP) and diastolic aortic pressure were all significantly lower than the respective control values. When ribose which has no hemodynamic or vasoactive properties (23) was administered concomitantly in a dose of 200 mg/kg/h, the decline in the measured functional parameters was not affected. In view of this result, the question immediately arises as to whether ribose may retain its stimulating metabolic effects when heart function is depressed by metoprolol.

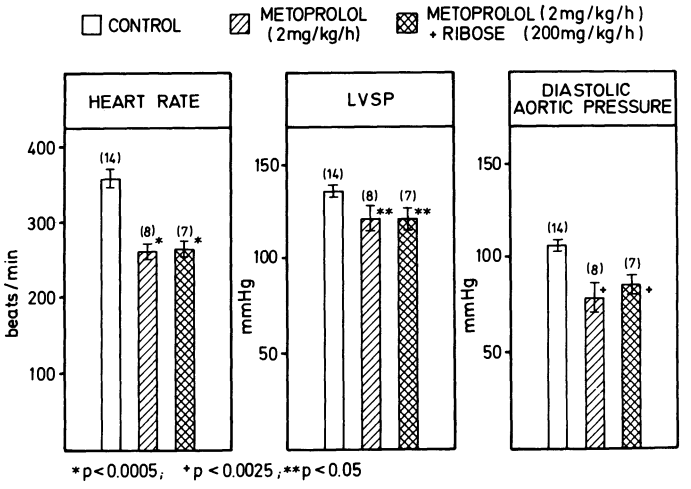

Fig. 3. Effect of continuous i.v. infusion of metoprolol alone and in combination with ribose for 24 hours on heart rate, LVSP and diastolic aortic pressure in rats. Data are mean values ± SEM; number of experiments in parentheses.

Fig. 4 shows the results of the metabolic studies which were obtained after 24 hours of continuous i.v.infusion of ribose and metoprolol alone and in combination in conscious, unrestrained rats. Two metabolic parameters were determined, the available pool of 5-phosphoribosyl-1-pyrophosphate as assessed by the incorporation of ^{14}C-adenine into cardiac adenine nucleotides and the rate of the de novo synthesis of myocardial adenine nucleotides. Both parameters were markedly enhanced by ribose. Metoprolol itself had no effect at all and did not interfere with the stimulation of the metabolic parameters elicited by ribose. Thus, ribose does not affect the negative hemodynamic changes brought about by metoprolol and retains its stimulating effect on cardiac metabolism in the presence of metoprolol. Combination of ß-adrenergic blockers like metoprolol with ribose is therefore entirely possible. When extrapolating these results obtained in rats to the human heart it would appear that ribose treatment may turn out to be compatible with $ß_1$-selective adrenergic blockers which are widely used in conventional cardiac therapy.

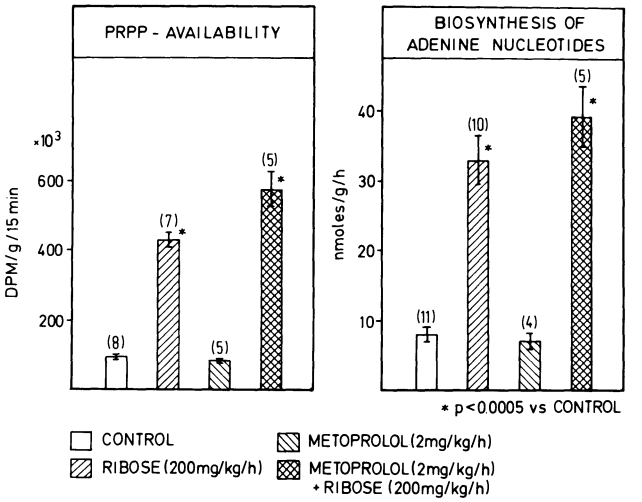

Fig. 4. The available pool of 5-phosphoribosyl-1-pyrophosphate (PRPP) and rates of cardiac adenine nucleotide biosynthesis (= de novo synthesis) in rats that had received continuous i.v. infusion of ribose and metoprolol alone and in combination for 24 hours. Infusions were done in the conscious, unrestrained animals. Mean values ± SEM; number of experiments in parentheses.

The situation is quite different with inosine. After 1 hour of continuous i.v. infusion in rats anesthetized with Inactin^{R}, metoprolol (10 mg/kg/h) had induced a decline in left ventricular systolic pressure and in LV dP/dt_{max} without significantly affecting cardiac output (Fig. 5). When inosine (400 mg/kg/h) in combination with adenosine deaminase (133 U/kg/h) was given together with metoprolol, LVSP and LV dP/dt_{max} were further reduced, and cardiac output index was markedly depressed. Adenosine deaminase was added to exclude any contaminating or endogenous adenosine. Previous studies had shown that inosine itself in a cardioprotective dose has acute negative hemodynamic effects in rats (24). These effects are characterized by a reduction in afterload, a diminution of LVSP and a concomitant drop in cardiac output index. It thus appears that inosine aggravates the negative hemodynamic effects of metoprolol in acute experiments on rats. Furthermore, it induces a depression in cardiac output index which is quite well maintained in the presence of even a high dose of metoprolol. When extrapolating these results to man, this combination would not appear to be advisable.

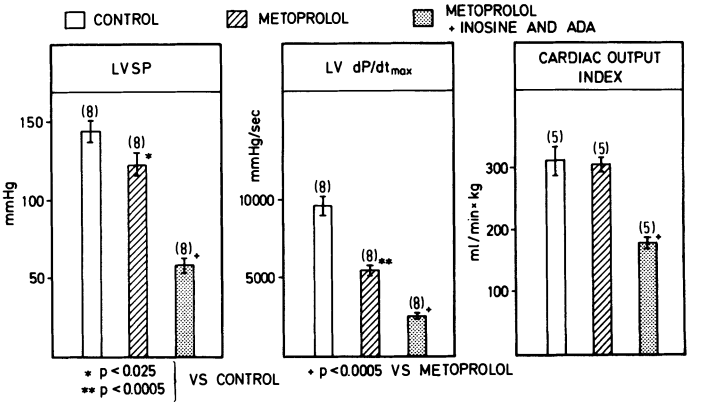

Fig. 5. Effects of continuous i.v. infusion of metoprolol (10 mg/kg/h) and inosine (400 mg/kg/h) in combination with adenosine deaminase (ADA, 133 U/kg/h) for 1 hour on hemodynamic parameters in rats. Data are means ± SEM; number of experiments in parentheses.

The last series of experiments deals with the interaction between metoprolol and triiodothyronine. It is well recognized that triiodothyronine has pronounced hemodynamic and metabolic effects, the latter resulting in increased RNA and protein synthesis associated with the development of cardiac hypertrophy. Fig. 6 demonstrates the increase in heart rate and in LV dP/dt_{max} after three days of daily s.c. administrations of triiodothyronine (o.2 mg/kg). When metoprolol was applied as continuous i.v. infusion in a dose of 1 mg/kg/h for three days, both functional parameters were entirely normalized. Thus, the triiodothyronine-induced increase in heart rate and cardiac contractility appears to be due to catecholamines. It was therefore interesting to examine whether the triiodothyronine-elicited metabolic changes and the development of cardiac hypertrophy may also be induced by catecholamines. Fig. 7 displays the enhancement in the 1-^{14}C-glycine incorporation into cardiac proteins and the increase in heart weight three days after daily administration of triiodothyronine.

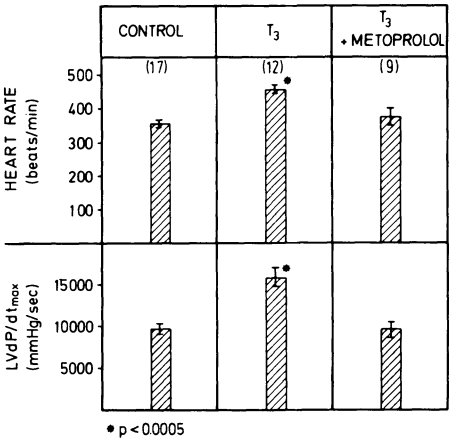

Fig. 6. Alterations in heart rate and in the maximal rate of rise in left ventricular pressure (LV dP/dt_{max}) in rats after 3 days of daily s.c. injections of triiodothyronine (0.2 mg/kg), and the effect of continuous i.v. infusion of metoprolol (1 mg/kg/h) for 3 days. Data are mean values ± SEM; number of experiments in parentheses.

Both the triiodothyronine-induced increase in ^{14}C-amino acid incorporation and the elevation in heart weight were not influenced by metoprolol given in a hemodynamically effective dose (see Fig. 6). To examine whether the increase in heart weight reflects actual cardiac hypertrophy, the RNA content and the RNA/DNA ratio were determined under these experimental conditions. As is evident from the data presented in Fig. 8, the triiodothyronine-elicited increase in both parameters was not affected at all by continuous i.v. infusion of metoprolol for three days. Thus, for the time period studied, RNA and protein synthesis is increased and cardiac hypertrophy develops in the presence of cardiac ß-blockade, which prevents the triiodothyronine-induced increase in left ventricular hemodynamic parameters. Thus, the triiodothyronine-elicited stimulation of myocardial metabolism and the development of cardiac hypertrophy can be dissociated by metoprolol from the triiodothyronine-induced enhancement of left ventricular function.

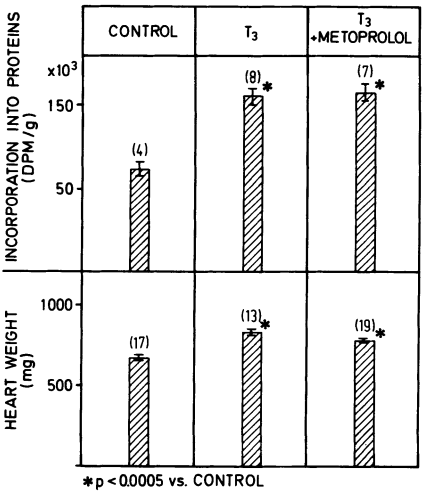

Fig. 7. Effect of daily s.c. injections of triiodothyronine (0.2 mg/kg) for three days on the incorporation of ^{14}C-glycine into cardiac proteins and on the heart weight. Also shown is the effect of continuous i.v. infusion of metoprolol (1 mg/kg/h) on these parameters in triiodothyronine-treated rats. The incorporation of 1-^{14}C-glycine reflects the rate of protein synthesis (10). Data are mean values ± SEM; number of experiments in parentheses.

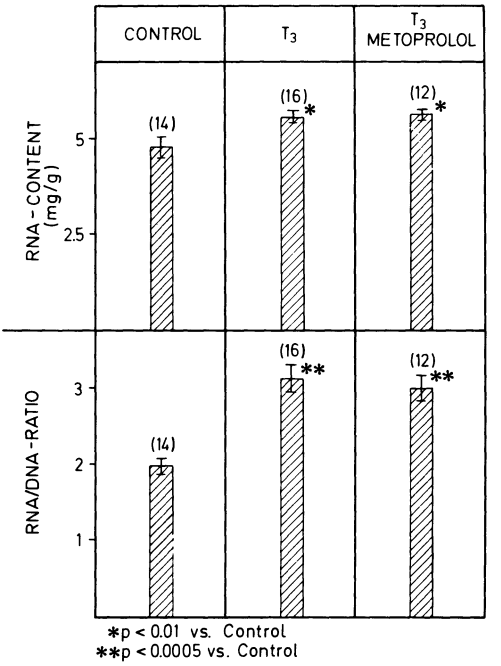

Fig. 8. The content of RNA and the RNA/DNA ratio in hearts of rats that were s.c. injected for three consecutive days with triiodothyronine (0.2 mg/kg), and the effects of metoprolol given as continuous i.v. infusion for three days. Mean values ± SEM; number of experiments in parentheses.

DISCUSSION

This study demonstrates that there are different interactions between the effects of the β_1-selective adrenergic blocker metoprolol and the metabolic interventions that we have studied in the rat in vivo. The situation is quite clear in the case of ribose. Ribose which has no hemodynamic or vasoactive properties of its own does not interfere with the negative inotropic and negative chronotropic effects of metoprolol. On the other hand, metoprolol does not interfere with the marked stimulation of ribose of the available pool of 5-phosphoribosyl-1-pyrophosphate and of adenine nucleotide de novo synthesis. A combination of ß-adrenergic

blockers with ribose may therefore be envisaged for therapeutic application in certain clinical conditions. One could imagine that successful recanalization of an occluded coronary artery with streptokinase (25) without or with subsequent percutaneous transluminal coronary angioplasty (26) is a situation that may be appropriate for ribose intervention. Since even brief periods of ischemia result in a long-term depression of the myocardial ATP pool that may last for days (13), the previously ischemic area of the heart needs urgently a metabolic support. In experiments on rats it has been shown that ribose accelerates considerably the restitution of the ATP pool during the recovery from a 15 min period of regional ischemia (13). Another situation could be heart failure due to myocardial energy deficiency. Also in patients with profound ventricular failure after open-heart surgery or heart transplantation who need temporary circulatory support with a ventricular assist pump (27) may benefit from ribose administration. That ribose is effective in man has been demonstrated recently in a patient with myoadenylate deaminase deficiency in skeletal muscle. This disease is characterized by a disturbance in the purine nucleotide cycle by which the adenine nucleotide content is conserved (28). During exercise, there is a marked alteration in the ATP content of muscle, and potentially, these changes in ATP content may account for muscle dysfunction (29). The typical symptoms of exercise-related muscle pain and cramps could be prevented by ribose (30).

In contrast to ribose, inosine and triiodothyronine have pronounced hemodynamic effects in rats. Inosine induces an acute negative inotropic effect, and triiodothyronine has positive chronotropic and positive inotropic effects. Inosine clearly has cardioprotective qualities, since it attenuates the isoproterenol-induced ATP decline to about the same extent as does ribose (31). It cannot be decided, however, to what extent this cardioprotective effect of inosine is due to the metabolic influence or to the negative inotropic effect. Since inosine aggravates the negative hemodynamic effects of metoprolol, it does not meet one of the essential criteria that should be fulfilled by a cardioprotective substrate. This requirement is that it should not have hemodynamic or vasoactive properties and should be compatible with conventional cardiac therapy.

The situation is quite complex in regard to ß-adrenergic blockers in combination with triiodothyronine. Metoprolol does not prevent the increase in cardiac RNA and protein synthesis and the development of cardiac hypertrophy. However, it abolishes entirely the left ventricular hemodynamic alterations such as the increase in heart rate and in contractility determined in this study as the enhancement in the maximal rate of rise in left ventricular pressure (LV dP/dt_{max}). From this result it appears that the functional changes observed under the influence of triiodothyronine are due to catecholamines. It has been shown previously that the number of cardiac ß-adrenergic receptors is increased (32), and this may be the mechanism for the increased functional state during triiodothronine administration. With the $ß_1$-selective adrenergic blocker metoprolol one can effectively dissociate the increase in metabolic alterations and the elevation in heart weight from the changes in left ventricular function. In this context it is interesting to mention that in thyroxine-treated mice, 6-hydroxydopamine failed to diminish the degree of cardiac hypertrophy (33). It has also been shown previously that the adenylate cyclase-cAMP-system is stimulated by thyroid hormone when the ß-receptors are blocked (34). These results have been extended in this study to the content of RNA, the RNA/DNA ratio and to the incorporation of 1-^{14}C-glycine into cardiac proteins. The stimulation of these metabolic processes as well as the development of cardiac hypertrophy seem to represent the results of the pure triiodothyronine-mediated effects on the myocardium.

SUMMARY

1. Ribose has no hemodynamic or vasoactive properties and does not affect the metoprolol-induced depression of left ventricular hemodynamic parameters. Metoprolol does not interfere with the ribose-elicited elevation of the cardiac 5-phosphoribosyl-1-pyrophosphate pool and the enhancement of myocardial adenine nucleotide biosynthesis. Combination of ribose with ß-adrenergic blocker therapy is therefore possible.

2. Inosine exerts an acute negative inotropic effect in rats and aggravates the metoprolol-induced decline in hemodynamic parameters. Combination of these two interventions is not compatible.

3. Triiodothyronine stimulates heart function and myocardial meta-

bolism and induces cardiac hypertrophy. Metoprolol prevents the triiodothyronine-induced increase in left ventricular functional parameters, but does not affect the metabolic stimulation and the development of cardiac hypertrophy induced by triiodothyronine. Thus, metoprolol dissociates the triiodothyronine-elicited enhancement of left ventricular function from the metabolic stimulation which results in the development of myocardial hypertrophy.

ACKNOWLEDGEMENTS

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REFERENCES

1. Sibley,D.R. and Lefkowitz,R.J. Nature 317: 124-129, 1985.
2. Robison,G.A., Butcher,R.W., Øye,I., Morgan,H.E. and Sutherland,E.W. Mol. Pharmacol. 1: 168-177, 1965.
3. St.Louis,P.J. and Sulakhe,P.V. Arch.Biochem. Biophys. 198: 227-240, 1979.
4. Reuter,H. Nature 301: 569-574, 1983.
5. England,P. Biochem.J. 160: 295-304, 1976.
6. Tada,M. and Katz,A.M. Ann. Rev. Physiol. 44: 401-423, 1982.
7. Zimmer,H.-G. and Ibel,H. Experientia 35: 510-511, 1979.
8. Zimmer,H.-G. and Ibel,H. FEBS Letters 106: 335-337, 1979.
9. Zimmer,H.-G. and Gerlach,E. Circ. Res. 35: 536-543, 1974.
10. Zimmer,H.-G. and Gerlach,E. Arzneim.Forsch./Drug Res. 30(II), 11a: 2001-2007, 1980.
11. Zimmer,H.-G. and Gerlach,E. Pflügers Arch. 376: 223-227, 1978.
12. Zimmer,H.-G., Ibel,H., Steinkopff,G. and Korb,G. Science 207: 319-321, 1980.
13. Zimmer,H.-G. and Ibel,H. J.Mol.Cell.Cardiol. 16: 863-866, 1984.
14. Wiedmeier,V.T., Rubio,R. and Berne,R.M. J.Mol.Cell.Cardiol. 4: 445-452, 1972.
15. Limas,C.J. and Chan-Stier,C. Circ. Res. 42: 311-316, 1978.

16. Sanford,C.F., Griffin,E.E. and Wildenthal,K. Circ.Res. 43: 688-694, 1978.
17. Hoh,J.F.Y. and Egerton,L.J. FEBS Letters 101: 143-148, 1979.
18. Siehl,D., Chua,B.H.L., Lautensack-Belser,N. and Morgan,H.E. Am.J. Physiol. 248: C309 - C319, 1985.
19. Zimmer,H.-G. Basic Res. Cardiol. 78: 77-84, 1983.
20. Zimmer,H.-G. and Gerlach,E. Basic Res. Cardiol. 72: 241-246, 1977.
21. Zimmer,H.-G., Trendelenburg,C., Kammermeier,H. and Gerlach,E. Circ. Res. 32: 635-642, 1973.
22. Zimmer,H.-G., Steinkopff,G. and Gerlach,E. Pflügers Arch. 336: 311-325, 1972.
23. Zimmer,H.-G. and Ibel,H. Am.J.Physiol. 245: H880-H886, 1983.
24. Zimmer,H.-G. and Westphal,R.C. Circulation 72 (Suppl. III): 1345, 1985.
25. Rentrop,P., Blanke,H., Karsch,K.R., Wiegand,V., Köstering,H., Rahlf,G., Oster,H. and Leitz,K. Dtsch.Med.Wschr. 41: 1438-1440, 1979.
26. Grüntzig,A.R. Lancet 1,263, 1978.
27. Pierce,W.S. Circulation 68: 883-888, 1983.
28. Goodman,M.N. and Lowenstein,J.M. J.Biol.Chem. 252: 5054-5060, 1977.
29. Sabina,R.L., Swain,J.L., Patten,B.M., Ashizawa,T., O'Brien,W.E. and Holmes,E.W. J.Clin. Invest. 66: 1419-1423, 1980.
30. Zöllner,N. and Gross,M. Klin. Wschr. 64 (Suppl. V): 68-69, 1986.
31. Zimmer,H.-G. In: Advances in Myocardiology, Vol. 6 (Eds. N.S.Dhalla and D.J.Hearse), Plenum Publishing Corporation, 1985, pp. 173-183.
32. Williams,L.T., Lefkowitz,R.J., Watanabe,A.M., Hathaway,D.R. and Besch,H.R. J.Biol.Chem. 252: 2787-2789, 1977.
33. Cohen,J. Circ. Res. 34,35: II49-II57, 1974.
34. Levey,G.S. and Epstein,S.E. J.Clin.Invest. 48: 1663-1669, 1969.

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LOCAL ADRENERGIC EFFECTS ON METABOLISM IN HEART AND SKELETAL MUSCLE IN MAN

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INTRODUCTION

Stimulation of adrenergic receptors, via sympathetic nerves or by circulating catecholamines, can enhance glycogenolysis in heart as well as in skeletal muscle (1,2). It is not well established, however, to what extent local adrenergic stimulation contributes to determine the utilization of glycogen in heart and skeletal muscle in intact man under physiological conditions, and to what extent a possible effect on glycogen utilization affects the utilization of other substrates. Studies in the dog (2) and the rat (3) suggest that adrenergic activity contributes significantly to the stimulation of the glycogenolysis during exercise. However, it has also been shown that epinephrine infusion did not affect glycogenolysis during a short period of intense muscle contractions in man (4).

We have therefore studied the effect of local adrenergic stimulation, by close intra-arterial infusion of epinephrine into one leg during two-legged exercise in healthy volunteers and by evaluating the effect of beta-adrenergic blockade on the metabolism of skeletal and heart muscle in healthy young men. A comparison of the conditions in heart and skeletal muscle were considered of interest, since basically similar mechanisms regulate the substrate metabolism in the two varieties of muscle tissue.

The adrenergic effects have been evaluated in three sets of experiments in which (1) the effect of adrenergic stimulation and (2) the effect of beta-adrenergic blockade on exerciseing skeletal muscle and (3) the effect of beta-adrenergic blockade on heart muscle metabolism in resting and exercising man have been studied.

EFFECT OF CLOSE INTRA-ARTERIAL INFUSION OF EPINEPHRINE ON LEG MUSCLE CARBOHYDRATE METABOLISM DURING EXERCISE

Subjects and methods

Nine healthy young men participated in the study. They performed two-legged exercise on an electrodynamically braked cycle ergometer for 45 min at a work load corresponding to 50% of individual maximal oxygen uptake ($\dot{V}O_2max$). Throughout the exercise period epinephrine was infused at constant rate, 0.4 $\mu g \times min^{-1} \times kg^{-1}$ bodyweight, into one of the femoral arteries. Arterio-venous differences of oxygen, glucose and lactate across the epinephrine stimulated and the non-stimulated leg was measured by simultaneous blood sampling from catheters in the brachial artery (a) and the two femoral veins (fv). The utilization of glycogen and accumulation of lactate and glycolytic intermediates in the epinephrine stimualted and non-stimulated leg was analyzed by simultaneous percutaneous biopsies from the right and left vastus lateralis of m. quadriceps femoris. An original report has been published previously (5).

Results

Epinephrine infusion produced a plasma epinephrine concentration in the femoral vein of the stimulated leg 2.6 fold higher than in the non-stimulated leg and about twice as high as in the brachial artery. There was no significant difference between the stimulated and non-stimulated leg in a-fv O_2 content difference. A negative a-fv lactate difference (net release) was at hand in both legs, which was greater in the stimulated than in the non-stimulated leg throughout the exercise period ($p<0.001$). Both the reduction in muscle glycogen ($p<0.1$) and the concentration of muscle lactate at the end of exercise tended to be greater in the stimulated leg. Similarly the accumulation of glucose ($p<0.05$) and the glycolytic intermediates glucose-6-phosphate and alpha-glycerophosphate ($p<0.1$) tended to be greater in the stimulated than in the non-stimulated leg.

Comments

Epinephrine infusion produced 2-3 fold higher epinephrine concentration in the vasculature of the infused leg, i.e. it was subjected to a significantly higher degree of adrenergic stimulation

than the non-infused leg, yet within a physiological range, 4-12 nmol/l, during heavy exercise. The same mechanical work performed by the two legs and no significant difference between legs in a-fv O_2 difference suggest no major side difference in leg blood flow, and consequently side differences in a-fv differences of substrates and metabolites would indicate differences between stimulated and non-stimulated leg in uptake or release of these substances.

Muscle glucose may have been derived from breakdown of intramuscular glycogen (6) or uptake from the blood. With the same arterial glucose consentration in the two legs and a smaller uptake in the epinephrine-stimulated leg, which was found, it seems less likely that the whole increase in muscle glucose concentration in the stimulated leg is derived from the blood. Consequently the increased muscle glucose concentration together with increased muscle concentrations of lactate, alpha-glycerophosphate and glucose-6-phosphate together with increased release of lactate and increased depletion of glycogen strongly suggest that epinephrine significantly enhances glycogenolysis during physiological modes of exercise. However, an effect of epinephrine on the membrane transport of glucose (7,8) or an inhibition of glucose phosphorylation (6) may have contributed to alter the intramuscular glucose concentration.

EFFECT OF BETA-ADRENERGIC BLOCKADE ON SKELETAL MUSCLE METABOLISM DURING EXERCISE

Subjects and Methods.

Two series of studies were performed. Six rather well trained young men took part in the first study. Since beta-adrenergic blockade affects not only local metabolism but also cardiac output and thereby the blood and oxygen supply to the exercising musculature, studies were performed both after the administration of a non-selective blocker (propranolol), which affects both circulation and metabolism and a $beta_1$-selective blocker (atenolol) which affects circulation only, and to be able to compare, from a circulatory point of view, equipotent doses of the blockers the subjects

were studied on 7 separate occasions after the acute administration orally on a doubleblind basis of either placebo or 40, 80 or 160 mg propranolol or 25, 50 or 100 mg atenolol. The exercise protocol consisted of a series of submaximal loads, stepwise increased by 30 W at 4-min intervals, followed after a short rest period by a work bout where the load was increased at 1-min intervals until exhaustion. Heart rate, $\dot{V}O_2max$ and performance time to exhaustion were measured. Blood lactate concentration was measured at all work intensities and a muscle biopsy was taken at exhaustion and analyzed for lactate, glucose-6-phosphate and glucose. An original report has been published previously (9).

In a second experiment twelve healthy, physically active men performed continuous work at a constant load corresponding to 65 % of $\dot{V}O_2max$ of each individual for 25 min with and without oral premedication by propranolol. Blood lactate as well as muscle lactate and glycogen concentrations were measured after 5 and 25 min of exercise. An original report has been published previously (10).

Results

Muscle concentrations of both glucose-6-phospahte and glucose at exhaustion in the first study were lower with propranolol than with either atenolol or placebo while there was no difference in concentrations between placebo and atenolol experiments. Blood lactate concentration at exhaustion was slightly lower with both blockers than placebo while muscle lactate tended to be higher with the blockers than placebo. Both blockers reduced maximal oxygen uptake and performance time. However, at any given reduction in maximal heart rate and oxygen uptake by beta-blockade the reduction in performance time was more pronounced by propranolol than by atenolol.

After 5 min of exercise at 65% of $\dot{V}O_2max$ (second experiment) muscle lactate was higher with than without beta-blockade while blood lactate concentration did not differ between the two conditions. With beta-blockade both muscle and blood lactate concentration decreased over the exercise period, while without blockade both blood and muscle lactate concentration tended to increase, such that at the end of exercise both blood and muscle lactate concentration was lower with than without blockade.

Comments

The protocol of the first study made it possible to compare doses of a $beta_1$-selective and a non-selective blocker which produced the same reduction in maximal heart rate and $\dot{V}O_2max$. On these dose levels the non-selective blocker produced a reduction in intramuscular concentration of the glycolytic intermediates glucose-6-phosphate and glucose, suggesting a reduced rate of glycogenolysis (for the interpretation of intramuscular glucose concentration, see above), while the non-selective blocker did not. The absence of a significant reduction in muscle lactate after the non-selective blockade was probably caused by a reduced rate of release from the muscle by a reduced muscle blood perfusion, as suggested by the reduced blood lactate concentration.

An interpretation of the results of the 25 min exercise at 65% of $\dot{V}O_2max$ is that in the beginning of exercise the reduced blood supply to the musculature by the effect of beta-blockade on cardiac output induces an enhanced anaerobic glyolysis. With prolongation of exercise, when a relative steady state with regard to blood flow is established at this submaximal work intensity, the anaerobic stimulus to glycogenolysis is reduced and the adrenergic activity, which increases with time, becomes a relatively more important stimulus of glycogenolysis, with lower lactate production after beta-blockade as a result. This interpretation is in accordance with findings in the exercising rat, suggesting that factors related to the contraction per se and the local oxygen supply are the most important stimuli of glycogenolysis at the onset of exercise, while sympathetic stimulation becomes more important the longer the duration of exercise (3).

EFFECT OF BETA-ADRENERGIC BLOCKADE ON CORONARY BLOOD FLOW AND MYOCARDIAL CARBOHYDRATE METABOLISM AT REST, DURING ATRIAL PACING AND DURING INTENSE EXERCISE

Subjects, Methods

Ten healthy young men were studied by arterial (a) and coronary

sinus (cs) catheterisation at rest, during atrial pacing at stepwise increased rate to a heart rate of 140 beats/min and during bicycle exercise in the supine position, at a submaximal load (heart rate about 150 beats/min) followed by a maximal load until exhaustion. A-cs differences of oxygen, glucose and lactate were measured at rest, during maximal pacing and during submaximal as well as maximal exercise. Coronary sinus blood flow was measured by thermodilution in connection with each blood sampling occasion.

On a separate occasion 8 subjects were studied with the same techique at rest and during atrial pacing before beta-blockade and at rest, during atrial pacing and during submaximal and maximal exercise after the administration i.v. of propranolol 0.15 mg/kg body weight. Thus, the effect of propranolol on myocardial metabolism at rest and during pacing could be studied in the same subject on the same occasion. The effect of propranolol on myocardial metabolism during heavy exercise, on the other hand, was studied by comparing data from 2 separate experiments because it was considered that heavy exercise could not be repeated on the same study occasion without an influence by the first exercise on the response to the second exercise. Preliminary data have been reported previously (11,12).

Results.

Propranolol did not affect myocardial glucose and lactate extraction at rest or during pacing. At rest coronary sinus blood flow was insignificantly lower after than before the administration of propranolol, probably because of a slightly lower myocardial oxygen consumption, related to the slightly lower heart rate.

Heart rate at the submaximal load was 45 beats/min lower and the maximal heart rate during exercise 35 beats/min lower with than without propranolol. The brachial artery pressure was at corresponding work loads about 10 mmHg lower with than without propranolol. The coronary sinus blood flow during the exercise was at corresponding work intensities substantially lower with than without propranolol. During maximal work it was 451 $\pm$ 55 (SE) ml/min without and 260 $\pm$ 15 ml/min with propranolol. At the submaximal work load the a-cs O_2 difference was the same with as without propranolol, but

during maximal work it tended to be greater with propranolol, the cs O_2 saturation being 22.7±1.6 % with and 25.1±1.9% without propranolol.

If the myocardial extraction of glucose and lactate is expressed as the oxygen extraction ratio, i.e. the fraction of the simultaneous oxidative metabolism which could have been covered by the substrate in question, there was no significant difference between exercise with and without propranolol. However, the arterial concentration of lactate during exercise was lower with propranolol, and consequently the fractional extraction of lactate at all work intensities greater with than without propranolol (Fig 1).

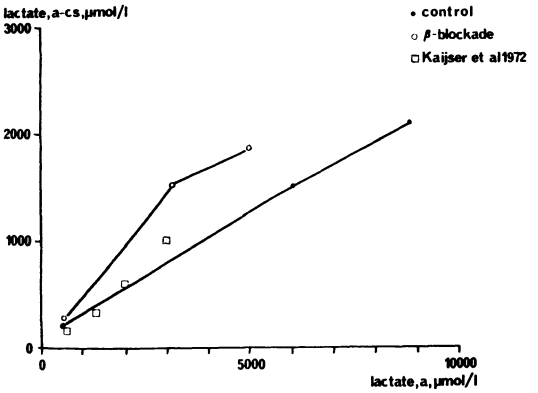

Fig 1. Myocardial lactate extraction in relation to arterial lactate concentration at rest, during submaximal and maximal exercise without (control) and with propranolol (beta-blockade). For comparison data from a previous study of submaximal exercise are given (13).

Comments

At rest and during increased myocardial work, produced by atraial pacing, propranolol did not affect myocardial carbohydrate metabolism. This is as expected, since the basal sympathetic nerve activity is low. Propranolol reduced the exercise heart rate and mean arterial pressure. As a consequence of this the myocardial oxygen consumption decreased - heart rate and arterial pressure are together

with the inotropic state the main determinents of myocardial oxygen consumption. The a-cs O_2-difference increased during exercise, more the higher the work load, as previously described (13), both with and without propranolol. It may be noted, however, that at any level of heart rate or myocardial oxygen consumption the a-cs O_2-difference was greater and the cs O_2-saturation as well as the cs blood flow lower with than without propranolol. This could indicate that under normal conditions beta-adrenergic stimulation may contribute to the vasodilatation of the coronary vascular bed during exercise, an effect which is inhibited by the beta-adrenergic blockade.

Myocardial utilization of carbohydrate substrates during exercise was little affected by beta-adrenergic blockade. However, under normal conditions the myocardial extraction of both lactate and glucose are well correlated with the arterial concentration of these substrates, i.e. the fractional extraction remains the same over a wide range of arterial concentrations (e.g. 13). In the present study the myocardial fractional extraction of lactate was substantially greater with than without beta-adrenergic blockade. One possible explanation of this could be that under influence of beta adrenergic blockade there is a reduced production of pyruvate from the breakdown of intramyocardial glycogen, which otherwise is at hand during heavy exercise. It is true that the normal heart muscle under most conditions covers its substrate supply for oxidative metabolism by uptake of bloodborne substrates, but at least during prolonged exercise it has previously been shown that intramyocardial substrates are being utilized (13).

CONCLUSIONS

During physical exercise the physiological activation of the sympathetic nervous system seems to contribute significantly to the stimulation of glycogenolysis in skeletal muscle of normal men. Factors other than the adrenergic stimulation seem to be quantitatively more important, however. Adrenergic stimulation seems to be less important for the metabolism of the healthy human heart muscle

than of the human skeletal muscle. The reason of this may be that the heart muscle contrary to the skeletal muscle derives its substrate for energy production to a rather insignificant degree from its endogenous glycogen store. However, there are some indications that adrenergic stimulation may contribute to the vasodilatation of the coronary vascular bed during heavy exercise and that adrenergic stimulation activates intramyocardial glycogenolysis during exercise of extremely heavy intensity.

REFERENCES

1. Dynarowicz, I. Acta Physiol Pol 25:145-150, 1974.
2. Issekutz, B. Jr. Diabetes 28:39-44, 1979.
3. Richter, E.A. Acta Physiol Scand. Suppl 528:1-42, 1984
4. Chasiotis, D. and Hultman, E. Acta Physiol Scand 123:55-60, 1985.
5. Jansson, E., Hjemdahl, P. and Kaijser, L. J. Appl. Physiol. 60: 1466-1470, 1986.
6. Saha, J., Lopez-Mondragon, R. and Narahara, H.T. J Biol Chem 243: 521-527, 1968.
7. Newsholme, E.A. and Randle, P.J. Biochem J 80:655-662, 1961.
8. Bihler, I., Sawh, P.C. and Solan, I.G. Biochem Biophys Acta 510: 349-360, 1978.
9. Kaiser, P., Tesch, P.A., Frisk-Holmberg, M., Juhlin-Dannfeldt, A. and Kaijser, L. Clin Physiol 6:197-207, 1986.
10. Kaiser, P., Tesch, P.A., Thorsson, A., Karlsson, J. and Kaijser, L. Acta Physiol Scand 123:285-291, 1985.
11. Kaijser, L. and Berglund, B. Clin Physiol 5, Suppl 4:102.
12. Kaijser, L. and Berglund, B. J Mol Cell Cardiol 17: Suppl 3:143, 1985.
13. Kaijser, L., Lassers, B.W., Wahlqvist, M.L. and Carlson, L.A. J Appl Physiol 32:847-858, 1972.

D. CARDIAC GLYCOSIDES AND SODIUM PUMP

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MULTIPLE FORMS OF THE CARDIAC GLYCOSIDE RECEPTOR WITH DIFFERENT AFFINITIES FOR CARDIAC GLYCOSIDES

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SUMARY

Cardiac glycosides bind specifically with high affinity to the external surface of $(Na^+ + K^+)$-ATPase thus inhibiting the sodium pump. In cardiac cell membranes, in ventricular heart tissue, and in cultured beating myocardial cells of the rat, two different types of receptors with high and low affinity for ouabain are present. These receptors mediate positive inotropic effects at low and high concentrations of ouabain. Arrhythmogenic effects never were seen when the high affinity receptor only was occupied by a drug molecule.

