INTRACELLULAR CALCIUM MOBILIZATION

IN CARDIOMYOCYTES OF RATS WITH EXPERIMENTAL DIABETES

by

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ABSTRACT

It is well known that alterations in intracellular calcium, $[Ca^{2+}]_i$, mobilization in the heart are among the major causes of cardiac dysfunction. To study the mechanism(s) involved in the development of diabetic cardiomyopathy, $[Ca^{2+}]_i$ (determined by fluorescence microscopy and Ca-indicators) and contraction of the cardiomyocytes (measured by a video-edge detector system) were studied in rats with experimental diabetes induced by streptozotocin (STZ). Ryanodine receptor (the calcium release channel) binding and sarcolemmal Na-Ca exchange activity were also investigated.

Cardiomyocytes were successfully isolated from rat hearts of diabetics and age-matched controls. The cell contractile function, determined by percentage of cell shortening, rate of shortening and relengthening (±dl/dt), and response to isoproterenol stimulation were significantly depressed in diabetic cells. These reflect similar features presented in intact diabetic heart and cardiac tissues.

The sarcoplasmic reticulum (SR) Ca^{2+} content assessed by rapid cooling contracture (RCC), caffeine contracture (CC), and caffeine-induced $[Ca^{2+}]_i$ transient was markedly reduced. Insulin treatment reversed depressed RCCs and caffeine-induced $[Ca^{2+}]_i$ transients, and also reversed the diabetes-induced depression of peak $-d[Ca^{2+}]_i/dt$ values which presumably are indications of depressed function of Na-Ca exchange.

Two binding sites for ryanodine were found in cardiac homogenates. The number of sites for high affinity and low capacity binding (B_{max}) were less in diabetic heart than in controls, while the binding affinity (K_d) was unchanged.

Alteration in SR calcium release channels may be involved in the SR dysfunction observed in diabetes.

Measurement of Na-Ca exchange activity in diabetic hearts showed a decreased V_{max} suggesting a reduced number of exchangers and a decreased K_m, an index of apparent affinity for calcium. These changes may occur as a result of the diabetes-induced membrane environmental change and/or adaptation.

In quiescent myocytes isoproterenol (1 μM) induced a sustained $[Ca^{2+}]_i$ decrease which was blocked by timolol (0.1 μM) and thapsigargin (1 μM) suggesting the SR Ca-ATPase was involved in this ß-adrenergic stimulated $[Ca^{2+}]_i$ response. In electrically-paced myocytes, isoproterenol caused a dose dependent increase of $[Ca^{2+}]_i$. The maximum response was depressed in diabetic cells. 8-bromo-cAMP caused a similar but less marked increase in $[Ca^{2+}]_i$ compared with isoproterenol. The maximum response to 8-bromo-cAMP was also decreased in diabetic cells. The data are in good agreement with the ß-adrenoceptor deficiency observed in diabetic hearts, and also suggest that post-receptor alterations occur in diabetes.

Paced diabetic myocytes showed a decreased maximum response but an increased sensitivity to ouabain stimulation. This suggests that diabetic cells are less tolerant to ouabain, which may be related to the depressed Na-pump activity and increased $[Na^+]_i$ in diabetic hearts.

ATP and KCl both caused an enhanced increase in $[Ca^{2+}]_i$ in diabetic myocytes (paced and quiescent, respectively). The exact mechanisms involved in the action of ATP is unknown although, like KCl, it activates the L-type Ca-channels. By using different blockers, $[Ca^{2+}]_i$ transients induced by KCl could be separated

into different components mediated by the L-type Ca-channel, Na-Ca exchange and SR release. An enhanced response to these agents might be related to the enhanced and altered Ca-channel activity reported in diabetic hearts.

In summary, diabetic cardiomyocytes provide a valuable model for studying cellular function of the diabetic heart. Myocytes from diabetic hearts retain the features of cardiac dysfunction seen in intact diabetic rat hearts. There appear to be multiple changes in the control of $[Ca^{2+}]_i$ in diabetic cardiomyopathy. The cardiac SR calcium content was reduced, which may consequently depress cardiac contraction. The $[Ca^{2+}]_i$ mobilization is altered in diabetes, notably a decreased response to isoproterenol and an enhanced sensitivity to ouabain. These observations provide some useful information about diabetic cardiomyopathy.

John H. McNeill, Ph.D.
Thesis Supervisor

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ATP	adenosine 5'-triphosphate
B_{max}	maximal number of binding sites
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine monophosphate
CC	caffeine contracture.
CON	control
DIA	diabetic
DCB	dichlorobenzamil
1,4-DHP	1,4-dihydropyridine
EC_{50}	the calculated concentration of an agonist that produces 50% of maximum effect
EGTA	ethyleneglyco bis(ß-aminoethylether)-N,N,N',N'-tetraacetic acid
E_m	membrane potential
FBS	fetal bovine serum
HEPES	N-2-hydroxyethylpiperazine-N'-2'ethanesulfonic acid
I_{Ca}	calcium current
i.h.	hypodermic injection
i.p.	intraperitoneal injection
IP_3	inositol 1,4,5,-triphosphate
ISO	isoproterenol
$[K^+]_i$	intracellular potassium
K_d	dissociation constant
K_m	Michaelis constant
K-pNPPase	K^+-stimulated p-nitrophenylphosphatase

M199	medium 199
MEM	minimum essential media
$[Mg^{2+}]_i$	intracellular magnesium
MOPS	3-(N-morpholino)-2-hydroxypropanesulfonic acid
ms	millisecond
n	number of observations
$[Na^+]_i$	intracellular sodium
p	statistical probability value
pD_2	negative log of the concentration of an agonist which produce 50% of maximum effect
RCC	rapid cooling contracture
SL	sarcolemma
SR	sarcoplasmic reticulum
STZ	streptozotocin
V_{max}	maximal velocity

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DEDICATION

To Jiansheng Zhao,

for his love and computer expertise

INTRODUCTION

I. $[Ca^{2+}]_i$ MOBILIZATION IN VENTRICULAR MYOCYTES

It is well known that the integrity of the circulatory system can be influenced by changes in the flux of intracellular calcium, $[Ca^{2+}]_i$, in heart. Calcium movements are closely related to cardiac electro-physiological events, contractile function, membrane integrity and energy metabolism. Maintaining the intracellular free calcium concentration requires a fine balance in the coordinated function of many organelles. Intracellular organelles which can incorporate calcium from and return calcium to the cytosol play a central role in determining $[Ca^{2+}]_i$ in the cell. In a mammalian ventricular cell, calcium moves across sarcolemma (SL), sarcoplasmic reticulum (SR), and mitocondrial membranes. SL contains Na-Ca exchangers, L-type calcium channels, Ca-ATPase, a calcium-leak pathway, and calcium binding sites. SR contains calcium release channels, Ca-ATPase and calcium binding sites. Mitochondria contain calcium uptake, Na-Ca exchange and Ca-binding proteins. The cytoplasm contains calmodulin, troponin C, and phosphate. It is assumed that the cytosolic concentration of calcium is uniform within the cell (Wier, 1990). Spatial distribution of the $[Ca^{2+}]_i$ transients is also uniform (Tamura et al., 1992).

During cardiac excitation, extracellular calcium enters into the cells through activated calcium channels and by means of reversed Na-Ca exchange (Noble, 1983; Sheu et al., 1986). This shift brings about a transient increase in sarcoplasmic calcium concentration which may directly activate contractile proteins, and induce release of stored calcium from SR (Fabiato, 1983). Calcium currents, I_{Ca}, may control the SR calcium release via a SR calcium release channel/ryanodine receptor/foot protein (Fleischer and Inui, 1989). Hence the transient calcium

increase originates from two sources, extracellular and intracellular. Contractile force is proportional to the amount of calcium released into the sarcoplasm from both sources (Yue, 1987). For relaxation to occur, calcium must be removed from the cytosol such that calcium will dissociate from troponin C. At least three processes are involved in removing calcium from the cytosol. Calcium may be transported either into the SR by SR Ca-ATPase or out of the cell by either the sarcolemmal Ca-ATPase or Na-Ca exchange. These three transport systems are thus in competition for cytoplasmic calcium. These processes act in concert both to regulate the intracellular calcium concentration and to modulate calcium dependent events (Janis et al., 1987).

It should be realized that the ability of the sarcolemmal membrane to maintain the intracellular free calcium concentration within specific limits is essential. Even a small imbalance would lead to progressive gains or losses of cellular calcium which could overload the cell (and SR) or deplete the cell (and SR) of calcium. The intracellular buffers are in a closed system and are consequently of finite capacity. In the steady state the calcium influx via I_{Ca} and Na-Ca exchange is balanced with the calcium extruded by Na-Ca exchange during a cardiac cycle (Wier, 1990).

A small leak of calcium out of the SR into the cytoplasm may be important at rest (Allen et al., 1976). Calcium may leak down its ≈10,000 -fold concentration gradient (≈1mM inside SR vs ≈100 nM in cytosol). A similar type of leak exists at the sarcolemma, since the electrochemical gradient between the extracellular space and cytoplasm is even greater than between the SR and cytoplasm (due to the negative membrane potential). The calcium leaked into the cytoplasm is then subject to the calcium transport system as described above (Bers, 1991). If the

same amount of calcium were re-accumulated by the SR there would be no net loss of SR calcium. However, if more of the calcium leaked by the SR is extruded from the cell by Na-Ca exchange or by sarcolemmal Ca-ATPase, it will represent a net loss of calcium from the SR and the cell. This is probably the basis for the phenomenon known as rest decay, or rest potentiation, that is, rat cardiac muscle exhibits larger post-rest contractions after longer rest intervals (Bers, 1985). Thus the resting muscle may not be at a true steady-state with respect to calcium influxes over a long time. It is believed that the calcium leak is not an important factor on the time scale of the physiologic $[Ca^{2+}]_i$ transient (Bers, 1991).

a. <u>Sarcolemma</u>

The surface sarcolemma is physically continuous with the membrane of the T-tubule and as such the two combine to form the permeability barrier of the myocytes. A major structural specialization of the sarcolemma is coupling with the SR by diads. In mammalian ventricular muscle 4-8% of the external sarcolemma and 20-50% of the T-tubular membrane are involved in junctional complexes (Page and Surdyk-Droske, 1979). Rat ventricles have a larger sarcolemmal fraction involved in SR junctions compared to rabbit ventricles. This structural observation may be related to physiological results which have suggested that the contraction of rat ventricle depends on SR calcium more strongly than does the contraction in ventricles of other species (Bers, 1987).

The two known mechanisms responsible for extrusion of calcium from cardiac myocytes are the sarcolemmal Ca-ATPase and Na-Ca exchange. Ca-ATPase transports one Ca^{2+} ion per ATP hydrolyzed and calcium extrusion by this pump appears coupled to proton influx (1Ca:1H) (Kuwayama, 1988). The turnover

rate of plasma membrane Ca-ATPase may approach ≈1,000/min with a $K_{m(Ca)}$ ≤1 μM (Schatzmann, 1989). Calmodulin had a profound effect on the $K_{m(Ca)}$ and V_{max}, while smaller effects were observed with cAMP-dependent protein kinase (Dixon and Haynes, 1989). It was reported that cAMP-dependent phosphorylation stimulated Ca-ATPase (≈3-fold) (Caroni and Carafoli, 1981). A $K_{m(ATP)}$ ≈30μM, $K_{m(Ca)}$ =0.3μM and V_{max} =31 nmol/mg protein/min in the presence of calmodulin was compared with a $K_{m(Ca)}$=11μM and V_{max} =10 nmol/mg protein/min in calmodulin depleted preparations (Caroni and Carafoli, 1981). While the sarcolemmal Ca-ATPase can have a high affinity for $[Ca^{2+}]_i$, the transport rate is too slow for it to be important to calcium fluxes during the cardiac cycle. It is suggested that the SL calcium ATPase plays a negligible role, both at high levels of $[Ca^{2+}]_i$ (calcium transients) and at low levels of $[Ca^{2+}]_i$ (diastole) (Dixon and Haynes, 1989).

L-type Ca-channels are the predominant type of calcium channel in the heart and have been found to be important for pacemaker depolarization. Blockade of the cardiac L-channel with dihydropyrodines or phenylalkylamines decreases, and can completely suppress, cardiac contractility (Janis et al., 1987). Cardiac I_{Ca} is rapidly activated by depolarization, reaching a peak in ≈2-7 ms depending on the temperature and E_m. Calcium entry via I_{Ca} would not normally be sufficient to activate cardiac muscle contraction (Fabiato, 1983). Variations on different species in calcium entry via I_{Ca} may generate 5-40% of maximal force (Bers, 1985, 1991, Fabiato, 1983). It is not entirely clear to what extent calcium influx via I_{Ca} participates in direct activation of the myofilaments (or to the induction of SR calcium release and/or reloading).

Calcium binding sites exist at the inner sarcolemmal surface (Bers, 1991). These sites possess a high affinity (K_d ≈10nM) at resting E_m and be almost fully saturated even at diastolic $[Ca^{2+}]_i$ levels (≈100 nM). There is no quantitative role for these inner sarcolemmal calcium binding sites in excitation-contraction coupling. When extracellular calcium is removed very quickly (<1 sec.) so that $[Ca^{2+}]_i$ and stores are not appreciably altered, depolarization does not produce measurable contraction or rise in $[Ca^{2+}]_i$ (Näbauer at al., 1989). Thus in the absence of calcium influx, depolarization does not lead to a significant calcium release from anywhere. In the end it would seem that the inner sarcolemmal sites may serve mainly as additional sites for intracellular calcium buffering (Bers, 1991).

Na-Ca exchange is the main means by which calcium is extruded from the cell, during both relaxation and diastole. Na-Ca exchange competes with the powerful SR Ca-ATPase for cytoplasmic calcium and thereby contributes to relaxation. The Na-Ca exchange can also mediate calcium influx sufficiently that contraction is activated, but this probably does not occur under normal physiological conditions (Reeves, 1990). Na-Ca exchange extrudes an amount of calcium responsible for ≈25% of developed tension in the presence of an intact SR (Bers and Bridge, 1989). In fact, Na-Ca exchange must extrude as much calcium as enters the cell via I_{Ca} in each cardiac cycle in order for a steady state to be achieved. With the use of cardiac sarcolemmal vesicle preparations it was established that the exchanger had a lower affinity for calcium than did the Ca-ATPase but had a greater transport capacity (≈30 times that of the Ca-ATPase) (Bridge and Bassingthwaighte, 1983). Reeves and Sutko (1983) found that calcium uptake into cardiac vesicles, driven by intravesicular Na, was competitively inhibited by extravesicular Na with a K_i for Na of 16 mM.

The stoichiometry of 3:1 (Na:Ca) is now generally accepted as the true stoichiometry of Na-Ca exchange in cardiac muscle (Reeves and Philipson, 1989). There is general agreement that the $K_{m(Na)}$ of the Na-Ca exchanger is ≈30 mM in sarcolemmal vesicles and in intact cardiac cells (Reeves and Philipson, 1989). There is less agreement about the $K_{m(Ca)}$. In isolated sarcolemmal vesicles the reported values of $K_{m(Ca)}$ range from 1.5-200 μM, although most values are in the 24-40 μM range (Reeves and Philipson, 1989). Estimates of $K_{m(Cai)}$ from intact cells have suggested values around 1μM (Miura and Kimura, 1989). The lower $K_{m(Cai)}$ in intact cells could be due to an effect of ATP, which has been shown to increase the $[Ca^{2+}]_i$ sensitivity of Na-Ca exchange (Reeves and Philipson, 1989). ATP-dependent stimulation is thought to be due to regulatory phosphorylation of the exchanger (Reeves, 1990).

Several physiological ligands and experimental procedures are known to influence Na-Ca exchange activity. They include nucleotides, intracellular calcium and magnesium, hydrogen ions, monovalent cations, orthophosphate, redox potential and modification of the lipid and protein compositions of the plasma membrane (Reeves 1990; DiPolo and Beaugé, 1990). The two major *in vivo* modulators are ATP and intracellular ionized calcium. ATP increases the apparent affinity of the transporting sites for external sodium and internal calcium without changes in the maximal rate of transport (DiPolo and Beaugé, 1986). The fact that V_{max} remains unchanged indicates that a direct coupling of ATP hydrolysis with transport is unlikely. On the other hand, there is indirect evidence suggesting that phosphorylation of either the carrier or other structures is required. The evidence relates to the need for magnesium and to selectivity. As is the case for most phosphorylating reactions, magnesium ions are also essential for ATP stimulation of the Na-Ca exchange. Stimulation by ATP is highly selective because other

phosphate compounds (GTP, GDP, cAMP, ADP, AMP, etc.) are ineffective (DiPolo and Beaugé, 1991). It was suggested that Na-Ca exchange was activated by a Ca-calmodulin-dependent phosphorylation in heart sarcolemma. The activation process increased the affinity of the exchanger (K_m decreased from approximately 10 μM to 2μM). (Caroni and Carafoli, 1983).

The Na-Ca exchanger can be inhibited by a number of drugs (Bers, 1991), but none of these agents is very potent and specific as an inhibitor. In addition even the most potent inhibitors among them (e.g. dichlorobenzamil K_i =4-17 μM, Garcia et al., 1988) are not very selective. They inhibit other ion transport systems and channels at even lower concentrations than those required to inhibit Na-Ca exchange. Dichlorobenzamil inhibits sarcolemmal Ca-channels, and related amiloride derivatives are potent inhibitors of Na-H exchange, Na-coupled sugar and amino acid transport, Na, K, and Ca-channels, and cholinergic and adrenergic receptors (Kaczorowski, 1986; Bers, 1991).

Na-Ca exchange has the capacity to transport a large amount of calcium. In sarcolemmal vesicles, calcium transport rates of ≈25 nmol/mg protein/sec are typically observed (Reeves and Philipson, 1989). This is more than enough in 1 sec to activate or relax a substantial contraction (Bers, 1991). It now appears that calcium entry via Na-Ca exchange can contribute quantitatively to the direct activation of the myofilaments.

b. Sarcoplasmic Reticulum

SR is an entirely intracellular membrane bound compartment which is not continuous with the sarcolemma. The main function of this organelle is to sequester

and release calcium to the myoplasm. The vast majority of the surface of the SR is likely to function primarily to remove calcium from the cytosol (Bers, 1991).

The SR has a well characterized Ca-ATPase which is the main route by which calcium is accumulated by the SR (Schatzmann, 1989). Calcium is released from SR into the cytoplasm via the SR Ca-release channel. This channel also appears to be the ryanodine receptor and the "foot protein" (Block et al., 1988). The SR Ca-ATPase has a high affinity for calcium (K_m of 1-5 μM) and a high V_{max}, relative to that of sarcolemmal Ca-ATPase (Levitsky et al., 1981). ß-adrenergic activation speeds the decline of the calcium signal in ventricular muscle by the cAMP-dependent phosphorylation of phospholamban (Kurihara and Konishi, 1987). This phosphorylation stimulates the pump by lowering the $K_{m(Ca)}$ 3-5 fold (Kranias and Solaro, 1982) and appears to be the main means by which ß-adrenergic agonists accelerate relaxation in the heart. It may also be supposed that catecholamines will bias the competition between the SR Ca-ATPase and the sarcolemmal Na-Ca exchange in favor of the former resulting in limited calcium extrusion from the cell via the exchanger. In combination with the potent stimulation of sarcolemmal I_{Ca} by ß-adrenergic agonists, this increase in SR calcium-pumping results in a substantial increase in SR calcium content available for release. Phospholamban can also be phosphorylated by calcium-calmodulin dependent protein kinase (Simmerman et al., 1986) and protein kinase C (Iwasa and Hosey, 1984). These kinases can increase calcium pumping but it is not clear whether phosphorylation by these enzymes is physiologically important (Carafoli, 1987; Bers, 1991).

The SR Ca-ATPase is sensitive to pH. Acidosis associated with ischemia depresses the rate of SR calcium-pumping and thus slows relaxation. Acidosis can

also reduce the calcium sensitivity of the myofilaments, calcium current, and Na-Ca exchange (Philipson, 1990).

Most of the calcium in the SR is probably bound such that the free $[Ca^{2+}]_i$ in the SR is only ≈1 mM (Carafoli, 1987). The intra-SR $[Ca^{2+}]$ is buffered to a significant extent by calsequestrin, an acidic Ca-binding protein primarily localized in the terminal cisternae of the SR (MacLennan and Wang, 1971) with a low affinity (K_m ≈0.1-1.0 mM) and high capacity for calcium (0.3-0.9 μmol/mg, Slupsky et al., 1987). Other SR calcium binding proteins include calreticulin (Fliegel et al., 1989), 170 kDa histidine-rich calcium binding protein (Hofmann et al., 1989), gp160 (sarcalumenin) and gp53 which is a major glycoprotein of SR (Campbell and Maclennan, 1981).

The permeability of the SR membrane to ions is very high. High conductance K- and anion-selective channels exist in the SR membrane (Hals et al., 1989). There do not appear to be any appreciable concentration gradients of monovalent ions between the inside of the SR and the cytosol (Somlyo and Somlyo, 1990). This implies that there is no membrane potential between the cytosol and the interior of the SR, which allows calcium release to proceed rapidly with monovalent fluxes compensating quickly for the divalent charges leaving the SR.

High concentrations of Ins(1,4,5)P_3 can induce calcium release from SR but the rate and extent was much lower than that for Ca^{2+}-induced Ca^{2+}-release (Fabiato, 1986). These effects of IP_3 may be physiologically important in the modulation of Ca^{2+}-induced Ca^{2+}-release from the SR. Photolysis of "caged Ca^{2+}" and "caged IP_3" are known to activate contraction in skinned rat ventricular muscle (Kentish et al., 1990). Activation of cardiac α_1-adrenergic and of muscarinic

receptors have also been reported to increase IP_3 production as well as contractile force (Gilmour and Zipes, 1985; Scholz et al., 1988).

c. Mitochondria

About 35% of the volume of mammalian ventricular muscle cells is occupied by mitochondria (Page, 1978). Mitochondria are the site of oxidative phosphorylation and the tricarboxylic acid cycle. The large mitochondrial content in mammalian ventricle meets the high demands for energy supplied by aerobic metabolism.

Calcium is continuously cycling across the inner mitochondrial membrane. This cycling is due to the presence of calcium efflux pathways and calcium uptake pathways. Maximal uptake of calcium by mitochondria in the absence of phosphate was approximately 100 nmol/mg mitochondial protein (Carafoli, 1987). The K_m of the uptake process was approximately 10 μM with V_{max} for calcium uptake to be approximately 0.5 μmol of calcium per minute per milligram of heart mitochondial protein.

Magnesium is a potent inhibitor of calcium uptake in heart mitochondia. This inhibition of calcium uptake occurs at magnesium concentrations which approximate those present in the normal cell suggesting that magnesium may play an important regulatory role in mitochondial calcium uptake in vivo (Carafoli, 1987). Ruthenium red and La^{3+} also inhibit the uptake. Under normal physiological conditions the calcium uptake pathway is inhibited at the normal cellular magnesium concentration. This, together with the relatively slow mitochondial Na-Ca exchange process, results in a slow calcium cycling across the mitochondria. This is not likely to contribute in a

major way to cytosolic calcium homeostasis. It has been proposed that a primary function of the mitochondrial calcium transport processes is to allow intramitochondrial matrix [Ca^{2+}] to follow that of the cytosol in order to modulate oxidative metabolism. Increase in cytosolic $[Ca^{2+}]_i$ under physiological or pathophysiological states would result in increased mitochondria matrix [Ca^{2+}], enhanced dehydrogenase activity, and correlation of the matrix free calcium with metabolic flux in the tricarboxylic acid cycle. Mitochondrial calcium transport is important in coping with cellular calcium overload (Crompton, 1985). When there is a slower increase in cytoplasmic $[Ca^{2+}]_i$ the mitochondrial calcium transport may play a critical role in increasing metabolism to meet increased metabolic demands. Severe cellular calcium overload, e. g. resulting from ischemia, may result in a massive amount of calcium accumulation in mitochondria to protect the cytosol from very high calcium levels.

The main route of calcium extrusion from the mitochondria is via electroneutral (i.e. 2:1) Na-Ca exchange (or antiport, Crompton et al., 1978). The mitochondrial Na-Ca antiporter is particularly active in heart. The $[Na^+]_i$ dependence of this Na-Ca antiporter is sigmoidal with half-maximal calcium extrusion at ≈5-8 mM Na which means that this system will be quite sensitive to changes of intracellular sodium in the physiological range (Crompton, 1985). The K_m for calcium on the matrix side of the membrane has been found to be 3-6 μM. Diltiazem and nifedipine inhibit this exchanger at high concentrations. There is also a Na-independent extrusion of calcium from mitochondia which is less prominent in the heart (Crompton et al, 1978). In general, tissues which exhibit high rates of Na-independent calcium extrusion are those which have low activities of Na-Ca exchange. No inhibitors of Na-independent calcium transport have been found (Crompton, 1985). The inner mitochondial membrane also has an active Na-H

exchange system which may be the means by which Na is extruded from the matrix and also completes the cycle (Carafoli, 1987). In this way the energy for calcium extrusion via Na-Ca exchange depends also on the proton movement during respiration and the consequently negative intramitochondrial potential. Thus the true energy source is respiration and the proton motive force it generates.

Isolated mitochondria can accumulate very large amounts of calcium. In intact cardiac muscle, when both the SR Ca-uptake and sarcolemmal Na-Ca exchange were inhibited, relaxation was slowed by more than an order of magnitude and was often incomplete (Bers, 1991). Thus, it is unlikely that mitochondria can compete effectively with the SR Ca-ATPase and sarcolemmal Na-Ca exchange under physiological conditions and probably do not contribute quantitatively to normal cardiac relaxation (Bers and Bridge, 1989)

d. Myofilaments

The myofilaments are the end effector of excitation-contraction coupling. They are responsible for transducing chemical energy into mechanical energy and work. The myofilaments occupy about 50% of the cell volume in mammalian ventricular myocardium. They are composed of the thick (or myosin) and thin (actin) filaments as well as associated contractile and cytoskeletal components (Swynghedauw, 1986).

When $[Ca^{2+}]_i$ increases, the myofilaments are activated in a Ca-dependent manner, thereby transducing the chemical signal and chemical energy (ATP) into mechanical force. The force of contraction is varied in large part by changes in the peak $[Ca^{2+}]_i$ reached during systole (Bers, 1991).

Cardiac troponin C has two Ca-specific binding sites ($K_{d(Ca)}$ ≈200-500 nM) which are responsible for regulating contraction, and two Ca-Mg sites at which calcium and magnesium bind competitively ($K_{d(Ca)}$ ≈2nM, $K_{d(Mg)}$ ≈25 μM) (Zot and Potter, 1987). If resting $[Ca^{2+}]_i$ = 100nM and $[Mg^{2+}]$ =1mM, the cardiac Ca-Mg sites would be ≈97% saturated (90% with calcium and 7% with magnesium). Thus these sites would always be nearly saturated. The Ca-specific site thus would be expected to respond to $[Ca^{2+}]_i$ changes which are likely to occur physiologically. The affinity to calcium was reduced ≈3-4 fold by adding ATP (Pan and Solaro, 1987; Swynghedauw, 1986)

Cardiac muscle exhibits different isoforms of several of the contractile proteins (myosin and myosin light chains) and can modulate the expression of various cardiac isoforms in vivo (Swynghedauw, 1986). For example, there are two main isoforms of the cardiac myosin heavy chain (α, ß), referred to as fast (α) and slow (ß) based on the myosin ATPase rate or shortening rate. Three different dimers can form (αα, or V_1, αß or V_2, and ßß or V_3). Adaptational effects (e.g. during hypertrophy) can shift rat ventricle to V_3 form and thyroid hormone can induce a switch from ß- to α- myosin heavy chain production (Swynghedauw, 1986).

In skinned cardiac muscle preparations, cooling, acidosis and shorter sarcomere length decrease the calcium sensitivity and the maximum force generated by the myofilaments. Acidosis may be particularly important during pathological conditions such as hypoxia or ischemia where intracellular pH (pH_i) is known to decline. While decreasing [ATP] as (Mg-ATP) increases myofilament sensitivity, it also decreases the maximum force (Best et al., 1977). The increase in PO_4 which accompanies the decline in high energy phosphate (ATP and creatine

phosphate) during ischemia can dramatically decrease both calcium sensitivity and maximum force (Kentish, 1986). The overall effect seems to be that the increase in PO_4 and, to a somewhat lesser extent, the decrease in pH_i during ischemia combine to produce a dramatic decrease in myofilament calcium sensitivity, which plays an important role in the early decline in mechanical function (Kentish, 1986).

Increase of the intracellular free magnesium also decreases myofilament calcium sensitivity (Best et al., 1977). Increased ionic strength (but not osmolarity) decreases both calcium sensitivity and maximum force of the myofilaments (Kentish, 1984). Imidazoles, which are naturally occurring in cardiac muscle cells, increase myofilament calcium sensitivity (Harrison et al., 1986; O'Dowd et al., 1988). These compounds are chemically related to caffeine, which can deplete the SR of calcium. However caffeine and other methylxanthines are potent myofilament calcium sensitizers. These compounds (e.g. 3-isobutyl-1-methylxanthine) are also phosphodiesterase inhibitors, but the effect on the myofilaments is not mediated by cyclic AMP (Wendt and Stephenson, 1983; Harrison et al. 1986).

In response to ß-adrenergic stimulation, a cyclic AMP dependent phosphorylation of cardiac troponin I occurs. This phosphorylation decreases myofilament sensitivity to calcium in intact ventricular muscle (Okazaki et al., 1990). Phosphorylation of troponin I (and shift in calcium sensitivity) could also be reversed by cyclic GMP or cholinergic agonists (Horowits and Winegrad, 1983). One of the myosin light chains (regulatory light chain) can also be phosphorylated by a Ca-calmodulin dependent protein kinase. The physiological role of this phosphorylation in cardiac muscle is not clear (England, 1984). Taken together, the control of the myofilaments by $[Ca^{2+}]_i$ is complicated and the relationship between $[Ca^{2+}]_i$ and the contraction cycle is not fixed.

e. Rest Potentiation and Post-rest Decay in Rat Myocardium

In rat myocardium, the resting $[Na^+]_i$ activity is high (aNa_i 12.7mM) (Shattock and Bers, 1989). The $E_{Na/Ca}$ would be near the resting membrane potential. A relatively small increase in aNa_i can have a large impact on the balance of calcium influxes mediated by Na-Ca exchange. During rest the high aNa_i favours calcium entry via Na-Ca exchange (Shattock and Bers, 1989). The calcium content of the SR can be gradually gained by sarcolemmal Na-Ca exchange during rest (Bers, 1985). During a potentiated post-rest contraction a large amount of calcium is released from SR and when $[Ca^{2+}]_i$ is high, $E_{Na/Ca}$ is positive to E_m so that there is a large driving force favoring calcium extrusion via Na-Ca exchange. In this way the competition between the SR Ca-ATPase and the Na-Ca exchanger is biased toward the latter and net calcium efflux occurs during the pulse. The SR calcium content will be lower at the next contraction until a new steady state is achieved, where calcium influx and efflux must be the same over a complete cardiac cycle (Bers, 1987).

II. DIABETIC CARDIOMYOPATHY

a. Cardiac Dysfunction in STZ Diabetic Rats

Diabetes mellitus has been associated with both clinical and experimental cardiac dysfunction. It has been suggested that there exists a cardiac disease peculiar to diabetes, resulting from a combination of microangiopathy, macroangiopathy, neuropathy and metabolic cardiomyopathy. This 'diabetic cardiomyopathy' has been characterized as myocardial failure independent of

hypertension or atherosclerostic coronary artery disease and has been strongly suggested by epidemiological, clinical and experimental research (Kannel 1978, Regan et al., 1977; Fein et al., 1980; Dhalla et al., 1985; McNeill and Tahiliani, 1986).

A major cardiac complication in diabetic patients with or without myocardial infarction consists of decreased contractility of the left ventricle (Jaffe et al., 1984; Smith et al., 1984). It has also been suggested that diabetic cardiomyopathy is characterized by both left ventricular systolic and diastolic dysfunctions without evidence of ongoing microvascular ischemia (Regan et al., 1977; Shapiro et al., 1980). The mechanisms involved in this cardiomyopathy are not well defined. Considerable data are presently available with regard to the metabolism, mechanics and function of heart preparations from experimental diabetic animals.

Rats made diabetic with STZ displayed depressed ventricular contractility, diminished ventricular compliance and generally decreased inotropic and chronotropic responses to certain drugs in an isolated cardiac preparation (Penpargkul et al., 1980; Fein et al., 1980; Vadlamudi and McNeill, 1983; Yu and McNeill, 1991a). Since the rat usually does not develop atherosclerosis, the *in vivo*/*in vitro* abnormalities in contractility and metabolism reflect changes in the function of the myocardial cell (Baandrup et al., 1981). Cardiac contractility and relaxation (represented by maximal rates of ventricular pressure development (+dP/dt), and decline (-dP/dt), respectively), and peak ventricular pressure development, were all reduced in hearts isolated from STZ-treated rats (Heyliger et al., 1982; Vadlamudi and McNeill, 1983; Rodgers, 1986). Similar changes in contractile properties of the heart have also been found *in vivo* in both anaesthetised (Dowell et al., 1986) and in conscious (Carbonell et al., 1987; Litwin

et al., 1990) STZ-treated rats. Isolated muscles preparations from diabetic hearts also showed delayed relaxation, slowed relaxation rate and increased peak relaxation time (Fein et al., 1980; Heyliger et al., 1982).

Diabetic rats treated with insulin, by both 'prevention' and 'reversal' regimens (Tahiliani et al., 1983), exhibited normal myocardial function while the untreated diabetic showed depressed function. Altered papillary muscle mechanics seen in diabetic rats can also be restored by insulin treatment (Fein et al., 1980). A metabolically determined abnormality in contraction thus seems to exist in experimental diabetes. The increased dependence on lipid metabolism with its possible consequences (changes in membrane integrity and intracellular calcium homeostasis) could serve as an explanation for this myocardial dysfunction (Katz and Messineo, 1981; Rodrigues and McNeill, 1986).

