EXERCISE-INDUCED ADAPTATIONS IN ISOLATED CARDIOMYOCYTES FROM RAT HEART

by

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ABSTRACT

The increase in stroke volume seen following endurance training has been attributed, in part, to an increase in myocardial contractility. Cellular adaptations attributed to the increase in contractility include, an altered sensitivity to β–agonists and altered calcium delivery by the sarcolemma (SL) and sarcoplasmic reticulum (SR). The objective of this investigation was to evaluate these cellular adaptations by assessing changes in the shortening properties of isolated Ca^{2+}-tolerant cardiomyocytes from trained and control rats.

Female Sprague-Dawley rats were randomly divided into running trained (T) and sedentary control (C) groups. The T group was placed on a 10-12 week endurance training program after which the T rats were running for 60 minutes on an 8% grade at 28 m/min. Cardiomyocytes were isolated using a retrograde (Langendorff) enzymatic perfusion technique and plated on laminin-coated cover slips. Experiments were performed in a Dvorak-Stotler tissue chamber located on the stage of an inverted phase contrast microscope at room temperature.

A video edge detector system (Steadman et al, 1988) was used to measure the shortening properties of isolated myocytes under pre-drug conditions and in the presence of four perturbations: 1) a β-agonist (isoproterenol-100 nM), 2) DHP calcium channel antagonist (nifedipine-10 nM), 3) DHP calcium channel agonist (Bay K 8644-10nM) and 4) a SR calcium release channel antagonist (ryanodine-100 nM).

Pre-drug measurements revealed T myocytes had significantly ($p<0.05$) greater resting cell length, maximum degree of shortening (dL_{max}) and time-to-peak shortening (TPS) compared to C. In the presence of isoproterenol dL_{max} and the maximum rate of shortening ($-dL/dt_{max}$) were significantly ($p<0.05$) greater in the C group compared to T. Although not significant, both nifedipine and ryanodine inhibited dL_{max} and $-dL/dt_{max}$ to a greater degree in C myocytes compared to T. No difference was seen in the responses of the T and C myocytes to Bay K.

In conclusion, distinct differences in myocytes isolated from the T and C rats were observed. The increase in TPS and dL_{max}. could be attributed to previous findings demonstrating endurance training prolonged the plateau phase of the cardiac action potential (Tibbits et al, 1981). The exact mechanism of the attenuated response to isoproterenol in the T group was not determined in this study. However, previous findings suggest this response may be attributed to a reduction in adenylate cyclase stimulation (Dohm et al, 1976 and Moore et al, 1982) through the β receptor.

Dedication

To my family and friends

and all my running and tennis partners.

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Abbreviations

AC:	adenylate cyclase
Bay K:	Bay K 8644: methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate
β-AR:	β-adrenergic receptor
BM:	body mass
$[Ca^{2+}]_i$:	intracellular calcium concentration
CICR:	calcium-induced calcium release
$\dot{Q}$:	cardiac output
cAMP:	adenosine 3',5' cyclic monophosphate
dL_{max} :	maximum magnitude of shortening
$-dL/dt_{max}$:	maximum rate of shortening
$+dL/dt_{max}$:	maximum rate of relaxation
DHP:	1,4 dihydropyridine
EDV:	end-diastolic volume
ESV:	end-systolic volume
FBS:	fetal bovine serum
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HR:	heart rate
HM:	heart mass
HMW:	high molecular weight
I:	current
IP_3:	inositol 1,4,5-triphosphate

Isopro:	isoproterenol: 4-[1-Hydroxy-2-[(1-methyl-ethyl)-amino]ethyl]-1,2-benzenediol
kDa:	kilodaltons
La^{3+}:	lanthanum
L-Type:	long-lasting
Mr:	molecular mass determined from SDS gel
m:	molecular mass determined from amino acid sequence
myosin P-LC:	phosphorylatable light chain of myosin
Na-Ca:	sodium calcium exchanger
NF:	nifedipine
pS:	picoSiemens
PK-A:	cAMP-dependent protein kinase
RCC:	rapid cooling contractures
Ryan:	ryanodine: ryanodol 3-(1H-pyrrole-2 carboxylate)
SR:	sarcoplasmic reticulum
SL:	sarcolemma
SV:	stroke volume
TPF:	time-to-peak force
TPS:	time-to-peak shortening
TPT:	time-to-peak-tension
T-Type:	transient
Tn I and Tn C:	Inhibitory and calcium subunits of troponin
V:	volts

INTRODUCTION

The typical response to endurance training includes a reduction in both resting heart rate (HR) and the heart rate at a given submaximal workrate. The reduction in heart rate is achieved by an increase in cardiac stroke volume (SV). Stroke volume is dependent upon a number of intrinsic (contractility) and extrinsic (preload and afterload) variables. Special attention has been given to intrinsic adaptations (altered contractile element and calcium delivery) suggested to increase stroke volume following endurance training. However, a number of methodological limitations such as cardiac geometry, elastic elements and diffusion limitations, have prevented a thorough assessment of these intrinsic adaptations. The objective of this study was to use shortening as an index of contractility, and identify a number of intrinsic exercise-induced adaptations in Ca^{2+}-tolerant myocytes.

LITERATURE REVIEW

Adaptation to Exercise in Humans

During dynamic exercise one may observe a large increase in oxygen consumption which can be attributed to an increase in stroke volume and heart rate (Andrew *et al.*, 1966). One response to endurance training in humans is an increased maximal oxygen

uptake which is, in part, due to an increase in arterial-venous O_2 (A-V O_2) difference, and an increase $\dot{Q}$. Although some change is seen in the A-V O_2 difference following training (Saltin, 1969) the increased $\dot{Q}$ is probably the most important factor contributing to the increase in $\dot{V}O_2$ max. Since maximal HR does not increase as a consequence of training, the increase in $\dot{Q}$ can be attributed to an increase in SV (Saltin *et al.* 1968 and Ekblom *et al.* 1968). At any given submaximal workrate, the $\dot{Q}$ will remain relatively unchanged however, a trained subject is able to sustain given $\dot{Q}$ at a lower HR. Therefore, under both submaximal and maximal conditions one can attribute the enhanced cardiac performance to an increase in SV (Andrew *et al.* 1966).

It has been difficult to determine the basis for the increased cardiac performance for there are at least three factors which regulate SV in the intact heart. Changes in any of these factors could contribute to the increase in SV observed following endurance training. Two factors regulating SV are extrinsic in nature, while the other is intrinsic. The two extrinsic variables are referred to as preload and afterload. Preload is defined as the degree of stretch placed on the ventricular fibres prior to systole. Afterload, on the other hand, is the pressure load due to the systemic resistance to flow which the myocardium must overcome during the pumping action. The third, or intrinsic factor determining SV is myocardial contractility. Contractility may be defined as a change in

developed tension or velocity of shortening independent of any change in resting fiber length. Research has indicated that an increased preload (Saltin *et al.* 1968) and more importantly contractility (Barnard *et al.* 1980 and Stein *et al.* 1980) are the primary factors contributing to a increased SV following endurance training.

Assessing Myocardial Function in Humans

Several techniques have been utilized in an attempt to assess myocardial function following endurance training in humans. One of these techniques is radionuclide angiography which allows determination of LV ejection fraction, end-diastolic volume, cardiac output and total blood volume (Anholm *et al.* 1982). The Doppler ultrasound technique is capable of determining SV and $\dot{Q}$ on a beat-to-beat basis (Eriksen *et al.* 1990), while volume and pressure transducers directly attached to the ventricular wall are able to assess the relative contribution of preload and contractility to determine stroke volume. Despite the relatively sophisticated techniques used to assess the change in myocardial contractility following endurance training there is a large amount of conflicting evidence on the topic. The basis for the equivocation stems from several extrapolations and assumptions made with respect to cardiac geometry and orientation. In addition, considerable variation may be observed in the type of training protocol and

intensity of the program. In order to overcome some of these experimental limitations, investigators have placed more emphasis on animal models.

Assessing Myocardial Function in Animals

Three different animals, the dog, rat and pig have been used traditionally in training studies. These animals show a similar response to endurance training as do humans such as increased myocardial oxygen consumption, stroke work and SV and a decreased resting HR (Ritzer *et al.* 1980 and Schaible *et al.* 1981).

In order to assess changes myocardial contractility the extrinsic variables of preload and afterload must be controlled. The indices traditionally used to assess the inotropic state of the myocardium include: peak rate of left ventricular pressure development (dP/dt), stroke volume (SV), stroke work (SW), stroke power (SP) and force-velocity changes during ejection (Ross and Peterson, 1973). Indices of inotropy or contractility have also been derived from isovolumic preejection-phase pressure which are less dependent on preload and afterload, these include dP/dt/developed pressure at 40 mm Hg or peak pressure dP/dt/total (Ross and Peterson, 1973)

The *in situ* heart preparation allows measurement of the pressure change in the aorta or ventricle during systole while keeping circulatory and neural pathways intact. In studies such as these, the rate of developed pressure (dP/dt) is commonly

used as an index of contractility. Several studies of this nature have reported an increase in ventricular performance following training (Barnard *et al.* 1980 and Ritzer *et al.* 1980) while others have not (Carew and Cowell, 1978). In addition to observing a significant increase in resting SV in trained rats, Karhunen *et al.*, (1988) also found stroke index, EDV and ESV significantly increased during $CaCl_2$ infusion. A factor which may complicate the results of this study and is apparent in many others, is exercise induced cardiac hypertrophy. The index often used to assess cardiac hypertrophy is the HW/BW ratio. Karhunen observed a significant increase in the HW/BW ratio of the trained rat. Cardiac hypertrophy was attributed to a relatively high running intensity (32 m/min on a 15% grade) which was used to train the animals. Despite the hypertrophy, stroke index (SI) was significantly higher in the trained group, and after isoproterenol infusion there was a more dramatic difference in SI.

Greater control over vascular and neurohumoral factors may be achieved by using *in vitro* preparations, such as the isolated perfused heart and the papillary muscle preparation (Scheuer, 1977). The *in situ* heart preparation does not allow direct assessment of myocardial contractility, but only allows predictions to be made based on changes in cardiac performance. Researchers incorporating the isolated heart and papillary muscle preparation have traditionally used hearts from rats.

Hearts isolated from run-trained rats demonstrate an increased pump performance as evidenced by the significant

increase in SV, SW and maximal power observed. For a similar end-diastolic volume, the ejection fraction, fibre extension and velocity of circumferential fibre shortening are significantly increased in trained animals (Mary, 1987). The difference between isolated hearts from the trained and control rats is even more pronounced as filling pressure is increased (Schaible and Scheuer, 1979). These findings are supported by the studies of Bersohn and Scheuer 1977, Schaible and Scheuer, 1981 and Schaible *et al.* 1981. In contrast to the findings by Schaible *et al.* (1981), Fuller and Nutter, (1981) found no significant improvement in peak systolic pressure, maximum rate of rise of LV pressure, $\dot{Q}$ and SW when plotted as a function of atrial pressure.

Studies using papillary muscle closely resemble studies assessing the mechanism regulating contractility in skeletal muscle. The preparation allows direct assessment of both passive and active tension (Nutter and Fuller, 1977). Indices used to assess contractility in the papillary muscle preparation include maximal developed force (F_{max}) or tension and the maximal rate of developed force (dF/dt_{max}). An increase in contractility has been demonstrated by a significant increase in peak isometric tension of papillary muscle isolated from trained rats compared with controls (Tibbits *et al.* 1978 and 1981).

Another heart preparation commonly used to assess myocardial function is the Ca^{2+}-tolerant isolated myocyte. This preparation offers a number of advantages over the multicellular

preparations previously discussed. These advantages include reducing the elastic influence of any non-contractile components and adjacent cells; reducing the diffusion limitation encountered in multicellular preparations and the assessment of mechanical events in a single cell. The main limitation of this preparation is the difficulty of cell attachment without injury or distortion, and the measurement of force generation directly.

Several methods have been developed to assess contractility in the single cell preparation. One approach taken is to measure changes in sarcomere length during contraction directly (Roos *et al.* 1982). Individual sarcomere lengths can be determined using high-resolution optical microscopy and digital image processing. This involves digitizing the striation pattern of the sarcomere profiled on a photodiode array which allows determination of individual and average sarcomere spacings. Sarcomere length determined using this technique ranged from 1.77-1.91 μm, with maximal shortening and lengthening of 1.51 and 1.93 μm respectively (Roos *et al.* 1982). The main limitations using sarcomere shortening as an index of contractility are: 1) the performance of the observed sarcomere bears little relation to overall muscle performance (i.e., a sarcomere in the central region shortens by up to 15% during an isometric contraction while those in other regions extend) and 2) a sarcomere in the central region shortens at a constant velocity even though force increases dramatically (Tarr, 1983).

Attempts have been made to measure force developed by an individual myocyte. However, this has only been successful in

frog myocardium (Brady et al. 1979 and Tung and Morad, 1988). The first attempt to measure force produced by an isolated myocyte used a photodiode system to detect the motion of a force beam attached to the myocyte by a suction pipette (Brady *et al.* 1979). Considerable inertia was associated with this technique making the frequency response very low. A modification of this technique used suction pipettes to fix both ends of the frog myocyte which was then draped across a force beam (Tung and Morad, 1988). The cantilever utilized the principle that displacement of an ultrasensitive force transducer (force beam) was proportional to the tension developed.