Evidence is presented suggesting that digitalis sensitive species also have different types of cardiac glycoside receptors in the heart.

After treatment with T_3 or after chronic hypokalaemia cultured myocardial cells increase the number of high and low affinity receptors concomitantly without any change in their affinity.

1. INTRODUCTION

Cardiac glycosides bind specifically and with high affinity to the $(Na^+ + K^+)$-ATPase at the external surface of the cell membrane. Todays most widely accepted concept is that this enzyme being the biochemical equivalent of the Na^+/K^+-pump of the cell is inhibited by the cardioactive steroid causing thereby a measureable increase in intracellular Na^+-activity (1-3,15).

Most pharmacological receptors (i.e. ß-adrenoceptor, α-adrenoceptor, histamine-receptor etc.) show slightly different affinities for certain drugs of the same groups of substances. Thereby, these receptors can be classified as distinct subtypes ($ß_1$- and $ß_2$-adrenoceptors etc.). Recently, multiple forms of cardiac glycoside receptors have been demonstrated to exist within one animal species or even within one organ

(for ref. see 4). In the following we intend to review the evidence for the existence of multiple forms of cardiac glycoside receptors and to discuss their possible role in vivo.

2. DIFFERENT BINDING SITES IN DIFFERENT ANIMAL SPECIES

The sensitivity of different species to the positive inotropic and toxic effects of cardiac glycosides varies widely. Thus, it is not surprising that binding sites for ^{3}H-ouabain have been found with rather different affinities. Usually the affinity is expressed with the dissociation constant (K_D) of the drug-receptor-complex, which can be measured reliably at constant and well defined conditions. The rat and the guinea pig are rather insensitive to cardiac glycosides ($K_D > 10^{-7}$M). Dog, cat, sheep, frog, pig, cow and humans are known to be sensitive ($K_D < 10^{-8}$M).

When the specific binding of ^{3}H-ouabain (or digoxin, digitoxin etc.) to isolated cell membranes is measured, in insensitive species several types of binding sites have been found. In the sensitive species the situation is not as clear - because of controversial reports.

2.1 the rat

In rat cardiac cell membranes two different binding sites for ouabain have been demonstrated by virtually all investigators ($K_{D1} \sim 10^{-7}$M, $K_{D2} \sim 10^{-5}$M) (for ref. see 4). An intoxication with digitalis in the rat leads to convulsions (i.e. cerebral side effects) rather than to cardiac arrhythmias. This suggests, that the rat brain contains a receptor with higher affinity than the heart. This in fact has been demonstrated (Table 1). As previously has been shown by gel electrophoresis (5), two ($Na^+ + K^+$)-ATPases exist in rat brain ($K_{D1} \sim 3x10^{-8}$M, $K_{D2} \sim 3x10-7$M).

In rat skeletal muscle we detected only one specific ouabain binding site ($K_D \sim 3x10^{-7}M$).

The same is true for rat kidney, with much lower affinity, however: $K_D \sim 10^{-5}M$. Thus, three different receptor populations seem to exist in the rat ($K_D \sim 10^{-8}M$, $10^{-7}M$ and $10^{-5}M$), of which heart and brain contain two types concomitantly (Table 1).

Table 1. Dissociation constants of the ^3H-ouabain-receptor-complexes of different rat organs.

tissue	apparent dissociation constant (M)		
heart	-	$3x10^{-7}$	$3x10^{-5}$
brain	$3x10^{-8}$	$3x10^{-7}$	-
skeletal muscle	-	$3x10^{-7}$	-
kidney	-	-	$3x10^{-5}$

In rat cell membranes isolated from Ca^{++}-free perfused heart a $(Na^+ + K^+)$-ATPase was detected, which was inhibited half maximally by $\sim 10^{-8}M$. If the cardiac cell membranes were prepared in the presence of traces of Ca^{++}, IC_{50} was $\sim 10^{-5}M$ (6). This probably can be taken as evidence for two different forms of the enzyme with high and low affinity. Moreover, several authors demonstrated that the concentration-response-curve for the positive inotropic effect of ouabain in isolated rat heart is biphasic corresponding to the two different receptor affinities (for ref. see 4). Thus, it is accepted today, that rat heart contains two functionally different forms of cardiac glycoside receptors.

Cultured beating heart cells from neonatal rats exhibit two distinct ouabain binding sites as well ($K_{D1} \sim 3x10^{-8}M$, $K_D 2 \sim 7x10^{-6}M$) (7).

There are about 0.2 pmoles/mg protein high affinity and 2.6 pmoles/mg protein low affinity binding sites. In these cells, concentration-response curves seem to be biphasic, too - suggesting two functionally different types of cardiac glycoside receptors (Fig.1).

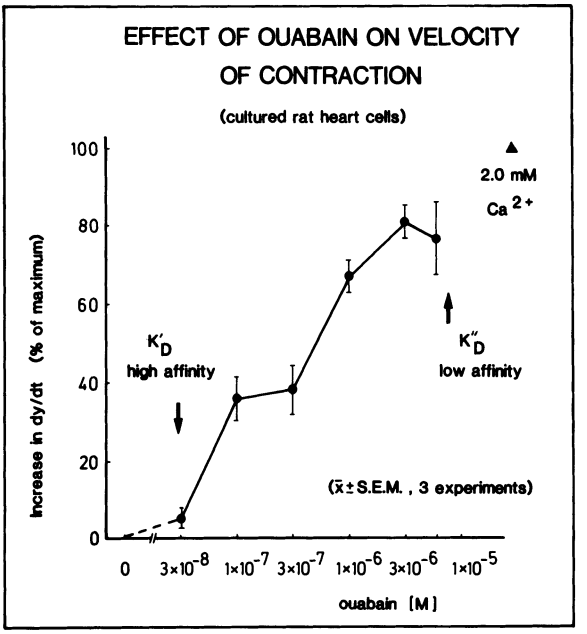


Fig. 1. Effect of ouabain on velocity of contraction in neonatal rat heart muscle cells in culture.
Contraction velocity of electrically driven (105/min; 100 V; 5 msec) rat heart muscle cells has been monitored at 37°C as described (7). Cells have been superfused (4.0 ml/min) with serum-free, Hepes-buffered CMRL medium (K^+ = 3.5 mM; Ca^{++} = 0.9 mM), with different concentrations of ouabain (see abscissa). Ouabain-induced increase in contraction velocity has been measured in a cumulative manner under equilibrium conditions (periods of 5-8 min), the increase by 2.0 mM Ca^{++} being set as 100% (see ordinate). The K_D-values indicated represent the dissociation constants of high- and low-affinity ouabain binding sites (3.2×10^{-8} and 7.1×10^{-6} M resp. (7)) in these cells.

2.2 the guinea pig

Specific ^{3}H-ouabain binding to partly purified guinea pig cardiac cell membranes showed a linear Scatchard plot ($K_D \sim 10^{-7}$M).

However, when these cardiac cell membranes were incubated in Tyrode

solution + ATP, two distinct binding sites could be analyzed ($K_{D1} \sim 4x10^{-7}M$), $K_{D2} \sim 6x10^{-6}M$). In electrically stimulated, contracting guinea pig left atria curvilinear Scatchard plots ($K_{D1} \sim 10^{-6}M$ with about 430 receptors/μm^2; $K_{D2} \sim 2x10^{-4}M$ with about 18.000 receptors/μm^2) were obtained (8).

Halfmaximal positive inotropic effects occurred at $4x10^{-7}M$. The relevance of the low affinity receptors remains unclear, as K_{D2} was well in the toxic range.

2.3 the frog (rana esculenta)

In cardiac cell membranes obtained from summer frogs, we could find one type of ouabain binding site only ($K_D \sim 3.3x10^{-9}M$). However, about 10 fold higher concentrations of ouabain were needed to inhibit ($Na^+ + K^+$)-ATPase halfmaximally under identical conditions.

2.4 the cat

Isolated cat cardiac cell membranes contain one type of specific ^3H-ouabain binding sites ($K_D \sim 6x10^{-9}M$) when analyzed without K^+ and Ca^{++}. In the presence of Tyrode solution + ATP two distinct binding sites can be shown ($K_{D1} \sim 10^{-8}M$, $K_{D2} \sim 10^{-7}M$).

In contracting papillary muscles from cat heart, we could not find evidence for a biphasic concentration-response curve. Koomen and coworkers (9) have published, however, a biphasic concentration-response curve for ouabain in a Langendorff-preparation of cat heart ($EC_{50/1} \sim 10^{-10}M$, $EC_{50/2} \sim 10^{6}M$). Although, as far as we know this experiment has not been reproduced by others, it offers a new way of explaining the unusually high ouabain concentrations necessary in vitro in contrast to the low concentrations useful in vivo. This will be discussed later.

2.5 the sheep

Sheep heart seems to contain only one type of ouabain receptor ($K_D \sim 2x10^{-9}M$) in isolated cardiac cell membranes. However, in isolated, electrically stimulated ventricular myocardium or Purkinje fibres, half maximal occupation of the receptors with ouabain occurred at $3x10^{-7}M$ and halfmaximal increase in force of contraction at $8x10^{-8}M$ (10). In this respect, it is of interest that Purkinje fibres apparently contain the same type of receptor as ventricular myocardim but about 50% less per g wet weight. This might explain the greater sensitivity of Purkinje fibres to toxic effects of cardiac glycosides (10).

2.6 chicken myocardial cells

In cultured rat and chicken beating myocardial cells, nerve fibres, fibrous tissue, endothelium etc. are absent. Thus, the existence of two types of receptors cannot be due to two different types of tissue. Werdan and coworkers (11), in contradiction to Lazdunski and coworkers (12), reported one type of receptor ($K_D \sim 1.5x10^{-7}M$) mediating the cardiac glycoside effects in chicken cells. Lazdunski and coworkers (12) find two receptors ($K_{D1} \sim 2.6x10^{-8}M$, $K_{D2} \sim 2.2x10^{-6}M$), of which the high affinity receptor apparently does not mediate any known effects. Further studies are needed as it is quite unusual that a high affinity receptor is not coupled to any effect.

2.7 the human heart

^{3}H-Ouabain binding to isolated human cardiac cell membranes follows a linear Scatchard plot if analyzed under steady state conditions and in the presence of Mg^{++} + Pi or Mg^{++} + ATP + Na^+ ($K_D \sim 3$ nM). If this specific drug-receptor binding is followed in Tyrode solution

(+ ATP, 3 mM), the Scatchard plot appears to be curved (Fig.2) indicative of two binding sites ($K_{D1} \sim 10^{-8}$M, $K_{D2} \sim 10^{-7}$M). One could evision that Tyrode solution being closer to in vivo conditions resembles a more physiological environment for the enzyme.

The therapeutic strophanthin (ouabain) concentrations in man are about 1-2 nM. This is in the range of the K_D of the drug-receptor-complex. As soon as human myocardium is used in vitro for analysing cardiac glycoside effects, however, one needs concentrations well above 10-50 nM to obtain reproducible positive inotropic effects. The reasons for this are not clear. It might be that the high affinity receptor is lost in vitro and the low affinity receptor mediated effect is seen only. This hypothesis remains to be substantiated.

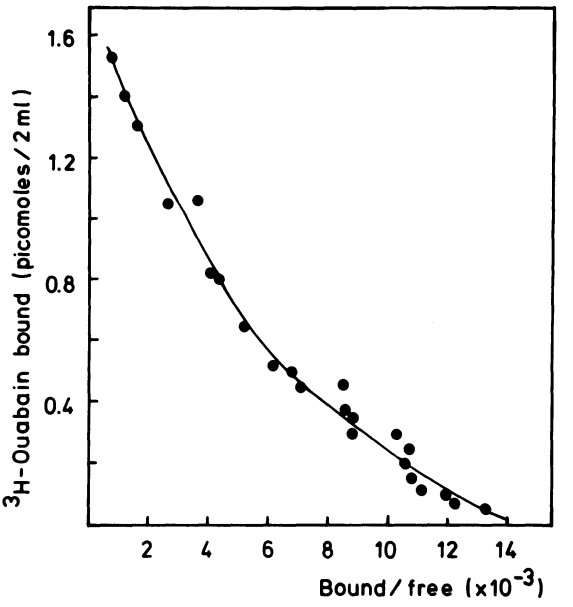


Fig. 2. ^{3}H-Ouabain binding to isolated cardiac cell membrane from human heart. The incubation medium (2 ml, 37°C) was Tyrode solution + 3 mM ATP and increasing amounts of ^{3}H-ouabain. ^{3}H-Ouabain bound in the presence of 10^{-4} unlabelled ouabain was considered as unspecific. Scatchard plot analysis.

Fig. 3. Effect of T_3 on high and low affinity ouabain binding in neonatal rat heart muscle cells in culture.
Rat heart muscle cells have been cultured at K^+ = 5.4 mM for 3 days at 37°C in serum-free, Hepes-buffered, bovine serum albumin supplemented (250 mg/l) CMRL medium (17), at different concentrations of T_3 (see abscissa). Thereafter, ^{3}H-ouabain binding has been measured (7) at $6x10^{-8}$ and $6x10^{-6}$M ouabain (K^+ = 0.7 mM). According to the dissociation constants (K_D) and binding capacities (B) of the high- and low-affinity ouabain binding sites (K_{D1} = $3.2x10^{-8}$M, B_1 = 0.2 pmoles/mg protein; K_{D2} = $7.1x10^{-6}$M, B_2 = 2.6 pmoles/mg protein; (7)), 86% (14%) of specifically bound radioactivity at $6x10^{-8}$M represent binding to the high- (low-) affinity site, while at $6x10^{-6}$M ouabain, 86% (14%) of cell-bound radioactivity reflects binding to the low- (high-) affinity site. ^{3}H-Ouabain binding is given as % of controls (cells grown in the absence of T_3). Data given as $\bar{x} \pm$ SD (n=3) from 3 different experiments. Acute exposure of the cells to 10^{-10}-10^{-6}M T_3 was without any effect on ^{3}H-ouabain binding.

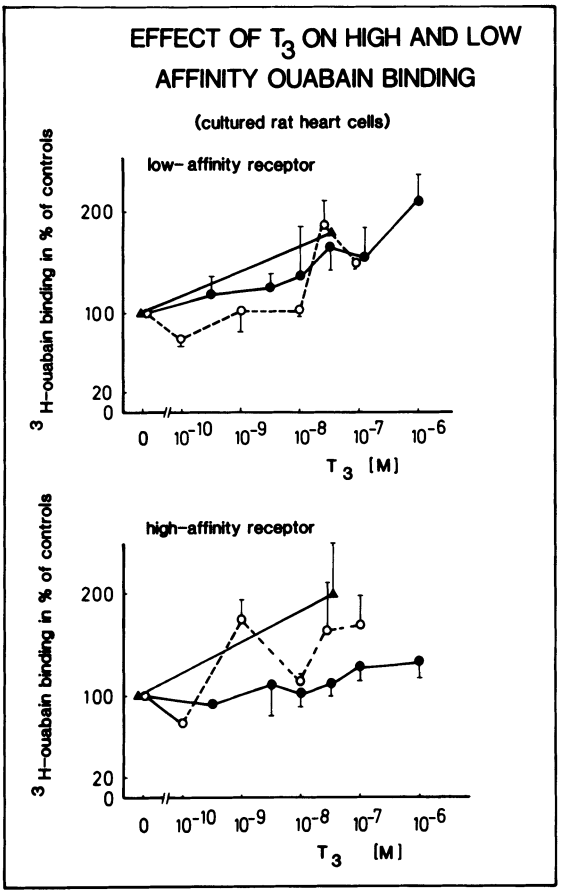
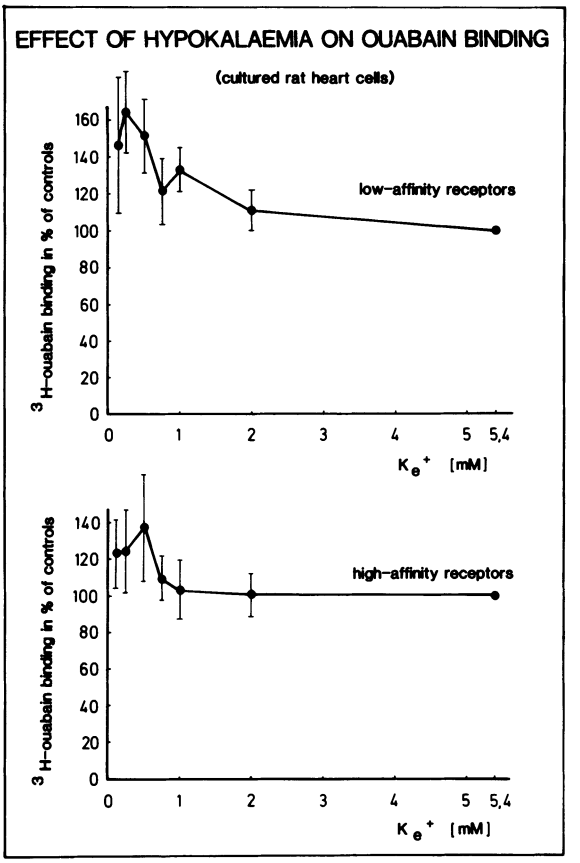


Fig. 4. Effect of extracellular K^+ on high- and low-affinity ouabain binding in neonatal rat heart muscle cells in culture.
Rat heart muscle cells have been cultured for 3 days in serum-free, Hepes-buffered CMRL medium (17), at different K^+ concentrations (see abscissa). Thereafter, specific ^{3}H-ouabain binding at 0.7 mM K^+ has been measured (7) at $6x10^{-8}$ and $6x10^{-6}$ M ouabain, representing ouabain binding to the high and low affinity sites resp. (see legend to Fig. 3). Data are given as % of ^{3}H-ouabain binding after cell culture at K^+ = 5.4 mM (x ± SEM, n=9, 3 different experiments).



3. UP-REGULATION OF CARDIAC GLYCOSIDE RECEPTORS

In animals treated with T_3 for several days, $(Na^+ + K^+)$-ATPase activity and ^3H-ouabain binding sites increase. The same is true for chronic hypokalaemia. In order to possibly differentiate between high and low affinity receptor regulation, we investigated thus conditions in cultured beating myocardial cells of neonatal rats. In chronic hypokalaemia and after T_3-treatment (for 3 days) both types of ouabain binding sites increased concomitantly (Fig. 3 and 4). In these cells, the positive inotropic effect is coupled to both receptors as well.

4. DIFFERENT TYPES OF CARDIAC GLYCOSIDE RECEPTORS - UNSOLVED QUESTIONS

Apparently, there is no doubt about the existence of two different receptors in the rat heart with a high affinity effect and a low affinity effect (14). The experiments in cultured beating myocardial cells prove that the distinct receptors are not due to artifacts from two different kinds of tissue (i.e. myocytes and fibroblasts). However, unless specific drugs are known with different affinities for either receptor population, the given evidence remains rather indirect. A site-to-site interaction and other mechanisms leading to such phenomena cannot be excluded (3,13). On the other hand, we all would wellcome a cardioactive steroid binding to the high affinity site only and thus mediating but the positive inotropic effect without causing the potentially dangerous arrhythmias - which invariably occur when a high proportion of $(Na^+ + K^+)$-ATPase molecules is inhibited.

Unfortunately, the situation seems to be very complex in the human heart, where the evidence for two receptors is rather weak. It is strange, however, that cardiac glycoside concentrations used in vitro are much too

high (by a factor of 50-100) as compared with the well known situation in the pantient. The appearance of two binding sites in isolated cell membranes in Tyrode + ATP may indicate different properties of two or more (Na^++ K^+)-ATPase molecules. If so, it is possible that the high affinity sites (10% of all binding sites) cannot be detected in isolated contracting human papillary muscles nor the effect mediated by these sites. For years, the small effects of the high affinity receptors of rat cardiac muscle had been overlooked, too. The isolation of the respective (Na^++ K^+)-ATPase molecules and their subunit structure analyses may give the answer (16).

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REFERENCES

1. Akera, T., Brody, T. Am. Soc. Pharmacol. Exp. Ther. 29 : 187-220, 1978.
2. Lee, C.O., Dagostino,M. J. Biophys. Soc. 40 : 185-198, 1982.
3. Herzig, S., Lüllmann, H., Mohr, K. J. Mol. Cell. Cardiol. 17 : 1095-1104,1985.
4. Erdmann, E. (edit.). Bas. Res. Cardiol. 79 : 1-162, 1984, Suppl.
5. Sweadner, K.J. J. Biol. Chem. 254 : 6060-6067, 1979.
6. Lelievre, L., Mansier, P., Charlemagne, D., Swynghedauw, B. In: Cardiac Glycoside Receptors and Positive Inotropy (ed. E. Erdmann), Steinkopff-Verlag Darmstadt, 1984, pp.11-31.
7. Werdan, K., Wagenknecht, B., Zwissler, B., Brown, L., Krawietz, W., Erdmann, E. Biochem. Pharmacol. 33 : 1873-1886, 1984.
8. Brown, L., Werdan, K., Erdmann, E. Biochem. Pharmacol. 32 : 423-435, 1983.
9. Koomen, J.M., van Gilst, W.H., Schevers, J.A.M., Wilting,J. In: Cardiac Glycoside Receptors and Positive Inotropy. (ed. E. Erdmann), Steinkopff-Verlag Darmstadt, 1984, pp. 102-109
10. Brown, L., Hug, E., Wagner, G., Erdmann, E. Biochem. Pharmacol. 34 : 3701-3710, 1985.
11. Werdan, K., Wagenknecht, B., Zwissler, B., Brown, L., Krawietz, W., Erdmann, E. Biochem. Pharmacol. 33 : 55-70, 1984.
12. Kazazoglou, T., Renaud, J.F., Rossi, B., Lazdunski, M. J. Biol. Chem. 258 : 12163-12170, 1983.
13. Wellsmith, N.V., Lindenmayer, G.E. Circ. Res. 47 : 710-720, 1980.
14. Erdmann, E., Werdan, K., Brown, L. TIPS 6 : 293-295, 1985.
15. Akera, T., Brody, T.M. TIPS 296-298, 1985.
16. Shull, G.E., Schwartz, A., Lingrel, J.B. Nature 316 : 691-695, 1985
17. Werdan, K., Reithmann, C., Erdmann, E. Klin. Wochenschr. 63 : 1253-1264, 1985.

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MODULATION OF GLYCOSIDE SENSITIVITY OF THE CARDIAC SODIUM PUMP

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INTRODUCTION

Glycoside sensitivity of isolated cardiac Na^+,K^+-ATPase, i.e., the enzymatic representation of the sodium pump, is dependent on the chemical structure of the glycoside and the animal species from which the enzyme is obtained (1). The sensitivity, or affinity, of the isolated enzyme for the cardiac glycoside can be estimated either from the K_D value (dissociation constant) for the [^{3}H]ouabain binding reaction or the IC_{50} value (concentration required to cause a 50% inhibition) for ouabain-induced inhibition of Na^+,K^+-ATPase activity. For example, the K_D value for Na^+,K^+-ATPase obtained from ventricular muscle of guinea-pig heart for [^{3}H]ouabain is 0.1 to 0.15 μM. The IC_{50} value is several-fold higher; 0.5 to 0.7 μM for the same combination of enzyme and glycoside. This difference results from the fact that [^{3}H]ouabain binding is usually estimated in the presence of Na^+, Mg^{2+} and ATP (or Mg^{2+} and inorganic phosphate) whereas Na^+,K^+-ATPase activity is assayed in the presence of these ligands plus K^+. All of these ligands influence the glycoside sensitivity of Na^+,K^+-ATPase.

Na^+, Mg^{2+} and ATP bind to the alpha-subunit of the enzyme from inside of the sarcolemma during the functional cycle of Na^+,K^+-ATPase or the sodium pump (Fig. 1). This binding triggers a conformational change of the enzyme into a "Na^+-induced" form, associated with translocation of Na^+ to the external surface. Phosphorylated enzyme in the Na^+-induced form is the form of the enzyme which preferentially binds the cardiac glycoside. Following the release of Na^+ into the extracellular medium, K^+ binds to the enzyme causing dephosphorylation and a conformational change into a "K^+-induced" form associated with the transport of K^+. Binding sites are not readily available to the glycoside when the enzyme is in the K^+-induced form. Because K^+ reduces the fraction of enzyme in the form available for glycoside binding, the presence of K^+ in the medium lowers apparent affinity of the Na^+,K^+-ATPase for the cardiac glycoside. Under the conditions of the [^{3}H]ouabain binding assay, i.e., in the presence of Na^+, Mg^{2+} and ATP but

without K^+, most enzyme molecules are in the Na^+-induced form. Therefore, the above observation that the presence of K^+ causes a several-fold decrease in estimates of apparent affinity for ouabain indicates that less than 20% of the enzyme is in the Na^+-induced form under the conditions of the Na^+,K^+-ATPase assay.

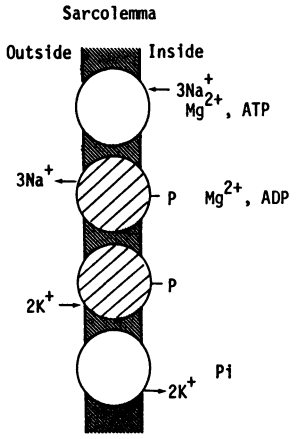

Fig. 1. Reaction scheme for the sodium pump. Binding sites are available to the glycoside in Na^+-induced form (shaded) but not in K^+-induced form (open). Several intermediate forms may exist with various degrees of availability to the glycoside.

In intact cells, the sodium pump is activated by intracellular Na^+ and extracellular K^+. Although extracellular K^+ concentration (4 to 6 mM) is several-fold higher than the Km value estimated using isolated enzyme (less than 1 mM), changes in extracellular K^+ concentrations apparently modulate glycoside sensitivity of the sodium pump in a manner predicted from the above model, i.e., an elevation of extracellular K^+ lowers glycoside sensitivity of the sodium pump. Intracellular Na^+ concentration is 6 to 9 mM (7, 8, 16). Because this value is lower than the estimated Km value for Na^+,K^+-ATPase activation (see below), modulating intracellular Na^+ concentration has a great influence on the glycoside binding to Na^+,K^+-ATPase. The effect of Na^+, however, is more complex than that which is predictable from the above model. Ca^{2+} also appears to influence glycoside sensitivity of the sodium pump.

RESULTS AND DISCUSSION

Increased Na^+ influx

Conditions that would increase the rate of Na^+ influx increase ouabain-sensitive $^{86}Rb^+$ uptake, an estimate of sodium pump activity (Table 1). In quiescent atrial muscle preparations of guinea-pig heart, the concentration of

ouabain required to cause a 50% inhibition of the ouabain-sensitive $^{86}Rb^+$ uptake was slightly higher than 1 μM (Table 1). Electrical stimulation increased the ouabain-sensitive $^{86}Rb^+$ uptake in the absence of ouabain and also reduced the concentration of ouabain needed to cause a 50% inhibition of the uptake; the IC_{50} value observed under 3-Hz stimulation was approximately 0.2 μM. A sodium ionophore, monensin, had similar effects. It should be noted that these experiments were performed in atrial muscle preparations which were not pre-loaded with Na^+. Na^+ loading of the cells increases ouabain binding to the sodium pump and makes it impossible to estimate the "normal" sensitivity of the sodium pump to the inhibitory action of the glycoside.

Table 1. Sodium pump activity and its ouabain sensitivity in isolated guinea-pig atrial muscle preparations estimated from ouabain-sensitive $^{86}Rb^+$ uptake: effects of electrical stimulation and monensin.

Treatment	Ouabain-sensitive $^{86}Rb^+$ uptake	IC_{50} for ouabain
	(nmol/mg tissue/35 min)	(μM)
Control (quiescent)	16.0 ± 0.6	1.50 ± 0.25
Electrical stimulation (3 Hz)	48.1 ± 4.1*	0.23 ± 0.03*
Monensin (2.5 μM)	24.7 ± 1.0*	0.52 ± 0.06*

All experiments were performed at 30°C without Na^+ pre-loading. Ouabain-sensitive $^{86}Rb^+$ uptake is the difference in values observed in the absence and presence of 0.3 mM ouabain. Value are mean of five experiments ± S.E. * Significantly different from corresponding control value ($P<0.05$).

When cells are not Na^+ loaded and are maintaining ionic equilibrium, activity of the sodium pump is apparently matched with the rate of Na^+ influx. An increase in Na^+ influx rate, therefore, increases sodium pump activity (Table 1). This means that the turnover rate of the sodium pump in these preparations is restricted by the availability of intracellular Na^+. When the rate of Na^+ influx is increased or the sodium pump is moderately inhibited, intracellular Na^+ concentration increases, causing the remaining sodium pump to turn-over more rapidly until the sodium pump activity again matches the rate of Na^+ influx. This concept is consistent with the findings that more than 100 mM Na^+ must be present for maximal activation of Na^+,K^+-ATPase and normal intracellular Na^+ concentration is insufficient to cause the maximal activation.

Cardiac muscle of senescent Fischer 344 rats appears to have less Na^+,K^+-

ATPase units per milligram tissue compared to that of younger animals (12). Although there are no differences in sensitivity of the isolated enzyme to ouabain, ouabain-sensitivity of the specific $^{86}Rb^+$ uptake observed with ventricular muscle slices is substantially higher in senescent heart muscle (IC_{50} value = 9 μM) compared to that of young adult rats (IC_{50} = 42 μM). It should be noted that these experiments were performed in rat hearts which have low sensitivity to the cardiac glycoside. These results illustrate a modulation of glycoside sensitivity of the sodium pump which cannot be assessed with isolated enzyme preparations.

It is generally accepted that an excess inhibition of the sodium pump by the cardiac glycoside results in toxicity, such as ventricular tachyarrhythmias. When Langendorff preparations of guinea-pig heart were perfused with 3 μM digoxin under 1-Hz stimulation at 32°C, arrhythmias were observed after 19.1 ± 2.7 min (13). Addition of 2.5 or 7.5 μM monensin to the perfusing solution shortened the time to onset of arrhythmias to 10.6 ± 1.4 and 6.7 ± 1.5 min, respectively. More rapid development of the arrhythmias observed in the presence of monensin can be explained partly from enhanced glycoside binding to the sodium pump resulting from an increase in Na^+ influx which is caused by this Na^+ ionophore. An increase in Na^+ influx causes the Na^+,K^+-ATPase to exist in the Na^+-induced form more frequently. These results and interpretation are consistent with the finding that an increase in Na^+ influx does promote glycoside binding to the sodium pump in isolated heart muscle preparations obtained from guinea pig heart (20). This, however, is apparently only a part of the reason for enhanced glycoside sensitivity of cardiac muscle which is observed in the presence of monensin.

Ventricular muscle of the above Langendorff preparations was rapidly homogenized at the time of onset of arrhythmias, and then incubated for 90 sec in the presence of 50 nM [^{3}H]ouabain, 100 mM NaCl, 5 mM $MgCl_2$ and 5 mM Tris-ATP. From the reduction in initial velocity of the [^{3}H]ouabain binding reaction, it is possible to estimate the fractional occupancy of the glycoside receptors by digoxin which occurred during perfusion of Langendorff preparations. This is because [^{3}H]ouabain binds only to free receptors and hence the initial velocity of binding is proportional to the concentration of free receptors. Thus, fractional occupancy can be calculated as the fractional decrease in the initial velocity of the [^{3}H]ouabain binding reaction. In preparations perfused with digoxin alone, the fractional occupancy of the glycoside receptor by digoxin was 38.3 ± 3.7 % at the onset of arrhythmias. Corresponding values observed in the presence of digoxin and 2.5 or 7.5 μM monensin were significantly lower; 28.6 ± 3.6 and 24.5 ± 3.5 %,

respectively. Assay of Na^+,K^+-ATPase activity in these homogenates also confirmed the finding that a smaller inhibition of the sodium pump precipitated arrhythmias when the rate of Na^+ influx is elevated.

These results support the following concepts. Sensitivity of the sodium pump to the cardiac glycoside is determined by the fraction of Na^+,K^+-ATPase in a Na^+-induced form and also by the degree of reserve capacity of the pump. Both are affected by changes in the rate of Na^+ influx, or more specifically the amounts of Na^+ to be pumped out per unit of the sodium pump. This results in several-fold differences in apparent sensitivity of the sodium pump to inhibitory action of the cardiac glycoside.

Decreased Na^+ influx

If an increase in Na^+ influx elevates glycoside sensitivity of the cardiac sodium pump, then a decrease in Na^+ influx is anticipated to lower glycoside sensitivity. Results obtained with a reduction in extracellular Na^+, which would decrease the rate of Na^+ influx, appear to support this hypothesis (21). In that study, lowering of extracellular Na^+ concentration from 145.2 mM to 27.2 mM reduced fractional occupancy of the glycoside binding sites on Na^+,K^+-ATPase by ouabain in left atrial muscle preparations obtained from guinea pig heart (Fig. 2).

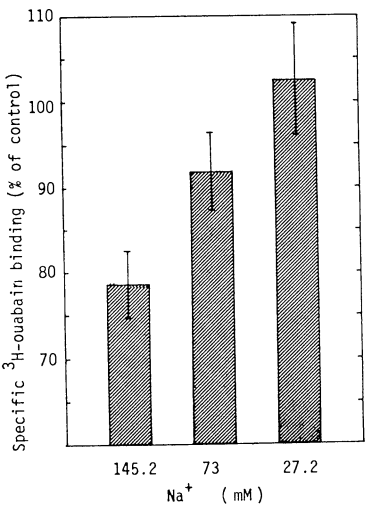

Fig. 2. Fractional occupancy of glycoside binding sites on the sodium pump by ouabain: effects of extracellular Na^+ concentration. Left atrial muscle preparations obtained from guinea-pig heart were exposed to 0.5 μM ouabain for 35 min at 30°C under 0.5-Hz stimulation in Krebs-Henseleit bicarbonate buffer solution. Na^+ concentration of the medium was adjusted as indicated using choline chloride as an osmotic substitute. Atropine (2 μM) was added to eliminate cholinergic effects. After incubation, the muscle was homogenized and incubated with 20 nM [^{3}H]ouabain in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris-HCl buffer (pH 7.5). Non-specific [^{3}H]ouabain binding observed in the presence of 0.1 mM unlabeled ouabain was subtracted. Percentage decreases from 100% in values for [^{3}H]ouabain binding correspond to fractional occupancy in percent. Mean of six or eight experiments. Vertical lines indicate S.E.

Several reports on the interaction between quinidine and digoxin, however, do not support the above concept (3, 14, 27). Pharmacokinetic interaction between quinidine and digoxin is well known; co-administration of quinidine increases plasma digoxin concentrations. This increase is not the result of quinidine displacing digoxin from its binding sites on Na^+,K^+-ATPase (15). Because quinidine reduces the rate of Na^+ influx in the beating cardiac muscle, it appears reasonable to anticipate that quinidine lowers glycoside sensitivity of the cardiac sodium pump and hence reduces the positive inotropic and toxic effects of the glycoside. If this occurs, higher glycoside concentrations observed in the presence of quinidine may be adequate for the treatment of patients, i.e., the dose of digoxin needs not to be lowered in patients receiving quinidine. This, however, is not the case. In isolated atrial muscle or Langendorff preparations in which digoxin concentration is maintained at a fixed value without influence of pharmacokinetic interactions between quinidine and digoxin, the rate of development and also the degree of the positive inotropic effect of digoxin at equilibrium were not altered by quinidine (14).

It may be argued that quinidine, which prolongs action potential duration, reduces the rate of Na^+ influx but may not reduce the total amount of Na^+ which enters the cell during membrane depolarization. Electrophysiological studies with the voltage clamp technique and biochemical studies examining ouabain-sensitive $^{86}Rb^+$ uptake in myocardial cells which were not Na^+ pre-loaded, however, indicate that quinidine does reduce net Na^+ influx in preparations which are electrically stimulated at a relatively high frequency, e.g., 3 Hz (4). Moreover, benzocaine which reduces the rate of Na^+ influx without prolonging action potential duration also failed to reduce the rate of development or the degree of the positive inotropic effect of digoxin. Failure of quinidine or benzocaine to reduce the positive inotropic effect of digoxin was associated with the failure of these agents to alter fractional occupancy of the glycoside binding sites on Na^+,K^+-ATPase by digoxin.

