IOWA STATE UNIVERSITY
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

1996

Utilization of fatigued and non-fatigued isolated guinea-pig papillary muscles and ventricular myocytes on the comparison of inotropic, chronotropic and intracellular calcium changes induced by monensin and digoxin

Ismail Meral
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

 Part of the Animal Sciences Commons, Cell Biology Commons, Medical Pharmacology Commons, Pharmacology Commons, Physiology Commons, and the Veterinary Physiology Commons

Recommended Citation
Meral, Ismail, "Utilization of fatigued and non-fatigued isolated guinea-pig papillary muscles and ventricular myocytes on the comparison of inotropic, chronotropic and intracellular calcium changes induced by monensin and digoxin " (1996). *Retrospective Theses and Dissertations*. 11121.
https://lib.dr.iastate.edu/rtd/11121

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Utilization of fatigued and non-fatigued isolated guinea-pig papillary muscles and ventricular myocytes on the comparison of inotropic, chronotropic and intracellular calcium changes induced by monensin and digoxin

by

Ismail Meral

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Physiology and Pharmacology
Major: Physiology (Pharmacology)

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate Collage

Iowa State University
Ames, Iowa

1996

UMI Number: 9620980

UMI Microform 9620980
Copyright 1996, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized copying under Title 17, United States Code.

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACV	Average contraction velocity
ATPase	Adenosinetriphosphatase
CA	Contraction amplitude
$[Ca^{2+}]_i$	Intracellular calcium concentration
$[Ca^{2+}]_o$	Extracellular calcium concentration
cAMP	Cyclic adenosine monophosphate
CF	Contraction force
CHF	Congestive heart failure
CNS	Central nervous system
COOH	Carboxyl
D	Dalton
DHP	Dihydrophyridine
DNA	Deoxyribonucleic acid
ICV	Initial contraction velocity
IRV	Initial relaxation velocity
KD	Kilodalton
$[K^+]_i$	Intracellular potassium concentration
$[K^+]_o$	Extracellular potassium concentration
kg	Kilogram

KRB	Krebs Ringer Bicarbonate (buffer solution)
L-Type	Long acting
mg	Milligram
Mg^{2+}	Magnesium
NH_2	Amino
$[Na^+]_i$	Intracellular sodium concentration
$[Na^+]_o$	Extracellular sodium concentration
P1	Early component of guinea-pig papillary muscle wave
P2	Late component of guinea-pig papillary muscle wave
PPS	Pulses per second
SDM	Standard deviation of mean
SE	Standard error
SL	Sarcolemma
pH_i	pH of intracellular compartment
SR	Sarcoplasmic reticulum
SRT	Stimulus to response time
TO	Equilibration time
T1	One hour after treatment
T2	Two hours after treatment
T3	Three hours after treatment
T4	Four hours after treatment

VDCCs	Voltage dependent calcium channels
V_{max}	Maximum velocity

GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains two separate manuscripts which have not been submitted for publication. Each individual manuscript is complete in itself and includes an Abstract, Introduction, Materials and Methods, Results, Discussion, and Reference section. The Ph.D. candidate, Ismail Meral, is the senior author for each manuscript. The first manuscript describes the functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin and the second manuscript describes digoxin- and monensin- induced changes of intracellular Ca^{2+} concentration in isolated guinea-pig ventricular myocytes. In addition, a current review of the literature relevant to this dissertation, a General Discussion section and an Appendix are included.

Research Objectives

The goals of this research were: 1) to compare the positive inotropic action of digoxin and monensin using fatigued and non-fatigued guinea-pig papillary muscles. Papillary muscle contractility was monitored for four hours following drug administration to evaluate the effects of these two drugs over time. Since the inotropic action of digoxin is much greater in severely depressed muscle than in normal muscle (Hoffman and Bigger, 1990), the intent of this research was to also compare non-fatigued and fatigued guinea-pig papillary muscles regarding their response to digoxin and monensin; 2) to investigate the

possible mechanisms of action of monensin and digoxin by using isolated guinea-pig ventricular myocytes. Because Ca^{2+} is a major signal for triggering contraction of cardiac muscle, another objective of this research was to determine whether monensin- or digoxin-induced enhancement of cardiac muscle contraction is mediated through an increase in Ca^{2+} influx or a release from intracellular stores; 3) to establish the feasibility for further investigation of monensin for the possibility of using it for the treatment of congestive heart failure.

LITERATURE REVIEW

This section provides concise background information for intracellular Ca^{2+} homeostasis and the best known actions of monensin and digoxin in cardiac myocytes.

Intracellular Ca^{2+} Homeostasis in Cardiac Myocytes

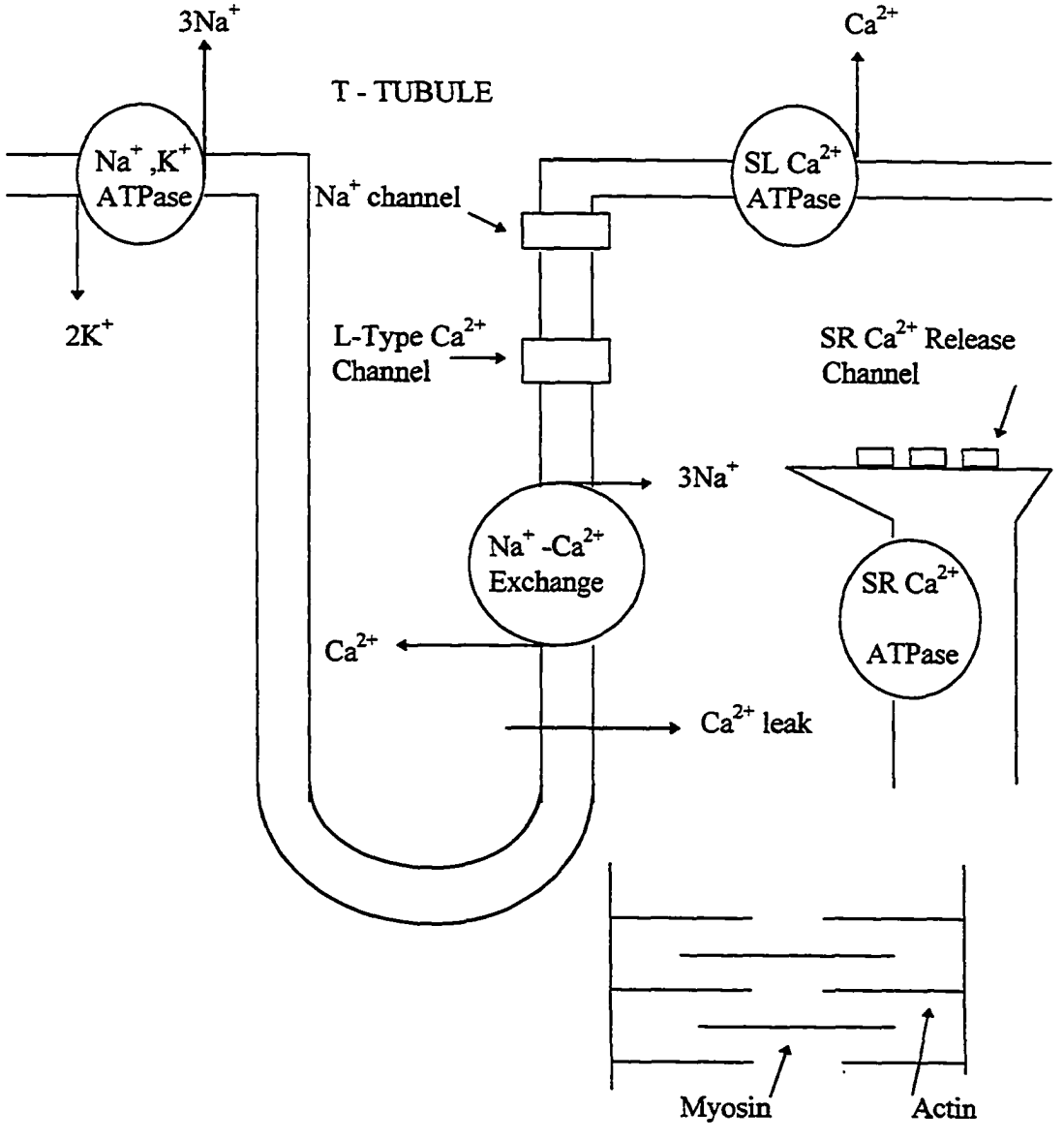
Ca^{2+} homeostasis in cardiac myocytes is functionally important for at least three reasons (Barry and Bridge, 1993): 1) control of resting intracellular calcium concentration ($[Ca^{2+}]_i$); 2) excitation-contraction coupling; 3) relaxation of the ventricular myocytes.

1). Control of Resting $[Ca^{2+}]_i$

Various ion channels and transport proteins involved in Ca^{2+} homeostasis in the cardiac myocyte are shown schematically in Fig. 1. In the resting ventricular myocyte, the $[Ca^{2+}]_i$ is influenced by a Ca^{2+} entry (influx) that is compensated for by an ATP-dependent sarcolemmal Ca^{2+} pump and the sarcolemmal Na^{+}-Ca^{2+} exchanger (Barry and Bridge, 1993) that cause a Ca^{2+} exit (efflux).

The Na^{+}-Ca^{2+} exchanger has recently been cloned by Nicole et al., 1990. The complementary DNA encodes a protein of 970 amino acids with a molecular mass of 108 KDa. The protein can be divided into three regions; a hydrophobic NH_2 terminal portion containing six potential membrane-spanning segments; a long hydrophilic region that is modeled as a large cytoplasmic loop; and a hydrophobic COOH terminal portion

Fig. 1. Schematic illustration of the sequence of the processes ongoing in the resting myocyte or at the end of diastole (Adapted from Barry and Bridge, Circulation, Vol. 87, 1993, page 1808). SR, sarcoplasmic reticulum; SL, sarcolemma.


3Na+
Ca2+
T - TUBULE
Na+ ,K+ ATPase
SL Ca2+ ATPase
Na+ channel
2K+
L-Type Ca2+ Channel
SR Ca2+ Release Channel
3Na+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
Ca2+
Ca2+ leak
Myosin
Actin

comprising six potential membrane-spanning segments (Barry and Bridge, 1993). At this time, little is known about relations between structure and function of this molecule.

Modern experiments investigating the binding of antibodies to the Na^+-Ca^{2+} exchanger with immunofluorescence and immunoelectron microscopy indicate that although the exchanger is detectable over the entire myocyte surface, antibody binding sites appear to be concentrated in the T-tubule region of the myocyte (Frank et al., 1992). The functional significance of this arrangement is not yet clear.

The Na^+-Ca^{2+} exchanger is a counterion transporter. Three Na^+ ions are exchanged for each Ca^{2+} ion which results in a positive charge movement that opposes the direction of Ca^{2+} transport (Reeves and Hale, 1984).

The Na^+-Ca^{2+} exchanger is also voltage sensitive and if the membrane potential is more negative than the reversal potential, as is normally the case in the resting myocyte, the exchanger functions in a Na^+ (in) /Ca^{2+} (out) mode and thus produces Ca^{2+} extrusion (Kimura et al., 1986).

The importance of the Na^+ -Ca^{2+} exchanger in regulating the resting level of Ca^{2+} within the cardiac myocytes was demonstrated most directly by the experiments of Sheu and Fozzard (1982), who measured intracellular sodium concentration ($[Na^+]_i$) and Ca^{2+} activities in cardiac purkinje cells with ion sensitive microelectrodes. These experiments revealed a direct relation between $[Na^+]_i$ and $[Ca^{2+}]_i$. Thus, increases in $[Na^+]_i$ cause dissipation of the electrochemical energy in the Na^+ gradient available to extrude Ca^{2+}, resulting in an increase in $[Ca^{2+}]_i$. The level of $[Na^+]_i$ in myocytes is controlled largely by

Na^+, K^+-ATPase, and thus the Na^+ pump is also an important indirect regulator of the Na^+-Ca^{2+} exchange (Barry and Bridge, 1993).

The activity of the Na^+ -Ca^{2+} exchanger can also be modulated by a variety of other mechanisms. The effects of pH on the Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles has been investigated by Philipson et al., 1982. It has been demonstrated that an acidosis inhibits the exchanger, whereas an alkalosis stimulates the exchanger. Haworth et al., 1987 demonstrated an inhibition of Ca^{2+} influx due to an inhibition in the Na^+-Ca^{2+} exchange in isolated adult rat heart cells by ATP depletion. It is likely that ATP increases the affinity of the exchanger for Ca^{2+} (dePolo and Beauge, 1986), but it has not yet been demonstrated that the protein kinase-mediated phosphorylation of the exchanger has any regulatory influences (Barry and Bridge, 1993).

Recently, a Na^+-Ca^{2+} exchanger inhibitory peptide which is identical to the calmodulin-binding sequence of the exchanger and effectively inhibits exchange has been synthesized (Li et al., 1991). It has not been established, however, that this calmodulin-binding peptide sequence within the exchanger exerts any autoinhibitory effect under normal physiological conditions (Barry and Bridge, 1993).

Another Ca^{2+} transport system that contributes to maintenance of the low $[Ca^{2+}]_i$ in ventricular myocytes during diastole is the sarcolemmal Ca^{2+}-ATPase. The structure and function of plasma membrane Ca^{2+}-ATPase have recently been reviewed by Carafoli, 1992. The plasmalemmal Ca^{2+}-ATPases are found in many cells and use the free energy released by the hydrolysis of one ATP to transport one Ca^{2+} out of the cell against its concentration

gradient. General properties of the plasmalemmal Ca^{2+}-ATPases include a molecular mass of 134,000 Da, stimulation by Ca^{2+} calmodulin and possibly a kinase-induced phosphorylation.

The Na^{+}-Ca^{2+} exchanger accounts for as much as 75% of Ca^{2+} efflux in the resting cell (Cannel, 1991). Thus, although the sarcolemmal Ca^{2+}-ATPase probably contributes to maintenance of $[Ca^{2+}]_i$ in resting myocytes, its importance in this regard relative to the Na^{+} -Ca^{2+} exchanger seems to be minor.

2. Excitation-Contraction Coupling

In cardiac myocytes , the transition from the resting relaxed state with low $[Ca^{2+}]_i$ to a contraction occurs because a small quantity of Ca^{2+} crosses the sarcolemma and induces a much larger release of Ca^{2+} from the sarcoplasmic reticulum (SR). This process is initiated by depolarization of the cell membrane, which causes opening of voltage-gated Na^{+} and Ca^{2+} channels. The initial upstroke of the action potential in ventricular cardiac myocytes is caused by a Na^{+} influx via the Na^{+} channels, whereas the subsequent inward current maintaining the plateau of the action potential is caused primarily by Ca^{2+} influx via the L-type Ca^{2+} channels. Cumulative Na^{+} influx via the Na^{+} channels also contributes to maintenance of the $[Na^{+}]_i$ in myocytes and thus can influence $[Ca^{2+}]_i$ via the Na^{+}-Ca^{2+} exchange (Barry and Bridge, 1993). It has been suggested that it is the Ca^{2+} release in response to increased $[Ca^{2+}]_i$ through opening of the L-type Ca^{2+} channels that is considered to be of greatest importance in excitation-contraction coupling (duBell and

Hauser, 1989 and Niggli and Lederer, 1990). The initial events that couple excitation to contraction are displayed in Fig. 2A.

The L-type Ca^{2+} channel is an oligomeric complex of five subunits (Catterall, 1991 and Singer et al., 1991). These subunits are designated as alpha1, alpha2, beta, gamma, and delta. The alpha1 subunit seems to provide the primary structural and functional basis for the assembled channel. For example, it contains the receptor for at least three classes of channel antagonists, including the dihydropyridines; it contains phosphorylation sites for cAMP-dependent protein kinase; and it is known to contain the pore of the channel (Barry and Bridge, 1993).

An important pharmacological characteristic of the L-type Ca^{2+}-channels is that they contain a high affinity receptor for 1,4-dihydropyridine ligands (Barry and Bridge, 1993). These ligands can function either as antagonists or agonists. Antagonists such as nifedipine, when bound to the channels, effectively block their activity (Barry and Bridge, 1993). Bay K 8644 is a dihydropyridine receptor agonist that increases Ca^{2+} current carried by the L-type Ca^{2+} channels (Sanguinetti et al., 1986).

It appears that Ca^{2+} influx via the Na^{+} -Ca^{2+} exchanger (reverse mode) can also occur during initial depolarization. This process may be further stimulated by $[Na^{+}]_i$ caused by the Na^{+} influx via the cardiac Na^{+} channels (Niggli and Lederer, 1990). However, it has been questioned whether or not this occurs during normal excitation-contraction coupling (Sham et al., 1992).

Fig. 2. Schematic illustration of the excitation-contraction coupling in an adult mammalian ventricular myocyte (Adapted from Barry and Bridge, Circulation, Vol. 87, 1993, page 1809). SR, sarcoplasmic reticulum; SL, sarcolemma. A. Initial phase.

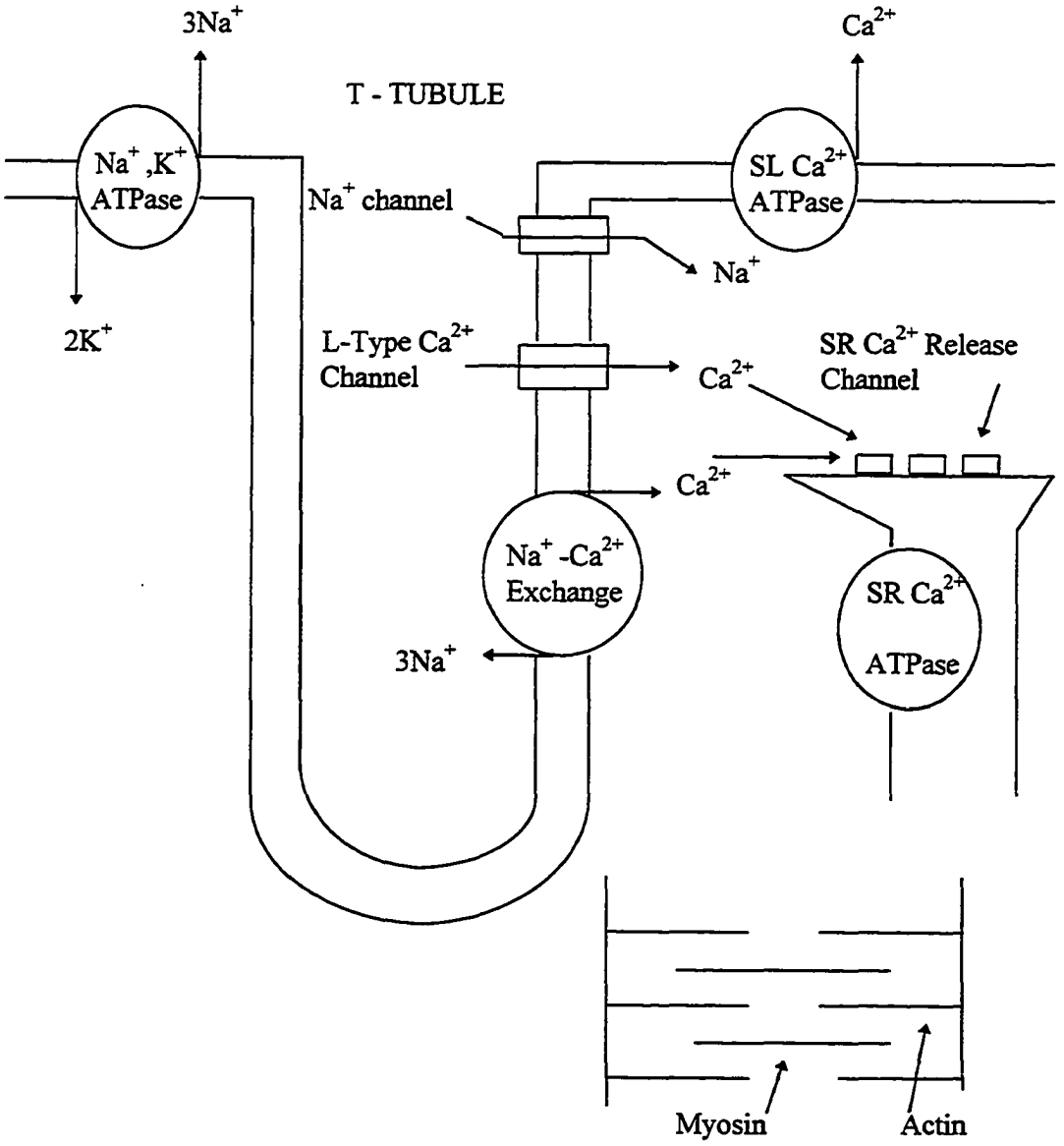
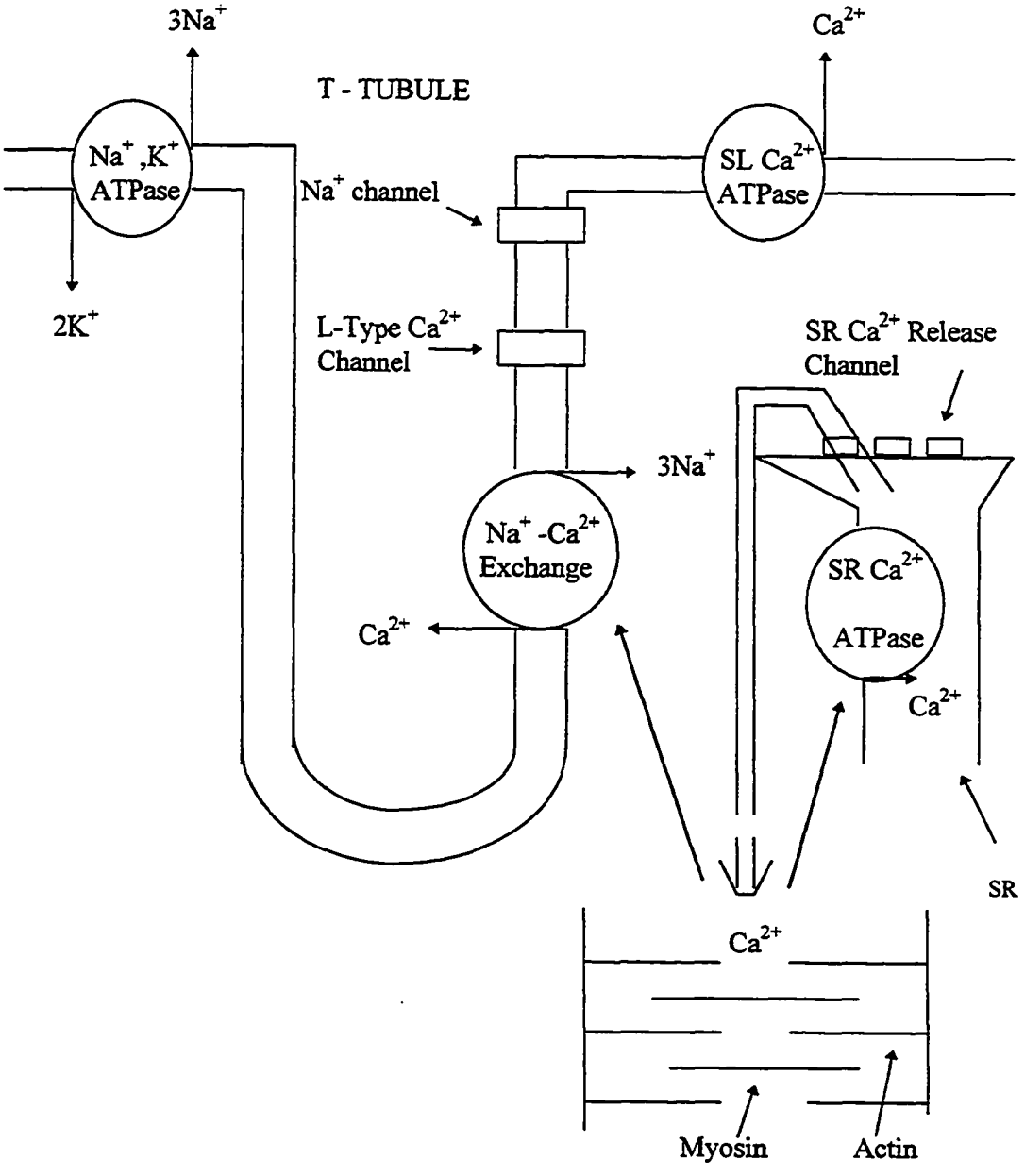

3Na+
Ca2+
T - TUBULE
Na+ ,K+ ATPase
SL Ca2+ ATPase
Na+ channel
Na+
2K+
L-Type Ca2+ Channel
SR Ca2+ Release Channel
Ca2+
Ca2+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
3Na+
Myosin
Actin

Fig. 2. (continued); B. Late phase.


3Na+
Ca2+
T - TUBULE
Na+ ,K+ ATPase
Na+ channel
SL Ca2+ ATPase
2K+
L-Type Ca2+ Channel
SR Ca2+ Release Channel
3Na+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
Ca2+
Ca2+
SR
Ca2+
Myosin
Actin

Ca^{2+} that enters the cell early after depolarization via the L-type Ca^{2+} channels, and possibly on the Na^{+} -Ca^{2+} exchanger, can bind to the Ca^{2+} release channels of the SR (fig. 2A) and activate these channels (Fabiato, 1983), causing release of Ca^{2+} from the SR (Ca^{2+}-induced Ca^{2+} release).

The Ca^{2+} release channels in the SR have been cloned by Otsu et al., 1990 and have been shown to be a 4,969-amino acid protein with a molecular mass of 564,711 Da. The probability of opening of these channels is markedly increased by exposure to micromolar concentrations of Ca^{2+} (Anderson et al., 1989 and Rousseau et al., 1987). These channels can also be opened by methylxanthines, such as caffeine (Meissner and Henderson, 1987). In low concentrations (<10 μM), ryanodine opens Ca^{2+} release channels of the SR (Rousseau et al., 1987) and at higher concentrations, ryanodine completely blocks the SR Ca^{2+} release channels (Meissner, 1986).