STZ induction of diabetes in rats is the most commonly used model in diabetic studies. STZ contains an N-methylnitrosourea moiety and is an alkylating agent. It is suggested that STZ initiates its cytotoxic action by alkylating effects (Fischer, 1985). The glucose moiety in the STZ molecule may have a specific affinity for the pancreatic ß-cell, because STZ concentrates in pancreatic islets (Fischer, 1985). Some evidence indicates that oxygen-free radicals may also be involved in STZ induced diabetes (Wilson et al., 1984; Fischer 1985; Sandler et al., 1983). STZ selectively destroys pancreatic ß-cells and produces a diabetic state, the severity of which can be varied by altering the dose of STZ. With time, e.g. 4-6 weeks after injection, rats so treated develop biochemical and functional myocardial abnormalities which are the result of the drug-induced metabolic changes rather than a direct effect of the drug itself (McNeill and Tahiliani, 1986).

b. Diabetes and Altered Calcium Mobilization in Heart

Evidence for abnormal myocardial cell functions in diabetes mellitus, influenced by metabolic changes, has appeared within recent years. Myocardial enzyme systems and subcellular organelles are affected. Experimental studies at the cellular level have provided data for several possible explanations. These are concerned with intracellular calcium homeostasis and trans-sarcolemmal receptor signalling (Dhalla et al., 1985; Tahiliani and McNeill, 1986).

There is evidence suggesting that intracellular calcium homeostasis is altered in the heart in chronic diabetes. The interactions of calcium with various subcellular organelles such as sarcolemma (SL), mitochondria, sarcoplasmic reticulum (SR) and contractile proteins are disturbed (McNeill and Tahiliani, 1986; Lopaschuk et al., 1983). SR, which acts as a calcium store and which takes up and releases calcium on a beat-to-beat basis, is impaired (Lopaschuk et al., 1983) in diabetes. Sarcolemmal calcium pump activity is also depressed (Heyliger et al., 1987; Tahiliani and McNeill 1986). The Na-Ca exchange activity in the SL is depressed (Makino et al., 1987), thus the efflux of calcium through SL is decreased. Mitochondrial abnormalities have also been reported (Dhalla et al., 1985).

Cardiac ß-adrenoceptor function in the diabetic animals is affected in terms of depressed ß-receptor number and response to agonist stimulation (Ramanadham and Tenner, 1987; Heyliger et al., 1982; Götzsche 1983). Study of the contractile function of isolated heart and cardiac tissue preparations from diabetic rats has revealed a significant alteration in the response and sensitivity to perfusate calcium and ß-adrenergic stimulation (Borda et al., 1988; Yu and McNeill, 1991a). Myocardial calcium flux in hearts from diabetic animals was found to be less

responsive to ß-adrenergic agonists than control preparations and thus it was concluded that membrane defects may alter calcium movements (Götzsche, 1983).

These results collectively support the hypothesis that diabetes mellitus leads to an alteration in calcium movements in the heart. Chronic insulin deficiency may cause a defect in the cell membrane and consequently intracellular calcium mobilization is altered. Until now there have been only a few direct measurements of intracellular calcium changes in heart or other cells from diabetic animals (Schaffer et al., 1989; Noda et al., 1992).

III. METHODOLOGY IN THE PRESENT STUDY

a. Isolation of Cardiomyocytes

The ability to isolate living cells from adult heart tissue has been a major prerequisite for cellular studies of myocardial functions such as intracellular calcium homeostasis, cell contractility, receptor properties, intracellular signalling mechanisms and excitation-contraction coupling. The advantage of achieving a primary cell digest is that the cells are fully differentiated and morphologically similar to cells in intact heart, but lack interstitial tissue and contaminating cell types which can complicate measurements in whole tissues.

The successful isolation of myocytes requires a period of exposure to low extracellular calcium concentration. The low calcium perfusion used to prepare viable myocytes must be limited to a certain time to avoid the calcium paradox in which cardiomyocytes become abnormally sensitive to physiological concentrations of calcium on subsequent re-exposure. This is characterized by an increase in the

permeability of the myocytes to calcium and quick initiation of hypercontracture on re-exposure to millimolar concentration of calcium. Quiescent myocytes, which respond to an electrical stimulation with a discreet contraction, are generally considered to be representative of healthy muscle. In this study, successful myocyte isolation techniques have been developed to minimize the calcium paradox while achieving the separation of cells with high viability (see Methods for details).

b. Measurement of [Ca2+]i by Fluorescent Indicators and Fluorescence Microscopy

Fluorescent Ca-indicators have been shown to be valuable in the measurement of $[Ca^{2+}]_i$. The use of Fura-2 and Indo-1 has been reported in many cell types including isolated myocytes. Fura-2 and Indo-1 are fluorescent derivatives of the commonly used chelator EGTA. Like EGTA, they have much higher affinities for calcium than for magnesium. Unlike EGTA and EDTA, the affinities of these compounds for calcium and magnesium are not very sensitive to changes in pH within the physiological range (Tsien, 1983). They differ significantly in the nature of their excitation and emission spectra and in the way in which those spectra are altered by the binding of calcium. They emit visible light and are best excited by ultraviolet light. They undergo a shift in either the excitation spectrum (fura-2) or the emission spectrum (indo-1), and this permits the dyes to be used in the "ratio-mode", which is a major advantage when it comes to calibrating dye signals (Grynkiewicz et al., 1985). Ratio-mode recording has the advantage that the concentration of dye and the depth of the specimen may be corrected in order to obtain a calibration (Tsien, 1983).

Fura-2 alters its excitation spectrum on binding calcium, but not its emission spectrum. The fluorescence intensity at the peak of the emission spectrum (≈510 nm) is measured while two excitation wavelengths are switched back and forth so that when calcium binds, the fluorescence intensity at one excitation wavelength falls while the other rises. From the ratio of the emission signals recorded at the two excitation wavelengths, one can calculate the calcium concentration. In the case of indo-1, a similar approach can be used except that a single excitation wavelength is used, and the fluorescence emission is recorded at two wavelengths. In principle, one would expect ratio-mode recording with dyes such as indo-1, which change their emission spectrum, to be easier than it is with those such as fura-2, because physically it is easier to record simultaneously at two different emission wavelengths than to alternate between two excitation wavelengths. The temporal resolution may also be limited by the speed of chopper which is required for the dual wavelength excitation. However, the emission spectrum of fura-2 is better separated from the fluorescence of intrinsic components of tissues and ratio-mode imaging is easier with fura-2 than indo-1. The signals from fura-2 and indo-1 have a longer duration than those of aequorin, therefore they are better for measuring calcium transients in muscle cells (Blinks, 1992).

The use of fluorescent indicator dyes for the measurement of $[Ca^{2+}]_i$ has increased our knowledge of cellular $[Ca^{2+}]_i$ regulation. With fluorescent dyes one may pursue studies on the following:

1. Basal $[Ca^{2+}]_i$ and the processes responsible for maintaining a steady state. Various experimental methods can be used to investigate these processes.

2. $[Ca^{2+}]_i$ response to Ca^{2+}-mobilizing stimuli. This is considered to be a major use of the dyes. The mechanism by which stimulation causes $[Ca^{2+}]_i$ change can be analyzed. For instance, by comparing the differences in agonist-induced rises in $[Ca^{2+}]_i$ in the presence and absence of external calcium, the role of calcium entry in the action of the agonist can be determined. The effects of other intracellular messengers on calcium transport can be studied, for example, by adding phorbol esters to stimulate protein kinase C or by applying agents which raise cAMP levels.

3. The restoration of $[Ca^{2+}]_i$ following stimulation-induced elevation. This is especially useful with rapidly desensitizing agonists which can produce a brief increase in calcium in the cytosol.

There is also some question about what the normal resting $[Ca^{2+}]_i$ is in cardiac muscle and how high $[Ca^{2+}]_i$ becomes during a normal contraction. This is largely due to questions about the *in vivo* calibration of the $[Ca^{2+}]_i$ indicators which have been used. Calcium microelectrodes can overestimate $[Ca^{2+}]_i$ because of imperfect impalements whereas fura-2 and indo-1 underestimate $[Ca^{2+}]_i$ because of binding of the indicator to intracellular constituents (Konishi et al., 1988). The situation is even more problematic when cells are loaded with Ca-indicators by incubation with their acetoxymethylester (AM) forms (rather than direct intracellular application of the free acid form). In this case, the cellular signals are complicated by the fluorescence of compartmentalization of the indicator (e.g. ≈50% may be trapped in the mitochondria, Spurgeon et al., 1990) and partial de-esterification. These questions place limitations on the quantitative detail with which we can currently understand intracellular calcium regulation. Therefore, possible problems in this present study will be:

1. Incomplete hydrolysis of dye ester bonds by cytosolic esterases.
2. Sequestration of dye in non-cytosolic compartments.
3. Dye loss, either active or passive, from loaded cells.

Solutions to these problems have been suggested (Wier, 1990):

1. Loading of cells with the dye's acid or salt form (by microinjection or ATP permeabilization or during isolation when gap junctions are open).
2. Loading the cells at low temperature or after chilling at 4°C to prevent intracellular organelle sequestration.
3. Performing experiments at lower (15-33°C) temperature and using an organic anion transport inhibitor, sach as probenecid, and ratio quantitation to remedy inaccuracies caused by dye leakage.

In order to obtain accurate results in this study, loading and experiments were performed at room temperature (23°C), and ratio quantitation was used in the fluorescence microscopy.

c. Measurement of Myocyte Contraction

Contraction of isolated myocytes is the characteristic that establishes the cells as the smallest functional units of the heart. Optical strategies for measuring contraction of isolated myocytes include laser diffraction, direct visual imaging, and photo diode array 'edge detectors'. Maximum shortening velocity for cardiomyocytes is 90-100 micron/sec. (measured with feline myocyte, Duthinh and Houser, 1988), or 10-12 micron/sec. for sarcomere shortening (Krueger et al., 1980; Tarr et al.,

1981). The peak shortening, equal to 10-20% of cell length, occurs in approximately 200 ms (Duthinh and Houser, 1988; Krueger et al., 1980; Tarr et al., 1981). In this study a video-based edge detector was used to noninvasively measure the extent and rate of shortening of isolated cardiac myocytes. The parallel orientation of the myocyte and the nature of the scan sweep allow the CCD (charge coupled device) camera to record the length of the cell with a time resolution of 16.7 ms (60 Hz). The high spatial resolution of the video-edge detector enables myocyte length to be determined with an error of ±0.5 μm (Steadman et al., 1988) thus temporal resolution of the edge-detector represents the only limitation on accurate analysis of myocyte shortening. To overcome this problem and ensure adequate sampling during cell contraction, values were obtained by averaging consecutive signals stored on the PTI (Deltascan-PTI, Photo Technology International Inc.) program.

Isometric tension and isotonic shortening velocity increase in parallel as intracellular calcium increases (Peeters et al., 1987). Thus the extent and rate of the shortening can provide a relative indication of the peak concentration of $[Ca^{2+}]_i$ that is producing the twitch. It should be possible to use the edge detector in conjunction with an intracellular calcium indicator to extend our understanding of the relationship between shortening and calcium transients (Steadman et al., 1988).

d. Other Measurements

Ryanodine, a specific ligand for SR calcium release channel, is known to be a very useful tool in elucidating SR function and SR release channel characteristics. It either activates or inhibits the calcium release channel in cardiac SR, depending on the concentration (Meissner, 1986). An SR ryanodine binding assay was carried out in diabetic and control cardiac SR. SR is the major organelle controlling the calcium

transient in the mammalian cardiac cell. In diabetic hearts ATP-dependent SR calcium uptake has been reported to be depressed (Lopaschuk et al., 1983) and SR releasable calcium is decreased in papillary muscles (Bouchard and Bose, 1990). However, the calcium release characteristics in diabetic heart are unknown. Since the SR ryanodine receptor is a calcium release channel as well as a component intimately involved in transverse tubule-SR communication, abnormalities in the cardiac muscle ryanodine receptor may be responsible for the abnormal SR calcium release and contractile properties. The measurement of ryanodine binding sites and affinity in diabetic heart may provide information on the change in handling of calcium by SR. The binding procedure was carried out according to the principle of radio ligand binding assays (see Methods for details).

Na-Ca exchange activity was measured in highly purified sarcolemmal membrane fractions. The enzymatic activity of K-pNPPase, which is a marker of sarcolemmal membrane fraction, was measured to estimate the membrane purification index and percentage of sarcolemma recovery. The assay was carried out as a Na-dependent ^{45}Ca uptake, i.e. ^{45}Ca uptake into Na-preloaded sarcolemmal vesicles as a function of calcium concentration. The uptake was ended at a time point in the linear uptake range. This *in vitro* measurement provided the information on calcium affinity and densities of sarcolemmal Na-Ca exchange in diabetic myocardium (see Method for details).

IV. THESIS OBJECTIVE

Experiments were designed to study the control of $[Ca^{2+}]_i$ in diabetic rat myocytes. The elucidation of the mechanism(s) of many biological processes has been accelerated by the use of pharmacological agents that selectively affect

specific steps of the process. Therefore, cardiac drugs, with different mechanism(s) of action, that influence calcium mobility were chosen to investigate the responses of $[Ca^{2+}]_i$ in isolated cardiomyocytes. The following were the specific objectives of this study:

1. $[Ca^{2+}]_i$ was measured in single cells by fluorescence microscopy using fura-2 and indo-1 as calcium indicators.

2. The extent and rate of myocyte shortening were measured by means of phase-contrast microscope video-edge detector system.

3. Isoproterenol effects, expressed by maximum response and sensitivity, on $[Ca^{2+}]_i$ and cell contractili function, were measured.

4. Other stimulants of $[Ca^{2+}]_i$ mobilization including caffeine, 8-bromo-cAMP, ouabain, ATP and KCl along with different blockers were also studied.

5. Characteristics of the SR calcium release channel, i.e. ryanodine binding sites and dissociation velocity, were investigated.

6. SR functionally releasable calcium in intact rat ventricular myocytes was assessed by both caffeine contracture and rapid cooling contracture.

7. Na-Ca exchange activity was characterized in purified sarcolemmal membrane.

8. The effect of insulin treatment on $[Ca^{2+}]_i$ in diabetic rats was examined. Comparisons were made between control, diabetic and diabetic treated groups.

METHODS

I. MATERIALS

a. Animals

Male Wistar rats weighing 200-225 g were obtained from the Animal Care Center, University of British Columbia.

b. Chemicals and Assay Kits

Chemicals and assay kits were purchased from the following sources:

BDH Chemicals, Vancouver, BC: calcium chloride, magnesium chloride, lithium chloride, sodium chloride, sodium bicarbonate, potassium chloride, nitric acid and d-glucose.

Boehringer-Mannheim, Laval PQ: Peridochrom^{R} Glucose GOD-PAP assay kit, bovine serum albumin (fraction V, fatty acid free).

Calbiochem, San Diego, CA: ryanodine.

Eli Lilly, Indianapolis, IN: Ultralente insulin zinc.

GIBCO, Burlington, Ont: Medium 199, fetal bovine serum.

ICN Inc, Costa Mesa, CA: $^{45}CaCl_2$, RSL ^{125}I insulin kit.

Miles Inc, Elkhart, IN: Glucometer^{R} II (Ames), and Glucostix^{R} (Ames); Etobicoke, Ont., netrendipine.

Merck Sharp & Dohme Canada, Kirkland, PQ: dichlorobenzamil.

Molecular Probes, Eugene, OR: fura-2/AM, indo-1/AM.

New England Nuclear - Dupont, Mississauga, Ont.: [^{3}H]ryanodine.

Sigma Chemical Company, St. Louis, MO: streptozotocin, d,l-isoproterenol hydrochloride, l-phenylephrine hydrochloride, timolol hydrochloride, ouabain octahydrate, 8-bromo-cAMP, adenosine 5'-triphosphate, penicillin G/streptomycin, laminin, thapsigargin, HEPES, MOPS, sucrose, caffeine, valinomycin.

Terry Fox Laboratory, Vancouver, BC: MEM.

Worthington Biochemicals, Freehold, NJ: collagenase, type II.

c. Media and Buffer Compositions

MEM: with Dulbecco's modified Eagle medium (D-MEM), 15 mM HEPES buffer, penicillin G 100 units/mL and streptomycin 100 µg/mL; without calcium.

M199: with Earl's salts, HEPES buffer 25 mM, penicillin G 100 units/mL and streptomycin 100 µg/mL; without $NaHCO_3$.

RCC, cold perfusing solution (pH 7.4 at 1°C): with NaCl 140.0 mM, KCl 6.0 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 1.8 mM, glucose 10.0 mM, HEPES 5.0 mM.

II. METHODS

a. Induction and Verification of Experimental Diabetes

Diabetes was induced by a single tail vein injection of STZ (55 mg/kg) dissolved in 0.9% NaCl solution. Control rats were injected with vehicle alone. The

injection was performed after anesthetizing the rat with 1-2% halothane inhalation. Animals were maintained with free access to food and water for up to six weeks.

Animals were tested for diabetes three days after the injection by checking for blood glucose using Glucometer^R II and Glucostix^R. Body weight, plasma glucose and insulin were also monitored as indications of diabetes. Blood samples were collected at the time of sacrifice. The measurements of glucose and insulin with commercial assay kit were performed according to the corresponding manual.

b. Insulin Treatment Protocols

Daily insulin treatment (Ultralente insulin zinc, 4-12 units/kg, i.h.) was given to a group of diabetic rats for four weeks. The injection was started two weeks after the STZ injection. Blood glucose and body weight were monitored continuously. The dose of insulin was adjusted daily according to the blood glucose level in order to maintain normoglycemia.

c. Isolation of Ventricular Myocytes

The isolation technique was modified from the protocols of Fischer et al. (1991). The rat was given an injection of heparin (2,000 units/kg, i.p.) 15 minutes before sacrifice. After an injection of sodium pentobarbital (65 mg/kg, ip), the heart was excised and immediately placed in cold-oxygenated (95% O_2 / 5% CO_2) MEM. Blood sample was collected from the chest cavity at the same time. The heart was then perfused via a Langendoff apparatus with MEM (nominally calcium free) at 37°C, pH 7.4, oxygenated with 95% 0_2 / 5% CO_2. In 3-5 minutes the coronary

arteries were cleared. The perfusion buffer was then changed to MEM-collagenase solution (collagenase 1 mg/mL, $CaCl_2$ 25 μM) for a total of 25-30 minutes, depending on the digestion of the connective tissue. At 15 and 20 minutes, the calcium concentration in the MEM-collagenase solution was brought up to 100 and then to 200 μM by adding $CaCl_2$ solutions.

After the perfusion, the ventricles were removed, cut open and teased apart with forceps and a scalpel so that the individual myocytes could be released. This procedure was completed by transferring the minced tissues into a water-jacketed beaker and incubating them with the above MEM-collagenase for 3-5 minutes.

The cell solution was then filtered through a 200 μm nylon mesh and centrifuged at 600 rpm for 2 minutes. The pellet was re-suspended in MEM (with 1% BSA) and left to settle by gravity. This procedure was repeated 3 times. After each sedimentation the MEM-BSA buffer was changed with step-wise increment in calcium concentrations, i.e. 200, 500 and 1,000 μM. Finally the cells were suspended in M199 (with BSA 1%, FBS 5%, $CaCl_2$ 1.8 mM) and were counted and plated.

Cell were plated on 22 x 22 mm glass cover slips (thickness 0.2 mm) previously etched with concentrated nitric acid, rinsed in distilled water and 70% ethanol and pre-incubated with laminin (diluted in M199, 2 μg/slip) for 30-40 minutes. The freshly isolated myocytes were plated on laminin-coated cover slips in polystyrene tissue culture wells (Corning, diameter 35 mm). At room temperature the viable cells attach to the slips within 20-30 minutes. There appeared to be no difference in the cell attachment between control and diabetic cells. The M199-BSA-

FBS solution was changed every 2 hours during experimentation. For cell counting, the cell suspension in M199-BSA-FBS was dripped into the hemocytometer (Baxter, Hausser Scientific, 0.2 mm deep). The cells were counted under the microscope according to standard counting procedures.

d. Measurement of Myocyte Contraction

The cover slip of the plated myocytes was mounted on the stage of an inverted microscope (Nikon DIAPHOT-TMD). The cell chamber was temperature controlled and continuously perfused by a peristaltic pump with a flow rate of 5 mL/minute or 15 mL/minute (for RCC and CC measurements). To elicit cell contraction, myocytes were field-stimulated with platinum electrodes at a frequency of 0.5 Hz, 3-8 volts (1.2 threshold), and a duration of 5 ms using a GRASS S11 dual output digital stimulator.

The criteria for selecting myocytes included: the myocytes were rod shaped with clear striations and edges, quiescent in the presence of 1.8 mM extracellular calcium without spontaneous wavelike contraction, and displayed a steady twitchlike contraction after 1 minute of continuous electrical stimulation, with minimum cell length of 60 μm, and minimum cell shortening of 5% of cell length.

Cell measurements were made at 400x magnification using phase-contrast optics. A charge-coupled device (CCD) television camera (COHU CCD 4815, San Diego, CA) attached to the microscope sideport allowed for video imaging of the cells in the cell chamber. The myocyte was focused on the light-sensitive element of the CCD camera and the longitudinal axis of the cell image was oriented parallel to

the horizontal scan lines by rotating the camera in the microscope side port. The CCD camera rapidly scanned the image area with 754 parallel horizontal lines from top to bottom at a vertical sweep rate of 60 Hz. The voltage along each scan line varied as the function of light intensity on the element, allowing for identification of the edge of the cell. Cell length was continuously updated with each vertical sweep. The video image of the myocytes was passed on line from the CCD camera to the video cassette recorder (Panasonic, PV-1730), through the video edge detector circuit board (Steadman et al. 1988) to a high resolution monitor (Sony, PVW-91). The edge detector uses the voltage changes to identify the left and right edges of the cell. A calibration factor for the edge detector was achieved by obtaining voltage outputs for a graticule graded in 10 μm increments (Figure 3). The output from the edge detector, change in cell length (expressed as volts) and its derivative (±dV/dt) were digitized by a Tekmar Labmaster board and connected to a personal computer (PC) monitor through Deltascan-PTI (Photo Technology International Inc.) program. PTI, at a sampling rate of 25 Hz, allowed on line viewing of the change in myocyte length and the rate of shortening and relaxation. The video tracking signals were stored for further analysis. The on line measurement also provided feedback about the quality of cell tracking by the edge detector. Prior to the experiments, the parameters which were recorded included: magnitude of cell shortening (expressed as S/L%), maximum rate of shortening (+dl/dt) and relaxation (-dl/dt), and time-to peak shortening.

e. Measurement of Rapid Cooling Contracture and Caffeine Contracture

The selection of control and cold buffer (or caffeine solution) was controlled with two solenoid valves (Lee Valves). After the cell contractions stabilized in control

buffer perfusion for 5 minutes, contractile tracings were recorded with the PTI program. For each cell, shortening (S/L%), peak shortening rate (+dl/dt) and peak relaxation rate (-dl/dt) were recorded. Then the stimulator was turned off and the bath solution was rapidly (in 1-2 seconds) switched to the cold (1°C) solution or buffer contained 20 mM caffeine. The cell contracture elicited by rapid cooling or caffeine was continuously recorded by the PTI program. In indo-1-am loaded cells the $[Ca^{2+}]_i$ transient that accompanied the contracture was recorded simultaneously as described below. The amplitude of RCCs decreased about 10% in indo-loaded control cells because of the buffering effects of indo-1. Therefore all the measurements of RCCs and CCs in control and diabetic cells were made in the absence of indo-1.

Rest potentiation was measured as follows: after the myocytes were stabilized at field stimulation of 0.5 Hz for 2 minutes, the stimulator was turned off, a resting period of 30 seconds was given before the stimulation of 0.5 Hz restarted. The first potentiated contraction upon the stimulation resumed was defined as rest potentiation.

f. Measurement of Intracellular Calcium, $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using fura-2 (when $[Ca^{2+}]_i$ was measured as the sole parameter) and indo-1 (when contractility was measured simultaneously) as fluorescent calcium indicators. Cardiac myocytes attached to the glass coverslip were loaded with fura-2 by incubating them with 5 μM fura-2/AM for 20-30 minutes at room temperature in M199 (1% BSA and 5% FBS). The loaded cells were then washed twice with fresh M199. For fura-2, the fluorescence signal was monitored at

505 nm with excitation wavelengths alternating between 335 and 385 nm using a spectrofluorometer (Deltascan, Photon Technologies Inc.) with a Nikon inverted microscope. The $[Ca^{2+}]_i$ was calculated as previously described with a K_d of 224 nM for the fura-2-Ca complex after correction for fluorescence from extracellular fura-2 and autofluorescence. The following equations was used to calculate $[Ca^{2+}]_i$:

$$[Ca^{2+}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times ß$$

Where R_{max} is the ratio of the fluorescence intensity with saturated calcium, measured in cells incubated in the solution containing 2 mM $CaCl_2$ and 20 µM digitonin, at excitation wavelengths of 335 and 385 nm; R_{min} is the fluorescence ratios with the dye not bound to calcium, measured in the solution of 100 mM EGTA (pH 8) and 20 µM digitonin at excitation wavelengths of 335 and 385 nm; ß is the ratio for fluorescence of reference wavelength (380 nm for fura-2, 480 nm for indo-1) with zero-Ca/saturated-Ca. The background fluorescence was the 335/385 ratio measured in the solution of 100-1000 µM digitonin and 100 mM EGTA (pH 8). We observed by cell imagining method that about 2-5% of the total flourescence could be detected at sarcolemma membrane by applying the background solution above which is presumebely the membrane trapped fura-2/AM. To ensure the accuracy of the calibration the R_{max}, R_{min} and background were measured during every experiment in each individual cell.

The measurement with indo-1 used the same equipment and procedures, while the excitation wavelength was set at 350 nm and emission fluorescence signals were monitored at 405 and 480 nm. The K_d value used for indo-1-Ca complex was 250 nM.

g. Measurement of Na-Ca Exchange

Sarcolemmal Isolation. The rat sarcolemmal vesicles were isolated by the procedure of Tibbits at al. (1989). The rat hearts from each group were obtained as described in the procedure for myocyte isolation. The heart was perfused with a homogenizing medium (sucrose 280 mM, MOPS 10 mM, pH 7.40) retrogradely through the aorta for 5 minutes. Eight to twelve ventricles from each group were pooled in homogenizing medium (4°C) and were then minced with scissors. The ventricles were disrupted further by homogenization using a Tekmar Tissumizer at setting 45 for two bursts of 3 seconds each. The homogenates were filtered through two layers of stainless steel mesh, and 1 mL aliquots were taken for further biochemical analyses as described below. The suspensions were then spun at 2000 g for 15 minutes. The supernatants were removed and then pelleted at 180,000 g for 1 hour. The resultant pellets were resuspended in 45% sucrose, and discontinuous sucrose gradients were layered on top. The gradients were spun in a swinging bucket rotor (SW 28) at 122,000 g for 16 hours. Four fractions from the gradient were collected and labeled F_1-F_4. Fraction 2, the most sarcolemma-enriched fraction, was harvested from the interface of the 27% and 30% sucrose layers. All fractions were diluted slowly with an ice-cold loading medium, consisting of NaCl 140 mM and MOPS 10 mM (pH 7.4). The suspensions were centrifuged at 180,000 g for 1 hour; the pellets were resuspended in 1.25 mL loading medium and then frozen in liquid nitrogen. The preparation were used within 3 weeks after the isolation. The sarcolemmal marker K-stimulated p-nitrophenylphosphatase (K-pNPPase) was measured in the crude homogenate and in all fractions derived from the sucrose gradient. The purification index was defined as the ratio of the specific

activity of K-pNPPase in the sarcolemmal fraction to that in the crude homogenate. Recovery was defined as the percentage of the total K-pNPPase activity (μmol/hour) in the sarcolemmal fraction compared with the crude homogenate.

Na-Ca exchange. Na dependent Ca uptake into sarcolemmal vesicles was performed as described (Shi et al. 1989). In brief, 5 μl of Na-loaded sarcolemmal vesicles (1.0-3.0 mg protein/mL) were suspended on the wall of a polystyrene tube containing 245 μl of uptake medium maintained at 37°C. The medium contained KCl or NaCl 140 mM, MOPS 10 mM (pH 7.4), $^{45}CaCl_2$ 2 μCi, valinomycin 0.5 μM, and $^{40}CaCl_2$ ranging from 10 to 80 μM. The uptake was initiated by vortex mixing and was stopped at a preset time by a custom-built rapid-quenching device. Exchange was quenched by the addition of 30 μl of a solution containing KCl 140 mM and $LiCl_3$ 20 mM. An aliquot of 220 μl was then filtered (Millipore, 0.45 μM), and the filters were dried and then counted. The uptake by vesicles diluted into NaCl represented blanks and was subtracted for all data points. This allows for the correction of counted $^{45}CaCl_2$ that is bound superficially to the sarcolemma or that has permeated the vesicle by some pathway other than Na-Ca exchange. The time course of exchange was determined with 5 μM $^{40}CaCl_2$ and the reaction was quenched at intervals ranging from 0.5 to 120 seconds. The apparent K_m of the exchanger for calcium was measured by quenching the uptake in media containing various ^{40}Ca concentrations at 2 seconds. The uptake at this time was confirmed experimentally to represent initial rates.

h. Ryanodine Binding Assay

The preparation of the heart homogenates was the same as the procedure for sarcolemmal isolation described above. The homogenates were freshly prepared daily and stored at 4°C. The [^{3}H]ryanodine binding was modified from the method of Chu et al. (1987). The reaction medium contained [^{3}H]ryanodine (New England Nuclear - Dupont, 60 Ci/mmol) 11 nM, nonradioactive ryanodine at various concentrations (0-50 µM), KCl 150 mM, MOPS 10 mM (pH 7.4), $CaCl_2$ 100 µM, and diluted homogenates (final protein concentration 0.8 mg/mL). A second set of tubes was set up with the same concentrations except for ryanodine which was added at a range of 11-500 µM giving a 1,000 fold unlabeled ryanodine to displace [^{3}H]ryanodine. The reaction, in a 1 mL volume, was allowed to proceed for 2 hours to equilibrium in duplicates at 23°C. The medium was then quickly filtered under vacuum through Whatman GF/C glass fiber filters. The filters were then rinsed twice with 4 mL cold washing buffer (KCl 150 mM, MOPS 20 mM (pH 7.4), and $CaCl_2$ 100 µM), dried and radioassayed by liquid scintillation counting.

The specific binding was determined by the difference between total binding and nonspecific binding obtained in the presence of 1-50 µM unlabeled ryanodine. Values for B_{max} and K_d were calculated by least squares linear regression and analysis of Scatchard plots using the computer software Ligand (Munson and Rodbard, 1984) and EBDA (McPherson 1985).

i. Dose Responses of $[Ca^{2+}]_i$ to Inotropic Agents

Myocyte preparation, $[Ca^{2+}]_i$ determination by fura-2 and cell stimulation were as described as above. After the calcium transient stabilized various experimental procedures were initiated. While the viability of the myocytes was generally unchanged throughout the first 24 hours after the isolation, the order of the drug exposure was randomized for each experiment to eliminate any cell viability change during the experiments. The cardiac myocytes were exposed to various inotropic agents, which included:

1. isoproterenol, a ß-adrenergic agonist producing inotropic effects through a cAMP dependent mechanism.

2. 8-bromo-cAMP, a membrane permeable form of cAMP, which is a second messenger of cAMP dependent cell function.

3. ouabain, a cardiac glycoside, which inhibits Na-K ATPase and, in turn, promotes calcium influx by activating Na-Ca exchange in the sarcolemma membrane.

4. ATP, a high energy phosphate compound, which acts on P_2 receptors of cardiac myocytes. The second messengers involved in the ATP-induced calcium increase and positive inotropic effect are not clear.

$[Ca^{2+}]_i$ responses to various dose of inotropic reagents were recorded at each concentration. $[Ca^{2+}]_i$ increments developed with each dose of the agent were

recorded when the responses were stabilized. The dose range and intervals were determined by preliminary experiments.

III. STATISTICAL ANALYSIS

The data are expressed as mean ± standard error (SE) of the mean. Statistical significance was determined by a multiple analysis of variance (MANOVA) followed by a one-way ANOVA and the Newman-Keuls multiple range test. In MANOVA, it is assumed that (1) the dependent variables are multivariately normally distributed and (2) the covariance matrices are equal for all groups. Differences on a set of dependent variables were analyzed by MANOVA. For example, if there were several measures of dose responses to a drug, a composite analysis was done to determine whether groups differ on them. The level of statistical significance was set at $p<0.05$.

RESULTS

I. GENERAL CHARACTERISTICS OF STZ-INDUCED DIABETES MELLITUS

STZ-induced diabetic rats, as expected, exhibited polyuria, polydipsia, and hyperphagia. Three days after the injection, glucometer tests for whole blood showed that all STZ injected rats had developed hyperglycemia. At six weeks the STZ-injected rats showed significantly less body weight gain, decreased plasma insulin and increased glucose (Table 1) in comparison with age-matched controls. Insulin treatment reversed impaired body weight gain in diabetic rats. Mean plasma glucose and insulin in the insulin-treated rats were partially restored but were not completely corrected to the control levels. The data are in good agreement with previous work done in our laboratory (Rodrigues and McNeill, 1986).

II. GENERAL CHARACTERISTICS OF CARDIOMYOCYTES FROM STZ-DIABETIC RATS

The technique for cardiomyocyte isolation developed in this laboratory yields a satisfactory population of myocytes. The percentage of viable myoctes, defined by the rod shape of the cell, was maintained at 70-80% in all groups (Table 2, Figures 1 and 2). At room temperature myocytes maintained a rod shape for more than 24 hours after isolation. Diabetic hearts were more easily digested by collagenase than control hearts and the isolation time was usually shorter than that for a control heart. There appeared to be more fat tissue in the diabetic heart. Cell viability was lower in the diabetic group than in controls, suggesting that diabetic myocytes may be more sensitive to the disruption of the isolation process. However the overall viability of 70% is usually indicative of a good quality isolation (Stemmer et al., 1992). The

measurements made in the diabetic myocytes, see below, also suggested that diabetic myocytes isolated by this protocol retained the features of diabetes-induced changes in the intact heart. It was also found that insulin treatment reversed some of the diabetes-induced changes.

a. Contractile Function of Myocytes from Control, Diabetic and Insulin Treated Rats

In response to electrical stimulation myocytes contracted regularly. Figure 4 shows cell shortening and $[Ca^{2+}]_i$ transients, measured by fluorescence microscopy and indo-1, in an electrically-stimulated control cell. The contractile data, measured without indo-1 loaded in cells, from the three groups are shown in Table 2. The myocyte contractile function in cells from diabetic hearts was depressed in comparison with those from controls. The cell shortening (S/L%, diabetic cells ≈56% of controls, Figure 5), velocities of shortening (+dl/dt, diabetic cells ≈42% of controls, Figure 6) and relengthening (-dl/dt, diabetic cells ≈44% of controls, Figure 7) were significantly lower than those in controls (Table 2). The time-to-peak shortening was prolonged in diabetic cells (≈147% of controls). Insulin treatment normalized the depressed cell shortening (insulin treated group ≈94% of controls), time to peak shortening (≈125% of controls) and rates of contraction and relaxation (≈70% and 79% of controls, respectively) in diabetic myocytes (Table 2), although the treatment did not completely restore all parameters to control values.