To date, the only viable approach for assessing contractility in the mammalian isolated myocyte is by measuring the magnitude and rate of shortening. Length changes have been assessed using a variety of techniques such as photodiode arrays (Philips *et al.* 1986), high speed film and video motion detectors (Harris *et al.* 1987). A recently published method uses a combination of phase-contrast microscopy and a video edge detector (VED) system. The VED technique provides a high spatial resolution and offers the flexibility of recording the myocyte image on a VCR cassette, allowing one to analyze the data at a later date.

Cellular Adaptation to Exercise

There are findings suggesting endurance training modifies two components influencing the mechanical properties of cardiac

muscle, the contractile element and calcium delivery. Modifications to the contractile element that have been reported include, changes in myosin isoform and ATPase activity. Changes in calcium delivery which have been investigated include, altered transmembrane calcium current (I_{Ca}) and SR calcium release.

One putative mechanism increasing myocardial contractility is an increase in cardiac myosin P-light chain (P-LC) phosphorylation (Resink et al. 1981b and Fitzsimons *et al.* 1990a). Increased phosphorylation following training is associated with an enhanced myosin Ca-ATPase activity which is correlated to changes in contractility (Resink and Gevers, 1981 and Resink *et al.* 1981a). Differences in P-LC phosphorylation have been demonstrated in the presence of isoproterenol and at elevated calcium concentrations (Resink *et al.* 1981b). In contrast, Fitzsimons *et al.* (1990b) associated endurance training with a 10% reduction in myosin ATPase activity, accounted for by a 2-fold increase in the V_3 (low ATPase) myosin isozyme. Tibbits *et al.* (1978 and 1981), although using a different protocol than Resink and co-workers, observed no change in myofibrillar ATPase following endurance training, despite an increase in contractility. Functionally, only phosphorylation of troponin I appears to have any clear importance in cardiac muscle. Phosphorylation of troponin I decreases the calcium sensitivity of activated myosin ATPase (Ray and England, 1976 and Stull *et al.* 1981). This factor, along with the enhanced SR Ca^{2+} uptake (Penpargkul *et al.* 1977), could

be two factors that increase the rate of myocardial relaxation following endurance training.

Myocardial performance during an exercise bout is enhanced in part, due to an increase in circulating catecholamines. However, it is unclear if the positive inotropic action of β-agonists is altered following endurance training. There is evidence that hearts of animals trained by swimming exhibit a greater response to catecholamines (Wyatt *et al.* 1978 and Noakes, 1979). This adaptation, however, has yet to be observed consistently in treadmill-trained animals. When β-receptor number and affinity was measured in membrane preparations from run-trained and control rats no significant difference was observed (Williams, 1980 and Moore *et al.* 1982). In addition, training appears to decrease the epinephrine-stimulated adenylate cyclase from trained heart (Dohm *et al.* 1976). The only increased response to β-agonists following treadmill training was shown by Resink *et al.* (1981a) where an increased phosphorylation of myosin P-light chain from run-trained animals was observed following perfusion with catecholamines. Again, this event was associated with improved cardiac function. In conclusion, the above studies demonstrate no clear relationship between training and β-agonists sensitivity.

A number of observations have been made suggesting calcium delivery is altered following endurance training. One finding suggests, the transmembrane I_{Ca} is increased due to a greater number of DHP sensitive (L-type) calcium channels. This is based on an increased maximal dihydropyridine binding in both

crude homogenate and sarcolemma enriched fractions from trained rat heart (Diffee and Tibbits, 1985 and Weymann and Tibbits, 1987). This increase was observed despite no change in ligand affinity, indicative of an increase in channel density. The putative increase in DHP channels finding is also supported by the finding that trained papillary muscle has 63% more SL Ca^{2+} binding sites (Tibbits *et al.* 1981). In addition, trained papillary muscle is more resistant to the action of La^{3+} (Tibbits *et al.* 1981) which competes for calcium binding sites and entry through the calcium channel (Reuter, 1973). It has been suggested that the Na-Ca exchanger may contribute to SL calcium influx. Activity of the Na-Ca exchanger which significantly increased in isolated SL preparations obtained from trained rats compared to the controls, may also serve as a mechanism increasing SL calcium influx (Tibbits *et al.* 1989).

To fully comprehend the putative exercise induced adaptations addressed in this study the following section will present a more detailed discussion of ECC. This will be followed by a discussion identifying the sites of action for isoproterenol, nifedipine, Bay K and ryanodine and the mechanisms by which these drugs influence ECC.

Excitation Contraction Coupling (ECC)

Excitation contraction coupling entails a series of events beginning with depolarization of the myocardium initiating the rise in intracellular calcium concentration ($[Ca^{2+}]_i$:), and ending

with the generation of force or shortening by the formation of myosin-actin cross-bridges. During depolarization, membrane potential of the myocardium decreases from a resting value of -80 mV to +30 mV. A rapid inward I_{Na} and to a lesser degree a rapid inward I_{Ca} are responsible for the action potential spike at the beginning of depolarization. This is based on the finding that the action potential spike can be abolished either by the sodium channel blocker tetrodotoxin, or through the removal of the extracellular Na ions (Reuter, 1979). The action potential spike is followed by the plateau phase which in part may be attributed to the slow inward I_{Ca} (Reuter, 1979). The I_{Ca} is attributed to the opening of the DHP calcium channels which have a threshold of approximately -35 mV (Hess, 1988). The slow inward I_{Ca} may then contribute directly to the observed rise in cytosolic calcium, and/or induce calcium release from the SR. It has been suggested recently that the slow inward I_{Ca} could be augmented by reversal of the Na-Ca exchanger (Leblanc and Hume, 1990). Cytosolic calcium in mammalian myocardium rises from approximately 100nM at rest (Cheung *et al.* 1989) to a peak value between 500 and 600 nM (O'Rourke *et al.* 1990). It has been suggested that the slow tail of the inward I_{Ca} contributes to the loading of the SR with calcium (Fehr and Fabiato, 1990). This would ensure an adequate amount of calcium is released during the subsequent beat.

In an attempt to identify the characteristics of I_{Ca}, one can alter the extracellular calcium concentration $[Ca^{2+}]_o$ or utilize a family of drugs known as DHP, which selectively act on the DHP

sensitive (L-type) calcium channel (Reuter, 1979). The ability to block the slow I_{Ca} has revealed the existence of a second I_{Ca} referred to as the fast inward I_{Ca}. The threshold potential for the fast inward I_{Ca} is more electronegative than the slow I_{Ca} and the current is of short duration (Reuter, 1979). The rapid Ca^{2+} influx has been attributed to a unique channel referred to as the T-type (DHP insensitive) calcium channel (Bean, 1985 and Nilius *et al.* 1985). The T-type channel is restricted mainly to atrial tissue and some ventricular tissue suggesting it may be more important for pacemaker activity than for ECC (Campbell and Giles, 1990).

It is generally accepted that the most important function of the secondary inward I_{Ca} in mammalian ventricular myocardium is to initiate the release of stored calcium from the SR (Fabiato, 1983). In the rat, the SR appears to be the most important source of activator calcium (Fabiato, 1982). In the dog and rabbit, a relatively greater contribution to the rise in $[Ca^{2+}]_i$ is made by SL calcium influx (Fabiato, 1982). In frog ventricular tissue, transsarcolemmal calcium influx appears to be the exclusive source of activator calcium. Findings suggest the slow I_{Ca} in frog is of sufficient magnitude to activate the myofilaments directly (Morad *et al.* 1983). In addition, frog myocardium is relatively insensitive to the blocking actions of ryanodine (Fabiato, 1982). In mammals the slow I_{Ca} induces calcium release from the SR not in an all-or-none manner but in a graded fashion. The graded release of calcium from the SR by the inward I_{Ca} is dependent upon the following three factors: 1) the rate of rise in cytosolic calcium around the SR release

channel 2) the magnitude of the inward I_{Ca} serving as the signal and 3) the preloaded state of the SR (Fabiato, 1982). It has been suggested that SR calcium release not only contributes to the rise in the $[Ca^{2+}]_i$, but serves as a negative feedback mechanism, inactivating both the DHP sensitive calcium channel and the SR calcium release channel (Fabiato, 1983). This initiates relaxation and the decline in the $[Ca^{2+}]_i$ through the action of the SR Ca-pump and Na-Ca exchanger. The processes involved in calcium transport are illustrated in Figure 1.

Attempts have been made to quantify the relative contribution of transsarcolemmal calcium influx and calcium release from the SR. An estimation of transsarcolemmal calcium influx has been made through monitoring Ca^{2+} depletion of the extracellular space. This has been attempted using a variety of techniques such as Ca^{2+}-selective electrodes (Bers, 1983 and 1987), calcium-sensitive dyes including antipyrylazo III and tetramethylmurexide, (Hilgemann *et al.* 1983) and ^{45}Ca isotope flux (Pierce *et al.* 1987). Measuring the depletion of the extracellular calcium indicates the maximum influx of calcium ranges between 10-45 μmol Ca^{2+}/kg tissue wet wt/beat. This influx alone would be insufficient to generate adequate tension. Estimations indicate the amount of calcium required to generate 50% of maximal steady-state tension ranges between 60-120 μmol/kg wet wt (Solaro *et al.* 1974 and Fabiato, 1982). The range of values obtained for transsarcolemmal Ca^{2+} influx suggests a considerable amount of activator calcium comes from SR release.

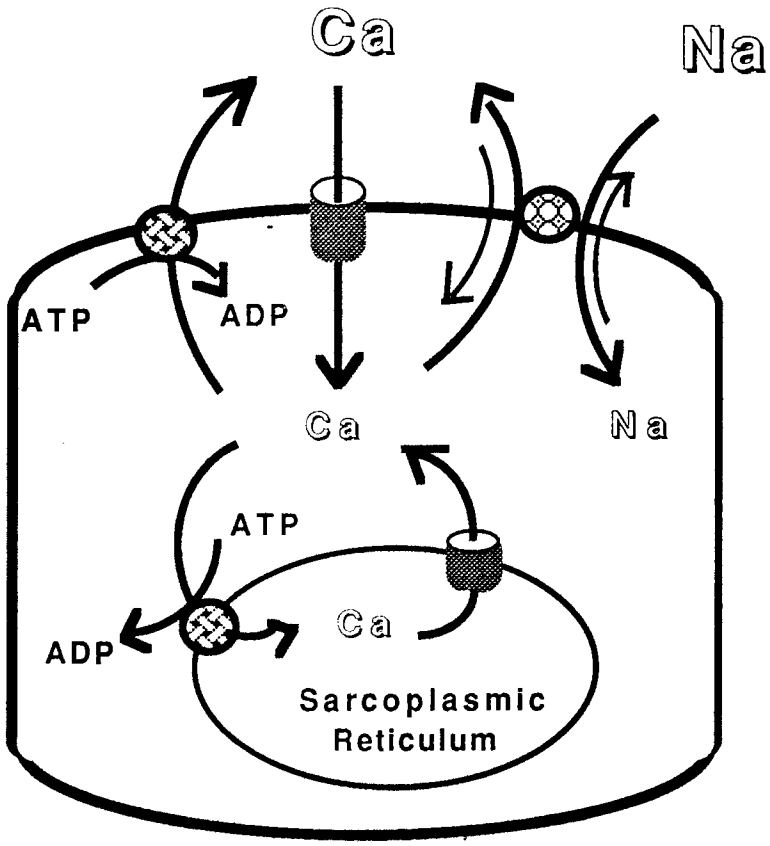


Figure 1. Summary of the excitation contraction coupling in adult mammalian myocardium. The above figure shows three sarcolemma (SL) transporting systems: DHP calcium channel, Na-Ca exchanger (electrogenic exchanger responsible for exporting calcium entering the cell during depolarization and under specific conditions undergoes reverse exchange) and the Ca-pump (a low activity ATP dependent calcium pump). Two calcium transporting systems are present in the SR: the Ca-pump (a high activity ATP dependent calcium pump) and the ryanodine calcium channel (calcium stimulated channel releasing SR calcium stores). Depolarization of the myocardium is followed by opening of the voltage gated DHP sensitive calcium channels. This allows the flux of calcium from the extracellular compartment (10^{-3} M) to the cytoplasm (10^{-7}M). The resulting inward I_{Ca} is capable of activating the myofilaments directly and/or initiate calcium release from the SR. In the mammalian myocardium calcium from the SR is a more important source of activator calcium. The release of calcium from the SR is graded depending upon the magnitude and rate of rise of the SL I_{Ca} and the preload state of the SR.

Rapidly cooling the myocardium from 30 to 1°C leads to the release of all the calcium in the SR (Bridge, 1986). The

contracture that results, can be used as an index for the amount of calcium in the SR. As one would expect based on the lack of SR, rapid cooling contractures (RCC) are absent in frog, although they are readily observed in rat and guinea pig myocardium (Harrison and Bers, 1990). The latter findings further support the theory that frog myocardium only depends upon extracellular Ca^{2+} influx for mechanical coupling (Harrison and Bers, 1990). Another feature observed during rapid cooling is a decrease in the calcium sensitivity of the myofilaments (Harrison and Bers, 1990). This was identified by tension spikes produced in the myocardium during the rewarming process. The decrease in calcium sensitivity or rewarming spike was greatest in the rat and guinea pig, and lowest in the frog.