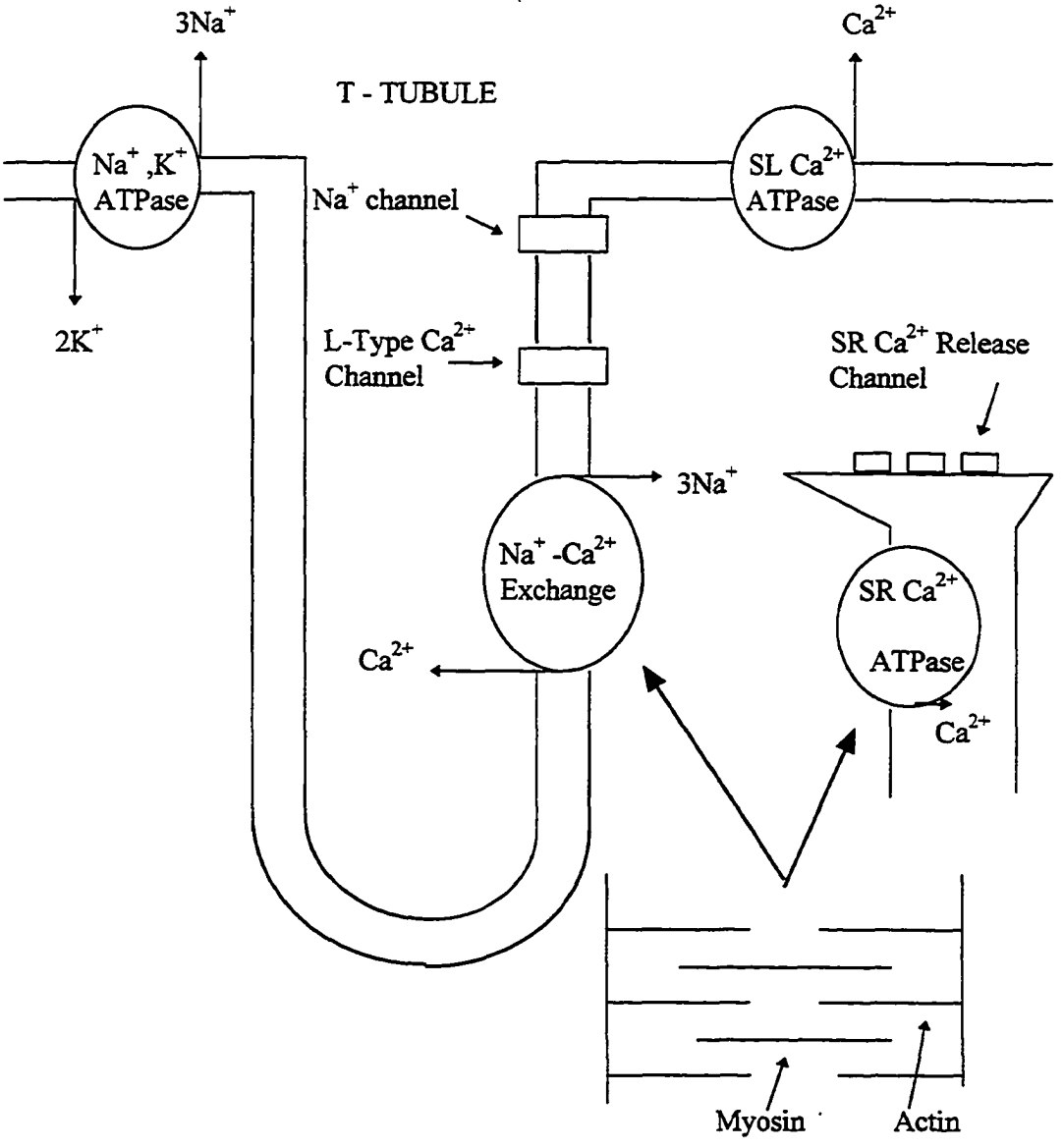
It has been suggested (Moravec and Bond, 1991) that not all the Ca^{2+} within the SR is released with each beat and that SR-Ca^{2+} release can be graded by the amount of trigger Ca^{2+} entering the cell. The evidence indicating a central role for Ca^{2+} channels in this process has recently been summarized by Fabiato, 1989. The Ca^{2+} spontaneously released from a Ca^{2+}-overloaded SR may induce adjacent SR Ca^{2+} release channels to release Ca^{2+}, resulting in an increased $[Ca^{2+}]_i$ and contraction (William et al., 1992). However, the Ca^{2+} released from the SR does not activate adjacent SR Ca^{2+} release channels under conditions of normal excitation-contraction coupling. This issue is still not satisfactorily resolved (Niggli and Lederer, 1990).

The Ca^{2+} that is released from the SR initiates contraction (Fig. 2B) by binding to the contractile proteins (Moss, 1992). In the resting state, the interaction of actin and myosin is inhibited by the troponin-tropomyosin complex, which is bound to actin. When Ca^{2+} binds to troponin C, a conformational change is induced that results in relief of this inhibition, with cross-bridge interaction and contractile element shortening. The affinity of troponin C for Ca^{2+} could be lowered by a decreased pH_i (Blanchard and Solaro, 1984). Also, the cAMP-dependent protein kinase-induced phosphorylation of troponin I decreases Ca^{2+} sensitivity of troponin C (McClellan and Winegrad, 1980 and Ray and England, 1976). Thus, changes in the affinity of the contractile elements for Ca^{2+} as well as the magnitude of the Ca^{2+} transient achieved during a single beat can regulate the force development by a myocyte.

3. Relaxation of the Ventricular Myocytes

The decay of the Ca^{2+} transient (Fig. 3) occurs because of uptake of Ca^{2+} into the SR mediated by the SR-Ca^{2+} ATPase and extrusion of Ca^{2+} from the myocyte primarily by the Na^{+}-Ca^{2+} exchange (Barry and Bridge, 1993). The SR Ca^{2+}-ATPase is concentrated in the longitudinal component of the SR (Jorgensen et al., 1982). Regulation of the SR Ca^{2+}-ATPase occurs primarily by phosphorylation of phospholamban which binds to and inhibits Ca^{2+} transport by the SR Ca^{2+}-ATPase (Barry and Bridge, 1993). When phospholamban is phosphorylated by cAMP-dependent protein kinase, the inhibition of SR Ca^{2+}-ATPase by phospholamban is

Fig. 3. Events associated with a fall in $[Ca^{2+}]_i$ and relaxation (Adapted from Barry and Bridge, Circulation, Vol. 87, 1993, page 1809). SR, sarcoplasmic reticulum; SL, sarcolemma.


3Na+
Ca2+
T - TUBULE
Na+ ,K+ ATPase
SL Ca2+ ATPase
Na+ channel
2K+
L-Type Ca2+ Channel
SR Ca2+ Release Channel
3Na+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
Ca2+
Ca2+
Myosin
Actin

removed, resulting in a greater V_{max} for Ca^{2+} transport (Sasaki et al., 1992).

Ca^{2+} is bound to calsequestrine after uptake by the SR Ca^{2+}-ATPase (Barry and Bridge, 1993). Calsequestrine is the major Ca^{2+}-binding protein in cardiac muscle. Binding and release of Ca^{2+} by calsequestrine is thought to be important in excitation-contraction coupling, but its exact role is not understood.

Digoxin

Introduction

Digitalis glycosides were introduced into the clinical practice of medicine by William Withering two hundred years ago and they have been a major impetus to cardiovascular phamacologic research since then (Sonberg et al., 1985). The inotropic effect of digitalis in a cat right ventricular papillary muscle was demonstrated by Cattel and Gold (1940). Since then, digitalis has remained a major part of the therapeutic approach to patients in congestive heart failure (CHF) and atrial fibrillation.

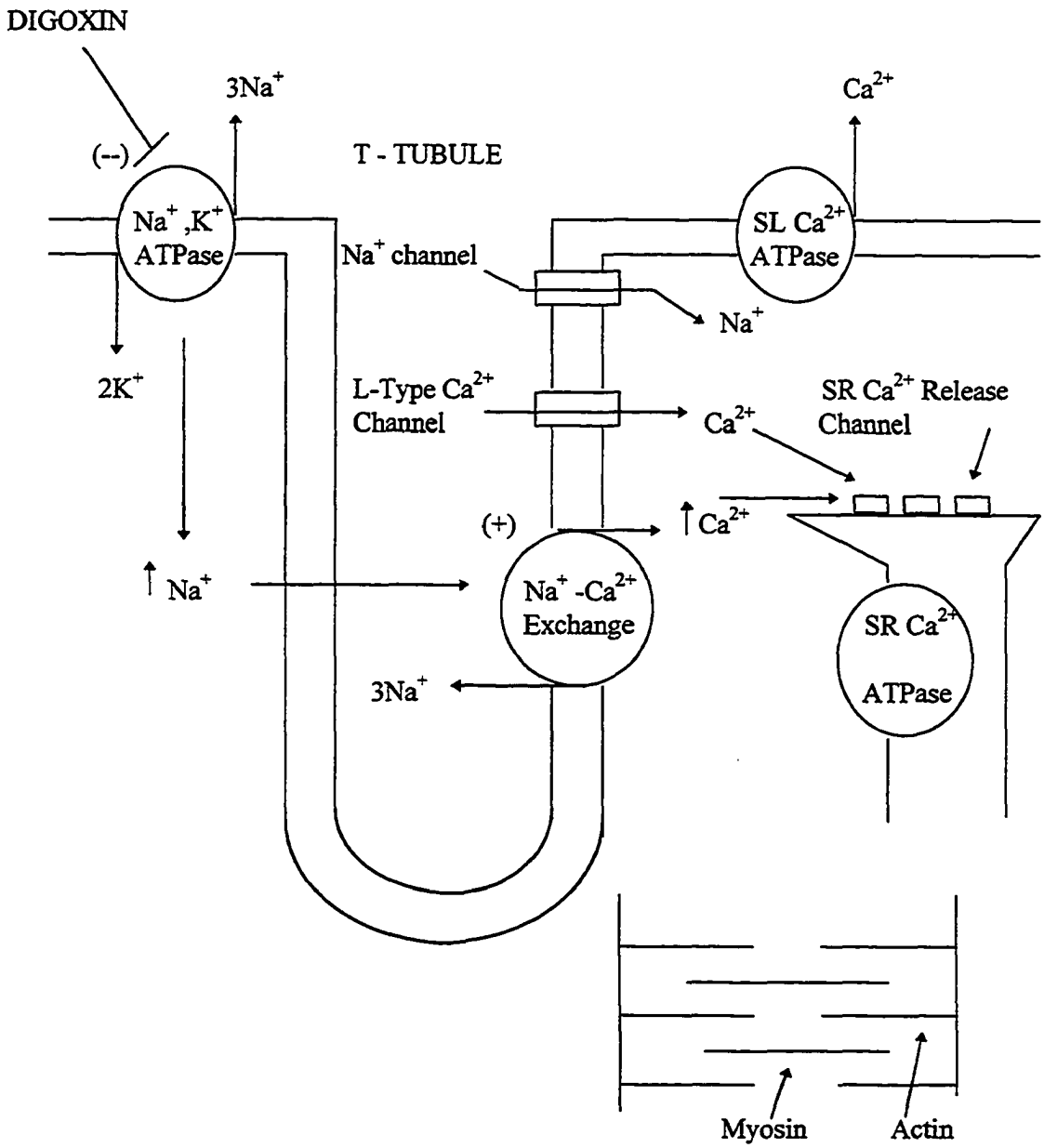
Digitalis glycosides have increased our understanding of cardiac cellular mechanisms involving contractility (Miura and Biedert, 1985). In the last fifty years many studies involving drug interactions, bioavailability, drug disposition, elimination, and clearance have had their origin in clinical problems relating to digitalis administration (Sonberg et al., 1985).

Cellular Mechanism of Digitalis Action

Although digitalis glycosides, especially digoxin, have been used in the treatment of arrhythmia and congestive heart failure for over two hundred years, the mechanism of their positive inotropic action remains controversial. The most widely accepted mechanism is their well-known inhibitory effect on the Na^+, K^+-ATPase (pump) resulting in an increase in $[Na^+]_i$ leading to an increase in $[Ca^{2+}]_i$ through the Na^+ (out) /Ca^{2+} (in) exchange.

Studies of the effects of digitalis on the electrophysiological properties of mammalian cardiac tissues have revealed a close relationship between digitalis and K^+ (Besch et al., 1970 and Ito et al., 1970). Since the cardiac glycosides inhibit the Na^+, K^+-ATPase transport system, the loss of $[K^+]_i$ would result in a decrease of cellular transmembrane potential (Cranefield and Hoffman, 1958 and Hoffman and Singer, 1964). This would account for many of the observed electrophysiological effects of cardiac glycosides. Measurements of $[K^+]_i$ activity have demonstrated that there is approximately a 14% decrease in the value of $[K^+]_i$ activity that occurs concomitantly with a decrease in measured transmembrane potential due to cardiac glycoside exposure (Miura and co-workers, 1976 and 1977). Using a recessed-tip, Na^+ -sensitive glass microelectrode, it has been demonstrated that the $[Na^+]_i$ was 7.2 mM at normal $[Na^+]_o$ for sheep heart purkinje fibers but application of a cardiac glycoside to the perfusate produced a rise in $[Na^+]_i$ that was only slowly reversible (Ellis, 1977).

Fig. 4. Schematic illustration of the cellular mechanism of the action of digoxin. Digoxin inhibits Na^+, K^+-ATPase causing an increase in $[Na^+]_i$. Increased $[Na^+]_i$ causes an increase in $[Ca^{2+}]_i$ by increasing Ca^{2+} influx on the Na^+ - Ca^{2+} exchanger during the excitation contraction coupling of cardiac muscle. Increased $[Ca^{2+}]_i$ provides more Ca^{2+} that is available for Ca^{2+} - induced Ca^{2+} release and thus an enhanced Ca^{2+} transient and positive inotropic effect.


DIGOXIN
3Na+
Ca2+
(--)
T - TUBULE
Na+ ,K+ ATPase
SL Ca2+ ATPase
Na+ channel
Na+
2K+
L-Type Ca2+ Channel
Ca2+
SR Ca2+ Release Channel
(+)
Ca2+
Na+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
3Na+
Myosin
Actin

Measurements of Na^+ and Ca^{2+} across the cell membrane were linked by the Na^+ - Ca^{2+} exchange mechanism. An increase in Ca^{2+} influx associated with an increase in $[Na^+]_i$ could account for a positive inotropic effect in cardiac tissue after inhibition of monovalent cation active transport (Baker et al., 1969). These data support the hypothesis that inhibition of monovalent cation active transport by cardiac glycosides is causally related to the development of their positive inotropic effect on heart muscle.

Modern theories have suggested a greater role of $[Ca^{2+}]_i$ in autoregulation (Marban and Tsien, 1982). There might be a regulation of Ca^{2+} entry itself by a Ca^{2+} -facilitated mechanism that might amplify the digitalis glycoside effect of positive inotropy (Miura and Biedert, 1983). These theories suggest a far more complex role of the cardiac glycosides than just inhibition of the monovalent cation transport mechanism. This role may include biochemical intracellular feedback mechanisms that could autoregulate intracellular cation movements.

The cardiac glycosides bind to an alpha subunit of the Na^+, K^+-ATPase and inhibit the translocation of Na^+ and K^+. Thus, partial inhibition of the Na^+ pump in the sarcolemma of the myocyte by cardiac glycosides causes a slight rise in $[Na^+]_i$ and a slight fall in $[K^+]_i$. An increase in $[Na^+]_i$ would be expected to increase the influx of Ca^{2+} on the Na^+-Ca^{2+} exchanger initially during the action potential and to impair extrusion of Ca^{2+} by the Na^+ -Ca^{2+} exchange subsequently during the Ca^{2+} transient. Both of these effects would be expected to cause the observed increase in diastolic $[Ca^{2+}]_i$ and the increased loading of the SR with Ca^{2+}, also providing more Ca^{2+} available for Ca^{2+}-induced Ca^{2+}

release and thus an enhanced Ca^{2+} transient and a positive inotropic effect (Barry and Bridge, 1993). The cellular mechanism of action of digoxin is schematically illustrated in Fig. 4.

Many of the effects of digoxin on the electrical and mechanical activity of the heart result from glycoside-induced modification of both automatic neural activity and the sensitivity of the heart to the vagal and sympathetic neurotransmitters (Hoffman and Bigger, 1990). Digoxin increases the vagal activity and decreases the sympathetic activity. The increase in vegal activity caused by digoxin appears to result from effects at several sites in the nervous system. The atrial baroreceptors are sensitized, probably because of an effect of digoxin on active transport of cations in the afferent nerve terminals. Digoxin also increases the sensitivity of the sinus node to the negative chronotropic effect of acetylcholine (Hoffman and Bigger, 1990).

Digoxin reduces the sensitivity of the sinoatrial and atrioventricular nodes to catecholamines. If the sinus rate is increased, as in CHF, the negative chronotropic effect of digitalis is usually quite prominent. Inhibition in the sympathetic activity by digoxin contributes to the net effect. The atrioventricular node is strongly influenced by the indirect action of digoxin. The enhanced vegal activity and the decrease in sensitivity to the catecholamines have pronounced effects on both the generation of the atrioventricular nodal action potential and the transmission of impulses through the node. Acetylcholine decreases the rate of rise and amplitude of action potentials at these sites. The impairment of conduction may progress to complete heart block (Hoffman and Bigger, 1990). The

most important result is to diminish the rate at which atrial impulses can be transmitted to the ventricles. Thus, in atrial tachycardias, atrial flutter, and atrial fibrillation, administration of digoxin will decrease the ventricular rate because of a blockage of an increased fraction of atrial impulses in the atrioventricular junction.

Clinical use of Digitalis

Digitalis glycosides, primarily digoxin are widely used in modern medicine for the control of the ventricular response in atrial fibrillation and the treatment of CHF. Recently, the use of digoxin for the treatment of CHF in patients with normal sinus rhythm has become quite controversial. Several studies suggest a small but definite hemodynamic or clinical improvement in patients treated with digoxin. These effects are limited by the onset of toxicity, which is at least partially mediated via the central nervous system (CNS). If the inotropic effect of the drug could be separated from the CNS effect, much higher doses of digitalis could be used and presumably a greater therapeutic effect could be obtained (Levitt and Keefe, 1985).

Gheorghiade and Beller (1983) studied 24 patients with ischemic heart disease, CHF and normal sinus rhythm. Eighty-eight percent of those patients were also taking a diuretic or a vasodilator. Withdrawal of digoxin from the regimen of these patients did not influence their heart rate, arterial pressure, weight, or ejection fraction. The data suggested that the withdrawal of digitalis failed to worsen the clinical manifestation of CHF in these patients.

Davidson and Gibson (1973) studied the effect of digoxin in CHF patients with normal sinus rhythm. They found a positive inotropic effect on the initial administration of digoxin but they were not able to demonstrate this effect with long-term administration. Since, this initial effect was less than that produced by mild exercise one question is not whether digitalis has an effect, but whether the effect in patients with normal sinus rhythm is clinically meaningful.

Lee and Dagostino (1982) studied 25 patients who were in normal sinus rhythm with CHF. In slightly more than half of the patients, the severity of CHF was reduced by digoxin. Responders, individuals whose CHF was improved by digoxin, had more chronic and more severe CHF, greater left ventricular dilation, and lower ejection fractions. The presence of a third heart sound (third heart sound due to rapid ventricular filling) was cited by the author as an important predictor of whether patients would respond to digitalis. About half of the patients who were in normal sinus rhythm responded clinically to digitalis.

Mechanism of Digitalis Toxicity

The molecular mechanism of digitalis toxicity is the same as that for its therapeutic action, just at a more intense level. Binding of digitalis glycosides to a large fraction of the sarcolemmal Na^+, K^+-ATPase molecules leads to a marked decrease in the activity of the Na^+-K^+ exchange pump across the cell surface. As a result, $[Na^+]_i$ and $[K^+]_o$ increase. The increased $[Na^+]_i$ causes an increase in Na^+-Ca^{2+} exchange, raising the $[Ca^{2+}]_i$. The

increased $[Ca^{2+}]_i$ is buffered in the usual intracellular storage sites to a point where ultimately, in marked cardiac glycoside toxicity, the $[Ca^{2+}]_i$ increases (Bigger, 1985).

Monensin

Introduction

Ionophores carry ions across lipid barriers as complexes soluble in the lipid phase of membranes. The potential use of ionophores as probes of biological function, or as potential therapeutic agents, was recognized very early, but major economic importance was not forthcoming until the discovery of monensin in 1967 (Mollenhauer et al., 1990).

Initially, Shumard and Callender (1969) recognized the effectiveness of monensin against experimental infections of coccidia in chickens and subsequently it was used widely in birds for control of coccidiosis. Monensin is also efficacious in the control of coccidiosis in lambs and calves (Bergstrom and Maki, 1978 and Fitzgerald, 1973). In beef cattle it improves the feed efficiency by 10-15% due to an increase in the production of more propionic acid which yields more energy than acetic or butyric acids following ruminant fermentation (Richardson et al., 1974).

Of the more than one hundred ionophores that have been reported, three, monensin, lasalocid, and salinomycin, have wide spread commercial use and of these, licensed monensin is probably used most widely (Mollenhauer et al., 1990). Among the domestic animals, horses are most sensitive to monensin toxicity (Whitlock et al., 1978). If

monensin is used as a coccidiostat or growth promoter in other species, the drug-treated feed would be dangerous if accidentally consumed. The medial lethal single dose (LD_{50}) of monensin in horses is 2-3 mg / kg body weight (Mitema et al., 1988). The clinical signs of monensin toxicity in animals include anorexia, ataxia, lethargy, diarrhea, and paresis (Van Vleet et al., 1983).

Cellular Mechanism of Monensin Action

One of the original interests in ionophores was their perceived potential for directly modifying intracellular ionic gradient, particularly Ca^{2+}, which would lead, hopefully, to the development of useful pharmacological agents or, alternatively, provide a tool for studying cellular functions mediated by changes in $[Ca^{2+}]_i$. It was soon realized, however, that many of the inotropic effects of the divalent ionophores could be duplicated with even greater efficiency by monovalent ionophores such as monensin which complexes Na^+ but almost no Ca^{2+} (Mollenhauer et al., 1990).

Monensin is a monovalent polyether antibiotic in which the oxygen functions are concentrated at the center of the structure where they are available for the complexation of a suitable cation, in this case Na^+. The alkyl groups are spread over the outer surface rendering the complex lipid soluble thus allowing the antibiotic to enter and diffuse through biological membranes. It is an open chain molecule that is capable of ion complexation through a cyclic form stabilized by hydrogen bonding between the carboxyl and hydroxyl

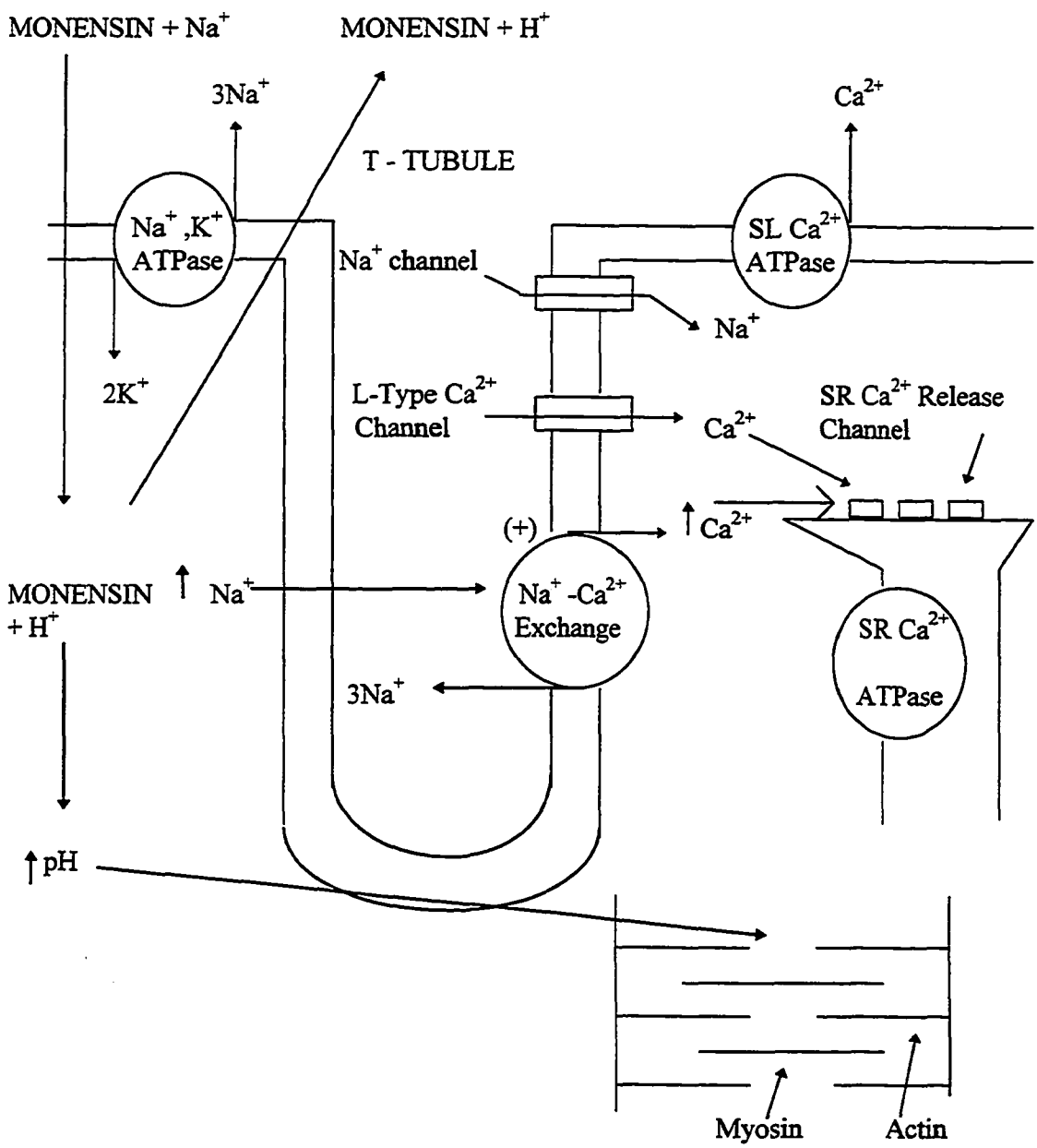
groups. Monensin mediates a Na^+ (in)/H^+ (out) exchange due to its affinity for Na^+ (Mollenhauer et al., 1990).

Once the ion transverses the membrane as a monensin-Na^+ complex, Na^+ is released, and the monensin molecule attracts a H^+ to form an undissociated molecule which can retraverse the membrane to release the H^+ to the outside of the cell, vesicle, organelle, or other subcellular compartment. Thus, the net effect for monensin is a transmembrane exchange (Fig. 5) of monovalent ions for protons (Mollenhauer et al., 1990).

Effects of ionophores on cation-transport and their distribution among different membrane-bounded compartments within the cell, will vary depending on the physical and chemical properties of the different membranes. Membrane fluidity, thickness, curvature, change and orientation of polar head groups of phospholipids, cholesterol content, and protein content, all influence solubility, penetration, and expression of the ionophore. The pharmacological effects of monensin will depend on the membrane composition and functional characteristics of the different compartments involved (Mollenhauer et al., 1990).

In cardiac tissue, monensin transports Na^+ across the sarcoplasmic membrane into the cytosol, thereby increasing $[Ca^{2+}]_i$ via a Na^+-Ca^{2+} exchange mechanisms (Gaide et al., 1984 and Sutko et al., 1977). An increase in $[Ca^{2+}]_i$ affects K^+ permeability, leading to shortening of the action potential (Isenberg, 1975). These results showed that, the increase in $[Ca^{2+}]_i$ increases K^+ conductance in cardiac tissue (Kass and Tsien, 1976 and McGuigan and Bassingthwaighte, 1974) through Ca^{2+}-activated K^+ channels.

Fig. 5. Schematic illustration of the cellular mechanism of action of monensin. Monensin binds Na^+ outside the myocyte and carries it into the cytosol. Inside the cell, monensin releases the Na^+ and binds a H^+ and carries it to the outside of the myocyte. The net effect is an increase in $[Na^+]_i$ and pH_i. Elevated $[Na^+]_i$ increases the $[Ca^{2+}]_i$ via the Na^+ -Ca^{2+} exchanger during the excitation contraction coupling. Both increased $[Ca^{2+}]_i$ and pH_i cause the positive inotropic action of monensin.


MONENSIN + Na+
MONENSIN + H+
3Na+
Ca2+
T - TUBULE
Na+ ,K+ ATPase
Na+ channel
SL Ca2+ ATPase
Na+
2K+
L-Type Ca2+ Channel
SR Ca2+ Release Channel
Ca2+
(+)
Ca2+
MONENSIN + H+
Na+
Na+ -Ca2+ Exchange
SR Ca2+ ATPase
3Na+
pH
Myosin
Actin

Monensin increases the resting tension in canine cardiac purkinje fibers and the tension developed decreases in Tyrodes solution with an increase in Ca^{2+} to 4.5 mM. This is due to a Ca^{2+} overload probably related to the high $[Ca^{2+}]_o$ (Tsuchida and Otomo, 1990). The most characteristic changes in the action potential elicited by monensin in canine purkinje fibers are the shortening of the duration and the suppression of automaticity. On the other hand, monensin does not significantly affect the action potential parameters, either in the Ca^{2+} -free solution or in the Na^{+} -free solutions. Therefore, either extracellular Na^{+} or Ca^{2+} is necessary for monensin to exert its effects on the action potential (Tsuchida and Otomo, 1990).

In 1989, Hugtenburg et al., studied the differential effect of Ca^{2+} channel antagonists on the positive inotropic effects induced by Ca^{2+} and monensin in cardiac preparations of rats and guinea-pigs. They found that monensin did not change the basal force of contraction in rat papillary muscle but it increased the force of contraction in guinea-pig papillary muscle. This difference indicates that the utilization of Ca^{2+} in rat and guinea-pig myocardium is different. They also used nifedipine which is an L-type Ca^{2+} channel blocker and ryanodine which is a ryanodine receptor blocker. They found that the positive inotropic effect of monensin in guinea-pig papillary muscles appeared nearly insensitive to inhibition by nifedipine and was only partially blocked by ryanodine. They concluded that monensin-generated Ca^{2+} may be through the Ca^{2+}-induced Ca^{2+} release mechanism.

