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# Evidence in Support of a Role for the Endocardium in the Functioning of the Mammalian Myocardium

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Evidence in Support of a Role for the Endocardium in the Functioning of the Mammalian Myocardium" submitted by Hamid Banijamali in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

An in vitro study of electrical and mechanical activities of the cat ventricular muscle has been performed in the presence and absence of an intact endocardium; the inner lining of the cardiac muscle. Upon damaging this layer by Triton detergent there is an immediate decrease in peak isometric contractile force with decrease in duration. This is followed by shortening of action potential duration and increase in amplitude. The diminution of contractile force is not a consequence of damage by Triton to the underlying myocardium as evident from electron micrographs, action potential characteristics, the absence of increased resting tension, and the time course of effects. Moreover, the experimental evidence presented in this thesis indicates that a loss of physical and chemical integrity is not the cause of decreased level of contraction.

Our observations suggest that endocardium acts not only as a passive barrier, but also as an active one that modulates the myocardial force. It is believed that damage to the endocardium changes (perhaps via decrease or loss of a chemical factor that would otherwise act on the myocardial sarcolemma) sarcoplasmic reticulum function and thus changes the contractile force. The delayed change in the shape of the action potential appears to be the consequence of a

feedback mechanism whose function becomes enhanced following the effects of endocardial damage on the contractile force. Therefore, we propose that mammalian endocardium constitutes a mechanism that actively influences the myocardial performance, and the observations point to involvement of a chemical in this process, although the identity of such a chemical remains to be determined.

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To My Parents,

for all their caring support

and for providing me with opportunities in life

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# Chapter 1. Introduction

## 1.1 Historical notes

Blood vessels along with their inner lining have been traditionally considered to be a passive system, acting only as a conduit for blood flow. This view is now being challenged, following an increased understanding of the complexity of the system.

The endothelial cells of the vascular system occupy a unique position, in that they are the only cells in the body that are in direct contact with blood and all its constituents. In addition, intimate structural relationships have been demonstrated between endothelial cells and the pericytes and smooth muscle cells on the ablumenal side [77,118] which suggests that the endothelial cells might have some influence on the underlying smooth muscle cells.

In 1977 Majno and Joris [72] proposed three groups of endothelial linings based on variation in the continuity of the lining:1) continuous, 2) fenestrated, and 3) endothelium with intercellular and transcellular openings. Gimbrone [43] described the functions of the endothelial lining which reflect its anatomical position: 1) a blood compatible "container" preserving fluidity of the blood by preventing clotting and thus participating in hemostasis; 2) a sensitive permeable barrier, protecting the medial layers of the vessel wall from injurious constituents of the

circulation and also maintaining the hemostasis by controlling the entry or egress of substances from the blood [43,68]; and 3) an active synthetic, metabolic, and secretory tissue [111].

Endothelial cells have a lumenal surface which does not bind platelets [13,125]. The endothelial lining also controls vascular wall permeability [76] and is refractory to growth factors in the blood [44]. These characteristics are highly dependent on the integrity of the endothelium, which can be damaged by such factors as hypercholesterolemia and hemodynamic stress [96,108]. It is due to the above features that an intact endothelial lining can protect underlying smooth muscle cells from proliferation and lesion formation [4,36,85,95].

The endothelial lining of different tissues differ not only in structure but also in function [55]. Some observations [71] underlie the great specificity of endothelial cells along the vascular tree, and the possibility that they possess different receptors, and reactivity, and peptidase enzyme activity depending on the locale, must be considered. The postulation of a secretory role for the endothelial lining is based on the observation that they synthesize and release factors such as heparin-like glycosaminoglycans [20,26,131], antithrombin III [27], prostacyclin ($PGI_2$) [19,46,48,62,63,82,83,84,94,115,126], Factor VIII, collagen, fibronectin, and laminin [6,54,56,105,120]. The release of substances such as EDRF (endothelial derived relaxing

factor) [22,40,41,121,122,132] is also observed. The actions of substances such as $PGI_2$ (i.e. a vasodilator as well as an inhibitor of platelet aggregation) has been suggested to work against the action of thrombaxane A2 (TXA2) derived from platelets [19,28,30,48,49,50,62,63,84,94,114]. In terms of metabolic functions of the endothelial cells, one of the interesting observations made is that pulmonary endothelial cells inactivate a blood pressure lowering substance (namely "bradykinin") [98,100] as well as forming angiotensin II (AII- a potent vasoconstrictor), as the venous blood is converted into arterial blood. The formation of AII occurs as a result of function of angiotensin converting enzyme (ACE) [23,44,101,124], situated on the lumenal surface of pulmonary endothelium [97,102,103,104]. Pulmonary endothelium also has been shown to metabolize serotonin (a vasoconstrictor) [127], thrombin [42] and prostaglandin $F_1$alpha [99]. All of these substances have well-defined effects on platelet function (adhesion/aggregation) and thus hemostasis [42]. Thus endothelial cells can participate in hemostasis in more than one way: by having a surface that does not bind platelets, and by inactivating substances such as thrombin and by $PGI_2$ release. Indeed it is these secretory and metabolic roles (in addition to playing the role of a barrier) that indicate the importance of endothelial cells and their influence on vascular smooth muscle function. Moreover, there is evidence that in bronchial smooth muscle, factors released from the

endothelial lining may be regulating the smooth muscle tone [37,38]. Obviously, the influence of endothelial cells is not limited to the vasculature system.