In an attempt to assess modifications to calcium delivery following endurance training, four drugs will be utilized which alter transsarcolemmal calcium influx and SR calcium release. Three of the drugs (nifedipine, Bay K and isoproterenol) alter transsarcolemmal calcium influx by influencing the DHP channel. Ryanodine will be used to modulate calcium release from the SR. The following section will identify the mechanisms of action for each drug in detail.

DHP Sensitive and Insensitive Calcium Channels

The existence of two distinct inward calcium currents (fast and slow) during depolarization was first demonstrated by Hagiwara in starfish oocytes (Hagiwara *et al.* 1975). More

recently, single channel recordings have shown a fast and slow calcium current carried by the T and L-type calcium channels respectively, in both atrial (Bean, 1985) and ventricular (Nilius *et al.* 1985) myocardium. T and L-type calcium channels can be found in most atrial myocardium, while only select mammalian species possess both T and L-type calcium channels in ventricular myocardium (Campbell and Giles, 1990).

Recordings taken from the T-type or DHP-insensitive calcium channel show the channel to be rapidly inactivated (within 10's of msec of onset), activated at a negative potential (-60 mV), insensitive to DHP and possessing a 8pS conductance for barium (Nilius et al. 1985 and Bean, 1985). Currents through both the T-type channel and the L-type (DHP sensitive) channel may be blocked by cobalt. In the presence of isoproterenol only the activity of the DHP channel is increased (Hess, 1988). The unique features of the DHP channel include its relatively large barium (Reuter *et al.* 1982) conductance (25 versus 8pS demonstrated by the T-type channel), a long lasting open time course (Reuter *et al.* 1982) at most potentials (with each channel having a mean open time of 1ms at 25°C), and its voltage dependent activation (-35 mV threshold) with a sigmoidal time course (Lee and Tsien, 1983). Unlike the T-type calcium channel, inactivation of the DHP channel is dependent upon both the membrane potential value and the rise in the $[Ca^{2+}]_i$ (Kass and Sanguinetti, 1984, Lee and Tsien, 1983 and Sanguinetti and Kass, 1984). The T-type channel is inactivated in a voltage dependent manner, but appears to have closing, inactivation and recovery

steps which are voltage independent (Chen and Hess, 1990). To date, there is no evidence to suggest T-type calcium channels exist in rat ventricular tissue. The remaining discussion will therefore focus on the properties of the L-type or DHP sensitive calcium channel.

The distinguishing feature of the L-type calcium channel is its sensitivity to DHP. Interestingly, DHP are capable of acting as either an agonist and antagonist in myocardial tissue (Kass and Sanguinetti, 1984), and in some instances DHP antagonists have the opposite effect on skeletal muscle (Kawata and Hatae, 1990). The mechanism of action of nifedipine (antagonist) and Bay K (agonist) is to increase the closing and opening times respectively of the DHP channel (Reuter *et al.* 1986). The net result is nifedipine and Bay K decrease and increase respectively, the slow inward I_{Ca} during the plateau stage of depolarization. DHP alters channel properties by specifically binding to the α_1-subunit of the DHP channel (Galizzi *et al.* 1986). Identification of the DHP binding site has been provided with the recent purification and cloning of the DHP channel.

The transverse-tubules of skeletal muscle possess the richest source of DHP receptors. Purification of the skeletal DHP receptor indicates it is made up of 5 subunits (illustrated in Figure 2), two high Mr (molecular mass determined from SDS gel electrophoresis) subunits and three low Mr (Takahashi *et al.* 1987). The α_1 is a 175kDa subunit containing the DHP binding site and a PK-A phosphorylation site(s). The second high Mr polypeptide is the α_2-subunit and is a 143kDa. It has none of the

properties of the α_1-subunit but binds lectins and is disulfide linked to the 24 and 27kDa forms of the δ-subunit (De Jongh *et al.* 1990). It appears both the α_2 and δ-subunits are encoded by the same gene (De Jongh *et al.* 1990). The β- and γ-subunits make up the remainder of the DHP channel and are 52 and 32kDa respectively.

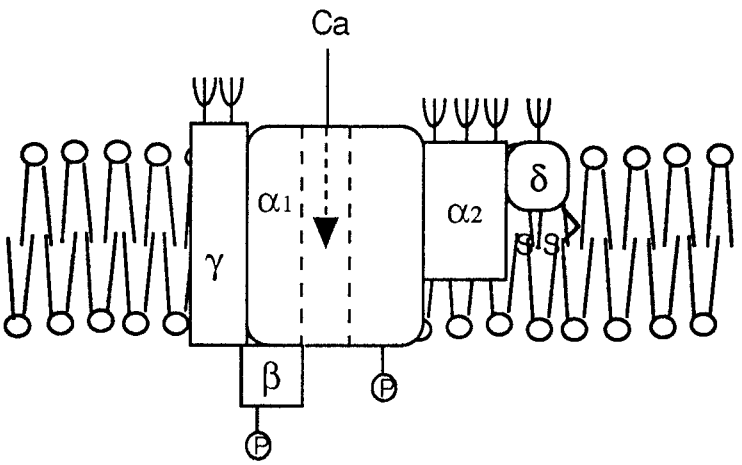


Figure 2. The five subunit (α_1, α_2, β, γ and δ) arrangement of the skeletal DHP sensitive calcium channel. The proposed channel structure for the cardiac DHP channel contains only four subunits (α_1, α_2, β, and γ) lacking the γ-subunit. Appendages on the α_2, γ and δ subunits indicates a glycosylated site. Adapted from Catteral *et al.* 1990.

The α_1 subunit was the first portion of the L-type calcium channel from skeletal muscle to be cloned and sequenced and contains 1873 amino acids giving it a *m* (molecular mass calculated from amino acid sequence) of 212kDa (Tanabe *et al.* 1987). This was followed by cloning of the α_2-subunit which contains 1106 amino acids giving it a *m* of 125kDa (Ellis *et al.* 1988). Cloning of the β-subunit reveals it is an important

regulatory subunit of the DHP channel. It is made of 524 amino acids (58kDa) and contains a number of concensus phosphorylation sites for Pk-C (12), PK-G (1), PK-A(1) and 3 N-glycosylation sites (Ruth et al. 1989). The β-subunit appears to possess no hydrophobic domains suggesting it resides on the cytoplasmic side of the channel bound to one of the subunits namely the α_1–subunit. The β-subunit may also possesses a low affinity calcium binding site (Ruth *et al.*, 1989) which could serve to inactivate the channel following SR calcium release.

Unlike the skeletal DHP channel, the cardiac DHP channel only possesses four subunits: α_1, α_2, β, and δ (Tuana and Murphy, 1990). Cloning and sequencing of the cardiac α_1-subunit shows it contains 2171 amino acids giving it a *m* of 242 kDa (Mikami et al. 1989). This is significantly larger than the *m* of 170-195 determined by SDS gel (Ferry *et al.*, 1987). The β- and δ–subunits from cardiac muscle have been purified and appear to have identical Mr as their skeletal muscle counter parts, 52 and 28 kDa respectively (Tuana and Murphy, 1990). The purified cardiac α_2-subunit is has a Mr of 170 kDa (Takahashi and Catterall, 1987) compared to 175 kDa (*m* 125 kDa) for the skeletal subunit (Tuana and Murphy, 1990).

The α_1-subunits from both skeletal and cardiac muscle have four repeated units of homology each repeat having 6 transmembrane spanning regions and 66% homology (Mikami *et al.* 1990). The four internal repeats of the α_1-subunits are highly conserved with the cytoplasmic domains less conserved. Ligand and reconstitution studies have confirmed the α_1-subunit of the

DHP receptor primarily functions as a calcium channel (Takahashi *et al.* 1987 and Perez-Reyer *et al.* 1989). The remaining subunits making up the native channel are responsible for regulating channel activity, as suggested by the number of phosphorylation and calcium binding sites (Ruth *et al.* 1989). Recently, it has been suggested the DHP channel may act as a voltage sensor and slow calcium channel in skeletal muscle. This was demonstrated when both ECC and slow I_{Ca} was restored in dysgenic muscle following the injection of cDNA for the DHP channel (Tanabe *et al.* 1988). Both normal muscle and dysgenic muscle responded to electrical stimulation under conditions where extracellular calcium was removed and 0.5 mM cadmium (Cd^{2+}) was included. Additional support for this hypothesis comes from the finding that the α_1-subunit from skeletal muscle is 29% homologous with the voltage-sensitive Na^+ channel (Tanabe *et al.* 1987) and contains a stretch of positively charged amino acid residues (S4) proposed to function as a voltage sensor (Catteral *et al.* 1988).

Ryanodine Channel

The primary function of the DHP channel in both mammalian cardiac or skeletal muscle is to initiate calcium release from the SR. Calcium release by the ryanodine-sensitive calcium channel has been demonstrated in reconstituted membrane and native SR preparations from both skeletal and cardiac muscle (Smith *et al.* 1988 and Nakai *et al.* 1990). Like the DHP calcium

channel, the ryanodine channel demonstrates a selective conductance for divalent ions. The high conductance of the purified ryanodine channel from cardiac (85pS) and skeletal muscle (110pS) demonstrates its importance as a calcium release channel (Rardon *et al.* 1990). The ryanodine channel can be activated by μM concentrations of calcium and mM concentrations ATP, and inhibited by ruthenium red (Smith *et al.* 1988). The unique properties of the channel are its apparent high and low affinity (K_d of 7nM and 1μM respectively) binding sites for ryanodine (Inui *et al.* 1988). The two binding affinities explain the conflicting observations that ryanodine may both inhibit and stimulate calcium efflux from the SR. It has been demonstrated that a high concentration of ryanodine (>1μM) inhibits calcium release by blocking the channel (Alderson and Fehr, 1987), while low concentration such as 20nM, causes a long term open state of lower conductance (Smith *et al.* 1988). This slow leak of calcium effectively depletes the SR of its calcium stores. The net effect of either low or high ryanodine concentrations is to decrease the calcium-induced release of calcium from the SR, which results in reduced peak $[Ca^{2+}]_i$ transients (Wier *et al.* 1985).

Cloning and sequencing of the ryanodine channel has provided important information with respect to the function, regulatory sites and nature of the transmembrane and cytoplasmic domains. The cloned ryanodine channel from skeletal muscle shows it contains 5,037 amino acids giving it an *m* of 565 kDa (Takeshima *et al.* 1989). Subsequent cloning of the

cardiac ryanodine channel indicates it contains 4,969 amino acids giving it a similar *m* of 564 kDa (Otsu *et al.* 1990). Electron micrographs have shown that the native ryanodine channel exists as a homotetramer giving it a total *m* of 2.26 x 10^6 (Lai *et al.* 1988). As was seen with the α_1 subunit from the DHP channel, the ryanodine channel from cardiac and skeletal muscle show 66% homology. Based on the predicted secondary structure and hydropathy plots, the two isoforms exhibit the same topology in both the transmembrane and cytoplasmic (foot) regions (Nakai *et al.* 1990). The highly conserved transmembrane or channel region consists of four highly hydrophobic domains (M1-M4) each comprising 20 amino acids, characteristic of transmembrane α–helices (Takeshima *et al.* 1989). In addition, to functioning as a channel, the transmembrane region contains the binding sites for the antagonists ryanodine and ruthenium red (Takeshima *et al.* 1989).

The cytoplasmic or foot region of the protein constitutes nine-tenths of the receptor and contains amino acid residues indicative of a possible nucleotide (ATP) binding domain, and 3 calmodulin binding regions (Otsu *et al.* 1990). Phosphorylation and calmodulin binding to the ryanodine channel has been shown in junctional SR vesicles from both cardiac and skeletal muscle (Seiler *et al.* 1984). This cytoplasmic region also shows a high degree of similarity to the IP_3 receptor (Furuichi *et al.* 1989). IP_3 is capable of inducing calcium release from both skeletal and cardiac SR, however, the physiological importance of this mechanism is not clearly defined in cardiac muscle (Fehr and

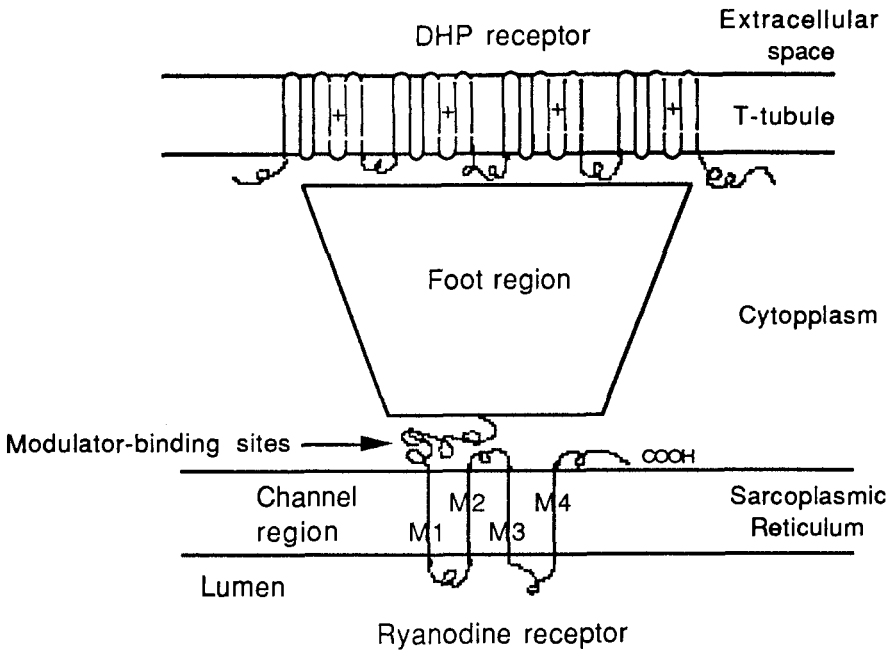


Figure 3. Transmembrane topology of the ryanodine channel and the DHP channel. The carboxy-terminal region of the ryanodine channel, including the transmembrane spanning segments M1-M4, the modulator binding sites, and the large cytoplasmic region corresponding to the foot structure. The transmembrane segments of the DHP channel (segments S1-S6 in each of the four repeated homology units) are also illustrated along with the possible voltage sensor segment S4 in each repeat indicated by plus signs. Adapted from Takeshima *et al.* 1989.