Cell shortening and ±dl/dt were also measured at different stimulation frequencies (Figures 5, 6 and 7). A negative staircase phenomenon was observed in control and diabetic rat myocytes (Figure 5). As the frequency decreased (at stimulation frequency of 2, 1, and 0.5 Hz, i.e. stimulation intervals of 0.5, 1 and 2

seconds), myocyte shortening increased (Figure 5) which is consistent with what is observed in intact rat cardiac tissues. With longer rest intervals the magnitude of post-rest contractions increases in rat cardiac muscle (Bers, 1985). Rest potentiation is indicative of releasable calcium from SR, although calcium release is not completed as in RCC, with a combination of small amount of calcium from Na-Ca exchange (Bers, 1987). In diabetic myocytes rest potentiation was found to be depressed (Table 2).

The high spatial resolution of the VED enables myocyte length to be determined with an error of ±0.5 μm (38) thus the temporal resolution of the VED represents the only limitation on accurate analysis of myocyte shortening. The latter parameter is limited by the resolution of the video camera which is only capable of scanning the myocyte image every 16.7 ms (60 Hz), and sampling rate of 25 Hz by A/D board in the present experiment. To overcome this problem and to ensure adequate sampling during cell contraction, values were obtained by averaging consecutive signals stored on the PTI program.

b. Contractile Response to Isoproterenol in Electrically-Stimulated Myocytes

The cell shortening (shortened length/resting length %) and time to peak shortening in response to the increased dose of isoproterenol were decreased in diabetic cells (Figures 8 and 9). The decreased response to isoproterenol is consistent with findings obtained from isolated heart or cardiac tissues of diabetic rats (Heyliger et al., 1982; Yu and McNeill, 1991b).

III. RAPID COOLING CONTRACTURE AND CAFFEINE CONTRACTURE

The magnitude of rapid cooling and caffeine-induced contracture is believed to be a relative index of SR calcium content (Bers, 1987). Rapid cooling contracture was measured by exposing single myocytes to cold (1°C) Tyrode buffer. A recording of RCC cell shortening and $[Ca^{2+}]_i$ of a single control cell is shown in Figure 10, and two individual control and diabetic cells is shown in Figure 11. The amplitude of RCCs decreased significantly in diabetic myocytes (≈68% of controls) compared with that in controls. Insulin treatment reversed this trend to some extent (≈86% of controls) but did not reverse RCC values back to the normal level (Figure 12).

Figure 13 is an example of caffeine contracture with recording of both cell shortening and $[Ca^{2+}]_i$ change. The amplitude of the peak caffeine (20 mM) contracture was higher in the control group than in the diabetic group (≈75% of controls) (Figure 14).

IV. INTRACELLULAR Ca^{2+} RESPONSE TO AGONISTS IN QUIESCENT MYOCYTES

a. Basal $[Ca^{2+}]_i$ in Myocytes from Control, Diabetic and Insulin-Treated Groups

The intracellular calcium concentration, $[Ca^{2+}]_i$, measured by fluorescence microscopy and fura-2, from 3 groups are listed in Table 1. Among control, diabetic and insulin treated groups $[Ca^{2+}]_i$ values were not different either in the quiescent or electrically stimulated states. Figure 15 shows a recorded $[Ca^{2+}]_i$ transient of a stimulated control myocyte at basal state and in response to isoproterenol (1 nM).

b. $[Ca^{2+}]_i$ Responses to Caffeine in Quiescent Myocytes

Fura-2 fluorescence is quenched by caffeine (data not shown), but in a wavelength-independent manner so that the fluorescence ratio used to estimate $[Ca^{2+}]_i$ is unaffected. In response to caffeine (20 mM), a $[Ca^{2+}]_i$ transient was recorded in myocytes (Figure 16). This transient was blocked by ryanodine (1 nM) or thapsigargin (1 μM), but was not influenced by the removal of extracellular calcium (data not shown). The transient was depressed in the diabetic cells (Figure 17, peak concentration 303±24 nM, n=22, 6 rats vs 208±20 nM, n=16, 4 rats). This indicates that SR content of calcium was decreased in diabetes. The decline of the $[Ca^{2+}]_i$ transient suggests the extrusion of calcium from the cytoplasm by Na-Ca exchange. The maximal rate of $[Ca^{2+}]_i$ decline (-peak $d[Ca^{2+}]_i/dt$) was decreased in diabetic cells (14±3 nM/sec, n=16 vs 38±4 nM/sec, n=22). Insulin treatment restored both the peak concentration of caffeine transients (269±10 nM, n=13 (***3*** rats)) and the rate of $[Ca^{2+}]_i$ decline (38±4 nM/sec, n=13).

c. $[Ca2+]_i$ Responses to KCl and Different Blockers in Quiescent Myocytes

KCl (3.125-50 mM) caused a dose-dependent, rapid $[Ca^{2+}]_i$ increase (Figures 18, 19, and 20). Figure 18 is an example of a KCl (50mM) induced $[Ca^{2+}]_i$ transient in a single control myocyte. The rates of increase and decline are relatively slower than the caffeine-induced $[Ca^{2+}]_i$ transients. The decline of $[Ca^{2+}]_i$ usually did not return to the basal level suggesting that a new balance was achieved in the presence of KCl. This increase in $[Ca^{2+}]_i$ was enhanced in diabetic cells, both the initial rate of $[Ca^{2+}]_i$ increase and the peak concentration of $[Ca^{2+}]_i$ were increased

(Figures 20-22). The dose response of $[Ca^{2+}]_i$ to KCl shows that the difference in diabetic cells could only be seen at 12.5 mM or above (Figure 22). Insulin treatment of the diabetic rats not only reversed the hyperresponse to KCl, but also caused the response to 25 mM KCl to fall below the control level (Figure 20).

In order to differentiate various portions of the $[Ca^{2+}]_i$ transient induced by KCl, different blockers were used (Figures 23-27). Removal of extracellular calcium abolished the $[Ca^{2+}]_i$ response to KCl (data not shown). Calcium entry via either Ca-channels or Na-Ca exchange may be necessary to trigger intracellular calcium release. Figures 23-24 show individual experiments of blocker effects on $[Ca^{2+}]_i$ transients. As shown in Figure 23, ryanodine decreased the initial part of the $[Ca^{2+}]_i$ transients and peak $[Ca^{2+}]_i$ suggesting that calcium release from SR contributes to the initial part of the calcium increase. When caffeine was used to deplete SR and nitrendipine to block L-type Ca-channels (Figure 24), the $[Ca^{2+}]_i$ transient was further decreased. At this time calcium entry is presumably mediated by Na-Ca exchange. When caffeine, nitrendipine and dichlorobenzamil were used together, the KCl-induced $[Ca^{2+}]_i$ transient was almost abolished (Figure 25). This suggests that the L-channel, Na-Ca exchange and SR all contribute to the KCl-induced $[Ca^{2+}]_i$ transients.

Blocker effects are summarized in Figures 26 and 27. Caffeine (20 mM) and dichlorobenzamil (10 μM) blocked $[Ca^{2+}]_i$ transients to a smaller degree in diabetic cells, which may suggest that SR $[Ca^{2+}]_i$ release and Na-Ca exchange play small roles in KCl induced $[Ca^{2+}]_i$ transients. Nitrendipine (5 μM) plus caffeine (20 mM) blocked more KCl-induced $[Ca^{2+}]_i$ transients in diabetic cells than in controls. Ryanodine, at the concentration used in this experiment (1 nM), would keep the SR

Ca-release channels in an open state, therefore depleting SR calcium. There was no difference in ryanodine effects between control and diabetic cells.

d. $[Ca2+]_i$ Responses to Isoproterenol in Quiescent Myocytes

As shown in Figures 28-30, after the cells were exposed to 1 μM isoproterenol/M199 solution, the $[Ca^{2+}]_i$ level decreased continuously. This decrease was blocked by timolol (0.1 μM) or thapsigargin (1 μM), suggesting the involvement of SR Ca-ATPase in ß-adrenergic stimulation and isoproterenol-induced $[Ca^{2+}]_i$ changes. Diabetic cells showed a blunted response to isoproterenol. Insulin treatment reversed the depressed response to isoproterenol in diabetic myocytes (Figures 29 and 30).

V. $[Ca^{2+}]_i$ RESPONSES TO ISOPROTERENOL, 8-BROMO-cAMP, OUABAIN AND ATP IN ELECTRICALLY STIMULATED MYOCYTES

The pharmacological parameters used in this study are defined as follows. Using classical pharmacological measurements, the sensitivity of a cell (expressed by the EC_{50} or pD_2 value) for an agonist as derived from the concentration-effect relationship is an estimate of the apparent affinity, usually defined as sensitivity, of the receptor for the agonist, and the maximum response is an estimate of the intrinsic activity of a drug, i.e. the inherent effect produced by an agonist after receptor occupancy (Ariens and Simonis 1964).

a. Isoproterenol

Figure 31 shows individual tracings of $[Ca^{2+}]_i$ in response to isoproterenol recorded from a control (panel A) and a diabetic (panel b) cell. In electrically-stimulated control myocytes, isoproterenol caused a dose-dependent $[Ca^{2+}]_i$ increase with a maximum response of 724±60 nM (increase of peak $[Ca^{2+}]_i$ transients, n=9, 4 rats; Figure 32). The change of $[Ca^{2+}]_i$ per second ($\pm d[Ca^{2+}]_i/dt$) also increased (data not shown). Stimulated diabetic myocytes showed a depressed response to isoproterenol (maximum response of 272±69 nM, n=8, 3 rats, $p < 0.05$, Figures 31 and 32). Figures 32 and 33 show the differences between the dose response curves of isoproterenol in control and diabetic cells. The maximum response to isoproterenol decreased in the diabetic group, but the mean pD_2 values (negative log concentration of the agonist which produce 50% of maximum effect) was not significantly different (Figure 33, and Table 5).

b. 8-Bromo-cAMP

Diabetic myocytes had a decreased maximum response to 8-bromo-cAMP (maximum response of 88±20 nM, n=8, 3 rats; vs 170±18, n=8, 3 rats) and they were less responsive to 8-bromo-cAMP at the concentrations of 10^{-5}-10^{-2} M. (Figures 34, 35). This may be related to the depressed response to isoproterenol as described above. Mean pD_2 values were not significantly different between control and diabetic cells (Figures 36 and Table 5).

c. Ouabain

Figure 37 shows individual $[Ca^{2+}]_i$ responses to ouabain from a control and a diabetic cell. In the control cell (panel A), from ouabain concentration of 10^{-7} M to 10^{-3} M, the peak $[Ca^{2+}]_i$ increased about 220 nM. In a diabetic cell (panel B), at concentrations of 10^{-9} M to 10^{-5} M, the peak $[Ca^{2+}]_i$ increase was about 100 nM. The highest toxic concentration to cause hypercontracture was 10^{-3} M in the control and 10^{-5} M in diabetic cell, respectively. This suggests that the diabetic cell was less tolerant to ouabain, and the absolute response to ouabain was decreased.

Figure 38 shows the ouabain dose response curves obtained from control and diabetic myocytes. The maximum response to ouabain was decreased in diabetic cells. In diabetic cells, the onset of the toxic event often occurred at lower concentration in comparison with control cells. Therefore it was not always possible to generate complete dose-response curves with ouabain. The significant leftward shift of the diabetic cell dose response curve (Figure 39) shows an increase in cell sensitivity to ouabain. The pD_2 values (negative log of the concentration of an agonist which produce 50% of maximum effect) increased in the diabetic group (Table 5) which is another index of increased sensitivity to ouabain.

Figure 40 shows an ATP-induced $[Ca^{2+}]_i$ change in control (panel A) and diabetic (panel B) myocytes. An increased maximal response to ATP was observed (Figure 40). This was also shown in diabetic cell group as compared to control (Figure 41). Figure 42 shows the percentage increase of $[Ca^{2+}]_i$ as a function of the increased concentration of ATP. The calculated pD_2 values of the two groups (shown in Table 2) was not significantly different. Therefore the sensitivity to ATP by diabetic myoctes was not changed.

VI. RYANODINE BINDING ASSAY

Two sets of binding site were determined by specific [^{3}H] ryanodine binding (Figure 43). The percentage of nonspecific binding over total binding was not significantly different between control and diabetic groups (Figure 44). The high affinity and low capacity binding site has been determined in both diabetic and control groups. The binding parameters are shown in Table 3, which indicates that the affinity of the calcium release channel in SR appears to be unchanged. However, the B_{max}, indicating the density of binding sites, was lower in diabetics than in controls. This suggests that the density of the release channel is reduced, which may be involved in the diabetic induced cardiac dysfunction.

VII. Na-Ca EXCHANGE IN SARCOLEMMAL MEMBRANE

Na-Ca exchange as a function of the extravesicular concentration of calcium is shown in Figure 45. The Na-dependent ^{45}Ca uptake was decreased in diabetic SR. Figure 46 shows the Eadie-Hofstee plots of ^{45}Ca uptake data from control and diabetic rats from Figure 45. K_m (-slope) and V_{max} (Y axis intercept) values were significantly decreased in diabetic rats compared to in controls (Table 4). This suggests a decrease in the exchanger number in the sarcolemma membrane and an increase in the affinity of exchangers to calcium in diabetic rats. These data are in partial agreement with data reported previously (Makino et al., 1987).

Table 1. Body weight, plasma glucose, and insulin levels of control, diabetic, and insulin treated diabetic rats

	Control	Diabetic	Treated
Body Weight (gm)	446±6 (63)	333±10* (37)	450±2 (10)
Plasma Glucose (mM)	6.0±0.1 (30)	19.3±0.4* (40)	7.8±0.3* (22)
Plasma Insulin (μunits/ml)	43.0±0.6 (10)	31.5±1.1* (9)	37.9±0.7* (10)

* $p < 0.05$ compared with control group.

Number of rats for each parameter is shown in parenthesis.

Table 2. General characteristics of myocytes from control, diabetic, and insulin treated diabetic rat hearts

	Control	Diabetic	Treated
Cell Viability (rod shape cells%)	79.7±2.9 (50)	70.2±1.2* (26)	86.2±0.8* (4)
Yield ($x10^6$ rod cells/heart)	25.2±6.1 (10)	18.8±2.7 (8)	21.8±2.2 (4)
Time to Peak Shortening (ms)	169±13 (36, 9)	248±27* (23, 8)	211±8* (40, 8)
Cell Shortening (S/L%)	14.52±1.03 (43, 9)	8.07±0.51* (48, 8)	13.64±0.36 (40, 8)
Max +dl/dt (micron/second)	138.4±2.9 (10, 3)	58.2±2.8* (11, 4)	96.5±2.1* (40, 8)
Max -dl/dt (micron/second)	107.5±1.8 (10, 3)	46.8±1.8* (11, 4)	84.9±1.6* (40, 8)
Rest Potentiation (S/L%)	15.87±0.77 (17, 3)	9.91±0.57* (17, 4)	
Resting $[Ca^{2+}]_i$ (nM)	79±1 (198, 42)	79±1 (141, 30)	80±2 (47, 17)
Stimulated $[Ca^{2+}]_i$ (nM)	370±28 (25, 10)	323±19 (36, 10)	406±30 (22, 6)

* $p < 0.05$ compared with control group. Contractile function and stimulated $[Ca^{2+}]_i$ were measured at 0.5 Hz, 3-8 V, and 5ms stimulation duration. Number of rats or cells followed by number of rats for each parameter is shown in parenthesis.

Rest potentiation was measured as follows: after the myocytes were stabilized at field stimulation of 0.5 Hz for 2 minutes, there was a resting period of 30 seconds, then stimulation at 0.5 Hz was restarted. The magnitude of shortening of the first potentiated contraction relative to that of last beat pre-rest was defined as rest potentiation.

Table 3. Parameters of Ryanodine Binding in Heart Homogenate from Diabetic and Control Rats

	Site 1		Site 2	
	Kd nM	Bmax fmol/mg	Kd μM	Bmax pmol/mg
Control (n=10)	9.7±1.5	660±49	51.1±18.5	57.3±12.2
Diabetic (n=8)	7.6±1.4	466±96*	30.4±15.6	47.3±15.4

* $p < 0.05$ compared with control group.

Number of rats for each group is shown in parenthesis.

Table 4. Characteristics of Sarcolemma and Na-Ca Exchange Activities in Diabetic and Control Rats

	Control	Diabetic
K^+pNPPase Activity µmol/mg/hr (n=3)	12.7±2.7	12.5±2.7
Sarcolemmal Recovery % (n=3)	11.0±2.6	9.2±2.6
Purification Index (n=3)	21.4±4.1	21.5±4.1
Km (Ca^{2+}) µmol (n=6)	55.2±4.5	38.9±4.5*
Vmax nmol/mg/sec (n=6)	4.05±0.39	2.78±0.39*

* $p < 0.05$ vs controls. All values are expressed as mean ± standard error.

K^+-pNPPase: K^+-stimulated p-nitrophenylphosphatase, n: number of preparations or experiments.

Table 5. pD_2 Values of inotropic agents in myocytes of diabetic and control rats (determined by $[Ca^{2+}]_i$)

	Control	Diabetic
Isoproterenol	10.7±0.4	11.0±0.3
(n)	(8)	(9)
8-bromo-cAMP	5.8±0.2	6.7±0.6
(n)	(8)	(8)
Ouabain	6.6±0.3	8.4±0.2*
(n)	(9)	(9)
ATP	7.8±0.5	8.5±0.4
(n)	(8)	(4)

* $p < 0.05$ vs controls. All values are expressed as mean ± standard error.

pD_2: negative log concentration of the agonist which produce 50% of maximum effect. n: number of cells, each drug was tested in at least 3 diabetic or control rats (except ATP diabetic group, n=4, 2 rats).

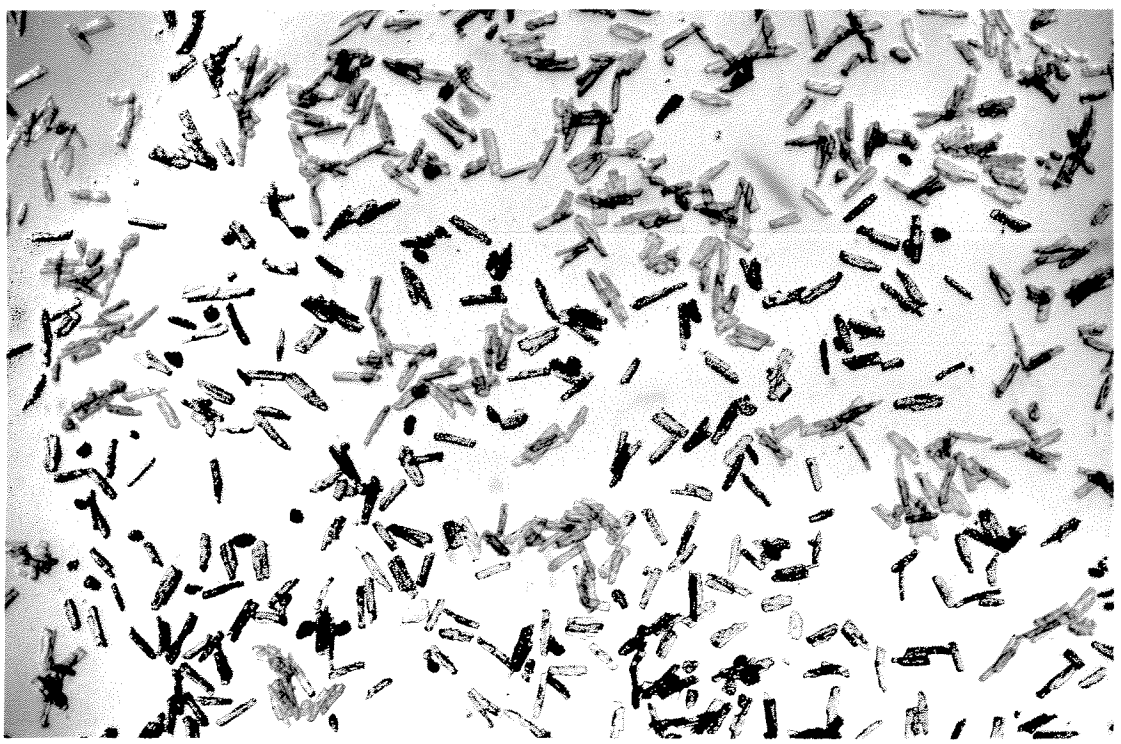

Figure 1. Isolated adult rat ventricular myocytes.

The photo was taken six hours after the isolation. The sample was randomly chosen. Cells were plated and attached to a laminin coated glass cover slip in M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C.

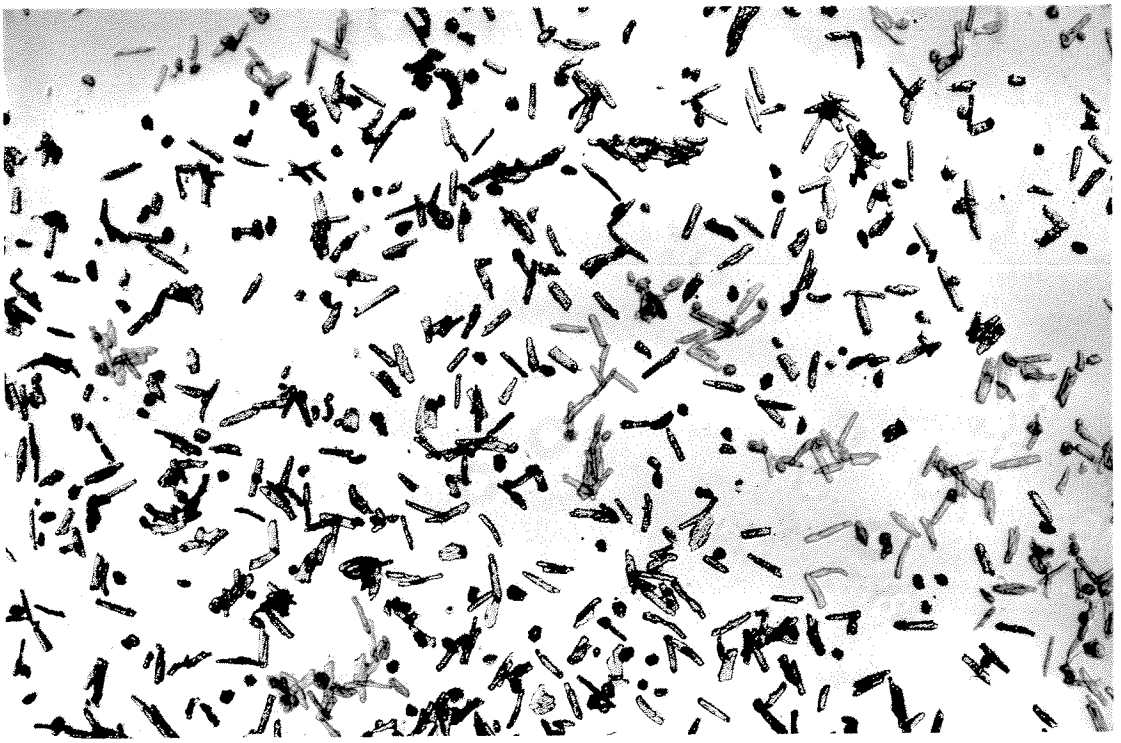

Figure 2. Isolated diabetic rat ventricular myocytes.

The photo was taken six hours after the isolation. The sample was randomly chosen. Cells were plated and attached to a laminin coated glass cover slip in M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C.

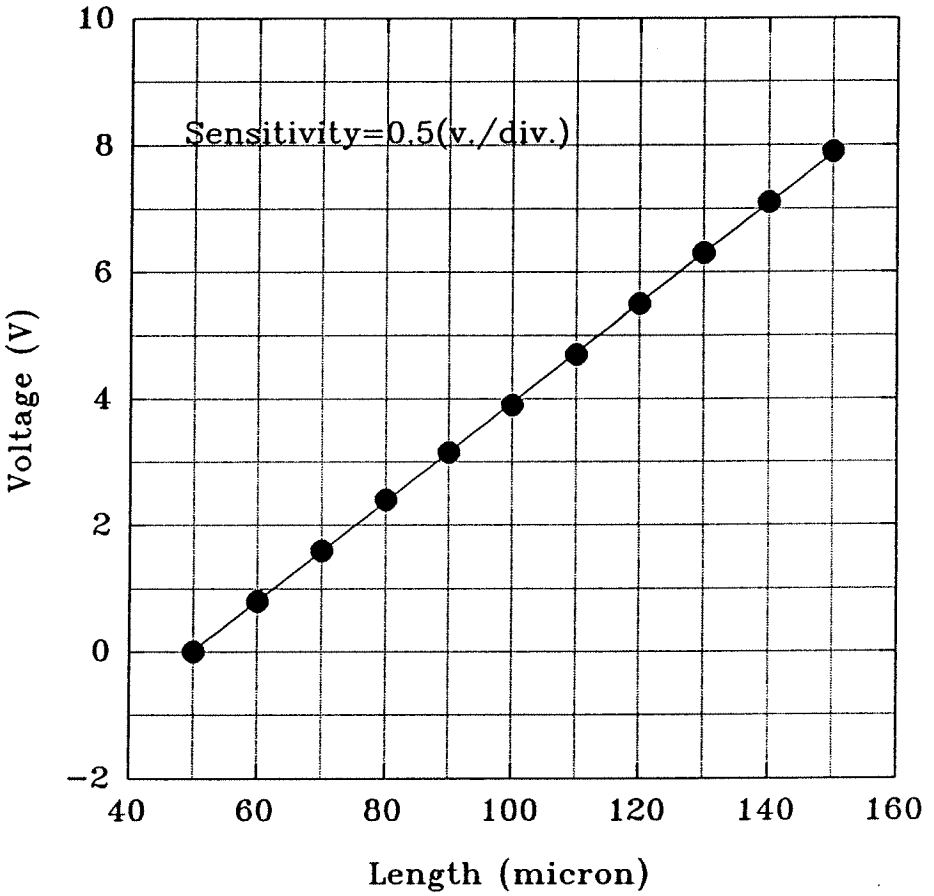

Figure 3. Calibration of edge detector.

The calibration factor was derived by obtaining voltage outputs from a graticule graded in 10 micron increments.

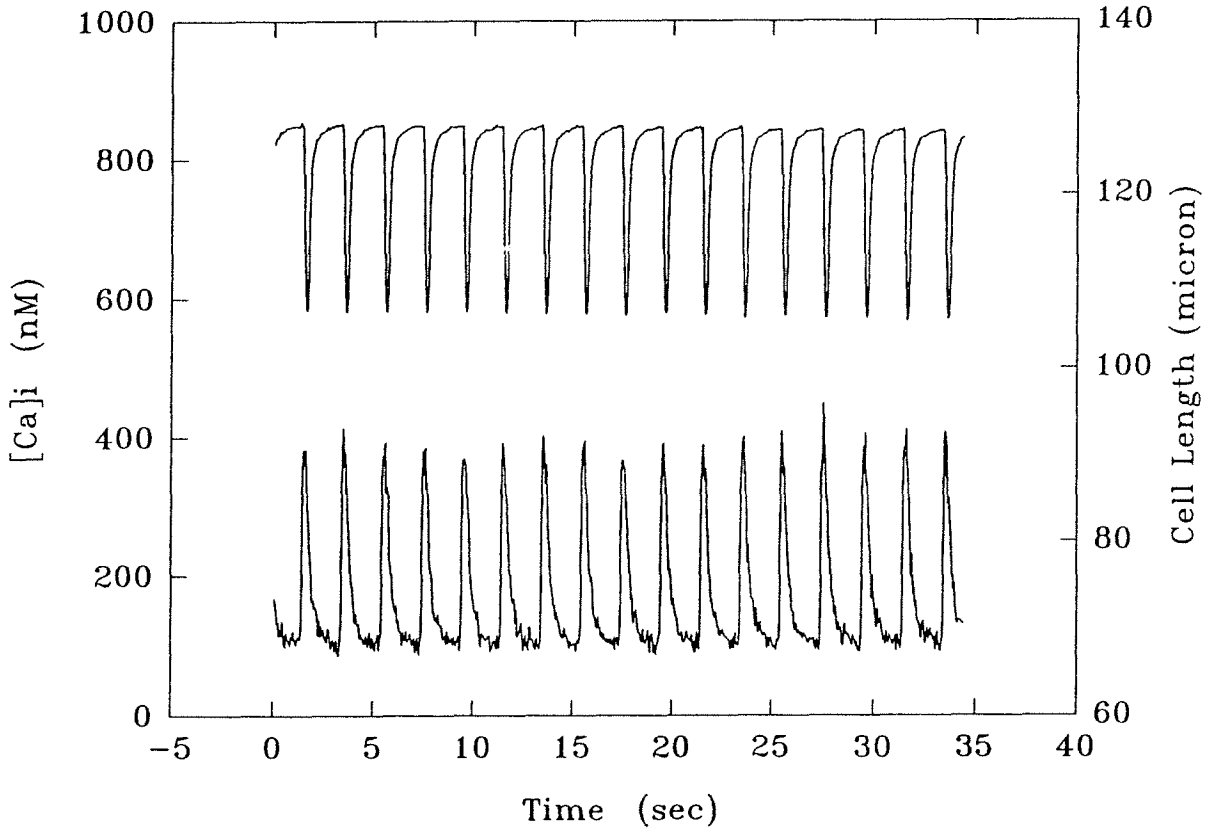

Figure 4. Cell shortening and $[Ca^{2+}]_i$ transients in an electrically-stimulated myocyte.

Myocytes were superfused with Tyrode buffer at 23°C and field stimulated at 0.5 Hz, 4 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and indo-1. Upper tracing is cell length change and lower tracing is $[Ca^{2+}]_i$ transients.

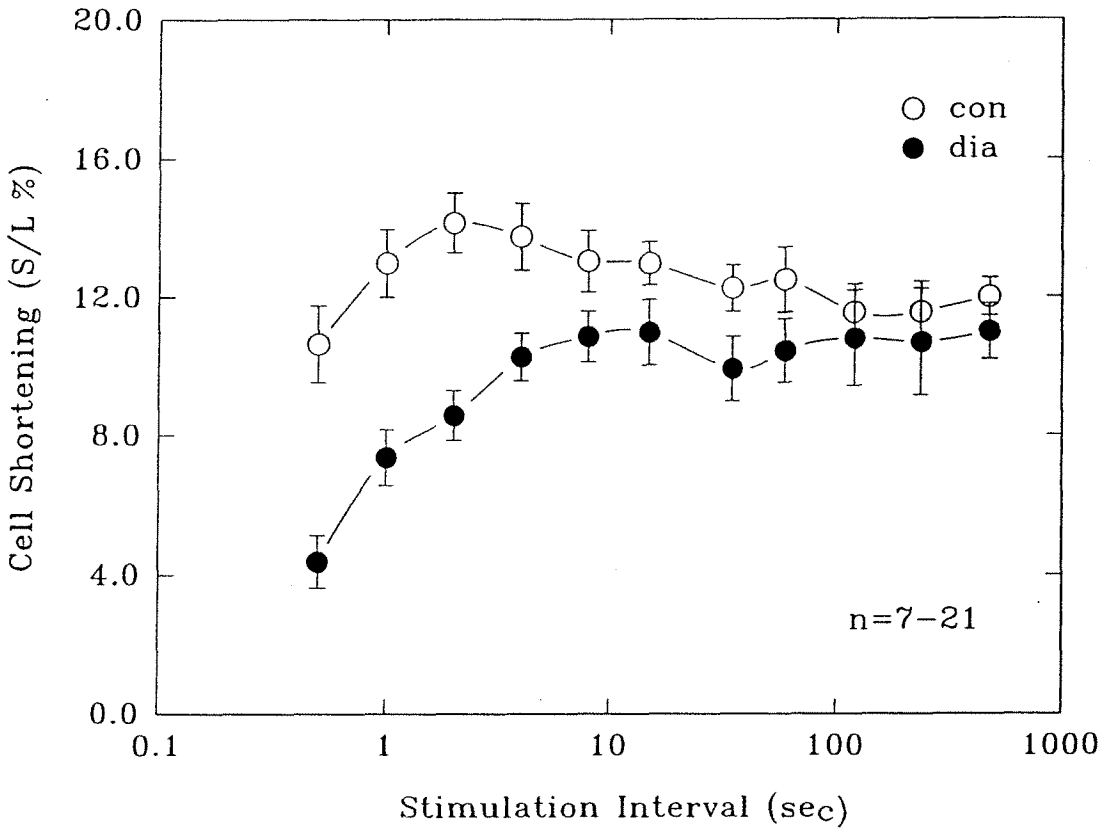

Figure 5. Effects of diabetes on the myocyte shortening-frequency relationship.

Myocytes were superfused with Tyrode buffer (calcium 1.8 mM) at 23°C and field stimulated at 0.5 Hz, 3-8 V, 5 ms stimulation and different stimulation frequencies. Data are expressed as mean ± SE. S/L %: shortened length / resting length %. n: number of cells.