In 1984, Ozaki et al., studied the action of monensin on smooth muscle of guinea-pig aorta. They found that monensin induced a contraction in isolated guinea-pig aorta in normal K^+ (5.4 mM) solution. They also observed that the monensin-induced contraction was markedly inhibited by α-adrenoceptor antagonists phentolamine (10^{-5} M) or prazosin (10^{-6} M). They concluded that the monensin-induced contraction resulted from the release of norepinephrine from the nerve endings.

Toxicity Studies of Monensin

The mechanism by which monensin interacts with coccidia and rumen microflora is well documented (Mollenhauer et al., 1990). However, the interaction between monensin and the tissue of the host animal is less well understood even though the clinical manifestations of monensin poisoning are well known. When used at recommended levels, either as a coccidiostat or a growth promoter, monensin seldom causes poisoning. Misuse of the product, usually from improperly mixed feed, may cause toxicosis and death. Horses, ponies and other equine species are particularly sensitive to monensin poisoning. In mammals, the physical signs of monensin toxicosis commonly include anorexia, diarrhea, depression, sweating, ataxia, palpitations of the heart, sudden death following exercise (Mollenhauer et al., 1990). Stiffness of hindquarters and swollen gluteus muscles, elevated pulse rate, and electrocardiograph abnormalities also have been reported (Mollenhauer et al., 1990). The most consistent microscopic observation in ponies, cattle,

pigs and fowl is cardiac myocyte degeneration and vacuolization (Mollenhauer et al., 1990).

A differential effect of monensin was noted by Van Vleet and Ferrans, 1983 who observed in swine severe necrosis in the diaphragm, vastus lateralis, semitendinosus, triceps and intercostal muscles; moderate necrosis in longissimus lomburum muscle; and little necrosis in the tongue. Necrosis was the greatest in muscles containing a high portion of Type 1 fibers. Monensin toxicosis in swine provides a unique example of acute drug-induced myotoxicity that is accompanied by rapid and complete regeneration of the widespread necrosis of skeletal muscle and the biochemical pathogenesis of monensin-induced myotoxicity is presumed to result from Ca^{2+} overloading.

Swollen mitochondria (a characteristic response to monensin) have not been observed in any of the nonmuscle cells of the heart, diaphragm, or appendicular tissues or in liver, adrenal or kidney cells (Mollenhauer et al., 1990). Thus, it appears that monensin administered to mammals invivo tends to induce mitochondrial changes only in selected tissues and / or types of muscle fibers. The mechanisms for these mitochondrial changes and reasons for specificity are not known.

FUNCTIONAL CHANGES IN ISOLATED GUINEA-PIG PAPILLARY MUSCLE INDUCED BY MONENSIN AND DIGOXIN

A paper to be submitted to the *Journal of Action and Agents*

Ismail Meral, Frederick B. Hembrough, Theodore B. Bailey, and Walter H. Hsu

Abstract

We investigated four different concentrations of digoxin (0.3, 1, 3 and 10 μmol/l) and three different concentrations of monensin (2, 7 and 12 μmol/l) on the contraction force of guinea-pig papillary muscles. The highest increase in contraction force was obtained by using 3 μmol/l digoxin or 7 μmol/l monensin. These concentrations were chosen for subsequent experiments to study the effects of these drugs on contraction force (CF), initial contraction velocity (ICV), average contraction velocity (ACV), initial relaxation velocity (IRV) and stimulus to response time (ST) change in fatigued (defined as the level at which papillary muscle contraction had lost 30% of its original contraction force with the elapse of time) and non-fatigued guinea-pig papillary muscles. The five hours of measurement in Experiment 3 were divided into five periods (T0 was equilibration, T1, T2, T3, and T4 were respectively one, two, three, and four hours after drug administration). We found that both monensin and digoxin increased the CF and ACV at T1 and increased the IRV at T2. Digoxin lost its effect with the elapse of time while monensin did not. Digoxin also

increased the ICV at T1 and decreased the ST at T2, T3 and T4. However, monensin increased the ICV at T4 and did not change the ST. We also found that although fatigued and non-fatigued guinea-pig papillary muscles behaved differently during four hours of experimentation, they did not respond to the drug treatment differently. We concluded that the initial effects of these two drugs on guinea-pig papillary muscles are similar regarding contractility but in time digoxin loses its positive inotropic effect while monensin does not. Thus, monensin should be studied further to determine if it is useful in the treatment of congestive heart failure

Introduction

Monensin is a carboxylic ionophore antibiotic which is derived from the fermentation of the fungus *Streptomyces cinnemonensis* [1]. It is used as a coccidiostat in birds, lambs and calves [2, 3, 7] and primarily as a growth promoter [4, 5] and as a bloat preventive [3, 6] in cattle. The biochemical pathogenesis of monensin-induced myotoxicity has been shown to be caused by intracellular calcium overloading, especially in mitochondria [8].

Monensin has a strong positive inotropic affect in cardiac muscle [9, 10, 11]. This effect occurs because, as an ionophore, monensin has strong affinity for $[Na^+]_o$. The affinity of monensin for Na^+ is ten times that for K^+, its nearest competitor in biological systems [8]. The monensin-induced increase in $[Na^+]_i$ facilitates the entry of Ca^{2+} into the cell by a Na^+ (out) / Ca^{2+} (in) exchange mechanism [12]. This Ca^{2+} shift is the primary

factor mediating the cellular response. Another factor modifying the cellular response includes the alteration of the pH of intracellular components (pH_i) since monensin increases the pH_i by transferring H^+ out of the cell [8].

Digitalis glycosides, including digoxin, have been used in the treatment of arrhythmia and heart failure for over 200 years [13]. Although cardiac glycosides augment contraction in the isolated heart or muscle preparations their effect in the normal mammalian heart remains in dispute. The inotropic action of digoxin in the myocardium depends on an inhibition of Mg^{2+}-dependent Na^+, K^+-ATPase which is necessary for the Na^+ (out) / K^+ (in) exchange. Increased $[Na^+]_i$ leads to an increase in $[Ca^{2+}]_i$ by a Na^+ (out) / Ca^{2+} (in) exchange mechanism and thus, increases force of contraction [14, 15].

In the present study, we compared the positive inotropic actions of digoxin and monensin over a four-hour experimental period. Many pharmacological experiments have compared different drugs according to their initial effects. Treatment of papillary muscles with these drugs for four hours gave us a better understanding of the effects of these two drugs with time. In the present experiment, we also compared non-fatigued (fresh) papillary muscles to "fatigued (tired) papillary muscles". "Fatigued muscles" had lost 30% of their original contraction force with the elapse of time before they were treated. It has been suggested that if the capacity of heart muscle to develop force is severely depressed, the inotropic action of digoxin is much larger than would occur in normal muscle [27]. This rationale suggested that the effects of these two drugs may be different on fatigued muscle when compared with non-fatigued muscle.

The objectives of this experiment were to utilize a papillary muscle preparation to compare monensin with digoxin using a fresh preparation and a preparation undergoing fatigue, and to establish the need for further investigation of monensin for the possibility of using it in the management of certain clinical syndromes such as congestive heart failure (CHF).

Materials and Methods

Preparation of Papillary Muscles

Thirty male guinea-pigs, 500-600 g, were heparinized (1000 IU per animal, IP injection) 30 minutes before being decapitated. One animal was used per day. Hearts were removed rapidly and put in a beaker filled with ice-cold KRB of the following composition [mmol/l]: Na^+, 115.9: Ca^{2+}, 2.2: K^+, 4.0: Mg^{2+}, 1.3: Cl^-, 126.9: $H_2PO_4^-$, 2.1: HCO_3^-, 21.7: glucose, 10.9. The pericardium was removed, and the aorta and the pulmonary artery were excised. The hearts were transferred to a second beaker of the same solution, and then rapidly put in a glass pan filled with Krebs solution and bubbled with 100 % O_2. Two papillary muscles with diameters of approximately 1.0-1.5 mm were dissected from the right ventricle of each guinea-pig and each muscle was mounted in an organ bath containing a Krebs solution with 2.2 mmol/l $CaCl_2$. The Krebs solution was perfused with a mixture of 95 % O_2 and 5 % CO_2 and maintained at 37 ^{0}C. The pH was maintained between 7.50 - 7.60. The muscles were attached to a capacitance transducer (Harvard isometric capacitance transducer, Harvard apparatus, inc., South Notick, MA) electrically

connected to a Beckman recorder (R611). The tendon end of each muscle was attached to the transducer by a silk suture and the opposite end was held by a plastic clamp placed in the muscle chamber. Muscles were stimulated at a frequency of 0.2 PPS by using a pair of platinum field effect electrodes. The transducers to measure force were calibrated for each experiment by using weights (1 g equaled a displacement of 40 mm). Frequency was calibrated by an oscilloscope.

Experiment 1: Effect of digoxin on the contraction amplitude of guinea-pig papillary muscle (Fig. 1)

A stock solution of 1.5 mmol/l digoxin (Sigma Chemical Co., St. Louis, MO) prepared with alcohol was used. Five treatments were used; control (0.18% alcohol), and digoxin at 0.3, 1, 3 and 10 μmol/l. Each treatment was replicated four times. Treatments were administered after an equilibration period of 35 min. Contraction force was measured one hour after treatment because the highest contraction force was obtained one hour after digoxin administration.

Experiment 2: Effect of monensin on the contraction amplitude of guinea-pig papillary muscle (Fig. 2)

A stock solution of 3.5 mmol/l monensin (Sigma Chemical Co., St. Louis, MO) prepared with alcohol was used. Four treatments were used; control (0.18% alcohol), and monensin at 2, 7 and 12 μmol/l. Treatments were administered after an equilibration period

of 35 min. Contraction force was measured two hours after treatment. Unlike the digoxin experiment, the highest force was obtained two hours after monensin administration.

Experiment 3: Contractility Experiment

Sixty papillary muscles were obtained from 30 guinea-pigs as described in the preceding section. Two different treatments were used on the same day on two different papillary muscles obtained from one guinea pig. With non-fatigued muscles, chemicals were administered to the organ bath after an equilibration period of approximately 35 min. With fatigued muscles, chemicals were administered to the organ bath after a fatigue period which was defined as the level at which papillary muscle contraction had lost 30% of its original contraction force with the elapse of time. Data were obtained from each muscle five times (before treatment application and 1, 2, 3, and, 4 hours after continuos treatment). Variables measured at each period included contraction amplitude, contraction angle, relaxation angle and stimulus to response time. Contraction force (CF), initial contraction velocity (ICV), average contraction velocity (ACV), initial relaxation velocity (IRV) and stimulus to response time (ST) were calculated as functions of the measured variables. Because there was no significant difference between fatigued and non-fatigued guinea-pig papillary muscles to the drug treatment over time, data obtained from fatigued and non-fatigued papillary muscles were combined.

Treatment design, experimental design and statistical analysis

Six treatments were used in this study (Table 1. shows the treatment mean ± standard deviation of the mean). Treatments corresponded to a 2 by 3 factorial with 2 methods and 3 drugs. Doses used were based on the results of the previous two experiments.

Treatment	Drug	Method
1	Control	Non-fatigued
2	Control	Fatigued
3	Digoxin	Non-fatigued
4	Digoxin	Fatigued
5	Monensin	Non-fatigued
6	Monensin	Fatigued

Because of the large variability among animals, a balanced incomplete block design was selected as the experimental design. Each animal provided two muscles, individual muscles were the experimental unit for the experiment. Blocks were size of two; with each block containing two muscles from the same animal. Experimental units (a single muscle) were randomly allocated to treatments [see appendix]. Two replications of the basic incomplete block design were completed. Analyses of variance were obtained using PROC MIXED of SAS (SAS institute, Inc., 1988, release 6.03, Cary, NC). Fixed effects in the

model included drug, method, time, the three two-factor interactions and the three factor interaction drug by method by time. Random effects in the model were block and block by treatment. Unbiased estimates of treatment means and their errors were obtained using the LSMEANS statement. The ANOVA indicated a significance when $P \leq 0.05$ [see appendix for ANOVA tables]. Contrasts [30] were used to test differences among means [see appendix].

Results

Dose response of digoxin and monensin

The effect of four different concentrations of digoxin on the contraction amplitude of guinea-pig papillary muscle is shown in Fig. 1. Three µmol/l digoxin caused the highest increase in contraction amplitude (contraction force). Fig 2 shows the effect of three different concentrations of monensin on the contraction amplitude of guinea-pig papillary muscle. Seven µmol/l monensin caused the highest increase in contraction amplitude (contraction force).

Effects of monensin and digoxin on contraction force, initial contraction velocity, average contraction velocity, initial relaxation velocity and stimulus to response changes of guinea-pig papillary muscles

Fig. 3 shows the changes in CF of guinea-pig papillary muscles during five time periods of the experiment. Digoxin caused a positive inotropic effect in guinea-pig

papillary muscles at T1. However, digoxin lost its positive inotropic action with the elapse of time and caused a negative rather than a positive inotropic action at T3 and T4. Monensin also caused a positive inotropic effect in guinea-pig papillary muscles at T1. However, monensin did not lose its positive inotropic action up to T4.

Fig. 4 shows the changes in the ICV of guinea-pig papillary muscles during five time periods of the experiment. Digoxin increased the ICV of guinea-pig papillary muscles at T1 and monensin increased the ICV at T4. Digoxin lost its effect between T2 and T4.

Fig. 5 shows the changes in the ACV of guinea-pig papillary muscles during five time periods of the experiment. Both monensin and digoxin increased the ACV of guinea-pig papillary muscles at T1. Digoxin lost its effect at T2 and T3 and decreased the ACV at T4. Monensin did not lose its positive effect up to T4.

Fig. 6 shows the changes in the IRV of guinea-pig papillary muscles during five time periods of the experiment. Both monensin and digoxin increased the IRV of guinea-pig papillary muscles at T2. Digoxin lost its effect at T3 and T4. However, monensin did not lose its positive effect up to T4.

Fig. 7 shows the changes in the ST of guinea-pig papillary muscles during five time periods of the experiment. Digoxin decreased the ST at T2, T3 and T4. However, monensin did not change the ST at any time period.

Fig. 8 shows the representative tracing of the monensin- or digoxin-induced changes in the CF of guinea-pig papillary muscles. Digoxin produced after-contractions

starting at T2. After-contractions and decrease in the positive inotropic effect of digoxin occurred at the same time. Monensin did not produce after-contractions with time.

Although fatigued and non-fatigued guinea-pig papillary muscles behaved differently during four hours of experimentation, they did not respond to the drug treatment differently over time.

Discussion

Effects of monensin and digoxin on contraction force, initial contraction velocity, average contraction velocity, initial relaxation velocity and stimulus to response change of guinea-pig papillary muscle

This study was undertaken to determine whether monensin and digoxin had the same effect on guinea-pig papillary muscles regarding contraction force, rate of contraction and relaxation and stimulus to response time. Because the contraction of heart muscle is affected by many factors, such as, pH_i, interaction of actin-myosin, changes in ionic concentration, this experiment, like many others, produced more questions than answers.

Contractility is the performance of a heart at a given preload and afterload. An increase in contractility (positive inotropic effect) is not only reflected by increments in developed force but also reflected by increments in velocity. Digitalis glycosides increase the contractile state of the myocardium by shifting the force-velocity relation upward and to the right in human subject [14].

The positive inotropic action of digoxin is due to the inhibition of Na^+, K^+-ATPase. As a result, there is a gradual increase in $[Na^+]_i$ and a gradual and small decrease in $[K^+]_i$ [16, 17]. An increase in $[Na^+]_i$ by digoxin increases the $[Ca^{2+}]_i$ due to the entry of Ca^{2+} into the cell by the Na^+ (out) / Ca^{2+} (in) exchange [18]. Increased $[Ca^{2+}]_i$ causes the release of additional Ca^{2+} from the SR [19] and, thus enhances the contractility . The Na^+-H^+ exchange also appears to play a role in amplifying the increase in $[Ca^{2+}]_i$ that results from inhibition of the Na^+, K^+-ATPase. The increase in $[Na^+]_i$ causes a fall in pH_i, as protons are exchanged for $[Na^+]_i$ to compensate for decreasing the increased $[Na^+]_i$ [20].

In our experiment we were able to demonstrate the positive inotropic effect of digoxin in guinea-pig papillary muscles. Digoxin's positive inotropic effect occurred gradually. It took 60 min to obtain a positive inotropic effect with digoxin treatment. Our result is consistent with that of Taubert and colleagues [24] who demonstrated a positive inotropic effect in cat papillary muscles by using 2 μmol/l digoxin over 110 min. We also demonstrated that continuous treatment of a papillary muscle with digoxin eliminated the positive inotropic action of digoxin. This is the first in vitro experiment that examined the action of digoxin on guinea-pig papillary muscle for four hours after treatment and demonstrated a decrease in digoxin induced positive inotropy with the elapse of time. Wier and Hess [25] have suggested that the negative inotropic effect of high concentrations of digoxin (> 10 μmol/l) may be related to lowering of pH_i [by a Na^+ (out)/H^+ (in) mechanism] which would decrease the affinity of the myofilaments for Ca^{2+}. However, the contraction of digoxin in our experiment was lower than 10 μmol/l. Our results indicated

that even a lower concentration of digoxin (3 μmol/l) may cause a negative rather than a positive effect with the elapse of time probably due to a decreased pH_i.

The 7 μmol/l monensin administration increased the CF of guinea-pig papillary muscles at T1 (60 min after treatment). Unlike digoxin, monensin did not lose its positive inotropic effect on guinea-pig papillary muscles with the elapse of time. This demonstrated that the positive inotropic effect that was obtained with monensin treatment lasted longer than digoxin. Monensin binds Na^+ outside of the cell and carries it into the cell and increases $[Na^+]_i$ and thus $[Ca^{2+}]_i$ [8]. Monensin also increases the pH_i due to the increased exit of a H^+ when monensin is transported out of the cell [8].

The action of these two drugs rely on an increase in $[Ca^{2+}]_i$ due to an increased $[Na^+]_i$. However, the mechanism is different. Although a Ca^{2+} shift is the primary factor mediating the cellular response of these two drugs, another factor modifying the cellular response is the pH_i change. Contraction of the heart is pH sensitive; classically an acidosis reduces contractions by inhibiting the Na^+-Ca^{2+} exchange [22, 23], whereas an alkalosis increases contractions by stimulating Na^+-Ca^{2+} exchange and the release of Ca^{2+} from the SR [21]. Digoxin induces an increase in $[Na^+]_i$ which in turn causes a fall in pH_i , as protons are exchanged for intracellular Na^+ to lower the increased $[Na^+]_i$ [20].

To be able to demonstrate whether digoxin- or monensin-induced increase in $[Ca^{2+}]_i$ is due to an influx or a release from the SR, we measured the ICV and the ACV. There are two components, early (P1) and the late (P2) components, of guinea-pig papillary muscle contraction wave [28]. The P1 is suggested to be generated by Ca^{2+} released from SR

whereas P2 is thought to result from Ca^{2+} influx [28]. The ICV would be affected by the change in P1 and the ACV would be affected by those factors that produce both P1 and P2 of the contraction wave [29]. An increase in the rate of Ca^{2+} release from the SR would increase the ICV. It has been demonstrated that 3 and 10 μmol/l digoxin strongly enhanced P1 which overlapped P2 [26]. When P1 is inhibited by using a Ca^{2+} -free solution, digoxin exerts only a negative inotropic effect [29]. Thus, in agreement with Adamantidis *et al.*, [26], the positive inotropic effect of digoxin is thought to be mediated by an increase in activator Ca^{2+} which enters the cell and causes the release of Ca^{2+} from the SR. Digoxin increased the ICV of guinea-pig papillary muscles at T1 and monensin increased the ICV at T4. Digoxin lost its effect with the elapse of time. This finding suggested that both digoxin and monensin increase the rate of Ca^{2+} release from the SR in guinea pig papillary muscles. However, digoxin lost its effect with time probably due to a decreased pH_i.

The ACV is not only influenced by the rate of Ca^{2+} release from the SR but also it is influenced by the Ca^{2+} influx, number of functional cross-bridges and affinity of troponin C for Ca^{2+} [29]. An increase in Ca^{2+} release from the SR would increase ACV by allowing increased binding of Ca^{2+} to the contractile proteins and by increasing ATPase activity of myosin [14]. When Ca^{2+} binds to troponin C, in an isotonic contraction, a conformational change is induced that results in the interaction of actin and myosin, with the cross-bridge interaction and contractile element shortening [14]. Both monensin and digoxin increased the average contraction velocity of guinea-pig papillary muscles at T1. Digoxin lost its effect with the elapse of time while monensin did not. This clearly indicated both digoxin

and monensin have a significant effect on the rate of Ca^{2+} release from the SR, the affinity of troponin C for Ca^{2+} or myosin-ATPase activity. However, digoxin had an inhibitory effect at T4 which could be due to a decrease in pH_i that inhibits the affinity of troponin C for Ca^{2+} [23].

The IRV is affected by two mechanisms. First, a SR Ca^{2+}-ATPase that pumps Ca^{2+} into the SR thus decreases $[Ca^{2+}]_i$. Second, a Na^+ (in) / Ca^{2+} (out) exchange [14]. Under steady-state conditions, the amount of Ca^{2+} entering the cell equals that which exits the cell, and the amount released by the SR equals that sequestered by the SR [14]. If an abrupt change in this balance of fluxes takes place, then the Ca^{2+} content of the SR will be affected. Both monensin and digoxin increased the IRV of guinea-pig papillary muscles at T2. Digoxin lost its effect with the elapse of time while monensin did not. This increase in IRV was due to the SR Ca^{2+}-ATPase and the Na^+ (in) / Ca^{2+} (out) exchange which would be stimulated when $[Ca^{2+}]_i$ increased in order to lower the increased $[Ca^{2+}]_i$.

Monensin did not change the ST at any time period. However, digoxin decreased the ST at T2, T3 and T4. This indicated that papillary muscles that were treated with digoxin became more sensitive to the electrical stimulus. This indicated that voltage dependent Ca^{2+}-channels and voltage dependent Na^+-channels would become more sensitive and open earlier in muscles treated with digoxin.

The toxic effect (after-contractions) of digoxin was noted [31] at high doses (> 10 μmol/l) is due to an increase in $[Ca^{2+}]_i$. These after-contractions are produced by an oscillatory release of Ca^{2+} from SR by a Ca^{2+}-induced Ca^{2+} release mechanism [32] and can

be reversed by the addition of a Ca^{2+} channel blocking agent [31]. The concentration of digoxin (3 μmol/l) in our experiment was lower than 10 μmol/l. However, our result indicated that even lower concentration of digoxin may produce a toxic effect on guinea-pig papillary muscles with a long term treatment. Unlike digoxin, monensin did not produce a toxic effect with time. However, more studies are needed to evaluate the effect of monensin on living animals.

We concluded that although fatigued and non-fatigued muscles acted differently over time, they did not respond to the drugs differently. However, if fatigue was increased to 50%, instead of 30%, these papillary muscles might have responded to drug treatment differently than non-fatigued papillary muscles. More importantly the treatment with monensin demonstrated that the positive inotropic effects lasted longer than with digoxin treatment. This was also true for the effect of monensin on the average contraction velocity which was long lasting as well as being a potent positive inotrope. This increased contraction velocity, as compared to both the control and the digoxin treated papillary muscles, is important; as an increased contraction velocity acts as an decreased afterload to a functional heart and can be an important contributor to heart failure.

References

[1] M. E. Jr. Haney, and M. M. Hoehn, *Monensin, a new biologically active compound: I. Discovery and isolations.* Antimicrob. Agents Chemother. 349-352 (1967).

[2] M. Fahim, and B. Pressman, *Cardiovascular effects and pharmacokinetics of the carboxylic ionophore monensin in dogs and rabbits.* Life Sci. *29*, 1959-1966 (1981).

[3] J. F. Van Vleet, and V. J. Ferrans, *Ultrasonic alterations in skeletal muscle of pigs with acute monensin myotoxicosis.* Am. J. Pathol. *114(3)*, 461-471 (1984).

[4] T. H. Elsasser, *Potential interactions of ionophore drugs with divalent cations and their function in the animal body.* J. Anim. Sci. *59(3)*, 845-853 (1984).

[5] J. H. Thornton, F. N. Owens, R. P. Lemenager, and R. Tatusek, *Monensin and ruminant methane production.* J. Anim. Sci. *43*, 336 (Abstr) (1976).

[6] E. L. Potter, C. O. Cooley, L. F. Richard, A. P. Roun, and R. P. Rathmacher, *Effect of monensin on performance of cattle fed forage.* J. Anim. Sci. *43*, 665-669 (1976)

[7] E. S. Mitema, and S. Sangiah, *Effects of some calcium modulators on monensin toxicity.* Vet. Hum. Toxicol. *30(5)*, 409-413 (1988).

[8] H. H. Mollenhauer, D. J. Morre, and L. D. Rowe, *Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity.* Biochim. Biophys. Acta. *1031*, 225-246 (1990),

[9] B. C. Pressman, *Biological applications of ionophores.* Ann. Rev. Biochem. *45*: 501-529 (1976).

[10] J. L. Sutko, H. R. Besch, J. C. Bailey, G. Zimmerman, and A. M. Watanabe, *Direct effects of the monovalent cation ionophores monensin and nigericin on myocardium.* J. Pharmacol. Exp. Ther. *203*: 685-700 (1977).

[11] M. Shlafer, and P. Kane, *Subcellular actions and potential adverse cardiac effects of the cardiotonic ionophore monensin.* J. Pharmacol. Exp. Ther. *214:* 567-573 (1980).

[12] K. Tsuchida, and S. Otomo, *Electrophysiological effects of monensin, a sodium ionophore, on cardiac purkinje fibers.* Eur. J. Pharmacol.. *190,* 313-320 (1990).

[13] J. Somberg, and B. Greenfield, *Digitalis: Historical development in clinical medicine.* J. Clin. Pharmacol. *25,* 484-489 (1985).

[14] W. H. Barry, and J. H. B. Bridge, *Intracellular calcium homeostasis in cardiac myocytes.* Circulation. *87(6),* 1806-1815 (1993).

[15] D. S. Miura, and S. Biedert, *Cellular mechanisms of digitalis action.* J. Clin. Pharmacol. *25,* 490-500 (1985).

[16] J. W. Deitmer, and D. Ellis, *Intracellular sodium activity of cardiac purkinje fibers during inhibition and reactivation of the Na-K pump.* J. Physiol. (*Lond.*) *284,* 241-259 (1978).

[17] D. A. Eisner, W. J. Lederer, and R. D. Vaughan-Jones, *The control of tonic tension by membrane potential and intracellular sodium activity in the sheep cardiac purkinje fibre.* J. Physiol. (*Lond.*). *335,* 723-743 (1983).

[18] C. O. Lee, and M. Dagostino, *Effect of strophanthidin on intracellular Na ion activity and twitch tension of constantly driven canine cardiac purkinje fibers.* Biophys. J. *40,* 185-198 (1982).

[19] A. Fabiato, and F. Fabiato, *Calcium release from sarcoplasmic reticulum.* Circ. Res. *40,* 119-129 (1977).

[20] D. Kim, E. J. Cragoe, and T. W. Smith. *Relations among sodium pump inhibitions, Na-Ca and Na-H exchange activities, and Ca-H interaction in cultured chick heart cells.* Circ. Res. *60,* 185-193 (1987).

[21] P. A. Poole-Wilson, *Regulation of intracellular pH in the myocardium; relevance to pathology.* Mol. Cell. Biochem. *89,* 151-155 (1989).

[22] C. Orchard, *The effect of acidosis on excitation-contraction coupling in isolated ferret heart muscle.* Mol. Cell. Biochem. *89,* 169-173 (1989).

[23] E. M. Blanchard and R. J. Solaro, *Inhibition of the activation and troponin calcium binding of dog cardiac myofibriles by acidic pH.* Circ. Res. *55:* 382-391 (1984).

[24] K. Taubert, J. T. Willerson, W. Shapiro and G. H. Templeton, *Contraction and resting stiffness of isolated cardiac muscle: effects of inotropic agents.* Am. J. Physiol. *1 (3):* H275-H282 (1977).