Fabiato, 1990). Removal of the foot structure by degradation of the channel with calpain (formation of 315 and 150kDa fragments) significantly increases the opening times of the channel (Rardon *et al.* 1990). However, channel activity is still sensitive to a small concentration (μM) of Ca^{2+} and a high concentration of ryanodine, suggesting that the fragment formed is the foot region and is important for the inactivation of the channel. The proposed model for coupling between the ryanodine and DHP channel is presented in Figure 3 which illustrates how in skeletal muscle the foot structure could transmit the voltage

signal from the DHP channel to the ryanodine channel when its acting as a voltage sensor. In the case of cardiac muscle the close association of the DHP and ryanodine channel would provide an ideal environment for the localized inward I_{Ca} to induce calcium release from the SR.

β-Adrenergic Receptor

Isoproterenol, although not directly binding to the DHP channel, regulates the secondary inward I_{Ca} by a cAMP mediated pathway (Tsien et al. 1986). In addition to being positive inotropic agents, β-agonists effect a number of other metabolic pathways in cardiac muscle. Increased mechanical performance through β-adrenergic stimulation may be demonstrated by their effect upon a number of muscle performance parameters such as peak developed force (F_{max}), $-dF/dt_{max}$, $+dF/dt_{max}$ and TPF (Endoh and Blinks, 1988). When a muscle is stimulated with a β-adrenergic drugs these parameters increase as do the rate at which intracellular Ca^{2+} is increased and decreased, and the magnitude of the Ca^{2+} transients change (Endoh and Blinks, 1988). Despite β-agonists influencing a number of cellular processes, it is generally accepted that the increased rise in cytosolic Ca^{2+} is brought about by a greater inward I_{Ca} through the DHP channels (Tsien et al. 1986).

The positive inotropic actions of β-agonists are first initiated by ligand binding to β_1- or β_2-adrenergic receptors (AR). The β_1- and β_2-AR contain 477 and 413 amino acids respectively,

each having a *m* of 64kDa (Lefkowitz and Caron, 1990). Cloning of the β-receptors has shown that the protein contains 7 hydrophobic regions indicative of transmembrane spanning regions and several regions important in coupling with the guanine nucleotide binding protein (G_s).

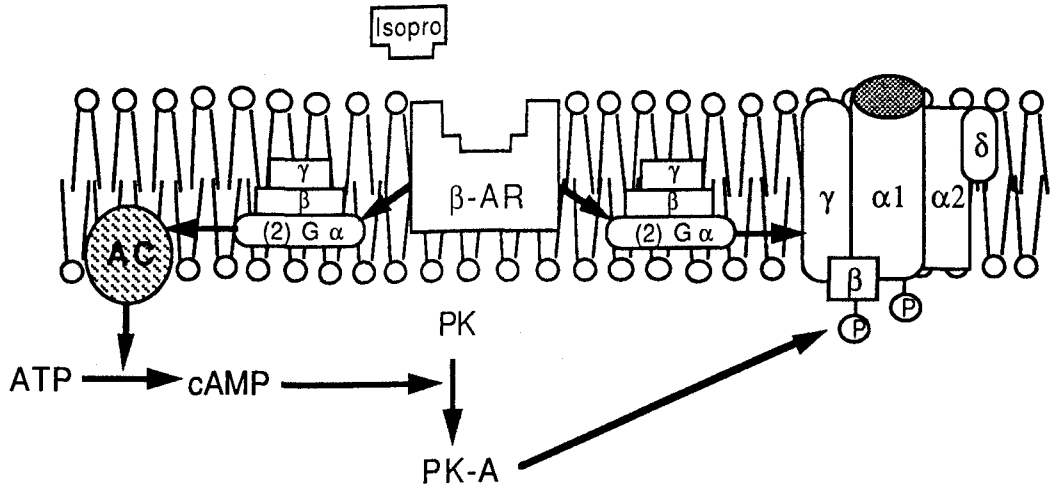


Figure 4. Direct (G_s mediated) and indirect (cAMP mediated) coupling of the β-receptor with the DHP calcium channel. Refer to text for discussion. Adapted from Brown, 1990.

The most widely recognized signal mechanism linking the β-AR and the DHP channel is a cAMP-mediated phosphorylation (summarized in Figure 4). Ligand binding to the β-receptor activates a (G_s) through the dissociation of the α-subunit from G_s. The α-subunit activates adenylate cyclase (AC) which leads to the formation of cAMP. The subsequent rise in cytosolic cAMP activates a cAMP-dependent protein kinase (PK-A). The activated PK-A selectively phosphorylates threonine and serine residues on the DHP-sensitive calcium channel (Tsien *et al.* 1986). It is not known whether phosphorylation of the calcium channel

directly or indirectly activates or inactivates the calcium channel. Two possible mechanisms proposed by Reuter and Scholz, (1977) include two phosphorylation sites, one acting to determine whether the channel is active or inactive and the other which regulates the channel (ie. channel kinetics). However, it is clear the regulatory site does not absolutely determine activity of the channel but merely regulates its activity.

In addition to the cAMP-mediated pathway, it has been recently suggested that a single DHP calcium channel can be directly activated by the α-subunit from the G_s protein (Yatani *et al.* 1987) associated with the β receptor (also illustrated in Figure 4). Support for this hypothesis has also been demonstrated in a whole cell preparation (Pelzer *et al.* 1990). Despite blocking cAMP mediated activation of the channel, β-agonists continued to produced a 50% increase in I_{Ca} (Pelzer *et al.* 1990). A similar increase in I_{Ca} was observed during intracellular activation of G_s and following intracellular application of preactivated G_s (Pelzer *et al.* 1990).

Modulation of the calcium channel by β-agonists does not appear to effect the single channel conductance but increases the probability of individual calcium channels being open (Reuter *et al.* 1982). This can be achieved by increasing the mean open time of the calcium channel, reducing the time interval between the bursts of channel openings, increase the burst length and reduce the failure of a channel opening upon depolarization (Tsien *et al.* 1986). Recent findings suggest β-agonists increase

the rate of cross-bridge cycling in rat cardiac muscle (Saeki *et al.* 1990), in addition to increasing contractility through an enhanced I_{Ca}.

Myocardial Contractility

Unlike skeletal muscle, the force velocity relationship of cardiac muscle is not constant and can be altered by changes in stimulation frequency (Chapman 1983). This enhanced inotropic state (contractility) of myocardium is associated with greater peak $[Ca^{2+}]_i$ which ultimately increases the formation of myosin and actin cross-bridges. Altering either the transsarcolemmal calcium influx or SR calcium release will ultimately effect the rise in $[Ca^{2+}]_i$. Several attempts have been made to correlate force development with $[Ca^{2+}]_i$. There is considerable support for a sigmoidal relationship between steady-state tension development and pCa in skinned cardiac cells (Solaro *et al.* 1974 and Fabiato, 1982). There is only a slight variation in the p50 ([Ca] inducing 50% maximal tension) of values for atrial and ventricular myocardium with the p50 averaging pCa 5.6 (Fabiato, 1982). This steady-state relationship provides only limited information with respect to the intact myocardium in which calcium levels are transient.

An alternate approach has been to establish the relationship between force development and $[Ca^{2+}]_i$ using intact myocardium injected with Ca^{2+}-sensitive dyes. Skinned trabeculae injected with aequorin demonstrated a close

relationship between peak force development and peak $[Ca^{2+}]_i$ during a twitch (Gwathmey and Hajjar, 1990). Likewise, the rate of rise in tension was linearly related over a large range of peak $[Ca^{2+}]_i$. A similar study using aequorin-injected papillary muscle observed a linear relationship between the rate of rise of tension (dF/dt) and $[Ca^{2+}]_i$ during a twitch contraction (Yue, 1987). The linear relationships held true for strengths of contractions approximately 70% of maximal activated force. An identical relationship held true for the complete rising phase of tension in contractions with $[Ca^{2+}]_i$ transients slowed (4-5x) by exposing the muscle to ryanodine (Yue, 1987). It appears a close correlation exists between peak $[Ca^{2+}]_i$ transients and F_{max} (Gwathmey and Hajjar, 1990 and Weir and Yue, 1986) and the rate of developed force (Weir and Yue, 1986 and Yue, 1987). For the sake of this study, it is not adequate to only relate changes in peak $[Ca^{2+}]_i$ to force production. This relationship must also be extended to myocyte shortening (ie. dL_{max} and dL/dt_{max}).

The relationship between peak $[Ca^{2+}]_i$ and myocyte shortening was previously investigated using a combination of high-speed imaging of fura-2-loaded myocytes and an edge detector system (O'Rourke *et al.* 1990). Findings demonstrated changes in the shortening and relaxation parameters were parallel with the alterations in Ca^{2+} transients. This was achieved by altering the stimulus frequency and exposing the myocytes to isoproterenol. A 100 nM dose of isoproterenol produced an increase in the peak $[Ca^{2+}]_i$ from approximately 500

nM to 800 nM with a corresponding increase (percent of control) in dL_{max} and dL/dt of 111% and 280% respectively.

Attempts have been made to relate the shortening characteristic and mechanical properties of isolated myocytes. To date this has only been done successfully in isolated frog myocytes. Tung and Morad, 1988 modified a previous design by Brady *et al.* 1979 by fixing both ends of the myocyte to suction pipettes and draping the cell over tubing of known compliance. Tension development was then calculated based on the degree of tubing displacement. A comparison of the frog myocyte and muscle strips revealed isometric twitches in the two preparations were qualitatively similar. Generally TPT was shorter and falling phase was prolonged in the single cell versus the muscle strip. Single cell force was measured in response to stimulus rate, resting length, $[Ca^{2+}]_o$ or by the addition of epinephrine and in all cases, force resembled the physiological response seen in the muscle strip. The only notable difference was the beat-to-beat variation in the single-cell twitches.

The previous discussion establishes the basis by which this study will relate the effect of the four perturbations on myocyte shortening with the rise $[Ca^{2+}]_i$. This enables one to gain insight into any putative exercise-induced adaptations related to ECC.

OBJECTIVES

The primary objective of this study was to address the many limitations encountered by multicellular preparations by assessing contractility in Ca^{2+}-tolerant isolated myocytes. This study addressed a number of cellular mechanisms suggested to increase myocardial contractility following endurance training. The first objective was achieved by assessing the shortening properties of trained and control myocytes under pre-drug conditions. The second objective was to evaluate three putative cellular modifications influencing myocardial performance following endurance training. The features of these cellular adaptations included; altered β-agonist sensitivity, transsarcolemmal I_{Ca} mediated by altered DHP channel density and SR calcium release. To assess the three putative exercise-induced adaptations, myocytes from endurance trained and control rat hearts were exposed to four drug perturbations. Changes in the shortening and relaxation properties of the trained and control myocytes were compared and used as an indicator for putative exercise-induced adaptations. Shortening parameters of the isolated myocytes were determined using a video edge detector technique.

METHODS

Training

The training protocol used in this study was described previously by Tibbits *et al.* (1989). Eighty female Sprague-Dawley rats were randomly divided into running trained and sedentary control groups. The rats were housed in an animal care facility and fed *ad lib.* Rats from the training group were run on a rodent treadmill five days per week for 10-12 weeks. Each training session was preceded by a 3-4 minute low intensity running warm-up. To ensure the rats continued running during the training session, an air jet was used as a shock stimulus. During the training program the workrate was progressively increased both in duration and intensity (as outlined in Table 1), until the ninth week of training. By the ninth week, the trained rats were running at a speed of 28 m/min on a 8% grade for 60 minutes. The body weights of the rats were measured once per week throughout the study.

Table 1: The progressive increase in running intensity and duration throughout the nine week training program.

PGM Lgth (Weeks)	Speed (m/min.)	Duration (min.)	Grade (%)
1-3	20	30	8
4-6	25	45	8
7-9	28	60	8
10+	28	60	8

Cell Isolation

Upon completion of the ten weeks of training ventricular Ca^{2+}-tolerant myocytes were isolated from trained and control rats on alternate days for a period of 3 weeks. A Langendorff perfusion apparatus (see **Appendix A**) was used modifying procedures previously described by Wittenberg and Robinson, 1981, Wittenberg *et al* 1986 and Langer *et al* 1987. Isolations were performed in a table top laminar flow hood (Nuair model Nu-201) using sterile filtered solutions. Trained rats were sacrificed 48-72 hours following the last training bout to minimize any acute responses to exercise.