The results with quinidine, therefore, are seemingly inconsistent with the hypothesis that the amount of Na^+ available to the sodium pump modulates glycoside-sensitivity of the pump. However, when the Na^+ activation curve for Na^+,K^+-ATPase was examined with the enzyme obtained from guinea pig heart, half-maximal activation required more than 50 mM Na^+. This assay was performed using choline chloride to maintain the sum of Na^+ and choline ion concentrations at 100 mM (4). The value of 50 mM is higher than that reported earlier by

Lindenmayer and Schwartz (18). The difference seems to result from the presence of a high concentration of cation (choline) which may compete for Na^+ in the study by Berlin et al. (4). It is desirable to use K^+ as a competing cation because activation of the sodium pump by intracellular Na^+ occurs in the presence of K^+. Studies of [^{3}H]ouabain binding to the isolated Na^+,K^+-ATPase in the presence of high concentrations of K^+, however, are meaningless because K^+ also acts at the binding sites on the external surface (K^+-loading sites on the sodium pump) in isolated enzyme preparations, reducing the fraction of enzyme taking a Na^+-induced form. This would markedly alter glycoside sensitivity of the sodium pump. Choline, which has no intrinsic activity for Na^+,K^+-ATPase stimulation, does not alter glycoside-sensitivity of the pump in a specific manner. Assuming that the half-maximal activation of Na^+,K^+-ATPase by Na^+ at the Na^+-loading site on the sodium pump occurs at 50 mM, and a Hill coefficient for Na^+ of 2.2, the theoretical curve for Na^+ activation of the sodium pump (Fig. 3) indicates that the sodium pump activity is markedly affected by concentrations of Na^+ higher than 10 mM but is relatively insensitive to changes in Na^+ below 8 mM. Because normal intracellular Na^+ concentration in the cardiac muscle is about 8 mM, it follows then that an increase in Na^+ influx activates the sodium pump and increases its glycoside sensitivity whereas a decrease in Na^+ influx would have relatively little effect on glycoside sensitivity of the pump.

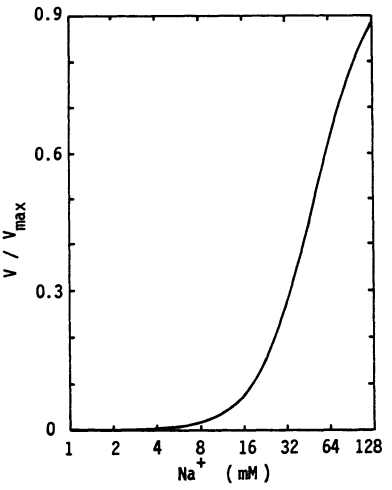

Fig. 3. Theoretical curve for Na^+ activation of the sodium pump. The curve represents the following equation:

$$\frac{V}{V_{max}} = \frac{[Na^+]^{2.2}}{50^{2.2} + [Na^+]^{2.2}}$$

How can we then explain a reduction in glycoside binding to the sodium pump observed when extracellular Na^+ concentration was lowered (21)? We do not have sufficient data to choose from several possibilities; however, one plausible explanation is the effect of extracellular Na^+ to stabilize the glycoside-Na^+,K^+-ATPase complex. It has been shown previously that high concentrations of Na^+ stabilize the glycoside-enzyme complex (2). Thus, the effects of Na^+ on the glycoside sensitivity of the sodium pump are apparently more complex than what is predicted simply from stimulation of the glycoside binding by intracellular Na^+.

Effects of Ca^{2+} on ouabain binding to the sodium pump

Intracellular Na^+ and extracellular K^+ regulate the glycoside-sodium pump interaction in intact cells as discussed above. The glycoside binding in intact cells, however, also appears to be regulated by additional factors. For example, enhancement of glycoside binding caused by catecholamine or insulin (6) does not appear to depend entirely on changes in Na^+ or K^+ concentrations. Whether the turnover of the enzyme and therefore glycoside binding is under the control of Ca^{2+} is an important question.

Ca^{2+} has substantial effects on the reaction sequence of isolated Na^+,K^+-ATPase and also affects [^{3}H]ouabain binding to the enzyme (22). For example, Ca^{2+} in relatively low concentrations (about 0.01 mM) supports the first step of phosphoenzyme formation in the absence of Mg^{2+}. Ca^{2+}, however, competes with Mg^{2+} for a subsequent step in which the ADP-sensitive phosphoenzyme (relatively low affinity for the glycoside) is converted to the K^+-sensitive phosphoenzyme (high affinity for the glycoside), thereby inhibiting [^{3}H]ouabain binding and blocking enzyme turnover. This effect of Ca^{2+} requires higher concentrations (approximately 0.5 mM). Therefore, Na^+,K^+-ATPase can be inhibited by millimolar concentrations of Ca^{2+}. In functioning cardiac cells, however, intracellular Ca^{2+} concentration does not reach millimolar concentrations. Because effects of Ca^{2+} on the Na^+,K^+-ATPase are either to substitute for or to compete with Mg^{2+}, and because it is intracellular Mg^{2+} which affects the sodium pump, it has been generally believed that Ca^{2+} has little physiological role in modulating sodium pump activity or its sensitivity to the cardiac glycoside.

In contrast to results obtained with isolated enzyme preparations, Yingst and his associates (23-26) have shown that the sodium pump is sensitive to physiological (micromolar) concentrations of intracellular Ca^{2+} in intact erythrocytes. This inhibition appears to require the presence of a calmodulin-like protein (not

calmodulin) which increases sensitivity of the sodium pump to Ca^{2+}. It has not been shown if a similar mechanism exists in the cardiac muscle; however, Lelievre and his associates reported that Ca^{2+}-free perfusion of Langendorff preparations obtained from rat heart using a solution containing EDTA markedly increases ouabain-sensitivity of subsequently isolated Na^+,K^+-ATPase (19). These investigators also reported that Ca^{2+} has a marked effect on the affinity of Na^+,K^+-ATPase for the glycoside under certain conditions (5, 17). Moreover, Huang and Askari (10, 11), demonstrated the presence of high affinity Ca^{2+} binding sites on the purified enzyme, in addition to the low affinity sites which apparently correspond to Mg^{2+} binding sites. Thus, there is a possibility that Na^+,K^+-ATPase in intact cells is modulated by Ca^{2+}.

In studies to determine glycoside binding to the sodium pump or ouabain sensitive $^{86}Rb^+$ uptake, isolated myocytes preparations have many advantages over intact muscles. Studies described here were performed using rod-shaped, viable cardiac muscle cells which have clear striation patterns. These cells exclude dyes having high molecular weight, are quiescent in a medium containing physiological concentrations of Ca^{2+}, maintain transmembrane potentials and respond to electrical stimulation by contraction. Advantages of these preparations over intact tissues include the lack of diffusion barriers outside the cells and the possibility for investigators to rapidly exchange the extracellular medium. When [^{3}H]ouabain binding was examined in myocyte preparations obtained from guinea-pig heart, however, the addition of high concentrations (0.3 to 1 mM) of unlabeled ouabain in order to estimate nonspecific binding resulted in all cells loosing their rod shape and striation patterns. These cells became round shaped with many of them apparently undergoing lysis and thus loosing the ability to exclude high molecular weight dyes.

Based on an assumption that myocytes are dying of Ca^{2+} overload as the result of a complete sodium pump inhibition, experiments were performed in a Ca^{2+}-free medium containing 0.25 mM EGTA using ventricular myocytes obtained from guinea pig heart. Removal of Ca^{2+} caused a marked increase in [^{3}H]ouabain binding when the concentration of [^{3}H]ouabain is relatively low (Table 2). The K_D value for [^{3}H]ouabain was not calculated because Scatchard plots of [^{3}H]ouabain binding data observed in the presence of Ca^{2+} are curved. The curved Scatchard plot is likely to result from the fact that intracellular Na^+ concentration is low in the absence or presence of low concentrations of ouabain, but increases as [^{3}H]ouabain concentration is elevated (9), rather than the possible presence of high

and low affinity binding sites. This is because the addition of 2.0 μM monensin increased [^{3}H]ouabain binding when its concentration was not saturating but had relatively small effects when [^{3}H]ouabain concentration was high (Table 2). The effect of 2.0 μM monensin, however, was smaller than that observed with Ca^{2+}-free incubation. In Ca^{2+}-free medium, monensin failed to further increase [^{3}H]ouabain binding. These results indicate that the presence of Ca^{2+} in the incubation medium inhibits glycoside binding to Na^+,K^+-ATPase in isolated myocytes.

Table 2. Effects of Ca^{2+} and monensin on [^{3}H]ouabain binding and ouabain-sensitive $^{86}Rb^+$ uptake in myocytes isolated from guinea-pig heart.

Treatment		Specific [^{3}H]ouabain binding		$^{86}Rb^+$uptake Specific uptake	IC_{50} for ouabain
Ca^{2+}	Monensin	0.5 μM	3.0 μM		
(mM)	(μM)	(pmol/mg protein)		(nmol/mg protein/6 min)	(μM)
1.8	0	3.4 ± 0.2	7.8 ± 0.1	56.8 ± 2.3	1.5
0	0	5.7 ± 0.2*	11.4 ± 0.4*	76.8 ± 2.8*	2.5
1.8	2	4.2 ± 0.1*	9.2 ± 0.2*	90.3 ± 3.8*	
0	2	5.7 ± 0.5	11.7 ± 0.6	111.8 ± 6.7*	
1.8	50			247.8 ± 12.4	0.5
0	50			278.5 ± 13.4	0.5

Values are mean ± S.E. of 4 experiments. * Significantly different from corresponding control value obtained in the presence of 1.8 mM $CaCl_2$ ($P<0.05$).

Ouabain-sensitive $^{86}Rb^+$ uptake by myocytes was also enhanced when extracellular Ca^{2+} was removed (Table 2). Again, the study was performed using myocytes which were not Na^+ pre-loaded. The effect of Ca^{2+}-free incubation to increase sodium pump activity, however, was smaller than that caused by 2.0 μM monensin. Moreover, the concentration of ouabain required to cause a 50% inhibition of ouabain-sensitive $^{86}Rb^+$uptake was increased in a Ca^{2+}-free medium, despite the fact that glycoside binding was enhanced. Monensin (50 μM) decreased the concentration of ouabain to cause a 50% inhibition of sodium pump activity as can be expected from the results with [^{3}H]ouabain binding studies. These results are indicative of the complexities of the effect of Ca^{2+} on glycoside sensitivity of the sodium pump. A simple explanation such as that an elevation of intracellular Na^+ concentration in a Ca^{2+}-free medium may occur via enhancement of Ca^{2+}

efflux, Na^+ influx exchange is not tenable. This is because Ca^{2+}-free incubation, which caused only a modest increase in ouabain-sensitive $^{86}Rb^+$ uptake, caused a greater increase in [^{3}H]ouabain binding compared to relative effects of monensin, yet sensitivity of ouabain sensitive $^{86}Rb^+$ uptake was decreased by Ca^{2+}-free incubation in contrast to the effect of monensin which increased ouabain sensitivity of the sodium pump. One possible explanation for the observed effects of Ca^{2+}-free incubation on [^{3}H]ouabain binding and ouabain-sensitive $^{86}Rb^+$ uptake is an increase in reserve capacity of the sodium pump. If this occurs without changes in the rate of Na^+ influx, an increase in $^{86}Rb^+$ uptake with a concomitant decrease in ouabain-sensitivity of the sodium pump may occur. This is consistent with the results of experiments reported here; however, more work is required to substantiate this hypothesis.

SUMMARY

Changes in the rate of Na^+ influx may alter glycoside sensitivity of the sodium pump by two mechanisms; an increase in glycoside binding caused by an increase in the fraction of sodium pump molecules in the Na^+-induced form which preferentially binds the glycoside, and a decrease in reserve capacity of the sodium pump. The combination of these two factors may cause a several-fold difference in glycoside sensitivity of the sodium pump in intact cells which may have identical intrinsic sensitivity to the glycoside when this parameter is estimated using isolated enzyme preparations. Glycoside sensitivity of the sodium pump in intact cells is further modified by K^+ and Ca^{2+} concentrations in the incubation medium. Effects of K^+ to reduce glycoside sensitivity of the sodium pump apparently results from a reduction in the fraction of Na^+,K^+-ATPase in a binding (Na^+-induced) conformation, whereas effects of Ca^{2+} are more complex and not well understood.

ACKNOWLEDGMENTS

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REFERENCES

1. Akera, T. Handbook of Experimental Pharmacology, vol. 56/I, Cardiac Glycosides (ed. K. Greeff), Springer-Verlag, Heidelberg, 287-336, 1981.

2. Allen, J.C., Harris, R.A. and Schwartz, A. Biochem. Biophys. Res. Commun. **42**: 366-370, 1971.
3. Belz, G.G. Doering, W., Aust, P.E., Heinz, M., Mattheus, J. and Schneider, B. Clin. Pharmacol. Ther. **31**: 548-554, 1982.
4. Berlin, J.R., Akera, T. and Brody, T.M. J. Pharmacol. Exp. Ther. 1986 (in press).
5. Charlemagne, D., Leger, J., Schwartz, K., Geny, B., Zachowski, B. and Lelievre, L.: Biochem. Pharmacol. **29**: 297-300, 1980.
6. Clausen, T. and Hansen, O. J. Physiol. (London) **270**: 415-430, 1977.
7. Cohen, C.J., Fozzard, H.A. and Sheu, S.S. Circ. Res. **50**: 651-662, 1982.
8. Ellis, D. J. Physiol. (London) **237**: 211-240, 1977.
9. Herzig, S., Krey, U., Lullmann, H. and Mohr, K. Trends Pharmacol. Sci. **6**: 432-433, 1985.
10. Huang, W.H. and Askari, A. Arch. Biochem. Biophys. **216**: 741-750, 1982.
11. Huang, W.H. and Askari, A. Arch. Biochem. Biophys. **231**: 287-292, 1984.
12. Katano, Y., Akera, T., Temma, K. and Kennedy, R.H. Europ. J. Pharmacol. **105**: 95-103, 1984.
13. Kennedy, R.H., Akera, T. and Brody, T.M. Eur. J. Pharmacol. **89**: 199-209, 1983.
14. Kim, D-H., Akera, T. and Brody, T.M. J. Pharmacol. Exp. Ther. **217**: 559-565, 1981.
15. Kim, D-H., Akera, T. and Brody, T.M. J. Pharmacol. Exp. Ther. **218**: 108-114, 1981.
16. Lee, C.O., Kang, D.H., Sokol, J.H. and Lee, K.S. Biophys. J. **29**: 315-330, 1980.
17. Lelievre, L., Zachowski, A., Charlemagne, D., Laget, P and Paraf, A. Biochim. Biophys. Acta **557**: 399-408, 1979.
18. Lindenmayer, G.E., and Schwartz, A. J. Biol. Chem. **248**: 1291-1300, 1973.
19. Mansier, P. and Lelievre, L.G. Nature (London) **300**: 535-537, 1982.
20. Temma, K. and Akera, T. J. Pharmacol. Exp. Ther. **223**: 490-496, 1982.
21. Temma, K. and Akera, T. J. Pharmacol. Exp. Ther. **225**: 660-666, 1983.
22. Tobin, T., Akera, T., Baskin, S.I. and Brody, T.M. Mol. Pharmacol. **9**: 336-349, 1973.
23. Yingst, D.R. Biochim. Biophys. Acta **732**: 312-315, 1983.
24. Yingst, D.R. and Hoffman, J.F. Fed. Proc. **40**: 543, 1981.
25. Yingst, D.R. and Marcovitz, M.J. Biochem. Biophys. Res. Commun. **111**: 970-979, 1983.
26. Yingst, D.R. and Polasek, P.M. Biochim. Biophys. Acta **813**: 282-286, 1985.
27. Zaman, L., Pastore, J.O. and Kosowsky, B.D. Clin. Res. **29**: 253A, 1981

23

UP AND DOWN REGULATION OF THE SODIUM PUMPING SITES IN MYOCARDIUM

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INTRODUCTION

It is well known that myocardial sodium pump plays an important role in the maintenance of the electrochemical activities of the heart, and it is now becoming apparent that an inhibition of its activity by cardiac glycosides is associated with both positive inotropic and arrhythmogenic effects (see 1 and 2). Many types of effector cells are known to undergo compensatory changes in sensitivity to stimuli and in receptor numbers, particularly the beta-adrenergic receptors, when there is a chronic increase or decrease in the contact between an agonist and the cells. The phenomenon of up and down regulation of sodium pumping sites in myocardium with chronic exposure to cardiac glycosides, as well as the subsequent myocardial sensitivity changes, however, are not well understood. Recently, we and a number of other investigators have also reported significant alterations in the steady-state activity of myocardial sodium pump in various pathological states, such as diabetic, hypertensive, hypothyroid and ischemic (3-7), as well as following various hormonal (insulin and thyroid) and chemical (reserpine) treatments (3, 7-8). The pathophysiological significance of these sodium pump activity alterations in relation to myocardial function and their responsiveness to cardiac glycosides, however, are not clear. Since it is becoming apparent that the remaining uninhibited sodium pump activity in the presence of cardiac glycosides ultimately determines the magnitude of increases in intracellular sodium concentration and the positive inotropic effect of cardiac glycosides, changes in the steady-state sodium pumping sites could thus modulate the sensitivity of cardiac glycosides. In this article we will briefly review the results of our studies on 1) the effects of chronic cardiac glycosides treatment as well as various pathological states, such as diabetes mellitus and acute myocardial ischemia, on myocardial sodium pumping sites, and 2) the sensitivity of these pathological hearts to the inotropic actions of cardiac glycosides.

RESULTS AND DISCUSSION

Sodium Pump in Chronic Digoxin Treatment

Although cardiac glycosides are commonly indicated as the drug of choice for improving mechanical failure of the heart muscle, the use of cardiac glycosides during long-term treatment of heart failure and in acute myocardial ischemia has recently been challenged. This uncertainty, in part, stems from published results of several clinical reports as well as experimental studies suggesting that long term treatment with cardiac glycosides results in an attenuation or even loss of the positive inotropic effect (9-11), and, in part, results from the apparent increased incidences of cardiac glycosides toxicity in patients and experimental animals with acute myocardial infarction (5, 12-13).

To investigate whether chronic treatment with non-toxic doses of cardiac glycosides, which cause a moderate inhibition of myocardial Na,K-ATPase and sodium pump, would result in a compensatory increase in the activity of that enzyme, we studied the relationship among serum digoxin concentration, binding of digoxin to the enzyme and cardiac Na,K-ATPase and sodium pump activities in dogs chronically treated (up to 4 weeks) with digoxin (14). As shown in Fig. 1, two hours after the intravenous injection of a single non-toxic dose (60 ug/kg) of digoxin, sodium pump activity, as estimated from the ouabain-sensitive ^{86}Rb uptake, as well as Na,K-ATPase (data not shown), were inhibited quantitatively in a manner corresponding to the binding of digoxin to the enzyme (Fig. 2). The magnitude of sodium pump inhibition was reduced 12 hr after the digoxin injection, with simultaneous decreases in serum digoxin concentration and the binding of digoxin to the enzyme. After 1 or 4 weeks of daily intravenous digoxin treatment with non-toxic doses (8-12 ug/kg/12 hr), the relationships among serum digoxin concentration, binding of digoxin to cardiac Na,K-ATPase and the degree of cardiac Na,K-ATPase or sodium pump inhibition remained unchanged (Figs. 1 & 2). The magnitude of the inhibition was closely related to serum digoxin concentrations and digoxin binding to Na,K-ATPase, and in a manner similar to that observed after a single digoxin injection. After 4 weeks of digoxin treatment with toxic doses (14-16 ug/kg/12 hr), these relationships were also unaffected. These results suggest that chronic digoxin treatment of dogs did not alter the total myocardial (inhibited plus uninhibited) Na,K-ATPase concentration and the number of sodium pumping sites. Furthermore, these results are consistent with the majority of clinical experience that neither tolerance nor "reverse tolerance" to cardiac glycosides develops during chronic cardiac glycosides treatment.

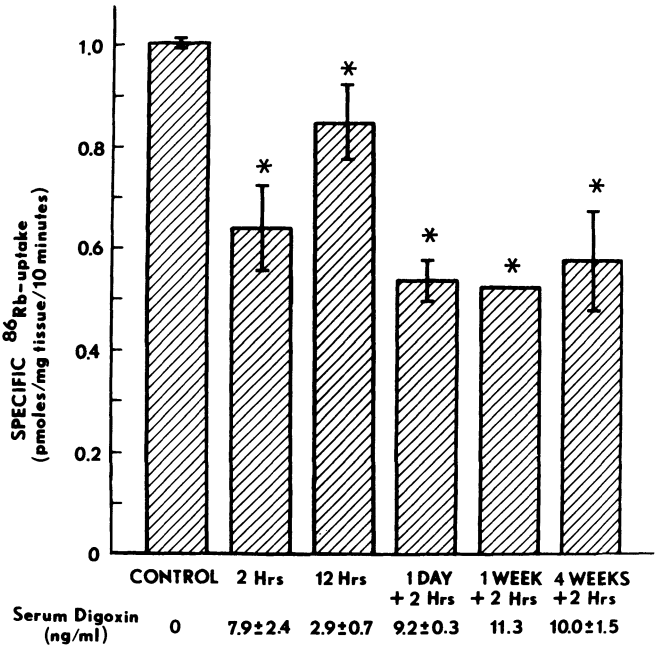

Fig. 1 Relationship between serum digoxin concentration and ouabain-sensitive ^{86}Rb uptake by ventricular slices obtained from control and digoxin-treated dogs. After various periods of saline (control) or digoxin treatments, animals were sacrificed and ventricular slices (0.5 mm thick) were prepared. Slices were incubated at 0-2°C in a K-free Krebs-Henseleit solution for sodium loading, transferred to a K-free solution containing 2 mM RbCl and tracer amount of ^{86}Rb and incubated at 37°C for 10 min. The amount of ^{86}Rb accumulated in the slices in the presence of 0.2 mM ouabain (non-specific uptake) was subtracted from that in the absence of ouabain to calculate the specific ^{86}Rb uptake due to sodium pump. Vertical lines indicate standard error. Serum digoxin levels were estimated with a radioimmunoassay method. Numbers in parentheses indicate the number of experiments. a=Time after digoxin (60 ug/kg) injection. *Significantly different from control; $P<0.05$ (Reprinted with permission from ref. 14).

Although a similar lack of adaptive changes in myocardial Na,K-ATPase was also reported in dogs following up to 4 months of digoxin treatment (15), other investigators, however, have reported a significant elevation of myocardial Na,K-ATPase occurring in guinea pigs and pigs as well as in cultured HeLa cells and chick heart cells (16-20). The cause of these contrasting results

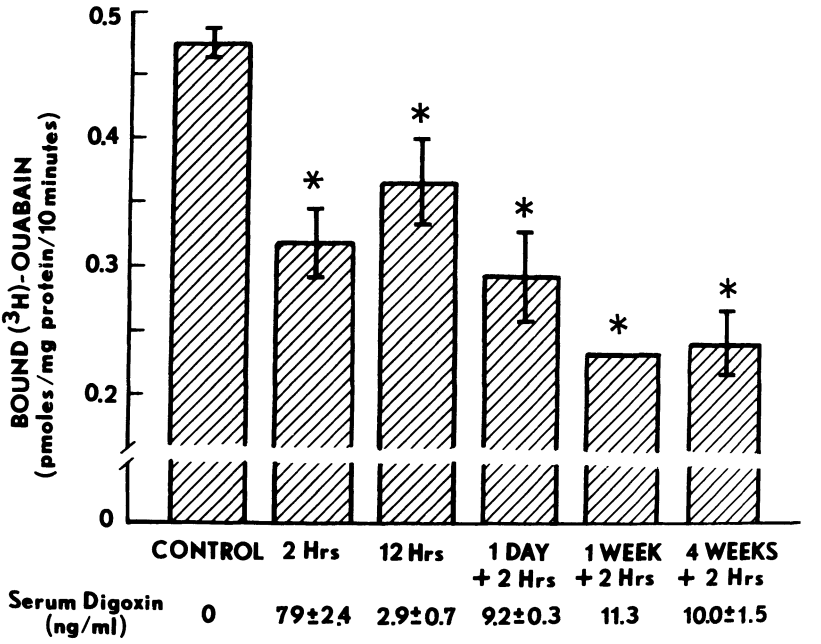

Fig. 2 Relationship between serum digoxin concentration and ATP-dependent ^{3}H-ouabain binding during digoxin treatment. Dogs were sacrificed 2 or 12 hr after receiving the single injection of digoxin (60 ug/kg) or 2 hr after challenge dose (60 ug/kg) in chronically digoxin-treated animals. Ventricular homogenates were prepared, and the binding of ^{3}H-ouabain to Na,K-ATPase was determined. Values obtained in the absence of ATP (non-specific binding) were subtracted from those obtained in the presence of ATP (total binding) to calculate the specific, ATP-dependent ^{3}H-ouabain binding. Numbers in parentheses indicate the number of experiments. The control values of 12 dogs were not significantly different in each group and were thus pooled. Vertical lines indicate standard error. * Significantly different from control; $P<0.05$. Serum digoxin levels at the time of sacrifice were estimated with a radio-immunoassay method. a=Time after digoxin (60 ug/kg) injection. (Reprinted with permission from ref. 14).

is not clear, but it may, in part, be related to differences in the experimental conditions and the animal models studied. More recently, Werdan et al (20) presented experimental evidence to suggest that this apparent controversy may also, in part, be related to the differences in the doses of cardiac glycosides used in the chronic treatments. These investigators

demonstrated that, in cultured chick heart cells, chronic treatment with low, therapeutic doses of cardiac glycosides did not induce any significant changes in sodium pump activity, binding characteristic of cardiac glycosides and the positive inotropic and toxic effects of cardiac glycosides. Whereas chronic treatment with higher, toxic doses of cardiac glycosides with apparent intracellular sodium accumulation did result in an increase in sodium pump activity and development of tolerance to cardiac glycosides. Results of these studies suggest that development of cardiac glycosides tolerance is not due to a simple up and down regulation of the cardiac glycoside receptor, as is more commonly seen with beta-adrenergic receptors, but rather it is coupled to the changes in the number of sodium pumping sites. The driving force for such changes is apparently related to the rise in intracellular Na ion. Increases in cellular sodium load have been shown to cause an induction of Na,K-ATPase in a variety of cells and experimental conditions. Results of these alteration in sodium pumping sites and the subsequent changes in sensitivity to cardiac glycosides, however, are not clear.

Sodium Pump in Experimental Diabetes

Insulin has been known for more than 50 years to alter the distribution of sodium and potassium ions across the cell membrane. The mechanism of the increase in intracellular potassium and decrease in intracellular sodium has been associated with the insulin-induced increases in membrane Na,K-ATPase and sodium pump activities. Such an enhancement has been reported to occur in a number of noncardiac tissues (21-23). The effects of insulin on cardiovascular sodium pump activities, as well as the effects of chronic diabetes mellitus on these hormone-mediated modulations of cardiovascular activities, however, have not been well studied. We recently reported that addition of insulin in incubation medium or subcutaneous injection of insulin for 1 week in normal rats did not induce any significant changes in the myocardial sodium pumping activity, as estimated from the specific ^{86}Rb uptake (3). Chronic depletion or reduction of circulating insulin by streptozotocin (STZ) treatment in rats, however, showed a significant reduction of myocardial as well as vascular sodium pumping activities as compared to the saline-citrate-treated controls (Fig. 3).

The decreases in cardiovascular sodium pump activities were apparently related to the reduction in circulating insulin in these experimental diabetic animals, such that addition of insulin, either <u>in vitro</u> (200 mU/ml in the

incubation medium) or in vivo (s.c. injection of insulin for 1 week), completely reversed the pump depression (Fig. 4). It should be noted that insulin re-administration resulted only in a reversal of myocardial sodium pump

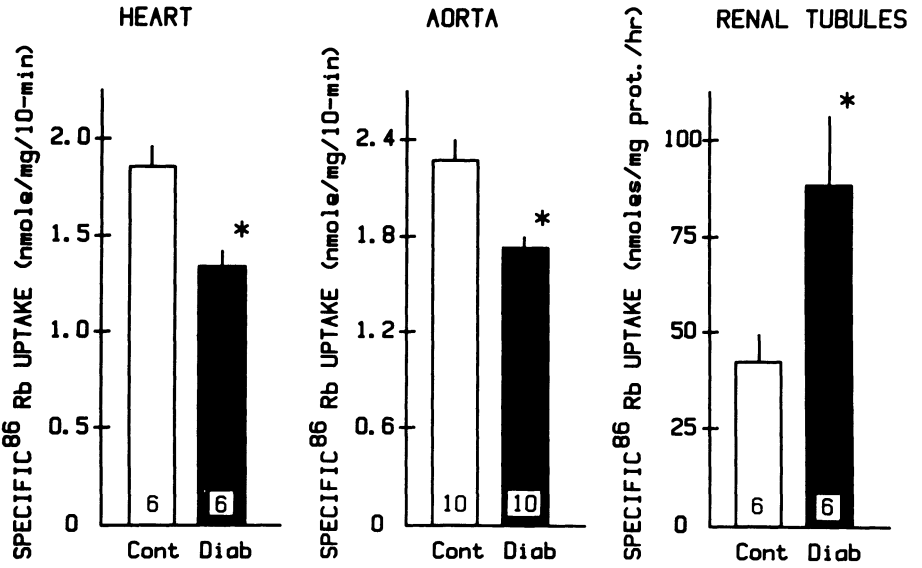

Fig. 3 Chronic effects of streptozotocin-induced diabetes on ouabain-sensitive ^{86}Rb uptake by myocardium, thoracic aorta and renal tubules of rats. Four to seven week after STZ (60 mg/kg) injection, ventricular slices and thoracic aorta were prepared and renal tubules were isolated nonenzymatically using a magnetic iron oxide method (26). The amount of ^{86}Rb accumulated by the tissues in the presence of 1.0 mM ouabain (non-specific uptake) was subtracted from that in the absence of ouabain (total uptake) to calculate the ouabain-sensitive ^{86}Rb uptake. The values shown are means $\pm$ SEM from the number of preparations indicated by the numbers at the base of each bar. *$P<0.05$

depression and that the pump activity did not increase higher than that of the controls. These results are consistent with our contention that in the control rats with normal circulating insulin, the hormone may have already exerted its optimal effect and that further addition of insulin did not produce an additional effect, as we have reported earlier.

The effect of chronic depression of myocardial and vascular sodium pump on

myocardial function of the STZ-diabetic rats has not been extensively studied. An inhibition or reduction of myocardial sodium pump by cardiac glycosides or other inhibitors has been shown to result in an increase in myocardial contractile force (see ref. 1-2). However, in contrast to an enhancement of myocardial contractile activity, the basal force of contraction

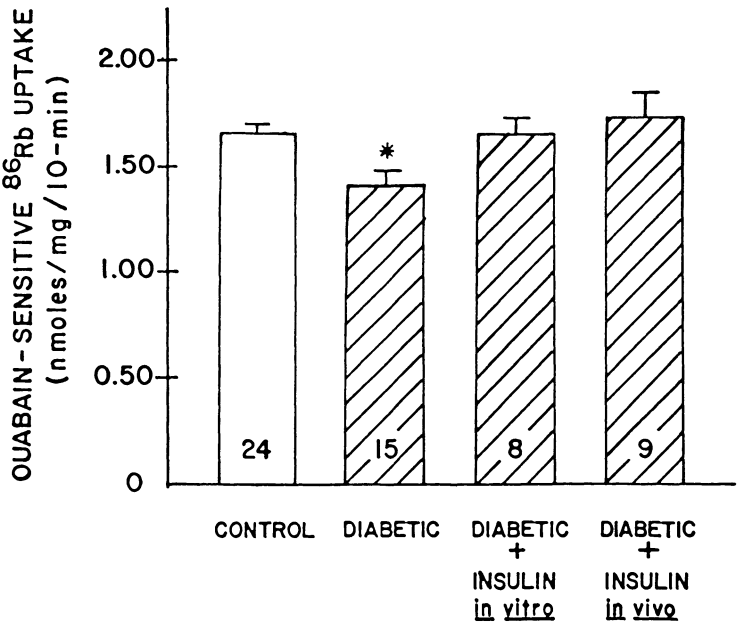

Fig. 4 Effects of insulin on ouabain-sensitive ^{86}Rb uptake by STZ-diabetic rat heart slices. Insulin administration either directly into the incubation medium (200 mU/ml) during the 10 min ^{86}Rb uptake studies or injected s.c. with a loading dose of 10 units following by two divided doses of 5 units each per day for 1 week. Experimental protocol for the determination of specific, ouabain-sensitive ^{86}Rb uptake was same as that described in legend to Fig.3. All values shown are mean $\pm$ SEM from the number of animals indicated at the base of each bar. * $P<0.05$

of the isolated, electrically-driven left atrial preparations of the STZ-diabetic rats was actually less than that of the controls (3). Similar decreases in myocardial contractile performance have also been reported in experimental diabetic animals (24-25). Subcutaneous injection of insulin in

these STZ-diabetic rats for 1 week was capable of partially reversing the myocardial contractile dysfunction. These results suggest that other factors, such as an altered myocardial energy production and utilization, calcium metabolism and/or cardiac contractile proteins, may contribute to the observed myocardial depression in STZ-diabetic rats. More importantly, however, the sensitivity of the STZ-diabetic heart to the positive inotropic effects of ouabain was not significantly altered from that of the controls. Similarly, the sensitivity of the myocardial sodium pump to the inhibitory effect of ouabain in vitro was not different between the two groups. Thus, these results clearly demonstrate that chronic depletion of circulating insulin by streptozotocin treatment in rats is accompanied by a reversible depression of cardiovascular sodium pump activity and myocardial contractile force. The sensitivity of these diabetic hearts to the inotropic effects of ouabain and its inhibitory effect on myocardial sodium pump, however, was not altered.

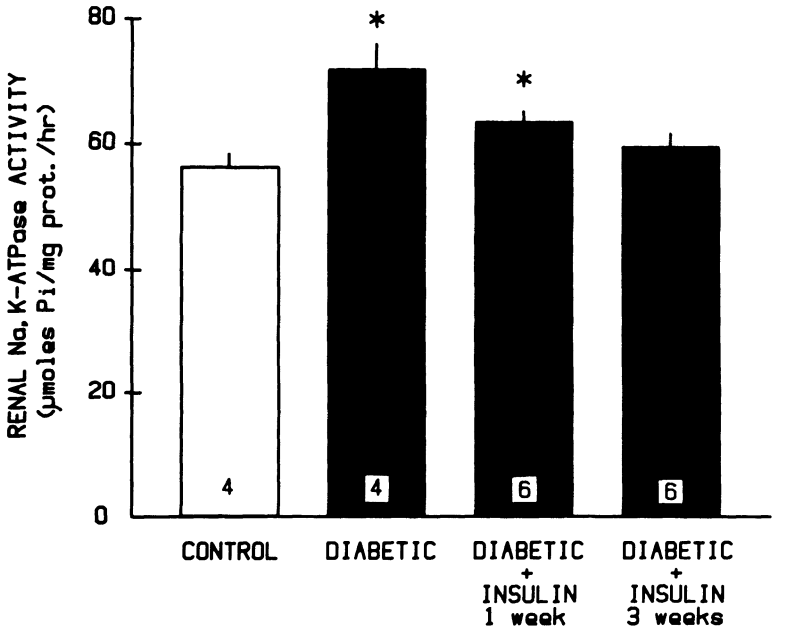

Fig. 5. Effects of s.c. insulin injection on renal Na,K-ATPase activities of control and STZ-diabetic rats. Experimental protocol for the s.c. injection of insulin was similar to that described in legend to Fig. 4. The specific Na,K-ATPase activities were measured in freshly prepared whole kidney homogenates in the presence of 0.1% sodium deoxycholate as previously described (26). The values shown are means ± SEM from the number of animals indicated at the base of each bar. * $P<0.05$

One of the interesting observations made during the course of our studies on the sodium pump activities in experimental diabetes was the finding of a significant increase in the renal tubular sodium pump activity (Fig. 3) and the renal cortical and outer medullary Na,K-ATPase activities of the STZ-diabetic rats (26-27). The cause of these paradoxical changes in sodium pump activities of the renal and cardiovascular tissues, as mentioned above, is not clear. If insulin exerts a similar direct stimulatory effect on the renal Na,K-ATPase, one would expect that chronic depletion of this hormone in STZ-diabetes should result in a generalized reduction of the enzyme activity as was the case with the cardiovascular sodium pump activity. Our findings of a gradual development of increased renal Na,K-ATPase activity following induction of STZ-diabetes, the gradual regression of this activity upon insulin treatment (Fig. 5) and their parallelism with the development and regression of renal hypertrophy and osmotic diuresis in these animals suggest that renal growth rather than a direct effect of insulin is the primary factor controlling the Na,K-ATPase activity in the diabetic kidneys. This conclusion is further supported by the analogous increase in Na,K-ATPase activity in the remnant kidney of uninephrectomized animals, a condition in which renal hypertrophy occurs without alterations in insulin levels. Thus, results of these studies of experimental diabetes on sodium pump activity clearly demonstrate that this monovalent cation transport system is highly flexible and capable of adjusting its activity with changes in either hormonal (insulin) levels or hyperfunctioning of the cells.