[25] W. G. Wier and P. Hess, *Excitation-contraction coupling in cardiac purkinje fibers. Effects of cardiotonic steroids on the intracellular (Ca) transient, membrane potential, and contraction.* J. Gen. Physiol. *83:* 395-415 (1984)

[26] M. M. Adamantidis, E. R. Honore and B. A. Dupuis, *Electrical and mechanical effects of new aminosteroids on guinea-pig isolated ventricular muscle.* Br. J. Pharmacol. *95:* 1063-1074 (1988).

[27] F. H. Brian and B. Jr. Thomas, *Digitalis and allied cardiac glycosides.* Goodman and Gilman's the pharmacologic basis of therapeutics. Eighth edition. 815.

[28] E. Honore, M. M. Adamantidis, B. A. Dupuis, C. E. Challice and P. Guilbault, *Calcium channels and excitation-contraction coupling in cardiac cells. I I. A pharmacological study of the biphasic contraction in guinea-pig papillary muscles* Can. J. Physiol. *65:* 1832-1839 (1987).

[29] E. Honore, M. M. Adamantidis, B. A. Dupuis, C. E. Challice and P. Guilbault, *Calcium channels and excitation-contraction coupling in cardiac cells. I. Two components of contraction in guinea-pig papillary muscles.* Can. J. Physiol. *65:* 1832-1839 (1987).

[30] G. W. Snedecor and W. G. Cochran, *Statistical methods.* Eight edition. 227 (1989).

[31] J. P. Morgan, *The effects of digitalis on intracellular calcium transients in mammalian working myocardium as detected with aequorin.* J. Mol. Cell Cardiol. *17:* 1065-1075 (1985).

[32] E. Marban and R. W. Tsien, *Enhancement of calcium current during digitalis inotropy in mammalian heart: Positive feed-back regulation by intracellular Calcium?* J. Physiol (Lond). *329:* 589-614 (1982).

TABLE 1. Means of contraction force, initial contraction velocity, average contraction velocity, initial relaxation velocity and stimulus to response time of guinea-pig papillary muscles treated with digoxin (3 μmol/l), monensin (7 μmol/l) and control (alcohol). Data are presented as means ± SDM (n=20). CF; contraction force, ICV; initial contraction velocity, ACV; average contraction velocity, IRV; initial relaxation velocity, ST; stimulus to response time.

		DRUG		
Variable	Time	Digoxin	Monensin	Control
CF (g)	0	0.44 ± 0.05	0.40 ± 0.05	0.43 ± 0.05
	1	0.66 ± 0.05	0.64 ± 0.05	0.48 ± 0.05
	2	0.51 ± 0.05	0.64 ± 0.05	0.50 ± 0.05
	3	0.29 ± 0.05	0.62 ± 0.05	0.46 ± 0.05
	4	0.16 ± 0.05	0.61 ± 0.05	0.40 ± 0.05
ICV (g/sec)	0	1.8 ± 0.4	1.7 ± 0.4	1.5 ± 0.4
	1	3.9 ± 0.4	3.3 ± 0.4	1.8 ± 0.4
	2	2.6 ± 0.4	3.1 ± 0.4	2.3 ± 0.4
	3	2.1 ± 0.4	3.8 ± 0.4	2.3 ± 0.4
	4	1.0 ± 0.4	3.8 ± 0.4	1.9 ± 0.4
ACV (g/sec)	0	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
	1	2.5 ± 0.2	2.4 ± 0.2	1.4 ± 0.2
	2	2.2 ± 0.2	2.9 ± 0.2	1.7 ± 0.2
	3	1.3 ± 0.2	2.8 ± 0.2	1.6 ± 0.2
	4	0.7 ± 0.2	3.0 ± 0.2	1.4 ± 0.2
IRV (g/sec)	0	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
	1	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.1
	2	2.0 ± 0.1	1.7 ± 0.1	1.2 ± 0.1
	3	1.0 ± 0.1	1.8 ± 0.1	1.1 ± 0.1
	4	0.6 ± 0.1	1.7 ± 0.1	1.1 ± 0.1
ST (msec)	0	54 ± 3.8	47 ± 3.8	53 ± 3.9
	1	44 ± 3.8	38 ± 3.8	48 ± 3.9
	2	31 ± 3.8	33 ± 3.8	45 ± 3.9
	3	28 ± 3.8	35 ± 3.8	45 ± 3.9
	4	17 ± 3.8	36 ± 3.8	40 ± 3.9

FIG. 1. Dose response curve of digoxin. The largest contraction force was obtained by using 3 μmol/l digoxin. Lsmean + SDM is shown (n=4).

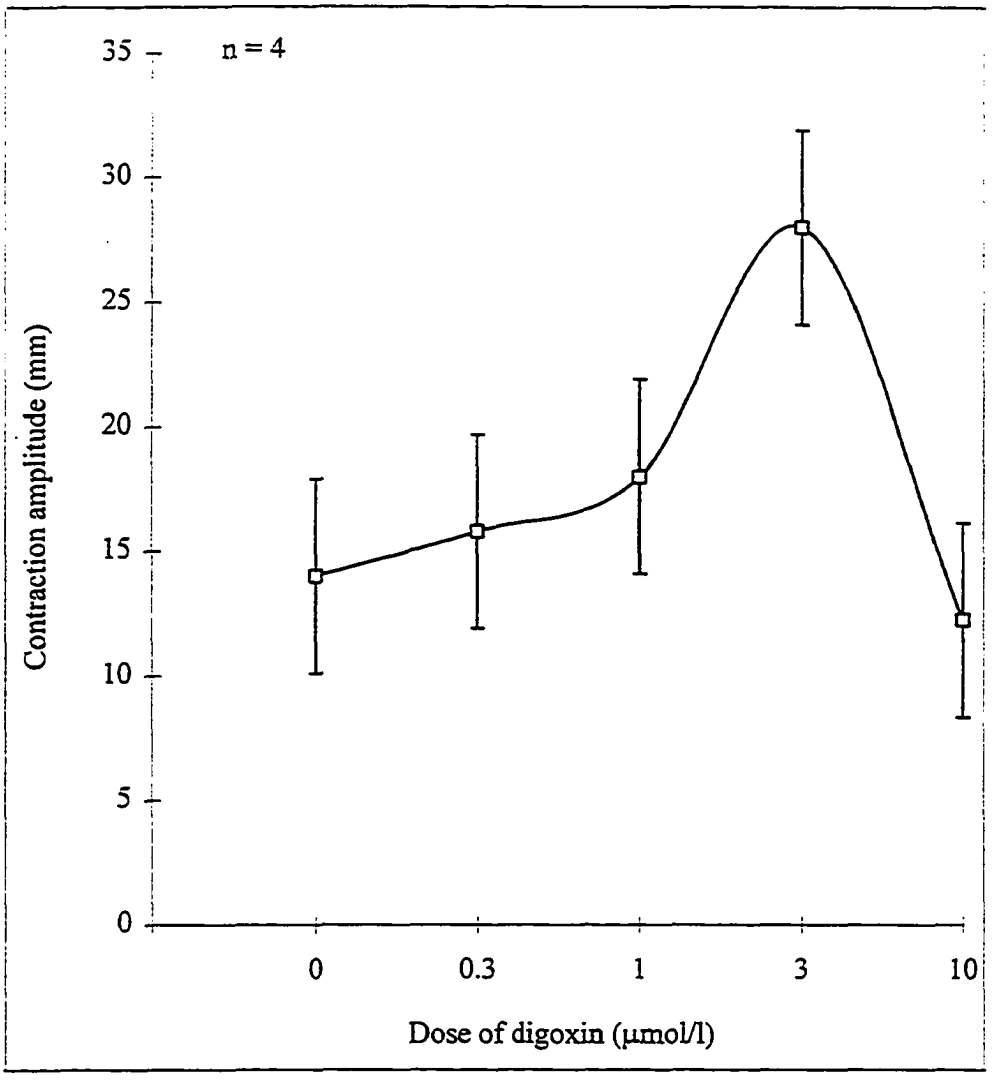

n = 4
35
30
25
20
15
10
5
0
Contraction amplitude (mm)
0
0.3
1
3
10
Dose of digoxin (µmol/l)

FIG. 2. Dose response curve for monensin. The largest contraction force was obtained by using 7 μmol/l monensin. Lsmean + SDM is shown (n=4).

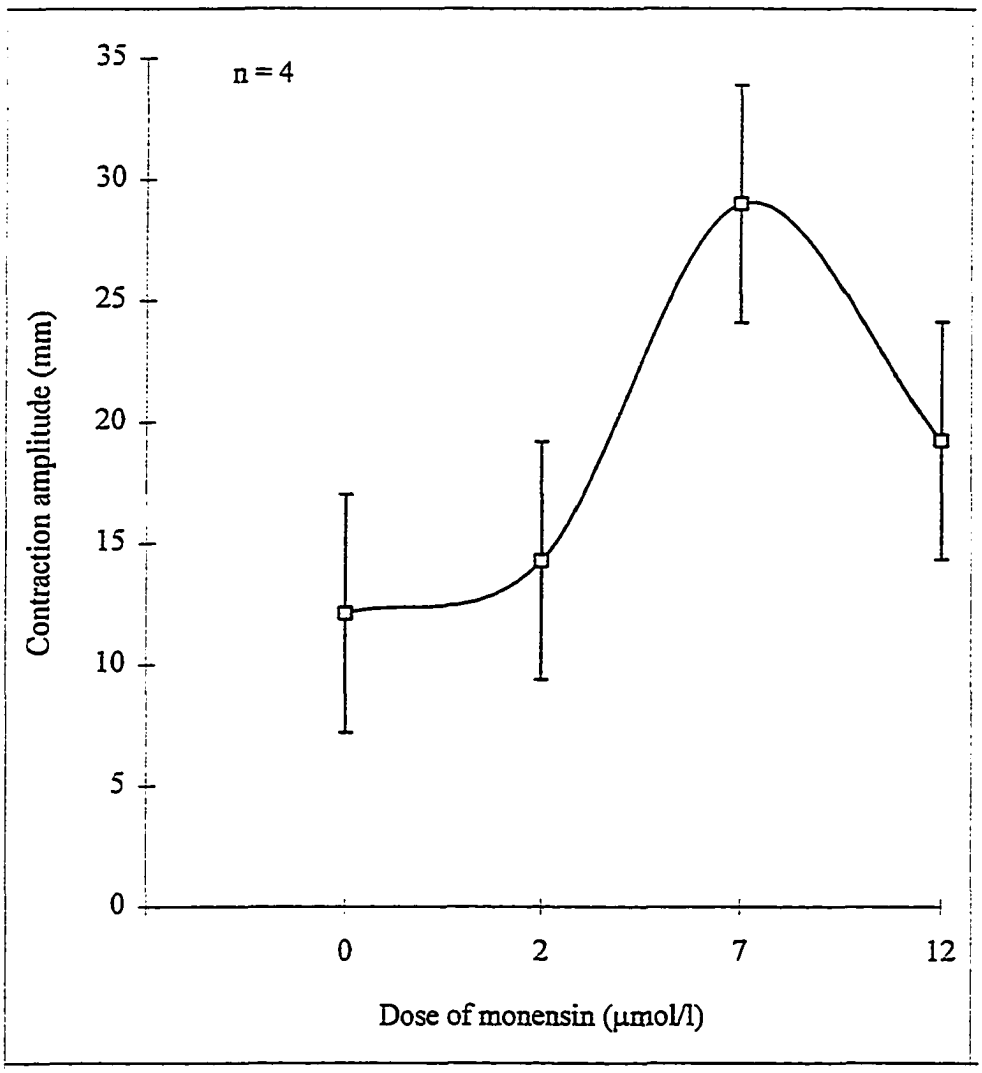

n = 4
Contraction amplitude (mm)
35
30
25
20
15
10
5
0
0
2
7
12
Dose of monensin (µmol/l)

FIG. 3. Plot of the CF means of guinea-pig papillary muscles in five time periods for three drugs (control, 3 μmol/l digoxin and 7 μmol/l monensin). The drug by time interaction was significant, $P < 0.05$. * indicates that comparison of drug mean with the control mean is significant at $P \leq 0.05$. Lsmean + SDM is shown (n=20).

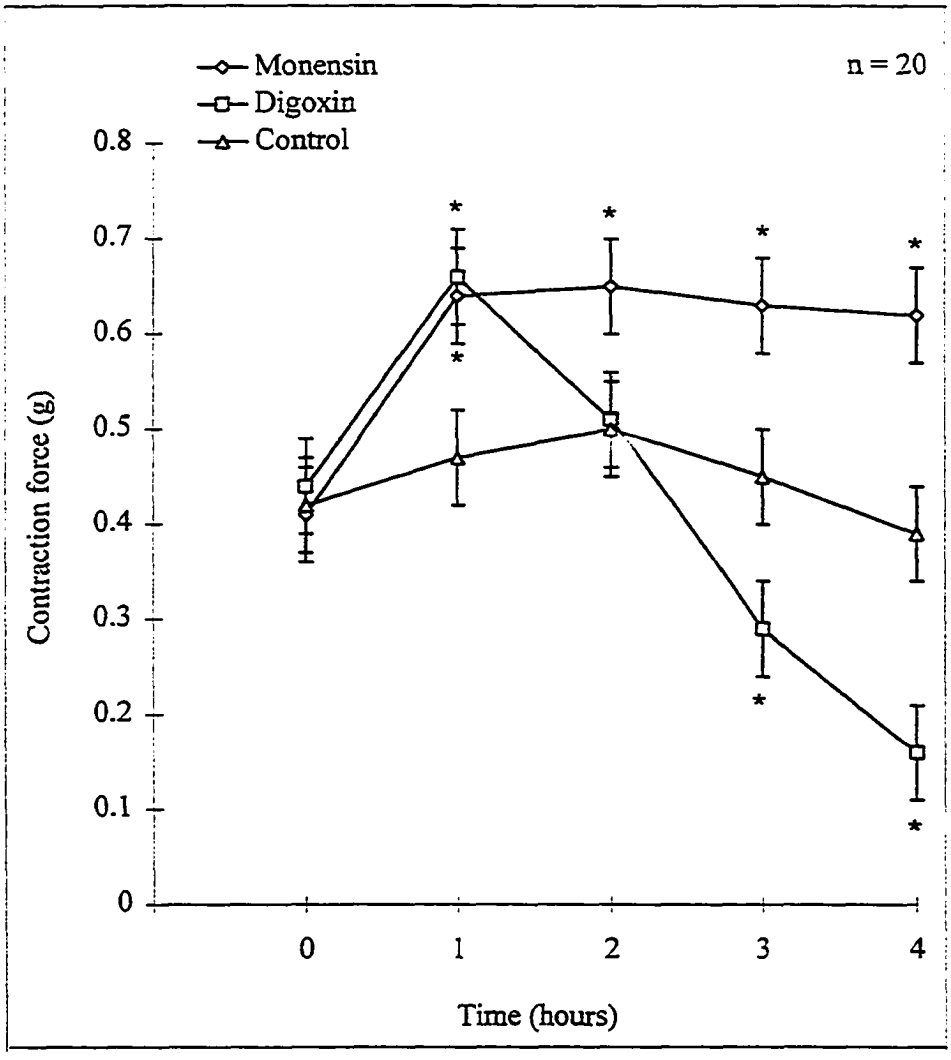

Monensin
Digoxin
Control
n = 20
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Contraction force (g)
0
1
2
3
4
Time (hours)

FIG. 4. Plot of the ICV means of guinea-pig papillary muscles in five time periods for three drugs (control, 3 μmol/l digoxin and 7 μmol/l monensin). The drug by time interaction was significant, $P < 0.05$. * indicates that comparison of drug mean with the control mean is significant at $P \leq 0.05$. Lsmean + SDM is shown (n=20).

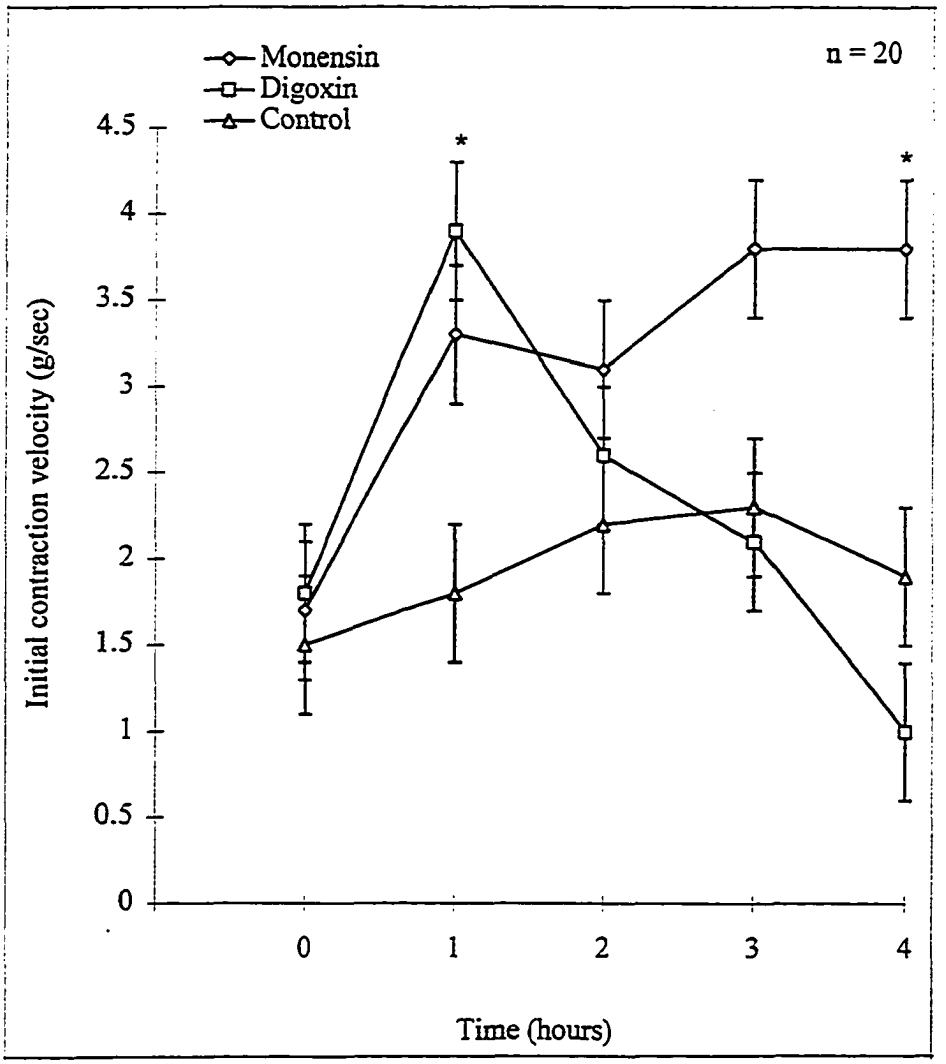

Monensin
Digoxin
Control
n = 20
Initial contraction velocity (g/sec)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
0
1
2
3
4
Time (hours)

FIG. 5. Plot of the ACV means of guinea-pig papillary muscles in five time periods for three drugs (control, 3 μmol/l digoxin and 7 μmol/l monensin). The drug by time interaction was significant, $P < 0.05$. * indicates that comparison of drug mean with the control mean is significant at $P \leq 0.05$. Lsmean + SDM is shown (n=20).

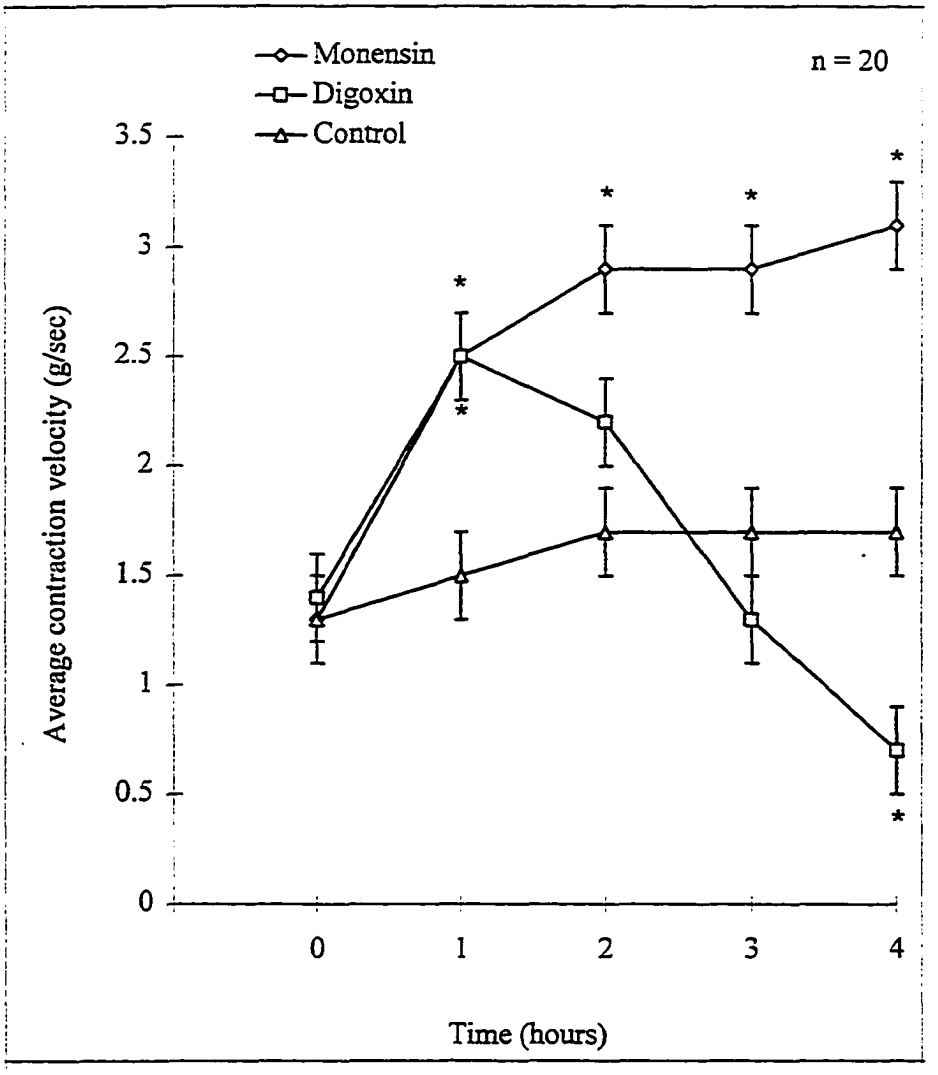

Monensin
Digoxin
Control
n = 20
Average contraction velocity (g/sec)
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
Time (hours)
*

FIG. 6. Plot of the IRV means of guinea-pig papillary muscles in five time periods for three drugs (control, 3 μmol/l digoxin and 7 μmol/l monensin). The drug by time interaction was significant, $P < 0.05$. * indicates that comparison of drug mean with the control mean is significant at $P \leq 0.05$. Lsmean + SDM is shown (n=20).

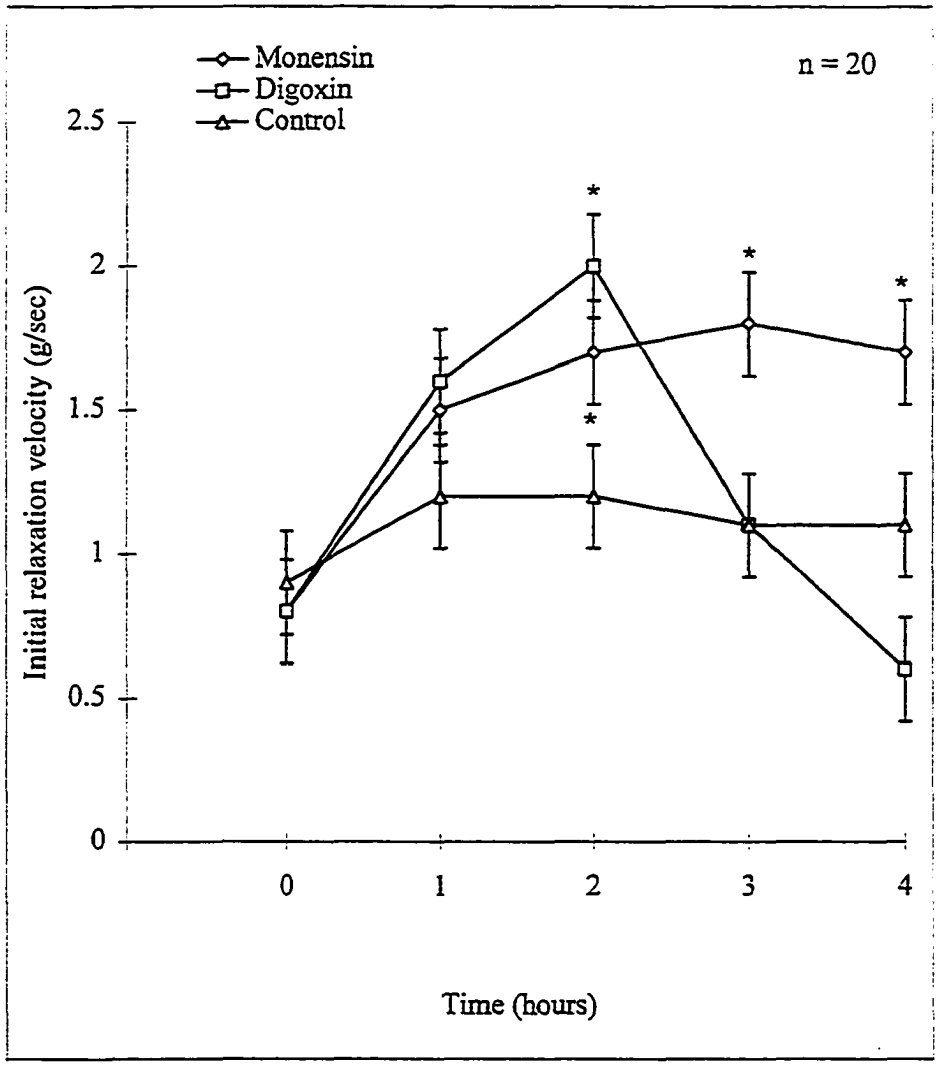

Monensin
Digoxin
Control
n = 20
Initial relaxation velocity (g/sec)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
Time (hours)
*
*
*
*

FIG. 7. Plot of the ST means of guinea-pig papillary muscles in five time periods for three drugs (control, 3 μmol/l digoxin and 7 μmol/l monensin). The drug by time interaction was significant, $P < 0.05$. * indicates that comparison of drug mean with the control mean is significant at $P \leq 0.05$. Lsmean + SDM is shown (n=20).