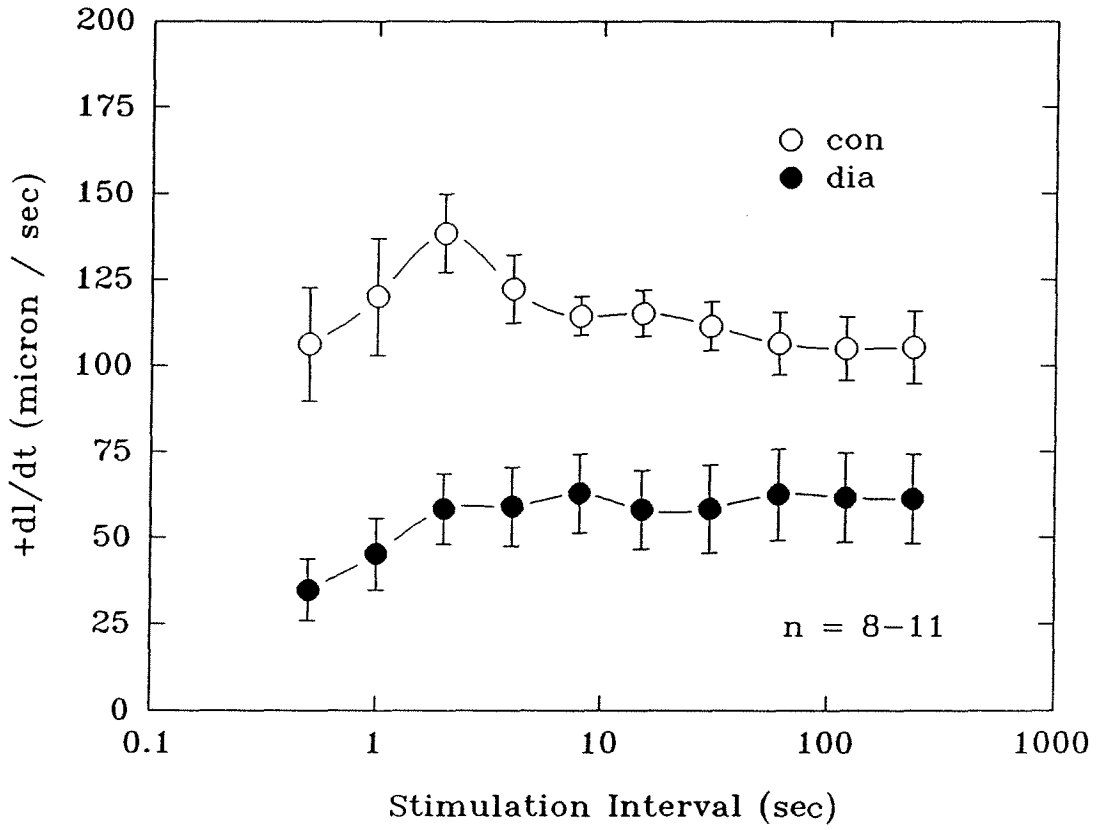

Figure 6. Effects of diabetes on maximum shortening rate of myocytes as a function of frequency.

Myocytes were superfused with Tyrode buffer (calcium 1.8 mM) at 23°C and field stimulated at 0.5 Hz, 3-8 V, 5 ms stimulation and different stimulation frequencies. Data are expressed as mean ± SE. n: number of cells.

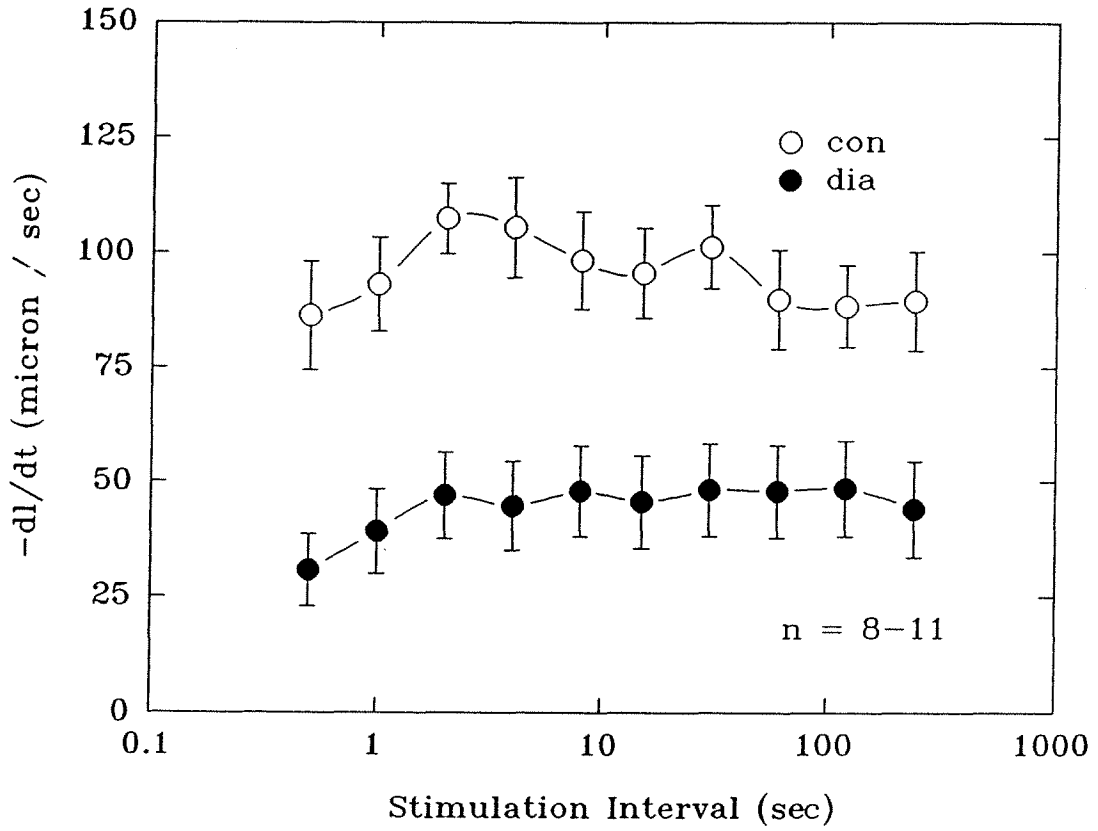

Figure 7. Effects of diabetes on the maximum relengthening rate of myocytes as a function of frequency.

Myocytes were superfused with Tyrode buffer (calcium 1.8 mM) at 23°C and field stimulated at 0.5 Hz, 3-8 V, 5 ms stimulation and different stimulation frequencies. Data are expressed as mean ± SE. n: number of cells.

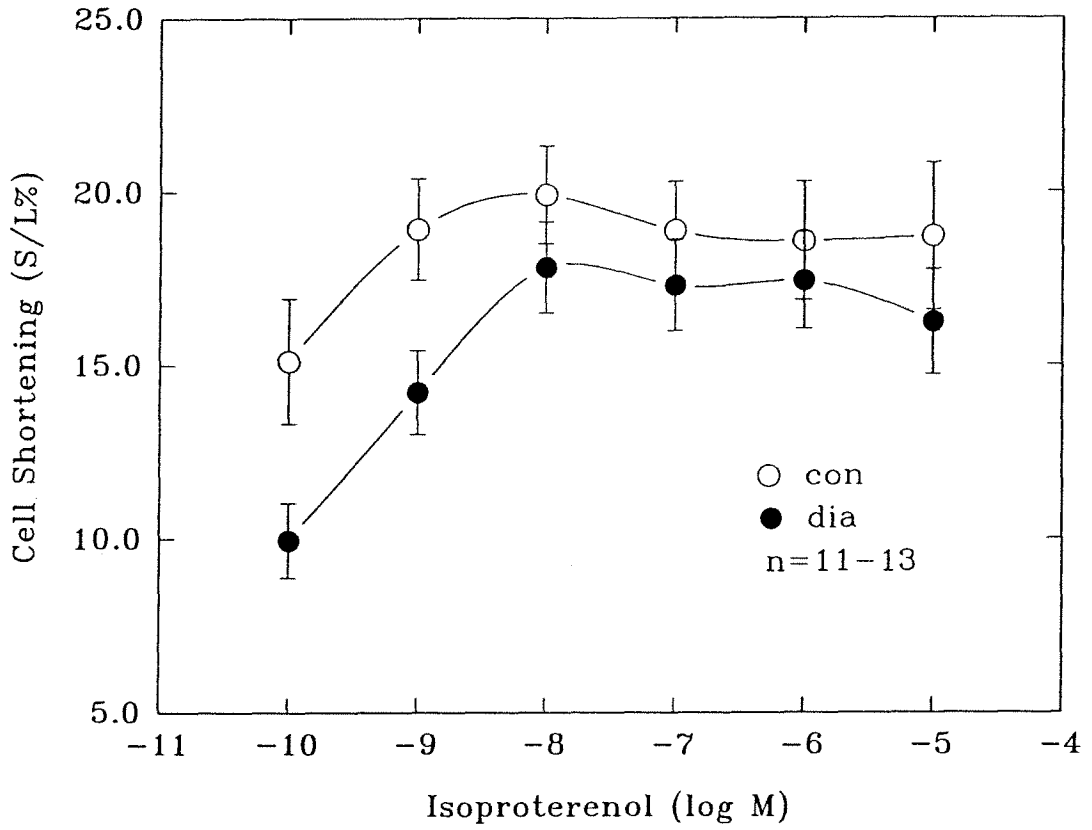

Figure 8. Effects of isoproterenol on cell shortening in control and diabetic myocytes.

Myocytes were superfused with Tyrode buffer (calcium 1.8 mM) at 23°C and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. Data are expressed as mean ± SE. S/L %: shortened length / resting length %. n: number of cells.

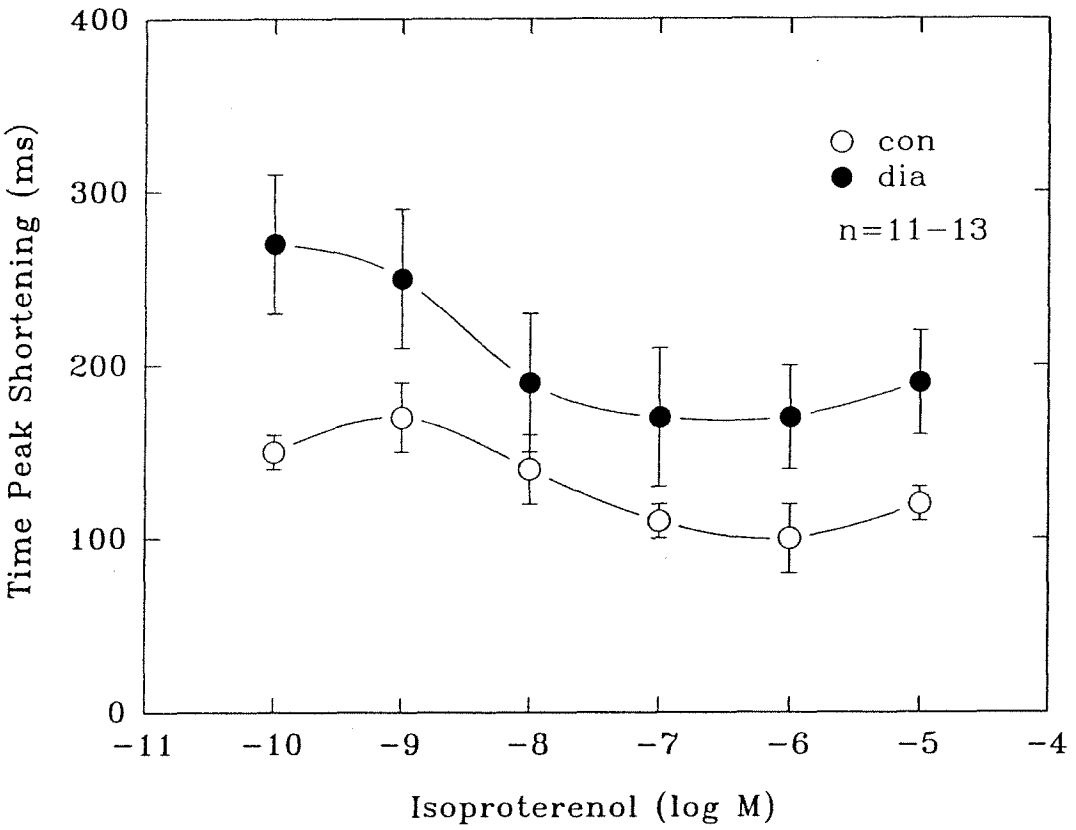

Figure 9. Effects of isoproterenol on time to peak shortening in control and diabetic myocytes.

Myocytes were superfused with Tyrode buffer (calcium 1.8 mM) at 23°C and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. Data are expressed as mean ± SE. S/L %: shortened length / resting length %. n: number of cells.

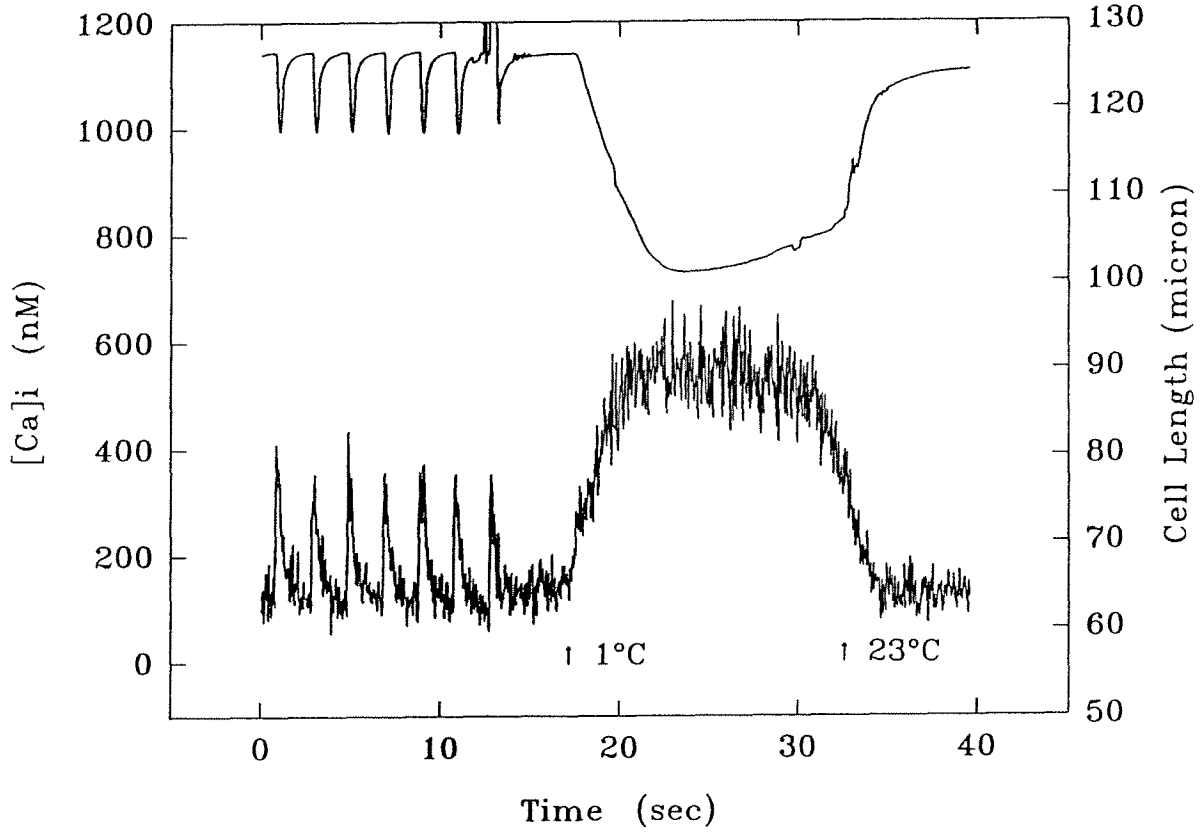

Figure 10. Recording of rapid cooling contracture (RCC) in a control myocyte.

Myocytes were superfused with Tyrode buffer at 23°C and field stimulated at 0.5 Hz, 5 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and indo -1. The normal or cold buffer (1°C) was rapidly (within 1-2 sec) delivered to the cell chamber by the control of two solenoids.

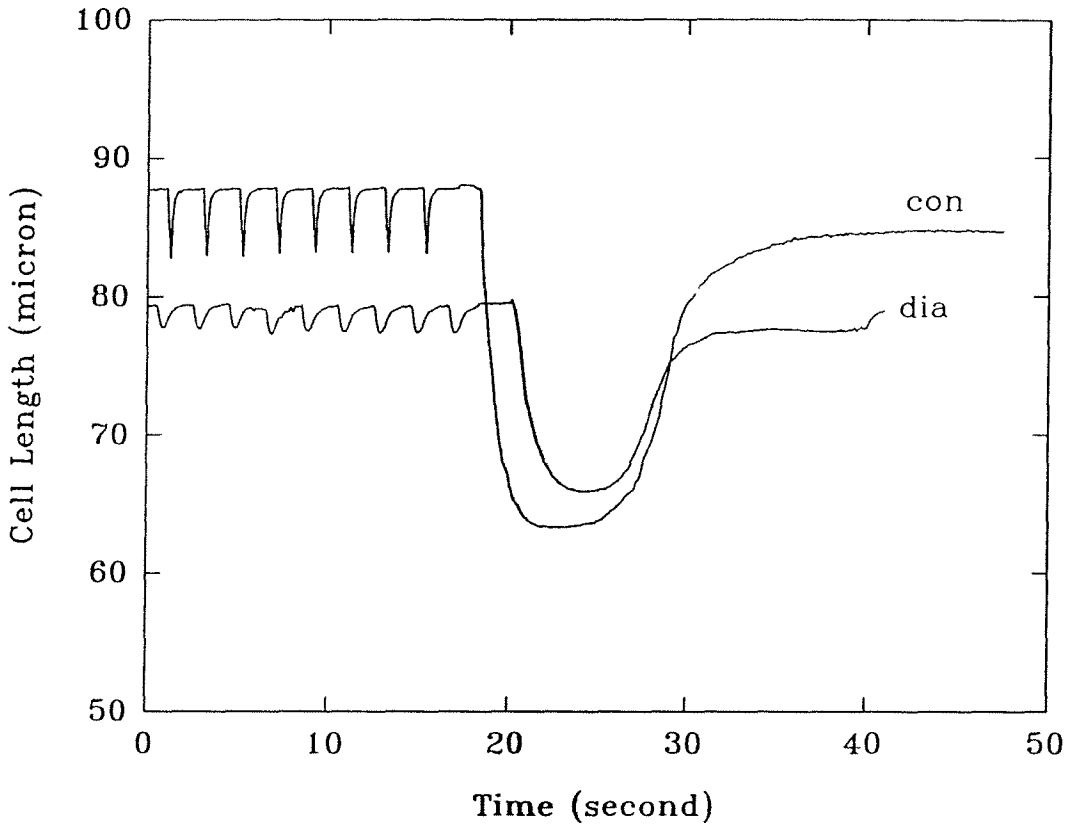

Figure 11. RCCs of control and diabetic myocytes.

Myocytes were superfused with Tyrode buffer at 23°C and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. The normal or cold buffer (1°C) was rapidly (within 1-2 sec) delivered to the cell chamber by the control of two solenoids.

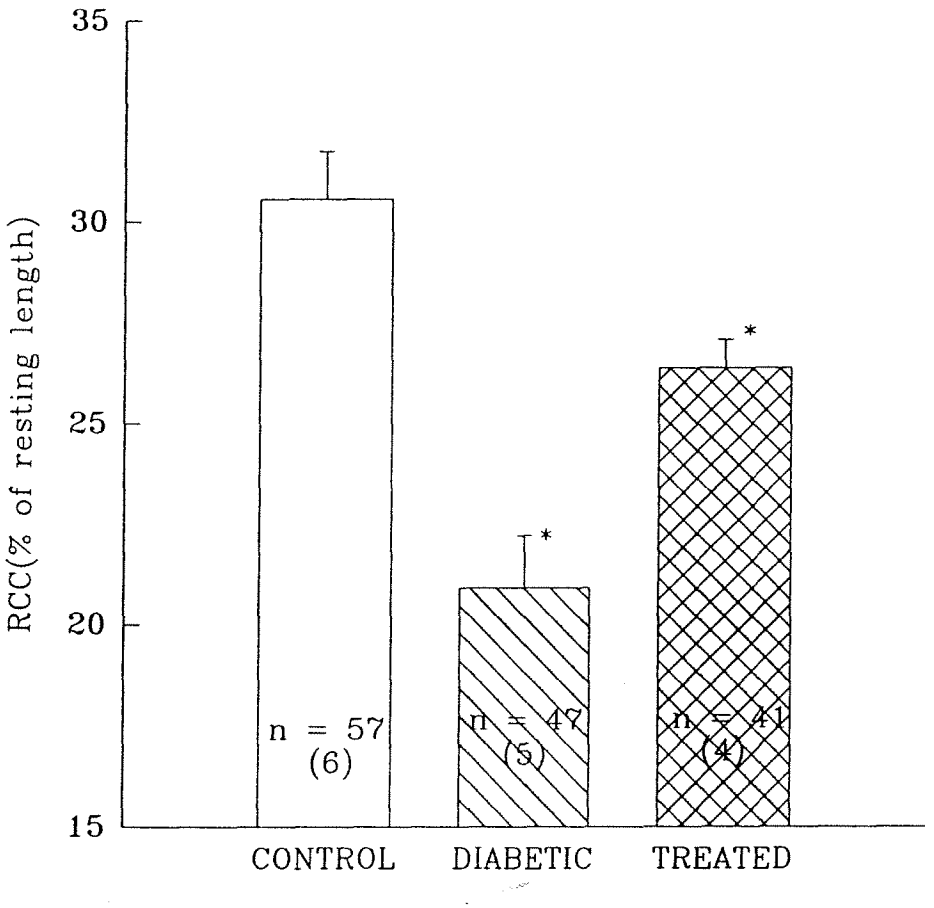

Figure 12. Effects of diabetes and insulin treatment on rapid cooling contractures.

Data are expressed as mean ± SE. * $p < 0.05$ vs controls. n: number of cells. Number of rats for each group is shown in parenthesis.

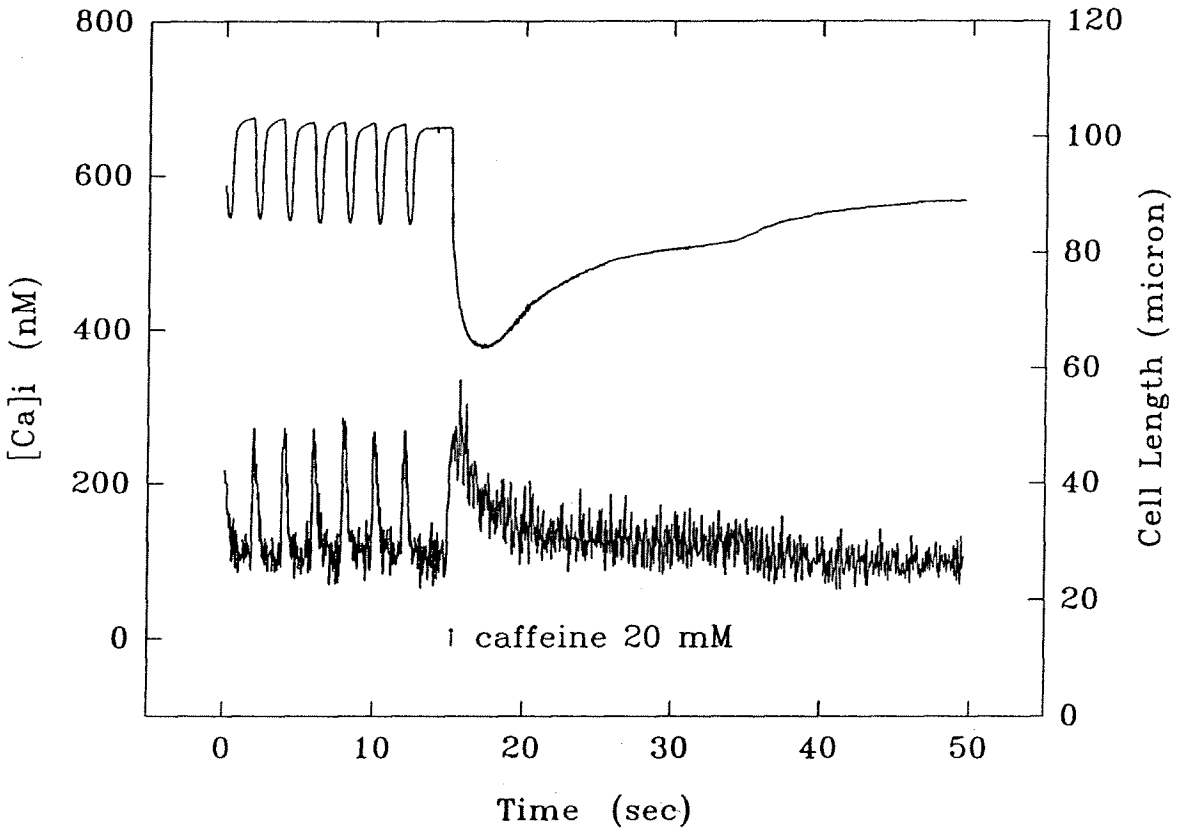

Figure 13. Recording of caffeine contracture (CC).

Myocytes were superfused with Tyrode buffer at 23°C and field stimulated at 0.5 Hz, 5 V, and 5 ms stimulation duration. The normal buffer or caffeine (20 mM) was rapidly (within 1-2 sec) delivered to the cell chamber by the control of two solenoids.

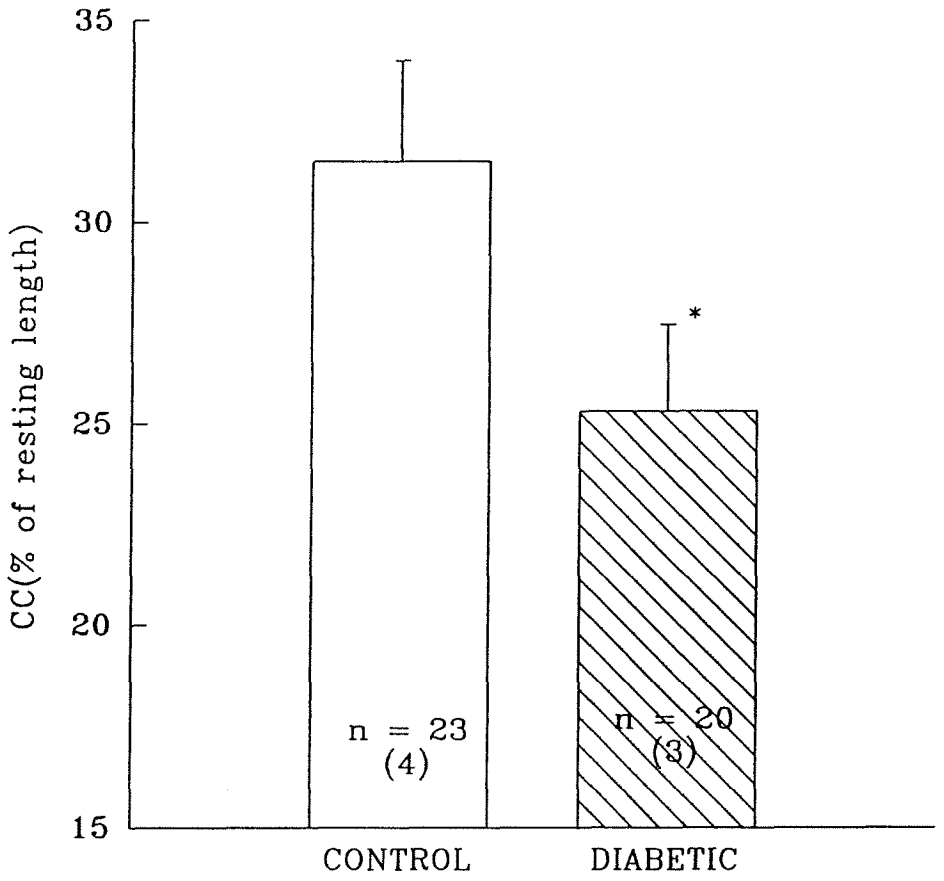

Figure 14. Effects of diabetes on caffeine contracture.

Data are expressed as mean ± SE. * $p < 0.05$ vs controls. n: number of cells. Number of rats for each group is shown in parenthesis.

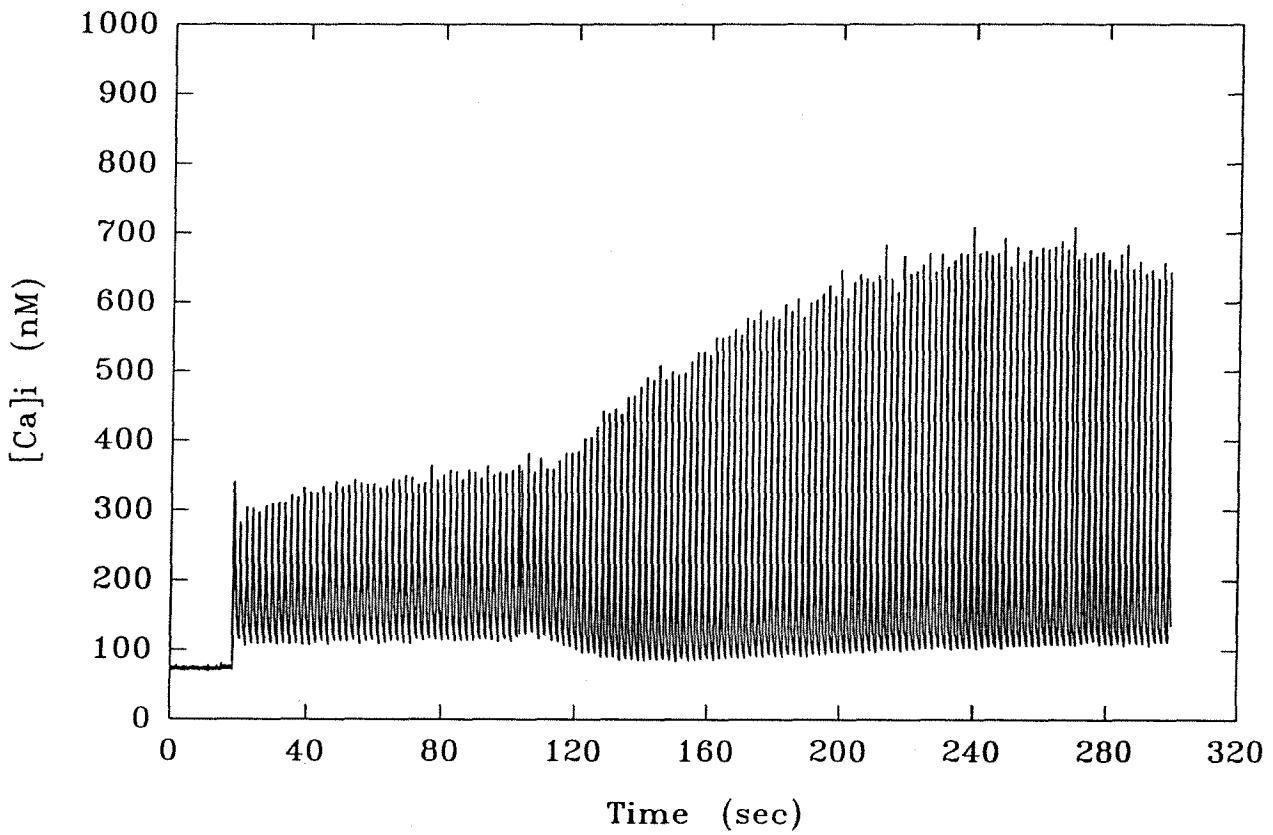

Figure 15. Isoproterenol (1 nM) induced $[Ca^{2+}]_i$ increase in an electrically-stimulated myocyte.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 4 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Isoproterenol (1 nM) was added to the media at 105 sec, which caused $[Ca^{2+}]_i$ change in the cell.

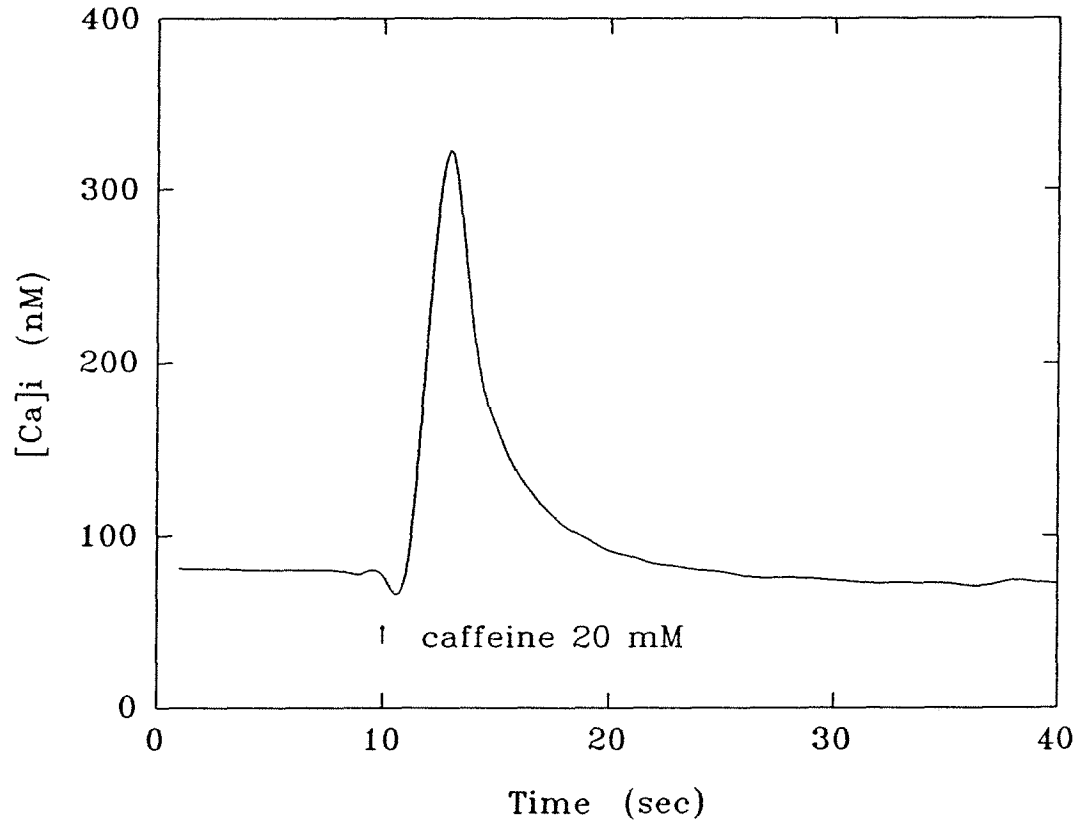

Figure 16. Response of $[Ca^{2+}]_i$ to caffeine (20 mM) in a quiescent control myocyte.