Sodium Pump in Myocardial Ischemia

The effects of acute myocardial ischemia on myocardial sodium pump or Na,K-ATPase activity and its interaction with cardiac glycosides have not been well studied. We and a number of other investigators have previously reported that myocardial sensitivity to cardiac glycosides-induced ventricular arrhythmias was significantly increased following an acute myocardial ischemia in dogs (5, 12) as well as in man (13). The mechanism of this increase in cardiac sensitivity to cardiac glycosides, however, remains controversial. Many recent studies have attempted to relate cardiac glycosides distribution and uptake in the ischemic myocardium to these increased cardiac glycosides toxicities. It has been demonstrated that ischemic myocardial tissue accumulated significantly less radio-labeled cardiac glycosides than normal, non-ischemic tissues. Beller et al (28) has previously suggested that the decreased glycosides uptake

by the ischemic tissue may be related to a defect or reduction in cardiac Na,K-ATPase. Although significant reduction in myocardial Na,K-ATPase activity had been reported after an acute myocardial ischemia (29), other investigators have reported that prolonged ischemia in skeletal muscle has actually resulted in an increase in Na,K-ATPase activity (30). In addition, it is also not clear whether these alterations in the sodium pump activity in the ischemically jeopardized, but still reversible, myocardial cell are reversible during coronary reperfusion.

To further determine the effects of acute myocardial ischemia on sodium pump activity, ouabain-sensitive ^{86}Rb uptake in ventricular slices obtained

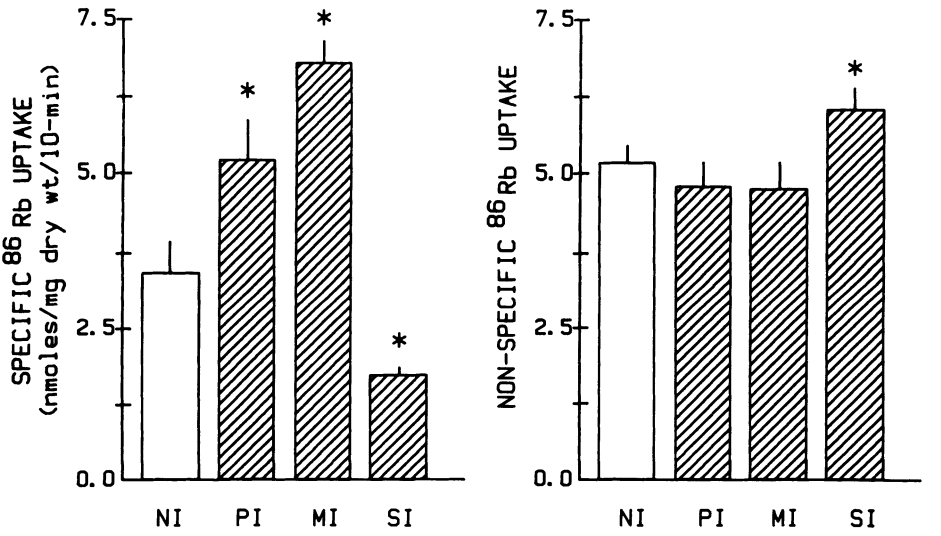

Fig. 6 Effects of temporary myocardial ischemia on ^{86}Rb uptake by canine ventricular slices. After a 90-min left anterior descending coronary occlusion and 2 hr of reperfusion in open-chested anesthetized dogs, ventricular slices obtained from the non-ischemic (NI), peri-ischemic (PI), moderately ischemic (MI) and severely ischemic (SI) regions were pre-incubated for 5 min at 0-2°C in a K-free and Ca-free Krebs-Henseleit solution for sodium loading. Various regions of ischemic injury were identified from the epicardial electrocardiographic techniques (ST-segment elevation and Q-waves development during coronary occlusion and reperfusion, respectively). Left panel depicts the changes in specific, ouabain-sensitive ^{86}Rb uptake, while the right panel indicates the changes in non-specific uptake observed in the presence of 0.1 mM ouabain. The values shown are means $\pm$ SEM from 5 different dogs. * $P<0.05$

from normal and various ischemic regions were studied after a 90-min coronary occlusion and 2 hr of reperfusion in dogs. The severity of myocardial ischemic injury was estimated from the development of epicardial ST-segment elevation 15 min after occlusion and the subsequent development of Q-waves after 2 hrs of reperfusion. As shown in Fig. 6, the ouabain-sensitive ^{86}Rb uptake by the severely ischemic or infarcted myocardial slices was significantly decreased (65%), as expected, from that of the non-ischemic control. In contrast, the ouabain-sensitive ^{86}Rb uptake by the peri-ischemic and moderately ischemic ventricular slices showed a significant increase, +43.4 and +99.4%, respectively, as compared to the non-ischemic controls. The ouabain-insensitive ^{86}Rb uptake, which generally represents the non-specific or passive uptake, was not altered in the peri- and moderately ischemic tissues, but was significantly increased (18.5%) in the severely ischemic tissue. This latter finding suggests that an alteration in the passive membrane permeability must have occurred in these severely ischemic or infarcted tissues.

The mechanism of these increases in myocardial sodium pump activity in the peri- and moderately ischemic tissues is not clear. Since these increases were observed in myocardial slices with sodium loading in vitro prior to ^{86}Rb uptake, these results suggest that the myocardial ischemic process might have a direct stimulatory effect on sodium pump activity. It is well known that myocardial ischemia is generally accompanied by an increase in membrane permeability and in intracellular sodium accumulation, thus, it is conceivable that this ischemia-induced alteration of Na/K homeostasis might lead to a compensatory change in myocardial sodium pump activity during coronary reperfusion of the reversibly injured myocardium. These results are consistent with previously reports of an induction of Na,K-ATPase during increased cellular sodium load.

Since the extent of cardiac glycoside binding is closely related to the activity of sodium pump or Na,K-ATPase, it would not be unreasonable to postulate that an increased sodium pump in this border of moderately ischemic myocardial tissues would result in an increase in cardiac glycoside binding. Determination of total tissue uptake of ^{3}H-digoxin after temporary myocardial ischemia, however, consistently demonstrated a marked reduction in cardiac glycoside uptake in these ischemic or infarcted myocardium. As shown in Fig.7, a reciprocal correlation was observed in ^{3}H-digoxin uptake in the crude homogenate with increasing severity of ischemic injury, as estimated from the loss of nitro-blue-tetrazolium (NBT) staining of the myocardium. A 20% and 80%

loss of NBT stain was associated with a 13.3% and 63.5% reduction in ^{3}H-digoxin uptake, respectively. ^{3}H-digoxin uptake in the particulate fraction (100,000xg pellet), which generally represents specific binding and which is associated with the pharmacologic effects of cardiac glycoside, however, was not altered in tissues with a loss of up to 50% of NBT stain. In fact, an 80% loss of NBT stain was associated with only a 33.9% decrease in digoxin uptake. Our results

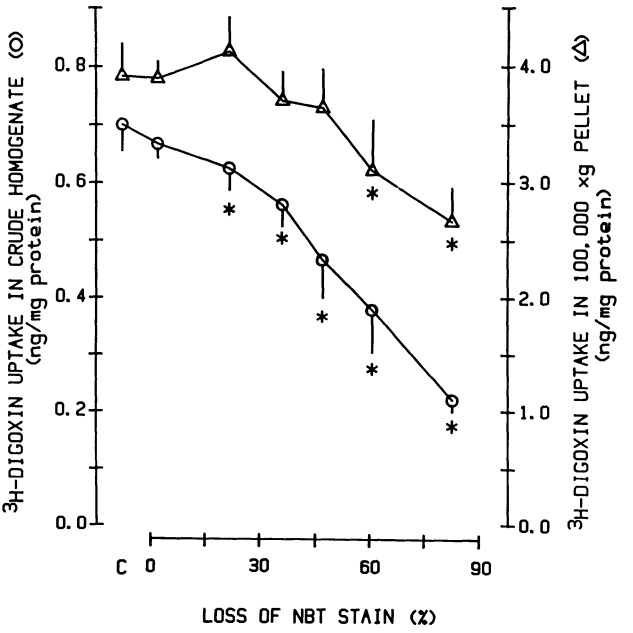

Fig. 7 Effects of temporary myocardial ischemia on regional subcellular distribution of ^{3}H-digoxin. Radiolabeled digoxin (0.03 mg/kg) was administered 15 minutes after complete release of the occluded coronary artery and was allowed to equilibrate for an additional 2 hr prior to the determinations of subcellular distribution of ^{3}H-digoxin. Ventricular tissues (approx. 1-2 gm) from both non-ischemic and various ischemic regions were minced, homogenized and subjected to differential centrifugation as we have previously described (4). The results are expressed as ng ^{3}H-digoxin bound per mg protein. Vertical lines represent the SEM of 12 dogs. * $P<0.05$

clearly demonstrate that measurement of total tissue digoxin uptake does not provide an accurate measure of the effects of acute ischemia on specific digoxin binding. More importantly, the ability of the peri- and moderately ischemic tissues to specifically bind digitalis was not decreased and was in

fact slightly increased after temporary myocardial ischemia. Thus, our results provide a means to explain the enhanced sensitivity of ischemic myocardium to cardiac glycosides. It will be interesting to explore exact mechanisms that regulate, and therapeutic interventions that may prevent, enhanced cardiac glycosides toxicity in ischemia.

SUMMARY

Chronic administration of non-toxic doses of digoxin in dogs did not alter the total myocardial (inhibited plus uninhibited) Na,K-ATPase content or sodium pump activity. These findings are consistent with clinical experience that neither tolerance nor "reverse tolerance" to cardiac glycosides develops during chronic cardiac glycosides treatment. Significant down regulation of myocardial sodium pumping sites, however, was observed in the streptozotocin-induced diabetic animals and was accompanied by a decrease in myocardial contractile function. Sensitivity of these hearts to the positive inotropic effect of cardiac glycosides and their inhibition of myocardial sodium pump, however, were not altered. In contrast, an up regulation of myocardial sodium pump in the ischemically jeopardized tissues, following a temporary coronary artery occlusion, was accompanied by an increase in the in vivo binding of cardiac glycosides. This latter finding could be attributed to the observed increased incidences of cardiac glycosides toxicity in ischemic heart disease. Thus, it appears that modulation of sodium pumping sites in myocardium could and would alter the efficacy of cardiac glycosides.

ACKNOWLEDGEMENTS

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REFERENCES

1. Akera, T. and Brody, T.M. TIPS 6:296-298, 1985
2. Akera, T. and Brody, T.M. Pharmcol. Rev. 29:187-220, 1978.
3. Ku, D.D. and Sellers, B.M. J. Pharmacol. Exp. Ther. 222:395-400, 1982.
4. Ku, D.D. Europ. J. Pharmacol. 88:329-338, 1983.
5. Ku, D.D. and Lucchesi, B.R. Europ. J. Pharmacol. 57:135-147, 1979.
6. Lee, S.-W., Schwartz, A., Adams, R.J., Yamori, Y., Whitmer, K., Lane, L.K. and Wallick, E.T. Hypertension 5:682-688, 1983.
7. Curfman, G.D., Crowley, T.J. and Smith, T.W. Circ. 53:II-112, 1976.
8. Ku, D.D. Life Sciences 30:277-284, 1982.

9. Davidson, C. and Gibson, D. Br. Heart J. 35:970-976, 1973.
10. Ford, A.R., Aronson, J.K., Grahame-Smith, D.G. and Carver, J.G. Br. J. Clin. Pharmacol. 8:135-142, 1979.
11. Vogel, R. Frischknecht, J. and Steele, P. Am. J. Cardiol. 40:171-176, 1977.
12. Hood, W.B. Jr., McCarthy, B. and Lown, B. Circ. Res. 21:191-199, 1967.
13. Morrison, J. and Killip, T. Clin. Res. 19:353, 1971.
14. Ku, D.D., Akera, T., Brody, T.M. and Weaver, L.C. N.-S. Arch. Pharmacol. 301:39-47, 1977.
15. Nechay, B.R., Jackson, R.E., Ziegler, M.G., Neldon, S.L. and Thompson, J.D. Circ. Res. 49:655-660, 1981.
16. Bluschke, V., Bonn, R. and Greeff, K. Europ. J. Pharmacol. 37:189-191, 1976.
17. Bonn R. and Greeff, K. Arch. Int. Pharmacodyn. 233:53-64, 1978.
18. Whittaker, J. and Swaminathan, R. IRCS Med. Sci. 11:846, 1983.
19. Aiton, J.F., Lamb, J.F. and Ogden, P. Br. J. Pharmacol. 73:333-340, 1981.
20. Werdan, K., Reithmann, C. and Erdmann, E. Klin. Wochenschr. 63:1253-1264, 1985.
21. Erlij, D. and Grinstein, S. J. Physiol. (Lond.) 259:13-31, 1976.
22. Clausen, T. and Hansen, O. J. Physiol. (Lond.) 270:415-430, 1977.
23. Hougen, T.J., Hopkins, B.E. and Smith, T.W. Am. J. Physiol. 234:C59-c63, 1978.
24. Fein, F.S., Kornstein, L.B., Strobeck, J.E., Capasso, J.M. and Sonnenblick, E.H. Circ. Res. 47:922-933, 1980.
25. Ingebretsen, C.G., Moreau, P., Hawell-Johnson, C. and Ingebretsen, W.R. Jr. Am. J. Physiol. 239:H614-H620, 1980.
26. Ku, D.D. and Meezan, E. J. Pharmacol. Exp. Ther. 229:664-670, 1984.
27. Ku, D.D., Sellers, B.M. and Meezan, E. Endocrinology (August), 1986.
28. Beller, G.A., Conroy, J. and Smith, T.W. J. Clin. Invest. 57:341-350, 1976.
29. Willerson, J.T., Scales, F., Mukhertee, A., Platt, M., Templeton, G.H., Fink, G., Maximulian, S. and Buja, L. Amer. J. Pathol. 87:159-181, 1977.
30. Kohama, A., Boyn, W.A., Ballinger, C.M. and Veda, I. J. Surg. Res. 11:297-302, 1971.

24

SENSITIVITY OF THE HYPERTROPHIED HEART TO OUABAIN.
AN in vivo AND in vitro STUDY.

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INTRODUCTION

Mechanical cardiac overload is associated with the development of several adaptational factors, both in the myocardium itself and in the vessels. Two fundamental processes adapt the heart to a chronic enhancement of work : (a) hypertrophy due to a multiplication of sarcomeres, and (b) an increased efficiency of contraction of each sarcomere. Calcium movements and shortening velocity are slower, and oxygen is more efficiently used. This represents a better adaptation to an increased pre- or after load.

There is no longer any doubt that chronic overload, at least in rats, induces a myosin heavy chain isoenzyme redistribution which favors the low ATPase forms, and can explain the decreased activity reported by several authors (for a review see ref 1). This mechanical modification was adaptational, since heat production was depressed and therefore energy metabolism improved.

The predominance of the isomyosin during overloading in rats corresponded to the reappearance of the fetal type isoenzyme.

At the sarcolemma level, electrophysiological and pharmacological alterations have been reported. The action potential is lengthened and the amplitude of the slow inward current is diminished (for a review, see 2). Note that the lengthened duration of the action potential was

also observed in newborn rat hearts. This reveals a remarkable feature, the resurgence of neonatal properties in hypertrophied rat hearts.

The concentration of the low-affinity muscarinic receptors (3), both the affinity and the density of β-adrenoreceptors (4, 5) and the inotropic effect of catecholamine (6, 7) were depressed in hypertrophied hearts.

An important enzyme in the sarcolemma is the Na^+, K^+-ATPase, generally accepted as the primary target of the cardiac glycosides. (for review, see ref 8). Although cardiac glycosides are the oldest drugs used to treat chronic failure, very few papers have so far dealt with the sensitivity of hypertrophied hearts to these drugs. Clinical studies (reviewed in 9), have shown that the drug was inotropic in both normal and failing hearts. Practically all the informations available on this subject come from Newman and coworkers (6, 7) who showed a normal inotropic response to ouabain in failing canine heart, in contrast to the depressed responsiveness to isoproterenol. Regarding the sensitivities of the cardiac Na^+,K^+-ATPase to inhibition by ouabain, they were in the same range of concentrations than the pharmacologically active doses in various species (8, 10) including rat (11, 14).

The question arises as to whether modifications of the responsiveness to ouabain of hypertrophied rat hearts may be related to alterations of Na^+, K^+-ATPase properties. From <u>in vitro</u> studies of the Na^+, K^+-ATPase activities in hypertrophied cardiac muscle preparations, highly conflicting results have been reported. Enzymic activity was found to be either increased, normal, or decreased. This would be due to technical problems, these

preparations having very low Na^+, K^+-ATPase activities.

The purpose of the present article is to study both in vivo the inotropic effect of ouabain on hypertrophied isolated rat heart and in vitro its inhibitory effect on a highly active Na^+, K^+-ATPase and its specific binding on receptors.

MATERIALS AND METHODS

Aortic stenosis.

Male Wistar rats with body weights ranging between 180 and 220 g were operated and paired to sham-operated rats of the same weight. Abdominal aortic stenosis was induced by Weck hemoclips placed above the renal artery near the diaphragm column using Weck forceps modified according to Cutilletta (15). The diameter of the clips was calibrated so as to reproduce cardiac hypertrophy with a reasonnably low mortality (around 20 %). A moderate hypertrophy (+ 40 to + 60 %) was obtained after two weeks in most of the animals and much more pronounced hypertrophy, (around 100 %), in one or two out of every ten. For experimental purpose, these animals with a rather high degree of hypertrophy have been selected on the basis of a ventricular weight/body weight (mg/g) above 3. For the sham-operated animals, the procedure consisted of dissection around abdominal aorta without banding.

Physiological study.

An isolated rat heart preparation perfused at a constant coronary pressure was used. In this preparation, a small cannulated fluid-filled balloon was placed in the left ventricle of the isolated heart and attached to a pressure transducer to monitor ventricular pressure. Since the balloon was non compressible, contraction was

isovolumic.

Perfusion technique. Retrograde coronary perfusion at 37°C was quickly started from a reservoir at a level above the heart equivalent to a pressure of 90 mm Hg. The perfusate consisted of modified Krebs-Henseleit buffer containing 118 mM NaCl, 4.69 mM KCl, 25 mM CO_3H Na, 1.2 mM Mg SO_4, 1.17 mM KH_2 PO_4, 11 mM glucose and 0.25 mM $CaCl_2$. The total K^+ content was 5.86 mM. After prolonged oxygenation in 5 % CO_2, 95 % O_2, the pH was 7.4 and the pO_2 around 600 mm Hg. Hearts were paced at 360 beats per min (6 Hz) using two atrial electrodes attached to a PHILIPS TP 300 stimulator adjusted to 4 mA. A one-msec rectangular unipolar impulse was used.

The measurement of mechanical function was performed as previously described (17). Measurements were made every 5 min. The collapsed balloon was slowly filled with water in order to obtain a diastolic pressure of 10 to 15 mm Hg which usually corresponds to 110 to 120 µl of water. In this model, records were made in isovolumetry with balloon volumes and diastolic pressures similar from heart to heart. Under these conditions (+dP/dt) max and +dP/dt max/Psys represent very satisfactory index of contractility.

Inotropic effects. Protocol 1. 9 sham-operated rats and 8 with aortic stenosis were compared. After 20 minutes of equilibration, ouabain (from 10^{-9} to 10^{-4} M) was continuously infused for sequential 5 min periods. This protocol was chosen to rapidly get a dose-response curve using a rather rare material. Slight elevation of the contractile performances was observed, indicating progressive accumulation of the drug. In sham-operated animals inotropy was achieved in 5 minutes.

Protocol 2. 8 sham-operated rats, and 8 rats with aortic stenosis were compared. After a stabilization period of 20 min, 10^{-5} M ouabain was continuously infused for 20 min.

Recovery. The recovery was studied for 30 minutes. It was expressed in % below the last measurement made in the presence of either 10^{-4} M or 10^{-5} M ouabain.

Biochemical study.

Sarcolemma preparations. In order to isolate the sarcolemma vesicles from hearts maintained under the experimental conditions used to measure inotropism (see above), both normal and hypertrophied hearts were submitted to a coronary perfusion with the Krebs-Henseleit solution containing 0.25 mM Ca. The left ventricles were used to prepare microsomes highly enriched in sarcolemma vesicles. The same isolation procedure was followed for hypertrophied and normal hearts (14, 16). On newborn (6 hr after birth) rat hearts, this isolation procedure was used again except that the 18-min hypotonic lysis was reduced to 2 min in order to maintain enzyme activity. The microsomal fraction was then subjected to repeated freezings and thawings (16) or to SDS treatment (0.2 mg/mg of protein, 30 min at 20°C) (16) to make the vesicles permeable to substrates and ligands before (^{3}H)ouabain binding studies and Na^{+}, K^{+}-ATPase assays.

Na^{+}, K^{+}-ATPase inhibition. The enzymatic activity in the absence or presence of various concentrations of ouabain was determined using the coupled assay method previously described (17). The final concentrations of ouabain in the assay medium variated from 10^{-10} M to 10^{-4} M. Inhibition percentage was calculated by comparing the activity in the presence of ouabain with that in the control after correcting for the ouabain-insensitive ATPase activity

measured in the presence of 2 x 10^{-3} M ouabain.

The Na^+, K^+-ATPase activities accounted for about 70 % of the total ATPase activities of the preparations from either normal or hypertrophied hearts. There was no significant difference in either the yield of sarcolemmal proteins (0.3 mg/g of heart) or in the average specific activity of Na^+, K^+-ATPase (105 $\pm$ 16 µmol. of phosphate liberated per mg of protein per hour) for sarcolemma preparations from normal and hypertrophied hearts. The activities is lower in neonatal cardiac preparations (48 $\pm$ 7 µmol.Pi hr^{-1} x mg^{-1}).

(^{3}H) ouabain binding assays (18). Equilibrium binding of (^{3}H) ouabain was measured after 60 min at 37°C in 1 ml of incubation medium containing 0.04 mg of protein, 4 mM $MgCl_2$, 4 mM ATP, 100 mM NaCl, and 40 mM imidazol-HCL, pH 7.4 (buffer I), with increasing concentrations of ouabain (6 x 10^{-9} M up to 3 x 10^{-6} M and specific radioactivity varying from 19 to 0.6 Ci/mmol). (For details, see ref 18).

Kinetics of (^{3}H) ouabain dissociation. Cardiac cell membrane preparations (0.04 mg of protein/ml) were incubated in the presence of 10^{-8} M (^{3}H) ouabain for 60 min at 37°C as for (^{3}H) ouabain binding assays. Dissociation of (^{3}H) ouabain was started by a 10-fold dilution of the incubation medium with buffer I containing unlabeled ouabain (10^{-3} M final). At the appropriate points of time, aliquots of 4.5 ml were filtered and the radioactivity that remained bound to the filters after washes was measured under the same conditions as above (18). Unspecific binding amounted to less than 10 % of total radioactivity bound to the membranes.

RESULTS

Animals have been selected on the basis of a ventricular/body weight (mg/g) ratio above 3, as a criterion of an indisputable chronic overload.

Physiological study

At 0.25 mM external Ca, ouabain concentrations as low as 10^{-6} M exerted significant inotropic effects.

In both hypertrophied and normal hearts, the responses to ouabain (in % above control values) did not significantly differ whatever the criteria, developed pressure, (+dP/dt max) or (+dP/dt max)/Psys used to evaluate inotropy.

At the end of the last sequential drug infusion, i.e. after 5 min perfusion with 10^{-4} M ouabain, hearts were washed with the Krebs-Henseleit buffer. The recovery of a normal developed pressure was significantly slower in hypertrophied than in normal hearts (Fig.1). This observation is also valid according to the (+dP/dt max)/Psys index expressed in % below either the control or the last measurement made in the presence of ouabain.

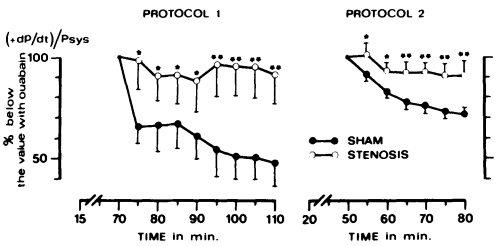

Fig. 1. Recovery periods studies according to protocol 1 (10^{-4} M) or 2 (10^{-5} M ouabain). (Reproduced from Am. J. Physiol. (17) with the permission of the Publishers).

Like the protocol 1, protocol 2 evidenced the inotropic effect of 10^{-5} M ouabain in low $(Ca)_o$. After the single 30 minute perfusion with 10^{-5} M ouabain, the return to the original values of the systolic, diastolic and developed pressures was slowered in the hypertrophied heart group when the recoveries were expressed as % below the last values measured with ouabain, the slowing down became statistically significant for the group with hypertrophy according to the (+dP/dt max/Psys index (Fig 1).

Biochemical study.

Inhibition of Na^+, K^+-ATPase by ouabain. The steady state levels of enzyme inhibition were achieved in 3-5 min and 5-15 min in normal or hypertrophied cardiac preparations, respectively. In both preparations, this inhibition remained stable over a 60 min incubation period. As depicted in Fig. 2, the dose-response curves were similar for both types of rat cardiac sarcolemma vesicles. These curves exhibited a complex pattern. This pattern has been analyzed assuming the existence of i) high-sensitivity enzyme forms associated with a 66

+ 9 % inhibition of activity and ii) low-sensitivity forms with an IC_{50} value equal to 1 x 10^{-6} M and associated with a 33 + 9 % inhibition. The dissociation processes could be accurately determined for both types of sites in each type of cardiac preparations. Indeed, the time course of relief from inhibition parallels the release of ouabain from the enzyme. As the dissociation is a first-order reaction, the relief from inhibition may be evaluated by the t 1/2 value which represents the time required to recover 50 % of the Na^+, K^+-ATPase activity (Table 1).

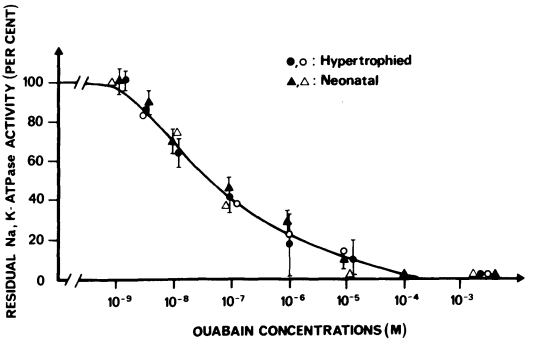

Fig. 2. Dose response curve of Na^+, K^+-ATPase activity versus ouabain concentrations in sarcolemmal vesicles isolated from hypertrophied and neonatal rat hearts. (Reproduced from J. Biol. Chem. (18) with the permission of the Publishers).

	Rat Hearts		
Receptor type	Normal	Hypertrophied	Neonatal
High-affinity	12.8*/11.66°	96.2*/41.2°	NT/52.5°
Low-affinity	1.05*/0.36°	3.2*/3.2°	NT/2.96°

Table 1. Half-times (in minutes) for ouabain-dissociation according to Na^+,K^+-ATPase assays (*) and ^{3}H-ouabain binding measurements (°).
NT : not tested.

The Na^+, K^+-ATPase forms present in hypertrophied cardiac preparation differ from those in normal heart by slower rates of ouabain release from their respective high- (7 fold) and low-sensitivity forms (3-fold). In spite of these different dissociation rate constants, the apparent affinities (10^{-8} and 10^{-6} M) were similar in both preparations. This would suppose the rates of ouabain binding to be slowered by similar factors in hypertrophied hearts. This assumption is confirmed by the longer period of time required to reach equilibrium in hypertrophied than in normal cardiac preparations (5-15 min instead of 3-5 min).

We examined (^3H) ouabain binding to sarcolemma vesicles from hypertrophied and newborn hearts with parallel analysis of cardiac preparations from normal adult rats. In all cases, the receptors for digitalis were heterogeneous with high and low affinity sites (Table 1). Previous communications have also described such a heterogeneity in normal rat ventricles (12, 13). In all three types of cardiac sarcolemmal preparations, the high affinity binding sites were indistinguishable in their respective affinities for ouabain.

The rates of dissociation were measured after equilibrium had been reached in the presence of 10^{-8} M (^3H) ouabain. Time course of (^3H) ouabain dissociation from Na^+, K^+-ATPase was not a simple exponential process (Fig. 3) and analysis of the curves according to Choi and Akera (19) gave two exponentials : two half times for dissociation could be calculated (Table 1).

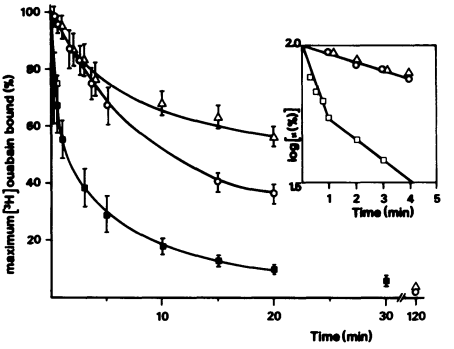

Fig. 3. Kinetics of (^{3}H)ouabain dissociation from normal (■), hypertrophied (●) and new born (▲) rat cardiac sarcolemma preparations. Inset : Details of the first 6 minutes. (Reproduced from J. Biol. Chem. (18) with the permission of the Publishers).

The same experiments carried out with hypertrophied and neonatal cardiac sarcolemma preparations clearly showed 4-5 fold slower rates of dissociation from high and low affinity sites. Hypertrophied and neonatal cardiac preparations had similar dissociation rate constants.

Because we found no significant difference between the experimental values of the equilibrium dissociation constants (K_d) for the three sarcolemma preparations, it appears that their association rate constants should differ by a similar factor. Indeed, at 10^{-7} M ouabain, equilibrium binding was reached after 15 min for both hypertrophied and neonatal cardiac preparations instead of 2-5 min for normal heart preparation.

DISCUSSION

The results presented here clearly show that the prolonged inotropic effect of ouabain on hypertrophied rat heart is associated with parallel slowered releases of ouabain from its specific receptor

sites. The physiological adaptation of the cardiac muscle to pressure overload is associated, in rat, with a change in the digitalis receptors which represents the expression of the neonatal forms. These new forms of ouabain receptors differed from those of normal rat heart in that time to reach steady state binding of ouabain was increased and release of ouabain occurred by 3 to 7-fold slower processes.

This receptor switch secondary to a myocardial adaptation could result from the expression of isoforms of the digitalis receptor as detected in rat brain (20) and dog heart (21). Although kinetic properties of the hypertrophied hearts were clearly different from those of the normal adult heart, these receptors could not be physically discriminated (18). An alternative explanation could reside in modifications of the membrane environment in relationship with Ca^{2+} (14, 22, 23).

We cannot yet explain the presence of neonatal forms of digitalis receptors in hypertrophied hearts. Nevertheless, it is worthy to note that neonatal and hypertrophied hearts have slowered movements of intracellular Ca^{2+} (24) associated with the presence of Na^{+}, K^{+}-ATPase forms insensitive to a Ca^{2+}-free perfusion (17, 18). In contrast, in normal hearts with a functional sarcoplasmic reticulum, the Na^{+}, K^{+}-ATPase sensitivity to ouabain was a function of the Ca^{2+}-free perfusion (14). We suggest that, in the adaptational process of the hypertrophied rat hearts, there might be a relationship between intracellular concentrations of Ca^{2+}, the presence of a functional sarcoplasmic reticulum, and the expression of the neonatal forms of the Na^{+}, K^{+}-ATPase.

SUMMARY

The inotropic effect of ouabain was evaluated on an isolated rat heart preparation at 0.25 mM $(Ca^{2+})_o$. The recovery of a normal contractile function after the inotropic response was significantly slowered in hypertrophied hearts obtained one month after aortic stenosis. A highly purified sarcolemma preparation (Na^+,K^+-ATPase = 105 $\pm$ 16 umol.h.mg^{-1}) was used to study both the Na^+, K^+-ATPase and the ^3H ouabain binding. The release of ouabain from both the high and low affinity sites, whether it is estimated in washout experiments by the rates of enzyme relief from inhibition or by ^3H ouabain release from membrane, was significantly slowered in hypertrophied heart as for newborn rat heart preparations.

ACKNOWLEDGEMENTS

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REFERENCES

1. Swynghedauw, B. and Delcayre, C. In : Pathobiol. Ann. (Ed. H.L. Ioachim), Raven Press, New York, 1982, pp. 137-183.
2. Ten Eick, R.E. and Bassett, A.L. In : Function of the Heart in Normal and Pathological States. (Ed. N. Sperelakis). Martinus Nijhoff Pub., Boston, 1983, pp. 245-267.
3. Robberecht, P., Walbroeck, M., Clacys, M., Nguyen Huu, A., Chatelain, P. and Christophe, J. Mol. Pharmacol. 21 : 589-593, 1982.
4. Bristow, M.R., Ginsburg, R., Minobe, W., Cubicciotti, R.S., Sageman, W.S., Lurie, K., Billingham, M.E. and Stinson, E.B. N. Engl. J. Med. 307 : 205-211, 1982.
5. Cervoni, P., Herzlinger, H., Lai, F.M. and Tanikella, T. Brit. J. Pharmacol. 74 : 517-523, 1981.
6. Newman, W.H. and Webb, J.G. Cardiovasc. Res. 14 : 530-536, 1980.
7. Newman, W.H., Frankis, M.B. and Webb, J.G. In : Functional Aspects of the Normal, Hypertrophied and Failing Hearts. (Eds. F.Abel and W.H. Newman). Martinus Nijhoff Pub., Boston, pp.189-209, 1984.
8. Akera, T. and Brody, T.M. Ann. Rev. Physiol. 44 : 375-388, 1982.
9. Mason, D.T. In : Cardiac Glycosides.I. Experimental Pharmacology. (Ed. K.Greeff) Springer-Verlag-Berlin, pp.497-515, 1981.
10. Schwartz, A., Whitmer, K., Grupp, G., Grupp, I., Adams, R.J. and Lee, S.W. N.Y.Acad. Sci. 402 : 253-261, 1982.
11. Adams, R.J., Schwartz, A., Grupp, G., Grupp, I., Lee, S.W., Wallick, E.T., Powell, T., Twist, V.W. and Gathiram, P. Nature 296 , 167-169, 1982.
12. Erdmann, E., Philipp, G. and Scholz, H. Biochem. Pharmacol. 29 : 3219-3229, 1980.
13. Finet, M., Godfraind, T. and Noel, F. Brit. J. Pharmacol. 80 : 751-759, 1983.
14. Mansier, P. and Lelièvre, L.G. Nature 300 : 535-537, 1982.
15. Cutiletta, A.F., Dowell, R.T., Rudnick, M., Arcilla, R.A. and Zak, R. J. Mol. Cell. Cardiol. 7 : 767-781, 1975.
16. Mansier, P., Charlemagne, D., Rossi, B., Preteseille, M., Swynghedauw, B. and Lelièvre, L. J. Biol. Chem. 258 : 6628-6635, 1983.
17. Lelièvre, L.G., Maixent, J.M., Lorente, P., Mouas, C., Charlemagne, D. and Swynghedauw, B. Am. J. Physiol. in press, 1986.
18. Charlemagne, D., Maixent, J.M., Preteseille, M. and Lelièvre, L.G. J. Biol. Chem. 261 : 185-188, 1986.
19. Choi, Y.R. and Akera, T. Biochim. Biophys. Acta 481 : 648-653, 1977.
20. Sweadner, K. J. Biol. chem. 254 : 6060-6067, 1979.
21. Matsuda, T., Iwata, H. and Cooper, J.R. J. Biol. Chem. 259 : 3858-3863, 1984.
22. Geny, B., Paraf, A., Fedon, Y. and Charlemagne, D. Biochim. Biophys. Acta, 692 : 345-354, 1982.
23. Lelièvre, L.G., Potter, J.D., Piascik, M.T., Wallick, E.T., Schwartz, A., Charlemagne D. and Geny, B. Eur. J. Biochem. 148 : 13-19, 1985.
24. Alpert, N.R. and Mulieri, L.A. Circ. Res. 50 : 491-500, 1982.

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MITOCHONDRIAL CALCIUM OVERLOAD IN DIGITALIS-INDUCED MECHANICAL TOXICITY

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INTRODUCTION

An existence of a correlation between inhibition of Na^+, K^+-ATPase and the positive inotropic response of cardiac glycosides has been documented by several investigators (1-3). This concept has been further examined and recently reviewed (4-6). In toxic concentrations, cardiac glycosides can provoke oscillatory potentials that are coupled to and triggered by preceding action potentials (7). The oscillatory potentials are increased in magnitude by an increase in extracellular Ca^{2+} (8) and can be abolished altogether by verapamil, tetrodotoxin (9) and Mn^{2+} (10). Tsien et al (11) have, however, suggested that a rise in intracellular Na^+ due to an inhibition of Na^+, K^+-ATPase, may cause an increase in intracellular Ca^{2+} through Na^+-Ca^{2+} exchange and consequently increase in inward oscillatory current. Oscillatory potentials, in presence of toxic concentrations of cardiac glycosides, are infact considered as an expression of cellular Ca^{2+} overload (12,13).