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg) supplemented with heparin (150 U/kg). Following removal of the heart from the chest cavity, 10 ml of solution #1 (calcium-free HEPES buffer, contents listed in **Appendix B**) maintained at 4°C was forced through the coronary arteries with a syringe. This was followed by arresting the heart in 30 ml of 4°C buffer. The aorta of the heart was cannulated with an 18 gauge blunt needle using silk thread and an electronic clip, and any remaining blood was washed out following perfusion of the coronary arteries with solution #1, in a non-recirculating manner for 3-5 minutes (flow rate 12ml/min). This was immediately followed by a 40-45 minute perfusion with solution #2; solution #1 supplemented with 50 μM $CaCl_2$ and 1.5 mg/ml (189 U/mg) collagenase (Worthington Biochemical Cooperation Type II Lot#89B-844).

During the perfusion procedure the perfusion medium was oxygenated (100% O_2) and maintained at 37°C. Following perfusion with solution #2, the ventricles and septum were detached from the cannula and teased apart in 10 ml of fresh, aerated solution #2 (37°C). To facilitate tissue breakdown further a 2-3 mm bore plastic Pasteur pipette was used to agitate the solution for 5-10 seconds at 2 minute intervals, for 8-10 minutes. The cell suspension was then passed through a nylon mesh (300 μm) into a 15 ml centrifuge tube. The centrifuge tube was placed on its side for 5-7 minutes and then turned upright to allow the cells to form a pellet at 1 x g. The supernatant was removed and the cells resuspended in solution #1 supplemented with 200 μM $CaCl_2$. The washing process was then repeated with solution #1 containing 500 μM $CaCl_2$. One final washing was performed with solution #1 containing 1 mM $CaCl_2$. The final pellet (0.3-0.6 ml) was resuspended in 35-40 ml of M-199 (contents listed in **Appendix B**) containing 1.8 mM $CaCl_2$ and supplemented with 5% fetal bovine serum (FBS). Myocytes were seeded for 60 minutes at room temperature on glass cover slips (located in 35 mm Petri dishes) previously etched with concentrated nitric acid and coated with laminin (M-199 containing 20 μg/ml laminin) as previously described by Haddad *et al.* 1988. The cover slips were washed twice with M-199 to removed any dead cells or debris. The cover slips were finally bathed in fresh M-199 containing no FBS for 2-4 hours at room temperature.

Myocyte Viability Criteria

To ensure the viability of cells during experiments, myocytes were selected according to the following criteria. Viability was assessed following the transfer of the plated myocytes to the tissue chamber.

1) A cell showed no spontaneous wave-like contraction in the presence of 1.8 mM $CaCl_2$.

2) A myocyte possessed clear striations and a clearly distinguishable cell membrane with no blebs or invaginations.

3) A cell responded to a 0.5 Hz electrical stimulation in a twitch-like manner during a five minute period prior to any experimental manipulation.

4) A cell was uniformly anchored to the laminin-coated glass cover slips and exhibited no bending during contraction.

5) A minimum and maximum rates of shortening and relaxation were used to select cells for experiments. A selected cell had a shortening rate no slower than 0.14 V and shortening rates no greater than 0.36 V, respectively. The corresponding minimum and maximum rates of relaxation were 0.10 V and 0.36 V, respectively.

6) All cells were used within 4-6 hours of the final isolation step.

Experiments were carried out on the myocytes following the transfer of the glass cover slips from the 35 mm tissue culture dish to a Dvorak-Stotler tissue chamber (Nicholson Precision Instruments) mounted on the stage of an inverted phase contrast microscope (Nikon Diaphot). Cells were viewed at

x400 magnification. An oxygenated (100%) bathing solution (room temperature) was delivered to the tissue bath by a Masterflex pump (model 7550-60) and removed by aspiration. The flow of the control and drug solutions through the tissue chamber was regulated using an electrically controlled valve. Myocytes were field stimulated with 20 volt pulses of 7 ms duration by a platinum wire electrode connected to a Grass S11 stimulator.

The 0.2 mm thick glass cover slips formed the bottom of the tissue chamber and allowed viewing of the cells through either the ocular or by the high resolution charge-coupled device (CCD) television camera (Cohu model 4815) attached to a side port of the inverted microscope. The video image of the myocytes was passed on line from the CCD camera to the video cassette recorder (Panasonic model PV-1730), through the video edge detector circuit board (Steadman *et al.* 1988) to a high resolution black and white monitor (Sony model PVM-91). The output from the edge detector: change in cell length (dL) and its derivative (dL/dt) were directed to a two channel storage oscilloscope (Tektronix model D11) which allowed on line viewing of the change in myocyte length and the rate of shortening and relaxation, respectively. The on-line measurement also provided feedback about the quality of cell tracking by the edge detector. The oscilloscope sweep was synchronized with the Grass stimulator which provided time course information with respect to the shortening and lengthening cycle of the isolated myocyte. For each myocyte prior to drug exposure, the parameters which

were recorded included: magnitude of cell shortening (dL_{max}), maximum rate of shortening ($-dL/dt_{max}$) and relaxation ($+dL/dt_{max}$) and time-to-peak shortening (TPS). The order of drug exposure was randomized for each experiment to eliminate any cell variability during the 4-6 hours of experiments. The maximal response to isoproterenol could be seen within 30 seconds after changing from the control solution to the isoproterenol solution. Values for each parameter were recorded five minutes after switching solutions. In the case of Bay K and nifedipine the maximal response could be seen within minutes. A 5-7 minute equilibration period was allowed for these two drugs. Ryanodine required 15 minutes of equilibration before measurements could be taken. After recording the response of 2-3 different cells to a drug, the tissue bath and perfusion lines were washed out with the control solution. The line was then primed with the next drug. On a given day the drug response of 8-12 cells (one cell per cover slip) were recorded.

Operation of the Video Edge Detector

The video edge detector system used in this study was devised by Steadman *et al* 1988. This system utilizes phase contrast imaging of an isolated myocyte where a signal representing a change in light intensity from the surrounding medium to cell body is picked up by a CCD camera. Measurement of cell length is achieved by the light-sensitive element of the CCD scanning the image area with 754 parallel horizontal lines

from top to bottom sweeping left to right. The voltage along these scan lines varies in relation to light intensity encountered by these elements enabling the edge detector to identify the darker cell boundaries (edges).

The parallel orientation of the myocyte, and the nature of the scan sweep allows the length of the cell to be quantified at 16.7 ms (60 Hz) intervals. As the scan line sweeps left to right, the left and right edges are represented as voltage spikes, as

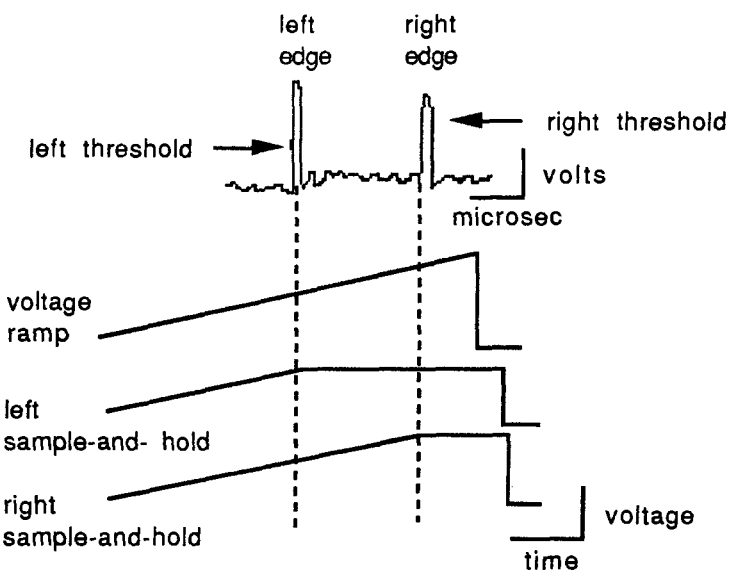
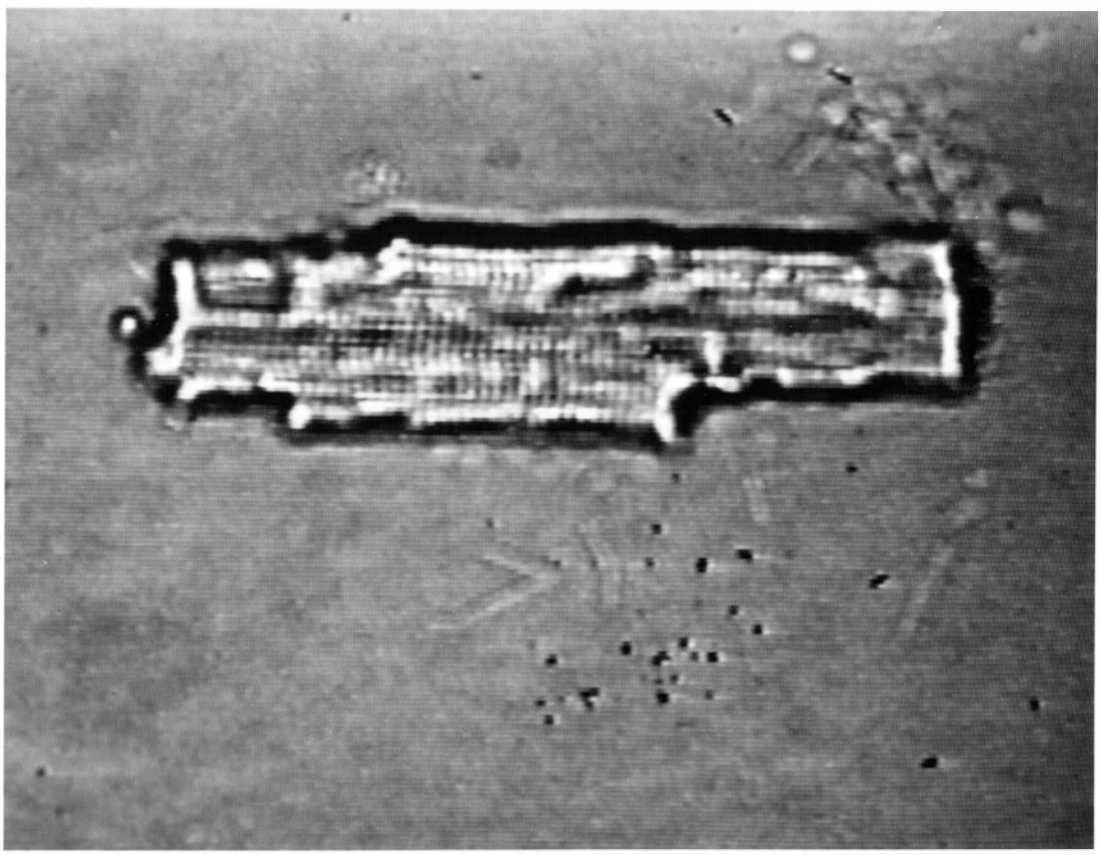


Figure 5: Video edge detector output. The upper tracing illustrates the scan line signal as it crosses the right and left edges of the cell. The lower traces show the voltage ramps used to obtain cell length. The left and right voltage ramps are achieved when the scan line achieves the pre-set threshold. Figure adapted from Steadman *et al.* 1988.

illustrated in the top tracing of Figure 5. The portion of the cell to produce the most significant voltage change along the raster line are the dark boundaries of the cell achieved using phase-contrast microscopy (illustrated in Figure 6). Figure 5, also illustrates the voltage ramp which serves as a reference signal

Figure 6. Video image of a resting rat myocyte with the edge detector windows (black line) and tracking dots (white dots overlaying the ends of the cell) in position. Contraction of the myocyte along its longitudinal axis results in movement of the ends within the window allowing visual assessment of the quality of tracking.



for the left and right sample-and-hold circuits. The ramp and sample-and-hold circuits are activated at the beginning of each sweep. When the scan line voltage at the left cell edge reaches the left preset voltage threshold, the left sample-and-hold circuit retains the ramp voltage. Similarly, when the scan line reaches the right edge of the cell, the right sample-and-hold retains its voltage. The length of the cell during a single sweep is determined by the difference between the right and left sample-and-hold voltages which are updated each time the camera sweeps the cell image. A calibration factor for the edge detector was achieved by obtaining voltage outputs for known graticule widths. The resulting slope calculated for the curve in Figure 7 was 0.04 volt/μm which is identical to the slope obtained by Steadman *et al.* 1988.

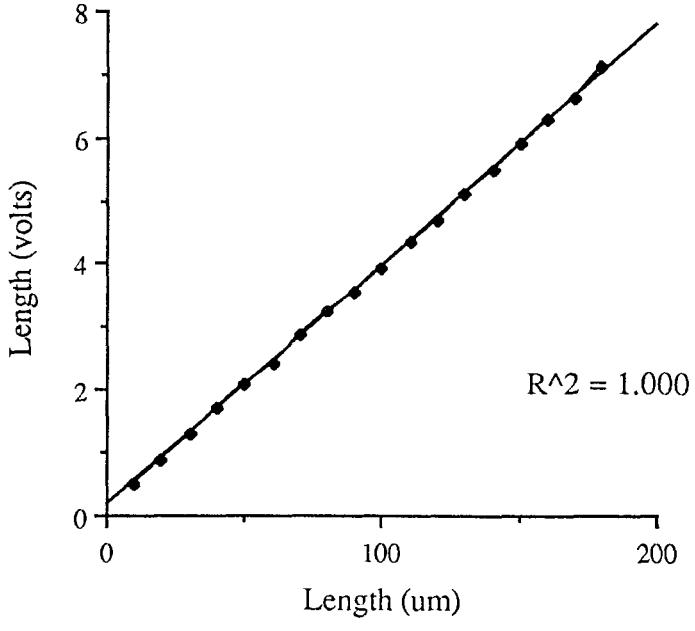


Figure 7. Calibration curve for the video edge detector output in volts and the corresponding graticule length in microns.