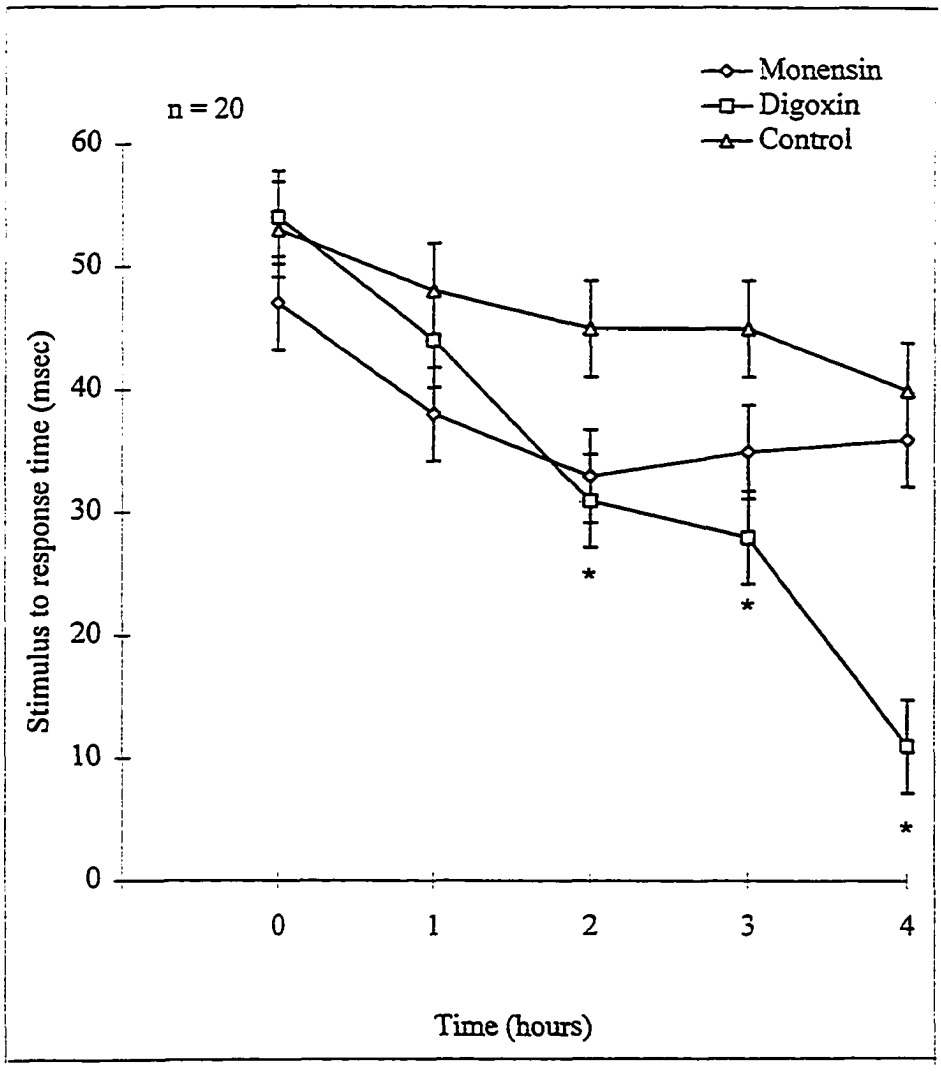
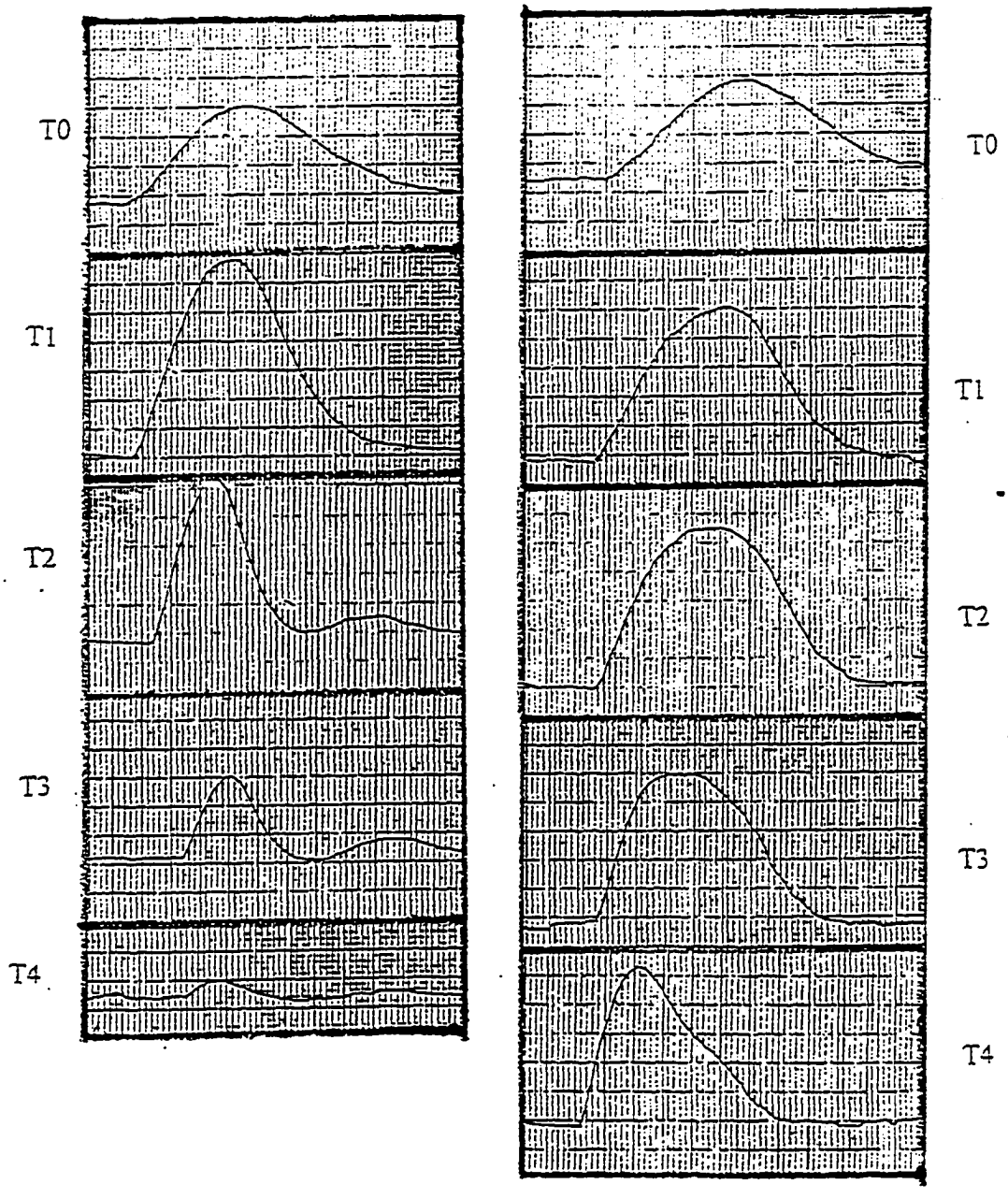

Monensin
Digoxin
Control
n = 20
60
50
40
30
20
10
0
Stimulus to response time (msec)
0
1
2
3
4
Time (hours)
*
*
*

FIG. 8. Representative tracing of monensin- and digoxin-induced increase in CF of guinea-pig papillary muscles with time. Digoxin produced after contractions starting at T2.

Digoxin

Monensin



T0 = Equilibration
T1 = One hour after drug addition
T2 = Two hours after drug addition
T3 = Three hours after drug addition
T4 = Four hours after drug addition

1 gram = 40 mm

DIGOXIN AND MONENSIN INDUCED CHANGES OF INTRACELLULAR Ca^{2+} CONCENTRATION IN ISOLATED GUINEA-PIG VENTRICULAR MYOCYTES

A paper to be submitted to the *Journal of Action and Agent*

Ismail Meral, Frederick B. Hembrough and Walter Hsu

Abstract

This study was undertaken to determine the possible mechanisms of action of monensin and digoxin by using isolated guinea-pig ventricular myocytes. Since Ca^{2+} is the major signal for triggering contraction of cardiac muscle, the objective of this study was to determine whether monensin- or digoxin-induced cardiac muscle contractions are mediated through an increase in Ca^{2+} influx. We used three different concentrations of digoxin (0.3, 1 and 3 μmol/l) and three different concentrations of monensin (0.3, 1 and 3 μmol/l) in Experiment 1. Each treatment was monitored for two hours by using computerized fluoroscopy. Both digoxin and monensin increased the $[Ca^{2+}]_i$ and accelerated the onset time of $[Ca^{2+}]_i$ increase in a dose-dependent manner. The largest increase in $[Ca^{2+}]_i$ and shortest onset time of $[Ca^{2+}]_i$ increase were obtained by using 3 μmol/l monensin and 3 μmol/l digoxin. In Experiment 2, we compared normal myocytes (these cells were loaded with fura-2 for 30 min before the treatment) to "weakened" myocytes (these cells were loaded with fura-2 for 3 hours before the treatment to create a "weakened" condition). We

found that although 0.3 μmol/l monensin and digoxin did not change the $[Ca^{2+}]_i$ in normal myocytes, they increased the $[Ca^{2+}]_i$ in "weakened" myocytes. In Experiment 3, we used a Na^+-free medium to demonstrate the effect of $[Na^+]_o$ on both monensin- and digoxin-induced increases in $[Ca^{2+}]_i$. We found that digoxin did not increase the $[Ca^{2+}]_i$ in the Na^+-free medium. Although monensin increased the $[Ca^{2+}]_i$ in the Na^+ -free solution, this increase was not as large as in the Na^+-containing medium. In Experiment 4, we used a Ca^{2+}-free medium to demonstrate whether monensin- or digoxin-induced increases in $[Ca^{2+}]_i$ is mediated through an increase in Ca^{2+} influx or release. Most of the myocytes did not respond to caffeine control treatment after two hours of experimentation. However, myocytes which responded to caffeine treatment after two hours of experimentation did not respond to either digoxin, monensin or control treatment. We concluded that the positive inotropic effect of digoxin depends on $[Na^+]_o$. However, monensin increases $[Ca^{2+}]_i$ in Na^+-dependent and -independent manners. We also concluded that "weakened" myocytes are more sensitive to the monensin and digoxin treatment than normal myocytes.

Introduction

Contraction of cardiac muscle is initiated by increased intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. It has been suggested [4, 12] that many positive inotropic agents, such as the digitalis glycosides (e. g. digoxin) and a carboxylic ionophore antibiotic, monensin, act by increasing $[Ca^{2+}]_i$.

Although digitalis glycosides have been used in the treatment of arrhythmias and congestive heart failure (CHF) for over two hundred years, the mechanism of their positive inotropic action remains controversial [3]. The most widely accepted mechanism is their well-known inhibitory effect on the Na^+, K^+-ATPase resulting in an increase in intracellular Na^+ concentration ($[Na^+]_i$) which leads to an increase in $[Ca^{2+}]_i$ through the Na^+ (out)/ Ca^{2+} (in) exchange resulting in an increased CF [1, 4, 5, 6, 7].

Monensin is a strong positive inotrope for cardiac muscle [8, 9, 10]. This effect occurs because as an ionophore monensin has a strong affinity for $[Na^+]_o$. The affinity of monensin for Na^+ is ten times that for K^+, its nearest competitor in biological systems [11]. The movement of Na^+ into a cellular compartment by monensin facilitates the entry of Ca^{2+} into the cell by a Na^+ (out) / Ca^{2+} (in) exchange [12]. This Ca^{2+} shift is the primary factor mediating the cellular response. Other factors modifying cellular responses include the alteration of pH of intracellular fluid since monensin increases the pH_i by transferring protons out of the cell [11].

The positive inotropic effect of digoxin depends strongly on the initial condition of the muscle [2]. If the capacity of heart muscle to develop CF is greatly depressed, the effects of digoxin are much larger than would occur in normal muscle [2]. In a preliminary experiment we observed that after three hours of exposure to fura-2, the $[Ca^{2+}]_i$ of myocytes gradually decreased by 20% (unpublished data). This observation gave us the idea that these cells could be used as "weakened" cells.

This experiment is the first one that compares monensin to digoxin regarding their effects on $[Na^+]_i$ and $[Ca^{2+}]_i$ in guinea-pig ventricular myocytes. The present study is also the first one that compares monensin to digoxin without electrically stimulating ventricular myocytes and monitors effects of these drugs on $[Na^+]_i$ and $[Ca^{2+}]_i$ in guinea-pig ventricular myocytes for two hours. Without using an electrical stimulus, we avoided the involvement of stimulating the voltage-dependent Ca^{2+} channels in our experiment. This experiment was designed to examine whether: 1) the monensin- or digoxin-mediated increase in $[Ca^{2+}]_i$ is due to Na^+ entry; 2) "weakened" cells are more responsive to monensin and digoxin than normal cells with regard to their effects on $[Ca^{2+}]_i$; and 3) the monensin- or digoxin-mediated increase in $[Ca^{2+}]_i$ is due to an increase in Ca^{2+} influx or release.

Materials and Methods

Isolation and Loading of Myocytes

Cardiac ventricular myocytes were isolated from the right ventricle of guinea-pig hearts according to a previously described method [13, 14]. In brief, guinea-pigs, 400-600 g, were heparinized (2000 IU per animal, IP injection) thirty minutes before being decapitated. Hearts were removed rapidly and put in a beaker filled with ice-cold non-standard Krebs-Ringer bicarbonate solution (KRB) of the following composition [mmol/l]: Na^+, 116; Ca^{2+}, 2.2; K^+, 4.0; Mg^{2+}, 1.3; Cl^-, 126.9; $H_2PO_4^-$, 2.1; HCO_3^-, 21.7; glucose, 10.9 and HEPES, 10. The pH was adjusted to 7.4 at room temperature. The hearts were then rapidly put in a glass pan filled with KRB and perfused with 100 % O_2 at room

temperature. The aorta was rapidly cannulated to perfuse the coronary arteries on a modified Langendorff apparatus (Fig. 1). The heart was first perfused with KRB at 34 °C for about 5 min. at a hydrostatic pressure of 90 cm of H_2O, then with a Ca^{2+}-free Tyrodes solution of the following composition [mmol/l]: Na^+, 116; Ca^{2+}, 0; K^+, 4.0; Mg^{2+}, 1.3; Cl^-, 126.9; $H_2PO_4^-$, 2.1; HCO_3^-, 21.7; glucose, 10.9; HEPES, 10 (pH was adjusted to 7.3 at 37 °C) for 5 min., and finally with 10 mg collagenase (Worthington Biochem. Co., Freehold, New Jersey) and 1.7 mg protease (Sigma Chemical Co., St. Louis, MO) in 25 ml of Ca^{2+}-free Tyrodes solution which was recirculated using a pump for 10-15 min. The heart was washed with a high K^+ solution (storage solution) of the following composition [mmol/l]: KOH, 70; L-glucuronic acid, 50; KCl, 40; taurine, 20; KH_2PO_4, 20; $MgCl_2$, 3; glucose, 10; HEPES, 10; EGTA, 0.5; pH was adjusted to 7.3 at 37 °C and ventricles were minced and poured into a beaker. The myocytes were dispersed by gently shaking the beaker. The myocytes were centrifuged at 1000 X g for 2 min and washed with KRB. The dispersed myocytes were allowed to attach to dishes at room temperature for 15 min. Some of the cells were stored in a storage solution at 4 °C for use on the second day. The attached myocytes were washed with KRB and loaded with 3 μmol/l fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) for 30 min at 37 °C, aerated with 5% CO_2 and 95% air. Then, the loaded myocytes were rinsed with KRB. Two ml of KRB was added to each dish. The fura-2 fluorescence was monitored by using a computerized fluoroscope (Attofluor™ Ratio Vision™ Version 6.00, Atto Instruments, Torrance, CA.).

Experiment 1. Dose response curve for digoxin and monensin

Seven treatments were used; control (0.18% alcohol), monensin (0.3, 1 and 3 μmol/l) and digoxin (0.3, 1 and 3 μmol/l). Each treatment was monitored for two hours and replicated three times. Twenty mmol/l KCl was added two hours after each experiment to test whether the cells would respond to this control treatment by increasing Ca^{2+} influx through VDCCs.

Experiment 2. Differences between "weakened" and normal myocytes

This experiment tested whether there were differences between "weakened" myocytes and normal myocytes in the action of monensin and digoxin. Myocytes were loaded with fura-2 three hours before treatment (this caused a gradual decrease in $[Ca^{2+}]_i$ producing a "weakened" cell). Three treatments were used: control, monensin (0.3 μmol/l) and digoxin (0.3 μmol/l). Twenty mmol/l KCl was added two hours after the control treatment to test whether the cells would respond to this control treatment.

Experiment 3. Effects of monensin and digoxin on $[Ca^{2+}]_i$ of cardiac myocytes in Na^+-free medium

To investigate whether an increase in $[Ca^{2+}]_i$ induced by monensin or digoxin is due to Na^+ influx, a Na^+-free medium was used. The cell preparation and loading were the same as in Experiment 1. The loaded cells were washed with KRB. Two ml of Na^+-free medium of following composition [mmol/l]; choline chloride, 116; $MgCl_2$, 1.3; K_2HPO_4, 2;

glucose, 10.9; $CaCl_2$, 2.2; HEPES, 10 (pH was adjusted to 7.4 at room temperature) was added to each dish and fura-2 fluorescence was monitored. In this experiment we replaced NaCl with CHCl (LiCl was also used to replace NaCl but it caused simultaneous contraction of myocytes). Twenty mmol/l KCl was added two hours after each experiment to test whether cells would respond to this control treatment. Three treatments were used: control, monensin (3 μmol/l) and digoxin (3 μmol/l).

Experiment 4. Effects of monensin and digoxin on $[Ca^{2+}]_i$ of cardiac myocytes in Ca^{2+}-free medium

To investigate whether an increase in $[Ca^{2+}]_i$ induced by monensin or digoxin is due to Ca^{2+} influx or a Ca^{2+} release from the SR, a Ca^{2+}-free medium of the following composition [mmol/l]: Na^+, 116; EGTA, 0.01; K^+, 4.0; Mg^{2+}, 1.3; Cl^-, 126.9; $H_2PO_4^-$, 2.1; HCO_3^-, 21.7; glucose, 10.9; HEPES, 10 (pH was adjusted to 7.4 at room temperature) was used. The cell preparation and loading were the same as in Experiment 1. Two ml of Ca^{2+}-free medium was added to each dish and fura-2 fluorescence was monitored. Three treatments were used; control, digoxin (3 μmol/l) and monensin (3 μmol/l). Ten mmol/l caffeine was added two hours after each experiment to test whether these cells would respond to this control treatment by increasing Ca^{2+} release from intracellular stores..

Data expression and Analysis

$[Ca^{2+}]_i$ is expressed by the ratio of the emission of fura-2 at 340 to that at 380 nm.

There are two different forms of fura-2: Ca^{2+}-binding form that is excited at 340 nm and Ca^{2+}-free that is excited at 380 nm. If there is an increase in $[Ca^{2+}]_i$, this would increase the 340/380 ratio by increasing the emission at 340 nm and decreasing the emission at 380 nm. Onset time of $[Ca^{2+}]_i$ is expressed as the starting time of the increase in 340/380 ratio. The data were expressed as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA). Tukey's test was used to test for differences among means for which ANOVA indicated a significant ($P \leq 0.05$) F ratio [see appendix for ANOVA tables].

Results

Experiment 1. Dose response curve for digoxin and monensin

The effect of three different concentrations of digoxin on $[Ca^{2+}]_i$ of guinea-pig ventricular myocytes is shown in Fig. 2. Digoxin increased the $[Ca^{2+}]_i$ and decreased the onset time of $[Ca^{2+}]_i$ increase in a dose-dependent manner (Table 1). 0.3 μmol/l digoxin did not change the $[Ca^{2+}]_i$ in the two hours of the experimental period. One μmol/l digoxin increased the $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 1.04 ± 0.39) 75 ± 5.47 min after treatment. However, a significant increase in $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 1.85 ± 0.13) occurred earlier (58 ± 3.2 min) when using 3 μmol/l digoxin.

The effect of three different concentrations of monensin on $[Ca^{2+}]_i$ of guinea-pig ventricular myocytes is shown in Fig. 3. Monensin also increased the $[Ca^{2+}]_i$ and decreased the onset time of $[Ca^{2+}]_i$ increase in a dose-dependent manner (Table 1). 0.3 μmol/l monensin did not change the $[Ca^{2+}]_i$ in two hours of exposure. One μmol/l

monensin increased the $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 1.13 ± 0.49) 80 ± 4.2 min after treatment. However, a significant increase in $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 2.80 ± 0.90) and shorter onset time (62 ± 4.2 min) were obtained by using 3 μmol/l monensin (Fig. 3). $[Ca^{2+}]_i$ gradually decreased by 15% over the two-hour period in the control myocytes. However, these cells responded to 20 mmol/l KCl treatment by increasing $[Ca^{2+}]_i$ (from 0.58 ± 0.03 to 1.20 ± 0.69) at the end of the experiment.

Representative tracing of monensin- and digoxin-induced increase in $[Ca^{2+}]_i$ and onset time for this increase is shown in Fig. 6. Both monensin and digoxin increased the $[Ca^{2+}]_i$ of guinea-pig ventricular myocytes.

Experiment 2. Differences between "weakened" and normal myocytes

Although both digoxin (0.3 μmol/l) and monensin (0.3 μmol/l) did not change the $[Ca^{2+}]_i$ of normal guinea-pig ventricular myocytes (Fig. 4), both of these drugs (0.3 μmol/l) increased the $[Ca^{2+}]_i$ of "weakened" guinea-pig ventricular myocytes (Fig. 4). An increase in $[Ca^{2+}]_i$ (from 0.63 ± 0.03 to 2.76 ± 0.87) by digoxin occurred 93 ± 4.5 min after treatment and it (from 0.63 ± 0.03 to 2.81 ± 0.16) occurred 92 ± 5.4 min after treatment when using monensin. In control myocytes, $[Ca^{2+}]_i$ gradually decreased by 15% over a two-hour period (Table 1). However, these cells responded to a treatment with 20 mmol/l KCl by increasing $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 0.94 ± 0.04) at the end of the experiment.

Experiment 3. Effects of monensin and digoxin on $[Ca^{2+}]_i$ of cardiac myocytes in Na^+-free medium

Although 3 μmol/l digoxin increased the $[Ca^{2+}]_i$ of myocytes in a Na^+-containing medium (Fig. 2), it did not increase the $[Ca^{2+}]_i$ of myocytes in a Na^+-free medium during the two hours of treatment (Fig. 5). However, application of KCl (20 mmol/l) after digoxin experiment, increased $[Ca^{2+}]_i$ (from 0.55 ± 0.04 to 0.97 ± 0.06) of myocytes in a Na^+-free medium. Monensin, on the other hand, increased the $[Ca^{2+}]_i$ (from 0.70 ± 0.08 to 2.06 ± 1.07) of myocytes in a Na^+-free medium 68 ± 5.6 min after treatment (Fig. 5). However, the increases in $[Ca^{2+}]_i$ by monensin in a Na^+-free medium was less than that in a Na^+-containing medium. In control myocytes, $[Ca^{2+}]_i$ gradually decreased by 15% over a two-hour period (Table 1). However, these cells responded to the treatment with 20 mmol/l KCl by increasing $[Ca^{2+}]_i$ (from 0.60 ± 0.03 to 1.06 ± 0.21) at the end of the experiment.

Discussion

This study is the first one to show that digoxin and monensin increase the $[Ca^{2+}]_i$ in guinea-pig ventricular myocytes. Both digoxin and monensin increased the $[Ca^{2+}]_i$ and decreased the onset time of $[Ca^{2+}]_i$ increase in a dose-dependent manner. The positive inotropic action of digoxin is caused by the inhibition of Na^+, K^+-ATPase. As a result, there is a gradual increase in $[Na^+]_i$ and a gradual small decrease in $[K^+]_i$ [15, 16]. It is the increase in $[Na^+]_i$ that can be judged to be crucially related to the positive inotropic effect of digoxin. An increase in $[Na^+]_i$ by digoxin raises the $[Ca^{2+}]_i$ due to the entry of Ca^{2+}

into the cell by Na^+ (out) / Ca^{2+} (in) exchange [17]. It has been shown that the Na^+-H^+ exchange also appears to play a role in amplifying the increase in $[Ca^{2+}]_i$ that results from inhibition of the Na^+, K^+-ATPase. The increase in $[Na^+]_i$ causes a fall in pH_i, as $[H^+]_o$ are exchanged for intracellular Na^+ to lower the increased $[Na^+]_i$ [18]. In experiment 1, we were able to demonstrate that 3 μmol/l digoxin increased $[Ca^{2+}]_i$ of myocytes two hours after treatment. However, the increase in $[Ca^{2+}]_i$ by digoxin was gradual. It took about 70 min for digoxin to reach plateau. This result suggested that it takes time for digoxin to inhibit Na^+, K^+- ATPase and increase $[Ca^{2+}]_i$ by increasing $[Na^+]_i$. Further studies measuring changes in $[Na^+]_i$ would be needed to confirm our hypothesis. This result is consistent with the results of our papillary muscle contraction experiment showing that digoxin caused a significant increase in contraction force of guinea-pig papillary muscles 60 min after treatment (see chapter 1).

Monensin has been characterized as a Na^+ ionophore by virtue of its ability to form a lipid-soluble cation complex that can rapidly traverse cell membranes. Monensin binds Na^+ outside of the myocytes and carries it into the cell and increases $[Na^+]_i$ and thus $[Ca^{2+}]_i$ [11]. Monensin also increases the pH_i due to the transferring of protons to outside the cell [11]. Contraction of the heart is pH sensitive; classically an alkalosis increases $[Ca^{2+}]_i$ by stimulating the Na^+ / Ca^{2+} exchange [19, 20]. An increased pH_i by monensin may also increase $[Ca^{2+}]_i$ by stimulating the Na^+ / Ca^{2+} exchange. In this experiment we were able to demonstrate that 3 μmol/l monensin increased $[Ca^{2+}]_i$ in normal myocytes. The increase in $[Ca^{2+}]_i$ by monensin was also gradual. It took about 70 min for monensin to reach a

plateau. This result suggests that it takes time for monensin to increase $[Ca^{2+}]_i$ of myocytes by increasing $[Na^+]_i$. Further studies measuring changes in $[Na^+]_i$ and pH_i would be needed to confirm our hypothesis. This result is consistent with the results of our papillary muscle contraction experiment (unpublished data) showing that monensin caused a significant increase in contraction force of guinea-pig papillary muscles 60 min after treatment.

"Weakened" cells were different from normal cells because of the decreased basal $[Ca^{2+}]_i$. There may be two reason for the decreased $[Ca^{2+}]_i$ of "weakened" cells. Although both digoxin (0.3 μmol/l) and monensin (0.3 μmol/l) did not change the $[Ca^{2+}]_i$ of normal guinea-pig ventricular myocytes, both of these drugs (0.3 μmol/l) increased the $[Ca^{2+}]_i$ of "weakened" guinea-pig ventricular myocytes (Fig. 4). This result is consistent with the following concept: When the capacity of heart muscle to develop force is greatly depressed (such as due to the decreased $[Ca^{2+}]_i$), the effect of these two drugs is much greater than would occur in normal muscle [2].

Although 3 μmol/l digoxin increased the $[Ca^{2+}]_i$ of myocytes in Na^+-containing medium two hours after the treatment, it did not increase $[Ca^{2+}]_i$ of myocytes in Na^+-free medium two hours after the treatment (Fig. 5). However, KCl (20 mmol/l) addition, after digoxin exposure, still increased $[Ca^{2+}]_i$ of myocytes in a Na^+-free medium indicating that voltage dependent Ca^{2+}-channels (VDCCs) function normally in these cells. This result clearly indicated that digoxin needs $[Na^+]_o$ to increase $[Ca^{2+}]_i$. This result also clearly demonstrated that digoxin does not directly effect either the release of Ca^{2+} from the SR or influx of the Ca^{2+} through VDCCs in the absence of extracellular Na^+. Our result is

consistent with the concept that it is the increase in $[Na^+]_i$ that is related to the positive inotropic effect of digoxin. An increase in $[Na^+]_i$ by digoxin increases the $[Ca^{2+}]_i$ due to the entry of Ca^{2+} into the cell by Na^+ (out) / Ca^{2+} (in) exchange [4, 5, 6, 7]. Monensin, on the other hand, increased $[Ca^{2+}]_i$ of myocytes in both the Na^+-containing and the Na^+-free medium two hours after treatment. However, the increase in $[Ca^{2+}]_i$ by monensin in a Na^+-free medium was not as large as the increase in a Na^+-containing solution (Fig. 5). The movement of Na^+ into the cellular compartment by monensin facilitates the entry of Ca^{2+} by a Na^+ (out)/ Ca^{2+} (in) exchange [12]. However, our result indicated that this increase in $[Ca^{2+}]_i$ by monensin in Na^+-free medium may be due to three reasons. First, monensin may be a Ca^{2+} ionophore as well as a Na^+ ionophore (the ion specificity of monensin is Ag > Na > K > Rb > Cs > Li > Ca [11] with approximately a 10-fold selectivity for Na^+ over K^+). Second, monensin may open the VDCCs of guinea-pig myocytes by increasing $[K^+]_i$ in Na^+-free medium. In the absence of $[Na^+]_o$, monensin might work as a K^+ ionophore and increase the $[K^+]_i$ and thus, opening of VDCCs. Further studies by measuring $[K^+]_i$ are needed to demonstrate that in a Na^+-free medium, monensin increases the $[K^+]_i$ by acting as a K^+ ionophore. By using a Na^+-containing medium Hugtenburg. *et. al.* [21] suggested that the positive inotropic effect of monensin in guinea-pig papillary muscles is nearly insensitive to inhibition by nifedipine (an L-type Ca^{2+} channel blocker) and is only partially blocked by ryanodine (a ryanodine channel blocker). He also suggested that the partial inhibitory effect of ryanodine suggests that a part of the monensin-induced $[Ca^{2+}]_i$ is taken up by the SR. This findings and our findings support the hypothesis of Fabiato [22] that

Ca^{2+} introduced into the cell by the Na^{+}/ Ca^{2+} exchange, in addition to the influx through VDCCs, might also contribute to the reloading of the SR. However, there may be an influx of Ca^{2+} through channels other than VDCCs [23].