In vitro experiments indicate that the toxic effects of cardiac glycosides on Purkinje fibers include both electrical and mechanical changes. For the mechanical changes, the cardiac glycosides first increase (positive inotropic effect) and then decrease the contractile force (mechanical toxicity) until Purkinje fiber become inexcitable (14). Knowledge about the mechanism(s) which underlie the process(es) by which cardiac glycosides cause mechanical toxicity is still not well understood. It has been postulated that the deterioration in the contractile force (mechanical toxicity) is due to an excessive uptake of Ca^{2+} by the cardiac cell (15,16). Recently, a 1.24 uM dose of ouabain which causes mechanical toxicity in rabbit heart, has been shown to cause significant elevation of myocardial calcium concentration (17). This increase in the myocardial

calcium concentration was found to be associated with ultrastructural changes such as swollen mitochondria, hypercontracted sarcomeres and autolysis. Similar mitochondrial damages have been shown to occur in an isolated guinea pig heart perfused with toxic doses of cardiac glycosides (18). Calcium overload is also known to occur under other conditions such as hypoxia, ischemia reperfusion and catecholamine administration. Concomitant decline in contractile force, under these conditions, was demonstrated to be due to an uptake of calcium into the mitochondria at the expense of oxidative phosphorylation (19,20).

It is evident from these reports that cardiac dysfunction, due to toxic dose of cardiac glycosides, may be related to a subcellular Ca^{2+} overload and further suggests that mitochondria may occupy a pivotal role in the mechanical toxicity induced by cardiac glycosides. If such is the case, then the use of agents which reduce myocardial calcium influx should protect the heart from mechanical toxicity induced by cardiac glycosides.

MATERIAL AND METHODS

Chemicals. Pyruvic acid, maleic acid, mannitol, sucrose, EDTA, Tris, were obtained from Fisher Chemical Company. Bovine serum albumin, nagarse, B-NADP, ADP, ouabain, hexokinase (ATP: D-hexose-6-phosphotransferase; EC 2.7.1.1; type C-130), Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1 49, Type VII) and ouabain were purchase from Sigma Chemicals Company, St. Louis, Mo. $^{45}CaCl_2$ specific activity of 1mCi/g, and aquasol were purchased from New England Nuclear Corporation, Lachine, Quebec, Canada. Nifedipine capsules (10 mg) and verapamil ampoules 2.5 mg/ml were obtained from the Pharmacy Department of the Health Sciences Centre, Winnipeg, Manitoba, Canada.

Perfusion technique. Guinea pigs of either sex weighing between 250-300 g were used. Hearts were quickly isolated after decapitation, atria removed, paced at 180 beats/min (SRI stimulator model 50 4969), perfused through the aorta as previously described (21). The hearts were perfused with Krebs-Hensleit buffer of the following composition in (mM): 120 NaCl; 4.7 KCl; 1.5 glucose; 1.2 $MgSO_4$; 25 $NaHCO_3$; 1.2 NaH_2PO_4; 1.25 $CaCl_2$; 95% O_2 + 5% CO_2; pH 7.4. The rate of perfusion was maintained between 6-8 mL $minute^{-1}$ using a four channel roller pump (Gilson minipuls 2). The heart was mounted under 2 g tension (Grass instruments) and left ventricular force of

contraction was measured using Beckman four channel physiograph (model R511A).

All hearts were equilibrated for 20 minutes with non-drug containing normal perfusion solution followed by a perfusion medium containing 1 uM ouabain administered through a two-way stop-cock (baker's double coil condenser) until the contracture developed 35-40 min. The same procedure was followed when hearts were perfused simultaneously with either 1 uM ouabain plus 50 nM nifedipine or 1 uM ouabain plus 500 nM verapamil. Control hearts were perfused with "normal" perfusate for the same total length of time as drug perfused hearts. In some experiments, isolated non-perfused hearts were used to be sure that the perfusion did not alter the mitochondrial functions under examination. At the end of the perfusion, hearts were quickly chilled on ice and only left ventricles were used for all the experiments.

Calcium content. Approximately 500 mg of left ventricle was dried to constant weight at 100°C after which time Ca^{2+} was extracted for 72 hours using 10 ml solution of 0.1% $LaCl_3$, 30 mM TCA and 100 mM NaCl at room temperature (22).

Preparation of mitochondria. Mitochondria was prepared by the standard method (23), with all solutions being prepared in 20 mM Tris-HCl pH 7.4. Mitochondrial protein was determined as described earlier (24).

Mitochondrial $^{45}Ca^{2+}$ uptake. Energy dependent $^{45}Ca^{2+}$ uptake was studied by incubating 2 mg of mitochondria (which were given final two washes in EDTA-free medium) with 10 mM Tris-pyruvate and 10 mM Tris-maleate, 5 mM KH_2PO_4 and 20 mM Tris HCl pH 7.4 for 5 minutes at 37°C. At the end of the incubation period, 1 umol of $^{45}CaCl_2$ containing $50x10^3$ cpm was added to a final volume of 2.5 mL. After one minute the reaction was stopped by the addition of 1 mL 280 mM sucrose solution containing 1 mM $LaCl_3$, quickly filtered and then given a final 2xl mL wash with the "stopping" solution. The filter papers were air dried then counted for radioactivity.

Polarographic studies. This was carried out at 25°C by incubating 2 mg mitochondrial protein with 225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 5 mM KH_2PO_4. 10 mM Tris-pyruvate and 10 mM Tris-maleate, 20 mM Tris-HCl, pH 7.4. Three minutes after state 4 respiration has started 0.3 mM ADP was added to a final volume of 2.5 ml to induce state 3 respiration. QO_2 (n atom oxygen consumed min^{-1} mg^{-1} mitochondrial protein), ADP/O ratio and RCI (respiratory control index) were calculated from the tracings as

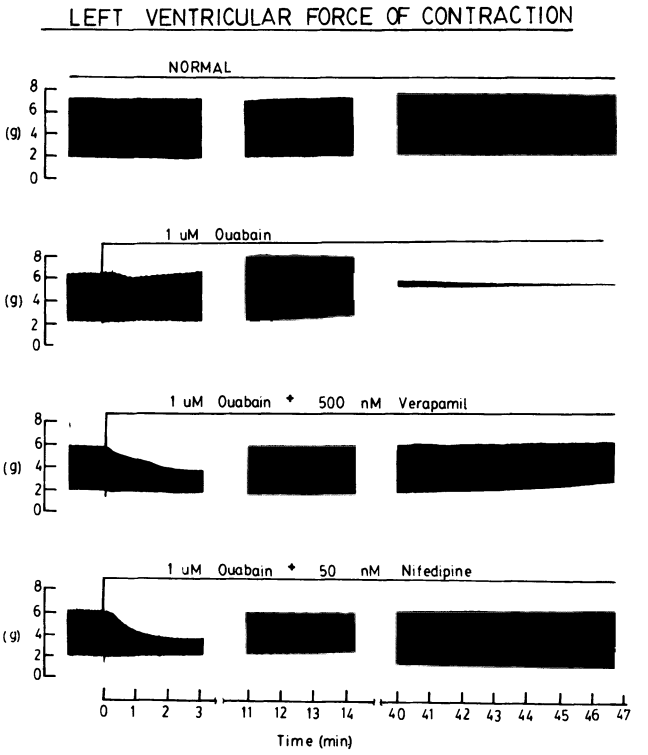

Fig 1. Typical recordings from 12 isolated paced guinea pig hearts. The tracings on the left show tension development in normal and the drug perfused hearts during the first 3 min of perfusion. Middle tracings represent the inotropic state during 11 to 14 min and the tracings on the right during 40 to 47 min of continuous perfusion.

described earlier (25).

Rate of mitochondrial ATP generation. Two mg of mitochondrial protein was incubated at 25°C with 225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 5 mM

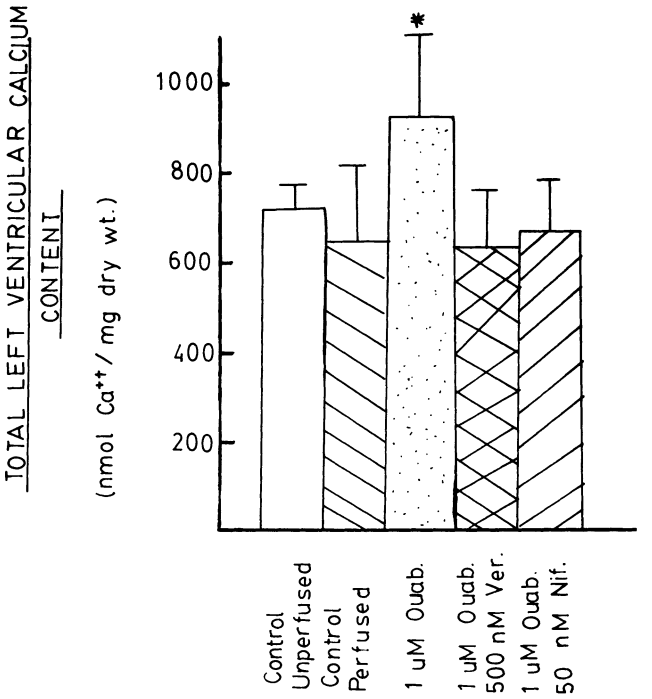

Fig 2. Left ventricular tissue Ca^{2+} in the isolated guinea pig hearts perfused with ouabain alone and combined with verapamil or nifedipine. The data represents mean $\pm$ S.E. from 6-8 experiments. * $P < 0.05$.

KH_2PO_4, 10 mM Tris-pyruvate and 10 mM tris-maleate, 20 mM Tris-HCl, pH 7.4. After 5 min of incubation, 0.5 mM ADP was added to a final volume of 3.0 mL. At 0.25, 0.5, 0.75, 1 and 4 min, 0.5 mL aliquot was removed, deproteinized by boiling for 5 min, cooled to room temperature and centrifuged at 2,000 g x 5 min. One hundred and fifty uL of the supernatant was then used for ATP analysis using hexokinase-glucose-6-Phosphate dehydrogenase coupled system (26).

RESULTS

Protective effect of nifedipine and verapamil against ouabain toxicity. A representative recording of isolated perfused hearts is shown in Fig 1. The first panel shows a normal perfused heart which had no significant change in contractile force or resting tension throughout the perfusion period. However, when hearts were perfused with 1 uM ouabain (panel 2), a positive inotropic response was observed within the first few minutes, showing a peak contractile force by the tenth minute. A decline in contractile force and an increase in the resting tension started by the thirteenth minute and finally, the development of contracture by 40-45 minutes. However, when hearts were perfused simultaneously with either ouabain and verapamil (panel 3), or ouabain and nifedipine (panel 4), we observed an initial negative inotropic effect which was then followed by a positive inotropic effect of ouabain. The development of a mechanical toxicity of ouabain was either significantly delayed or abolished in the presence of verapamil and nifedipine. These effects of verapamil and nifedipine are dose dependent on these drugs, as demonstrated earlier (16).

Effect of ouabain on left ventricular tissue calcium content. The results presented in Fig 2 indicate that: a) the total left ventricular tissue Ca^{2+} content was increased by 40% in ouabain perfused hearts compared to control unperfused or perfused hearts, b) that the Ca^{2+} content of left ventricular tissue was the same as in normal hearts when these hearts were perfused simultaneously with either ouabain and nifedipine or ouabain and verapamil.

Effect of ouabain on mitochondrial Ca^{2+} uptake. Energy dependent $^{45}Ca^{++}$ uptake by mitochondria, prepared from ouabain prefused hearts, was found to be 60% higher than those from control hearts (Fig 3). This mitochondrial Ca^{2+} uptake was the same as in normal hearts, when mitochondria was prepared from hearts perfused with ouabain in the presence of either nifedipine or verapamil.

Effect of ouabain on mitochondrial QO_2, ADP:O ratios, RCI. With respect to mitochondrial oxygen rates (QO_2), there was a 50% reduction in the oxygen rates in ouabain perfused hearts (Fig 4), compared to control perfused or unperfused hearts. This decrease was not observed when hearts were perfused simultaneously with either ouabain and nifedipine or ouabain and verapamil.

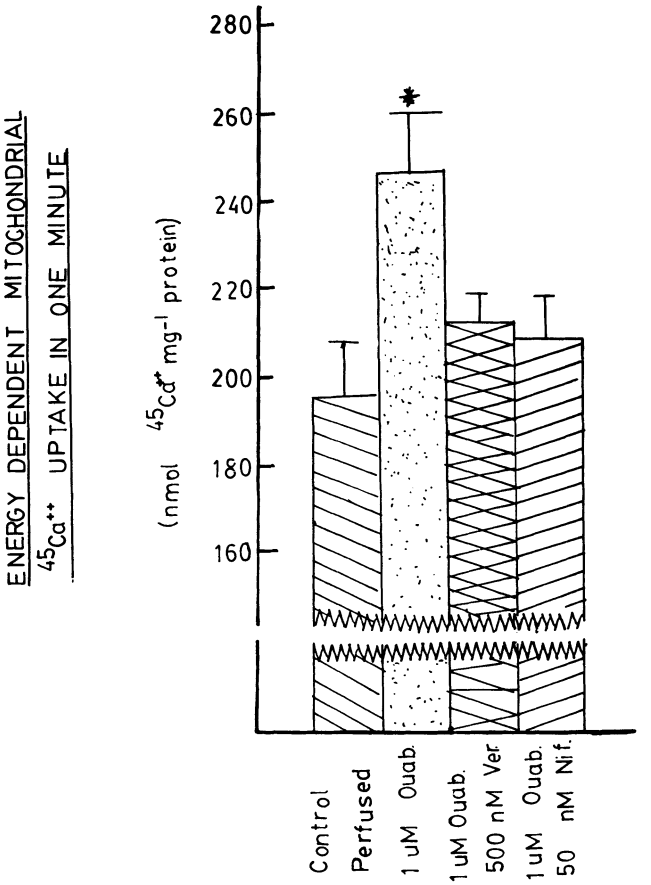

Fig 3. Energy dependent mitochondrial $^{45}Ca^{2+}$ uptake isolated from hearts perfused with ouabain alone and combined with verapamil or nifedipine. The data represents mean $\pm$ S.E. from 6-8 experiments. *P < 0.05.

A similar change was observed for the ADP:O ratios (Fig 5) where there was a 33% decrease in ouabain intoxicated hearts compared to control hearts. Again, with the mitochondria derived from hearts perfused

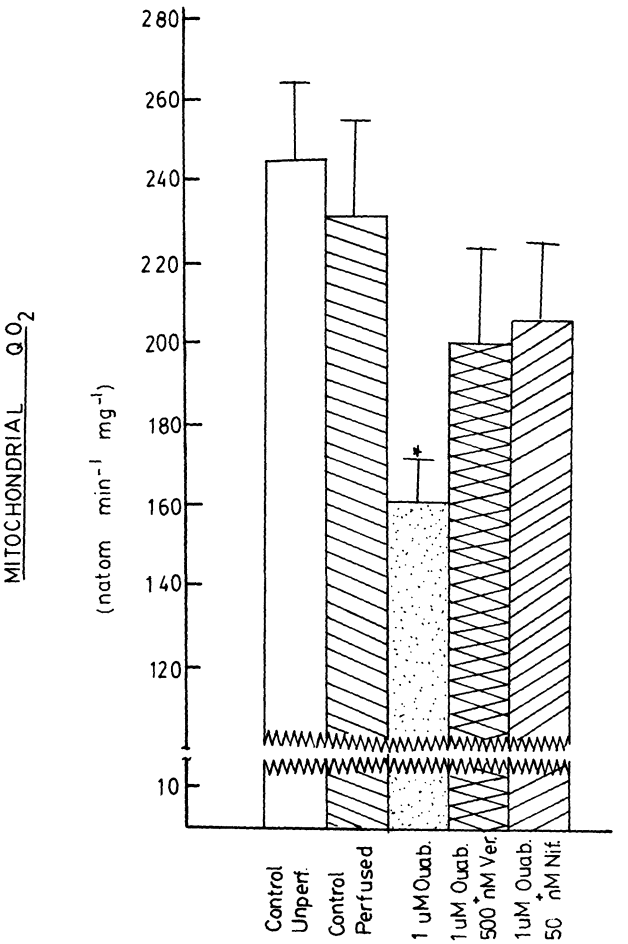

Fig 4. Mitochondrial QO_2 in isolated hearts perfused with ouabain alone and combined with verapamil or nifedipine. The data represents mean ± S.E. from 6-8 experiments. *P < 0.05.

simultaneously with either ouabain and nifedipine or ouabain and verapamil, the ADP:O ratios were the same as with mitochondria from normal perfused hearts. Despite the decrease in the QO_2 and ADP:O ratios, no significant

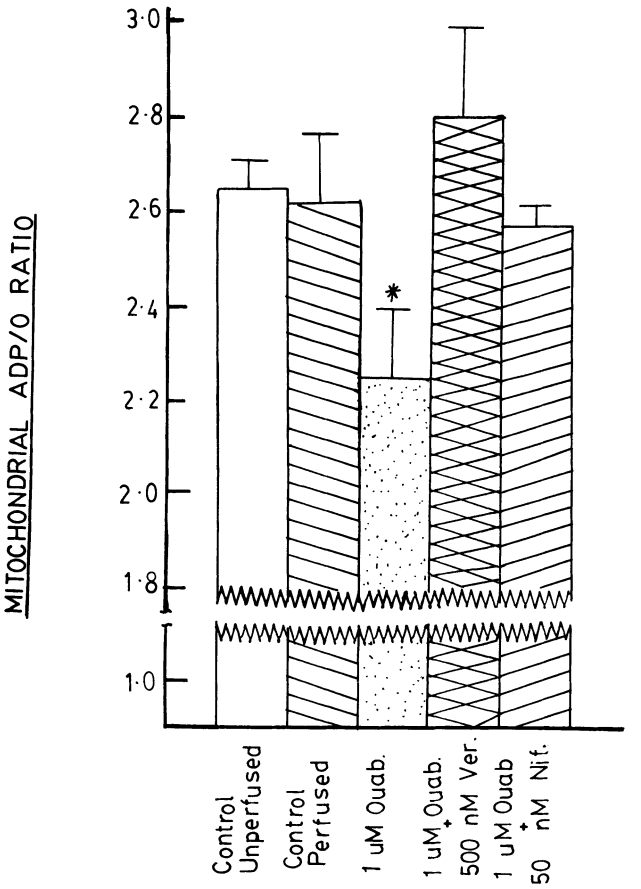

Fig 5. Mitochondrial ADP/O ratios from isolated hearts perfused with ouabain alone and combined with verapamil or nifedipine. The data represents mean ± S.E. from 6-8 experiments. *P < 0.05.

change in RCI (Fig 6) was observed for mitochondria prepared from a) perfused or unperfused controls, b) perfused with ouabain alone, c) perfused with either ouabain and verapamil or ouabain and nifedipine.

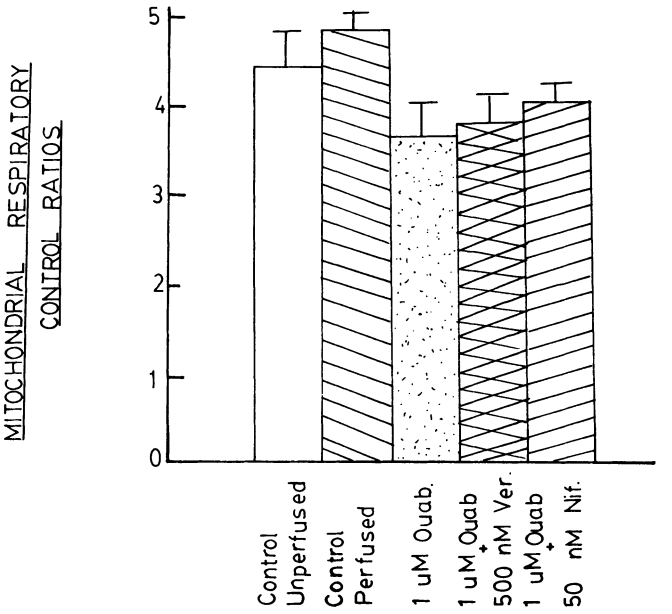

Fig 6. Mitochondrial respiratory control ratios (RCI) from isolated hearts perfused with ouabain alone and combined with either verapamil or nifedipine. The data represent mean ± S.E. from 6-8 experiments. *P < 0.05.

Effects of ouabain on mitochondrial rate of ATP generation. In the ouabain perfused hearts the rate of mitochondrial ATP generation was found to decrease by 50% relative to control hearts (Fig 7). However, when the hearts were perfused simultaneously with either ouabain and verapamil or ouabain and nifedipine, the mitochondrial rate of ATP generation was found to be the same as seen in control perfused hearts (see Fig 7).

DISCUSSION

The results demonstrate that ouabain intoxication in the isolated perfused guinea pig heart causes alterations in mitochondria as manifested

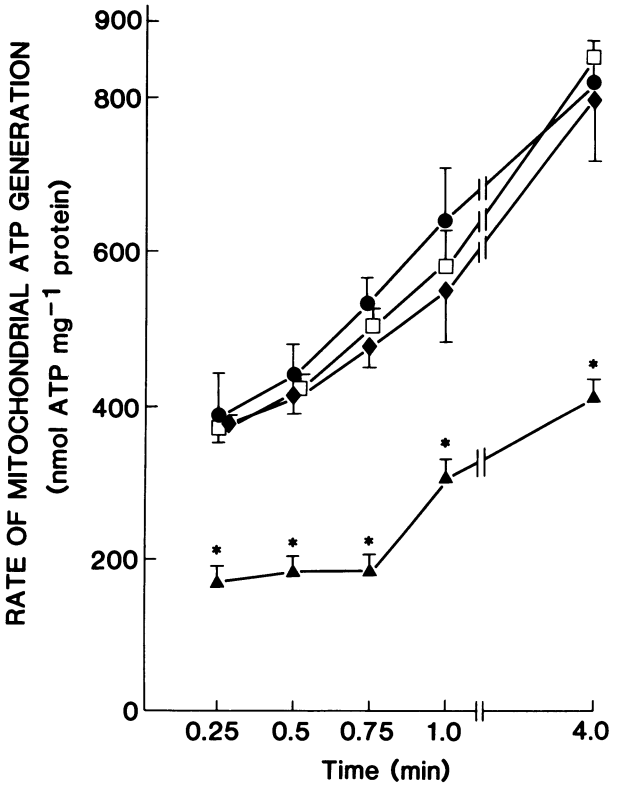

Fig 7. Mitochondrial rate of ATP generation isolated from hearts perfused with normal Kreb's (□—□); 1 uM ouabain (▲—▲); 1 uM ouabain and 500 nM verapamil (●—●); 1 uM ouabain and 50 nM nifedipine (◆—◆). The data represents mean ± S.E. from 6-8 experiments. *P < 0.05.

by an impaired rate of oxygen consumption, oxidative phosphorylation and partial inhibition of the rate of ATP generation. Although, Gervais et al (27) have shown an increase in the mitochondrial O_2consumption without

uncoupling of oxidative phosphorylation, in their study, the mitochondria was isolated at earlier stages of electrical toxicity (EKG disturbances). At this early stage, it might represent a compensatory mechanism of energy-producing process of mitochondria to quantitatively increase ATP production via increased production of ADP in the cytosol. Furthermore, toxic concentrations of ouabain have been shown earlier to be associated with a decrease in O_2 consumption in the intact heart muscle (28).

It is generally accepted that digitalis compounds specifically inhibit membrane Na^+, K^+-ATPase (3). Thus the primary effect of digitalis may be an alteration of the ionic concentrations across the cell membrane. Tsien et al (11) have suggested that a rise in intracellular Na^+_i, due to an inhibition of Na^+, K^+ATPase, may cause an increase in intracellular Ca^{2+} through Na^+-Ca^{2+} exchange. Contractile deterioration in ouabain induced mechanical toxicity in the Purkinje fiber (15) and isolated heart (16) have been postulated to be due to an excessive uptake of Ca^{2+} by the cardiac cell. The left ventricular cellular Ca^{2+} was recently shown to increase in the isolated rabbit heart exposed to toxic doses of ouabain (17). Tower et al (29) have indicated that most of the calcium gained by the tissue in presence of ouabain, is accumulated by mitochondria. Our data has clearly demonstrated an increase in the total tissue Ca^{2+} and on increased rate of Ca^{2+} uptake in the isolated mitochondria from heart perfused with 1 uM ouabain. These observations suggest that an impairment of mitochondrial rate of O_2 consumption, oxidative phosphorylation and inhibition of ATP generation may be a result of mitochondrial Ca^{2+} overload in the presence of toxic doses of ouabain. Vasington and Murphy (30) have suggested that when calcium is undergoing energy dependent accumulation by mitochondria, phosphorylation does not take place, the respiratory energy being diverted into calcium uptake. Others (31), however, have shown that preferential accumulation of calcium over the process of oxidative phosphorylation is not absolute. Calcium overload and an impairment of mitochondrial oxidative phosphorylation activity has been reported in pathological conditions such as hypoxia, ischemia and heart failure (32-34).

The postulate, that implicates mitochondrial Ca^{2+} overload in the development of mechnical toxicity, is further supported by the fact that in the present study when Ca^{2+} channel blockers such as verapamil or nifedipine are included in the perfusion medium, significant protection from the development of cardiac mechanical toxicity is observed. Under

these conditions, the rate at which the ATP reserves are depleted during ouabain induced mechanical toxicity should be slowed, leaving sufficient ATP to maintain intracellular homeostasis. In addition, in the presence of Ca^{2+} channel blockers, the inhibition of Na^+, K^+-ATPase by ouabain and the consequent increase in cytosolic Ca^{2+} due to Na^+-Ca^{2+} exchange may be sufficient to induce positive inotropic response but may not be enough to induce Ca^{2+} overload (16).

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REFERENCES

1. Akera, T. Science, New York. 198: 569-574, 1977.

2. Akera, T., Larsen, F.S. and Brody, T.M. J. Pharmacol. Exp. Ther. 170: 17-26, 1969.

3. Schwartz, A., Lindenmayer, G.E. and Allen, J.C. Pharmacol. Rev. 27: 3-134, 1975.

4. Godfraind, T. Eur. Heart J. 5 (Suppl F): 303-308, 1984.

5. Akera, T. and Brody, T.M. Ann. Rev. Physiol. 44: 375-388, 1982.

6. Schwartz, A., Whitmer, A.K., Grupp, G., Grupp, I., Adams, R.J. and Sin-Woong Lee. Ann. N.Y. Acad. Sci. 402: 253-271, 1982.

7. Ferrier, G.R. Prog. Cardiovasc. Dis. 19: 459-474, 1977.

8. Ferrier, G.R., Saunders, J.H. and Mendez, C. Circ. Res. 32: 600-609, 1973.

9. Rosen, M.R. and Danilo, P. Circ. Res. 46: 117-124, 1980.

10. Ferrier, G.R. and Moe, G.L. Circ. Res. 33: 508-515, 1973.

11. Tsien, R.W., Kass, R.S. and Weingart, R. Ann. N.Y. Acad. Sci. 307: 483-490, 1978.

12. Kass, R.S., Lederer, W.J., Tsien, R.W. and Weingart R. J. Physiol. (London), 281: 187-208, 1978.

13. Kass, R.S., Tsien, R.W. and Weingart, R.J. Physiol. (London), 281: 209-228, 1978.

14. Lin, C.I. and Vassalle, M. Am. J. Physiol. 234: H477-H486, 1978.

15. Vassalle, M. and Lin, C.I. Am. J. Physiol. 236: H689-H697, 1979.

16. Khatter, J.C., Agbanyo, M., Hoeschen, R.J., Navaratnam, S. and Bains, R. J. Pharmacol. Exp. Ther. (In Press), 1986.

17. Pilati, C.F. and Paradise, N.F. Proc. Soc. Exp. Biol. Med. 175: 342-350, 1984.

18. Tanz, R.D. and Russell, N. J. Fed. Proc. 42: 2470-2474, 1983.

19. Mukherjee, A., Wong, M., Templeton, G., Buja, L.M. and Willerson, J.T. Am. J. Physiol. 237: H224-H238, 1979.

20. Wrogerman, K. and Nylen, E.G. J. Mol. Cell. Cardiol. 10: 185-195, 1978.

21. Langendorff, O. Pflugers Arch. Ges. Physiol. 61: 291, 1895.

22. Polimeni, P.I. In: Techniques in the Life Sciences Vol. P3/II, "Techniques in Cardiovascular Physiology" (Ed. R.J. Linden), Elsevier/North Holland Biomedical Press, Amsterdam, 1984, pp. 1.

23. Tyler, D.D. and Gonze, J. In: Methods in Enzymology Vol. 10 (Eds. R.W. Estbrook and M.E. Pullman), Academic Press, New York/London, 1967, pp. 75-77.

24. Lowry, O.H., Rosebrough, J.N., Farr, A.L. and Randall, R.J. J. Biol. Chem. 193: 265-275, 1951.

25. Estabrook, R.W. In: Methods in Enzymology Vol. 10 (Eds. R.W. Estabrook and M.E. Pullman), Academic Press, New York/London, 1967, pp. 41-47.

26. Lamprecht, W. and Trauchold, I. In: Methods of Enzymatic Analysis Vol. 4 (Ed. Bergmeyer, H.U.), Verlag Chemie Weinheim, Academic Press, New York/London, 1974, pp. 2101-2110.

27. Gervais, A., Busch, V., Wood, J.M. and Schwartz, A. J. Mol. Cell. Cardiol. 10: 1003-1015, 1978.

28. Covell, J.W., Braunwald, E., Ross, J. Jr. and Sonnenblock, E.H. J. Clin. Invest. 45: 1535-1542.

29. Tower, D.B. Exp. Brain Res. 6: 273-283, 1968.

30. Vasington, F.D. and Murphy, J.V. J. Biol. Chem. 237: 2670-2677, 1962.

31. Jacobus, W.E., Tiozzo, R., Lugli, G., Lehninger, A.L. and Carafoli, E. J. Biol. Chem. 250: 7863-7870, 1975.

32. Ganote, C., Seabra-Gomes, R., Nayler, W.G. and Jennings, R.B. Am. J. Patho. 80: 419-450, 1975.

33. Ito, Y. and Chudney, C.A. J. Mol. Cell. Cardiol. 4: 507-517, 1972.

34. Nayler, W.G., Ferrari, R. and Williams, A. Am. J. Cardiol. 46: 242-248, 1980.

E. NEWER INOTROPIC AGENTS AND CALCIUM

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CLASSIFICATION AND MECHANISM OF ACTION OF THE CARDIAC INOTROPIC AGENTS: AN OVERVIEW

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Introduction

The cardiac inotropic agents are substances that modify cardiac muscle contraction by acting directly on the myocardium (1). These agents could be categorized into two major classes; positive inotropic agents that stimulate cardiac muscle contraction such as the cardiac glycosides (2), the catecholamines (3) and the bipyridines (4), and negative inotropic agents that depress cardiac contraction such as the β-adrenergic blockers, the antiarrhythmics and the barbiturates (5).

The primary use of the cardiac positive inotropic agents is in the treatment of heart failure, a disease state in which the myocardium fails to contract sufficiently to generate adequate pressure to pump blood to the peripheral tissues (6). The causes and pathophysiological changes responsible for heart failure are summarized in Table I.

Table I

Causes and Pathophysiology of Congestive Heart Failure

Cause		Pathophysiology
Hypertension	→	Pressure Overload
Valvular Insufficiency Congenital Heart Disease	→	Volume Overload
Coronary Artery Diseases	→	Loss of Muscle Mass
Cardiomyopathy	→	Impaired Contractility
Constricted Pericarditis Pericardial Tamponade Stiffening of the Ventricle	→	Restricted Filling

In early stages of congestive heart failure, three primary compensatory mechanisms (Table II) become operative but at late stages of heart failure all compensatory mechanisms surpass their potentials and fail to compensate (6).

Table II

Primary Compensatory Mechanisms in Congestive Heart Failure

1.	Frank-Starling Mechanism
2.	Increased Adrenergic Nerve Activity
3.	Myocardial Hypertrophy

Treatment of Heart Failure

Historically, the management of heart failure could be chronologically subdivided into four periods (Table III).

1. The William Withering period that began around 1780 and continues to the present time.
2. The diuretic period that began in the 1920's with the discovery of the mercurial diuretics and later with the introduction of the thiazides.
3. The vasodilator period that began in the early 1970's in which cardiac performance was manipulated by changes in preload and afterload.
4. The new inotropic agents period that began in the late 1970's with the discovery of the bipyridine amrinone.

Table III

Treatment of Congestive Heart Failure

I 1780	Digitalis Reduced salt intake Mechanical removal of excess fluids
II 1920	Parenteral diuretics Oral diuretics
III 1970	Vasodilators
IV 1977	New inotropic agents - bipyridines, imidazoles, benzoimidazoles

The search for new inotropic agents originated from the need for a safe and effective orally active inotropic agent for the treatment of chronic heart failure. For the past two centuries, the only orally active inotropic drugs available have

been the digitalis glycosides. These agents have questionable long-term efficacy and low therapeutic range (7).

In the early 1970's Goldberg and his associates reported on the advantages of the endogenous catecholamine, dopamine, in the treatment of acute heart failure (8). Soon after, the synthetic catecholamine, dobutamine, was introduced as an intravenous therapy for acute heart failure (9). Since then several intravenous and orally active β-adrenergic agonists have been used in clinical trials but were not proven to be useful for long-term therapy due to the chronotropic and arrhythmogenic side actions and the development of tolerance after chronic medication (7). A general classification of known natural and synthetic inotropic agents, with their putative mechanism of action is summarized in Tables IV and V, respectively. It should be emphasized that in most cases, the exact mechanism of action of these agents (based on a cause and effect relationship) has not been completely established. The rest of this brief overview focuses on the pharmacological profile and possible mechanism of action of the new, non-glycoside and non-catechol inotropic agents.

Table IV
Classification of Naturally Occurring Inotropic Agents

Chemical Class	Example	Mechanism
Ions	Calcium	Activation of TNC
Glycosides	Digitalis	*↓ Na, K-ATPase
Catecholamines	Dopamine	**↑ β-Adrenergic Receptor
Imidazoles	Histamine	↑Histaminergic Receptor
Methylxanthines	Caffeine	↓Phosphodiesterase ↑Ca Sensitivity
Alkaloids	Veratridine	↑Fast Na Channels
Diterpenes	Forskolin	↑Adenylate Cyclase
Peptides	Glucagon	↑Adenylate Cyclase
Antibiotics	X537A	↑Ca-Permeability

*Inhibit
**Stimulate

Table V
Classification of Synthetic Inotropic Agents

Chemical Class	Example	Mechanism?
Bipyridines	Amrinone	↑Ca Influx ↓Phosphodiesterase
Imidazopyridines	Sulmazole	↓Phosphodiesterase ↑Ca Sensitivity
Imidazolones	MDL 17043	↓Phosphodiesterase
Quinolinone	OPC-8212	↓Phosphodiesterase
Piperazinyl-indole	DP1201-106	↑Fast Na Channels
Pyridazinones	CI914	↓Phosphodiesterase

PHARMACOLOGICAL PROFILE OF THE NEW INOTROPIC AGENTS

In the search for a new inotropic agent, a well defined set of criteria has to be established to characterize what could be considered as an "acceptable" inotropic agent. These characteristics (Table VI) should be demonstrated in the pharmacological test systems listed in Tables VII and VIII.

Table VI
Characteristics of an "Acceptable" Inotropic Agent

1. Potent positive inotropic activity without chronotropic or dromotropic effects.
2. Wide therapeutic index.
3. Intravenous and oral activity in patients with congestive heart failure at rest and during exercise.
4. Moderate duration of action.
5. No tachyphylaxis after prolonged therapy.
6. Compatible with other cardiovascular drugs.

Table VII
Effects on the Normal Heart

In Vitro Activity

Positive inotropic activity should be demonstrated in:

Isolated atria and papillary muscles
Isolated blood-perfused papillary muscle
Langendorff preparation
Isolated cardiac myocytes

In Vivo Activity

Positive inotropic activity should be demonstrated after:

Intravenous administration as -

Bolus injection
Intravenous infusion

Oral administration as -

Single dose
Repeated medication

Table VIII
The Effects on the Failing Heart

In Vitro Action

The agent has to be effective in:

Cardiac tissues from animals with experimentally-induced heart failure

Cardiac tissues from humans with congestive heart failure

In Vivo Activity

The agent has to demonstrate a reversal of heart failure in:

Animals with experimental heart failure

Animals with naturally-occurring heart failure

Patients with chronic congestive heart failure of multiple etiologies

MECHANISM OF ACTION OF INOTROPIC AGENTS

In general, muscle contraction is the result of binding of calcium to the regulatory protein troponin C on the thin filament that leads to the activation of actin and its interaction with myosin and ATP hydrolysis (10). Phosphorylation of troponin I, which is regulated by a cAMP-dependent protein kinase, reduces the affinity of troponin C to Ca^{2+}, while phosphorylation of myosin light chain, a Ca^{2+}-activated calmodulin-dependent kinase may govern the rate of crossbridge cycling (11).