The high spatial resolution of the VED, enables myocyte length to be determined with an error of ±0.05 μm (Steadman *et al.*1988). Thus 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 (ie. 60 Hz). To overcome this problem and ensure adequate sampling of dL_{max}, $-dL/dt_{max}$ and $+dL/dt_{max}$ during cell contractile activity values were obtained by averaging consecutive signals stored on an oscilloscope.

Statistical Analysis

All data is reported as means ± SE. A Student's *t* -test was used and statistical significance was set at $P \leq 0.05$.

RESULTS

The body weight of animals in the control and trained groups were monitored throughout the course of the study as illustrated in Figure 8. By the final week of the program the run-trained rats had body weights 105.5±1.0% of the control group, with the mean body weights of the trained and control groups being 308±3 and 292±3 grams, respectively.

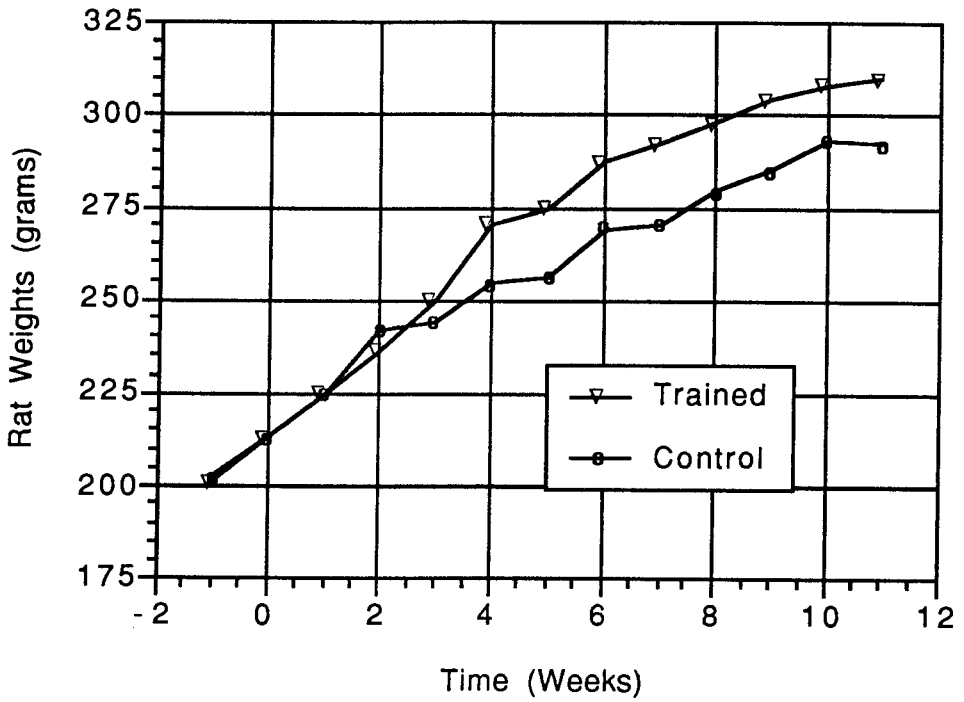


Figure 8. The mean group weight gain of the trained and control rats one week prior to and during training regimen. Error bars have the same width as the symbols.

The mean pre-drug value for cell length, TPS, $-dL/dt_{max}$ dL_{max}, and $+dL/dt_{max}$ for cardiomyocytes from trained and control rats are summarized in Table 2. Cardiomyocytes from the trained group on average had a mean cell length 6.8 μm longer than the

control group. This increase in cell length is equivalent to 3 additional sarcomere units in series. TPS and dL_{max} were also significantly greater in myocytes from trained animals. No difference was seen in either $-dL/dt_{max}$ or $+dL/dt_{max}$ between groups of animals. Both groups had identical ratios $-dL/dt_{max}$ and $+dL/dt_{max}$ of 1.05.

Table 2. Pre-drug dimensions and shortening properties of myocytes isolated from trained and control rats. Values are means ± S.E. and NS represents not significant.

	Resting Length (μm)	Mean Shortening (% cell length)	$-dL/dt_{max}$ (V)	$+dL/dt_{max}$ (V)	TPS (ms)
Trained (n=79)	128.8±2.2	12.1±0.4	0.22±0.01	0.20±0.01	122±3
Control (n=75)	122.0±1.9	11.2±0.2	0.22±0.01	0.21±0.01	113±2
Training Effect	p≤0.05	p≤0.05	NS	NS	p≤0.05

Table 3. Time-to-peak shortening in the control solution and in the presence of isoproterenol, nifedipine, Bay K and ryanodine for the trained and control groups. Values are means ± S.E. and * represents the drug value is significantly different than the predrug response.

TPS (ms)	Predrug	Isopro	NF	Bay K	Ryan
Trained	122±3	122±5	131±5*	137±8*	130±7
Control	113±3	107±5	94±5*	113±3	128±5*

Four perturbations were used in this study and isoproterenol produced the most significant finding. In the presence of isoproterenol the change (represented as a percent

of control) in dL_{max} and $-dL/dt_{max}$ was significantly greater in the control myocytes compared with the trained myocytes (as illustrated in Figure 9). The change in $+dL/dt_{max}$ was also greater in control myocytes however, this difference was not significant. In the presence of isoproterenol, TPS for trained and control groups were identical to the values recorded prior to drug exposure (Table 3).

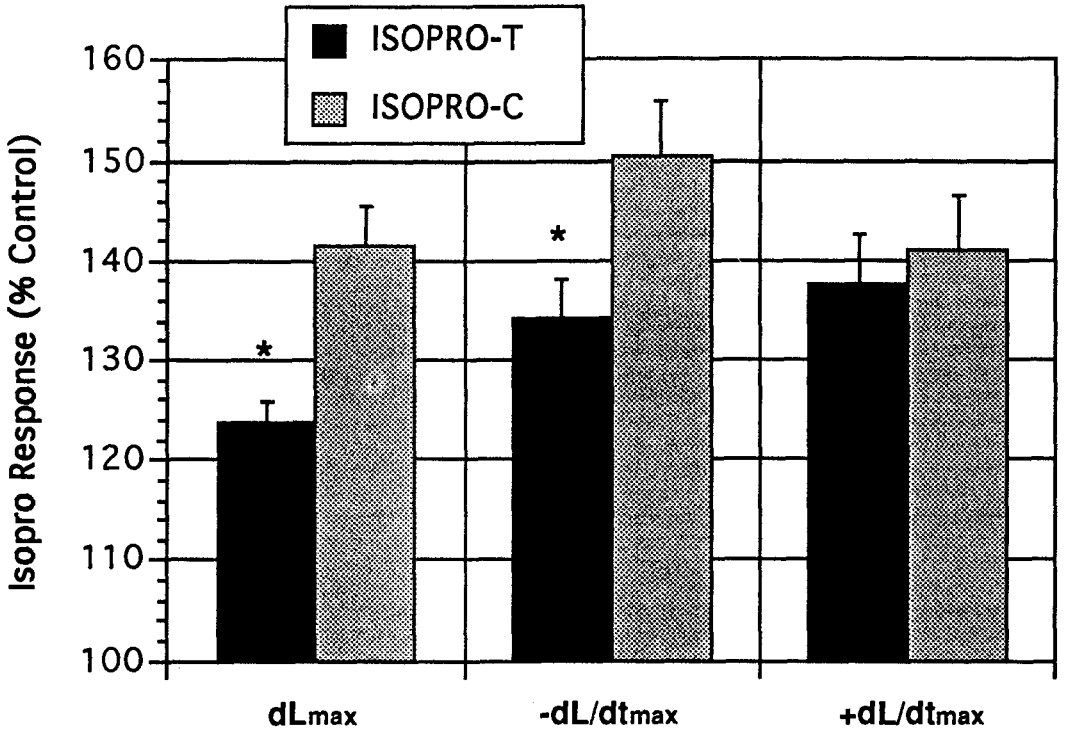


Figure 9. Change in dLmax, -dL/dtmax, and +dL/dtmax of myocytes from the trained and control group in the presence of 0.1 mM isoproterenol. Changes are represented as a percent of control. Values are means ± S.E. and * represents $p \leq 0.05$.

Training did not produce any significant modifications in dL_{max}, $-dL/dt_{max}$ and $+dL/dt_{max}$ in the presence of nifedipine, Bay K and ryanodine. However, the findings suggest training did induce an adaptation which enabled the myocytes from the

trained group to resist the negative inotropic effects of nifedipine compared to controls. Figure 10 shows dL_{max}, $-dL/dt_{max}$ and $+dL/dt_{max}$ were inhibited to a lesser degree in myocytes from trained animals compared with controls. In the presence of nifedipine, myocytes from trained animals showed significantly prolonged TPS (131±5 ms versus the pre-drug value of 122±3 ms) while in cardiomyocytes from control animals TPS was significantly shorter (94±5 ms versus pre-drug value 113±5 ms).

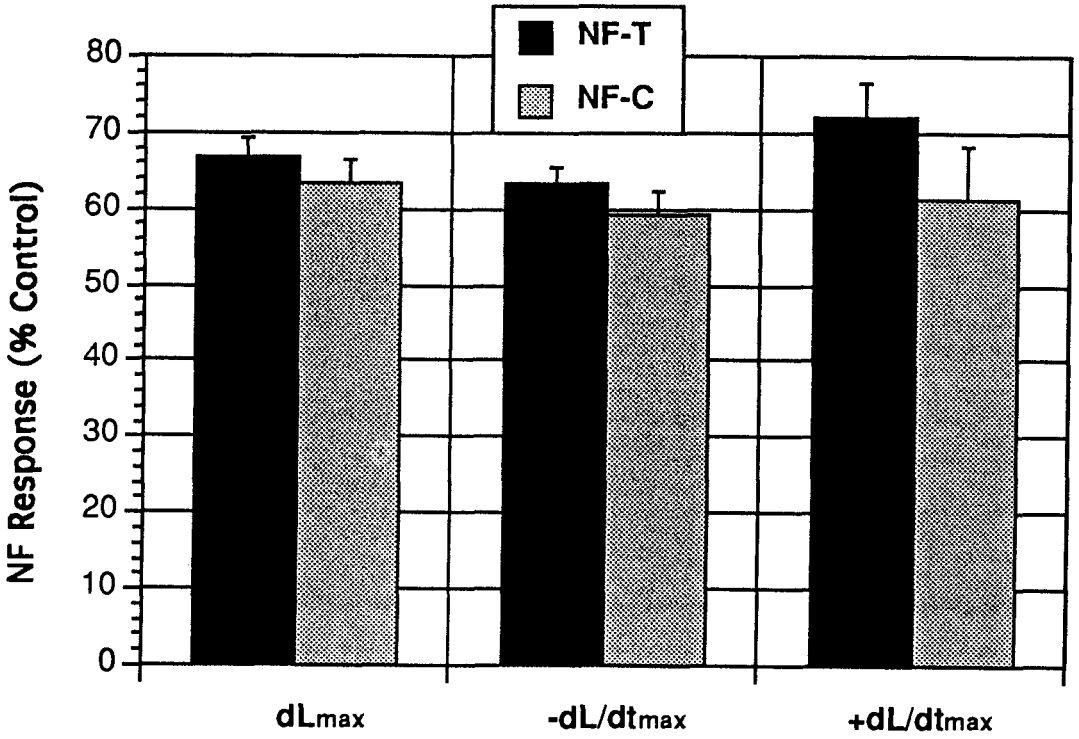


Figure 10. Changes in dL_{max}, $-dL/dt_{max}$ and +dL/dt of trained and control myocytes in the presence of 10nM nifedipine. Changes are represented as a percent of control. Values are means ± S.E. and * represents $p \leq 0.05$.

A DHP analog Bay K (Ca^{2+} channel agonist), had a dramatic positive inotropic affect on both trained and control myocytes as

illustrated in Figure 11. The change in dL_{max}, $-dL/dt_{max}$ and $+dL/dt_{max}$ were almost identical in the two groups. The only significant observation made in the presence of Bay K was the increase in TPS of the trained group (137±8 ms versus the pre-drug value of 122±3 ms) compared to the pre-drug state (Table 3). TPS was identical in the control group prior to, and following drug exposure. The changes in dL_{max} and $-dL/dt_{max}$ produced by Bay K were similar to the values obtained for myocytes from control animals in the presence of isoproterenol.