Representative of 10 cells. Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

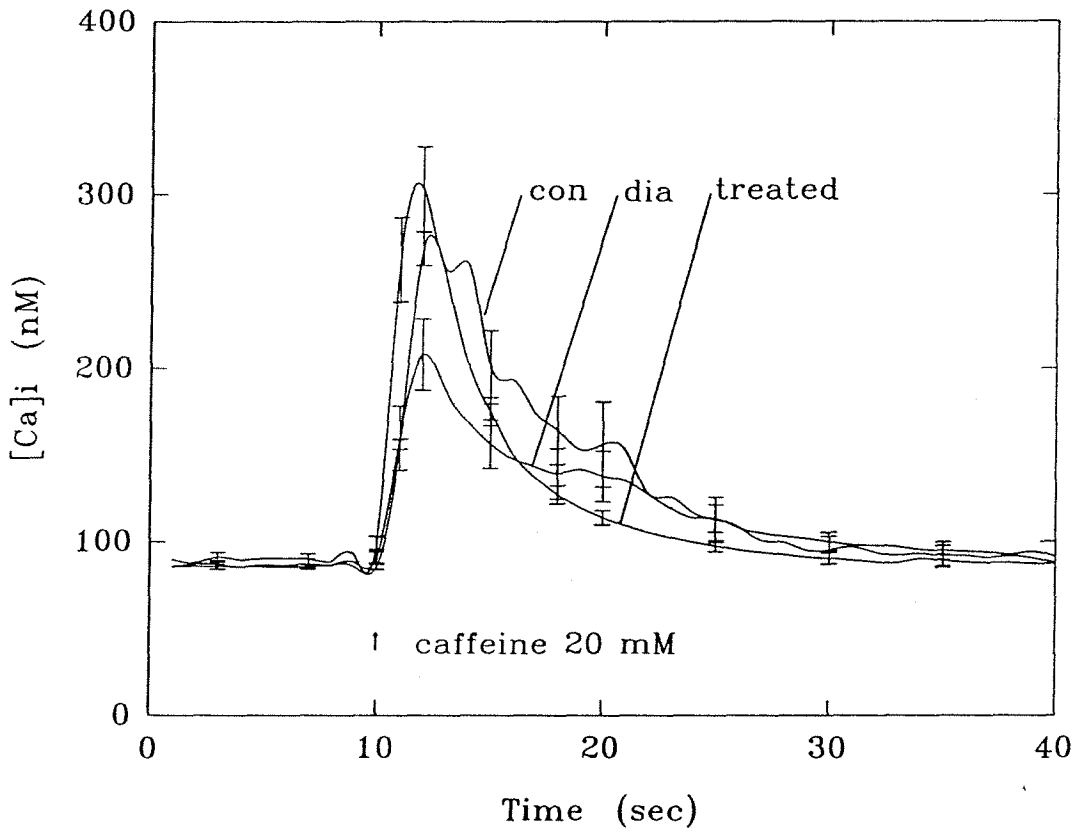

Figure 17. Response of $[Ca^{2+}]_i$ to caffeine in quiescent myocytes from control, diabetic and insulin treated diabetic rats.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Data are expressed as mean ± SE. The ratios of $[Ca^{2+}]_i$ decline ($d[Ca^{2+}]_i/dt$) for each group are listed in 'Results'. con: control, n=22(4 rats), dia: diabetic, n=16(4), treated: insulin treated diabetic, n=13(3).

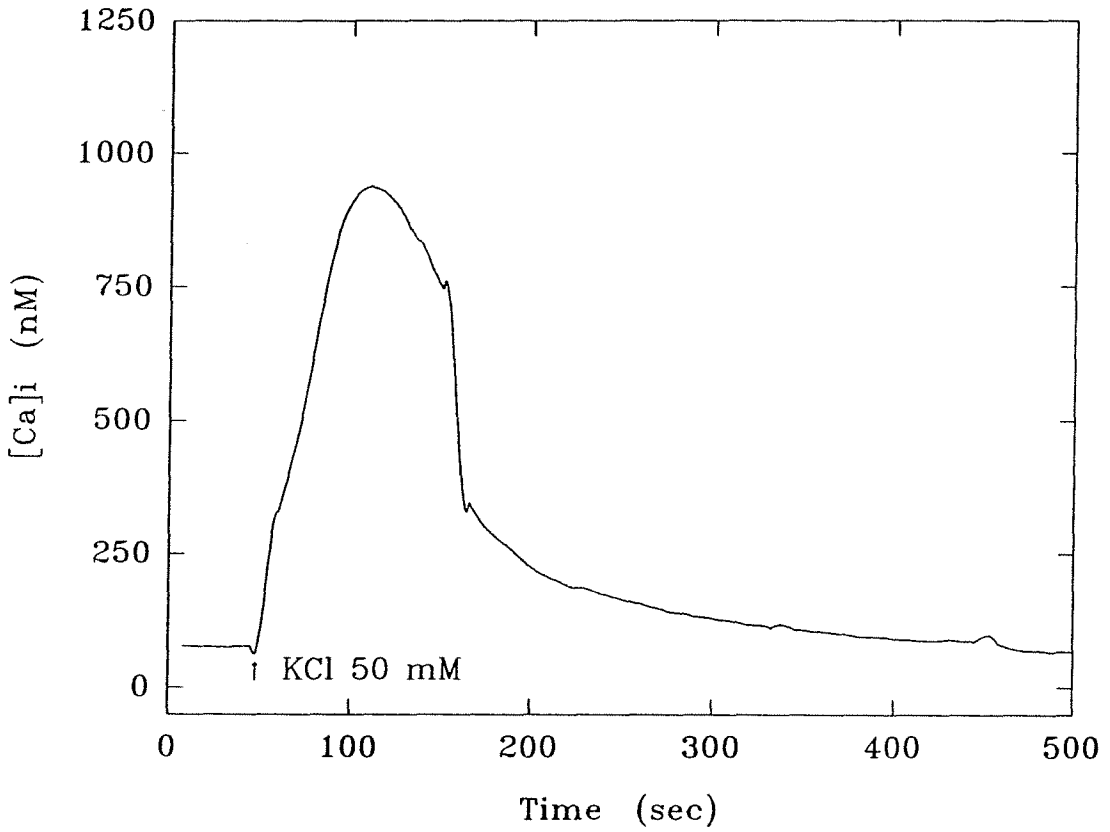

Figure 18. Response of $[Ca^{2+}]_i$ to KCl (50 mM) in a quiescent control myocyte .

Representative of 6 cells. Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

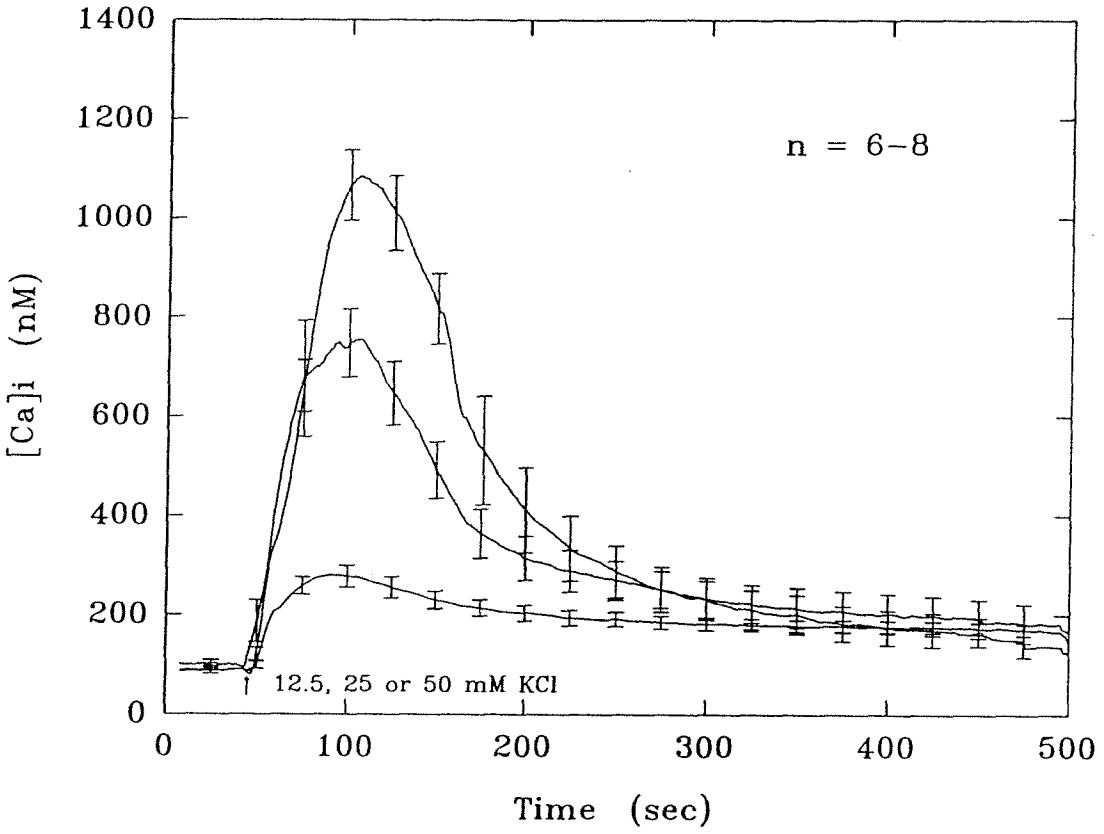

Figure 19. Response of $[Ca^{2+}]_i$ to KCl (12.5-50 mM) in quiescent control myocytes .

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. n: number of cells.

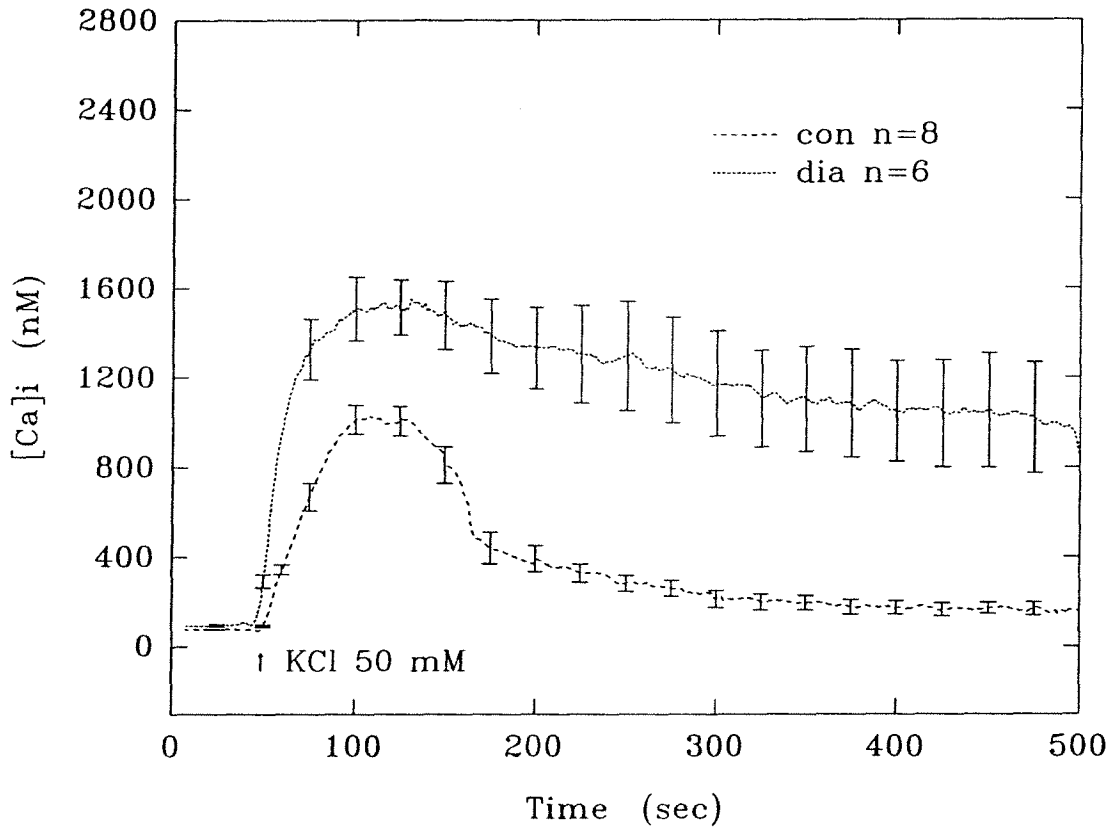

Figure 20. Response of $[Ca^{2+}]_i$ to KCl (50 mM) in quiescent myocytes from control and diabetic rats.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. n: number of cells.

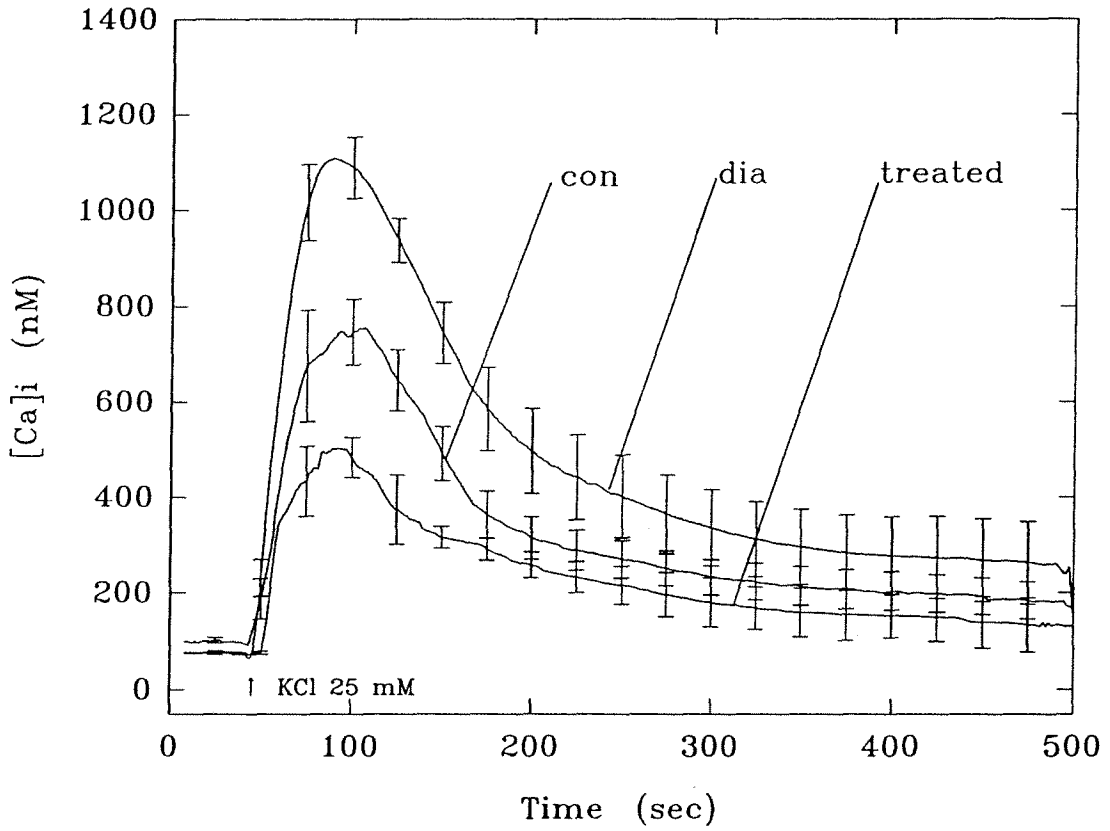

Figure 21. Response of $[Ca^{2+}]_i$ to KCl (25 mM) in quiescent myocytes from control, diabetic and insulin treated diabetic rats.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Data are expressed as mean ± SE, con: control, n=8(3 rats), dia: diabetic, n=7(3), treated: insulin treated diabetic, n=14(3).

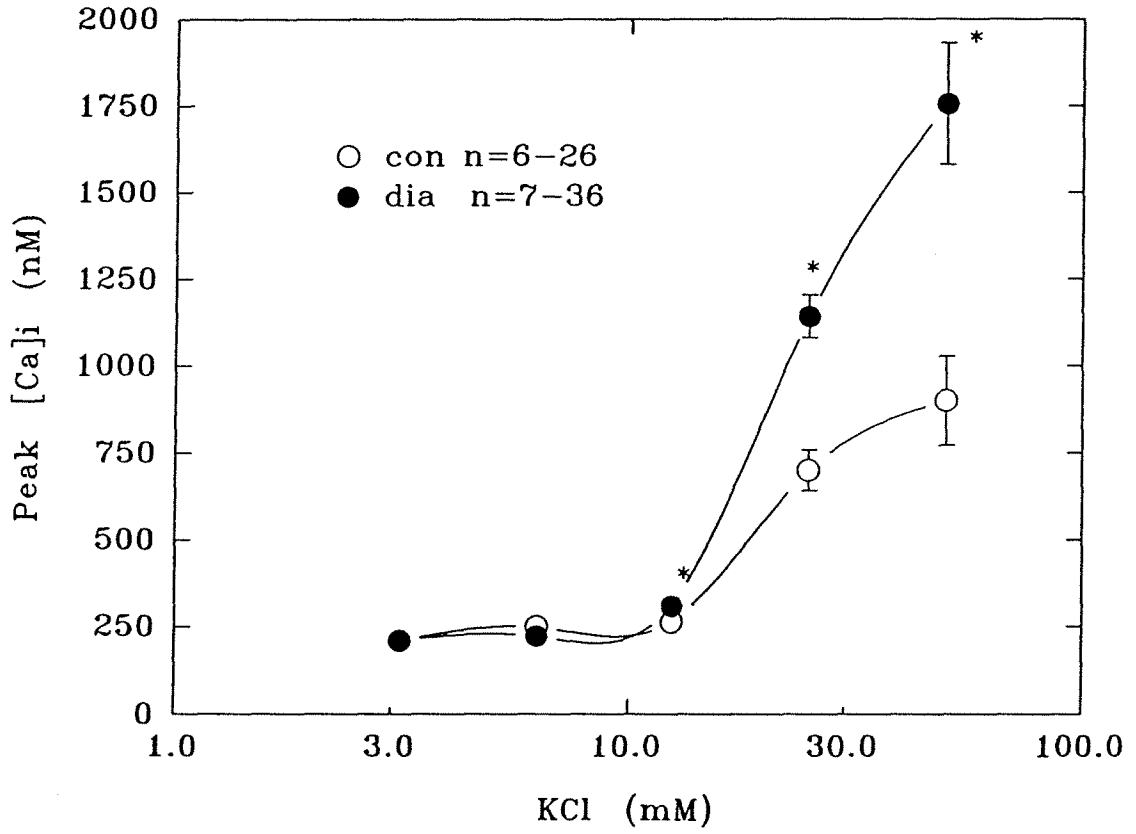

Figure 22. Dose-response curves of $[Ca^{2+}]_i$ to KCl (3.125-50 mM) in quiescent myocytes from control and diabetic rats.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. n: number of cells.

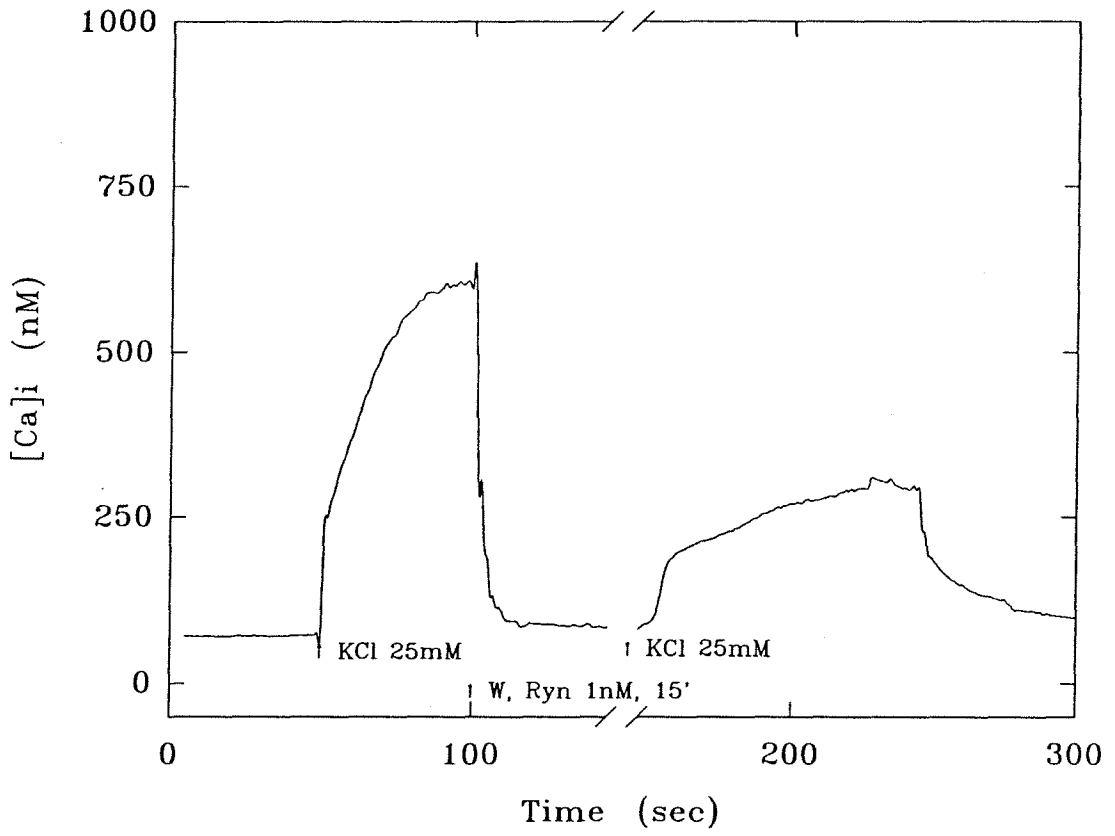

Figure 23. Effects of ryanodine on KCl induced $[Ca^{2+}]_i$ transient in a quiescent control myocyte.

Representative of 5 cells. Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Please note the two phase response to KCl 25 mM which was different from Figure 24. Cells were incubated with ryanodine (Ryn) for 15 min before the second KCl stimulation. W: wash.

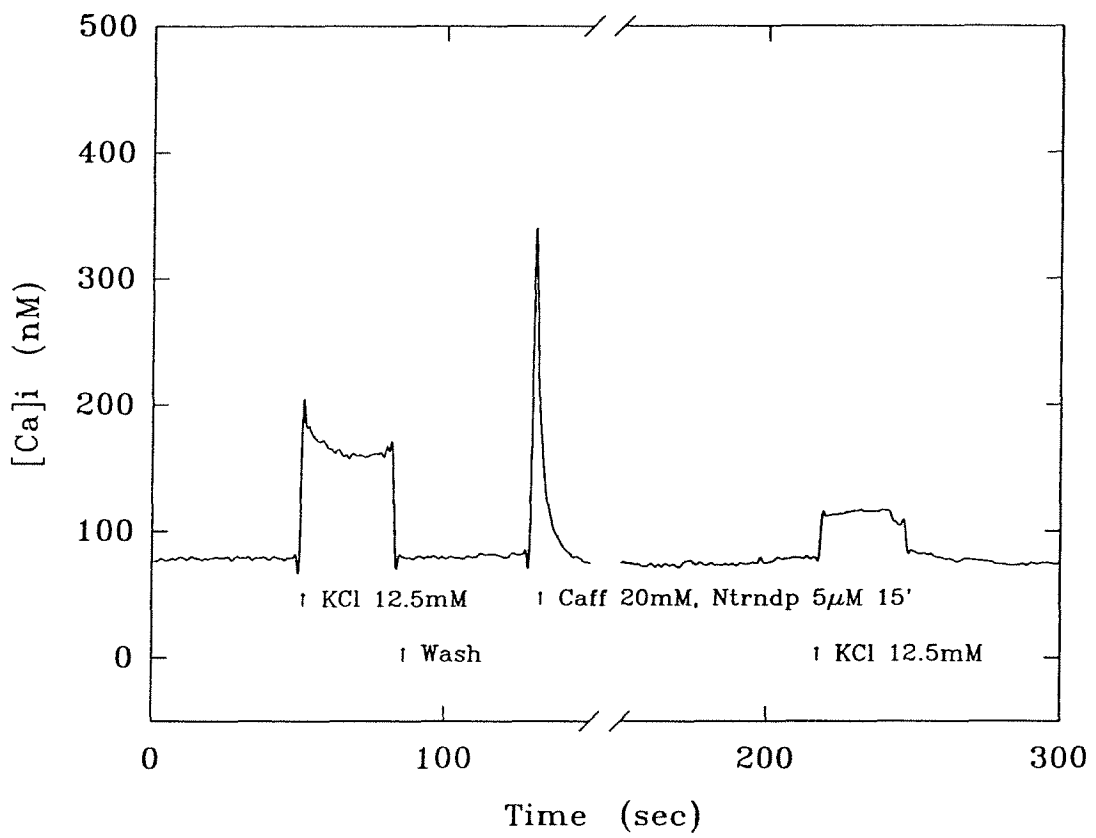

Figure 24. Effects of caffeine and nitrendipine on KCl induced $[Ca^{2+}]_i$ transient in a quiescent control myocyte.

Representative of 8 cells. Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Cells were incubated with caffeine (Caff) and nitrendipine (Ntrndp) for 15 min before the second KCl stimulation.

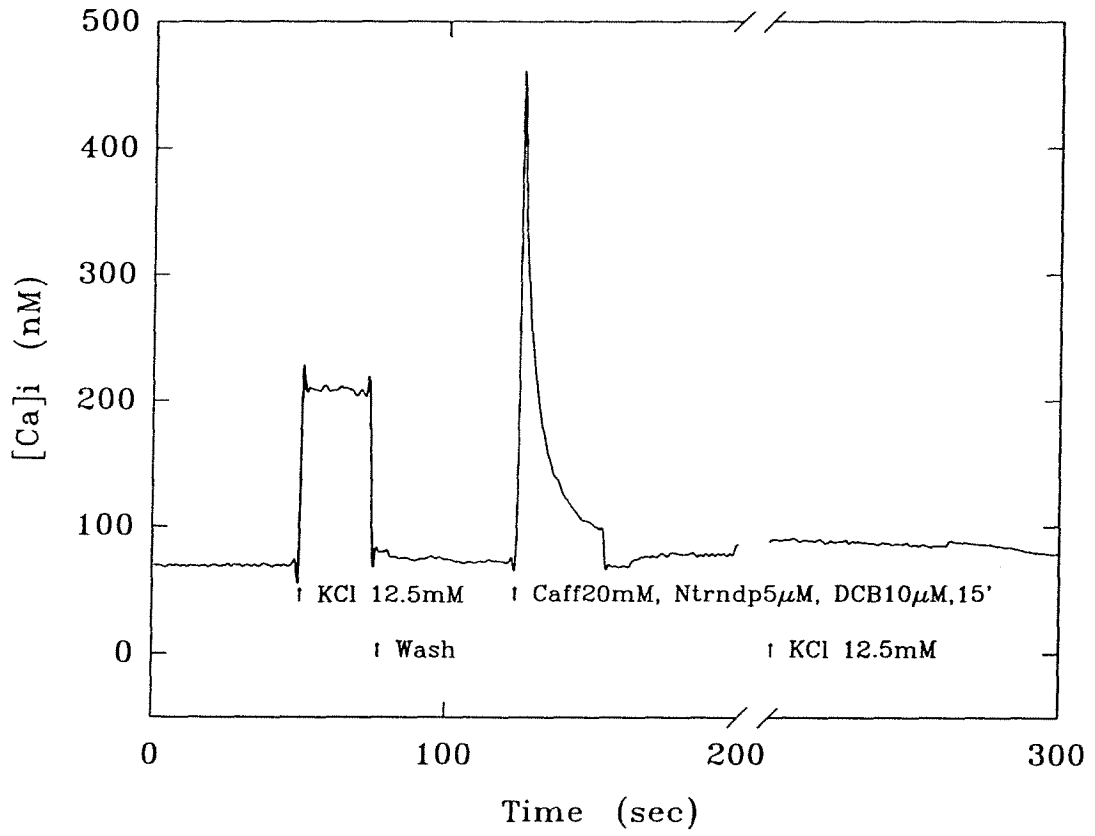

Figure 25. Effects of caffeine, nitrendipine and dichlorobenzamil on KCl induced $[Ca^{2+}]_i$ transient in a quiescent control myocyte.

Representative of 8 cells. Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Cells were incubated with caffeine (Caff), nitrendipine (Ntrndp) and dichlorobenzamil (DCB) for 15 min before the second KCl stimulation.

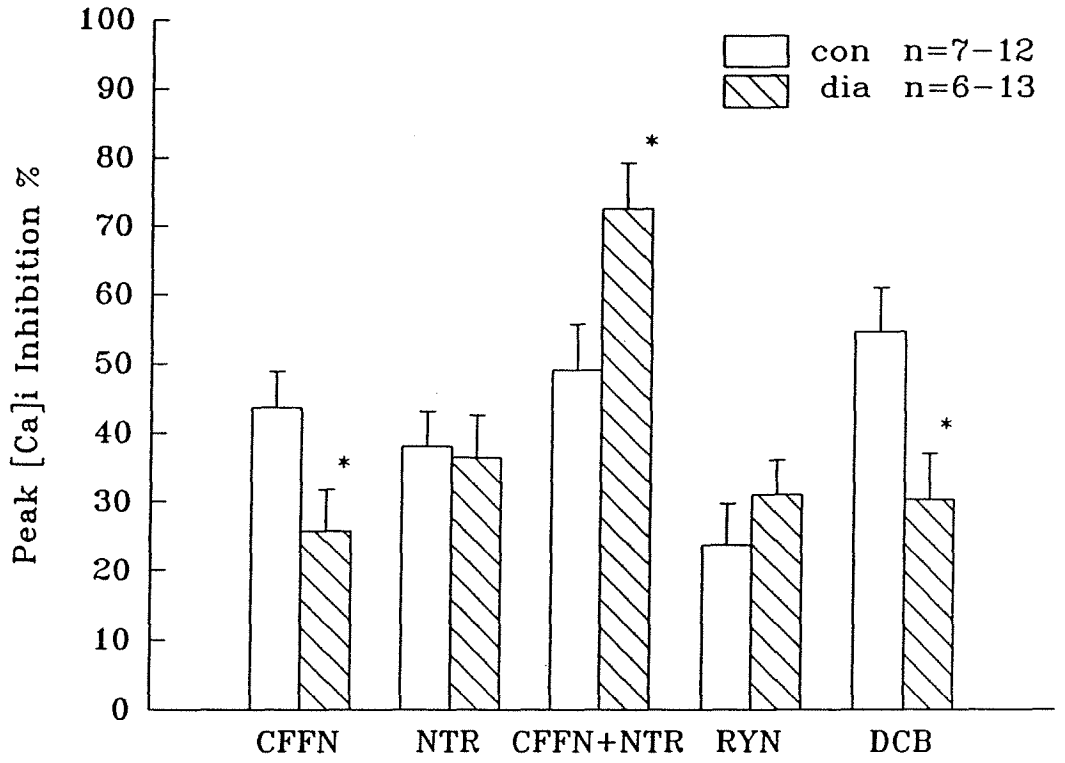

Figure 26. Effects of blockers on KCl (12.5 mM) induced $[Ca^{2+}]_i$ transient in quiescent myocytes from control and diabetic rats.

con: control, dia: diabetic, CFFN: caffeine (20 mM), NTR: nitrendipine (5 μM), RYN: ryanodine (1 nM), DCB: dichlorobenzamil (10 μM). Data are expressed as mean ± SE. * $p < 0.05$ vs controls.

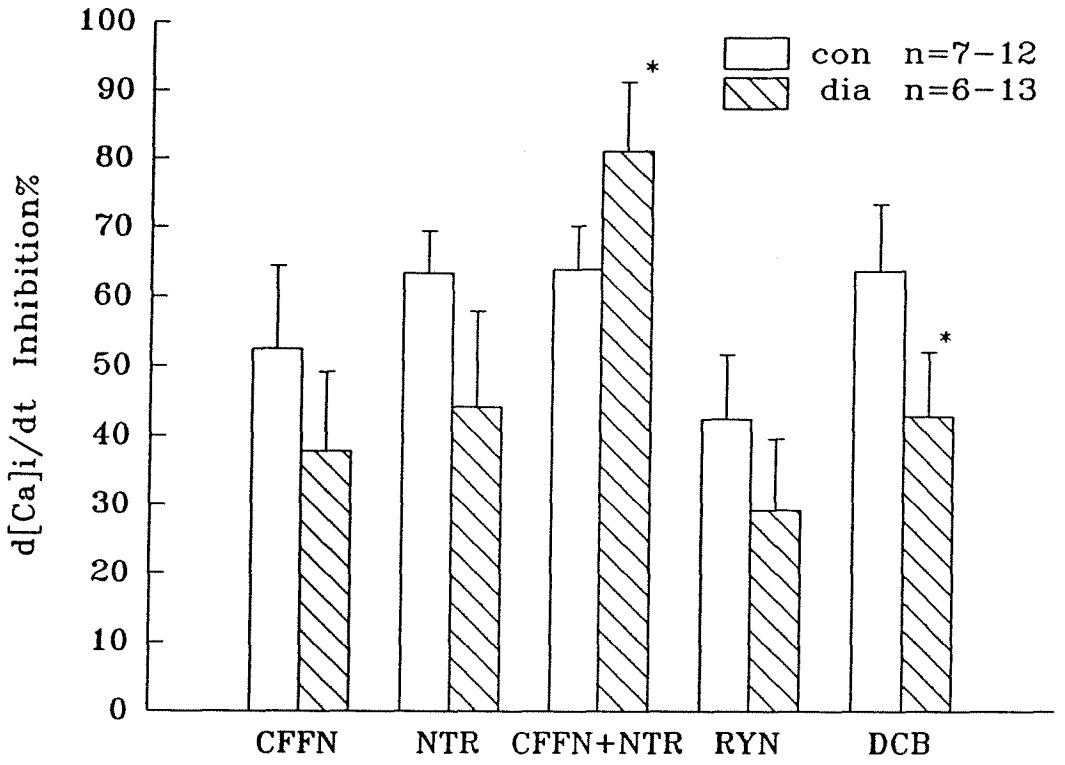

Figure 27. Effects of blockers on KCl (12.5 mM) induced $[Ca^{2+}]_i$ influx rate ($d[Ca^{2+}]_i/dt$) in quiescent myocytes from control and diabetic rats.

con: control, dia: diabetic, CFFN: caffeine (20 mM), NTR: nitrendipine (5 μM), RYN: ryanodine (1 nM), DCB: dichlorobenzamil (10 μM). Data are expressed as mean ± SE. * $p < 0.05$ vs controls.