The inotropic agents could modulate muscle contraction by exerting their actions on one or more of the mechanisms listed in Table IX.

Table IX
Possible Mechanism of Action of Inotropic Agents

Increase calcium availability
Increase myofibrillar sensitivity to calcium
Modify myosin ATPase isozyme activity
Modify the regulatory interaction between thick and thin filament proteins.

1. Increase Calcium Availability

A large number of inotropic agents cause increases in calcium availability to the myofibrils during systole. This is usually accomplished by affecting Ca^{2+}-movement across the several intracellular membranous structures that regulate intracellular Ca^{2+}-concentration (Table X). The sarcolemma and its transverse-tubules change their permeability to Ca^{2+} during the excitation-contraction coupling and are capable of regulating cytosolic Ca^{2+}-concentration by: voltage-dependent Ca^{2+}-channels, receptor-operated channels, MgATP-dependent Ca^{2+}-pump, Na^{+}-Ca^{2+} exchange mechanism and Na^{+}, K^{+}-ATPase pump.

Pharmacological agents that increase sarcolemmal Ca^{2+}-influx via any of the mechanisms listed in Table X could cause an increase in cardiac muscle contraction. Agents that stimulate the slow Ca^{2+} channels by either direct stimulation, such as the calcium agonist Bay K 8644 or by increasing intracellular cAMP level, are potent inotropic agents. Increase in cardiac cAMP levels have been demonstrated after cyclic nucleotide phosphodiesterase inhibition with the methyl xanthines and bipyridines, direct stimulation of adenylate cyclase with glucagon, histamine or forskolin and after direct stimulation of β-adrenergic receptors with

the sympathomimetic amines.

Intracellular Ca^{2+}-influx could also be stimulated by agents that act on specific receptor-operated channels or by increasing the permeability of the sarcolemma to calcium as in the case of the calcium ionophores. Increasing extracellular calcium by the parathyroid hormone has been shown to increase intracellular calcium and the induction of inotropy. There is also some evidence to indicate that the activation of a sarcolemmal Ca^{2+}-calmodulin dependent system could result in enhanced intracellular calcium fluxes.

A second possible mechanism that could lead to enhanced availability of Ca^{2+} to the myofibrils is by an increase in intracellular sodium concentration and stimulation of Na^{+}-Ca^{2+} exchange mechanism. This could be achieved either by the inhibition of Na^{+}, K^{+}-ATPase as in the case of the digitalis glycosides or by stimulation of the fast sodium channels as in the case of veratrum alkaloids and DPI 129. It is also possible that an agent could decrease Ca^{2+}-efflux and hence increase Ca^{2+}-availability to the contractile proteins.

The second important regulator of cytosolic Ca^{2+}-concentration, the sarcoplasmic reticulum, also could be affected by the inotropic agents and result in changes in Ca^{2+}-availability to the myofibrils. It is well established that agents that increase intracellular cAMP concentration via inhibition of phosphodiesterase, stimulation of adenylate cyclase or stimulation of β-adrenergic receptors, increase Ca^{2+}-uptake and subsequent release by the sarcoplasmic reticulum. Other substances such as the methylxanthines have direct effect on the sarcoplasmic reticulum causing an increase in Ca^{2+}-release and elevation of cytosolic Ca^{2+}-concentration.

The mitochondrion is a complex organelle with high capacity to store Ca^{2+}. However, its slow rate of Ca^{2+}-release prevents it from playing an important role in regulating Ca^{2+} during systole. Agents that cause an increase in intracellular Na^{+} concentration may stimulate Ca^{2+} release from the mitochondria and exert positive inotropic action.

Finally, pharmacological agents that interfere with Ca^{2+}-binding to the Ca^{2+} buffering proteins may also increase Ca^{2+}-availability to the myofibrils.

Table X

1. Agents That Could Increase Calcium Availability
   - A. Effects on sarcolemma
     1. Increase Ca influx
        - a. Stimulate Slow Ca^{2+}-Channels
          - i. direct stimulation
          - ii. indirect stimulation via increase in $[cAMP]_i$
            - stimulate β-receptors
            - stimulate adenylate cyclase
            - inhibit phosphodiesterase
        - b. Stimulate receptor-operated channels
        - c. Increase Ca^{++} permeability
        - d. Increase $[Ca^{++}]_e$
        - e. Activate Ca-calmodulin-dependent system
     2. Increase $[Na]_i$ - Stimulate Na^+-Ca^{++} Exhange Mechanisms
        - a. Inhibit Na,K-ATPase
        - b. Stimulate fast Na^+-channels
     3. Decrease Ca^{++}-Efflux
   - B. Effects on Sarcoplasmic Reticulum
     1. Increase calcium uptake
        - a. increase $[c\text{-}AMP]_i$
        - b. stimulate phospholipid-dependent protein kinase
     2. Increase calcium release
   - C. Effects on Mitochondria
     1. Stimulate calcium relase by increasing $[Na^+]_i$
   - D. Effects on Buffering Proteins

2. Increase Myofibrillar Sensitivity to Calcium

A second major possible mechanism by which inotropic agents may affect the magnitude of muscle contraction is by increasing the sensitivity of the myofibrils to calcium. Agents such as caffeine, AR-L115 (sulmazole), pimobendan, APP201-533 and DPI 201 were shown to increase the calcium sensitivity of skinned cardiac muscle cell and shift the calcium-tension curve to the left (12). The usefulness of these agents in the treatment of the failing heart will depend on their ability to increase the sensitivity of the myofibrils to calcium concentration achieved only during systole (10^{-6}-10^{-5}M) rather than those maintained during diastole (10^{-7}M). An increase in calcium sensitivity during diastole will result in incomplete myocardial relaxation, increased energy utilization and reduction in cardiac efficiency. On the other hand, an increase in tension as a result of increased calcium sensitivity without an increase in intracellular calcium concentration may be beneficial in the treatment of the diseased heart.

3. Modify Myosin ATPase Isozyme Activity

Changes in myosin ATPase activity after prolonged physiological or pharmacological manipulation have been demonstrated in several experimental animals (13). This tonic form of control of myocardial contractility results in a change in V_{max} and a shift in the force-velocity curve. Pharmacological agents such as thyroxine have been shown to increase V_1-myosin isozyme, ATPase activity and speed of muscle shortening (14).

4. Modify the Regulatory Interaction Between Thick and Thin Filament Proteins

Although no known pharmacological agents have yet been identified under this class of inotropic agent, it is possible that such an agent could be developed.

In summary, at the present time the newly developed positive inotropic agents constitute an important component of the therapeutic armamentarium used in the management of congestive heart failure. We hope that the thorough investigation of the mechanism of action of these agents results in better understanding of the multiple pathways by which cardiac contractile machinery could be activated. Our future attention should focus on investigation of the molecular defects that lead to the precipitation of myocardial cell failure and the development of a more rational approach for the design of pharmacological agents capable of modifying the course of the disease and hence a better therapeutic endpoint.

References

1. Alousi, A.A. and Farah, A.E., Trend Pharmacol. Sci. 1: 143-146, 1980.
2. Grupp, G., Grupp, I.L., Hickerson, T., Lee, S.W. and Schwartz, A. In: Cardiac Glycosides 1785-1985. (Eds. E. Erdmann, K. Greef and J.C. Skou), Stinkopff Verlag Darmstadt, Springer-Verlag, New York, 1986, pp. 99-108.
3. Scholtz, H. In: Handbook of Experimental Pharmacology, Adrenergic Activators and Inhibitors, (Ed. L. Szekeres), Springer-Verlag, Berlin/ Heidelberg/New York, 54(I): 651-733, 1980.
4. Alousi, A.A., Fabian, R.J., Baker, J.F. and Stroshane, R.M. In: New Drugs Annual: Cardiovascular Drugs (Ed. A. Scriabine), Raven Press, New York, 3:245-283, 1985.
5. Alousi, A.A., Canter, J.M. and Fort, D.J. Cardiovas. Res. 19: 483-494, 1985.
6. Braunwald, E. In: Heart Disease (Ed. E. Braunwald), W.B. Saunders Comp., Philadelphia, London, Toronto, 1984, pp. 453-471.
7. Colucci, W.S., Wright, R.F. and Braunwald, E. New. Engl. J. Med. 314: 349-358, 1986.
8. Goldberg, L.I. Pharmacol. Rev. 24: 1-29, 1972.
9. Tuttle, R.R., Mills, J. Circ. Res. 36: 185-196, 1975.
10. Katz, A. In: Physiology of the Heart (Ed. A. Katz), Raven Press, 1977, pp. 107-119.
11. Stull, J.T. and Mayer, S.E. In: Handbook of Physiology, The Cardiovascular System, Vol. I. The Heart. (Eds. R.M. Berne, N. Sperelakis and S.R. Geiger). The Physiological Soc., Bethesda, Maryland, 1979, pp. 741-774.
12. Ruegg, J.C. Cir. Res. Supp. 73: III, 78-84, 1986.
13. Hoh, J.F.Y., McGrath, P.A. and Hale, P.T. J. Mol. Cell. Cardiol. 10: 1053-1076, 1978.
14. Pagani, E.D. and Julian, F.J. Circ. Res. 54: 586-594, 1984.

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NEW APPROACHES FOR THE TREATMENT OF THE FAILING MYOCARDIUM: MYOFIBRILLAR PROTEINS AS POTENTIAL TARGETS FOR PHARMACOTHERAPY

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Introduction

A new approach for the development of pharmacological agents to treat myocardial cell failure that is fundamentally different from the more traditional approaches used in the development of positive inotropic agents is beginning to emerge. The traditional approaches, in general, rely heavily on screening chemical compounds in a biological system with the aim of identifying compounds that have inotropic activity. The new approach is different in that a specific enzyme or cellular process that is identified to be defective in failing heart muscle is targeted for chemical modification with the goal of improving diastolic and/or systolic performance of the heart and raising cardiac output. Associated with the new approach is the possibility that by reversing the defect in failing heart muscle cells the progression of the disease may be slowed or even prevented. Although in both the new approach and traditional approach the final objective in treating myocardial cell failure may be the same, the pathways leading to the objective are very different, as could be the potential long range result.

The success of this new approach toward the development of pharmacological agents is dependent on numerous factors. Two key factors are the identification of intracellular defects in the failing muscle cell and the ability to target, chemically modify, and correct the defective intracellular process. Results obtained in studies of animal models of heart failure have identified defects in both membrane and protein systems, including defects in the contractile proteins (1), which are involved in the excitation-contraction-relaxation processes of the heart. Moreover, there is evidence that hormones, like thyroxine and insulin, are capable of inducing changes in the contractile proteins and sarcoplasmic reticulum (2, 3, 4, 5, 6) of cardiac muscle and that these changes translate into alterations in cardiac muscle performance (7, 8, 9).

The discussion in this paper will focus on the contractile proteins in the cardiac muscle cell as potential targets in the treatment of myocardial failure.

Factors that influence the number of cycling myosin crossbridges and factors that influence the rate of myosin crossbridge cycling during muscle contraction will be described. Results obtained from in vitro studies of isolated myofibrillar proteins and chemically skinned and intact muscle preparations will be used to delineate mechanisms that could regulate the interaction of myosin crossbridges with actin in the intact myocardium. Finally, a description will be given as to the ways in which controlling both the number of myosin crossbridges interacting with actin and the rate of myosin crossbridge cycling could possibly be utilized to improve the pump performance of the failing heart.

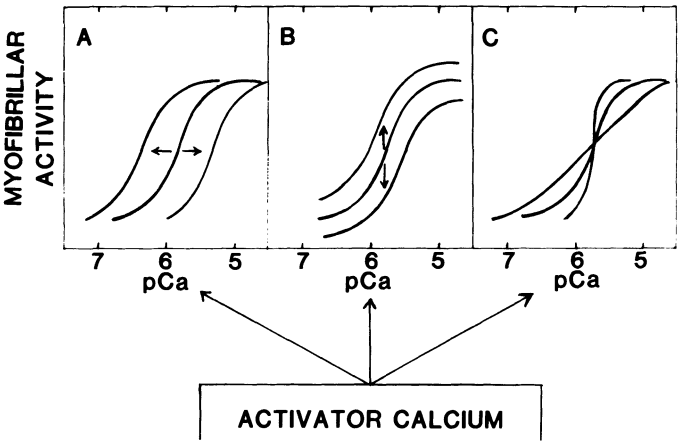
Recruitment of Myosin Crossbridges in Cardiac Muscle

During skeletal muscle activation the recruitment of myosin crossbridges occurs both at the motor neuron level, via the selection and activation of muscle cells and at the muscle cell level. However, recruitment of myosin crossbridges during a contraction of the heart only occurs at the level of the heart muscle cells. Stated another way, force development by the heart is regulated by recruiting myosin crossbridges within cells rather than through the recruitment of individual cardiac muscle cells. This concept is relevant to the regulation of force development by the heart since the number of myosin crossbridges interacting in parallel with the actin filament, at a fixed muscle length, is a major determinant of total force development.

When the concentration of myoplasmic calcium is less than about 10^{-7}M in cardiac muscle, the probability that myosin crossbridges will interact with the actin filament is minimal due to an inhibitory process involving the calcium sensitive regulatory complex troponin (TNC, TNI, TNT) and the strand-like protein tropomyosin, both of which are situated along the actin filament (10). When the myoplasmic calcium concentration rises above 10^{-7}M, calcium occupies the calcium specific binding site associated with the troponin C subunit and a conformational change occurs in the troponin complex (11) which is thought to induce the movement of tropomyosin to a position on the actin filament that favors myosin interaction with actin (12).

There are three general ways in which the relation between myofibrillar protein activation and intracellular ionized calcium (activation-pCa curve) can be modified (Figure 1) (13). Panel A shows a rightward shift in the activation-pCa curve which may indicate a decrease in the calcium sensitivity of the myofibrillar proteins, whereas a leftward shift in the activation-pCa curve may indicate an increase in the calcium sensitivity of the myofibrillar proteins. Panel B, on the

other hand, illustrates an upward and downward shift in the ATPase activity of the myofibrillar proteins without a change in calcium sensitivity. This type of change appears to be related to alterations in the ATPase activity of myosin alone or resulting from the interaction of myosin crossbridges with the actin filament. Panel C shows a combination of the effects illustrated in Panels A and B. This curve is shifted along both the ordinate and absissca and relates to a change in both the calcium sensitivity and intrinsic ATPase activity of the myofibrillar proteins. When interpreting data from experiments in which myofibrillar ATPase activity is measured over a range of calcium concentrations, it should be noted that changes in ATPase activity can occur either through alterations in the sensitivity of the myofibrillar regulatory proteins or through alterations in the intrinsic ATPase activity of myosin. Normalizing the data as a percentage of the maximal ATPase activity has been used as a way to separate these phenomena.



(Figure 1)

The relation between the concentration of calcium surronding the contractile proteins and the number of myosin crossbridges that are recruited, via the availability of actin binding sites, has been suggested to change in response to a variety of conditions (14). Some of these conditions which have been reported for in vitro studies of myofibrillar proteins and for chemically skinned and intact segments of heart muscle are listed in Table 1.

Table 1

Factors Which Influence the Calcium Sensitivity of Cardiac Myofibrillar Proteins

	Reference
Sarcomere length	14
Troponin I phosphorylation	15,16
Intracellular pH	17,18
Myofilament Protein Isoforms	40
Active Force	41

Of the conditions listed in Table 1 only some of them may be relevant to the phasic, beat-to-beat regulation of the cardiac contraction-relaxation cycle, while some would appear to be more relevant in the long-term tonic control of the heart. For example, the influence of sarcomere length on the sensitivity of the myofibrillar proteins to calcium would appear to influence both systolic and diastolic function of the heart on a beat-to-beat basis in the following ways. The sarcomere length at end-diastole (preload) may influence the sensitivity of the myofibrillar proteins at the start and throughout the duration of the subsequent systolic cycle (Figure 1, Panel A). The extent of muscle cell shortening during and at end-systole determines the sarcomere length of the muscle cells and thus influences the sensitivity of the myofibrillar proteins at the start of and throughout the duration of the subsequent diastolic cycle (Figure 1, Panel A).

In addition to the influence of sarcomere length on calcium sensitivity is the reported effect of troponin I (TNI) phosphorylation and its effect on the sensitivity of the myofibrillar proteins to calcium. Phosphorylation of TNI has been shown by in vitro myofibrillar ATPase studies (15) and by troponin calcium binding studies (16) to desensitize the contractile proteins to calcium (Figure 1, Panel A). This phenomenon has been suggested to be responsible, in part, for the observed increase in the rate of relaxation of cardiac muscle in response to β-agonist stimulation. Thus, theoretically, β-agonists could increase the rate of relaxation of the heart through both TNI phosphorylation and by promoting an increase in the extent of muscle cell shortening at end-systole, both of which decrease the sensitivity of the contractile proteins to calcium.

Results of in vitro studies of isolated myofibrils (17) and chemically skinned cardiac muscle preparations (18) examined over a range of pH values has indicated that the sensitivity of the contractile proteins to calcium decreases as pH

decreases. Under conditions in which the metabolic demand of the heart for oxygen exceeds the delivery of oxygen, for example in the case of coronary artery disease, there is a tendency for the intracellular pH of the cardiac muscle to decrease (19). A reduction in the intracellular pH would tend to reduce force development by the heart through this desensitization phenomenon.

The examples described above illustrate ways in which the Ca^{2+} sensitivity of the myofibrillar proteins could influence the number of myosin crossbridges that interact with the actin filament during diastole and systole. Some of the conditions which affect the sensitivity of myofibrillar proteins, for example a change in intracellular pH, also influence other processes in the muscle cell, such as calcium transport by the sarcoplasmic reticulum, that take place during the contraction and relaxation cycle of the heart. Therefore, the observed changes in the calcium sensitivity of the myofibrillar proteins per se, in certain instances, may not be predictive in determining cardiac muscle performance.

Before evidence appeared suggesting that sarcomere length influences the calcium sensitivity of the myofibrillar proteins it had been thought that muscle length would influence isometric force development by the heart. This idea came from studies of isolated skeletal muscle fibers in which a relation between sarcomere length and active force development was found and was explained to be a result of the physical structure of the myofibrillar proteins in terms of the lengths of the myosin and actin filaments and interfilament spacing (20). Julian and Sollins (21) showed that the sarcomere length-force relation in cardiac muscle is similar, but does not exactly resemble the relation reported by Gordon, et. al. (20) for single frog muscle fibers. Thus, the basis for the Frank-Starling mechanism of the heart involves, to some extent, the effect of filament overlap in setting the maximum number of crossbridges which are able to interact with the actin filament and also involves other phenomena like the influence of sarcomere length on calcium sensitivity (14), interfilament steric hindrance of myosin crossbridge interaction with actin (21) and calcium release from the sarcoplasmic reticulum (22).

This section only touched on the numerous ways in which the recruitment of myosin crossbridges can be modulated in cardiac muscle. The observation that the sensitivity of the myofibrillar proteins can change under normal physiological conditions suggests that the pathways for control of myofilament calcium sensitivity do exist and recruitment of myosin crossbridges could be modulated by targeting the regulatory proteins for chemical modification.

Myosin Crossbridge Cycling and Muscle Performance

The relation between force and speed of muscle shortening is best described by a rectangular hyperbola. The speed of muscle shortening is the highest, and force is the lowest, when cardiac muscle is shortening against a zero load. Conversely, force development by cardiac muscle is the highest and the speed of shortening is the lowest when cardiac muscle is contracting against an infinite load (isometric contraction). These two mechanical properties of cardiac muscle, i.e. maximum speed and maximum force, set the end points of the hyperbolic curve for the force-velocity relation of cardiac muscle.

A number of conditions have been reported to influence the shape of the force-velocity relation (23). In fact, an entire family of force-velocity curves can be obtained for a single segment of cardiac muscle by altering the preload (sarcomere length) or afterload or changing the contractile state of the muscle cells. Moreover, disease, aging and pharmacological agents have also been shown to alter the shape of the force-velocity relation of cardiac muscle. In many of these cases, changes in the shape of the force-velocity curve have been suggested to be associated with changes in the ATPase activity of myosin which has been used as an *in vitro* index of the rate of myosin crossbridge cycling (24). Some examples of the ways in which the rate of myosin crossbridge cycling has been altered and the ways in which this type of alteration influences cardiac muscle performance will be discussed below.

Cardiac Myosin Isozymes

It has been firmly established that hearts of rats, rabbits and a few other small animals contain multiple isozymes of myosin. An excellent and detailed review of myosin isozymes in muscle has been recently published (25). Cardiac myosin isozymes, within a species, appear to differ in respect to the structure of the myosin heavy chains rather than the structure of the myosin light chains. For example, rabbit ventricular muscle cells contain two distinct myosin heavy chains, α and β, and up to three myosin isozymes, composed of $\alpha\alpha$, $\beta\beta$, or $\alpha\beta$ heavy chains (26). Cardiac myosin isozymes can be separated under non-denaturing conditions by polyacrylamide gel electrophoresis and have been denoted V_1, V_2 and V_3 based on their decreasing electrophoretic mobility on polyacrylamide gels and Ca^{2+}-activated ATPase activity (27).

The types of myosin isozymes present in hearts of small animals have been shown to change with age, in response to stressing the heart by chronic swimming exercise or by imposing a volume or pressure overload on the heart (26). The relative amounts of the myosin isozymes in the heart have also been shown to be

sensitive to the level of serum thyroxine (27). Thyroid hormone deficiency induces the net synthesis of cardiac V_3 myosin while an excess of thyroid hormone induces the net synthesis of cardiac V_1 myosin. In vitro analyses of the Ca^{2+}-activated myosin ATPase activity have indicated that, in general, a linear relation exists between myofibrillar myosin ATPase activity and the percentage of V_1 myosin present (4). However, there appears to be instances when an increase in Ca^{2+}-myosin ATPase activity or changes in the proportion of myosin isozymes do not correspond to a parallel change in the myofibrillar MgATPase activity (28, 29).

Myosin Crossbridge Cycling and the Velocity of Muscle Shortening

Changes in the Ca^{2+}-activated ATPase activity of myosin in ventricular muscle, as a result of a redistribution in the proportions of myosin isozymes, have been suggested to be responsible for alterations in the velocity of cardiac muscle shortening. In studies of intact rabbit papillary muscles in which the speed of muscle shortening was measured for muscles that contained various proportions of V_1 and V_3 myosin, Pagani and Julian (9) found that a linear relation exists for the speed of muscle shortening and proportion of V_1 myosin present. This relation was observed for muscles shortening over a wide range of external loads from a near maximum load to zero load. Similar results were obtained for intact rat papillary muscles (30) and chemically skinned segments of rat heart (31) in which the speed of unloaded muscle shortening was compared for muscles that contained either V_1 or V_3 myosin. The skinned cardiac muscle studies by Ebrecht et. al. (31) are especially important because in these studies it was demonstrated that under conditions where the activating calcium concentration is controlled the speed of muscle shortening is still dependent on the proportions of myosin isozymes present. The regulation or effect of crossbridge cycling in cardiac muscle also appears to be independent of the calcium sensitivity of the myofibrillar proteins. For cardiac muscles in which the maximum rate of crossbridge cycling can be very different, for example in cardiac muscles that contain either V_1 or V_3 myosin, the calcium sensitivity of the myofibrillar proteins appear to be the same (32).

Myosin Crossbridge Cycling and the Efficiency of Cardiac Muscle Contraction

There may be certain physiological or pathological conditions when the supply of oxygen to the heart is reduced and the amount of ATP used for pumping blood approaches the point of becoming rate limiting for normal cardiac contraction and relaxation. Since a large percentage of the ATP hydrolyze during the cardiac cycle is due to crossbridge cycling, alterations in the rate of crossbridge cycling could affect the intracellular levels of the high energy phosphate compounds. Alpert and Mulieri (33) reported that the contraction of papillary muscles which contain V_3

slow-cycling myosin crossbridges is more economical than ones which contain more V_1 myosin based on results from studies of rabbit papillary muscles made to contract isometrically while in contact with a heat-sensitive thermopile. However, in the intact myocardium, the issue of economy turns to a question of efficiency and becomes more complicated. In the walls of the heart, muscle cells contract isotonically and perform work. Therefore, an estimate of the efficiency of contraction for muscle cells which contain fast or slow cycling crossbridges must take into consideration the segment of the force-velocity curve along which the muscle cells are working (34). The shape of the force-velocity curve, as well as the segment along which any given muscle cell is working, may be quite different and variable between beats of the heart for working cells belonging to the different layers (endo, middle and epicardial) and regions (apex to base) of the ventricular free wall. Numerous factors affect the efficiency of cardiac contraction, such as wall stress and its related parameters, segmental ventricular wall shortening, heart rate and contractility (35). Under certain conditions it is possible that force development and shortening of muscle cells containing a relatively larger percentage of fast cycling crossbridges could be more efficient than muscle cells that contain relatively higher percentages of slow cycling crossbridges.

Acute Control of Myosin Crossbridges

Conditions which influence the rate of myosin crossbridge cycling and speed of muscle shortening over a long-term basis were described. The control of crossbridge cycling has also been suggested to be controlled on a beat-to-beat basis through the specific recruitment of either fast or slow cycling crossbridges (36) or via myosin light chain phosphorylation (37).

In studies of hyperpermeable segments of rat cardic muscle, Winegrad et. al. (36) interpreted their results to suggest that a cAMP-dependent mechanism is present in heart cells that is more selective in activating V_1 myosin crossbridges than V_3 myosin crossbridges in response to β-adrenergic stimulation. These results suggest a novel mechanism for the control of force development and also suggests that the speed of cardiac muscle shortening is regulated on at least two levels, one which involves the relative proportions of V_1 and V_3 isozymes present and the second which involves the selection of specific myosin isozymes via neural, humoral and pharmacological stimulation of the heart.

The role that myosin light chain phosphorylation plays during cardiac force development and muscle shortening is not entirely understood. However, recently, Sweeney and Stull (37) reported results from studies of chemically skinned cardiac muscles in which phosphorylation of the 20,000 dalton cardiac P-

light chain was suggested to be responsible for an increase in the number of attached crossbridges in the present of submaximal concentrations of ionized calcium. The mechanism did not apparently involve direct changes in the calcium sensitivity of troponin associated with TNI phosphorylation since the phosphorylation of protein was restricted to the myosin light chain by using the light chain specific myosin light chain kinase. No measurement for the speed of muscle shortening was made, therefore is not known from these studies if light chain phosphorylation influences the rate of myosin crossbridge cycling.

Myofibrillar Proteins as a Target for Pharmacological Agents in the Treatment of Myocardial Cell Failure

In the preceding sections, descriptions were given as to the ways in which the calcium sensitivity of the contractile proteins and the rate and number of cycling crossbridges can be altered. These types of changes in the myofibrillar proteins could influence cardiac muscle contraction and relaxation and significantly affect the systolic and diastolic function of the heart. The extent of systolic and diastolic dysfunction in cardiac pump failure varies in respect to both the severity and etiology of the disease. Moreover, in man, it is not clear as to what extent the myofibrillar proteins are responsible, if at all, for myocardial cell failure. There is some question regarding the role played by myosin isozymes in the human heart and whether changes in the proportions of the myosin isozymes can account for the abnormal contraction and relaxation of the ventricular wall. Takeda, et. al. (38) reported that two forms of myosin isolated from human papillary muscles can be separated by polyacrylamide gel electrophoresis and that differences in papillary muscle myofibrillar ATPase activity among papillary muscles correlate with differences in the relative proportions of myosin isoforms present. However, results from studies of normal and diseased human ventricular muscle, in which the proportions of myosin isoforms was quantitated with the use of monoclonal antibodies to myosin, have indicated that the amount of the V_1 type myosin does not significantly change in hypertrophied human ventricle (39). Nevertheless, valuable information has been obtained regarding the influences of myosin isozymes and the rate of myosin crossbridge cycling on the mechanical performance of small animal hearts. These data suggest that drugs which either slow down or speed up the rate of crossbridge cycling in human heart muscle should induce a parallel change in the speed of human cardiac muscle shortening and have an effect on the pump performance of the heart.

The issue of which type or combination of pharmacological interventions (changes in calcium sensitivity of the regulatory proteins or in myosin crossbridge

interaction with actin) will be the most effective in improving the pump performance of the failing heart can only be answered after more data is generated regarding the intracellular defects associated with human heart disease. Sensitizing the myofibrillar proteins to calcium could potentially have an adverse effect on diastolic filling depending upon the segment of the activation-pCa curve (Figure 1, Panel A) that is made more sensitive to calcium. For example, if the myofibrillar proteins are made more sensitive to calcium at the lower calcium concentrations, diastolic wall stiffness may increase and impair ventricular filling. However, if the myofibrillar proteins are made more sensitive to calcium only at the higher concentrations of calcium, then possibly systolic function would be improved with no adverse effects on diastolic function and ventricular filling.

Drugs which change the rate of crossbridge cycling either by altering the proportions of myosin isozymes or through a direct effect on the myosin crossbridges could have an influence on both diastolic and systolic function by affecting the efficiency of the heart as a pump. However, the overall efficiency of the heart as a pump is determined by many factors which determine the segment of the force-velocity curve along which muscle cells of the heart work. Although it has been suggested that isometric contraction by cardiac muscle cells which contain slow cycling V_3 crossbridges is more economical than cardiac cells which contain fast cycling V_1 myosin, no data is yet available regarding the efficiency of contraction for cardiac muscle cells, which contain either V_1 or V_3 myosin isozymes, in the normal or failing myocardium. Moreover, the efficiency of muscle contraction may not be a critical issue in all types of human heart disease, especially in those types where oxygen supply far exceeds the oxygen demands of the heart or when the ability of muscle cells to synthesize ATP is not compromised.

Whichever myofibrillar component of the muscle cell is target for modification, it is important to consider the implications that it will have on both contraction and relaxation of the heart. Agents which improve systolic function at the expense of worsening diastolic function may ultimately impair pump performance and lower cardiac output.

References

1. Perspectives in Cardiovascular Research; Myocardial Hypertrophy and Failure, Vol. 7, ed. Norman R. Alpert, Raven Press, NY 1983.
2. Malholtra, A., Penpargkul, Fein, F. Sonnenblick, E.H. and Scheuer,J. Cir. Res. 49: 1243-1250, 1981.

3. Penpargkul, S., Fein, F., Sonnenblick, E.H. and Scheuer, J. J. Mol. Cell. Cardiol., 13: 303-309, 1981.
4. Martin, A.F., Pagani, E.D., Solaro, R.J. Cir. Res. 50: 117-124, 1982.
5. Suko, J. J. Physiol. 228: 563-582, 1973.
6. Pagani, E.D. and Solaro, R.J. Am. J. Physiol. 247: 909-915, 1984.
7. Penpargkul, S., Schaible, T., Yiptinsoi, T., and Scheuer, J. Circ. Res. 47: 911-921, 1980.
8. Fein, F.S., Miller-Green, B. and Sonnenblick, E.H. Am. J. Physiol. 248: 729-736, 1985.
9. Pagani, E.D. and Julian, F.J. Circ. Res. 54: 586-594, 1984.
10. Potter, J.D., Holroyde, M.J., Robertson, S.P., Solaro, R.J., Kranias, E.G. and Johnson,J.D. In: Muscle and Non-Muscle Motility, eds. Dowben, R.M. and Shay,J.W., Plenum, New York, 1982.
11. Holroyde, M.J., Robertson, S.P., Johnson, D.J., Solaro, R.J. and Potter, J.D. J. Biol.Chem. 255: 11688-11693, 1980.
12. Hasselgrove, J.C. Cold Spring Harbor Sym. Quant. Biol. 37: 361-376, 1972.
13. Solaro, R.J., Martin, A.F., Pagani, E.D. In: Myocardial Hypertrophy and Failure (Perspectives in Cardiovascular Research), Vol. 7, ed. N.R. Alpert, Raven Press, New York, 1983, p. 541-552.
14. Allen, D.G. and Kentish, J.C. J. Mol. Cell Cardiol. 17: 821-840, 1985.
15. Holroyde, M.J., Howe, E. and Solaro, R.J. Biochim. Biophys. Acta 586: 63-69, 1979.
16. Robertson, S.P., Johnson, J.D., Holyrode, M.J. Kranias, E.G., Potter, J.D. and Solaro, R.J. J.Biol. Chem. 257: 260-263, 1982.
17. Blanchard, E.M. and Solaro, R.J. Circ. Res. 55: 382-391, 1984.
18. Ricciardi, L., Bucx,J.J.J., and Ter Keurs, HEDJ, Cardio. Res. 20: 117-123, 1986.
19. Matthews, P.M., Taylor, D.J. and Radda, G.K. Cardiol. Res. 20: 13-19, 1986.
20. Gordon, A.M., Huxley, A.F. and Julian, F.J. J. Physiol. 184: 170-192, 1966.
21. Julian, F.J. and Sollins, M.R. Circ. Res. 37: 299-308, 1975.
22. Allen, D.G., Jewell, B.R. and Murray, J.W. Nature, 248: 606-607, 1974.
23. Brutsaert, D.L. and Paulus, W.J. Cardio. Res. 11: 1-16, 1977.
24. Barany, M. J. Gen. Physiol. 50: 197-218, 1967.
25. Swynghedauw, B. Physiol. Rev. 66: 710-711, 1986.
26. Chizzonite, R.A., Everett, A.W., Clark, W.A., Jakovoic, R., Rabinowitz, M., and Zak, R. J. Biol. Chem. 257: 2056-2065, 1982.
27. Hoh, J.F.Y., McGrath, PA., Hale, P.T. J. Mol. Cell. Cardiol. 10: 1053-1076, 1978.
28. Pagani, E.D., Solaro, R.J. Am. J. Physiol. 245: 713-720, 1983.
29. Nakanishi, T., Nagar, M. and Takao, H. Circ. Res. 58: 890-895, 1986.
30. Schwartz, J., LeCarpentier, Y., Martin, J.L., Lompre, A.M., Mercardier, J.J., and Swynghedaww, B. J. Mol. Cell. Cardiol. 13: 1071-1075, 1981.
31. Ebrecht, G., Rupp, H. and Jacob, R. Basic Res. Cardiol. 77: 220-234, 1982.
32. Pagani, E.D., Shemin, R., Julian, F.J. J. Mol. Cell. Cardiol. 18: 55-66, 1986.
33. Alpert, N.R. and Mulieri, L.A. Fed. Proc. 41: 192-198, 1982.
34. Woledge, R.C., Curtin, N.A. and Homsher, E. Energetic aspects of muscle contraction; Monographs of the Physiological Society, No. 41, Academic Press, London, 1985.
35. Suga, H., Igarashi, Y., Yamada, O. and Goto, Y. Heart and Vessels, 1: 3-8, 1985.
36. Winegrad, S., McClellan, G., Tucker, M. and Lin, L.E. J. Gen. Physiol. 81: 749-765, 1985.

37. Sweeny, H.L. and Stull, J.T. Am. J. Physiol. 250: 657-660, 1986.
38. Takeda, N., Rupp, H., Fenchel, G., Hoffmeister, H-E. and Jacob, R. Jpn. Heart J. 26(6): 909-922, 1985.
39. Mercadier, J.-J., Bouveret, P., Gorza, L., Schiaffino, S., Clark, W.A., Zak, R., Swynghedauw, B. and Schwartz,K. Circ. Res. 53: 52-62, 1983.
40. Solaro, R.J., Kumar, P., Blanchard, E.M. and Martin, A.F. Circ. Res. 58: 721-729, 1986.
41. Allen, D.J. and Kurihara, S. J. Physiol. 27: 79-94, 1982.

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THE ROLE OF THE LIPID BILAYER IN AMPHIPHILE-MEMBRANE/RECEPTOR INTERACTIONS: A UNIFYING HYPOTHESIS

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Introduction

The precise nature of the interaction of amphiphilic[1] substances, such as drugs, with native biological membranes is complex for a variety of reasons, not the least of which is the number of potential membrane sites. Certainly, small molecular substances with different chemical constituents may interact preferentially with one or more different regions of a biomembrane. These regions include but are not limited to (1) the bulk lipid matrix; (2) the lipid annular region of an integral membrane protein; (3) the hydrophobic and (4) the hydrophilic regions of a membrane protein; and (5) the glycolsylated moieties attached to both lipid and protein components.

The interaction of substances, like drugs, may represent a nonspecific binding of the drug to one or more of these sites or it may involve a site selective specific interaction such as the binding of a drug to a protein receptor site. Whatever the nature of this interaction, it is crucial to determine molecular parameters governing the mechanisms of drug entry into the membrane and perturbations of membrane structure following this event. With the advent of such biophysical probes as x-ray and neutron diffraction, spectroscopy and

[1]Amphiphile, as used in this text, refers to small molecular substances which possess both hydrophilic and hydrophobic properties.

molecular modeling, it is now possible to obtain these molecular parameters.