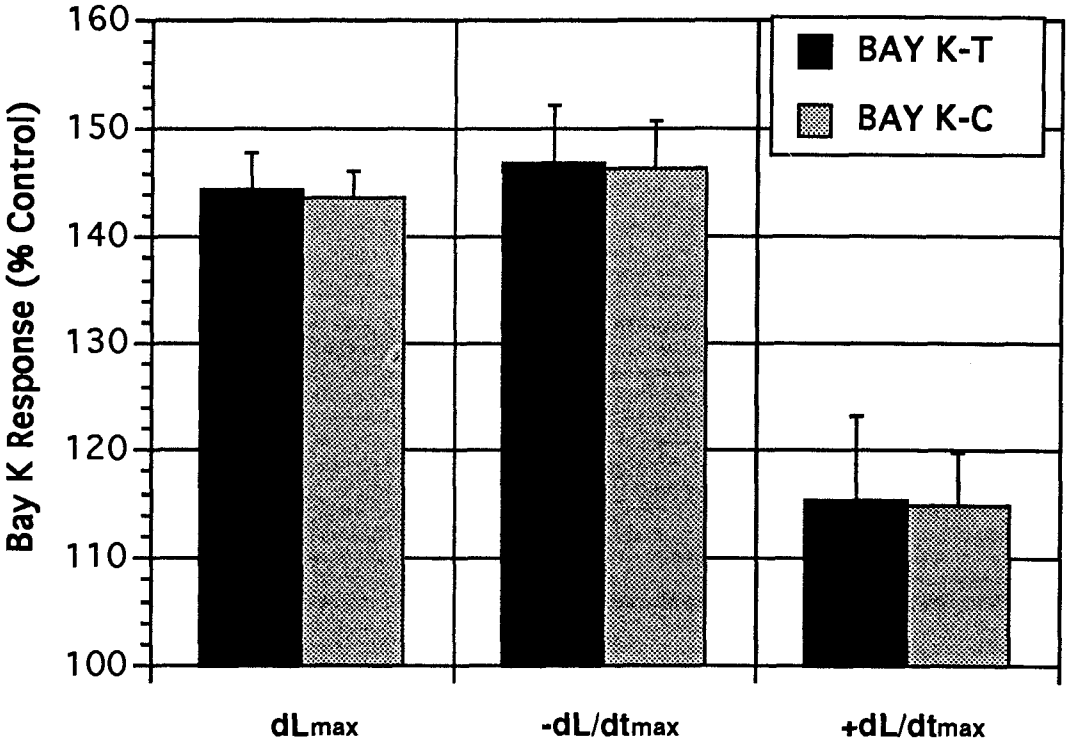


Figure 11. Changes in dL_{max}, $-dL/dt_{max}$, and $+dL/dt_{max}$ of trained and control myocytes in the presence of 10nM Bay K. Changes are represented as a percent of control. Values are means ± S.E. and * represents $p \leq 0.05$.

Ryanodine prevents the release of calcium by preventing the SR calcium channel from opening. Similar to the observations

made in the presence of nifedipine, trained myocytes also appear to resist the negative inotropic action of ryanodine (illustrated in Figure 12). Although not significant, the reduction in dL_{max}, $-dL/dt_{max}$ and $+dL/dt_{max}$ respectively were all consistently greater in myocytes isolated from the control animals compared with myocytes from the trained group. The most dramatic change in the presence of ryanodine was a large increase in the mean TPS of control myocytes (Table 3). In the presence of ryanodine, TPS for the control myocytes was significantly increased from 113±2ms (pre-drug) to 128±5 ms, while the TPS for trained cells increased from 122±2 pre-drug to 130±7 ms.

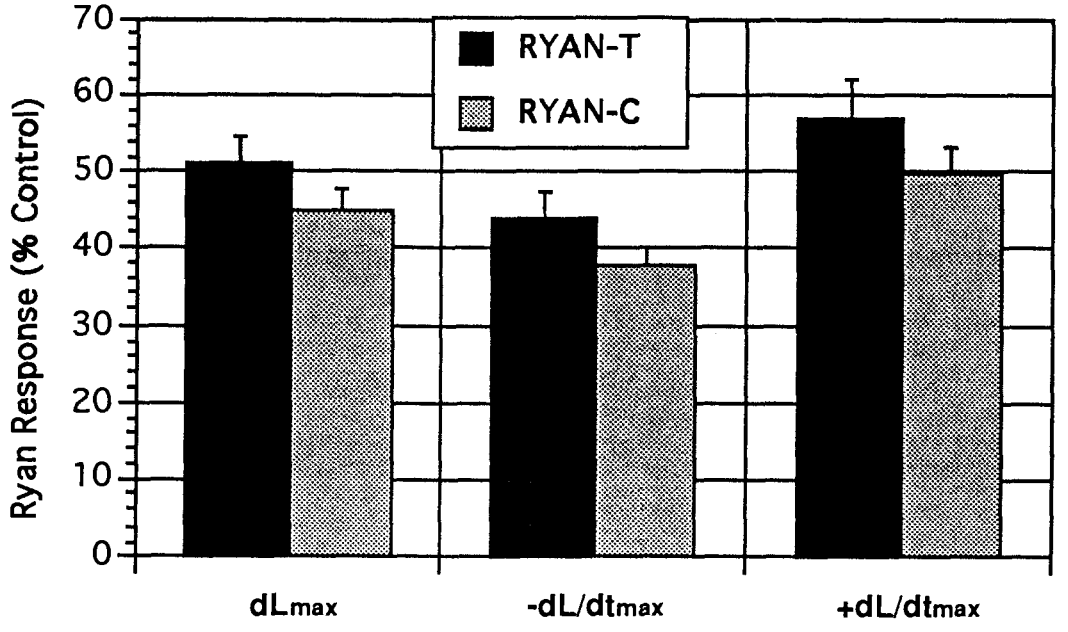


Figure 12. Changes in dL_{max}, $-dL/dt_{max}$, and $+dL/dt_{max}$ of trained and control myocytes in the presence of 0.1μM ryanodine. Changes are represented as a percent of control. Values are means ± S.E. and • represents $p \leq 0.05$.

DISCUSSION

Hypertrophy

The level of training intensity used in this study was set to limit the degree of exercise-induced cardiac hypertrophy. Cardiac hypertrophy is often associated with more intense continuous running and interval training programs (Mary, 1987). A parameter commonly used as an indirect index for cardiac hypertrophy is the HW/BW ratio. Heart weight was not measured in this study in order to ensure optimal myocyte yield. Results obtained from previous studies (Tibbits *et al.* 1981 and 1989) using a similar training intensity found only a slight increase (104-107% of control) in the HW/BW ratio. In this study, assessment of hypertrophy was made by measuring cell length directly. Mild hypertrophy was seen in the myocytes from trained which were on average 6.8 μm (104.9% of control) longer than the myocytes from the control group. The additional 6.8 μm would correspond to approximately 3 sarcomere units in series (Loud *et al.* 1984).

In addition to the length differences between the myocytes from the trained and control groups, the training regimen also significantly increased dL_{max} and TPS without altering either $-dL/dt_{max}$ or $+dL/dt_{max}$. The increase in TPS could be attributed to a prolonged plateau phase of the cardiac action potential. Support for this conclusion comes from the finding that the action potential measured in papillary muscle was significantly

prolonged at 0 mV in trained rats compared with controls (Tibbits *et al.* 1981). A prolonged plateau of the action potential would contribute to a greater inward I_{Ca} increasing rise in $[Ca^{2+}]_i$ directly, and indirectly by triggering a greater SR calcium release (Fabiato, 1983). The increase in dL_{max} of trained myocytes observed in this study and peak isometric tension (Tibbits *et al.* 1981) observed in trained papillary muscle also suggests there is an increase in the peak $[Ca^{2+}]_i$.

Isoproterenol

Desensitization of the heart to β agonist has been demonstrated following chronic exposure of the heart to catecholamines. This form of desensitization results in a reduced inotropic response due to a decrease in the number of β-adrenergic receptors on the cell surface (Masini *et al.* 1990). Three processes associated with desensitization of the β-adrenergic receptors include (Collins et al. 1990): 1) a rapid uncoupling of the receptor from effector units, 2) rapid sequestration of the receptor away from the cell surface and 3) down regulation which takes place over several hours and results in a net decrease in receptor number.

This study demonstrated an attenuated response to isoproterenol in myocytes from trained rat heart. However, this study did not go beyond assessing the mechanical response of the trained and control myocytes in the presence of a β-agonist. It appears, however, that treadmill training does not involve a

reduction in β-receptor number or affinity as one would expect if training did desensitize the myocardium (Moore *et al.* 1982). Membrane preparations from treadmill trained rats were found to show no change in β-receptor number or affinity compared to controls. One possible explanation for the attenuated response of trained myocytes could be attributed to the findings of Dohm *et al.* 1976 and Moore *et al.* 1982. Dohm and his colleagues observed a decrease in epinephrine stimulated adenylate cyclase (AC) in trained rat heart. Moore and colleagues observed a 47% reduction in the flouride stimulated AC activities of crude homogenate obtained from endurance trained rat. Fluoride is typically used to maximally stimulate AC which suggests the maximal ability of AC to generate cAMP is reduced following endurance training. This reduction in cAMP would translate into a reduced cAMP mediated phosphorylation of the DHP calcium channel. As discussed previously, phosphorylation of the DHP channel namely at the α_1 and β-subunits is very important in the regulating channel activity (Catteral *et al.* 1990). An increased phosphorylation of the DHP calcium is one putative mechanism enhancing channel current (Tsien *et al.* 1986). The attenuated response of the trained myocytes could be attributed to the reduction in DHP sensitive calcium channel phosphorylation as a result of the decreased AC activity. The reduced I_{Ca} would lead to a decreased CICR from the SR and ultimately to a reduced peak $[Ca^{2+}]_i$.

Nifedipine

In cardiac muscle, nifedipine selectively blocks the slow inward calcium current through binding to the α_1-subunit (*m* 242) of the DHP calcium channel. Single channel recordings indicate this reduced current can be attributed to a reduction in the number of channel openings per sweep (Tsien *et al.* 1986). In addition, the action of nifedipine is independent of the cAMP mediated response seen with β-agonists (Tsien *et al.* 1986). This is supported by the observation that inhibitors of cAMP-mediated phosphorylation caused no reversal in antagonistic action of nifedipine.

In the presence of nifedipine, shortening of the trained and control myocytes was reduced to approximately 65% of the pre-drug values. In addition, there is a trend suggesting the trained myocytes have a greater resistance to the actions of nifedipine. One explanation for the lack of a significant finding in the presence of nifedipine, is the large degree of variability between myocytes. The variability between the groups could potentially be overcome by doubling the sample number for the drug. As will be discussed below, there is evidence to support the hypothesis that training increases the resistance of the myocardium to calcium channel antagonists.

If trained myocardium does demonstrate a greater resistance to the negative inotropic effects of nifedipine, this could be attributed either to a decrease in the affinity (K_m) to nifedipine or an increase in the number of receptor sites (i.e.,

increased number of DHP sensitive channels) in the trained group. Previous findings indicate the affinity for DHP is unchanged by endurance training but the number of binding sites for this drug is increased. This is based on two studies in which DHP binding was significantly increased in both crude and purified sarcolemmal homogenates from endurance trained rats (Diffee and Tibbits, 1985 and Weymann and Tibbits 1987).

The increase resistance of the trained myocardium to nifedipine is similar to the observations made when lanthanum (La^{3+}) was used to block calcium entry. Isometric tension developed by papillary muscles from endurance trained rats was found to be more resistant to the negative inotropic actions of La^{3+} compared to controls (Tibbits *et al.* 1981). The drawback of La^{3+}, unlike nifedipine, is that it also blocks the Na-Ca exchanger. The main interest in using La^{3+} is its ability to prevent calcium entry through the DHP channel (Reuter, 1973).

The greater degree of shortening demonstrated by the trained myocytes would reflect a greater peak $[Ca^{2+}]_i$ is achieved during the cardiac cycle. The slow I_{Ca} is an important component of ECC and determines the degree of calcium release from the SR. Since a greater number of functioning DHP channels (i.e., greater I_{Ca}) would initiate greater SR calcium release one could correlate this event with a greater magnitude and rate of cell shortening.

Bay K

Bay K shows the opposite effects of nifedipine despite having a similar chemical structure (structures illustrated in Appendix C). As a calcium channel agonist, Bay K increases contractility and prolongs the ventricular action potential by increasing the frequency of calcium channel opening. The increase in calcium channel opening by Bay K is mediated independent of cAMP. In addition, unlike isoproterenol Bay K does not effect the speed of relaxation nor does it increase HR (Thomas *et al.* 1985).

The action of Bay K to increase twitch tension without increasing the time course of tension development is highly dependent upon stimulation frequency (max at 0.5-1.0 Hz) of the muscle (Thomas *et al.* 1985). The increase in action potential duration produced by Bay K is sensitive to DHP antagonists such as nisoldipine. This is also supported by the ability of Bay K to increase the Sr^{3+} current by altering the opening time of the voltage dependent channel (Thomas *et al.* 1985). In addition to increasing the I_{Ca}, Bay K appears to increase the duration of the action potential by decreasing the net outward current (Thomas *et al.* 1985). These changes in channel gating are inconsistent with cAMP or Ca^{2+} mediated changes suggesting Bay K directly acts on the calcium channel. Other actions of Bay K which have been ruled out include changing the myofilament sensitivity, elevation of $[Na^+]_i$ and alteration of the Na^+ or K^+ channels which could indirectly increase $[Ca^{2+}]_i$.

Experiments involving single channel recordings suggest Bay K increases the probability of a channel being open. Bay K appears to increase the length of time a channel remains open (20 ms versus the normal 1 ms), and reduce the closed time (1 ms versus 1-5 ms) and the interval between channel openings (Hess, 1988). Bay K has also been shown to increase the slow tail current following repolarization which serves to enhance calcium loading of the SR (Cohen and Chung, 1984). The properties of Bay K therefore lead to an enhanced calcium entry during depolarization and increased the calcium loading of the SR. As discussed previously, these factors would correlate to a greater rise in cytosolic Ca^{2+} and therefore contractility. One would expect that the trained cells, with a putative increase in the number of calcium channels, would exhibit larger increases in shortening. The data obtained from the trained and control groups of myocytes show no difference in the response to the 10nM dose of Bay K. One possible explanation is the 10 nM dose of Bay K induces maximal calcium induced calcium release from the SR in both groups of myocytes. The Bay K would therefore produce similar rise in the $[Ca^{2+}]_i$ in the trained and control groups.