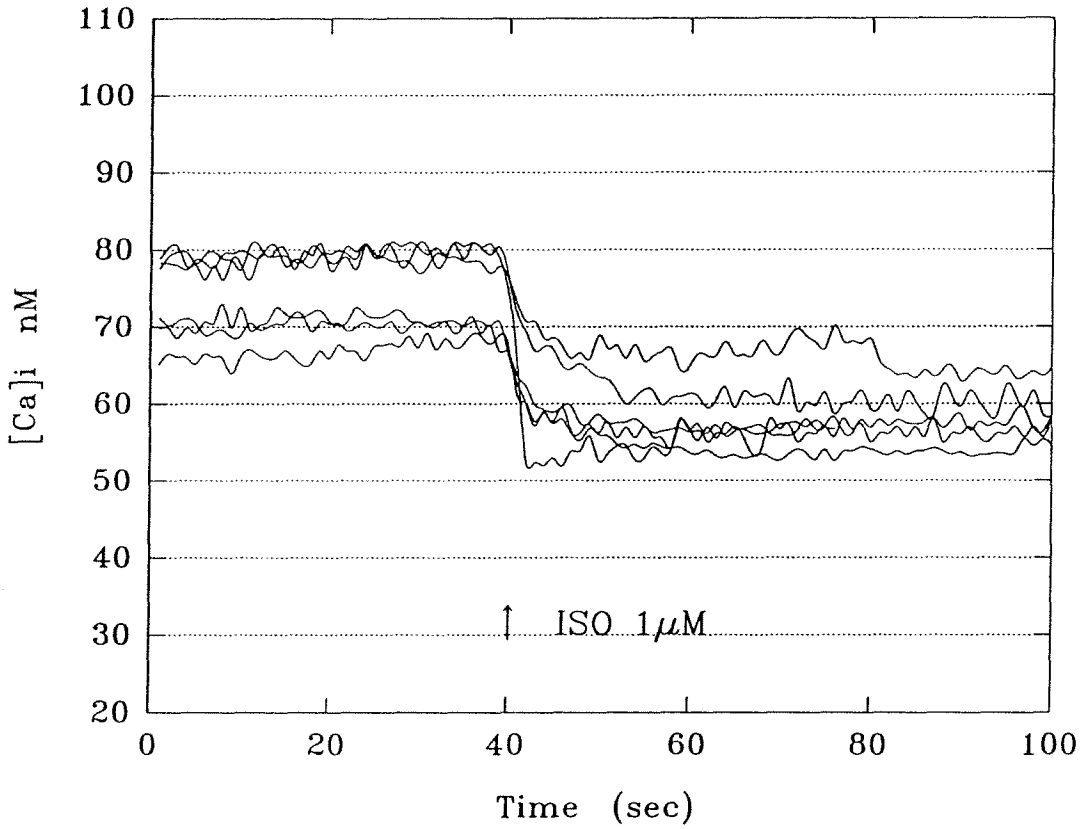

Figure 28. Isoproterenol-induced $[Ca^{2+}]_i$ changes in quiescent control myocytes

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Tracings were from six individual cells. ISO: isoproterenol.

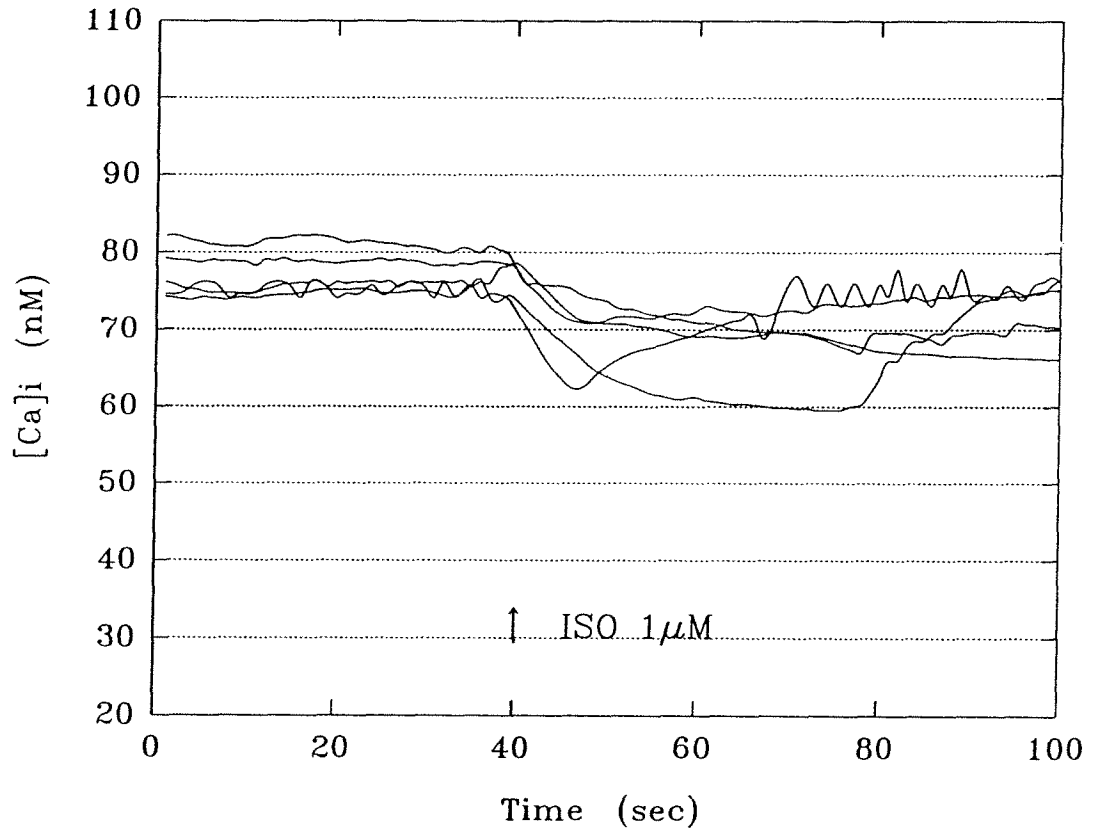

Figure 29. Isoproterenol-induced $[Ca^{2+}]_i$ changes in quiescent diabetic myocytes.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Tracings were from five individual cells. ISO: isoproterenol.

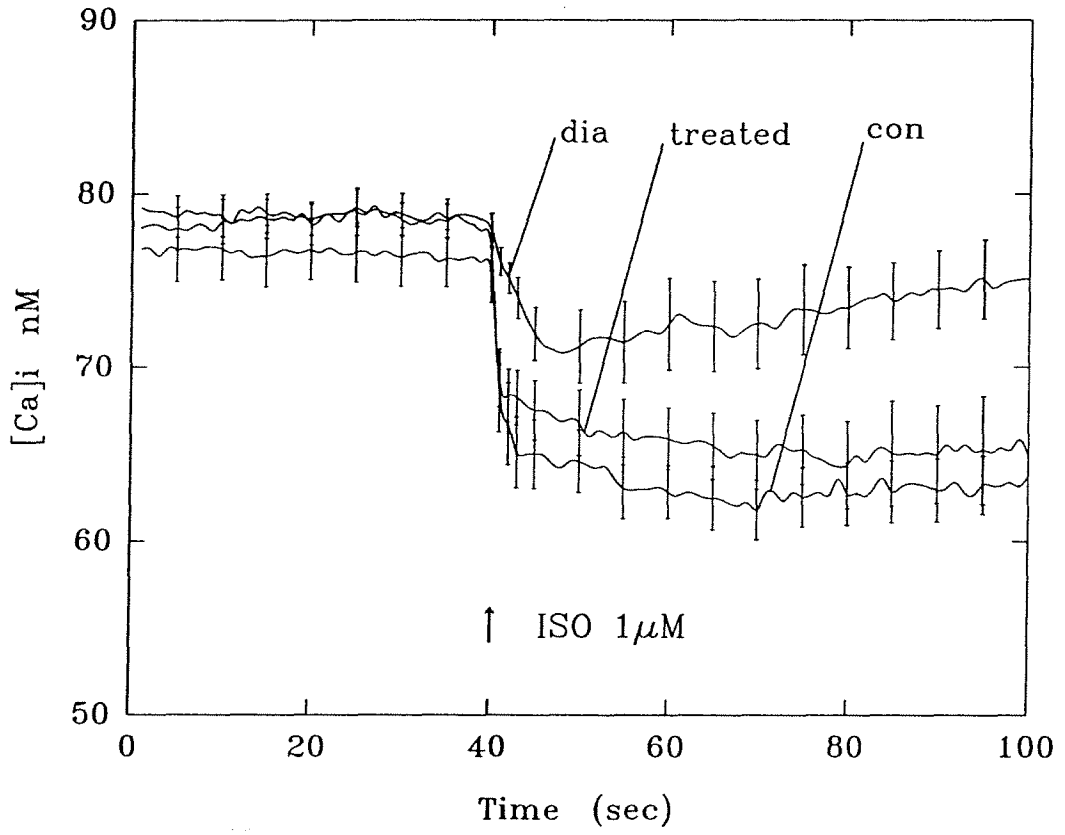

Figure 30. Response of $[Ca^{2+}]_i$ to isoproterenol (1 μM) in quiescent myocytes from control, diabetic and insulin treated diabetic rats.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Data are expressed as mean ± SE. con: control, n=16(4 rats), dia: diabetic, n=14(4), treated: insulin treated diabetic, n=20(5). ISO: isoproterenol.

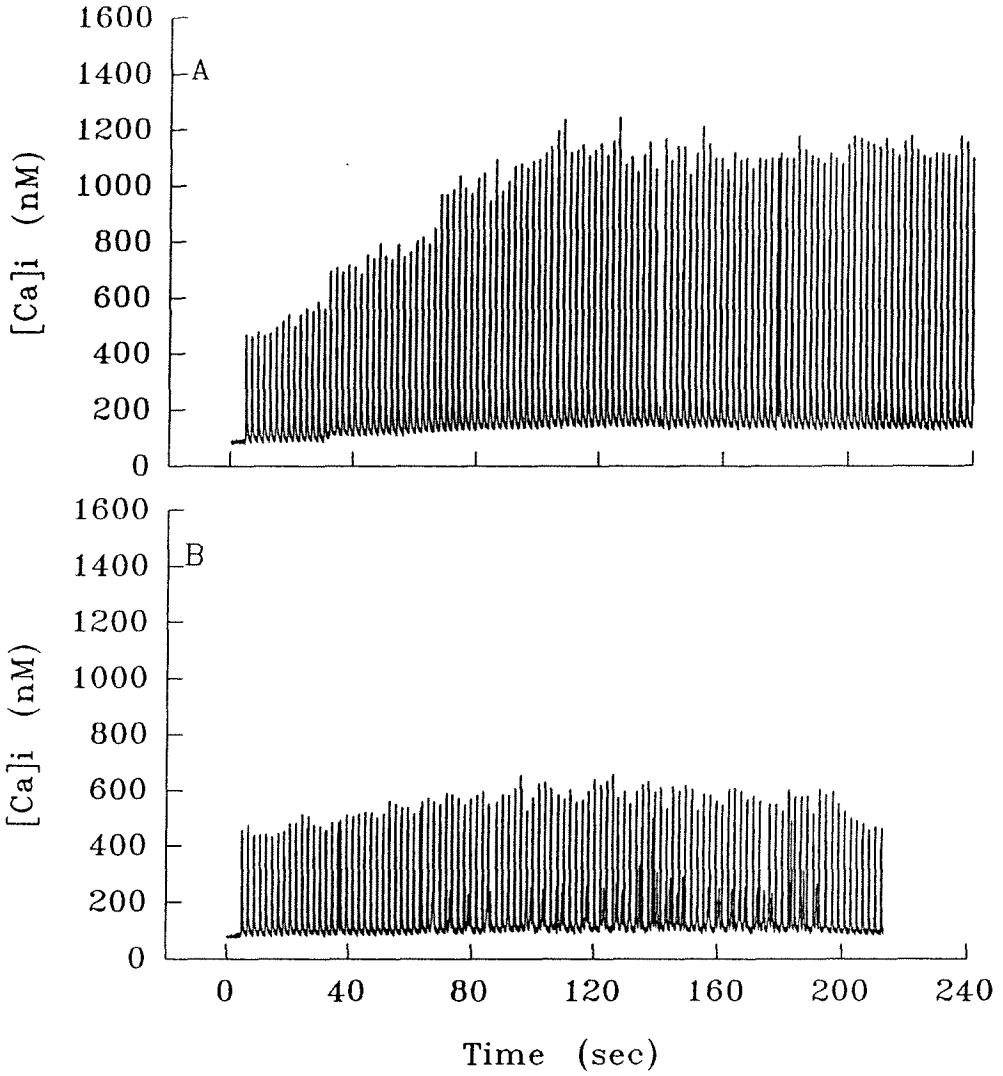

Figure 31. Isoproterenol-induced $[Ca^{2+}]_i$ increase in electrically-stimulated myocytes.

$[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. A: a control myocyte. B: a diabetic myocyte. In A, isoproterenol 10^{-15} M was added at time 20 sec. Then the concentration of isoproterenol was increased gradually to 10^{-8} M at 230 sec. In B, isoproterenol 10^{-9} M was added at time 18 sec. Then the concentration of cAMP was increased gradually to 10^{-6} M at 215 sec.

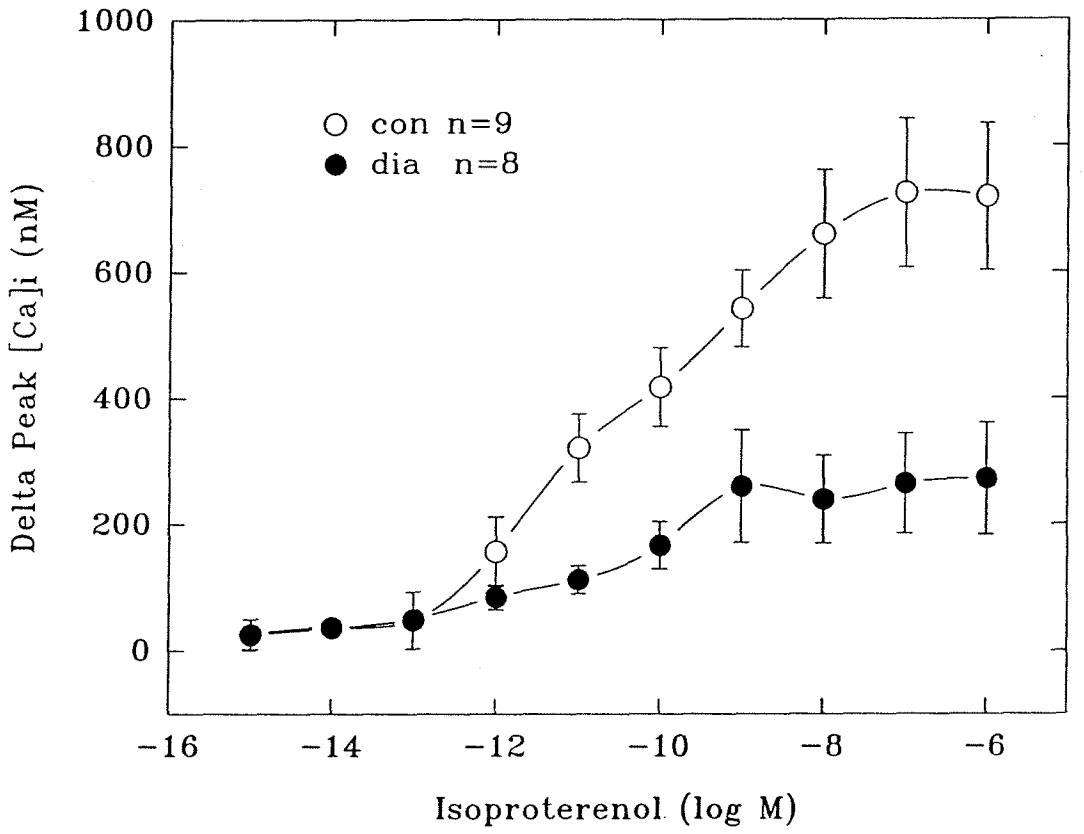

Figure 32. Isoproterenol dose-response curves obtained in stimulated control and diabetic myocytes. $[Ca^{2+}]_i$ increase as a function of the concentration of isoproterenol.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Data are expressed as mean ± SE. con: control, n=9(3), dia: diabetic, n=8(3).

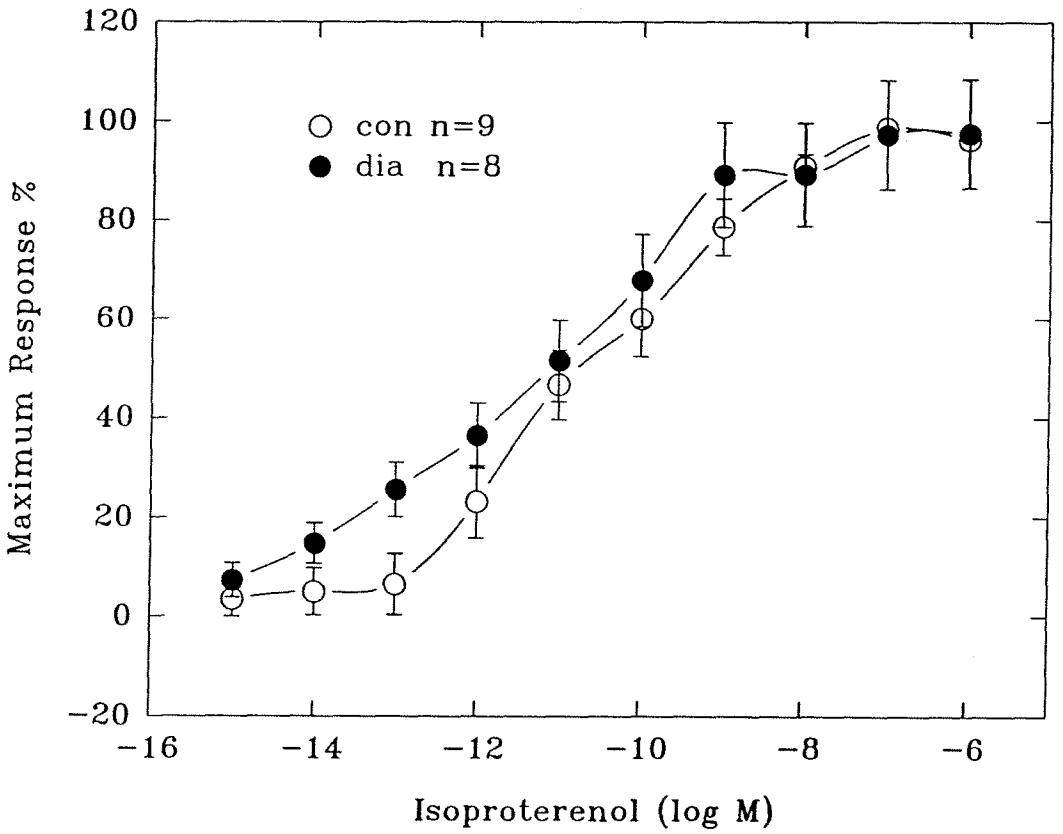

Figure 33. Isoproterenol dose-response curves obtained in stimulated control and diabetic myocytes. Percentage of the maximum response of $[Ca^{2+}]_i$ increase as a function of the concentration of isoproterenol.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. Data are expressed as mean ± SE. con: control, n=9(3), dia: diabetic, n=8(3).

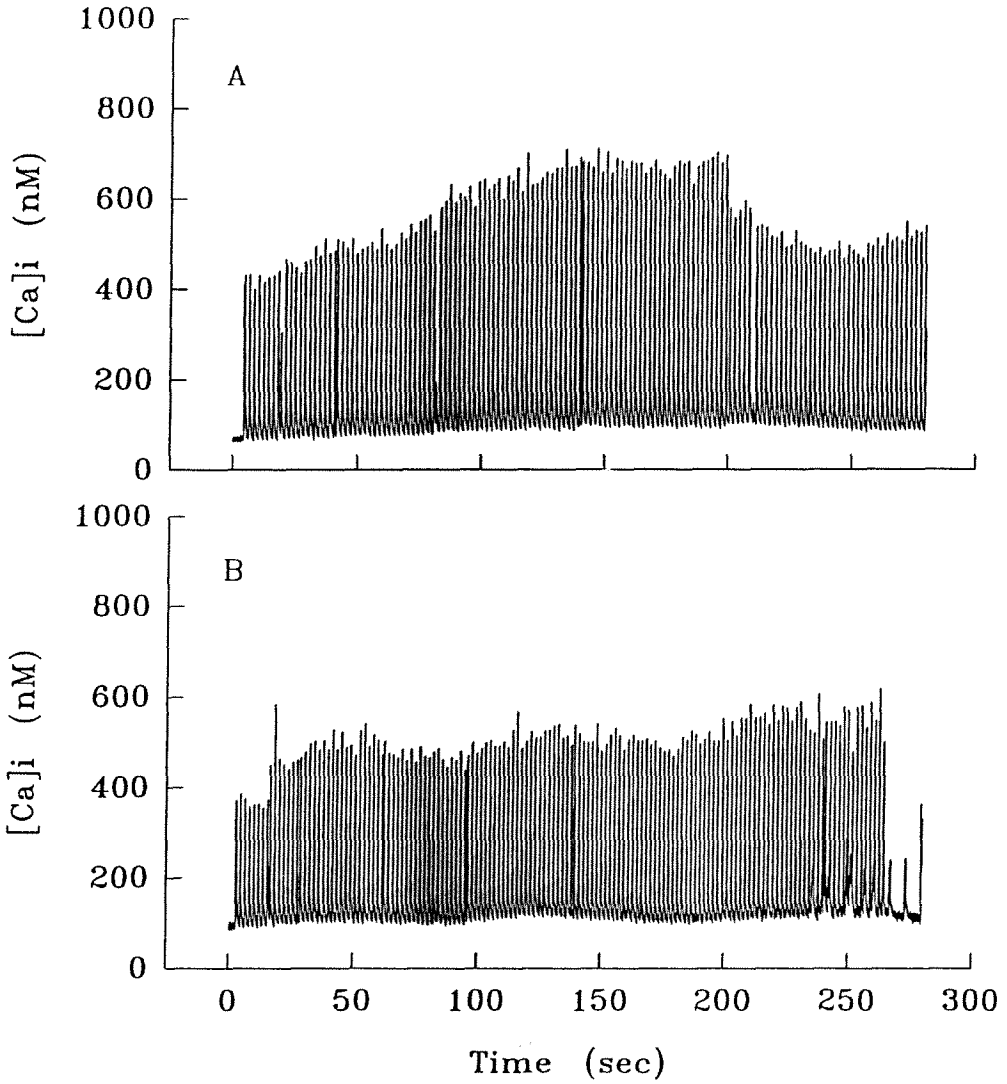

Figure 34. 8-bromo-cAMP-induced $[Ca^{2+}]_i$ increase in electrically-stimulated myocytes.

$[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. A: a control myocyte. B: a diabetic myocyte. In A, 8-bromo cAMP 10^{-7} M was added at time 18 sec. Then the concentration of 8-bromo cAMP was increased gradually to 10^{-4} M at 210 sec. In B, cAMP 10^{-9} M was added at time 15 sec. Then the concentration of cAMP was increased gradually to 10^{-3} M at 240 sec.

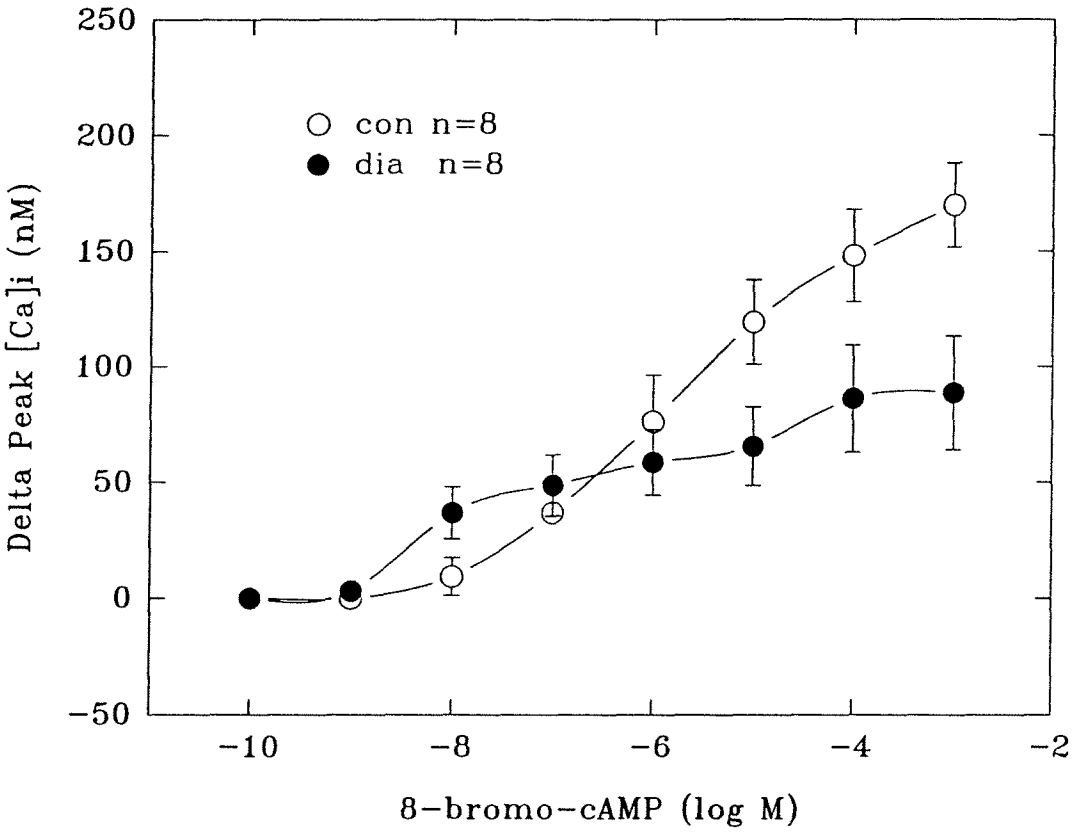

Figure 35. 8-bromo-cAMP dose-response curves obtained in stimulated control and diabetic myocytes. $[Ca^{2+}]_i$ increase as a function of the concentration of 8-bromo-cAMP

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

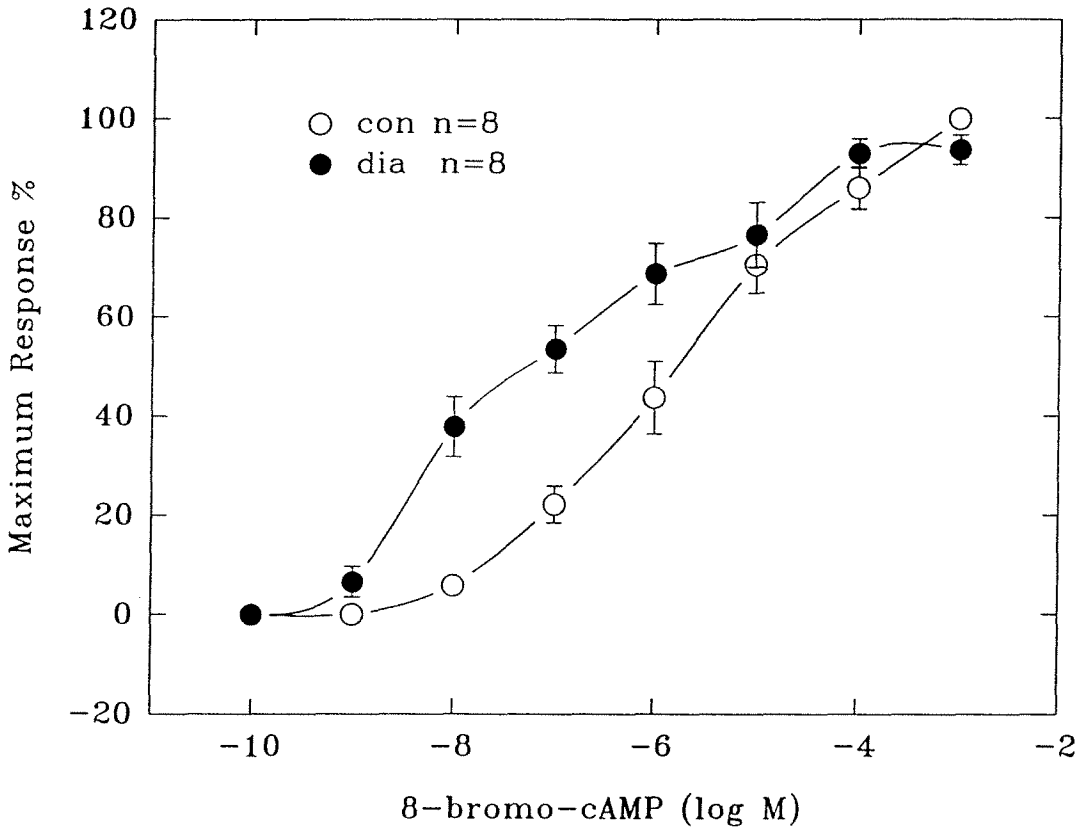

Figure 36. 8-bromo-cAMP dose-response curves obtained in stimulated control and diabetic myocytes. Percentage of the maximum response of $[Ca^{2+}]_i$ increase as a function of the concentration of 8-bromo-cAMP

Myocytes were superfused with 199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

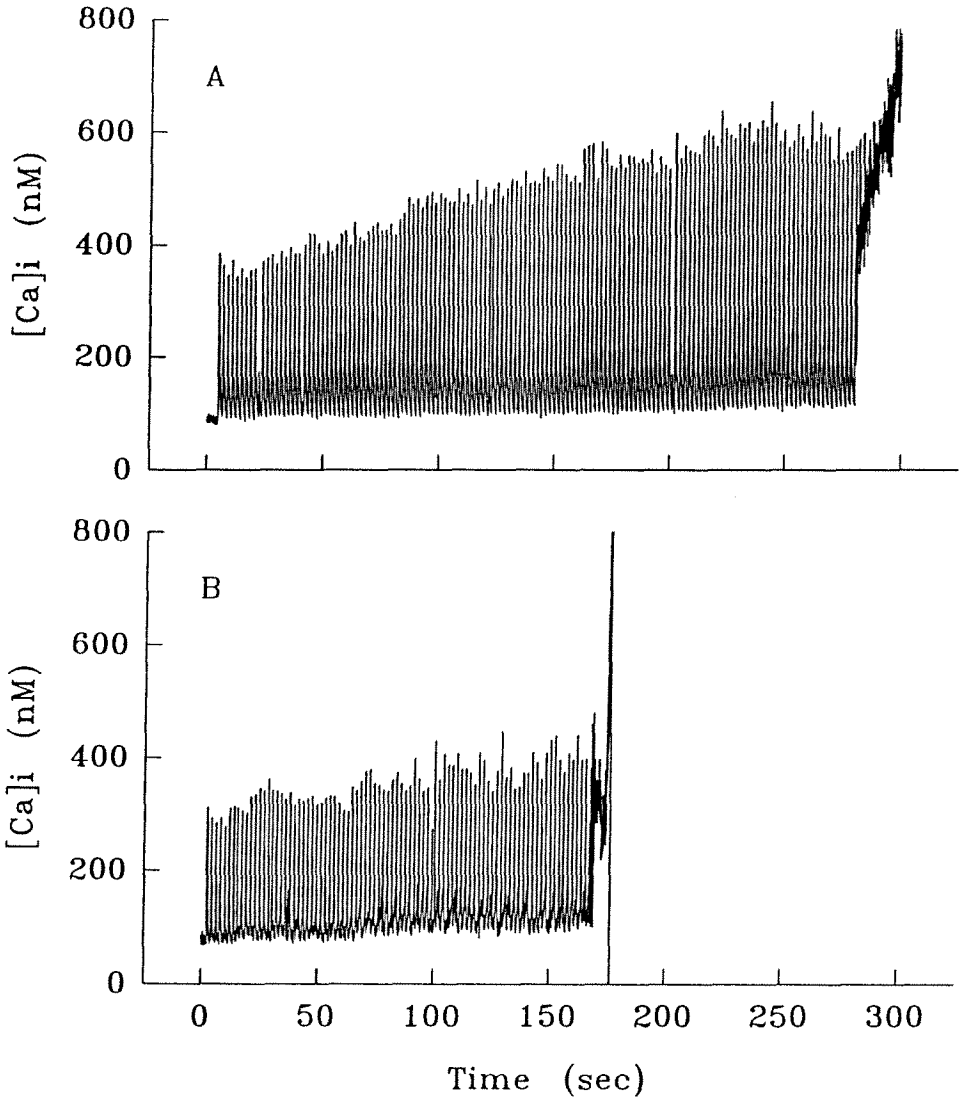

Figure 37. Ouabain-induced $[Ca^{2+}]_i$ increase in electrically-stimulated myocytes.

$[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. A: a control myocyte. B: a diabetic myocyte. In A, ouabain 10^{-7}M was added at time 20 sec. Then the concentration of ouabain was increased gradually to 10^{-3}M at 280 sec, at this concentration the cell hypercontracted. In B, ouabain 10^{-9}M was added at time 15 sec. Then the concentration of ouabain was increased gradually to 10^{-5}M at 170 sec, at this concentration the cell hypercontracted.