Because the effects of monensin (3 μmol/l) and digoxin (3 μmol/l) to increase $[Ca^{2+}]_i$ in guinea-pig ventricular myocytes took a long time (around 60 min), our Ca^{2+} -free experiment did not give us consistent results as most of the myocytes did not respond to the caffeine (10 mM) treatment after the two hours of experimental period due to the depletion of SR. However, we observed that the cells which responded to caffeine treatment after two hours of experimentation did not respond to either digoxin, control or monensin treatment. Further studies are needed to confirm the idea that these drugs do not affect the SR to release Ca^{2+} in Ca^{2+}-free medium. It has been shown that ouabain, another cardiac glycoside, used at a higher concentration (1 mmol/l) did not increase the $[Ca^{2+}]_i$ of rat ventricular myocytes in the Ca^{2+} -free medium [24].

We also attempted to use thapsigargin (200 nmol/l) which is a SR Ca^{2+} ATPase blocker [25] to deplete the SR. However, thapsigargin did not deplete the SR without electrical stimulation. Since we did the experiments without stimulating the cells, it was not proper for us to compare the stimulated myocytes to un-stimulated myocytes with regard to $[Ca^{2+}]_i$ changes. Thapsigargin experiments need to be performed with electrically stimulated guinea-pig ventricular myocytes.

We concluded that, it is the increase in $[Na^{+}]_i$ that is crucially related to the positive inotropic effect of digoxin. An increase in $[Na^{+}]_i$ by digoxin increases the $[Ca^{2+}]_i$ due to

the entry of Ca^{2+} into the cell by Na^{+} (out) / Ca^{2+} (in) exchange. Although $[Na^{+}]_i$ is important for the inotropic action of monensin, this agent may also increase $[Ca^{2+}]_i$ due to either carrying Ca^{2+} inside the cell, opening VDCC or releasing Ca^{2+} from the SR.

Because both monensin and digoxin change the pH_i and increase the $[Ca^{2+}]_i$ by increasing $[Na^{+}]_i$ of myocytes, further studies are needed: 1) for monitoring pH_i, $[Na^{+}]_i$ and $[Ca^{2+}]_i$; and 2) for comparing unstimulated myocytes vs stimulated myocytes to evaluate whether $[Ca^{2+}]_i$ and onset time of increased $[Ca^{2+}]_i$ of stimulated myocytes, induced by monensin and digoxin, are different.

References

[1] D. A. Eisner, *The role of intracellular Ca ions in the therapeutic and toxic effects of cardiac glycosides and catecholamines.* J. Cardiovasc. Pharmacol. *8 (supp. 3):* S2-S9 (1986).

[2] F. H. Brian and Jr. B. Thomas, *Digitalis and allied cardiac glycosides.* Goodman and Gilman's the pharmacologic basis of therapeutics. Eighth addition 815.

[3] J. Somberg, and B. Greenfield, *Digitalis: Historical development in clinical medicine.* J. Clin. Pharmacol. *25,* 484-489 (1985).

[4] T. Akera and T. M. Brody, *The role of Na^{2+} -K^{+} ATPase in the inotropic action of digitalis.* Pharmacol. Rev. *29:* 187-217 (1978).

[5] G. E. Lindenmayer, *Mechanism of action of digitalis glycosides at the subcellular level.* Pharmacol. Ther. 843-861 (1976).

[6] K. J. Sweadner and S. M. Goldin, *Active transport of sodium and potassium ions.* N. Engl. J. Med. *302:* 777-783 (1980).

[7] P. A. Poole-Wilson, E. Galindez and C. H. Fry, *Effect of ouabain in therapeutic concentrations of K^+ exchange and contraction of human and rabbit myocardium.* Clin. Sci. *57:* 415-420 (1979).

[8] B. C. Pressman, *Biological applications of ionophores.* Ann. Rev. Biochem. *45:* 501-529 (1976).

[9] J. L. Sutko, H. R. Besch, J. C. Bailey, G. Zimmerman, and A. M. Watanabe, *Direct effects of the monovalent cation ionophores monensin and nigericin on myocardium.* J. Pharmacol. Exp. Ther. *203:* 685-700 (1977).

[10] M. Shlafer, and P. Kane, *Subcellular actions and potential adverse cardiac effects of the cardiotonic ionophore monensin.* J. Pharmacol. Exp. Ther. *214:* 567-573 (1980).

[11] H. H. Mollenhauer, D. J. Morre, and L. D. Rowe, *Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity.* Biochim. Biophys. Acta. *1031,* 225-246 (1990),

[12] K. Tsuchida, and S. Otomo, *Electrophysiological effects of monensin, a sodium ionophore, on cardiac purkinje fibers.* Eur. J. Pharmacol. . *190,* 313-320 (1990).

[13] J. Yazawa, K. Muneshige, M. Ohara and M. Kameyama, *An improved method for isolating cardiac myocytes useful for patch-clamp studies.* Jpn. J. Physiol. *40:* 157-163 (1990).

[14] M. Artman, H. Ichikawa, M. Avkiran and W. A. Coetzee, *Na^{2+}/Ca^{2+} exchange current density in cardiac myocytes from rabbits and guinea-pigs during postnatal development.* Am. J. Physiol. *37:* H1714-H1722 (1995).

[15] J. W. Deitmer, and D. Ellis, *Intracellular sodium activity of cardiac purkinje fibers during inhibition and reactivation of the Na-K pump.* J. Physiol. (*Lond.*) *284,* 241-259 (1978).

[16] D. A. Eisner, W. J. Lederer, and R. D. Vaughan-Jones, *The control of tonic tension by membrane potential and intracellular sodium activity in the sheep cardiac purkinje fiber.* J. Physiol. (*Lond.*). *335,* 723-743 (1983).

[17] C. O. Lee, and M. Dagostino, *Effect of strophanthidin on intracellular Na ion activity and twitch tension of constantly driven canine cardiac purkinje fibers.* Biophys. J. *40,* 185-198 (1982).

[18] D. Kim, E. J. Cragoe, and T. W. Smith. *Relations among sodium pump inhibitions, Na-Ca and Na-H exchange activities, and Ca-H interaction in cultured chick heart cells.* Circ. Res. *60,* 185-193 (1987).

[19] P. A. Poole-Wilson, *Regulation of intracellular pH in the myocardium; relevance to pathology.* Mol. Cell. Biochem. *89,* 151-155 (1989).

[20] C. Orchard, *The effect of acidosis on excitation-contraction coupling in isolated ferret heart muscle.* Mol. Cell. Biochem. *89,* 169-173 (1989).

[21] J. G. Hugtenburg, M. J. Mathy, J. J. Beckeringh and P. A. van Zwiten, *The differential effect of calcium antagonists on the positive inotropic effects induced by*

calcium and monensin in cardiac preparations of rats and guinea-pigs. Naunyn-Schmiedeberg's Arch. Pharmaco.l *340:* 558-566 (1989).

[22] A. Fabiato, *Simulated calcium current can both cause calcium loading in and trigger calcium release from sarcoplasmic reticulum of a skinned canine cardiac purkinje cell.* J. Gen. Physiol. *85:* 291-320 (1985).

[23] T. Ehara, A. Noma and K. Ono, *Calcium-activated non-selective cation channel in ventricular cells isolated from adult guinea-pig hearts.* J. Physiol [Lond] *403:* 117-133 (1988).

[24] K. Yamamoto, U. Ikeda, Y. Seino, Y. Tsuruya, A. Oguchi, K. Okada, S. Ishikawa, T. Saito, K. Kawakami, Y. Hara and K. Shimada, *Regulation of Na, K-adenosine triphosphatase gene expression by sodium ions in cultured neonatal rat cardiocytes.* J. Clin. Invest. *92:* 1889-1895 (1993).

[25] B. Lewartowski, M. Rozycka and R. Janiak, *Effects of thapsigargin in normal and pretreated with ryanodine guinea-pig cardiomyocytes.* Am. J. Physiol. *35:* H1829-H1839 (1994).

Table 1. Means of ratio and onset time of $[Ca^{2+}]_i$ increase of ventricular myocytes treated with monensin and digoxin. Data are presented as mean ± standard deviation (SD).

TRT	CELL TYPE	ONSET TIME (Min)	RATIO (340/380)
Experiment 1			
Control	Normal	----	0.60 ± 0.03
Digoxin 0.3 μmol/l	Normal	----	0.70 ± 0.06
Digoxin 1 μmol/l	Normal	75 ± 5.4	1.04 ± 0.39
Digoxin 3 μmol/l	Normal	58 ± 3.2	1.85 ± 0.13
Monensin 0.3 μmol/l	Normal	----	0.71 ± 0.03
Monensin 1 μmol/l	Normal	80 ± 4.2	1.13 ± 0.49
Monensin 3 μmol/l	Normal	62 ± 4.2	2.80 ± 0.90
Experiment 2			
Control	"weakened"	----	0.63 ± 0.03
Digoxin 0.3 μmol/l	"weakened"	93 ± 4.5	2.76 ± 0.87
Monensin 0.3 μmol/l	"weakened"	92 ± 5.4	2.76 ± 0.16
Experiment 3			
Control	Na^+-free	----	0.70 ± 0.08
Digoxin 3 μmol/l	Na^+-free	----	0.55 ± 0.04
Monensin 3 μmol/l	Na^+-free	68 ± 5.6	2.06 ± 1.07

Fig. 1. Schematic illustration of a modified Langendorff apparatus.

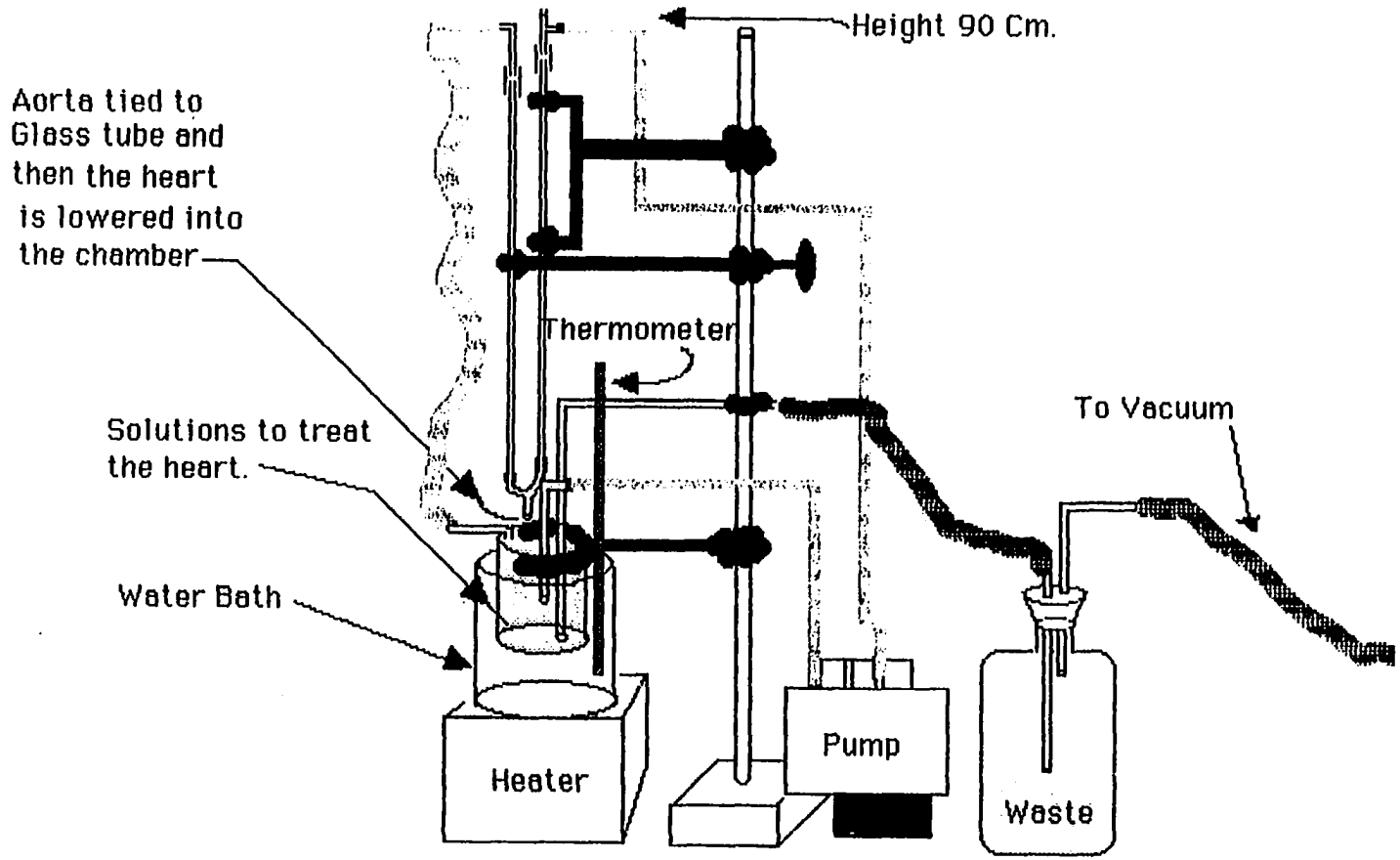

Height 90 Cm.
Aorta tied to
Glass tube and
then the heart
is lowered into
the chamber
Thermometer
To Vacuum
Solutions to treat
the heart.
Water Bath
Heater
Pump
Waste

FIG. 2. Dose response of digoxin. The largest increase in $[Ca^{2+}]_i$ was obtained by using 3 μmol/l digoxin. Means + SD is shown (n=3). * denotes a significant difference between control and treatment mean, $P < 0.0001$.

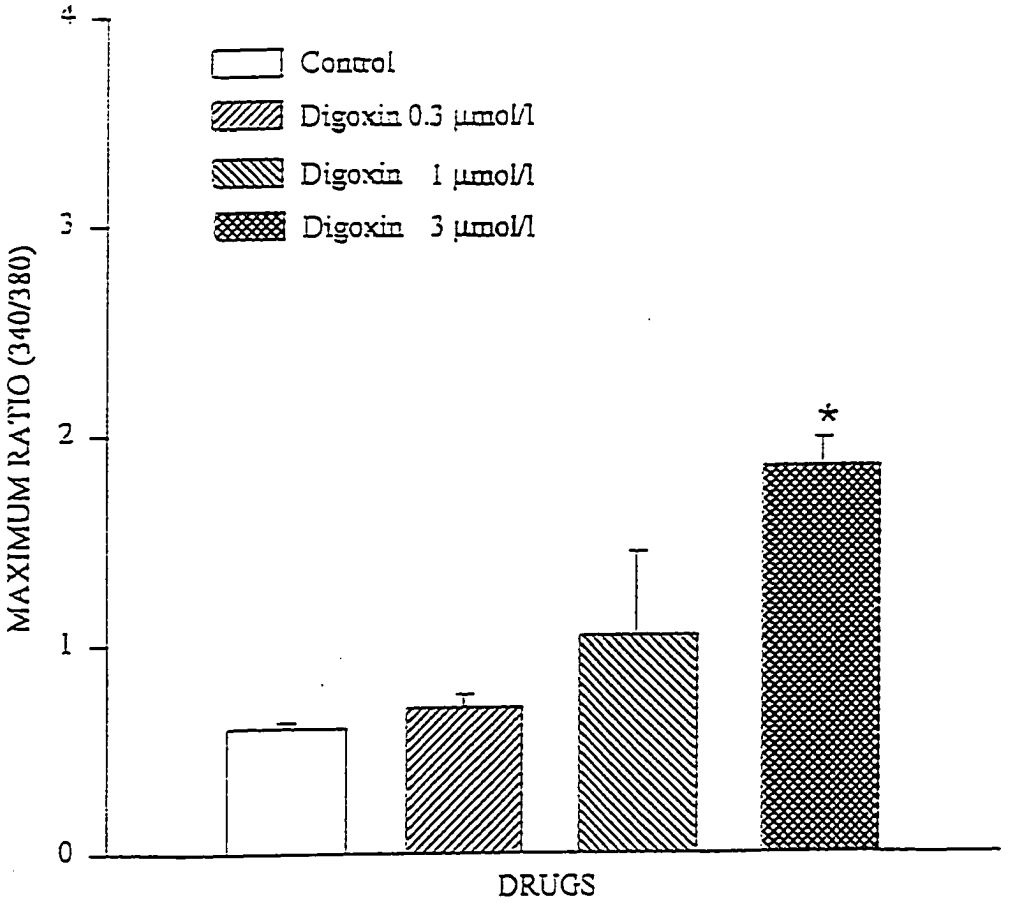

Control
Digoxin 0.3 µmol/l
Digoxin 1 µmol/l
Digoxin 3 µmol/l
MAXIMUM RATIO (340/380)
0
1
2
3
4
*
DRUGS

FIG. 3. Dose response of monensin. The largest increase in $[Ca^{2+}]_i$ was obtained by using 3 μmol/l monensin. Means + SD is shown (n=3). * denotes a significant difference between control and treatment mean, $P < 0.0001$.

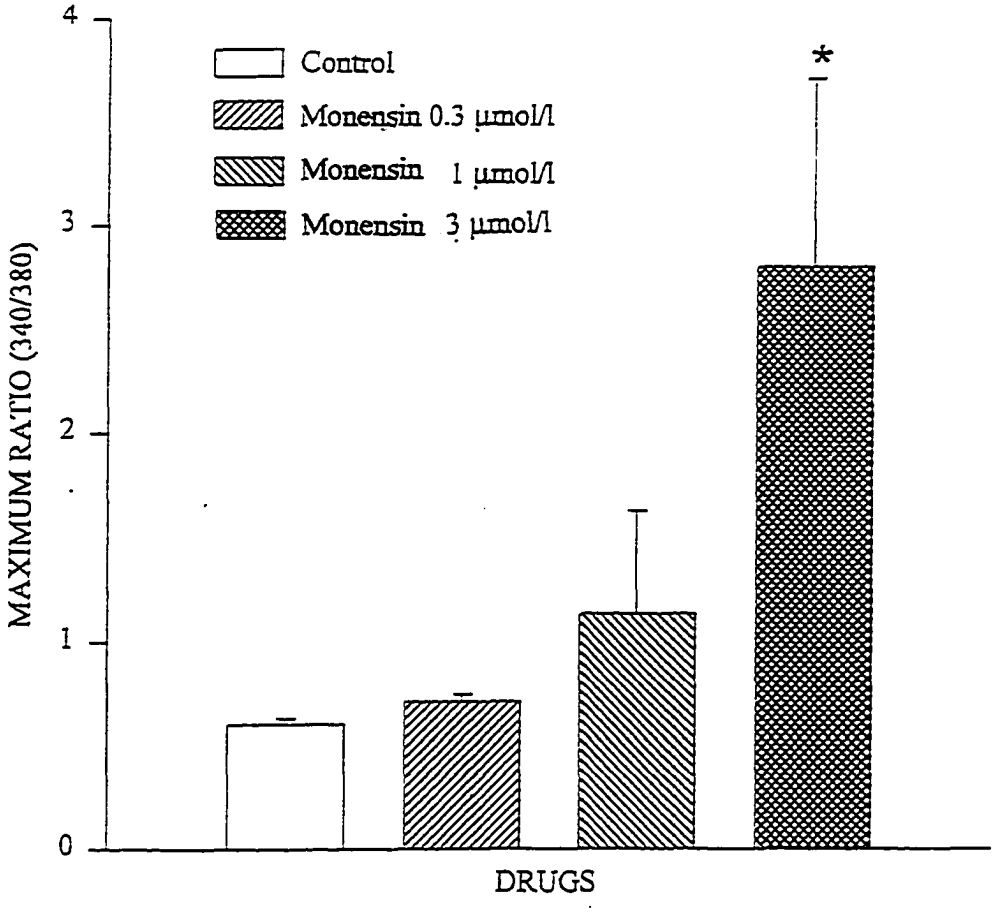

4
3
2
1
0
MAXIMUM RATIO (340/380)
Control
Monensin 0.3 µmol/l
Monensin 1 µmol/l
Monensin 3 µmol/l
*
DRUGS

FIG. 4. Effects of monensin (0.3 μmol/l) and digoxin (0.3 μmol/l) in $[Ca^{2+}]_i$ of "weakened" cells. Means + SD is shown (n=3). * denotes a significant difference between control and treatment mean, $P < 0.0031$.

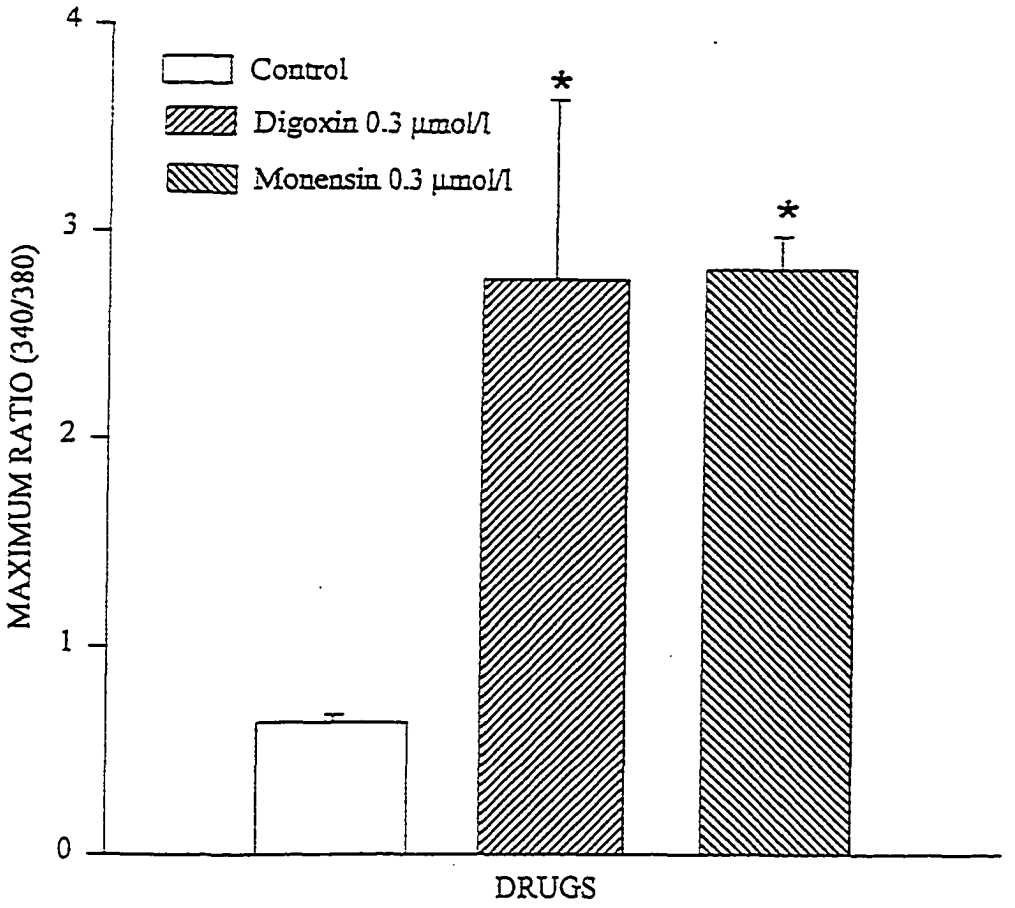

4
3
2
1
0
MAXIMUM RATIO (340/380)
Control
Digoxin 0.3 µmol/l
Monensin 0.3 µmol/l
*
*
DRUGS

FIG. 5. Effects of monensin (3 μmol/l) and digoxin (3 μmol/l) on $[Ca^{2+}]_i$ of cardiac myocytes in Na^+-free medium. Means + SD is shown (n=3). * denotes a significant difference between control and treatment mean, $P < 0.016$.

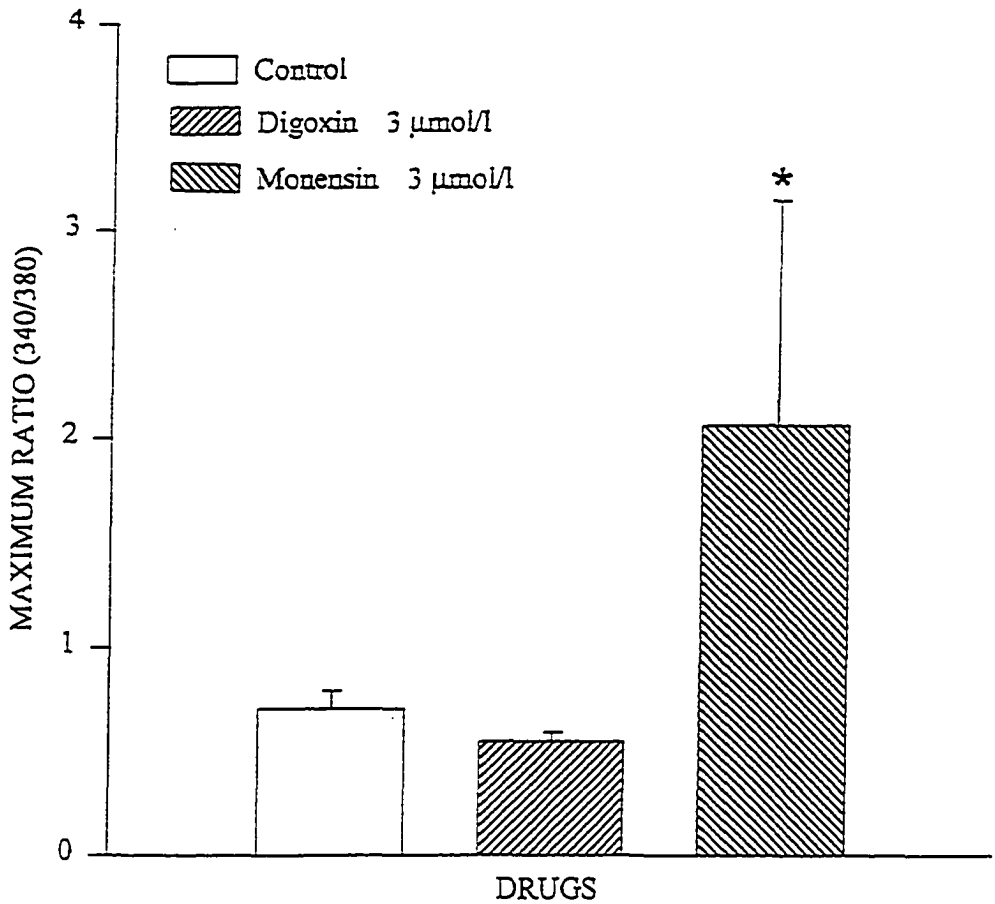
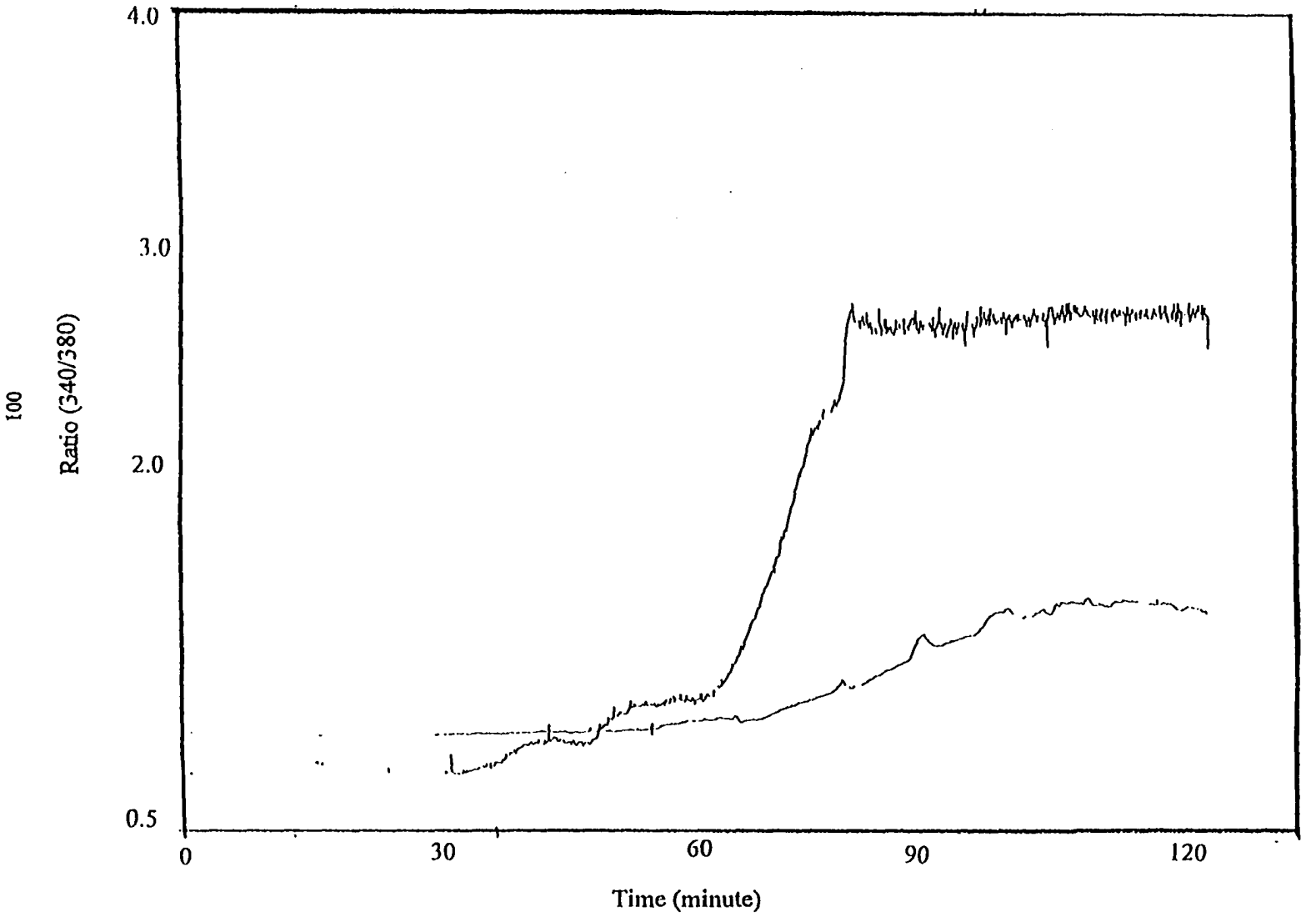

4
3
2
1
0
Control
Digoxin 3 µmol/l
Monensin 3 µmol/l
*
MAXIMUM RATIO (340/380)
DRUGS

FIG. 6. Representative tracing of monensin- and digoxin-induced increase in $[Ca^{2+}]_i$ of guinea-pig ventricular myocyte.