Ryanodine

Fabiato, (1983) demonstrated ryanodine inhibits three forms of calcium release from the sarcoplasmic reticulum in skinned cardiac fiber. They were, calcium-induced calcium

release, caffeine-induced release and spontaneous cyclic release. Investigations into the action of ryanodine indicate it binds to, and acts as a blocker of the SR calcium release channel (Sutko and Willerson, 1980). The order of species sensitivity to ryanodine was rat>dog=cat>rabbit which relates to the relative dependence of these species to intracellular calcium for the development of force (Sutko and Willerson, 1980). In the rat, the effect of ryanodine is similar to decreasing $[Ca]_o$, while the positive inotropic response of paired stimulation is prevented by ryanodine. The effect of ryanodine is to decrease the availability of stored calcium thereby reducing the peak rise in the $[Ca^{2+}]_i$.

Results from this study suggest myocytes from trained rats have a greater resistance to the negative inotropic effects of ryanodine. Recent findings suggest there is a close coupling between the DHP channel and the ryanodine channel (as illustrated in Figure 3). If this is true, one would expect a parallel increase the density of the ryanodine channel as suggested by the increase DHP binding in trained rat heart (Diffee and Tibbits, 1985 and Weymann and Tibbits 1987). However changes in receptor affinity for ryanodine following training cannot be ruled out. If training does increases ryanodine channel density it would account for the greater resistance of myocytes from run-trained rats. This would allow a greater number of SR calcium channels to release SR calcium. Myocytes from trained rat heart would therefore achieve a greater peak $[Ca^{2+}]_i$, and obtain a greater degree of shortening.

CONCLUSION

An increase in myocardial contractility is one important factor contributing to an increase in stroke volume following endurance training. This study investigated three mechanisms postulated to increase contractility, altered sensitivity to β-agonists, and modified transsarcolemmal calcium influx and SR release. The close relationship established between myocyte shortening and $[Ca^{2+}]_i$ was used to assess possible changes in ECC following endurance training.

Myocytes isolated from the trained and control rats exhibited significantly different shortening properties under pre-drug conditions. The relative increase in cell length of trained myocytes was similar in degree to the cardiac hypertrophy assessed using an index of HW/BW (Tibbits *et al.* 1981 and 1989). One possible explanation for the greater magnitude of shortening and time to peak shortening following training is a prolonged action potential (Tibbits *et al.* 1981).

In response to isoproterenol both groups demonstrated a significant increase in $-dL/dt_{max}$ and in dL_{max} compared to control solutions. The percent change in both $-dL/dt_{max}$ and dL_{max} in response to the isoproterenol were significantly lower in the trained versus control. This study did not assess any of the intermediate steps of the β-receptor/cAMP transduction pathway, therefore no conclusions as to the mechanisms of this observation can be made. However, previous findings suggest that endurance treadmill training does little to alter the β-

receptor number or affinity (Williams, 1980 and Moore *et al.* 1982), but significantly depresses fluoride stimulation of AC (Moore *et al.* 1982). In respect to the mechanical properties of the cell, depressed AC activity would lead to a decrease in PK-A phosphorylation of the DHP sensitive calcium channel. Reduced phosphorylation of the calcium channel would decrease the probability of the channel being in an open state and ultimately decreasing the inward I_{Ca}. The decrease in the I_{Ca} would decrease both SR loading and calcium release which would lead to a reduced maximal rise in cytosolic calcium.

Both nifedipine and ryanodine significantly decreased dL_{max} $-dL/dt_{max}$ and $+dL/dt_{max}$ in the trained and control groups compared with the predrug values. However, there was no significant difference in the relative decrease in shortening between the two groups. A trend was seen suggesting trained myocytes have a greater resistance to both nifedipine and ryanodine. This would support previous findings in which DHP binding was increased following endurance training (Diffee and Tibbits. 1985 and Weymann and Tibbits. 1987). To improve the statistical significance of these findings a subsequent study would require a sample number approximately twice that used in this study.

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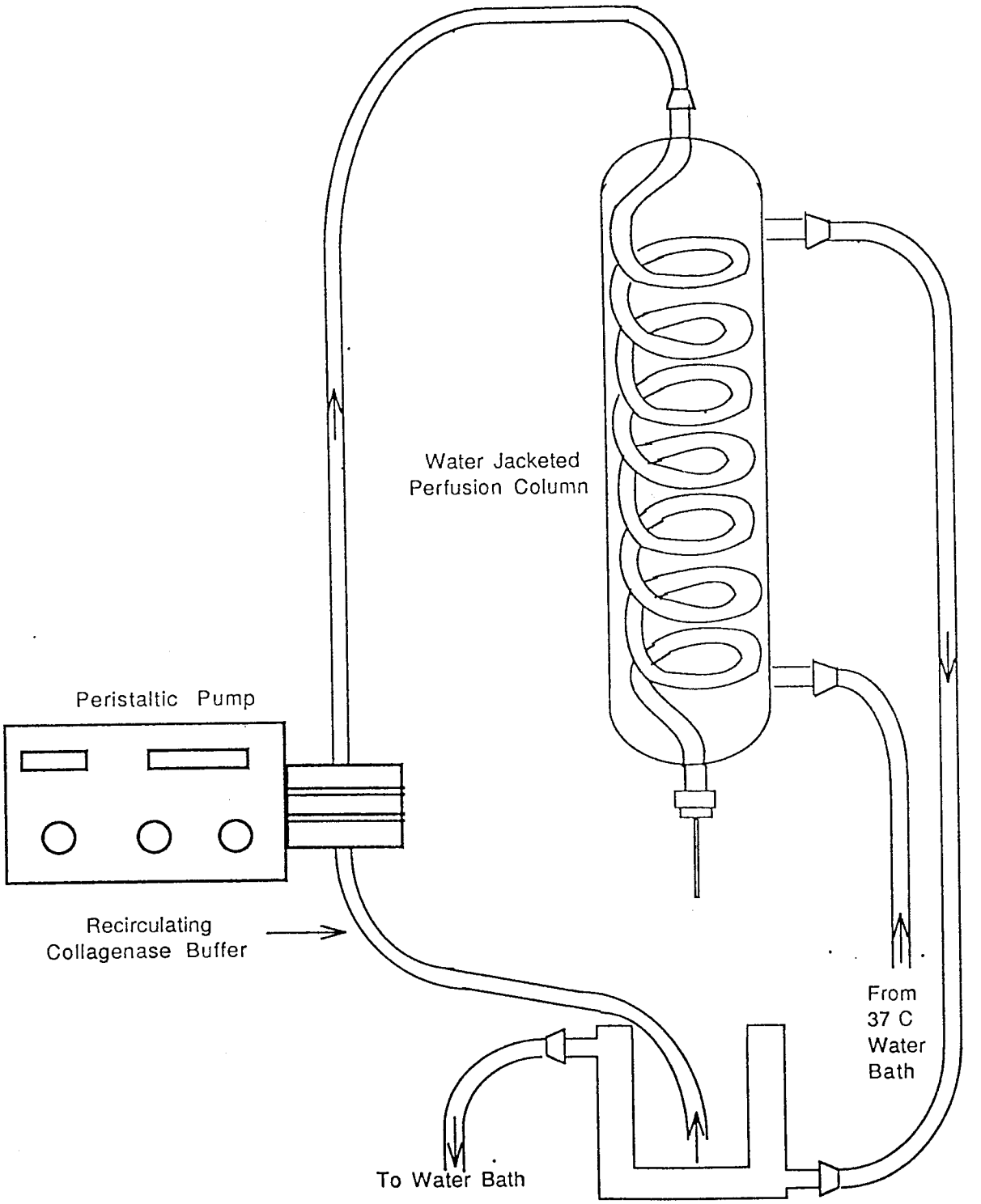
APPENDIX A

Diagram of Langendorff perfusion apparatus.



APPENDIX B

Contents of Joklik Modified-Minimum Essential Medium (MEM) HEPES Buffer (GIBCO Laboratories-Lot#61N2183)

Component	g/L	Component	g/L
Organic Salts			
KCl	0.400	$MgCl_2$	0.093
NaCl	6.500	NaH_2PO_4	1.327
Amino Acids			
L-Arginine	0.126	L-Cysteine	0.032
L-Glutamine	0.292	L-Histidine	0.032
L-Isoleucine	0.052	L-Leucine	0.052
L-Lysine	0.058	L-Methionine	0.015
L-Phenylalanine	0.032	L-Threonine	0.048
L-Tryptophan	0.010	L-Tyrosine	0.054
L-Valine	0.046	D-Ca pantothenate	0.001
Choline Chloride	0.001	Folic Acid	0.001
i-Inositol	0.002	Nicotinamide	0.001
Pyridoxal HCl	0.001	Riboflavin	0.0001
Thiamine HCl	0.001		
Other Components			
D-Glucose	2.000	Phenol Red	0.010

In addition to the 5.6 g/L of MEM the following agents were added: 21.1mM HEPES, 4.4 mM $NaHCO_3$, and 5% Bovine Serum Albumin (Lot # 11697123-04 Boeringer). The pH of this solution was adjusted to 7.3 at 37°C using NaOH.

Contents of Medium-199 (Gibco Laboratories-Lot# 62K 3395)

Component	mg/L	Component	mg/L
Inorganic Salts			
$CaCl_2$	200.00	$Fe(NO_3)_3\ 9H_2O$	0.72
KCl	400.00	$MgSO_4$	97.67
NaCl	6800.00	$NaH_2PO_4\ H_2O$	140.00
Amino Acids and Vitamins			
DL-Alanine	50.00	DL-Aspartic Acid	60.00
L-Arginine	70.00	Cysteine	0.11
L-Cystine	26.00	Glutamic Acid	150.00
L-Glutamine	100.00	Glycine	50.00
L-Histidine	21.88	L-Hydroxyproline	10.00
DL-Isoleucine	40.00	DL-Leucine	120.00
L-Lysine	70.00	DL-Methionine	30.00
L-Phenylalanine	50.00	L-Proline	40.00
DL-Serine	50.00	DL-Threonine	60.00
DL-Tryptophan	20.00	L-Tyrosine (salt)	57.66
DL-Valine	50.00	Ascorbic Acid	0.50
α-Tocopherol phosphate	0.01	d-Biotin	0.01
Calciferol	0.10	D-Ca pantothenate	0.01
Choline Chloride	0.50	Folic Acid	0.01
i-Inositol	0.05	Menadione	0.01
Niacin	0.025	Nicotinamide	0.025
Para-aminobenzoic Acid	0.05	Pyridoxal HCL	0.025
Pyridoxine HCl	0.025	Riboflavin	0.01
Thiamine HCl	0.01	Vitamin A (acetate)	0.14
Other components			
Adenine sulfate	10.00	ATP (sodium salt)	1.00
Adenylic Acid	0.20	Cholesterol	0.20
Deoxyribose	0.50	D-Glucose	1000.0
Glutathione	0.05	Hypoxanthine	0.345

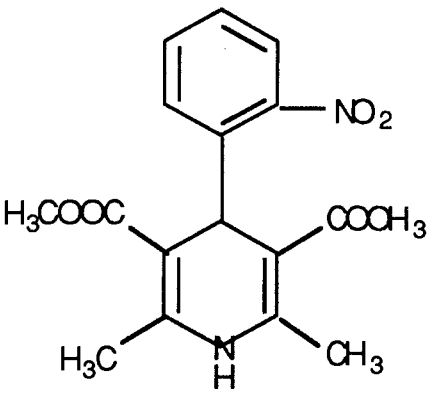
Phenol Red	20.00	Ribose	0.50
Sodium Acetate	50.00	Thymine	0.30
Tween 80®	20.00	Uracil	0.30
Xanthine (Na salt)	0.344		

In addition to the 10.0 g/L of M-199 powder the following agents were added: 21.1 mM HEPES, 4.4 mM $NaHCO_3$ penicillin (100 μg/ml) and streptomycin (100 μg/ml). The pH of this solution was adjusted to 7.3 at 37°C using NaOH.

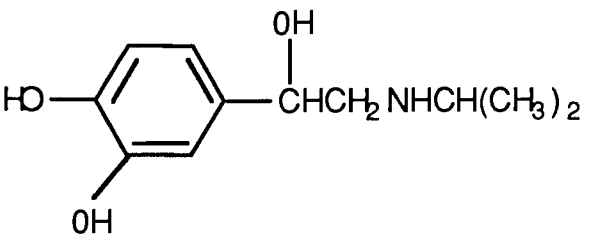
APPENDIX C

Chemical Structures of Drugs:

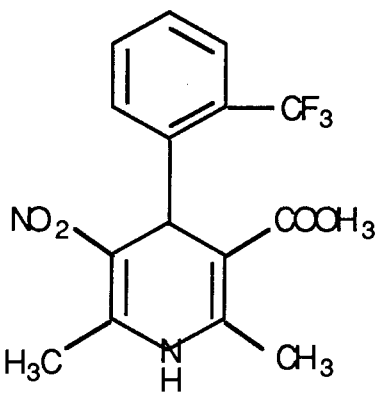
Nifedipine



Isoproterenol



Bay K 8644



Ryanodine