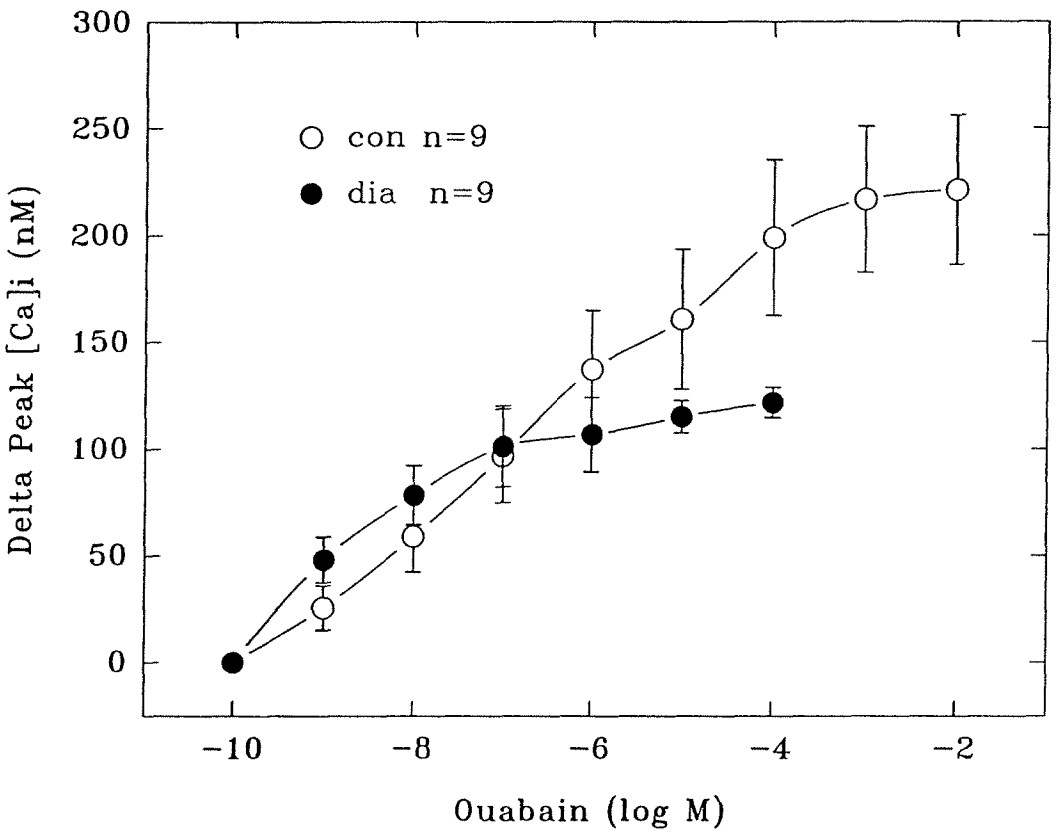

Figure 38. Ouabain dose-response curves obtained in stimulated control and diabetic myocytes. $[Ca^{2+}]_i$ increase as a function of the concentration of ouabain

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

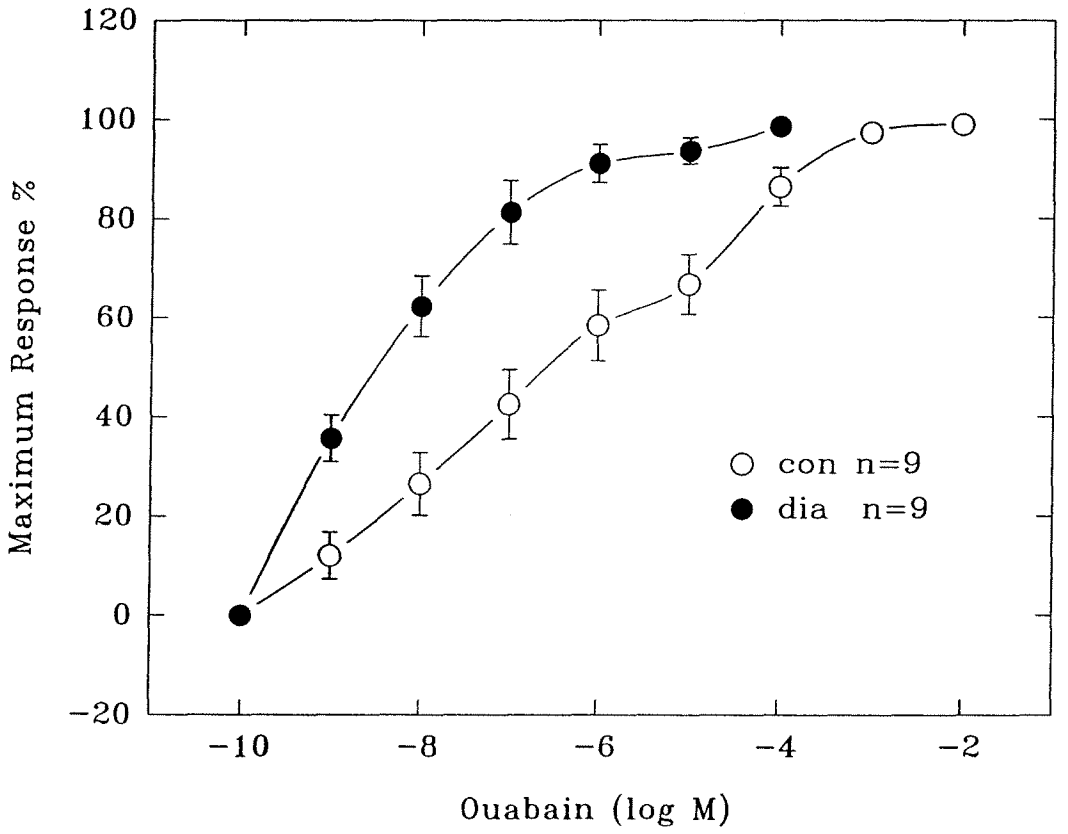

Figure 39. Ouabain dose-response curves obtained in stimulated control and diabetic myocytes. Percentage of the maximum response of $[Ca^{2+}]_i$ increase as a function of the concentration of ouabain.

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

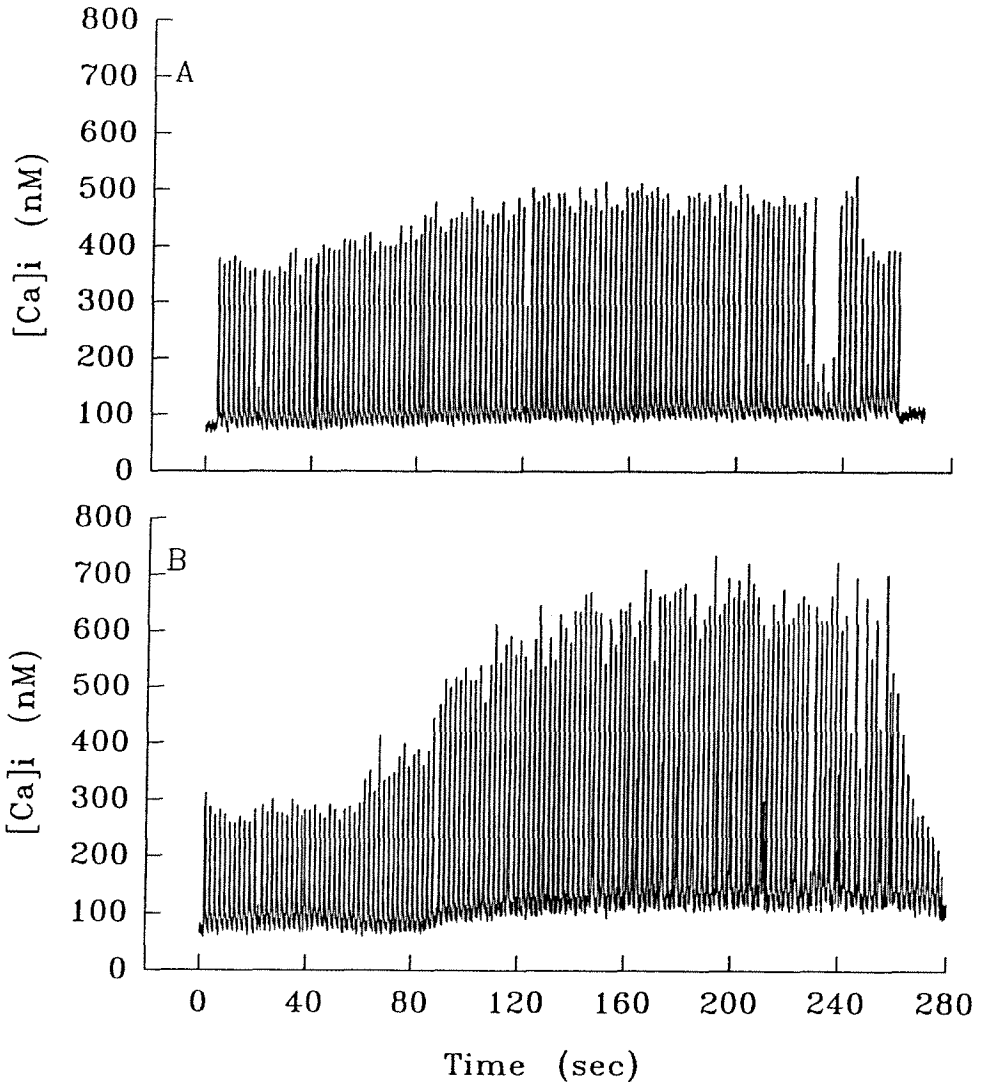

Figure 40. ATP-induced $[Ca^{2+}]_i$ increase in electrically-stimulated myocytes.

$[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2. A: a control myocyte. B: a diabetic myocyte. In A, ATP 10^{-7}M was added at time 40 sec. Then the concentration of ATP was increased gradually to 10^{-2}M at 245 sec. The stimulator was off at 253 sec.In B, ATP 10^{-9}M was added at time 40 sec. Then the concentration of ATP was increased gradually to 10^{-4}M at 260 sec.

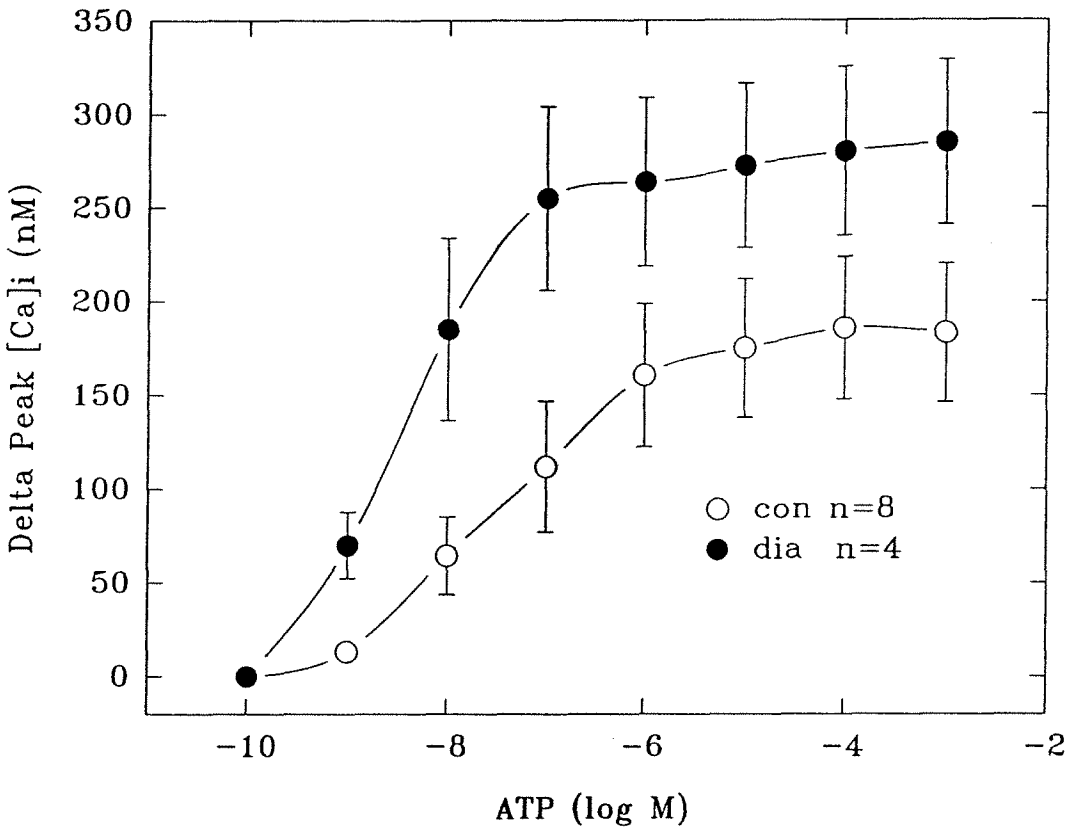

Figure 41. ATP dose-response curves obtained in stimulated control and diabetic myocytes. $[Ca^{2+}]_i$ increase as a function of the concentration of ouabain

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

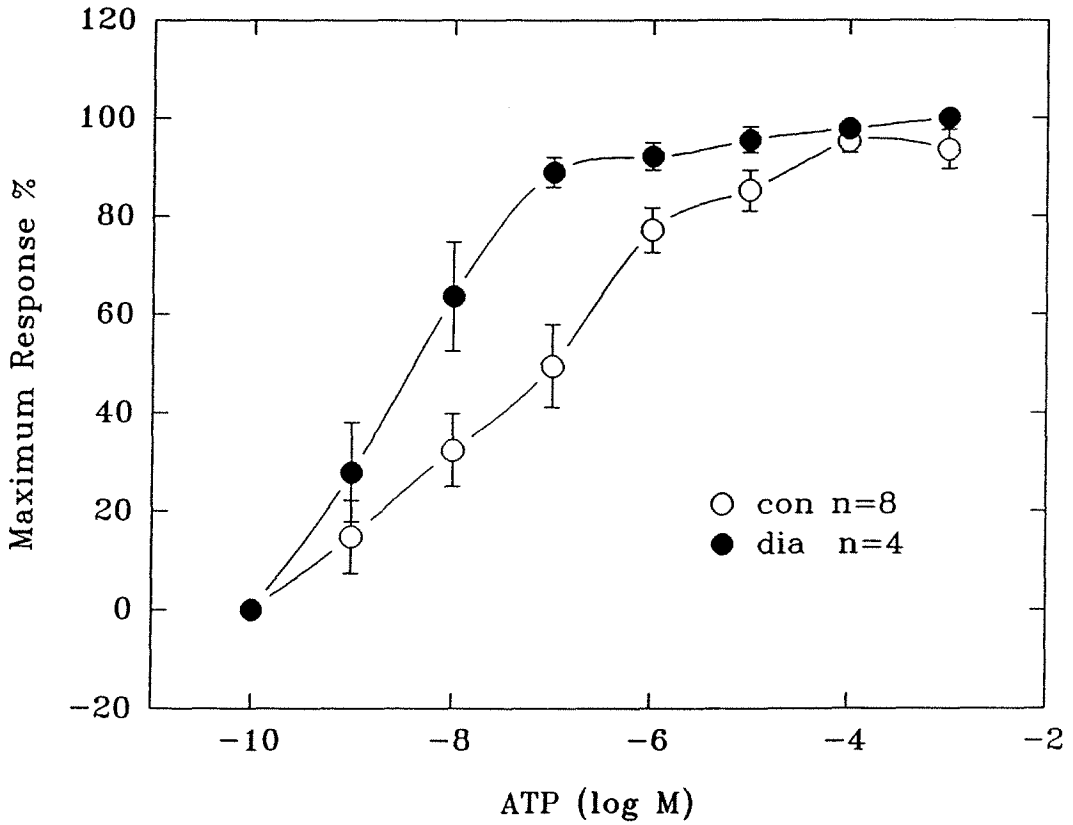

Figure 42. ATP dose-response curves obtained in stimulated control and diabetic myocytes. Percentage of the maximum response of $[Ca^{2+}]_i$ increase as a function of the concentration of ouabain

Myocytes were superfused with M199 (with Earl's salts, 1.8 mM $CaCl_2$, 25 mM HEPES, 5% FBS and 1% BSA) at 23°C, and field stimulated at 0.5 Hz, 3-8 V, and 5 ms stimulation duration. $[Ca^{2+}]_i$ was measured by fluorescence microscopy and fura-2.

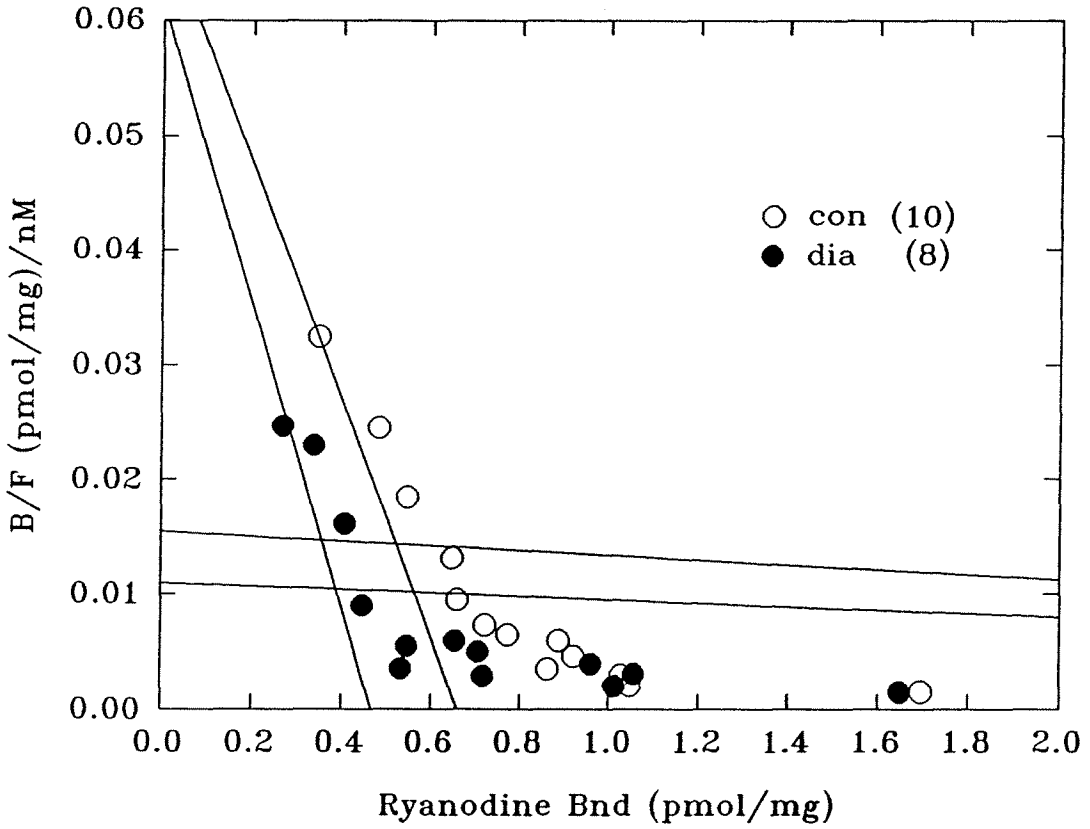

Figure 43. Scatchard plots for [^{3}H]-ryanodine binding.

Two straight lines were drawn according the high affinity binding site parameters (Km, Bmax, see Table 3. for each corresponding parameters). con: control, dia: diabetic. Number of rats for each group is shown in parenthesis.

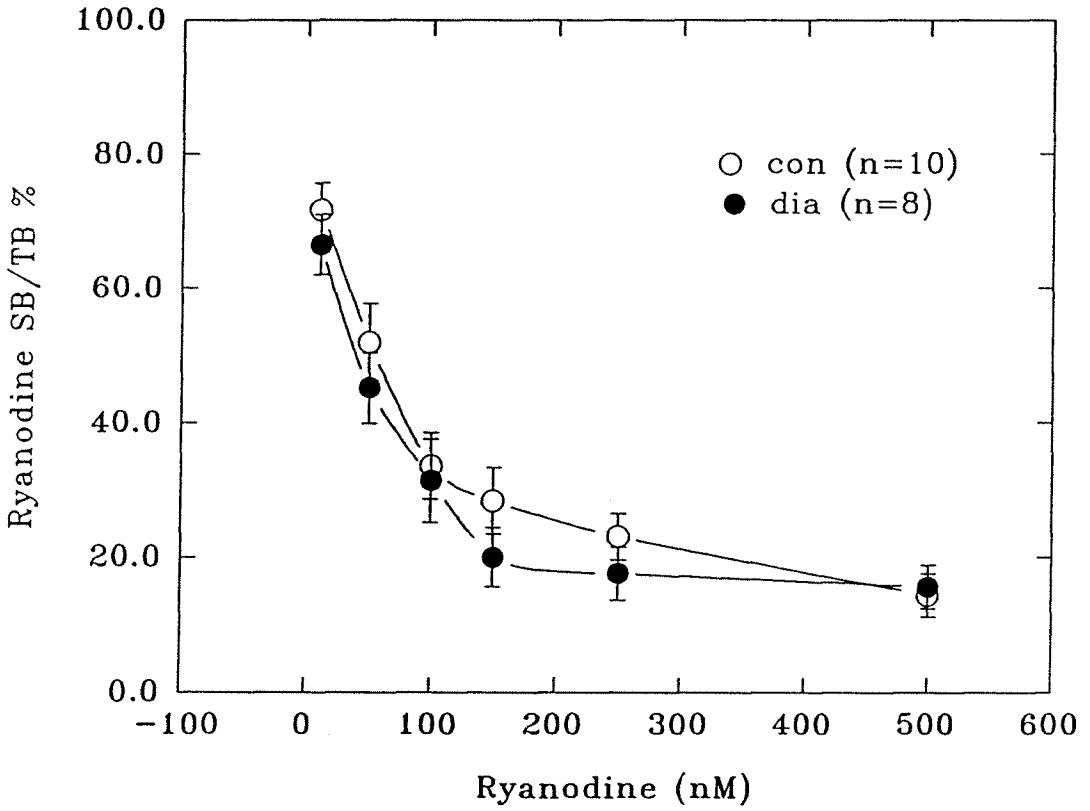

Figure 44. Specific ryanodine binding as the percentage of total binding.

Data are expressed as mean ± SE. con: control, dia: diabetic. n: number of rats.

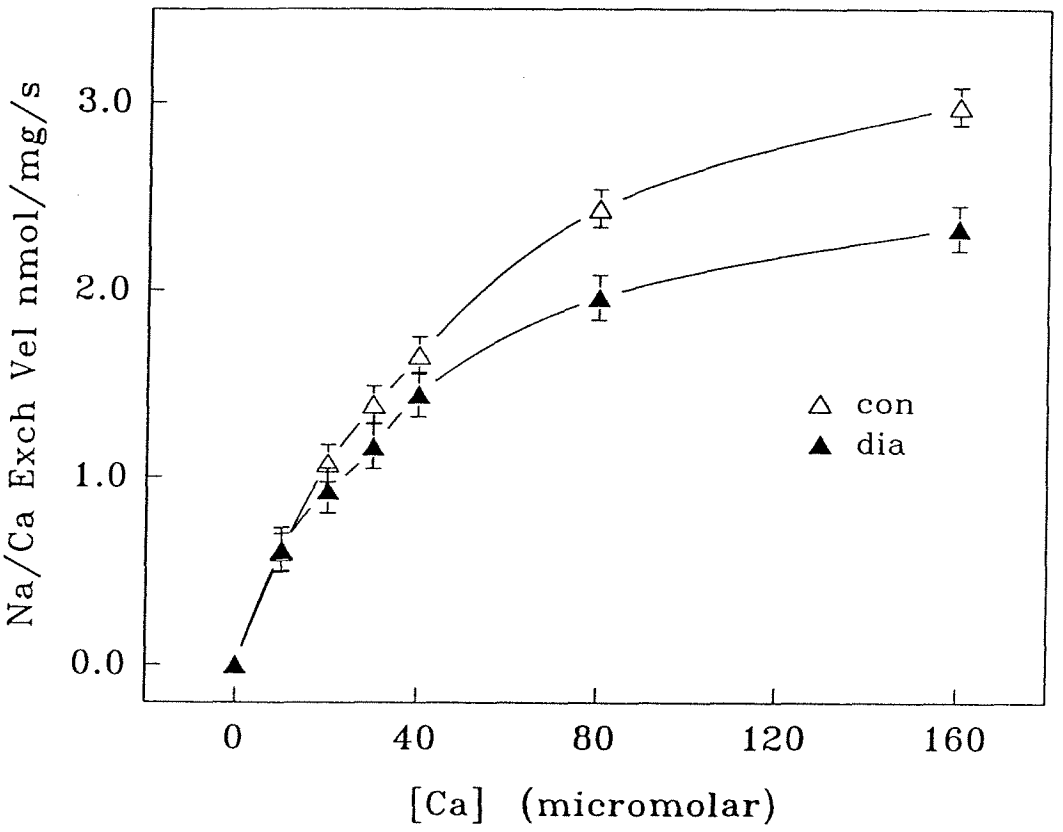

Figure 45. Na^+-Ca^{2+} exchange as a function of extravesicular concentration of Ca^{2+}.

Ca^{2+} uptake by sarcolemmal vesicles were quenched at 2 sec. Values are presented as mean ± SE of 6 experiments from 3 different sarcolemmal preparations of either control or diabetic rats.

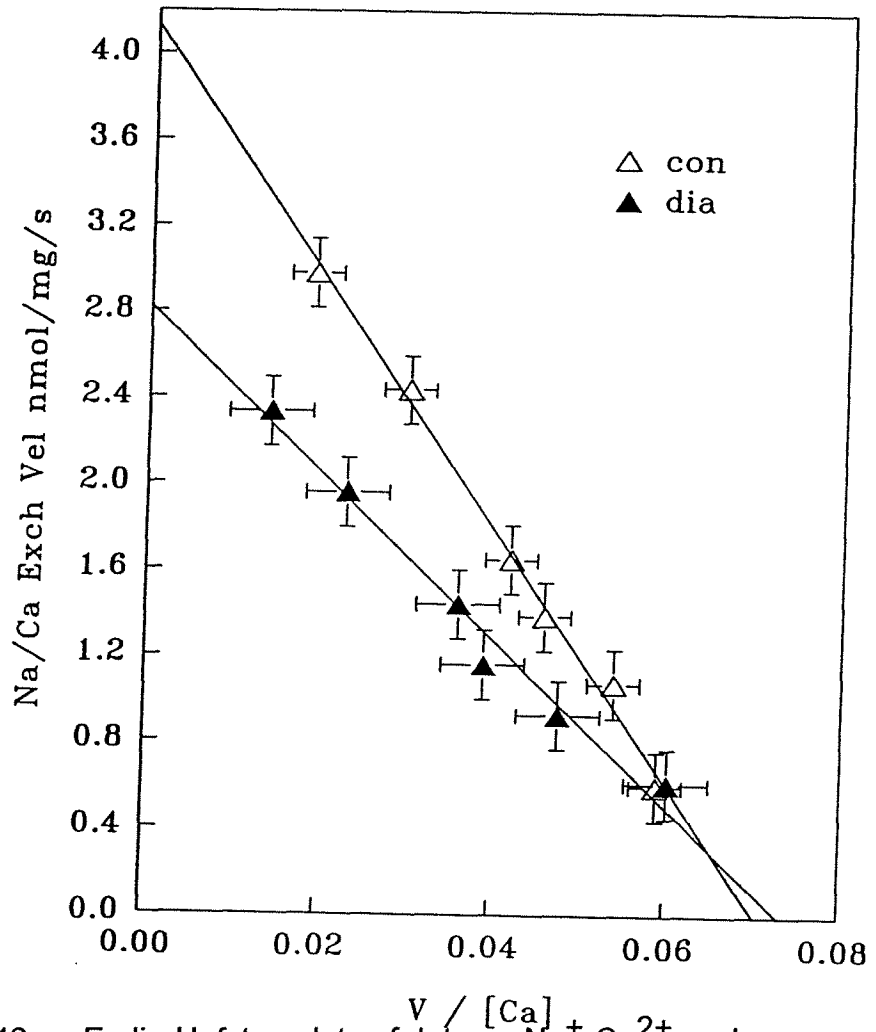

Figure 46. Eadie-Hofstee plots of data on Na^+-Ca^{2+} exchange.

Data are expressed as mean ± SE. Eadie-Hofstee plots of data shown in Figure 43. Km (-slope) and Vmax (Y axis intercept) values are significantly decreased in diabetic (dia) compared with control (con) rats.

DISCUSSION

I. STZ-INDUCED DIABETES MELLITUS AND INSULIN EFFECTS

As stated earlier, the mechanism by which STZ elicits its specific action on the ß-cell is largely unknown. However the close approximation of STZ-induced diabetes to the human insulin dependent diabetes mellitus state warrants its use in diabetic studies. In this study decreased body weight gain, hyperglycemia and hypoinsulinemia were successfully induced in STZ-treated rats. Other indications of experimental diabetes, including hyperlipidemia and hypothyroidism, were also evident in this model (Yu and McNeill, 1991a). The effects noted in this study are due to the diabetic state and not to any direct toxic effect of STZ on the cardiovascular system. At the dose used in this study, the effect of STZ is highly specific in that destruction of the ß-cells of the pancreas occurs without damage to other organ systems (Junod et al. 1967). McGrath and McNeill (1986) showed that no detectable abnormalities appear in the heart in 6 week STZ-treated rats. Results of the insulin treatment of STZ rats suggested that changes in rat heart function are due to chronic insulin deficiency and not to STZ itself. In isolated perfused heart and papillary muscle from diabetic rats the cardiac performance defects can be prevented and reversed by insulin treatment. Corrections of the hyperglycemic state by insulin administration have proven to be the most successful means of correcting cardiac dysfunction (Tahiliani et al. 1983). Daily insulin administration to diabetic rats is capable of reversing all myocardial ultrastructural defects associated with short-term diabetes (Reinilä and Akerblom 1984). In this study insulin treatment reversed most of the abnormalities in cardiomyocytes from diabetic rats. This

suggests that insulin deficiency plays an important role in the pathophysiology seen in STZ diabetic heart.

$[Ca^{2+}]_i$ in diabetic myocytes has been reported to be increased (Schaffer et al., 1989) or decreased (Noda et al., 1992). In this study *in vivo* calibration and subtraction of autofluorescence were applied to assure accurate calibration of $[Ca^{2+}]_i$. The basal $[Ca^{2+}]_i$ in both quiescent and electrically-stimulated cells showed that there was no difference between diabetic and control myocytes. This suggests that resting $[Ca^{2+}]_i$ and regular $[Ca^{2+}]_i$ transients may not reflect changes brought about by diabetes until the system is challenged by certain stimuli. In this experiment the ratio of the $[Ca^{2+}]_i$ change was not calculated, which may be a better representation of the $[Ca^{2+}]_i$ in diabetic cells.

II. DEPRESSED CARDIOMYOCYTES CONTRACTILITY IN DIABETES

a. Myocyte Contractility and Shortening-Interval Relationship

As shown in Figure 4, the overall shape of a single cell twitch (isotonic contraction) is reminiscent of that obtained in ventricular papillary muscles or muscle strips (isometric contraction) (Yu and McNeill, 1991b), although the time courses of the contraction are different. The time to peak shortening was usually shorter, and the falling phase was prolonged in the single cell compared with the muscle strip (Tung and Morad, 1988). This may be due to the cells being externally unloaded and isotonically contracted. The shortening response resembled the physiological responses seen in the muscle strip. At a constant stimulation rate, the steady-state amplitude of the single-cell twitch exhibited beat-to-beat variations, which were also seen in accompanied $[Ca^{2+}]_i$ transients (Figure 4). These may be due to the

sampling rates of CCD camera (60Hz) and A/D board (controlled by PTI at 25Hz). As well, fluctuations in intracellular ionic composition may occur in the single cell and can alter force directly by $[Ca^{2+}]_i$ or indirectly by $[Na^+]_i$ or $[K^+]_i$, (Tung and Morad, 1988). However by averaging the cell shortening and $[Ca^{2+}]_i$ transient data, the single cell preparation is suitable as a model for intact tissue.

The depressed contractility (expressed as reduced cell shortening, ±dl/dt and prolonged time-to-peak shortening) in diabetic myocytes agrees with the results in intact heart and tissue preparations of diabetic rats which show decreased ventricular pressure development and the rates of ventricular pressure development and decline (±dP/dt). Insulin treatment normalized the depressed cell shortening in diabetic myocytes, which suggests these changes are specific to diabetes and that insulin deficiency may play a causal role in these changes. Insulin treatment, however, did not reverse the ±dl/dt and time to peak shortening to control levels. This may be related to the fact the correction of plasma glucose was incomplete in some diabetic rats (Table 1).

In rat myocardium, the resting $[Na^+]_i$ activity is high (aNa_i ≈12.7mM, activity of intracellular sodium, measured by sodium microelectrode) (Shattock and Bers, 1987). During rest, high aNa_i is in favour of calcium entry via Na-Ca exchange. The calcium content of the SR can be gradually raised by sarcolemmal Na-Ca exchange during rest. During a potentiated post-rest contraction a larger amount of calcium is released from SR. Rest potentiation is considered an indication of releasable calcium from SR in combination with a small amount of calcium from Na-Ca exchange (Bers, 1987) although this SR calcium release is not completed. Therefore, depressed rest potentiation from diabetic myocytes may be indicative of a depleted SR in the diabetic myocardium. Rest potentiation measured in papillary

muscle of diabetic rats was also depressed (Yu and McNeill, 1991b) which is consistent with the data from the present study.

As shown in Figure 5, a 'negative staircase' (Haworth et al., 1980) effect can be seen in control and diabetic rat myocytes with the degree of shortening increased as the frequency is decreased. This again suggests that the cardiomyocytes have the characteristics of intact rat cardiac tissue, i.e. SR calcium gain during the rest and SR calcium decline during high frequency stimulation (Bers, 1989) and net calcium uptake is favoured during longer rest. This can explain the rest potentiation and rest-dependent increase in SR calcium in rat ventricle. Depending on the calcium loading at different conditions, a positive staircase is also demonstrable, e.g. rat myocytes placed at low $[Ca^{2+}]_o$ and exposed to high frequency (Frampton et al., 1991; Bers 1991). The force-frequency relationship seems to be an intrinsic mechanism regulating the mechanics of the heart. It is accomplished under physiological conditions and without any important contribution on the part of the extracardiac regulatory system.

b. <u>Isoproterenol Inotropy in Diabetic Myocytes</u>

A depressed response to isoproterenol is one of the important features in diabetic heart. Data from isolated myocytes are consistent with results shown in isolated heart or cardiac tissues (Vadlamudi et al., 1982; Yu and McNeill, 1991a). Decreased ß-adrenoceptor numbers and unchanged receptor affinity have been reported (Heyliger et al., 1982; Nishio et al., 1988), which may be due to the increased catecholamine turnover in diabetic heart and down regulation of the ß-adrenoceptor (Ganguly et al., 1986). If sufficient isoproterenol does not bind to the receptor, a decrease in the stimulation of adenylate cyclase activity will occur.

Theoretically, less cAMP will be generated and phosphorylation will be less than optimal. This may explain the attenuated positive inotropic response of the diabetic myocytes to isoproterenol. The depressed response to ß-adrenergic stimulation suggests that an altered ß-adrenergic pathway is present in diabetic myocytes.