Recently, two emerging hypotheses have been described which, taken together, offer the possibility of providing a molecular framework to test different aspects of both nonspecific and specific interactions of small amphiphilic substances with biomembranes. The first of these hypothesis is currently being quantitatively evaluated and describes how a small amphiphile (e.g. a drug) may reach specific sites on proteins contained within the membrane bilayer. The membrane bilayer pathway hypothesis (1,2), stated simply, describes how a lipophilic drug substance could locate both nonspecific and specific sites on membrane proteins embedded in a lipid bilayer matrix. The drug substance could partition into the membrane bilayer where it adopts a well defined position along the membrane bilayer axis.[2] At this position, the drug could assume both a well defined orientation (with respect to the bilayer axis) and conformation. Lateral diffusion of the drug along a plane at this depth within the membrane bilayer, to a protein also contained in the bilayer, could then lead to either a nonspecific or specific interaction (see Figure 1). If the drug binds

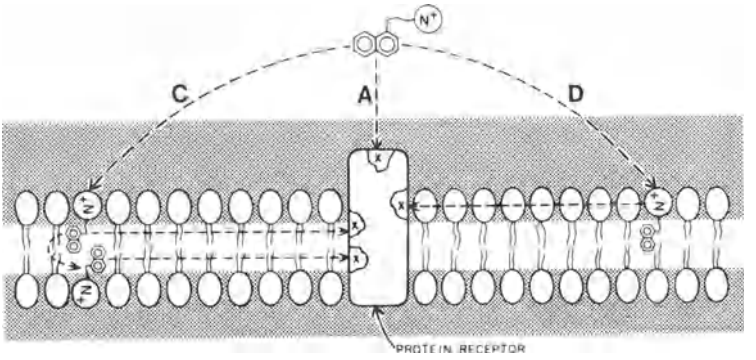

Figure 1

[2]The membrane bilayer axis is taken to be that axis perpendicular to the plane of the phospholipid bilayer.

to a specific site on a protein receptor, the interaction is completed and other events that modulate or regulate the protein's function may be essentially internal to the protein. If, however, there is no specific site on a protein to which the drug will bind, it may still exert some effect on the functioning of the protein in a nonspecific fashion. What happens in this case may be understood in light of the second hypothesis currently being tested, namely, the intrinsic radius of curvature hypothesis (3). Bilayers are characterized by a parameter, called the intrinsic radius of curvature, which basically measures an internal stress in the lipid layer (4). This parameter is related to the proclivity of the lipid system to assume non-bilayer configurations, such as the H_{II} phase (Figure 2), and may be experimentally measured, via x-ray and neutron diffraction, by determining the structure of lipid phases (5,6). By altering the composition of the bilayer, the intrinsic radius of curvature may be varied over a wide range. Experiments which correlate the composition of bilayers with the operation of certain intrinsic membrane proteins suggest that the proteins require a limited range of values of the bilayer intrinsic radius of curvature for optimal function. Amphiphilic substances are potent modifers of the bilayer intrinsic radius of curvature and may act, via this parameter, to non-specifically perturb

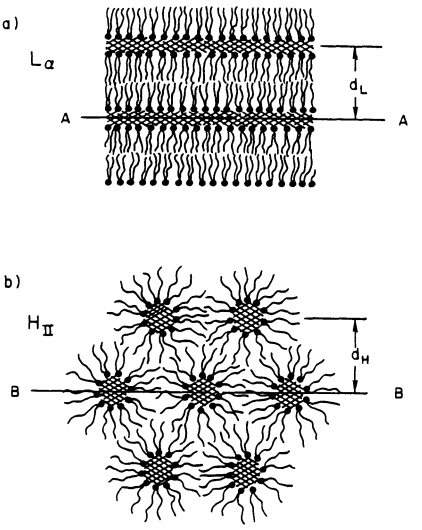

Figure 2

membrane protein function. The intrinsic radius of curvature hypothesis simply stated, identifies a hitherto unrecognized internal parameter of the bilayer which correlates with membrane function and which is readily altered by amphiphilic compounds.

Thus, the combination of these two hypotheses may allow a molecular definition of amphiphile/membrane/receptor interactions. The membrane bilayer pathway hypothesis describes how an amphiphile may use a nonspecific medium to interact with a protein, whereupon the protein's function may be modulated by a specific event or by a nonspecific mechanism according to the intrinsic radius of curvature hypothesis. This conceptual framework could be applicable to the membrane interactions of drugs, toxins, small polypeptides and other small molecular substances.

Implications of the Membrane Bilayer Pathway Hypothesis

The membrane bilayer pathway model as a mechanism for the way in which calcium antagonists and other lipophilic cardiovascular agents "locate" and bind to their protein receptors in the heart has been previously discussed (1,2,7). It may also be applicable to other ligand/membrane receptor systems where the ligand is lipophilic. Presently, whether or not this is the actual mechanism for such interactions, the membrane bilayer model does offer a conceptual framework for testing new concepts and rationalizing drug/membrane effects.

For example, in Figure 3, a comparison of the ability to remove amiodarone (a sodium channel inhibitor), nimodipine (a 1,4-dihydropyridine calcium channel antagonist), and propranolol (a beta adrenergic antagonist) from the sarcoplasmic reticulum membrane following incubation of these drugs with this membrane is shown. Under

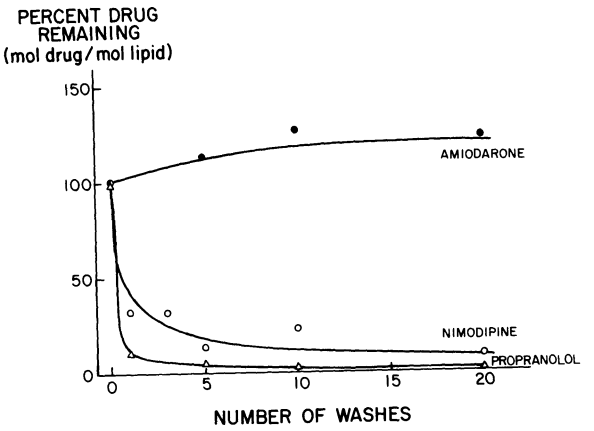

Figure 3

these conditions, amiodarone remains in the membrane bilayer whereas nimodipine and propranolol can be removed to negligible levels. Figure 4 provides a summary of the locations of these drugs in lipid bilayers utilizing either x-ray or neutron diffraction. Interestingly, amiodarone is buried deep within the lipid bilayer near the terminal methyl region, consistent with its negligible removal from the bilayer as indicated by the "washout" experiment in Figure 3. Nimodipine and propranolol are located near the hydrocarbon core/water interface, and can be easily washed out of the lipid bilayer. By comparing the

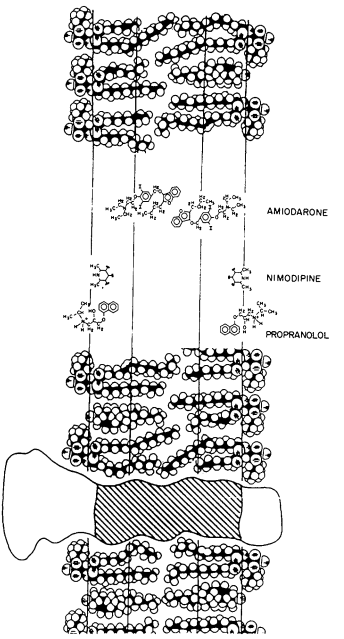

Figure 4

clinical half lives for activity in Table 1 with these results, it is reasonable to conclude that the pharmacokinetic properties of the drugs at the molecular level which may be related to the duration of clinical activity may at least depend upon the location of the drug in the lipid bilayer.

Table 1 Clinical Halflives of Activity

Amiodarone	>1,000[1]
Nimodipine	5.5[2]
Propranolol	3.9[1]

[1]Goodman and Gilman, 7th Edition
[2]Janis, R.A., Personal Communication

X-ray and neutron diffraction can provide valuable information regarding the interaction of drugs with membranes with molecular resolution. Apart from the significance in determining mechanisms for drug-membrane and drug-receptor actions, these molecular data can be used to define the activity and selectivity of drug substances. A drug that does not penetrate to the proper depth within the membrane bilayer (drug Y in Figure 5B) would not be as active or possibly inactive compared to drug X which, at the proper depth within the membrane bilayer, can diffuse to the receptor site and participate in a successful interaction. This feature of the membrane bilayer model may also accomodate a selection for activity, whereby only a portion of the drug structure is the "active site" so that a hydrophobic (that portion of the drug within the hydrocarbon core) or a hydrophilic (that portion of the drug within the aqueous region of the membrane bilayer) interaction may occur. The limited amount of information to date indicates that cardiovascular agents have a precise location (i.e. depth of penetration) within the membrane bilayer. The membrane bilayer model imparts another form of selectivity related to the optimal orientation (Figure 5C) and conformation (Figure 5E) within the

membrane. Drug Y in Figure 5C and E would not be active since its orientation or conformation respectively does not match that of the receptor site whereas drug X does. The limited amount of structure data to date also points to a precise orientation and conformation of these cardiovascular agents within the membrane bilayer. Finally, the partial reactivity of some drugs may be rationalized as a distribution of the drug along the profile axis where the most probable location

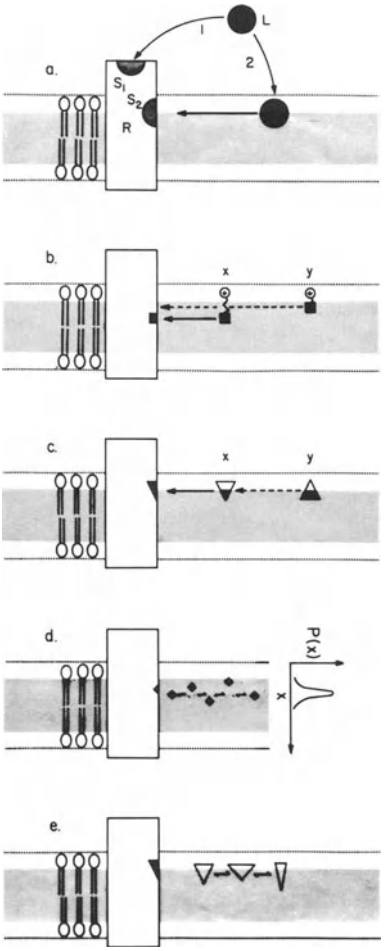

Figure 5

(center of the gaussian distribution) may not coincide precisely with the location of the protein receptor site (Figure 5D).

These approaches to understanding drug/membrane/receptor interactions may eventually play a role in allowing new breakthroughs in drug design (see Figure 6). Single crystal studies of drug

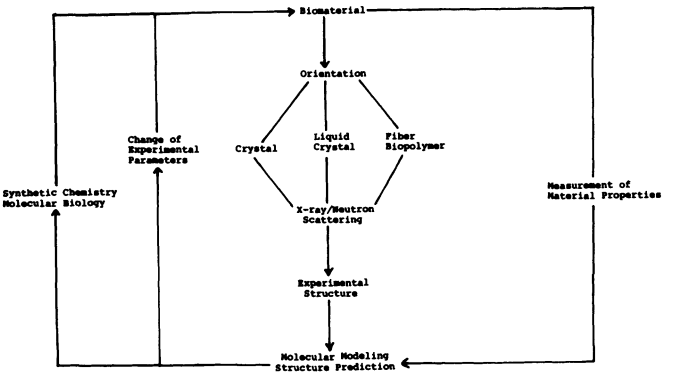

Figure 6

structures (obtained by both x-ray and neutron diffraction) could be combined with drug structures (again obtained by x-ray and neutron diffraction) as determined directly in biological membranes. These experimentally determined structures for the drug in crystal and membrane bound form could be inputed into molecular modeling schemes. Based on the study of several drug substances in a particular class, similarities and/or differences of the drug structures in the crystal and membrane bound forms in addition to their structure when bound at a protein receptor site, will allow calculations for potential site reactivity and selectivity. Optimized drug structures calculated in this manner would then be determined, completing the cycle. This cyclic scheme of inputting experimentally determined drug structures

(crystalline vs. membrane bound form) into molecular modeling programs and predicting new structures should allow a rapid conversion of optimizing therapeutic agents within a particular class of drugs and, possibly, the prediction of new drug classes with clinical potential.

Implications of the Radius of Curvature Hypothesis

The intrinsic radius of curvature, R_o, is a lipid layer parameter which essentially measures the average mismatch between the minimum free energy projected areas of the hydrophilic and hydrophobic portions of the lipid molecules. For many common membrane lipids, such as unsaturated phosphatidylethanolamines, this mismatch is large, corresponding to small values of R_o and large internal strains in the bilayer. For other common lipids, such as phosphatidylcholines, R_o is large, i.e., the bilayer is relaxed. Morever, R_o is a bulk parameter of the lipid layer and results from contributions of all the molecules in the layer. Thus, there are numerous lipid mixtures which result in a given value of R_o. Bilayers characterized by widely different R_o values present qualitatively and quantitatively different environments to intrinsic membrane proteins. The important implication is that, insofaras proteins are sensitive to the environmental variations measured by R_o, the bilayer composition non-specifically affects the functioning of the membrane proteins. It is also likely that the relative partitioning of amphiphiles between different membranes is dependent on their R_o values.

Amphiphilic drugs which partition into bilayers also contribute to R_o and substantially alter its value. This is the case for many anesthetics at physiologically relevant concentrations. Moreover, the affinity of the bilayer for the amphiphile is itself dependent on R_o. This suggests a rational approach for the design of lipophilic drugs.

There are two cases to be considered. The first case is where the target proteins exhibit a function which is sensitive to the environmental conditions measured by R_o. In this case, an understanding of the way different molecules affect R_o outlines a set of molecular design criteria and a specific set of measurements to be performed in vitro to optimize efficacy of the drug. This is also the case of non-specific drug interactions which typically requrie non-trivial (>1 mole %) membrane concentrations of the amphiphile. Moreover, such drugs may be expected to exhibit a spectrum of side effects resulting from the alteration of R_o and the concommitant effects on a variety of proteins.

A second case is where a drug-membrane protein site-specific interaction is involved, but where the site is accessible only after the drug has partitioned into the bialyer in a specific orientation. Because of the specificity of the interaction, this case may require only very small membrane concentrations of the amphiphile. Here, an understanding of the relationship between R_o and the relative bilayer affinity of the drug can be used to optimize insertion of the drug into the target membrane. This couples to the membrane pathway hypothesis discussed, above.

Summary

The interaction of amphiphiles with biomembranes at the molecular level is complex. This molecular interaction is further complicated by the fact that amphiphiles, such as drugs, have both nonspecific and specific interactions which may lead to different effects. By definition, specific interactions usually involve binding of ligands to site selective regions on protein receptors; nonspecific interactions may be classified as all other types of interactions that occur in

biomembranes (amphiphile-lipid bilayer or non-receptor protein interactions). We have proposed two emerging molecular hypotheses to aid our understanding of both nonspecific and specific amphiphile/-membrane interactions. The membrane bilayer pathway hypothesis stated simply, describes how amphiphiles, such as drugs, may locate specific receptor proteins by first partitioning into the membrane lipid bilayer, where the amphiphile (drug) assumes a well defined location, orientation and conformation, and then diffuses laterally through a plane in the bilayer to a protein receptor site. The intrinsic radius of curvature hypothesis, stated simply, describes how amphiphiles incorporated into the membrane bilayer, may distort the intrinsic radius of curvature from some optimal value to cause perturbations which may affect membrane protein operation. Taking these two hypothesis together, an amphiphile could either enter the bilayer and laterally diffuse to a protein receptor (specific interaction) or non-receptor protein (nonspecific interaction) and/or alter bilayer parameters to cause either specific or nonspecific perturbations to the functioning of the protein. At the very least, we can begin to rationalize the diverse effects of amphiphiles with membranes by involving a role for the membrane bilayer such that amphiphiles may follow nonspecific pathways to elicit both nonspecific and specific effects.

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1. Rhodes, D.G., Sarmiento, J.G., and Herbette, L.G. Mol. Pharmacol. 27:612-623, 1985.
2. Herbette, L.G., Chester, D.W., and Rhodes, D.G. Biophys. J. 49:91-94, 1986.
3. Gruner, S.M. Proc. Natl. Acad. Sci. 82:3665-3669, 1985.
4. Kirk, G.L., Gruner, S.M., and Stein, D.L. Biochem. 23:1093-1102, 1984.
5. Kirk, G.L. and Gruner, S.M. J. de Phys. 46:761-769, 1985.
6. Tate, M. and Gruner, S.M. Biochem. (in press).
7. Herbette, L.G. In: The Heart and Cardiovascular System (Eds. Fozzard, H.A., Haber, E., Jennings, R.B., Katz, A.M., and Morgan, H.E.), Raven Press, 1986.

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FORSKOLIN, A NEW INOTROPIC AGENT

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Forskolin is a labdane diterpene obtained from the plant Coleus Forskohlii. Forskolin probably acts by activation of adenylcylase. Forskolin was infused intravenously in patients with low cardiac output, commencing with 0.1 ug/kg/minute in Hemaccel solution. The rate was doubled every 2 minutes to a maximum of 1.6 ug/kg/Min. Ventricular functions was evaluated by a nuclear stethoscope before infusion and 5, 10, 15 and 50 Minutes after stopping the infusion. The cardiovascular parameters included heart rate, systolic blood pressure, diastolic blood pressure, ejection fraction, cardiac output, peak ejection rate, peak filling time and overall filling time. The ejection fraction, in 8 patients, rose from the mean basal value of 23.10± 2.37 to 38±3.59($P < 0.001$). There were also significant increases in ejection velocity and filling velocity. These effects were seen at a dose of 8-16 ug/kg/10minutes. No significant chronotropic or barotropic effects were noted. Forskolin was extremely well tolerated at this dose.

INTRODUCTION:

It has been recognised for a long time that the strength of heart muscle contraction is controlled by changes in cytosolic Ca^{++} levels which are in turn governed by the interaction of endogenous catecholamines with cardiac β -receptors. Increase in cellular cyclic AMP level normally accompany β - receptor activation. Indeed, inotropic action is closely connected with cyclic APM or the second messenger.

Forskolin is a labdane diterpene compound isolated from Coleus Forskohlii.(Fig. 1) In isolated papillary muscles of the rabbit Rodger and Shahid showed that Forskolin induces concentration -related positive inotropic response and that this was related to the intracellular cyclic AMP levels (2). Metzger and Lindner showed in 1981 that Forskolin is an activator of adenylate cyclase in rabbit heart membranes (1). Furthermore, Seamon and Daly were able to show that Forskolin could activate adenylate cyclase directly in the absence of guanine nucleotide regulatory protein(3).

FORSKOLIN

(A LABDANE DITERPENE)

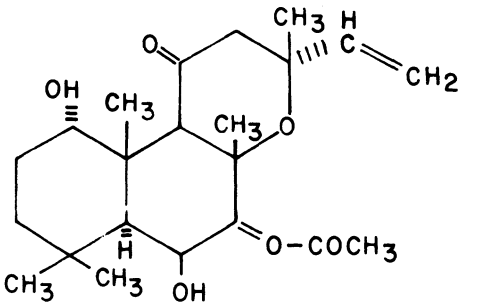

Fig. 1.

The aim of the present study was to confirm in humans the positive inotropic properties of Forskolin. In ten patients with clinical and echo-cardiographic proof of poor LV function and low cardiac output, the drug was given intravenously by drip method with graded doses increasing every 10 minutes.

METHOD:

The nuclear stethoscope (Bios, New York) consists of an ECG-gated scintillation probe with a dedicated microprocessor programmed to measure hemodynamic data in real time. Red cells are labelled with 99^{m} technetium and various parameters of cardiac function are assessed on a beat-to-beat basis. These include heart rate, stroke volume, end-diastolic volume, ejection fraction, relative cardiac output, peak ejection and the peak filling rate (Fig.2).

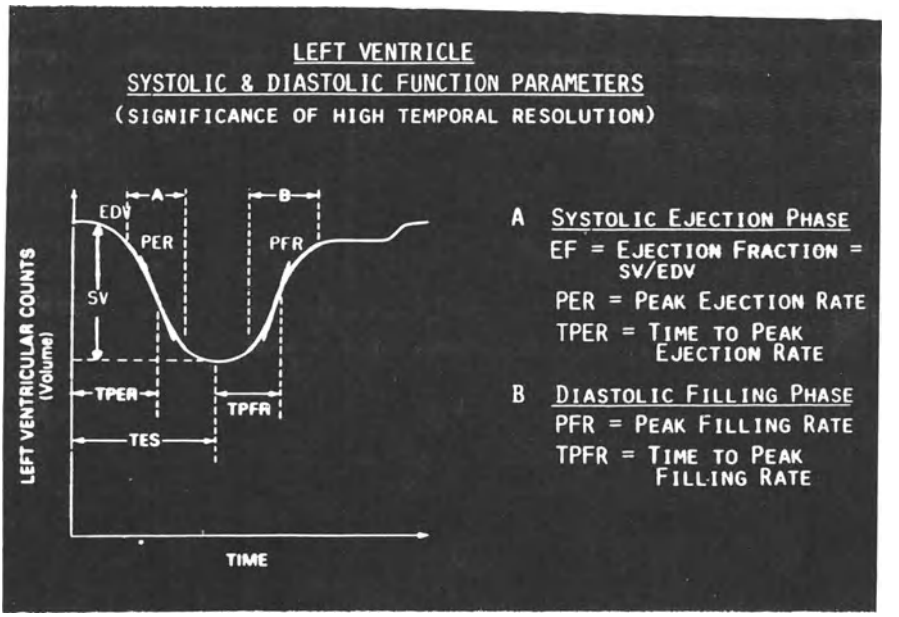

Fig. 2.

The inclusion criteria for the study were as follows:

1. Patients suffering from congestive cardiac failure due to ischemic heart disease, cardiomyopathy or rheumatic heart disease.
2. The diagnosis was established by clinical examination, X-ray Chest, ECG, 2-D Echo studies and lab. tests.
3. The age limit was 15-75 years.

Cases excluded from the study were those who were in renal or hepatic failure. Patients with concurrent serious illness like severe infection or cancer were excluded. Our list of selected patients contained two with dilated Cardiomyopathy, six with ischemic heart disease and two with rheumatic heart disease(Table I).

FORSKOLIN – INOTROPIC EFFECT

DEMOGRAPHY

PT. NO.	DIAGNOSIS	AGE (YRS.)	WEIGHT (Kg)	SEX
1.	CARDIOMYOPATHY	66	50	M
5.	CARDIOMYOPATHY	65	63	M
7.	I. H. D.	56	50	M
9.	I. H. D.	47	70	M
10.	I. H. D.	56	70	M
2.	C.C.F. [VALVULAR DISEASE]	22	40	M
3.	C.C.F. [VALVULAR DISEASE]	29	45	M
4.	I.H.D. + C.C.F.	59	70	M
6.	I.H.D. + C.C.F.	55	60	M
8.	I.H.D. + C.C.F.	61	52	F

Table I

Forskolin was given intravenously by a micro-drip set. The starting dose was 0.1 ug/kg/min. Every ten minutes the dose was doubled to a maximum of 1.6 ug/kg/min. The compound was dissolved in Haemaccel® (Hoechst polymer from degraded protein).

RESULTS:

Table II gives the effect of Forskolin infusion on the ejection fraction (EF). In all ten patients there was a significant increase in the EF (mean + SE increase from 23.10$\pm$ 2.37 to 38.30$\pm$3.59 $p < 0.001$ Table II). Fig. 3 & 4 depict the nuclear stethoscope derived data from a typical case before and after Forskolin (1.6 ug/kg/10Min.).

FORSKOLIN - INOTROPIC EFFECT
EJECTION FRACTION

PT. NO.	BASAL [%]	INCREASE [%]
1	14	32
5	24	34
7	19	32
9	31	56
10	30	52
2	22	29
3	20	38
4	10	20
6	29	40
8	32	50
MEAN + S.E.	23·10 ± 2·37	38·30 ± 3·59
	P < 0·001	

Table II

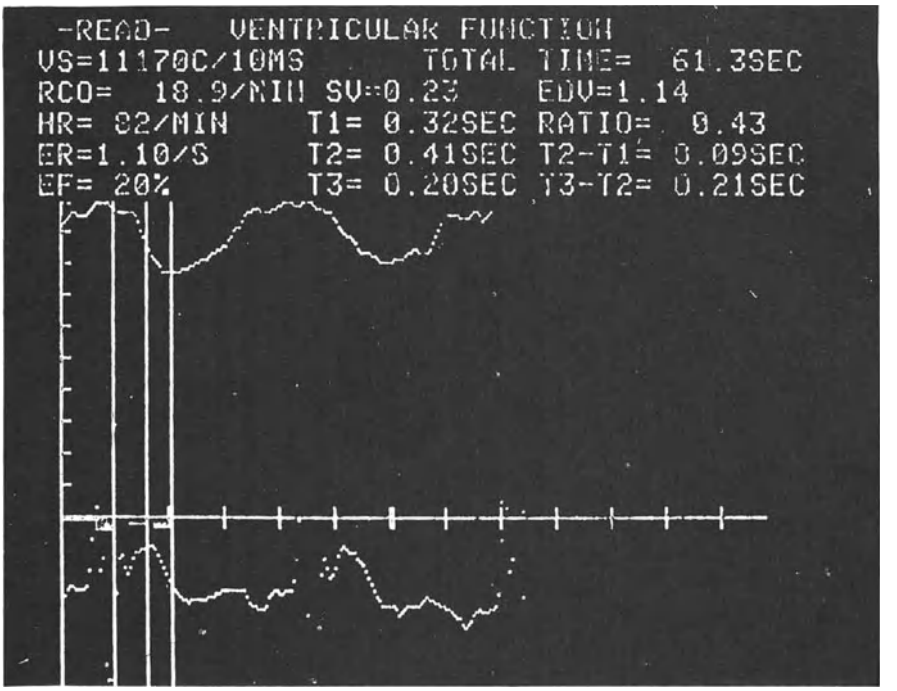
-READ- VENTRICULAR FUNCTION
VS=11170C/10MS TOTAL TIME= 61.3SEC
RCO= 18.9/MIN SV=0.23 EDV=1.14
HR= 82/MIN T1= 0.32SEC RATIO= 0.43
ER=1.10/S T2= 0.41SEC T2-T1= 0.09SEC
EF= 20% T3= 0.20SEC T3-T2= 0.21SEC

Fig. 3 & 4.

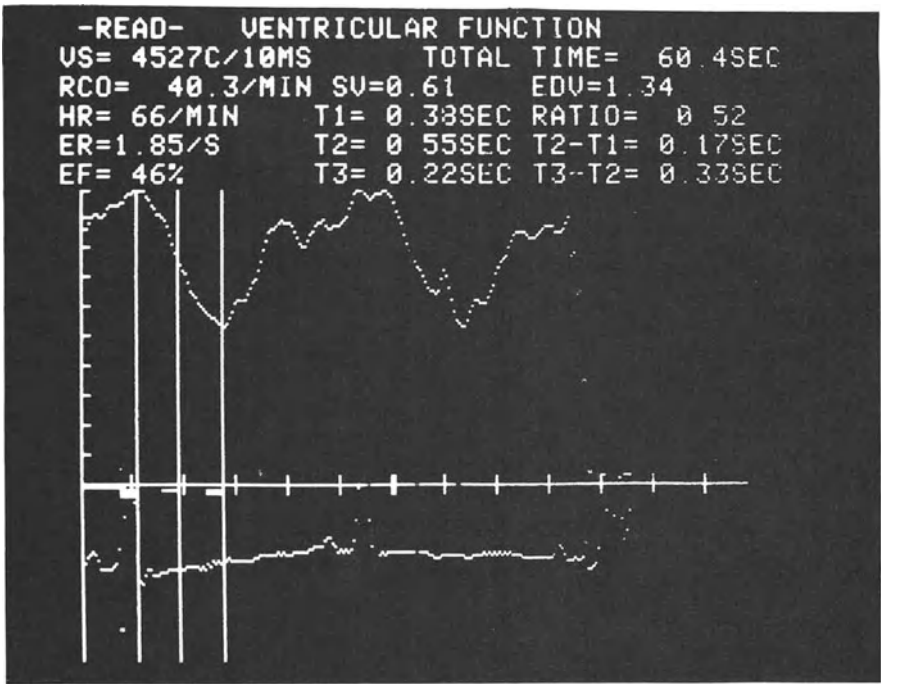
-READ- VENTRICULAR FUNCTION
VS= 4527C/10MS TOTAL TIME= 60.4SEC
RCO= 40.3/MIN SV=0.61 EDV=1.34
HR= 66/MIN T1= 0.38SEC RATIO= 0 52
ER=1.85/S T2= 0 55SEC T2-T1= 0.17SEC
EF= 46% T3= 0.22SEC T3-T2= 0.33SEC

Table III gives the details of a typical study. Note the excellent increase in ejection fraction and stroke volume with a slight increase in end-diastolic volume and heart rate. In every case there was also an increase in peak ejection rate and peak filling rate. In all of the ten cases there was no appreciable change in systolic or diastolic blood pressure from control values.

NUCLEAR STETHOSCOPE FINDINGS AFTER FORSKOLIN

PATIENT MRS. MARGARET

	BASAL	0·8 µg/Kg	1·2 µg/Kg	1·6 µg/Kg
HR	108	112	112	118
RCO	50·4	71·7	71·7	72·3
SV	0·42	0·64	0·72	0·76
EDV	1·29	1·30	1·41	1·41
EF	32	42	50	55
ER	1·8	2·05	3·00	3·05
FR	2·77	3·45	3·40	3·36

Table III

None of the patients suffered from any side effects. Forskolin was very well tolerated.

DISCUSSION:

The pronounced increase in ejection fraction stroke volume and peak filling rate without significant changes in blood pressure or heart rate clearly show that Forskolin has a definite positive inotropic effect in patients who have an impaired LV function with low cardiac output. The nuclear stethoscope has been shown to be a useful device in estimating the effect of a new inotropic drug during a continuous infusion[1].

CONCLUSIONS:

1. Forskolin produces clinically significant increase in ejection fraction, ejection velocity, and filling velocity.

2. Forskolin has significant inotropic effect at doses of 0.8 - 1.6 ug/kg/10 mts.

3. At the dose of 0.8 ug/kg/10mts, no chronotropic effect was seen and at 1.6 ug chronotropic effect was present.

4. The volume of infusion with the dose is very small. (approx. 0.5 - ml/mt.)

5. The drug was well tolerated at the dosage studied.

ACKNOWLEDGEMENTS:

We thank the Trustees of the Jaslok Hospital and Research Centre for the use of the nuclear stethoscope and Ms. Hoechst Pharmaceuticals for the generous supply of Forskolin ampoules.

REFERENCES:

1. Metzger H. and Lindner F. The Positive Inotropic - Acting Forskolin, a Potent Adenylatecyclase Activator. Arzneimittel-Forschung 31(11)8, 1248-1250 (1981).

2. Rodger, I.W. and Shahid, M. Forskolin, cyclic nucleotides and positive inotropism in isolated papillary muscles of the rabbit Brit. J. Pharmac 81, 151-159 (1984).

3. Seamon, K.B. and Daly, J.W. Forskolin: A Unique Diterpene activator of Cyclic AMP-Generating Systems. Journal of Cyclic Nucleotide Res. 7(4) : 201-224 (1981).

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DEPENDENCE OF VASOCONSTRICTOR RESPONSES ON EXTRACELLULAR CALCIUM

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INTRODUCTION

Vascular tone is regulated by metabolic, hormonal, ionic, and neuronal mechanisms. The adrenergic nervous system plays a major role in regulating vascular tone in physiologic and pathophysiologic states. Adrenergic stimulation increases vasoconstrictor tone by stimulating alpha adrenoceptors on the plasma membrane of resistance vessels. Recent studies have provided evidence that postsynaptic receptors with pharmacological properties similar to those of alpha-1 and alpha-2 adrenoceptors are present in the systemic and pulmonary vascular beds (1-6). It has been shown in in vivo studies that pressor responses to exogenous and neurogenically released norepinephrine may be mediated by different alpha adrenoceptor subtypes (2,7,8). In the canine hindlimb and pithed rat, responses to sympathetic nerve stimulation are selectively blocked by alpha-1 adrenoceptor antagonists whereas responses to exogenous norepinephrine are attenuated by alpha-2 adrenoceptor antagonists (2,7-9). It was, therefore, postulated that alpha-1 adrenoceptors are located intrasynaptically and are preferentially activated by neurogenically released norepinephrine whereas exogenous norepinephrine acts mainly on alpha-2 receptors which are located extrasynaptically (2,7,8). There are, however, exceptions to this concept in that neuronally released norepinephrine does not preferentially activate postjunctional alpha-1 adrenoceptors in isolated human blood vessels (10).

In addition to a possible different anatomic location of alpha-1 and alpha-2 adrenoceptors on the postjunctional membrane, it has been postulated that these receptor subtypes may be distinguished by the source of calcium ions utilized to elicit a pressor response (3,11-13). Calcium entry blocking agents have been reported to selectively inhibit pressor responses to agonists which stimulate alpha-2 adrenoceptors (3,11-13).

The purpose of the present investigation was to characterize the alpha adrenoceptor subtypes present in the feline mesenteric vascular bed in vivo and to determine which receptor subtypes are stimulated by exogenous and nerve released norepinephrine and which responses are influenced by calcium entry blockade. The results of these studies demonstrate that both receptor subtypes are present and that vasoconstrictor responses elicited by activation of both receptor subtypes are inhibited by calcium entry blockade.

METHODS

Experiments were performed in cats under conditions of controlled superior mesenteric blood flow so that changes in intestinal perfusion pressure directly reflected changes in intestinal vascular resistance. Briefly, animals were sedated with pentobarbital sodium, intubated, and femoral arterial and jugular venous catheters were positioned for measurement of systemic arterial pressure and administration of subsequent anesthetic. An extracorporeal circuit was created so that blood withdrawn from a common carotid artery was pumped under constant flow into the superior mesenteric artery (14). All agonists studied were administered as an intra-arterial injection in small volumes directly into the perfusion circuit whereas all antagonists were administered intravenously. Calcium entry blockers were infused directly into the perfusion circuit as well. Agonists used in the study were phenylephrine, a selective alpha-1 agonist; UK 14304, a selective alpha-2 agonist; norepinephrine; tyramine; angiotensin II; U46619, a thromboxane mimic; and Bay K 8644, a calcium channel agonist.

In experiments in which the sympathetic nerves were stimulated electrically, the nerve plexus of the superior mesenteric artery was carefully isolated and placed on a shielded Palmer electrode. The nerve was decentralized by crushing it proximally to the electrode and was stimulated with a Grass stimulator (SCMG) in a random sequence with square wave pulses, 2 msec duration, 12 volts, at 1, 3, and 10 Hz for periods of 30 sec.

In animals in which 6-hydroxydopamine was used to destroy the integrity of adrenergic terminals, the animals were treated with 6-hydroxydopamine HCl (Sigma), 100 mg/kg i.p., for 3 days and were studied 3-6 days later.

RESULTS

Intra-arterial injections of phenylephrine and UK 14304 caused dose-related increases in mesenteric perfusion pressure (Fig. 1). The

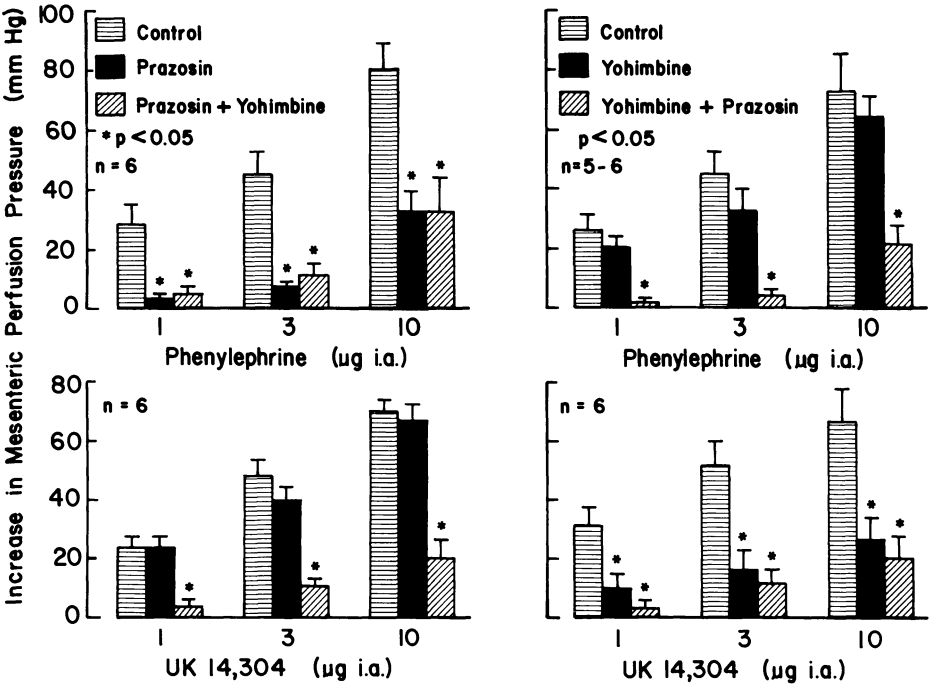

Fig. 1. Top left panels: effects of prazosin and of prazosin + yohimbine on increases in mesenteric arterial perfusion pressure in response to phenylephrine. Top right panels: effects of yohimbine and of yohimbine + prazosin on vasoconstrictor responses to phenylephrine. Lower left panels: influence of prazosin and of prazosin + yohimbine on responses to UK 14304. Lower right panels: effects of yohimbine and of yohimbine + prazosin on responses to UK 14304. n indicates number of animals and the asterisk indicates that the response is significantly different than the corresponding control.

effects of prazosin, an alpha-1 adrenoceptor antagonist, and yohimbine, an alpha-2 adrenoceptor antagonist, on responses to the alpha-adrenoceptor agonists were investigated and these data are shown in the left-hand panels of Fig. 1. Prazosin, 0.1 mg/kg i.v., significantly reduced the increases in mesenteric arterial pressure in response to phenylephrine

but had no significant effect on responses to UK 14304 (Fig. 1, top left panel). The administration of yohimbine, 1 mg/kg i.v., to the same animals that had received prazosin significantly reduced the mesenteric vascular response to UK 14304 but had no additional blocking effect on responses to phenylephrine (Fig. 1, left panel).