100
Ratio (340/380)
4.0
3.0
2.0
0.5
0
30
60
90
120
Time (minute)

GENERAL DISCUSSION

Contraction velocity is used as an indicator of how well cardiac tissue functions. It can be used to assess the function of the sarcomere, the myocyte, a papillary muscle, ventricular strips, or an intact heart.

The inverse relationship between load and velocity of contraction was one of the first to be established in cardiac mechanics. This is usually associated with afterload. When the load is zero the V_{max} derived from an afterload curve, with a constant preload, is a theoretical value which is considered to be a maximum velocity with a given state of contractility. However, with an increasing preload (on the ascending limb of a preload curve) the velocity is directly related to the preload (or to the length of muscle), if the afterload is constant theoretically, V_{max} does not change. The unloaded (afterload) velocity of cardiac muscle contraction is strongly $[Ca^{2+}]_i$ dependent. This is not true of skeletal muscle (Sauders, 1989).

If an adult (intact) heart is considered, the velocity of contraction is a variable that gives important information in regard to function. Of the many examples available, cardiac enlargement serves well to demonstrate the value of the velocity term. Some authors classify cardiac enlargement as "hypertrophy" and "dilatation". Hypertrophy is then subdivided into two parts as: "concentric hypertrophy" (caused by a pressure overload) and "eccentric hypertrophy" (caused by a volume overload). With eccentric hypertrophy the wall thickness may increase just enough to compensate for the increased volume (increased

diameter) of the chamber. In this case the velocity term can fall into the normal range of function. Whereas, with a concentric hypertrophy there is a large increase in wall thickness. If the increase in wall thickness equals the increase in the afterload or exceeds it, then the velocity term again falls within the normal range. With a true dilatation, force and velocity are decreased at every point along the entire ascending limb of the preload curve.

Time is the enemy of a contracting heart. One of the better correlates with oxygen consumption is the time-tension index. When the velocity of contraction is low, the heart must contract longer (and over a longer period of the normal systolic phase because it no longer functions as an impulse producer giving momentum to the blood). This requires more oxygen, more energy consumption and in a sense this becomes an added afterload on the heart.

The velocity of contraction is directly related to the crossbridge cycling rate and this is directly related to the myosin-ATPase (a weak ATPase) and to the more important acto-myosin ATPase (a strong ATPase). Therefore: anything that influences the enzyme systems of cardiac muscle will also influence the velocity of contraction.

In the first study, we evaluated the effects of monensin and digoxin on contractility changes in fatigued and non-fatigued guinea-pig papillary muscles. We found that both monensin (7 μmol/l) and digoxin (3 μmol/l) caused a positive inotropic effect in guinea-pig papillary muscles. However, the positive inotropic effect of monensin lasted longer than the positive inotropic effect of digoxin. This was also true for the effect of monensin on the contraction velocity, as compared to both the control and the digoxin treated papillary

muscles. These findings are very important; as a decreased contraction velocity acts as an increased afterload to a functional heart and is an important consideration in congestive heart failure (CHF).

In cardiac myocytes, a small quantity of Ca^{2+} crosses the sarcolemma and induces a much larger release of Ca^{2+} from the sarcoplasmic reticulum (SR). The Ca^{2+} that is released from the SR initiates contraction by binding to the contractile proteins (Barry and Bridge, 1993). The cross bridges that project from the thick (myosin) filaments and interact with the thin (actin) filaments constitute the molecular motors that generate force in cardiac muscle (Fuchs, 1995). There are two routes of thin filament activation. The first route is based on the binding of Ca^{2+} to the troponin C subunit of the regulatory complex. The second route is the cross bridges themselves (Fuchs, 1995). Under conditions that favor a strong actin-myosin binding affinity (low Mg^{2+}-ATPase concentrations, low ionic strength), contraction can occur in the virtual absence of Ca^{2+} (Fuchs, 1995). The affinity of troponin C for Ca^{2+} is affected by several factors. Low intracellular pH (pH_i) decreases the affinity of troponin C for Ca^{2+} (Blanchard and Solaro, 1984). cAMP-dependent protein kinase-induced phosphorylation of troponin I also decreases Ca^{2+} sensitivity of troponin C (McClellan and Winegrad, 1980). Thus, not only the rate of increased $[Ca^{2+}]_i$ but also the affinity of the contractile elements for Ca^{2+} regulates the force developed by a myocyte.

The positive inotropic action of digoxin is due to the inhibition of Na^+, K^+-ATPase. As a result, there is a gradual increase in $[Na^+]_i$ and a gradual small decrease in $[K^+]_i$ (Deitmer and Ellis, 1978). It is the increase in $[Na^+]_i$ that is related to the positive

inotropic effect of digoxin. An increase in $[Na^+]_i$ by digoxin would raise the $[Ca^{2+}]_i$ due to the pumping out this increased intracellular Na^+ for the exchange of extracellular Ca^{2+} by a Na^+ (out) / Ca^{2+} (in) exchanger (Lee and Dagostino, 1982). Increased $[Ca^{2+}]_i$ causes the release of additional Ca^{2+} from the SR (Fabiato, 1983). The increase in $[Na^+]_i$ is adjusted by a Na^+-H^+ exchanger that pumps a Na^+ outside and a H^+ inside the myocyte. This causes a decrease in pH_i (Kim et al., 1987). In our first study, we were able to demonstrate that 3 μmol/l digoxin caused a positive inotropic action. To be able to demonstrate whether the digoxin-induced positive inotropic action was due to an increase in $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ of digoxin (3 μmol/l) treated guinea-pig ventricular myocytes by loading them with fura-2. We found that digoxin significantly increased the $[Ca^{2+}]_i$ of myocytes. We concluded that the positive inotropic action of digoxin is due to an increase in $[Ca^{2+}]_i$. However, digoxin lost its positive inotropic action and caused a negative rather than a positive inotropic effect with the elapse of time. This may be due to several reasons. First, a toxic effect of digoxin occurred followed by a positive inotropic effect due to an excessive amount of $[Ca^{2+}]_i$. Second, an assumed decreased pH_i decreases the affinity of troponin C for Ca^{2+}. To be able to demonstrate whether digoxin-induced increases in the force of contraction and increases in $[Ca^{2+}]_i$ were due to $[Na^+]_o$, we used a Na^+ -free solution and measured the $[Ca^{2+}]_i$ of myocytes. We found that digoxin did not increase the $[Ca^{2+}]_i$ in a Na^+ free solution. We concluded that the digoxin-induced increase in the force of contraction and $[Ca^{2+}]_i$ was directly related to the $[Na^+]_o$.

Monensin binds Na^+ outside of the cell and carries it into the cell and increases $[Na^+]_i$ and thus $[Ca^{2+}]_i$ (Mollenhauer et al., 1990). Monensin also increases the pH_i due to the transfer of a H^+ ion outside the cell (Mollenhauer et al., 1990). The 7 μmol/l monensin also caused a positive inotropic effect in guinea-pig papillary muscles. To be able to demonstrate whether monensin's induced positive inotropic action was due to an increase in $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ of monensin (3 μmol/l) treated guinea-pig ventricular myocytes by loading them with fura-2. We found that monensin significantly increased the $[Ca^{2+}]_i$ of myocytes and concluded that the positive inotropic action of monensin is due to an increase in $[Ca^{2+}]_i$. Unlike digoxin, monensin did not lose its positive inotropic effect on guinea-pig papillary muscles with the elapse of time. This demonstrated that the positive inotropic effect that was obtained with monensin treatment lasted longer than digoxin. Elevated pH_i which increases the affinity of troponin C for Ca^{2+} may partially be responsible for the long lasting positive inotropic action of monensin. To be able to demonstrate whether the monensin-induced increase in force of contraction and $[Ca^{2+}]_i$ is due to the $[Na^+]_o$, we used a Na^+ -free medium and measured $[Ca^{2+}]_i$ of myocytes. We found that monensin increased the $[Ca^{2+}]_i$ in Na^+-free medium. We concluded that monensin can increase $[Ca^{2+}]_i$ in both the presence of $[Na^+]_o$ and in the absence of $[Na^+]_o$, which increases the force of contraction.

There are two components, the early and late components, of the guinea-pig papillary muscle contraction wave (Vierling, 1988). The early component is suggested to be generated by Ca^{2+} released from the SR whereas the late component is thought to result

from Ca^{2+} flowing into the cell via the inward Ca^{2+} current (Adamantidis et al., 1988). The ICV would be affected by the change in the early component and the ACV would be affected by those factors that produce both the early and late components of the contraction wave (Adamantidis et al., 1988). Velocity is not affected by increased $[Ca^{2+}]_i$ alone. An increase in heart rate or temperature increases the velocity of the contraction by increasing ATPase activity of myosin without changing $[Ca^{2+}]_i$ or contraction force of the myocardium. Both digoxin and monensin increased the velocity of contraction of guinea-pig papillary muscles suggesting that they have a significant effect on the rate of Ca^{2+} release from the SR, the affinity of troponin C for Ca^{2+} or myosin ATPase activity. Digoxin lost its effect with the elapse of time probably due to decreased pH_i which decreases the affinity of troponin C for Ca^{2+}. Further studies are needed to measure pH_i when using monensin and digoxin to confirm our hypothesis.

REFERENCES

Adamantidis, M. M., E. R. Honore, and B. A. Dupuis. Electrical and mechanical effects of new aminosteroids on guinea-pig isolated ventricular muscle. Br. J. Pharmacol. 95: 1063. 1988.

Anderson, K., F. A. Lai, Q. Y. Lin, E. Rousseau, H. P. Ericson, and G. Meissner. Structural and functional characterization of the purified cardiac ryanodine receptor-Ca^{2+} release channel complex. J. Biol. Chem. 204: 1329. 1989.

Barry, W. H., J. H. B. Bridge. Intracellular calcium homeostasis in cardiac myocytes. Circulation. 87: 1806. 1993.

Baker, P. F., M. P. Blaustein, and A. L. Hodgkin. The influence of Ca^{2+} on Na^{+} efflux in squid axons. J. Physiol. (Lond). 200: 431. 1969.

Bergstrom R. C., and L. R. Maki. Effect of monensin in young crossbred lambs with naturally occurring coccidiosis. J. Am. Vet. Med. Assoc. 165: 288. 1978.

Besch, H. L., J. C. Allen, and G. Glick. Correlation between the inotropic action of ouabain and its effect on subcellular enzyme systems from canine myocardium. J. Pharmacol. Exp. Ther. 171: 1. 1970.

Bigger J. T. Digitalis toxicity. J. Clin. Pharmacol. 25: 514. 1985.

Blanchard, E. M., and R. J. Solaro. Inhibition of the activation and troponin calcium binding of dog cardiac myofibrils by acidic pH. Cir. Res. 55: 382. 1984.

Cannel, M. B. Contribution of sodium-calcium exchange to calcium regulation in cardiac muscle. Ann. N. Y. Acad. Sci. 639: 428. 1991.

Carafoli, E. The Ca^{2+} pump of the plasma membrane. J. Biol. Chem. 267: 2115. 1992.

Catterall, W. A. Functional subunit structure of voltage-gated calcium channels. Science. 253: 1499. 1991.

Cattel H., and M. Gold. Mechanism of digitalis action abolishing heart failure. Arch. Intern. Med. 65: 263. 1940.

Cranefield, P. F., and B. F. Hoffman. Electrophysiology of single cardiac cells. Physiol. Rev. 38: 41. 1958.

Davidson C., and D. Gibson. Clinical significancy of positive inotropic action of digoxin in patients with left ventricular disease. Br. Heart. J. 35: 970. 1973.

Deitmer, J. W., and D. Ellis. Intracellular sodium activity of cardiac purkinje fibers during inhibition and reactivation of the Na-K pump. J. Physiol. (Lond.) 284: 241. 1978.

dePolo, R., and L. Beauge. Reverse Na^{+} -Ca^{2+} exchange requires internal Ca^{2+} and / or ATP in squid axons. Biochim. Biophys . Acta 854: 296. 1986.

duBell, W. H., and S. R. Hauser. Voltage and beat dependence of the Ca^{2+} transient in feline ventricular myocytes. Am. J. Physiol. 257: H746. 1989.

Ellis, D. The effects of external cations and ouabain on the intracellular sodium activity of sheep heart purkinje fibers. J. Physiol. (Lond). 273: 211. 1977.

Fabiato, A. Calcium induced release of calcium from the cardiac sarcoplasmic reticulum. Am. J. Physiol. 245: C1. 1983.

Fabiato, A. Appraisal of the physiological relevance of two hypotheses of the mechanism of Ca^{2+} release from the cardiac sarcoplasmic reticulum: Calcium induced calcium release versus charge coupled release. Mol. Cell. Biochem. 89: 135. 1989.

Frank, J. S., G. Mottino, D. Reid, R. R. Molday, and K. D. Philipson. Distribution of the Na^{+} -Ca^{2+} exchange protein in mammalian cardiac myocytes: An immunofloresence and immunocolloidal gold-labeling study. J. Cell. Biol. 117: 337. 1992.

Fitzgerald P. R. Efficacy of monensin against bovine coccidiosis in young Holstein-Friesian calves. J. Protozool. 20: 121. 1973.

Fuchs, F. Mechanical modulation of the calcium regulatory protein complex in cardiac muscle. Am. Physiol. Soc. 10: 6. 1995.

Gaide, M. S., J. J. Lehr, S. S. Wong, F. A. Lattanzio and A. L. Basset. Comparative study of the effects of salinomycin and monensin on electrophysiologic and contractile properties of canine myocardium. Eur. J. Pharmacol. 100: 321. 1984.

Gheorghiade M., and G. A. Beller. Effects of discontinuing maintenance digoxin therapy in patients with ischemic heart disease and cardiac heart failure in sinus rhythm. Am. J. Candiol. 51: 1243. 1983.

Hoffman B. F., and Bigger. Jr. T. Digitalis and allied cardiac glycosides. Goodman and Gilman's the pharmacologic basis of therapeutics. Eighth edition. 814. 1990.

Hoffman, B. F., and D. H. Singer. Effects of digitalis on electrical activity of cardiac fibers. Prog. Cardiovasc. Dis. 7: 226. 1964.

Haworth, R. A., A. B. Goknur, D.R. Hunter, J.O. Hegge, and H. A. Berkoff. Inhibition of Ca^{2+} influx in isolated adult rat heart cells by ATP depletion. Cir. Res. 60: 586. 1987.

Hugtenburg, J. G., M. J. Mathy, J. J. Beckeringh, and P. A. var Zwieten. The differential effect of calcium antagonists on the positive inotropic effects induced by calcium and monensin in cardiac preparations of rats and guinea-pigs. Naunyn-Schmiedeberg's Ach. Pharmacol. 340: 558. 1989.

Isenberg, G. Is potassium conductance of cardiac purkinje fibers controlled by intracellular calcium concentration ? Nature. 253: 273. 1975.

Ito, M., P. B. Hollander, and B. H. Marks. The effects of six cardiac glycosides on the transmembrane potential and contractile characteristics of the right ventricle of guinea-pigs. J. Pharmacol. Exp. Ther. 172: 188. 1970.

Jorgensen, A. O., A. C. Y. Shen, P. Daly, and D. H. MacLennan. Localization of Ca-Mg ATPase of sarcoplasmic reticulum in adult rat papillary muscle. J. Cell. Biol. 93: 883-892. 1982.

Kass, R. S., and R. W. Tsien. Control of action potential duration by calcium ion in cardiac purkinje fibers. J. Gen. Physiol. 67: 599. 1976.

Kim, D., E. J. Cragoe, and T. W. Smith. Relations among sodium pump inhibitions, Na-Ca and Na-H exchange activities, and Ca-H interaction in cultured chick heart cells. Circ. Res. 60: 185. 1987.

Kimura, J., A. Noma, and H. Irisawa. Na^{+} -Ca^{2+} exchange current in mammalian heart cells. Nature 319: 596. 1986.

Lee, C. O., and M. Dagostino. Effect of strophanthidin on intracellular Na ion activity and twitch tension of constantly driven canine cardiac purkinje fibers. Biophys. J. 40: 185. 1982.

Levitt B., and D. L. Keefe. Clinical use of digitalis materials. J. Clin. Pharmacol. 25: 507. 1985.

Li, Z., D. A. Nicoll, A. Collins, D. W. Hilgerman, A. G. Filoteo, J. T. Penniston, J. N. Weiss, J. M. Tomich, and K. D. Philipson. Identification of a peptide inhibitor of the cardiac sarcolemma Na^{+}-Ca^{2+} exchanger. J. Biol. Chem. 266: 1014. 1991.

Marban, E., and R. W. Tsien. Enhancement of calcium current during digitalis inotropy in mammalian heart: Positive feed-back regulation by intracellular calcium? J. Physiol. (Lond). 329: 589. 1982.

McClellan, G. B., and S. Winegrad. Cyclic nucleotide regulation of contractile proteins in mammalian cardiac muscle. J. Gen. Physiol. 75: 283. 1980.

McGuigan, J. A. S., and T. B. Bassingthwaighte. Possible relationship between intracellular calcium concentration and P_K in ventricular muscle. Experientia. 30: 680. 1974.

Meissner, G. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. J. Biol. Chem. 261: 6300. 1986.

Meissner, G., and J. S. Henderson. Rapid Ca^{2+} release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. J. Biol. Chem. 262: 3065. 1987.

Mitema E. S., S. Sangiah, T. Martin. Effects of some calcium modulators on monensin toxicity. Vet. Hum. Toxicol. 30 (5): 409. 1988.

Miura, D. S , S. Biedert. Does calcium control the transmembrane cation fluxes in cardiac muscle (abstract). J. Gen. Phyisol. 82: 19A. 1983.

Miura, D. S., and S. Biedert. Cellular mechanisms of digitalis action. J. Clin. Pharmacol. 25: 490. 1985.

Miura, D. S., B. F. Hoffman, and M. R. Rosen. The effect of extracellular potassium on the intracellular potassium ion activity and transmembrane potentials of canine cardiac purkinje fibers. J. Gen. Physiol. 69: 463. 1977.

Miura, D. S., B. F. Hoffman, and M.R. Rosen. Effect of ouabain on intracellular potassium activity and transmembrane potentials of canine cardiac purkinje fibers. Fed. Proc. 35: 320. 1976.

Mollenhauer H. H., J. D. More, D. L. Rowe. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Brachium. Trophy. Acta. 1031: 225. 1990.

Moravec, C. S., and M. Bond. Calcium is released from the junctional sarcoplasmic reticulum during cardiac muscle contraction. Am. J. Physiol. 260: H989. 1991.

Moss, R. L. Calcium regulation of mechanical properties of striated muscle. Circ. Res. 70: 865. 1992.

Nicole, D. A., S. Longoni, and K. D. Philipson. Molecular coloning and functional expression of the cardiac sarcolemmal Na^+ -Ca^{2+} exchanger. Sci. 250: 562. 1990.

Niggli, E., and W. J. Lederer. Voltage independent calcium release in heart muscle. Sci. 250: 565. 1990.

Otsu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. Maclennan. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J. Biol. Chem. 265: 13472. 1990.

Ozaki, H., T. Kismhimato, S. Chihara, U. Umeno, and N. Urakawa. Action of the sodium ionophore monensin on vascular smooth muscle of guinea-pig aorta. Eur. J. Pharmacol. 100: 299. 1984.

Philipson, K. D., M. M. Bersohn, and A. Y. Nishimato. Effects of pH on Na^+ -Ca^{2+} exchange in cardiac sarcolemmal vesicles. Circ. res. 50: 287. 1982.

Ray, K. P., and P. J. England. Phosphorylation of the inhibitory subunit of troponin and its effect on the calcium dependence of cardiac myofibril adenosine triphosphatase. FEBS. Lett. 70: 11. 1976.

Reeves, J. P., and C. C. Hale. The stoichiometry of the cardiac Na^+ -Ca^{2+} exchange system. J. Biol. Chem. 259: 7733. 1984.

Richardson, L. F., A.P. Raun, E. L. Potter, C.O. Cooley, R. P. Rathmatiher. Effects of monensin on ruminal fermentation in vitro and in vivo. J. An. Sci. 39: 250. 1974.

Rousseau, E., J. S. Smith, and G. Meissner. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channels. Am. J. Physiol. 253: C364. 1987.

Sanguinetti, M. C., D. S. Krafte, and R. S. Kass. Voltage dependent modulation of Ca^{2+} channel current in heart cells by Bay K 8644. J. Gen. Physiol. 88: 369. 1986.

Sasaki, T., M. Incis, Y. Kimura, T. Kuzuya, and M. Tada. Molecular mechanism of regulations of Ca^{2+} pump ATPase by phospholamban in cardiac sarcoplasmic reticulum. J. Biol. Chem. 267: 1674. 1992.

Sauders, W. B. Textbook of physiology 2: 21st edition 820. 1989.

Sham, J. S. K., L. Cleemann, and M. Morad. Gating of the cardiac Ca^{2+} release channel: The role of Na^{+} current and Na^{+} -Ca^{2+} exchange. Sci. 255: 850. 1992.

Sheu, S. S., and H. A. Fozzard. Transmembrane Na^{+} and Ca^{2+} electrochemical gradients in cardiac muscle and their relationship to force development. J. Gen. Physiol. 80: 325. 1982.

Shumard R. F., and M. E. Callender. Effectiveness of monensin against experimental injections of coccidia in chickens. Proc. 18 th Western Poultry Disease Conference, Davis, CA: 20, 1969.

Singer, D., M. Biel, I. Loton, V. Flockerzi, F. Hofmann, and N. Dascal. The roles of subunits in the function of the calcium channel. Sci. 253: 1553. 1991.

Sonberg, J., B. Greenfield, and D. Tepper. Digitalis: Historical development in clinical medicine. J. Clin. Pharmacol. 25: 484. 1985.

Sutko, J. L., H. R. Bescl, J. C. Bailey, G. Zimmerman, and A. H. Watanahe. Direct effect of monovalent cation ionophores monensin and nigericin on myocardium. J. Pharmacol. Exp. Ther. 203: 685. 1977.

Tsuchida, K., and S. Otomo. Electrophysiological effects of monensin, a sodium ionophore, on cardiac purkinje fibers. Eur. J. Pharmacol. 190: 313. 1990.

Van Vleet, J. F., and V. J. Ferrans. Ultrastructural alterations in skeletal muscle of pigs with acute monensin myotoxicosis. Am. J. Pharmacol. 114 (3): 461. 1983.

Van Vleet J. F., N. E. Amstutz, W. E. Weirich, A. H. Rebar, and U. J. Ferrans. Clinical, clinicopathologic and pathologic alterations in acute monensin toxicosis in cattle. Am. J. Vet. Res. 44: 2133. 1983.

Vierling, W. Ryanodine in mammalian heart ventricular muscle: indication for the introduction of calcium leakage from the sarcoplasmic reticulum. Eur. J. Pharmacol. 145: 329. 1988.

Whitlock R. H., N. A. White, G. N. Rowlond, and R. Plue. Monensin toxicosis in horses: Clinical, manifestations. Proc. 24 th Ann. Conv. Am. Assoc. Equine Pract, St Louis. MO: 473-486. 1978.

William, D. A., L. M. Delbridge, S. H. Cody, P. J. Harris, and T. D. Morgan. Spontaneous and propagated calcium release in isolated cardiac myocytes viewed by confocal microscopy. Am. J. Physiol. 262: C731. 1992.

ACKNOWLEDGMENTS

I am very grateful to Dr. Frederick B. Hembrough for his constant guidance and support throughout my doctoral career. His encouragement and enthusiasm made the process rewarding and insightful.

I would like to express my sincere gratitude to Dr. Walter H. Hsu, Dr. Richard L. Engen, Dr. Theodore B. Bailey and Dr. Wendy Ware for serving on my POS committee and giving me important advice, criticism and friendship during my graduate study at Iowa State University.

I also owe my thanks to my fellow graduate students Ronghua ZhuGe, Ter-Hsien Chen and Bumsup Lee for their friendship and help in various ways during my study.

I deeply appreciate that my employer, Yuzuncu Yil University, Turkey, allowed me to come to the United States to pursue my Ph.D. study and that Turkish government provided me the financial support.

I would like to dedicate this dissertation to my parents, Ibrahim and Nazmiye Meral, and parents-in law, Mr. and Mrs. Cansiz, in appreciation of all love and sacrifices made so I could reach my goals.

Finally, I would like to thank my wife, Didem Meral. In spite of the hardships she has had to bear these past two years, she has always been a source of support and encouragement.

APPENDIX

TABLE 1. Original data of manuscript one. M; muscle, ACV; average contraction velocity, ICV; initial contraction velocity, IRV; initial relaxation velocity, CF; contraction force, ST; stimulus to response time, NF; non-fatigued papillary muscles, F; fatigued papillary muscles.