III. REDUCTION OF SR Ca^{2+} CONTENT IN DIABETIC CARDIOMYOCYTES

a. Rapid Cooling Contractures and Caffeine Contractures

The calcium content of the SR which is available for release is clearly an important determinant of the contractile state, but is difficult to measure in myocytes. Two approaches used in this study to assess SR calcium in cardiomyocytes are rapid application of caffeine or cold solution (≈1°C) to induce SR calcium release. The resulting contracture can be used as an index of the SR calcium available for release. The advantages of using these approaches is that they can be done 'on line' with a contracting cell (Bers, 1991). The actual amount of calcium released is not easy to determine since the calcium released quickly equilibrates with intracellular buffers. Although the mechanisms of SR Ca-release channel open are similar the features of rapid cooling and caffeine contracture are not entirely identical. Both contractures were used in this study to assess the SR calcium content.

Rapid cooling of cardiac muscle (to 1°C) results in a contracture which is attributed to the rapid release of the SR calcium to the cytosol. The amplitude of the contracture is indicative of the amount of calcium available for release from SR at the time of cooling. Generally, during the cooling process, the probability of the SR calcium release channel opening increases (Sitsapesan et al., 1991), action

potential duration increases (Shattock and Bers, 1987), peak calcium current decreases (Briggs and Bers, 1990), myofilament calcium sensitivity decreases (Harrison and Bers, 1989), and Na-Ca exchange activity also decreases (Debetto et al. 1990). In a RCC there is neither action potential generated nor calcium current across sarcolemma. The duration of $[Ca^{2+}]_i$ elevation during the RCC in unloaded conditions prolongs the active state and may allow the myocyte to shorten progressively during the RCC. As shown in Figure 10., the rise in $[Ca^{2+}]_i$ during RCC was less transient than that during caffeine-induced contractures. The $[Ca^{2+}]_i$ declined very slowly before the rewarming by 23°C buffer. This is due to the inhibition of membrane calcium transport (calcium pump and Na-Ca exchange). $[Ca^{2+}]_i$ is much higher during the RCC than during a normal twitch, thus it seems that rapid cooling can release all of the available SR calcium (Bers, 1991), while only a fraction of the SR calcium available for release is, in fact, released during a normal twitch. However in this study, it appeared that the intracellular indo-1 was saturated by the Ca released during RCCs so that $[Ca^{2+}]_i$ was not dramatically increased.

Caffeine increases SR calcium channel opening and, therefore, promotes a calcium leak into the myoplasm which effectively prevents the SR from accumulating calcium and leaves the SR calcium channels open (Rousseau and Meissner, 1987). Calcium pumped into the SR is immediately reintroduced into the myoplasm via the open SR channels, and can be removed from the cell by Na-Ca exchange. This may explain the finding that caffeine inhibits SR calcium uptake without direct effects on the SR calcium pump. This is a useful approach, especially in isolated myocytes where diffusional limitations are minimal. However, the other effects of caffeine such as myofilament sensitization and phosphodiesterase inhibition which can increase cAMP and cAMP-dependent protein kinase can

complicate interpretation (Fabiato, 1981). The fact that the calcium released is immediately subjected to transport systems such as Na-Ca exchange is also a limitation.

As shown in Figure 13, the $[Ca^{2+}]_i$ rise is transient and the decline may represent calcium extrusion from the cell via Na-Ca exchange. The second component of the contraction developed along the same time course as the rise in intracellular caffeine concentration (O'Neill et al., 1990) and is likely to be due primarily to this myofilament sensitizing effect. The other effects of caffeine such as myofilament sensitization and phosphodiesterase inhibition which can increase cAMP and cAMP-dependent protein kinase can complicate interpretation (Bers, 1991). Indo-1 fluorescence is strongly quenched by caffeine, but in a wavelength-independent manner so that the fluorescence ratio used to estimate $[Ca^{2+}]_i$ is unaffected (O'Neill et al., 1990).

b. Caffeine Induced $[Ca^{2+}]_i$ Transients in Quiescent Myocytes

The SR content of calcium was also assessed by measuring $[Ca^{2+}]_i$ response to caffeine with the fluorescence Ca-indicator, fura-2. As mentioned in the "Introduction", fura-2 fluorescent signals generally separate better from the background auto fluorescence (Blinks, 1992), which is also observed in this study. Distinct fura-2 fluorescent tracing without the interruption of background signals makes it easier to calibrate $[Ca^{2+}]_i$.

As shown in Figure 17. the peak $[Ca^{2+}]_i$ transient in response to caffeine was significantly decreased in diabetes suggesting decreased calcium storage in SR. Insulin treatment can prevent this depressed response, which is probably related to

the fact that insulin treatment of STZ diabetic rats can reverse the depression of Ca-ATPase activity and calcium uptake by the SR (Lopaschuk et al. 1983). Caffeine is known to release calcium from SR and, thus, to prevent its accumulation in the SR of heart cells (Bers and Bridge, 1989). Brief exposure to caffeine induces a transient inward current which reflects the electrogenic extrusion of calcium across the membrane by the Na-Ca exchange (Callewart and Morad, 1989). The decreased peak negative ratio of $[Ca^{2+}]_i$ decline (-peak $d[Ca^{2+}]_i/dt$) suggests that Na-Ca exchange activity is depressed in myocytes. The markedly depressed $-d[Ca^{2+}]_i/dt$ was successfully corrected by insulin treatment, suggesting again that insulin deficiency plays an important role in SR dysfunction in diabetes.

There are opposite theories about altered $[Ca^{2+}]_i$ in diabetic hearts, i. e. calcium is overloaded or underloaded and whether the decrease in cardiac contractility in STZ induced diabetes in the rat is accompanied by reduced or excessive loading of calcium into SR. The results from this study indicate both a decreased SR calcium store and release as assessed by RCC, CC, and caffeine-induced $[Ca^{2+}]_i$ transients. The data agree with Bouchard and Bose (1991) who reported a reduction in SR calcium stores and decreased fractional release of calcium during stimulation in papillary muscles from STZ-treated rats. The marked reduction of developed tension in diabetic tissues was suggested to be a consequence of depleted SR calcium stores, rather than a result of chronic SR calcium overloading. It has been suggested that reduced calcium uptake by SR (Lopaschuk et al., 1983) could diminish calcium stores within SR and hence impair calcium release, with a consequent reduction in cardiac contraction. Therefore the decreased uptake and storage of calcium in SR may result in reduction of myocyte contraction.

IV. ALTERED MODULATION OF $[Ca^{2+}]_i$ TRANSIENTS IN DIABETIC CARDIOMYOCYTES DURING MEMBRANE DEPOLARIZATION

KCl-induced membrane depolarization is a well known phenomenon, but the quantitative definition of each component of the $[Ca^{2+}]_i$ transient is obscure. The degree of membrane depolarization is a linear function of the log concentration of KCl which may explain the dose-dependent $[Ca^{2+}]_i$ changes observed in the study. The influx of $[Ca^{2+}]_i$ is a prerequisite of the KCl-induced $[Ca^{2+}]_i$ transient because EGTA abolished the transient. The $[Ca^{2+}]_i$ influx is triggered by membrane depolarization and thus the activation of L-type Ca-channels and 'reversed' mode operation of Na-Ca exchange may be involved. Calcium influx via Ca-channel and Na-Ca exchange may directly contribute to the $[Ca^{2+}]_i$ increase and, more importantly, induce calcium release from SR.

It should be noted that the blocking effect of DCB is not very specific for Na-Ca exchange. It may block the Ca-channel as well at high concentration. Caffeine and DCB when used alone blocked $[Ca^{2+}]_i$ transients at a lower degree in diabetic cells. This suggests that calcium released from SR and possibly the influx from Na-Ca exchange play less of a role mediating the $[Ca^{2+}]_i$ increase in diabetic cells. If these two mechanisms are less functional in diabetic cells, one would assume that extracellular calcium enters the diabetic cell by Ca-channels. Caffeine plus nitrendipine blocked a greater degree of the $[Ca^{2+}]_i$ increase in diabetic cell. This supports the assumption that L-type Ca-channel activity might increase in diabetic cells. However nitrendipine (5 μM) applied alone did not show any difference in blocking effect between control and diabetic cells. It is possible that nitrendipine due to its structural feature of light sensitivity was decomposed more or less during the exposure to the UV light and causing inconsistency in the results. However this is

difficult to prove with the present experiment. A similar enhanced $[Ca^{2+}]_i$ response to KCl (30 mM) in a myocyte suspension from diabetic rats has been reported by Schaffer et al.(1989), except that the basal level of $[Ca^{2+}]_i$ was higher in diabetic cells than in controls. There are slight differences in the preparation of the STZ-diabetic rat model between the two studies which may account for the enhanced $[Ca^{2+}]_i$ level in the measurement in Schaffer's study. Increased L-type Ca-channel density determined by $[^3H]$PN-200-110 binding sites in cardiac membrane has been reported in diabetic heart (Nishio et al., 1990). Interestingly the $[^3H]$PN-200-110 binding to control cardiac membrane was dose-dependently inhibited by verapamil, but this was not the case in diabetic cardiac membranes. This suggests that L-type Ca-channels are quantitatively and qualitatively altered in diabetes, which may be related to the nitrendipine effects found in this study.

Ryanodine, at a concentration of 1nM, keeps the SR calcium release channel in a subconducting state, therefore there would be no SR-releasable calcium response to KCl -induced membrane depolarization (Meissner, 1986). Theoretically ryanodine effects should be similar to the effects of caffeine. However this was not the case in Figures 25 and 26. It has been reported earlier that ryanodine effects on diabetic papillary muscle as determined by rest-potentiation were not different from controls (Yu and McNeill, 1991b). The exact mechanism involved is not clear. Taken together, the mechanism(s) of KCl-induced $[Ca^{2+}]_i$ transients is complicated. There may exist a causal or permissive connection between calcium influx and intracellular calcium release. Because of the dynamic change in $[Ca^{2+}]_i$ during membrane depolarization, the cause of the disparity could not be derived from current data. Further studies are needed to fulfill this task.

V. ALTERATIONS OF CALCIUM RELEASE CHANNELS IN SR OF DIABETIC CARDIOMYOCYTES

Ryanodine, a selective and specific ligand for the SR calcium channel was found to produce a progressive decline in cardiac muscle contraction. At low concentrations, 1-30nM, ryanodine accelerates calcium loss from heavy SR vesicles. It opens the SR calcium release channel to a stable subconducting state where the channel no longer responds to Ca, ATP, Mg or ruthenium red (Meissner, 1986). This probably corresponds to the occupation of the high affinity ryanodine site (K_d ≈10 nM). Ryanodine also accelerates SR calcium leakage, but to a lesser extent than caffeine, as channels are in an open subconducting state. At high concentrations (>100 μM), ryanodine slows calcium efflux and the release channel appears to be locked in a closed state (Meissner, 1986). This may result from ryanodine binding to low affinity sites. There were one high affinity ryanodine binding site and three low affinity binding sites per tetramer (Lai et al., 1989) which exhibit negative cooperativity depending on the concentrations of calcium and ATP. Under conditions where no high affinity ryanodine binding is observed (e.g. low $[Ca^{2+}]_i$, all four sites per tetramer are of the low affinity type.

The binding parameters obtained from this study are similar to the previously reported data. K_d values of 4-36 nM have been found and the numbers of sites in SR preparations vary from 0.5-14 pmol/mg protein. In whole heart homogenates an average of ≈300 fmol/mg protein has been reported (Bers, 1991). Many studies of ryanodine binding in heart have revealed low affinity sites with an apparent K_d in the μM range (Inui et al., 1987).

The reason for the decreased ryanodine binding sites in diabetic heart is not clear. It may be due to the change in the environment of the membrane in diabetes in which hyperlipidemia may have an influence, or to the adaptation to an altered SR calcium loading as shown by decreased SR calcium content in the present study. The impact of the decreased binding sites is also unknown. It may have an influence on depressed myocyte shortening and the rate of shortening as shown above and therefore may be involved in the diabetic induced cardiac dysfunction. However further studies are needed to understand the role of SR release channel changes in the diabetic heart.

VI. ALTERATION OF Na-Ca EXCHANGE ACTIVITY IN SL OF DIABETIC MYOCARDIUM

K-pNPPase activity, a measure of the dephosphorylation phase of the Na-K ATPase was not changed between control and diabetic groups. It has been reported that Na-K ATPase activity was decreased in diabetes (Pierce and Dhalla, 1983). The discrepancy here may be due to the experimental error and different experimental conditions.

Depressed Na-Ca exchange activity without change in affinity to calcium has been reported before (Makino et al, 1987). In this study, increase of affinity to Ca may be due to membrane environmental changes in diabetes. Membrane composition changes have been reported in diabetes before. It is possible that diabetes-induced hyperlipidemia could influence membrane bound enzyme activity (Katz, et al., 1981). In addition, Na-Ca exchange is remarkably sensitive to alteration of the lipid bilayer. Exchange is increased by reconstitution with acidic phospholipids either by the addition of exogenous negatively charged amphiphiles or by

phospholipase cleavage of native phospholipids to yield negatively charged membrane lipids. Certain anionic head groups are more stimulatory for Na-Ca exchange than others and several cationic amphiphiles are inhibitory (Philipson, 1984). We have data showing that hypertensive-diabetic rats show a more significant increase of affinity to calcium in Na-Ca exchange (Tibbits et al., unpublished data). The hypertensive-diabetic rat model is known for its severe hyperlipidemia and metabolic changes (Rodrigues and McNeill, 1986).

VII. CARDIAC DRUG RESPONSES IN DIABETIC MYOCYTES

In the present study, using classical pharmacological measurements, the sensitivity of a tissue (expressed by the EC_{50} or pD_2 value) for an agonist as derived from the concentration-effect relationship is an estimate of the apparent affinity, usually defined as sensitivity, of the receptor for the agonist, and the maximum response is indicative of the intrinsic activity of a drug, i.e. the effect produced after receptor occupancy (Ariens and Simonis 1964).

a. Isoproterenol and 8-bromo-cAMP

In this study isoproterenol produced a decrease in $[Ca^{2+}]_i$ in quiescent myocytes. This agrees with the data of Sheu et al. (1987) measured in myocyte suspensions with quin-2. This effect is mediated by ß-adrenoceptors because the response was blocked by timolol. In beating myocytes isoproterenol increases I_{Ca} due to its effects in accelerating calcium influx via L-type Ca-channels (Tsien et al., 1986). In quiescent myocytes, the L-channels are not open because of the E_m is below the threshold of action potential. This excludes the possibility that isoproterenol causes large $[Ca^{2+}]_i$ changes via the L-channel under this condition.

We demonstrated that thapsigargin, which specifically inhibits Ca-ATPase in SR of myocytes (Wrzosek et al., 1992), abolished the isoproterenol-induced $[Ca^{2+}]_i$ decrease. Therefore isoproterenol effects are most likely mediated by SR calcium uptake by Ca-ATPase. It could be argued that isoproterenol has some other effects on the quiescent myocyte, such as a decrease in sensitivity of the myofilaments to calcium, which would cause a $[Ca^{2+}]_i$ increase or a stimulation of sarcolemma calcium efflux, which would cause a $[Ca^{2+}]_i$ decrease (Lee and Vassalle, 1983; Desilets and Baumgarten, 1986; Okazaki et al., 1990). However the change of sensitivity of myofilaments to calcium may not contribute to the absolute values of $[Ca^{2+}]_i$ (Endoh and Blink, 1988) especially in quiescent myocytes, and calcium efflux accelerated by isoproterenol occurs mostly during condition of increased $[Ca^{2+}]_i$ (Desilets and Baumgarten, 1986).

In beating myocytes, isoproterenol produced large increases in the amplitude and in the rate of the $[Ca^{2+}]_i$ transients and cardiac contraction ($d[Ca^{2+}]_i/dt$, data not shown). It is known that ß-adrenergic agonists increase calcium current, enhance SR calcium uptake and decrease myofilament calcium sensitivity (Bers 1991). Isoproterenol stimulates adenylate cyclase with a consequent elevation of cyclic AMP, activation of cyclic AMP-dependent protein kinase (PKA) and phosphorylation of a number of cellular proteins including sarcolemmal calcium channels, troponin I, and phospholamban (associated with the SR calcium pump). ß-adrenergic agonists can also increase calcium current by a more direct G protein-mediated pathway (Yatani et al., 1987; Pelza et al., 1990). In the presence of a ß-adrenergic agonist, more calcium enters the cell at each excitation and the SR accumulates a larger fraction of the cytosolic calcium pool during relaxation. Thus a larger amount of calcium loads into the SR. The increased I_{Ca} might also increase the amount of calcium released from SR via Ca-induced Ca-release. The SR Ca-

ATPase stimulation also accelerates relaxation and leads to an earlier peak and more rapid decline of the $[Ca^{2+}]_i$ transient. The higher $[Ca]_i$ peak will also tend to stimulate calcium extrusion via Na-Ca exchange (Okazaki et al., 1990). The dramatic increase in the $[Ca^{2+}]_i$ transient with ß-adrenergic stimulation more than compensates for the decrease in myofilament calcium sensitivity, therefore contraction increases substantially. A defect in all or any of the steps in adrenergic-induced inotropy may explain the attenuation in stimulation by adrenergic agents in diabetic rat hearts.

There may be two kinds of calcium- dependent deficiency that affect the contractility of diabetic heart: changing the availability of calcium to the myofilaments, and changing the responsiveness of the myofilaments to activation by intracellular calcium. The availability of intracellular calcium is regulated by the sarcolemma and sarcoplasmic reticulum, and calcium responsiveness is controlled by the myofilaments and the regulatory troponin-tropomyosin complex (Morgan, 1991). There have been several reports of diminished cardiac ß-adrenoceptor numbers, with no changes in affinity, in cardiac tissues taken from STZ-treated rats (Heyliger et al., 1982 and Nishio et al., 1988). Kashiwagi et al. (1989) measured concentrations of cell surface and total cell ß-adrenoceptors of cardiac myocytes 10 weeks after STZ injection. Although there was a 41% reduction in cell surface binding sites, there was no difference between STZ-treated and control rats in total cell receptor concentration, suggesting abnormalities in ß-adrenoceptor recycling. Nishio et al. (1988) found no change in basal cAMP production following STZ treatment and suggested that the defect was in the coupling of ß-adrenoceptors to adenylate cyclase. This also suggests that changes occur in regulatory guanosine triphosphate-binding proteins because there was evidence for an increase in G_i proteins, which inhibits adenylate cyclase, in cardiac tissue from STZ-treated rats

(Nishio et al., 1988). The sympathetic nervous system appears to be activated during diabetes because plasma catecholamines have been reported to be elevated (Berkowitz et al., 1980). Norepinephrine turnover, uptake, release and synthesis in the heart from diabetic animals are markedly increased (Ganguly et al., 1986). It is possible that enhanced turnover of catecholamines in the myocardium could contribute to adrenoceptor down-regulation.

The functional depression in catecholamine sensitivity of hearts from diabetic rats may be closely associated with the receptor defect. The decreased number of ß-adrenoceptors will cause a depression in the stimulation of adenylate cyclase activity, theoretically less cAMP will be generated, and phosphorylation will be less than optimal. This may explain the attenuated positive inotropic response of the diabetic myocardium to isoproterenol and the decrease in SR calcium uptake after stimulation by a ß-adrenergic agonist in diabetic heart (Lopaschuk et al., 1983; Götzsche, 1983).

Additionally diabetic myocytes showed less $[Ca^{2+}]_i$ response to 8-bromo-cAMP at 10^{-5} M and higher concentrations. This suggests that at steps distal to the ß-adrenoceptor and adenylate cyclase, diabetic myocytes exhibit a deficiency. Therefore there may be some alteration between cAMP and the $[Ca^{2+}]_i$ increase such as PKA activation and the phosphorylation process of proteins. Depressed SR Ca-ATPase activity and calcium uptake to SR are well known in diabetic heart (Lopaschuk et al., 1983). In the beating myocardium SR mediated $[Ca^{2+}]_i$ transient accounts for 90% of $[Ca^{2+}]_i$ changes in the rat myocardium (Bers, 1985). Bouchard and Bose (1991) reported a reduction in SR calcium stores and a decreased fractional release of calcium during stimulation in STZ-treated rat papillary muscles.

However the characteristics of the phosphorylation process in the SR of diabetic myocytes is unknown.

The possibility of depressed sensitivity to $[Ca^{2+}]_i$ by contractile protein also can not be excluded. Treatment with STZ resulted in a decrease in cardiac myosin-ATPase activity due to a shift from the more active V_1 myosin isoenzyme to less active V_3 form (Dillmann, 1982). Since contractile function is thought to be related to myosin-ATPase activity, it is possible that this shift contributed to the diminished cardiac contractility of STZ-treated rats.

Hypothyroidism following STZ-treatment has been suggested to contribute to the diminished number of cardiac ß-adrenoceptors, and the depression of myosin-ATPase (Dillmann, 1982). Chronic administration of T_3 to rats following STZ treatment prevented the reduction in myosin-ATPase activity and the shift in isoenzyme distribution (Dillmann, 1982) and the depression in myocardial ß-adrenoceptor density was normalized (Sundaresan et al., 1984). However the depressed cardiac contraction was not normalized (Tahiliani and McNeill, 1984). Insulin treatment, which reversed the effects of STZ on cardiac ß-adrenoceptors, had no effect on plasma thyroid hormone levels (Nishio et al., 1988). Therefore the role that hypothyroidism plays in diabetes-induced ß-adrenergic deficiency is not totally clear.

b. <u>Ouabain</u>

It is known that the actions of ouabain are due to an inhibition of sarcolemmal Na-K ATPase (Adams et al., 1982). Ouabain binds to the outward-facing site of the Na-K ATPase in the SL (Smith, 1988). At high concentration this binding leads to

inhibition of the Na-K ATPase activity and consequently to an elevated level of $[Na^+]_i$ which in turn leads, via the Na-Ca exchanger, to higher levels of $[Ca^{2+}]_i$ and thus a positive inotropic effects (Vemuri et al., 1989).

The inotropic response to ouabain was reported to be biphasic (Adams at al., 1982). This is correlated with two sets of binding sites (Adams at al., 1982; Fawzi and McNeill, 1985; Hallaq at al., 1991), one of high affinity (K_d ≈3-5 x 10^{-8} M, and B_{max} ≈0.2-0.5 pmol/mg) and one of low affinity (K_d ≈1-6 x 10^{-6} M, and B_{max} ≈1-6 pmol/mg) sites. The high affinity response correlates with the high affinity [^{3}H]-ouabain binding sites but does not correlate with an inhibition of the enzyme Na^+-K^+ ATPase. The low affinity response to ouabain in the rat heart is accompanied by an inhibition of the enzyme (Adams et al., 1982).

It has been previously reported that the concentration of ouabain associated with inotropic effects is about 10^{-7}-10^{-4} M in rat heart (Fein et al., 1983). In sarcolemma vesicles isolated from normal rat heart Na-K ATPase was inhibited by ouabain with the half-maximal inhibitory effects occurred at 1-2 x 10^{-8} M (Lelievre et al., 1984). In cultured chick-embryo heart cells, EC_{50} of ouabain for $[Na^+]_i$ was between 10^{-9}-10^{-8} M. A second increase in $[Ca^{2+}]_i$ was observed between 10^{-6} - 10^{-5} M. (Ahlemeyer et al., 1992). Stimers et al. (1985) reported that the increase in $[Na^+]_i$ caused a 10-fold decrease in the values of the K_m of Na-K pump for ouabain.

It was also reported that the maximum number of binding sites of the high and low affinity binding sites in membrane preparations obtained from chronically diabetic rats was significantly reduced to 60 and 49% of controls, respectively. The dissociation constant of the high (53±0.4 nM vs 39.2±2.5 nM) and the low (0.8±0.2 μM vs 2.1±0.2) affinity binding sites in the diabetic heart, compared to controls, was

significantly increased and decreased, respectively (Fawzi and McNeill, 1985). This means that the sensitivity to ouabain was lower at the high affinity site, but higher in the low affinity site compared with controls. The response to ouabain in papillary muscle and left atria from hearts of diabetic rats was reported to be markedly depressed (Fein et al, 1984, McCullough and McNeill, 1983). These results suggest that the decreased inotropic response of ouabain in the intact cardiac tissue obtained from diabetic rats may be related to a decreased number of ouabain binding sites (Fawzi and McNeill, 1985). The altered K_d could be due to an alteration in the ouabain binding sites or due to an altered composition of the membrane in diabetes.

In this study, the concentrations of ouabain used to induce $[Ca^{2+}]_i$ increase correlates well with the data above, and fits especially well with the binding data in diabetic hearts. It has been shown that the activity of Na-K ATPase is dramatically reduced in the diabetic heart (Pierce and Dhalla, 1983). Because this enzyme indirectly regulates $[Ca^{2+}]_i$ levels through its modulation of $[Na^+]_i$, the diabetes-linked decrease in Na-K ATPase activity would be expected to mediate a net increase in both $[Na^+]_i$ and $[Ca^{2+}]_i$. Myocardium from the diabetic rats is susceptible to calcium loading by ouabain incubation as measured by the development of afterdepolarizations (Nordin et al., 1985). These data suggest that a decline in the reserve capacity of the sarcolemmal Na^+ pump in diabetic heart. The decrease in Na-K ATPase could enhance the sensitivity to digitalis-like compounds by reducing the reserve capacity of the Na-pump and thus the extent of digitalis-induced pump inhibition required before the onset of toxicity (in the case of single myocyte, hypercontraction). A reduction of reserve capacity may lower the tolerance to ouabain by decreasing the number of pump sites which the glycoside would have to

inhibit before eliciting a marked $[Na^+]_i$ accumulation and the resulting toxic effects believed to be mediated by Ca-overload.

Cardiac arrhythmias are frequent and serious complications of the clinical use of digitalis glycosides, and it is possible the tolerance to these cardiotoxic effects is reduced in diabetic patients, and the margin of safety for cardiotonic steroids is reduced.

Panet et al., (1990) reported that, at the therapeutic concentration of (10^{-7} M), ouabain induced an inotropic effect in neonatal-rat cardiomyocytes by increasing Na influx into the cells through a bumetanide-sensitive Na-K-Cl cotransporter which was suggested to play an essential role in the positive inotropic effect of low concentrations of ouabain. The bumetanide-sensitive potassium influx was found to be coupled to influxes of sodium and chloride with a $Na^+/K^+/Cl^-$ ratio of 1:1:2 (Atlan et al., 1984). Ouabain at low concentration (10^{-9}-10^{-7} M) stimulated the bumetanide-sensitive Rb^+ influx in rat myocytes (Heller et al., 1988). At this concentration, Na-K ATPase was not affected while the bumetanide-sensitive Rn^+ influx was stimulated by 300%. The relevance of this Na-K-Cl cotransporter to effects seen in diabetic heart is not clear.

In this study, ouabain started to increase $[Ca^{2+}]_i$ transients at a concentration of 10^{-9} M in cardiomyocytes. Thus it seems that the high affinity binding sites for ouabain are functioning. Whether ouabain binding to this site causes $[Ca^{2+}]_i$ increase via the Na-K-Cl cotransporter is not clear, however enhanced sensitivity to ouabain in diabetic cells might be related to this mechanism.

c. ATP

In cardiomyocytes ATP is the only known transmitter or agent (aside from KCl) to directly depolarize and stimulate $[Ca^{2+}]_i$ transients (De Young and Scarpa, 1989). The mechanism by which ATP increases $[Ca^{2+}]_i$ is not clear. The effects of ATP in modulating Ca-channels and intracellular calcium stores are very similar to KCl (De Young and Scarpa, 1989). They are both sensitive to BAY K 8644, nifedipine, and EGTA and both are partially inhibited by ryanodine and caffeine. These similarities suggest that the ATP mechanism is similar to the KCl effects of membrane depolarization (De Young and Scarpa, 1989) and direct activation of Ca-channels.

ATP has been shown to have positive inotropic effects on mammalian ventricular cells (Danzger et al., 1988). ATP increased I_{Ca} up to twofold (Alvarez et al., 1990) and enhanced the free $[Ca^{2+}]_i$ synergistically with ß-agonists in rat cardiomyocytes (De Young and Scarpa, 1987). These effects are generally attributed to P_2-purinergic receptor stimulation. ATP may have some physiological role in the regulation of cardiac cellular ion transport. It also can be released from hypoxic heart cells (Forrester and Williams, 1977).

ATP is known to depolarize cells by altering ion influx. Direct ATP-receptor activation of the L-type Ca- channel is possible or the cells may be depolarized by a different mechanism that would then activate voltage-sensitive Ca-channels. Also it is possible that activation of a receptor-operated Ca-channel could depolarize the cells, causing amplification of the calcium signal. It is known that the addition of extracellular ATP induces phospholipid hydrolysis and formation of IP_3 in ventricular cells and in intact heart, but the functional role of this in myocardial cellular calcium

kinetics is unclear. α_1-adrenergic stimulation also causess IP_3 formation, but no $[Ca^{2+}]_i$ transient results (De Young, 1989). Alvarez et al. (1990) showed that ATP induced I_{Ca} in frog ventricular cells by a pathway involved in phosphoinositide turnover. It has been suggested that IP_3 enhances release of calcium from cardiac SR, reinforcing the idea that, although calcium influx is apparently required for the $[Ca^{2+}]_i$ response to ATP or KCL, other intracellular messages may be modulatory.

In myocyte suspensions there are two phases of the ATP induced $[Ca^{2+}]_i$ response. The first phase is a P_i-independent phase elicited by ATP and the second phase is P_i-dependent. At 11.2 mM P_i the response to ATP requires extracellular Ca, Na, and P_i, suggesting coordinated transport between these ions. There may exist a P_i- and ATP-activated Na and/or Ca channel or an ATP stimulated a Na-Pi co-transport (Jack et al., 1989) which could in turn stimulate Na-Ca exchange and cause a sustained $[Ca^{2+}]_i$ increase. Or, the ATP receptor may directly stimulate both of these transporters. Whether any Na influx occurs under the normal P_i concentration is not clear.

Christie, et al., (1992) showed that in myocyte suspensions, ATP produced a concentration-, time-, and Mg-dependent, biphasic increase of $[Ca^{2+}]_i$. Extracellular calcium was required for the ATP-induced increase of $[Ca^{2+}]_i$ and pre-treatment of the cell with caffeine, ryanodine, verapamil or nitrendipine partially inhibited the $[Ca^{2+}]_i$ increase. ATP activated an ionic current and associated with the ATP-activated current was cellular depolarization. ATP stimulated the phosphorylation of several extracellular membrane-bound proteins. The phosphorylation of these proteins was concentration-, time- and Mg-dependent. The ATP-induced increase in $[Ca^{2+}]_i$ is a result of the activation, possibly by protein phosphorylation, of a novel ion channel carrying inward current (Christie, et al., 1992). The ATP activated

channel may be permeable to Na and Ca and causes $[Ca^{2+}]_i$ to rise. More importantly, this inward current depolarizes the cell to the threshold of inducing spontaneous firing of action potentials. The firing of action potentials results in the influx of calcium through L-type Ca-channels which would trigger calcium release from the sarcoplasmic reticulum and lead to the increase in $[Ca^{2+}]_i$. The recent report suggests that protein kinase C and A are involved in the ATP-induced $[Ca^{2+}]_i$ increase (Zheng, 1992)

Because of its complicated mechanism of inducing $[Ca^{2+}]_i$ transients, it is difficult to predict the mechanism by which ATP is involved. Enhanced ATP response in diabetic cells, similar to those seen with KCl, could be related to the enhanced response to KCl. They may share a similar mechanism or may just show similar characteristics.

SUMMARY AND CONCLUSIONS

The quality control indices of myocyte isolation (viability, yield, survival time, cell response, etc.) suggest that the adult rat myocyte model is stable and useful in $[Ca^{2+}]_i$ measurements and functional studies at the cellular level. Myocytes have certain of the features of intact cardiac tissues. The changes in myocytes from diabetic rats were found to be representative of the pathophysiological changes previously seen in the intact diabetic rat hearts.

Diabetic myocytes showed a decreased contractility. However, the basal $[Ca^{2+}]_i$ in both quiescent and electrically-stimulated cells were not changed. Resting levels of $[Ca^{2+}]_i$ and basal $[Ca^{2+}]_i$ transients may not reflect the abnormalities observed in diabetes until the system is challenged by certain stimuli.

$[Ca^{2+}]_i$ responses to isoproterenol were depressed in both resting and stimulated diabetic cells. This suggests that there is an alteration in the ß-adrenergic pathway, possibly related to the ß-adrenoceptor deficiency reported in the diabetic heart. SR Ca-ATPase is involved in the isoproterenol-induced $[Ca^{2+}]_i$ changes. The decreased maximum response to 8-bromo-cAMP noted in this study provides evidence of a post-receptor alteration in the pathway as well.

Diabetic myocytes were more sensitive to ouabain and the maximum response to ouabain was depressed. This may be the result of depressed Na-K ATPase and increased $[Na^{+}]_i$. If the reserve capacity of the Na+-pump was impaired in diabetic heart the ouabain would cause cytotoxic effects in diabetic cells at lower concentrations.

The results from the SL membrane Na-Ca exchange study suggest a decreased exchanger number and an increased exchanger affinity to calcium in diabetic myocytes. These changes may be due to diabetes-induced metabolic alteration in the membrane.

In diabetic myocytes, RCCs and CCs were depressed, and caffeine-induced calcium transients were decreased. Ryanodine binding parameters suggested a decreased number of high affinity binding sites in the SR of diabetic myocytes. The data provide indications that SR releasable calcium is reduced and that the major functions of SR, notably uptake, release and storage, may be depressed in diabetic myocytes.

The enhanced $[Ca^{2+}]_i$ response during KCl-induced membrane depolarization and ATP-induced $[Ca^{2+}]_i$ may be related to an altered L-channel activity and characteristics in sarcolemma of diabetic hearts. Further studies are needed in order to understand the mechanisms of the changes.

Insulin treatment normalized most of the diabetes-induced changes in cardiomyocytes suggesting that metabolic alterations due to insulin deficiency play an important role in the diabetic cardiomyopathy.

The data from the current study shows that in diabetes the function of major organelles which handle $[Ca^{2+}]_i$ in myocytes is depressed, which in turn causes the alteration of $[Ca^{2+}]_i$ mobilization in myocytes. Different second messenger systems involved in E-C coupling may also be altered due to the metabolic impairment.

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