In the second group of cats in this series, the order of administration of prazosin and yohimbine were reversed. Yohimbine, 1.0 mg/kg i.v., had no significant effect on mesenteric vascular responses to phenylephrine; however, responses to UK 14304 were reduced significantly (Fig. 1, right panel). Subsequent administration of prazosin, 0.1 mg/kg i.v., to the same animals that had received yohimbine resulted in a significant decrease in the mesenteric vascular responses to phenylephrine but no additional effect on responses to UK 14304 (Fig. 1, right panel).

The results from experiments with phenylephrine and UK 14304 suggest the presence of alpha-1 and postjunctional alpha-2 adrenoceptors in the feline mesenteric vascular bed. Since experimental evidence suggests that alpha-2 adrenoceptors are located at both pre- and postjunctional sites (15), it is important to preclude a prejunctional effect of the alpha-2 agonist by pretreating the animals with 6-hydroxydopamine. Pretreatment with 6-hydroxydopamine significantly reduced the mesenteric vasoconstrictor response to tyramine. Following treatment with 6-hydroxydopamine, phenylephrine and UK 14304 produced similar increases in mesenteric perfusion pressure as observed in untreated animals (Fig. 1). In 6-hydroxydopamine treated animals, prazosin, 0.1 mg/kg i.v., significantly decreased vasoconstrictor responses to phenylephrine whereas responses to UK 14304 were unchanged. Subsequent administration of yohimbine to these same animals significantly decreased vasoconstrictor responses to UK 14304 with no further change observed in the vasoconstrictor response to phenylephrine. The effects of reversing the order of administration of the alpha-adrenoceptor blocking agents on vasoconstrictor responses were investigated in another group of cats pretreated with 6-hydroxydopamine. When yohimbine was given first, vasoconstrictor responses to UK 14304 were decreased significantly whereas responses to phenylephrine were not changed. After administration of prazosin to these same animals, vasoconstrictor responses to phenylephrine were reduced significantly.

Recent studies have provided evidence that vasoconstrictor responses elicited by alpha-2 adrenoceptor agonists may be dependent on the influx of extracellular calcium ions whereas alpha-1 mediated responses are not sensitive to blockade by calcium entry antagonist. In order to test this hypothesis in the feline mesenteric vascular bed, the effects of nitrendipine, a dihydropyridine calcium entry blocking agent, on vasoconstrictor responses to phenylephrine and UK 14304 were investigated. As in previous experiments, intra-arterial injections of phenylephrine and UK 14304 increased mesenteric arterial perfusion pressure in a dose-related manner (Fig. 2). During the infusion of nitrendipine, 1.0 µg/min, into the mesenteric vascular bed, vasoconstrictor responses to phenylephrine and to UK 14304 were decreased significantly (Fig. 2). Moreover, 60 min after the end of the nitrendipine infusion, vasoconstrictor responses to phenylephrine and UK 14304 returned to control value (Fig. 2). Nitrendipine caused a significant reduction in mesenteric and systemic arterial pressures whereas infusion of the nitrendipine vehicle had no significant effect on vascular pressures.

It has been postulated that responses to sympathetic nerve stimulation result mainly from activation of alpha-1 adrenoceptors whereas responses to exogenous norepinephrine are due mainly to an effect on postjunctional alpha-2 receptors. In order to determine which receptor subtypes released by electrical excitation of the sympathetic nerves and exogenous norepinephrine act on, the effects of prazosin and yohimbine were investigated in two groups of cats and these data are presented in Fig. 3. In the first group of cats, responses to electrical stimulation of the sympathetic nerves were reduced significantly after administration of prazosin, 0.1 mg/kg i.v., whereas responses to norepinephrine were not changed significantly (Fig. 3, left panels). Subsequent administration of yohimbine, 1 mg/kg i.v., to these same animals resulted in a significant decrease in mesenteric vasoconstrictor responses to norepinephrine whereas no additional blocking effect was observed on responses to electrical stimulation of the sympathetic nerves (Fig. 3, left panels). In a second group of cats, administration of yohimbine, 1 mg/kg i.v., significantly decreased mesenteric vasoconstrictor responses to norepinephrine whereas responses to electrical stimulation of the nerves were not changed significantly (Fig. 3., right panels).

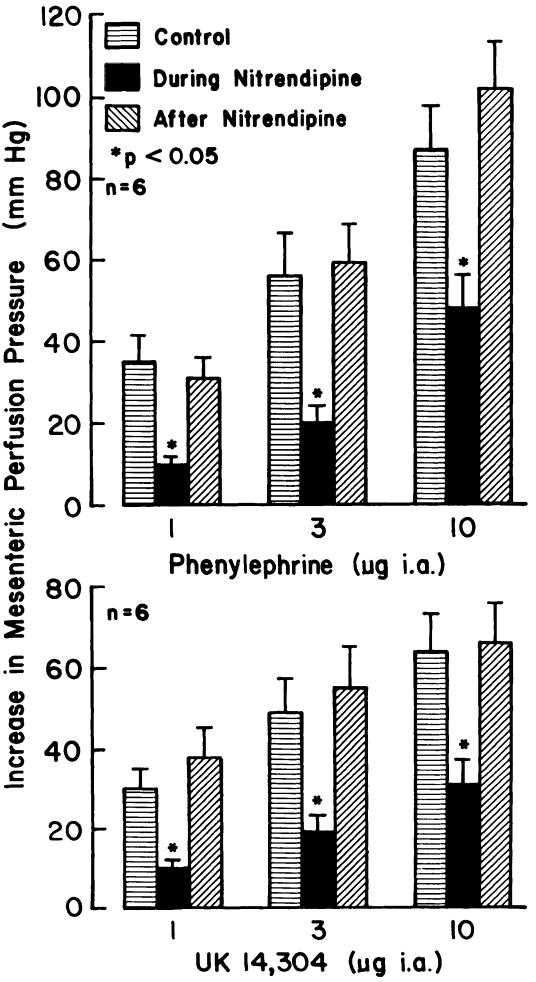

Fig. 2. Effects of intra-arterial infusion of nitrendipine, a calcium entry blocking agent, 1 µg/min, on increases in mesenteric perfusion pressure in response to phenylephrine (upper panel) and UK 14304 (lower panel). Responses to phenylephrine and UK 14304 were measured before, during, and 60 min after termination of the nitrendipine infusion. n indicates number of animals and the asterisk denotes that the response is significantly different than control.

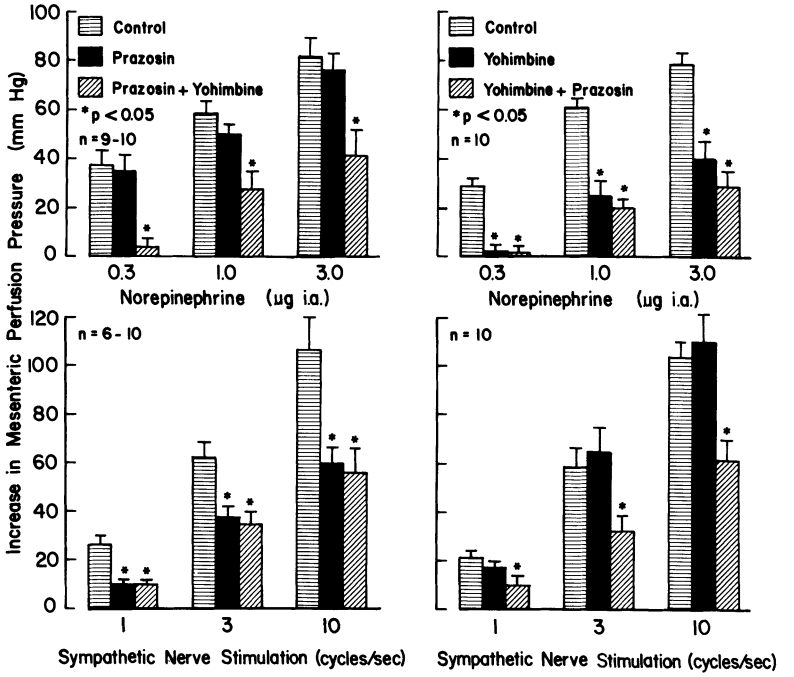

Fig. 3. Top left panels: effects of prazosin and of prazosin + yohimbine on increases in mesenteric arterial perfusion pressure in response to norepinephrine. Top right panels: effects of yohimbine and of yohimbine + prazosin on responses to norepinephrine. Lower left panels: influence of prazosin and of prazosin + yohimbine on responses to electrical stimulation of the sympathetic nerves. Lower right panels: effects of yohimbine and of yohimbine + prazosin on responses to electrical stimulation of the sympathetic nerves. n indicates number of animals and the asterisk denotes that the response is significantly different than control.

Administration of prazosin, 0.1 mg/kg i.v., to these same animals significantly decreased mesenteric vasoconstrictor responses to electrical excitation of the nerves. The release of norepinephrine from sympathetic nerve terminals by electrical excitation of the nerves can be modified by agonists and antagonists which act on presynaptic alpha-2 receptors. However, norepinephrine released by tyramine is not subject to autoreceptor regulation. Therefore, the effects of yohimbine

on responses to tyramine were investigated in the mesenteric vascular bed in another series of animals. Intra-arterial injections of tyramine caused dose-dependent increases in mesenteric perfusion pressure. Vasoconstrictor responses to tyramine were decreased significantly by yohimbine, 1 mg/kg i.v., and were decreased to an even greater extent by the administration of prazosin, 0.1 mg/kg i.v., in these same animals. Moreover, in other animals when prazosin was administered first, responses to tyramine were reduced significantly and were decreased to an even greater extent by yohimbine. To ascertain the extent to which responses to tyramine were indirectly mediated in the mesenteric vascular bed, the effects of cocaine were investigated. The administration of cocaine, 5 mg/kg i.v., markedly reduced responses to tyramine but had no significant effect on responses to UK 14304 or phenylephrine.

In order to ascertain if extracellular calcium is required for mesenteric vasoconstrictor responses to sympathetic nerve stimulation and norepinephrine, the effects of nitrendipine, a calcium entry blocking agent, were investigated. In these experiments, a dose-response curve for norepinephrine and a frequency-response curve for electrical stimulation of the sympathetic nerves were obtained, and nitrendipine was infused into the mesenteric perfusion circuit at a rate of 1 μg/min. Responses to nerve stimulation and norepinephrine were determined 5-10 min after the onset of the nitrendipine infusion and again 60 min after the end of the infusion. During infusion of the calcium entry blocking agent, mesenteric vasoconstrictor responses to nerve stimulation and exogenous norepinephrine were decreased significantly and responses returned to control value 60 min after the termination of the infusion.

The influence of the calcium entry blocking agent on responses to agonists which induce vasoconstriction by way of receptor-operated and voltage-dependent mechanisms and which release norepinephrine by a calcium-independent process were also investigated in another series of animals and these data are summarized in Fig. 4. During infusion of nitrendipine, 1 μg/min, mesenteric vasoconstrictor responses to KCl, U46619, tyramine, and angiotensin II were reduced significantly and responses to these agents returned to baseline value 60 min after the nitrendipine infusion was terminated (Fig. 4). The vasoconstrictor response to intra-arterially injected KCl was not dependent on the

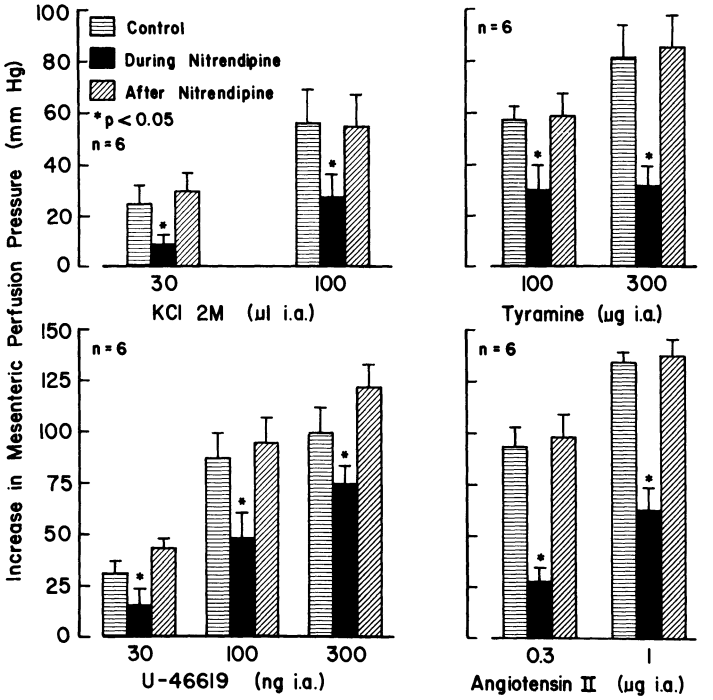

Fig. 4. Effects of intra-arterial infusion of nitrendipine, 1 g/min, on increases in mesenteric arterial perfusion pressure in response to KCl 2 M (top left), tyramine (top right), U46619 (bottom left), and angiotensin II (bottom right). Responses to KCl, tyramine, U46619, and angiotensin II were obtained before, during, and 60 min after termination of the nitrendipine infusion. n indicates number of cats and the asterisk denotes that the response is significantly different from control.

activation of alpha adrenoceptors since responses to depolarizing agent were not modified by phenoxybenzamine in a dose (5 mg/kg i.a.) which reversed the vasoconstrictor response to norepinephrine. Although the alpha blocking agent had no significant effect on the response to norepinephrine, subsequent infusion of nitrendipine, 1 µg/min, into the mesenteric vascular bed significantly reduced the responses to norepinephrine.

DISCUSSION

Results of the present study in the cat show that phenylephrine and UK 14304, alpha-1 and alpha-2 adrenoceptor agonists, increase mesenteric arterial perfusion pressure in a dose-related manner. Inasmuch as blood flow to the intestinal vascular bed was maintained constant, the increases in perfusion pressure indicate that both types of agonists cause vasoconstriction. Vasoconstrictor responses to phenylephrine were blocked in a selective manner by prazosin, whereas responses to UK 14304 were selectively blocked by yohimbine. Moreover, similar data were obtained in experiments in which 6-hydroxydopamine was used to destroy the integrity of adrenergic terminals and exclude a presynaptic action of the alpha-2 adrenoceptor agonists and antagonists (6,16,17). The present data suggest the presence of postjunctional receptors mediating vasoconstriction in the mesenteric vascular bed with pharmacologic characteristics similar to alpha-1 and alpha-2 adrenoceptors (1,4,18,19). These data support previous work showing the presence of alpha-2 adrenoceptors in the peripheral and pulmonary vascular beds and suggest that the magnitude of the alpha-2 receptor mediated vasoconstriction in the mesenteric vascular bed may be greater than observed in other organ systems (1,4,6,18,19).

In addition to suggesting the existence of postjunctional alpha-1 and alpha-2 adrenoceptors, the present data demonstrate that vasoconstrictor responses to electrical excitation of the sympathetic nerves are blocked by prazosin whereas responses to exogenous norepinephrine are blocked by yohimbine. These data support the hypothesis that responses to exogenous norepinephrine in the systemic vascular bed are mediated mainly by stimulation of postjunctional alpha-2 adrenoceptors whereas responses to norepinephrine released by nerve impulses are due, for the most part, from activation of alpha-1 receptors (2,7-9). However, the receptor subtype stimulated by exogenous norepinephrine may not be similar in all organ systems. Recent studies have shown that vasoconstrictor responses to exogenous norepinephrine in the lung are due mainly to activation of alpha-1 adrenoceptors (6,20). Thus, in the cat pulmonary and mesenteric vascular bed, responses to norepinephrine are mediated differently. Tyramine also releases norepinephrine from adrenergic terminals (21-23). However, the effects of prazosin on vasoconstrictor responses to tyramine and to sympathetic nerve stimulation

are different in the mesenteric vascular bed. Whereas prazosin, but not yohimbine, was effective in reducing responses to electrical excitation of the sympathetic nerves, prazosin and yohimbine had similar blocking effects on the response to tyramine. These data suggest that norepinephrine released by tyramine may act on both alpha-1 and postjunctional alpha-2 adrenoceptors whereas transmitter released by nerve excitation only acts on alpha-1 receptors. The explanation for the differences in the effects of the blocking agents on responses to tyramine when compared to responses to nerve stimulation is uncertain, but similar results have been obtained in the rat vas deferens (24). It is possible that the transmitter pool displayed by tyramine may differ from the stores released by electrical stimulation and that transmitter released by tyramine may diffuse to greater distances and thereby interact with postsynaptic alpha-2 adrenoceptors which are remote from the nerve terminal. Tyramine is an indirect acting amine which displaces norepinephrine from adrenergic terminals by a calcium-independent process (21,22). Although tyramine may possess some direct activity (25), vasoconstrictor responses to this agent are mainly indirect in the feline mesenteric vascular bed since they are almost entirely blocked by cocaine.

Although yohimbine reduced responses to tyramine by approximately 50%, the effects of this agent on responses to electrical stimulation of the sympathetic nerves are difficult to interpret since pre- and postsynaptic blocking actions of the antagonist would be expected to have opposing effects on the response (15).

The present studies suggest that the mesenteric vascular bed possesses alpha-1 and postsynaptic alpha-2 receptors mediating vasoconstriction. It has been reported that alpha-2 adrenoceptor-mediated vasoconstriction may be primarily dependent on the entry of extracellular calcium ions whereas alpha-1 mediated responses depend on the release of calcium from intracellular stores. This hypothesis is based on studies showing that calcium entry blocking agents have a differential effect on responses to alpha-1 and alpha-2 adrenoceptor agonists (3,11-13). Moreover, it has been suggested that alpha-2 mediated responses may be distinguished by the source of calcium utilized for vasoconstriction (3,11,13). Since the mesenteric vascular bed has alpha-1 as well as alpha-2 vasoconstrictor mechanisms, this would be an appropriate model

system to examine the sensitivity of alpha-1 and alpha-2 mediated responses to the effects of a calcium entry blocking agent. The results of these studies show that vasoconstrictor responses to alpha-1 and to alpha-2 adrenoceptor agonists are blocked to a similar extent and in a reversible manner by nitrendipine, a dihydropyridine calcium entry blocking agent. Similar results have also been obtained with other calcium entry blocking agents in the feline mesenteric vascular bed (26). These data are not in agreement with results obtained in the pithed rat or cat and in the ganglion-blocked rabbit in which calcium entry blocking agents had a selective blocking effect on alpha-2 mediated responses (3,11-13). The reason for the difference in results in the feline mesenteric circulation and in previous work is uncertain but is not related to species since it has been previously reported that nifedipine had a greater inhibitory effect on increases in diastolic pressure elicited by BHT 920 than by phenylephrine in the cat (27). However, cardiac output and regional vascular responses were not measured in the pithed animals, so that the effects of the alpha adrenoceptor agonists and their interactions with the calcium entry blocking agents on vascular resistance are difficult to assess (3,11,13,27). The present data provide support for an alternate hypothesis that similar sources of calcium are required for vasoconstriction elicited by selective alpha-1 and alpha-2 adrenoceptor agonists in the feline mesenteric vascular bed.

If calcium entry blockers are effective in inhibiting both alpha-1 and alpha-2 mediated vasoconstrictor responses, then responses to nerve released and exogenous norepinephrine should be reduced by calcium entry blocking agents. The present results show that responses to electrical stimulation of the sympathetic nerves and to injected norepinephrine are blocked in a reversible manner by nitrendipine. The finding that vasoconstrictor responses to electrical stimulation of the sympathetic nerves are reduced by calcium entry blocking agents is similar to the findings of Holck and Gerold (9) in the pithed rat. The present data extend the studies of Holck and Gerold (9) by showing that responses to electrical stimulation, tyramine, and alpha-1 agonists are attenuated by calcium entry blocking agents. Since the release of norepinephrine from adrenergic nerves by tyramine is not dependent on the influx of calcium (22), and since responses to nerve excitation and tyramine are reduced to a similar extent, these data suggest that the major effect of the

calcium entry blocking agent in resistance vessels of the intestine is postsynaptic. The inhibitory effect of nitrendipine on vasoconstrictor responses in the mesenteric vascular bed is nonspecific in that responses to U46619, a thromboxane A_2 mimic, angiotensin II, and potassium chloride, which depolarizes smooth muscle, require extracellular calcium. The response to potassium chloride is not reduced by an alpha adrenergic blocking agent, suggesting that it is not dependent on the release of norepinephrine from adrenergic terminals. The present data indicate that vasoconstrictor responses in the cat mesenteric vascular bed elicited by agents which stimulate receptors on the postsynaptic membrane or depolarize resistance vessels require an extracellular source of calcium and differ from studies in isolated vascular tissue (28,29).

The present results suggest that the inhibitory effects of calcium entry blocking agents on vasoconstrictor responses to both sympathetic nerve stimulation and exogenous vasoconstrictor hormones may be involved in the mechanism of the antihypertensive action of these drugs.

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REFERENCES

1. Drew, G.M. and Whiting, S.B. Br. J. Pharmacol. 67:207-215, 1979.
2. Langer, S.Z., Massingham, R. and Shepperson, N.B. Clin. Sci. 59: 225s-228s, 1980.
3. van Meel, J.C.A., de Jonge, A., Kalkman, O., Wilffert, B., Timmermans, P.B.M.W.M. and van Zwieten, P.A. Naunyn-Schmiedeberg's Arch. Pharmacol. 316:288-293, 1981.
4. Ruffolo, R.R., Jr., Nelson, W.L. and Yaden, E.L. Naunyn-Schmiedeberg's Arch. Pharmacol. 322:93-97, 1983.
5. Jie, K., van Brummelen, P., Vermey, P., Timmermans, P.B.M.W.M. and van Zwieten, P.A. Circ. Res. 54:447-452, 1984.
6. Hyman, A.L. and Kadowitz, P.J. Am. J. Physiol. 249:H891-H898, 1985.
7. Yamaguchi, I. and Kopin, I.J. J. Pharmacol. Exp. Ther. 214:275-281, 1980.
8. Langer, S.Z. and Shepperson, N.B. J. Cardiovasc. Pharmacol. 4: S8-S13, 1982.
9. Holck, M. and Gerold, M. Naunyn-Schmiedeberg's Arch. Pharmacol. 330:22-32, 1985.
10. Stevens, M.J. and Moulds, F.W. Circ. Res. 57:399-405, 1985.
11. van Meel, J.C.A., Towart, R., Kazda, S., Timmermans, P.B.M.W.M. and van Zwieten, P.A. Naunyn-Schmiedeberg's Arch. Pharmacol. 322:34-37, 1983.

12. Cavero, I., Shepperson, N., Lefevre-Brog, F. and Langer, S.Z. Circ. Res. 52(I):69-76, 1983.
13. van Zwieten, P.A., van Meel, J.C.A. and Timmermans, P.B.M.W.M. Circ. Res. 52(I):77-80, 1983.
14. Armstead, W.M., Lippton, H.L., Hyman, A.L. and Kadowitz, P.J. Can. J. Physiol. Pharmacol. 62:1470-1478, 1984.
15. Langer, S.Z. Br. J. Pharmacol. 60:481-497, 1977.
16. Kostrzewa, R.M. and Jacobowitz, D.M. Pharmacol. Rev. 26:199-288, 1974.
17. Kadowitz, P.J., Knight, D.S., Hibbs, R.G., Ellison, J.P., Joiner, P.D., Brody, M.J. and Hyman, A.L. Circ. Res. 39:191-199, 1976.
18. Timmermans, P.B.M.W.M., Kwa, H.Y. and van Zwieten, P.A. Naunyn-Schmiedeberg's Arch. Pharmacol. 319:189-193, 1979.
19. Timmermans, P.B.M.W.M. and van Zwieten, P.A. Naunyn-Schmiedeberg's Arch. Pharmacol. 313:17-20, 1980.
20. Hyman, A.L. and Kadowitz, P.J. Am. J. Physiol. (in press), 1986.
21. Trendelenburg, U., Muskus, A., Fleming, W.M. and de la Sierra, B. J. Pharmacol. Exp. Ther. 138:170-180, 1962.
22. Lai, F.M. and Hudgins, P.M. Toxicol. Appl. Pharmacol. 32:368-376, 1975.
23. Vanhoutte, P.M., Verbeuren, T.J. and Webb, R.C. Physiol. Rev. 61: 151-247, 1981.
24. Lucchelli, A., Barbone, S.M.G. and Grana, E. Pharmacol. Res. Comm. 17:787-801, 1985.
25. Miyahara, H. and Suzuki, H. Br. J. Pharmacol. 86:405-416, 1985.
26. Hyman, A.L., Lippton, H.L. and Kadowitz, P.J. Fed. Proc. (in press), 1986.
27. Timmermans, P.B.M.W.M., de Jonge, A., van Meel, J.C.A., Mathy, M.J. and van Zwieten, P.A. J. Cardiovasc. Pharmacol. 5:1-11, 1983.
28. Towart, R. J. Cardiovasc. Pharmacol. 4:895-902, 1982.
29. Towart, R. and Stoepel, K. Naunyn-Schmiedeberg's Arch. Pharmacol. 308:R18, 1979.

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CYTOSOLIC FREE CALCIUM TRANSIENTS INDUCED BY ERGONOVINE IN CULTURED VASCULAR SMOOTH MUSCLE CELLS

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INTRODUCTION

Ergonovine is a vasoconstrictor and now widely used as a diagnostic test in patients with suspected variant angina. However, the etiology underlying ergonovine-induced vasoconstriction remains uncertain. Constriction of vascular smooth muscle cells (VSMC) is regulated by changes in the concentration of cytosolic free calcium ((Ca)i). Using the microfluorometry of quin 2, the effects of ergonovine on (Ca)i in cultured rat VSMC were investigated.

MATERIALS AND METHODS

Rat aortic medial VSMC in primary culture were used throughout the experiments. On day 5 to 6, just before reaching confluence, the cultured VSMC on Lux chamber slides were loaded with quin 2, as the acetoxy-methyl ester (quin 2/AM, DOTITE), as described (1). Unless otherwise indicated, recording of (Ca)i transients were performed in normal physiological saline solution (normal PSS) containing 1 mM $CaCl_2$ at 25°C. The millimolar composition of the normal PSS (pH 7.4) was: NaCl 135; KCl 5; $CaCl_2$ 1; $MgCl_2$ 1; glucose 5.5; HEPES 10. A calcium-free version of this solution (Ca-free PSS) was prepared by replacing $CaCl_2$ with 2 mM EGTA.

Cytosolic fluorescence intensity of VSMC was recorded using a fluorescence microscope (model 18, Zeiss) equipped with a water immersion objective lens (Plan-Neofluor 63, Zeiss) and an appropriate combination of filters, in which VSMC were excited at wavelengths between 350 and 360 nm and analyzed at wavelengths between 470 and 560 nm. Using a pinhole diaphragm (Zeiss) in the light axis, the fluorescence intensity in a spot ($<1\ \mu m^2$) of the cytosol 3 μm apart from the nucleus was measured. Each cell was exposed to the excitation light, only once, for not longer than 2 sec in order to

avoid the photobleaching effect on the dye. VSMC showed no morphological change during the course of all experiments, as assessed in a phase-contrast microscope at 400X.

Drugs used were ergonovine maleate (SIGMA), ketanserin tartrate (JANSSEN), phentolamine mesylate (CIBA-GEIGY) and 2,4-dinitrophenol (WAKO). Drugs were dissolved in either normal PSS or Ca-free PSS.

The results obtained were expressed as the mean$\pm$S.D. of 5 experiments, and the number of cells counted in each experiments was 8, and statistical significance was assessed using an analysis of variance. Difference of $p < 0.05$ were considered significant.

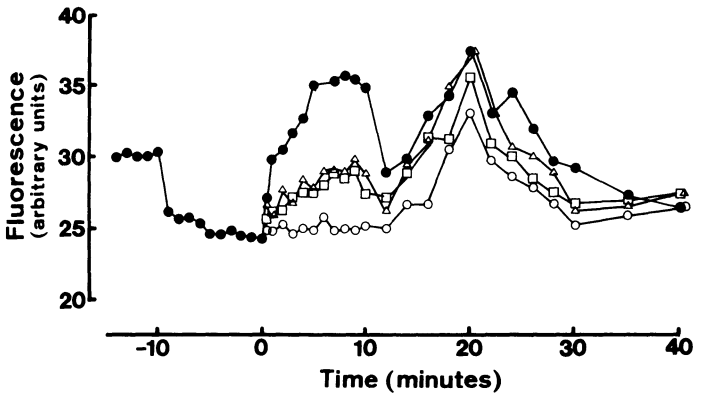
RESULTS

Both in the presence (in normal PSS containing 1 mM $CaCl_2$) and the absence (in Ca-free PSS containing 2 mM EGTA) of extracellular calcium, ergonovine induced transient, dose-dependent and double-peaked elevations of (Ca)i with similar time course and extent. The first and the second peaks were observed at 8 and 20 min, respectively.

In the presence of extracellular calcium, the first peak elevations were inhibited and finally abolished by phentolamine or ketanserin dose-dependently. However, the second peak elevations were not affected at all by ketanserin, and were only partially, if any, inhibited by phentolamine.

In contrast, in the absence of extracellular calcium, the first peak elevations in response of (Ca)i to ergonovine were not completely abolished by phentolamine or ketanserin alone. However, when VSMC stimulated with ergonovine were exposed simultaneously to phentolamine and ketanserin, the first peak elevations were completely abolished (Figure).

In the absence of extracellular calcium, dinitrophenol caused a transient elevations in (Ca)i; (Ca)i reached a maximal level at 20 min, and then declined to a pre-exposure level within 30 min, despite the continuous application of dinitrophenol. When VSMC were pretreated with 10^{-6} M ergonovine for 40 min, (Ca)i elevations elicited by 10^{-4} M dinitrophenol were completely abolished. Conversely, when VSMC were pretreated with 10^{-4} M dinitrophenol for 30 min, the second peak elevations of fluorescence elicited by 10^{-6} M ergonovine were attenuated by 50 % and the first peak elevations were not affected at all.



A typical example of the effect of 10^{-6} M ergonovine on fluorescence signal in VSMC in Ca-free PSS containing 2 mM EGTA. When VSMC were exposed to Ca-free PSS prior to the application of ergonovine, (Ca)i decreased gradually and reached a steady state level in 5 min. This level remained unchanged for at least 60 min, as previously noted (1,2,3). VSMC were pre-incubated with Ca-free PSS for 10 min, and then, exposed to ergonovine in Ca-free PSS (at time 0 in the figure). Ergonovine (●) caused transient elevations in the fluorescence with peaks at 8 and 20 min. The first peak elevations were inhibited only partially by ketanserin 10^{-10} M (Δ) or phentolamine 10^{-6} M (□) alone (doses sufficient to exert maximum responses of fluorescence decrease in VSMC stimulated with ergonovine. Combined blockade (○, 10^{-10} M ketanserin + 10^{-6} M phentolamine) completely abolished the first peak elevations.

DISCUSSION

The present study clarifies the partial mechanism of ergonovine action on VSMC. Both in the presence and the absence of exracellular calcium, ergonovine induced transient and double peaked elevations of (Ca)i with almost similar time course and extent in rat aortic VSMC in primary culture. This indicates that transient (Ca)i elevations induced by ergonovine are primarily due to a release of calcium from cellular store sites.

Although both phentolamine (alpha adrenoceptor antagonist) and ketanserin (serotonin S_2-selective antagonist) could completely abolish the first peak elevations of (Ca)i elicited by ergonovine in the presence of extracellular calcium, the abolition of the first peak elevations by each antagonist was partial and only combined blockade completely suppressed the first peak elevations in the absence of extracellular calcium. At present, since truly selective antagonists for alpha adrenoceptors and serotonin

receptors are not vailable, it is not possible to determine whether ergonovine preferentially activates the former or the latter. However, the present study demonstrates that the alpha and serotonergic blocking effects of these highly selective antagonists, phentolamine and ketanserin, on ergonovine action were additive. Thus, it is indicated that the first peak elevations of (Ca)i elicited by ergonovine are due to activation of the alpha afrenergic receptors and/or serotonin S_2-receptor as an agonist, and that alpha adrenoceptors and serotonin S_2-receptors, although distinct entities, have features in common.

Many investigators have considered that ergonovine is an alpha adrenoceptor agonist or constrict the vascular smooth muscle by a "direct" action (4,5). On the other hand, recent work in experimental animals has shown that coronary arterial contractions induced by ergonovine are mediated by serotonergic receptors (6,7). Species and organ differences may account for many of the diverse effects of ergonovine. Recently, it was reported that ergonovine constricted "normal" arteries by stimulating alpha adrenergic receptors, but the supersensitivity of "atherosclerotic" arteries to ergonovine was mediated predominantly by a serotonergic mechanism (8). The results of the present study that ergonovine can act both at alpha adrenoceptors and at serotonin S_2-receptors to induce the first peak elevations of (Ca)i may explain the diversity of the results of these previous studies of ergonovine-induced vasoconstriction in different tissues and animals.

Dinitrophenol is known to act mainly on mitochondria to induce a release of stored calcium (9). Our results demonstrated that dinitrophenol-sensitive calcium store sites were mostly depleted when VSMC were treated with ergonovine, and that the second peak elevations of (Ca)i were partially attenuated when VSMC were pretreated with dinitrophenol. This suggests that ergonovine acts directly at mitochondria to induce a release of stored calcium, which results in the second peak elevations of (Ca)i.

SUMMARY

Cytosolic calcium transients induced by ergonovine were recorded microfluorometrically in primary cultured rat aortic smooth muscle cells treated with quin 2. Both in the presence and the absence of extracellular calcium, ergonovine induced transient, dose-dependent and double-peaked elevations of cytosolic calcium with similar time course and extent, the

first and the second peaks being observed at 8 and 20 min, respectively. In the absence of extracellular calcium, the first peak elevations were partially inhibited by phentolamine or ketanserin dose-dependently, and completely inhibited when phentolamine and ketanserin were added at the same time. The second peak was partially abolished when cells were pretreated with dinitrophenol. When cells were treated with ergonovine, dinitrophenol-sensitive calcium store sites were mostly depleted. Thus, ergonovine activates both serotonin receptors and alpha adrenoceptors to induce a release of calcium from the cellular store sites, and also induces calcium release from mitochondria, which result in the transient and double-peaked elevations of cytosolic calcium in cultured rat aortic vascular smooth muscle cells.

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REFERENCES

1. Kobayashi,S.,Kanaide,H. and Nakamura,M. Science 229:553-556,1985.
2. Kobayashi,S.,Kanaide,H. and Nakamura,M. Biochem. Biophys. Res. Commun. 129:877-884,1985.
3. Shogakiuchi,Y., Kanaide,H.,Kobayashi,S.,Nishimura,J. and Nakamura,M. Biochem. Biophys. Res. Commun. 135:9-15,1986.
4. Cipriano,P.R.,Guthaner,D.F.,Orlick,A.E.,Ricci,D.R.,Wexler,L. and Silverman,J.F. Circulation 59:82-89,1979.
5. Curry,R.C.,Popine,C.J.,Sabom,M.B. and Conti,C.R. Circulation 59; 307-312,1979.
6. Brazenor,R.M. and Angus,J.A. J. Pharmacol. Exp. Ther. 218: 530-536,1981.
7. Suyama,A. and Kuriyama,H. Naunyn-Schmiedeberg's Arch. Pharmacol. 326:357-363,1984.
8. Henry,P.D. and Yokoyama,M. J. Clin. Invest. 66:306-313,1980.
9. Deth,R. and Casteels,R. J. Gen. Physiol. 69:401-416,1977.