M	Drug	Method	Time	ACV (mg/sec)	ICV (mg/sec)	IRV (mg/sec)	CF (mg)	ST (Msec)
1	D	NF	0	578.62	620.52	301.22	300	70.20
1	D	NF	1	1157.27	1363.20	642.65	475	54.01
1	D	NF	2	1793.80	2142.25	1577.07	625	43.55
1	D	NF	3	1683.33	2320.80	1111.55	400	43.20
1	D	NF	4	1403.15	1766.62	1289.25	275	43.55
2	M	NF	0	737.22	992.97	472.57	225	43.67
2	M	NF	1	2650.07	3279.12	1679.20	687.5	21.61
2	M	NF	2	3783.77	3836.02	2585.97	825	32.70
2	M	NF	3	5016.37	10937.15	2483.22	875	10.90
2	M	NF	4	6115.20	10937.15	3251.32	1000	16.35
3	C	NF	0	771.52	1002.24	485.54	400	64.80
3	C	NF	1	861.02	1230.98	626.42	450	65.32
3	C	NF	2	1267.50	1680.50	796.43	575	64.80
3	C	NF	3	1249.10	1729.38	620.52	512.5	64.80
3	C	NF	4	1424.35	1729.38	689.60	400	59.40
4	M	F	0	1624.35	2299.37	1125.16	425	32.70
4	M	F	1	2402.04	3251.34	1350.60	550	21.80
4	M	F	2	2637.18	3619.66	1455.40	575	21.80
4	M	F	3	3224.81	4079.23	1464.43	562.5	21.80
4	M	F	4	2436.52	3425.90	1287.65	425	21.80
5	M	F	0	1012.97	1203.45	835.19	375	48.99
5	M	F	1	1589.58	1937.84	994.22	450	32.66
5	M	F	2	1457.11	1667.06	955.32	412.5	32.66
5	M	F	3	1501.27	2486.33	1014.57	425	38.10
5	M	F	4	1504.47	2017.97	926.02	325	43.20
6	D	F	0	3654.78	4354.63	2139.58	637.5	43.60
6	D	F	1	1638.00	2387.96	1418.96	250	32.70
6	D	F	2	0	0	0	0	0
6	D	F	3	0	0	0	0	0
6	D	F	4	0	0	0	0	0
7	M	NF	0	717.32	1126.57	564.08	250	65.33
7	M	NF	1	1518.94	2506.38	1186.39	525	43.20
7	M	NF	2	2218.14	3281.65	1589.81	575	43.20
7	M	NF	3	2296.06	3097.11	2001.83	500	43.55
7	M	NF	4	2367.81	2821.30	1320.14	412.5	43.55

TABLE 1. Continued.

8	M	F	0	2770.95	3425.90	1013.30	725	43.60
8	M	F	1	3179.20	4079.23	1575.12	762.5	43.60
8	M	F	2	3439.80	2949.36	1999.33	750	43.60
8	M	F	3	3898.44	3619.66	2216.78	850	43.60
8	M	F	4	4231.42	3802.24	1372.23	837.5	43.98
9	C	F	0	819.74	1002.24	496.21	425	75.60
9	C	F	1	1132.13	1299.65	727.45	562.5	70.20
9	C	F	2	1918.66	2086.52	1240.90	787.5	70.20
9	C	F	3	1864.52	2320.82	1363.20	725	54.00
9	C	F	4	1339.37	1877.52	832.20	437.5	59.88
10	C	NF	0	1519.24	1664.98	760.79	662.5	54.51
10	C	NF	1	1815.45	2139.58	1101.29	712.5	54.51
10	C	NF	2	1629.41	1859.15	842.68	537.5	43.98
10	C	NF	3	1587.60	1875.17	683.23	450	43.61
10	C	NF	4	1136.80	1650.75	520.84	325	49.48
11	D	F	0	746.22	918.62	401.93	325	70.77
11	D	F	1	2831.81	2700.53	1820.54	925	48.99
11	D	F	2	3840.10	3457.84	6009.45	912.5	40.50
11	D	F	3	3278.99	4712.68	3281.65	637.5	43.20
11	D	F	4	1148.03	1667.06	1495.36	225	43.55
12	M	NF	0	1051.05	1575.12	695.46	412.5	65.41
12	M	NF	1	4371.41	3251.34	3251.34	762.5	59.96
12	M	NF	2	4299.75	4354.63	3251.34	750	32.70
12	M	NF	3	3663.02	4629.23	4988.77	725	32.98
12	M	NF	4	4586.40	3619.66	3093.24	600	32.70
13	C	F	0	1983.13	2339.38	1443.65	637.5	64.29
13	C	F	1	1929.29	2526.44	970.73	537.5	53.57
13	C	F	2	2136.53	2844.13	908.29	600	48.60
13	C	F	3	2296.06	3255.40	1079.56	700	48.99
13	C	F	4	2296.06	3430.18	974.48	700	48.99
14	M	NF	0	1289.92	2139.58	625.65	337.5	49.05
14	M	NF	1	2197.65	2949.37	1149.86	575	49.06
14	M	NF	2	2136.84	2697.16	1384.07	512.5	43.60
14	M	NF	3	1385.47	2483.23	1229.44	362.5	43.60
14	M	NF	4	1278.90	2067.25	1101.29	362.5	65.41
15	C	NF	0	771.52	1022.75	826.39	400	75.60
15	C	NF	1	956.02	1135.65	1213.15	475	70.20
15	C	NF	2	1041.56	1507.42	926.02	450	64.80
15	C	NF	3	1035.47	1432.19	714.56	425	70.20
15	C	NF	4	1092.99	1363.20	665.65	425	59.40
16	D	F	0	1989.40	2674.11	1004.64	437.5	43.98
16	D	F	1	1082.50	1618.94	683.23	212.5	43.98
16	D	F	2	0	0	0	0	0
16	D	F	3	0	0	0	0	0
16	D	F	4	0	0	0	0	0
17	C	F	0	1243.70	1457.22	574.01	487.5	76.21
17	C	F	1	1497.67	2237.45	944.26	550	70.20
17	C	F	2	1833.15	2744.09	614.64	550	64.29
17	C	F	3	1506.79	1877.52	804.74	525	76.21
17	C	F	4	1092.99	1835.22	826.39	425	64.80

TABLE 1. Continued.

18	D	NF	0	1223.04	1493.49	604.13	400	59.96
18	D	NF	1	3528.00	3836.03	2139.58	1000	49.05
18	D	NF	2	3439.80	3836.03	2139.58	600	32.70
18	D	NF	3	819.00	992.98	489.64	125	27.25
18	D	NF	4	0	0	0	0	0
19	C	F	0	1219.78	1577.09	775.73	425	65.33
19	C	F	1	1025.02	1352.29	696.33	312.5	65.33
19	C	F	2	991.96	1432.19	767.88	300	59.40
19	C	F	3	743.97	1065.73	578.64	225	64.80
19	C	F	4	765.35	1230.98	564.09	200	65.32
20	M	F	0	2361.04	2674.11	1372.23	675	54.98
20	M	F	1	4586.40	10939.17	2697.16	1100	38.16
20	M	F	2	3841.11	5031.78	2067.25	837.5	32.70
20	M	F	3	3410.40	4629.23	1650.75	750	38.48
20	M	F	4	3095.82	3425.90	1575.12	675	33.25
21	M	NF	0	612.68	857.87	320.74	225	59.40
21	M	NF	1	1683.78	2001.83	1176.90	550	48.99
21	M	NF	2	1942.82	2486.33	1457.22	550	54.44
21	M	NF	3	1986.98	2390.94	1385.79	562.5	43.55
21	M	NF	4	1958.49	2410.23	1680.50	550	54.00
22	C	NF	0	764.40	882.80	720.73	250	43.60
22	C	NF	1	716.62	789.08	1201.94	250	43.60
22	C	NF	2	899.96	1115.54	1218.93	237.5	38.48
22	C	NF	3	1255.80	1493.49	1055.88	287.5	38.15
22	C	NF	4	1605.24	2299.37	1229.44	350	43.60
23	C	F	0	2378.07	2700.53	1620.97	725	48.99
23	C	F	1	2050.06	2390.94	1385.79	625	43.55
23	C	F	2	2107.92	2506.38	1396.97	637.5	59.40
23	C	F	3	2487.40	2700.53	1667.06	650	54.44
23	C	F	4	2445.81	2821.38	1352.29	612.5	59.88
24	D	F	0	2178.54	2817.85	1034.26	475	49.60
24	D	F	1	6568.17	25050.00	3223.55	1300	21.99
24	D	F	2	0	0	0	0	0
24	D	F	3	0	0	0	0	0
24	D	F	4	0	0	0	0	0
25	D	NF	0	462.91	503.01	521.01	200	108.01
25	D	NF	1	1012.97	1203.45	1079.56	375	59.88
25	D	NF	2	1388.75	1547.65	1269.72	300	48.60
25	D	NF	3	1012.63	1363.20	677.50	175	43.20
25	D	NF	4	0	0	0	0	0
26	C	NF	0	1289.92	1713.41	1350.61	225	38.16
26	C	NF	1	1146.60	1618.94	563.38	250	32.70
26	C	NF	2	1136.80	1698.76	827.02	200	32.98
26	C	NF	3	891.80	1201.94	1055.88	175	38.15
26	C	NF	4	859.95	917.7	720.73	150	21.80
27	D	NF	0	1019.51	1043.90	599.20	462.5	64.80
27	D	NF	1	1937.30	1820.54	1352.29	675	65.32
27	D	NF	2	2224.31	2589.21	1577.09	775	54.44
27	D	NF	3	2645.23	2976.87	2017.97	800	48.60
27	D	NF	4	3156.25	4712.68	2086.52	750	43.20

TABLE 1. Continued.

28	D	F	0	2102.10	2585.98	1533.36	550	38.15
28	D	F	1	4267.90	4097.23	1818.27	837.5	38.15
28	D	F	2	3572.80	3588.73	5407.95	550	38.48
28	D	F	3	0	0	0	0	0
28	D	F	4	0	0	0	0	0
29	D	NF	0	1118.71	1589.81	476.99	362.5	48.60
29	D	NF	1	1414.46	2844.13	944.26	275	54.00
29	D	NF	2	0	0	0	0	0
29	D	NF	3	0	0	0	0	0
29	D	NF	4	0	0	0	0	0
30	M	F	0	805.23	1004.64	653.87	212.5	32.98
30	M	F	1	1089.43	1247.23	984.49	287.5	32.98
30	M	F	2	1719.90	1493.49	866.16	375	21.80
30	M	F	3	2078.21	2387.96	1149.86	362.5	54.50
30	M	F	4	1719.90	2299.37	992.98	300	43.60
31	M	NF	0	696.00	828.32	579.99	225	64.65
31	M	NF	1	1015.00	1302.70	560.10	262.5	53.88
31	M	NF	2	1508.00	1839.52	1366.39	325	43.10
31	M	NF	3	1392.00	1958.04	1114.17	300	53.88
31	M	NF	4	1682.00	2512.25	1272.69	362.5	48.49
32	M	F	0	1377.50	1807.80	861.17	362.5	43.86
32	M	F	1	1841.53	3598.83	1447.02	525	38.37
32	M	F	2	1805.00	1924.28	1143.24	475	32.89
32	M	F	3	1658.18	2286.14	948.63	400	27.41
32	M	F	4	2820.00	3232.62	1310.91	587.5	27.41
33	D	F	0	3132.00	4126.91	2242.69	675	48.49
33	D	F	1	3269.09	3465.94	2616.20	775	43.10
33	D	F	2	2615.29	3156.37	2875.36	475	42.73
33	D	F	3	1160.00	1733.43	1433.54	250	53.88
33	D	F	4	0	0	0	0	0
34	M	F	0	1177.08	1398.42	886.87	312.5	49.77
34	M	F	1	2486.00	2906.67	1907.40	550	38.71
34	M	F	2	2736.00	3406.18	3406.18	600	38.37
34	M	F	3	2260.00	2108.60	2353.39	550	33.18
34	M	F	4	2223.00	2286.14	2127.26	487.5	38.37
35	M	NF	0	882.60	1138.31	522.23	437.5	64.65
35	M	NF	1	1311.30	1785.05	965.28	650	48.49
35	M	NF	2	1537.00	2616.20	910.41	662.5	37.71
35	M	NF	3	2088.00	3465.94	742.36	675	48.49
35	M	NF	4	2871.81	3317.69	1345.40	675	37.39
36	C	NF	0	1533.57	2266.09	870.02	475	44.24
36	C	NF	1	2516.81	2777.06	1791.95	612.5	38.71
36	C	NF	2	2938.00	3376.30	1907.40	650	33.18
36	C	NF	3	2511.11	2906.67	1434.33	500	22.12
36	C	NF	4	2203.50	3204.27	1791.95	487.5	27.25
37	C	F	0	800.95	984.64	621.97	362.5	64.65
37	C	F	1	902.22	1243.81	859.88	350	59.26
37	C	F	2	793.68	1114.17	876.28	325	59.26
37	C	F	3	928.00	1400.23	798.30	300	53.88
37	C	F	4	981.53	1243.81	1046.34	275	48.49

TABLE 1. Continued.

38	M	NF	0	1789.16	2266.09	1211.64	475	33.18
38	M	NF	1	3295.83	4958.93	1713.41	875	38.71
38	M	NF	2	3806.31	4601.54	1713.41	800	22.12
38	M	NF	3	3076.11	4601.54	1331.05	612.5	22.12
38	M	NF	4	3138.88	4958.93	1364.03	625	22.12
39	C	NF	0	756.52	813.13	522.23	375	64.65
39	C	NF	1	501.42	595.30	448.88	225	96.15
39	C	NF	2	612.50	820.14	638.41	237.5	69.44
39	C	NF	3	658.12	867.29	805.18	225	69.44
39	C	NF	4	1015.00	1366.39	828.32	262.5	48.49
40	C	NF	0	1341.87	1907.40	723.16	475	49.77
40	C	NF	1	2362.72	3048.46	2184.69	575	27.65
40	C	NF	2	3446.50	4291.59	2777.06	762.5	27.65
40	C	NF	3	4449.37	4958.93	1511.16	787.5	22.12
40	C	NF	4	4308.12	3376.30	1970.38	762.5	22.12
41	D	F	0	1170.00	1226.47	425.02	325	53.41
41	D	F	1	1392.00	1897.09	984.64	300	48.49
41	D	F	2	1813.50	2262.02	1651.98	387.5	48.07
41	D	F	3	2513.33	2983.83	2326.25	487.5	43.10
41	D	F	4	1933.33	1637.86	1733.43	250	43.10
42	D	NF	0	1477.69	1907.40	940.31	425	22.12
42	D	NF	1	3012.85	3598.83	2932.39	462.5	27.41
42	D	NF	2	2568.18	3567.26	2548.54	312.5	22.12
42	D	NF	3	0	0	0	0	0
42	D	NF	4	0	0	0	0	0
43	C	F	0	720.00	776.32	735.44	300	69.44
43	C	F	1	640.71	805.18	762.38	287.5	64.10
43	C	F	2	920.00	1046.34	579.99	287.5	53.87
43	C	F	3	642.14	836.06	655.57	193.7	59.26
43	C	F	4	713.84	1057.20	729.16	200	48.49
44	M	F	0	1849.09	2447.28	978.60	450	38.71
44	M	F	1	3988.23	4958.93	1970.38	750	27.65
44	M	F	2	4331.66	5375.60	1848.03	575	22.12
44	M	F	3	3992.66	5867.73	1471.87	662.5	27.65
44	M	F	4	3955.00	4958.93	1791.95	525	22.12
45	M	F	0	1709.47	2164.58	1510.95	700	59.26
45	M	F	1	1755.00	2436.69	1345.40	712.5	42.73
45	M	F	2	2010.66	2415.87	1215.99	650	43.10
45	M	F	3	2449.68	3009.56	1855.38	837.5	53.41
45	M	F	4	2977.33	4126.90	2022.69	962.5	48.49
46	M	NF	0	1502.72	1924.28	877.72	362.5	32.89
46	M	NF	1	4161.00	4329.57	2571.10	912.5	21.93
46	M	NF	2	5660.00	5375.60	3567.26	1000	16.59
46	M	NF	3	4873.12	5867.73	2548.54	862.5	16.59
46	M	NF	4	4590.62	5375.60	2037.32	812.5	11.06
47	D	NF	0	1268.75	1839.52	876.28	437.5	59.26
47	D	NF	1	4350.00	5090.58	2728.68	937.5	48.49
47	D	NF	2	5026.66	5090.58	3661.97	975	32.32
47	D	NF	3	2900.00	9482.88	3661.97	500	32.32
47	D	NF	4	1667.50	4559.16	1733.43	287.5	32.32

TABLE 1. Continued.

48	C	NF	0	1251.07	2266.09	886.87	387.5	44.24
48	C	NF	1	2495.41	3567.26	1791.95	662.5	33.18
48	C	NF	2	2825.00	4958.93	2184.69	750	27.65
48	C	NF	3	2825.00	5375.60	1331.05	625	22.12
48	C	NF	4	1900.00	1655.40	1310.91	500	21.93
49	D	F	0	614.11	716.24	783.82	225	64.65
49	D	F	1	1284.28	1958.04	1785.05	387.5	48.49
49	D	F	2	1408.57	2242.69	1243.81	425	48.49
49	D	F	3	859.26	1243.81	1138.31	250	59.26
49	D	F	4	580.00	691.21	513.14	162.5	64.65
50	C	F	0	2636.66	2906.67	1970.38	700	38.71
50	C	F	1	2165.83	3376.30	1688.60	575	22.12
50	C	F	2	2486.00	4291.59	1040.60	550	22.12
50	C	F	3	2378.94	4020.17	1552.32	500	27.65
50	C	F	4	2542.50	3204.27	1552.32	450	27.65
51	D	F	0	2112.85	2164.58	1138.31	637.5	48.49
51	D	F	1	1923.15	3880.86	2164.58	787.5	43.10
51	D	F	2	3351.11	4405.53	4126.91	650	32.32
51	D	F	3	1015.00	2242.69	579.99	175	43.10
51	D	F	4	0	0	0	0	0
52	D	NF	0	1265.60	1791.95	616.59	350	27.65
52	D	NF	1	3333.50	3567.26	1158.42	737.5	22.12
52	D	NF	2	3712.85	4601.54	2037.32	575	22.12
52	D	NF	3	2280.00	3232.62	1807.80	300	10.96
52	D	NF	4	0	0	0	0	0
53	D	F	0	994.28	1333.90	1189.17	450	70.04
53	D	F	1	1707.77	2091.41	2164.58	662.5	59.26
53	D	F	2	3712.00	4126.90	3289.34	800	37.71
53	D	F	3	1098.94	1897.09	965.28	225	43.10
53	D	F	4	0	0	0	0	0
54	C	F	0	1356.00	1791.95	1108.87	300	44.24
54	C	F	1	1243.00	1434.33	959.18	275	27.65
54	C	F	2	977.14	1168.67	622.04	150	27.41
54	C	F	3	678.00	736.32	627.49	150	27.65
54	C	F	4	0	0	0	0	0
55	D	NF	0	1450.00	1684.44	691.21	625	48.49
55	D	NF	1	2289.47	2728.68	1733.43	937.5	43.10
55	D	NF	2	2706.66	2850.78	1684.44	700	43.10
55	D	NF	3	3306.00	4405.53	2326.25	712.5	32.32
55	D	NF	4	1950.91	2850.78	2512.25	375	32.32
56	M	NF	0	1390.77	1552.32	517.72	400	22.12
56	M	NF	1	3277.00	4020.17	1688.60	725	22.12
56	M	NF	2	6215.00	3048.46	1907.40	1100	16.59
56	M	NF	3	4520.00	4291.59	2658.11	800	16.59
56	M	NF	4	3884.37	4020.17	2266.09	687.5	22.12
57	D	NF	0	1133.63	1551.28	632.95	537.5	53.88
57	D	NF	1	2204.00	3129.39	1189.17	950	43.10
57	D	NF	2	1400.48	3661.97	1958.04	1237.5	43.10
57	D	NF	3	2552.00	2788.45	1733.43	825	43.10
57	D	NF	4	2734.28	3289.34	2512.25	825	43.10

TABLE 1. Continued.

58	C	NF	0	1669.28	1987.82	1250.75	512.5	21.93
58	C	NF	1	1892.75	2266.09	1848.03	837.5	22.12
58	C	NF	2	2422.50	2932.39	1864.38	850	21.93
58	C	NF	3	2260.00	3376.30	2777.06	650	33.18
58	C	NF	4	2373.00	2777.06	1907.40	525	22.12
59	M	F	0	828.57	1366.39	783.82	375	48.49
59	M	F	1	1037.89	1163.29	667.21	425	48.49
59	M	F	2	934.44	1189.17	798.30	362.5	43.10
59	M	F	3	2242.66	2850.78	1785.05	725	43.10
59	M	F	4	4429.09	5090.58	2850.78	1050	48.49
60	C	F	0	887.85	1085.35	536.16	275	22.12
60	C	F	1	2219.64	2266.09	1970.38	687.5	16.59
60	C	F	2	3154.58	2906.67	1552.32	837.5	33.18
60	C	F	3	2919.16	3376.30	1511.16	775	27.65
60	C	F	4	3107.50	3204.27	2548.54	687.5	22.12

TABLE 2. Experimental design for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin". Blocks were of size two, each containing two muscles from the same animal. Experimental units (a single muscle) were randomly allocated to the treatments. Two replications of the basic incomplete block design were completed. M; monensin, D; digoxin, C; control, NF; non-fatigued papillary muscle, F; fatigued papillary muscle, rep 1; replication 1, rep 2; replication 2.

BLOCK	C-NF	D-NF	M-NF	C-F	D-F	M-F
REP1						
1		X	X			
2	X					X
3					X	X
4			X			X
5	X			X		
6			X		X	
7			X	X		
8	X				X	
9		X		X		
10				X		X
11	X		X			
12				X	X	
13	X	X				
14		X			X	
15		X				X
REP 2						
16			X			X
17					X	X
18	X		X			
19			X	X		
20	X X					
21		X			X	
22				X		X
23			X			X
24	X	X				
25				X	X	
26		X			X	
27				X	X	
28		X	X			
29	X	X				
30				X		X

TABLE 3. Anova of the contraction force of guinea-pig papillary muscles for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin".

Source	df	Type III F	Pr > F
Method	1	0.17	0.6802
Drug	2	5.72	0.0038
Drug by method	2	0.85	0.4268
Time	4	18.14	0.0001
Method by time	4	3.36	0.0107
Drug by time	8	11.65	0.0001
Drug by method by time	8	0.78	0.6223

TABLE 4. Anova of the initial contraction velocity of guinea-pig papillary muscles for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin".

Source	df	Type III F	Pr > F
Method	1	0.17	0.6800
Drug	2	5.96	0.0030
Drug by method	2	0.03	0.9745
Time	4	5.09	0.0006
Method by time	4	2.87	0.0242
Drug by time	8	3.76	0.0004
Drug by method by time	8	0.74	0.6550

TABLE 5. Anova of the average contraction velocity of guinea-pig papillary muscles for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin".

Source	df	Type III F	Pr > F
Method	1	0.52	0.4701
Drug	2	9.03	0.0002
Drug by method	2	0.04	0.9588
Time	4	10.92	0.0001
Method by time	4	3.50	0.0086
Drug by time	8	9.12	0.0001
Drug by method by time	8	0.57	0.8002

TABLE 6. Anova of the initial relaxation velocity of guinea-pig papillary muscles for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin".

Source	df	Type III F	Pr > F
Method	1	0.05	0.8156
Drug	2	3.95	0.0207
Drug by method	2	0.25	0.7753
Time	4	12.56	0.0001
Method by time	4	2.65	0.0341
Drug by time	8	5.50	0.0001
Drug by method by time	8	1.26	0.2666

TABLE 7. Anova of the stimulus to response time of guinea-pig papillary muscles for the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin".

Source	df	Type III F	Pr > F
Method	1	0.15	0.8285
Drug	2	3.27	0.0401
Drug by method	2	0.57	0.5648
Time	4	35.44	0.0001
Method by time	4	0.83	0.5092
Drug by time	8	7.11	0.0001
Drug by method by time	8	0.27	0.9740

TABLE 8. Contrasts that were used to test differences among the means in the manuscript entitled "Functional changes in isolated guinea-pig papillary muscle induced by monensin and digoxin". Difference between drug mean and control mean at T1, T2, T3 and T4 was compared to the difference between drug mean and control mean at T0 (equilibration). M; monensin, C; control, D; digoxin and T0, T1, T2, T3 and T4 are time 0, 1, 2, 3 and 4.

Variable	Contrast	Pr > T
Contraction force	M-T0 / C-T0 — M-T4 / C-T4	0.0005
	M-T0 / C-T0 — M-T3 / C-T3	0.0062
	M-T0 / C-T0 — M-T2 / C-T2	0.0199
	M-T0 / C-T0 — M-T1 / C-T1	0.0093
	D-T0 / C-T0 — D-T4 / C-T4	0.0004
	D-T0 / C-T0 — D-T3 / C-T3	0.0115
	D-T0 / C-T0 — D-T2 / C-T2	0.9929
	D-T0 / C-T0 — D-T1 / C-T1	0.0153
Initial contraction velocity	M-T0 / C-T0 — M-T4 / C-T4	0.0314
	M-T0 / C-T0 — M-T3 / C-T3	0.0774
	M-T0 / C-T0 — M-T2 / C-T2	0.3533
	M-T0 / C-T0 — M-T1 / C-T1	0.0937
	D-T0 / C-T0 — D-T4 / C-T4	0.1346
	D-T0 / C-T0 — D-T3 / C-T3	0.5622
	D-T0 / C-T0 — D-T2 / C-T2	0.9025
	D-T0 / C-T0 — D-T1 / C-T1	0.0268
Average contraction velocity	M-T0 / C-T0 — M-T4 / C-T4	0.0003
	M-T0 / C-T0 — M-T3 / C-T3	0.0056
	M-T0 / C-T0 — M-T2 / C-T2	0.0044
	M-T0 / C-T0 — M-T1 / C-T1	0.0142
	D-T0 / C-T0 — D-T4 / C-T4	0.0034
	D-T0 / C-T0 — D-T3 / C-T3	0.1171
	D-T0 / C-T0 — D-T2 / C-T2	0.5088
	D-T0 / C-T0 — D-T1 / C-T1	0.0356

Table 7. Continued.

Initial relaxation velocity	M-T0 / C-T0 --- M-T4 / C-T4	0.0160
	M-T0 / C-T0 --- M-T3 / C-T3	0.0110
	M-T0 / C-T0 --- M-T2 / C-T2	0.0248
	M-T0 / C-T0 --- M-T1 / C-T1	0.1405
	D-T0 / C-T0 --- D-T4 / C-T4	0.1875
	D-T0 / C-T0 --- D-T3 / C-T3	0.8631
	D-T0 / C-T0 --- D-T2 / C-T2	0.0015
	D-T0 / C-T0 --- D-T1 / C-T1	0.0732
Stimulus to response time	M-T0 / C-T0 --- M-T4 / C-T4	0.8279
	M-T0 / C-T0 --- M-T3 / C-T3	0.3437
	M-T0 / C-T0 --- M-T2 / C-T2	0.1591
	M-T0 / C-T0 --- M-T1 / C-T1	0.3510
	D-T0 / C-T0 --- D-T4 / C-T4	0.0001
	D-T0 / C-T0 --- D-T3 / C-T3	0.0001
	D-T0 / C-T0 --- D-T2 / C-T2	0.0008
	D-T0 / C-T0 --- D-T1 / C-T1	0.2561

TABLE 9. Anova of dose response experiment for monensin and digoxin in the manuscript entitled "Digoxin and monensin induced changes of intracellular Ca^{2+} concentration in isolated guinea-pig ventricular myocyte".

Source	df	SS	MS	F	Pr > F
TRT	6	11.51	1.92	10.87	0.0001
Error	14	2.47	0.17		
Corrected Total	20	13.98			

TABLE 10. Anova of "weakened" cell experiment in the manuscript entitled "Digoxin and monensin induced changes of intracellular Ca^{2+} concentration in isolated guinea-pig ventricular myocyte".

Source	df	SS	MS	F	Pr > F
TRT	2	9.29	4.64	17.63	0.0031
Error	6	1.58	0.26		
Corrected Total	8	10.87			

TABLE 11. Anova of Na^{+}-free experiment in the manuscript entitled "Digoxin and monensin induced changes of intracellular Ca^{2+} concentration in isolated guinea-pig ventricular myocyte".

Source	df	SS	MS	F	Pr > F
TRT	2	5.19	2.59	8.92	0.0160
Error	6	1.74	2.90		
Corrected Total	8	6.93			