## 1.2 The Endocardium

The above historical background indicates the significance of endothelial lining in modulating the vascular and bronchial smooth muscle function by acting as an active as well as a passive barrier. With this in mind, it would seem reasonable to suspect that, like the vascular and bronchial endothelium, the endocardial endothelium (which also occupies a similar anatomic position to that of other endothelial linings), could exercise a modulatory function on the underlying myocardium. Mammalian endocardium covers the inner wall of the heart muscle, and consists of a single, very thin layer of closely apposed endothelial cells, adhering in places to a basement membrane, also overlying several layers of collagen and elastin fibres [61,67,80,92]. Microscopic evidence of surface structure of the endocardial endothelium (as in the case of vascular endothelium [80]) of normal monkeys have helped recognize endothelial cells by the presence of nuclear bulge and marginal folds. Microvilli covered the free cell surface. The study also led to the observation of projections that correlated with pinocytotic vesicles, thus suggesting a role

in the interaction with circulating biologically active substance [51]. The endocardium is cytologically not substantially different from the endothelial cellular organization of other animal tissues [109]. Recently, after examination of calf cardiac valvular endothelial cells in culture, Manduteanu et al. [73] observed the production of prostacyclin in similar amounts to those reported in the endothelial cells of bovine aorta or human umbilical vein. Furthermore, these cells secrete amounts of glycosaminoglycans (e.g. heparin sulphate) which relates them to bovine [25] and rabbit [20] cultured aortic endothelial cells. Although these observations are from valvular endothelial cells, the concept that the rest of the endocardium (i.e. that not involving valves) could also be important is very intriguing and any evidence that may point to such a possibility deserves examination. Indeed, the collection of information could prove to be useful in moving towards a better understanding of the significance of endothelial cells as well as realizing that their function may not be limited to the vascular smooth muscle only. The amount of attention paid to this possibility has not been substantial, and it was with this in mind that the present study of examination of a role for endocardium was undertaken.

## 1.3 Evidence for an endocardial role (Brutsaert's observations)

Recently Brutsaert et al. [17,18] have examined the mammalian heart muscle and have suggested an active role (e.g. secretory or an electrochemical barrier) for the endocardium. Their original suggestion [18] has been followed by a detailed study [16] containing a series of observations suggesting a possible involvement of mammalian endocardium in the modulation of the underlying myocardial performance by modulation of early tension decline. Brutsaert et al. [16] state that a chemical treatment (1 second in 1% Triton X100 detergent followed by thorough wash) or abrasion (3-4 times gentle rubbing) of cat and rat papillary muscles led to a damaged endocardial surface (perforation of the plasmalemma of the endothelial cells or denudation of the endothelial lining), concomitant with an irreversible decrease in the isometric and isotonic twitch contractions, both types of contractions being abbreviated. Although the twitch tension amplitude (at Lmax) was returned to control levels by raising the extracellular calcium concentration after endocardium damage, the duration of isometric tension and the time to the peak of this tension remained shortened. In the case of isotonic contractions, no significant difference in the velocity of shortening between the control and treated muscle was observed at two temperatures (29 $^{o}$C and 37 $^{o}$C) or by changing extracellular

calcium concentration (1.25-7.5 mM). Furthermore, there was a non-parallel shift in the tension-$[Ca^{2+}]_o$ curve towards increasing calcium, i.e. the higher the $[Ca^{2+}]_o$, the smaller the difference between a control and treated muscle (at Lmax) in terms of the mechanical performance. Additionally, there was a downward and rightward shift of length-tension relation. The changes observed after endocardial damage were similar in pattern to those changes observed after decreasing the length of the muscle at different extracellular calcium concentrations. Brutsaert et al. suggested a change in the calcium sensitivity of the myofilaments after the endothelium damage as the cause of these observations.

With regard to these observations and conclusions for an active role for mammalian endocardium, the following concerns can be raised which this thesis has tried to resolve, and thus determine if indeed a role can be attributed to the endocardium. There is a possibility of damage to the underlying myocardium with Triton or rubbing. In addition to failure of light and transmission electron microscopy to demonstrate any damage [16] further evidence is needed to confirm the absence of damage. Furthermore, with regard to the type or extent of damage (especially in a large papillary muscle), the possibility of a sarcoplasmic reticulum (SR -a tubular system for internal storage of calcium) calcium overload due to calcium leak into damaged

cells, which can lead to "overload-induced" calcium release, should be considered. It has been suggested [116] that calcium ions released by this process diffuse to adjacent sites and cause calcium-induced release of calcium, leading to spontaneous contractions, and also giving rise to a depolarization by the action of the Na/Ca exchanger. This would clearly induce concomitant changes in force and action potential. Spontaneous continuous fluctuations in force and membrane current have been observed and attributed to the SR [45,58]. Another important possibility which demonstrates the need for a broader but accurate definition of damage (other than that involving myocardium itself) is that since treatment (Triton or rubbing) can cause denudation of endothelial lining as well as perforation of its plasmalemma, there can be a reorganization of the immediate milieu surrounding the myocardial cells, i.e. the interstitium. This could even lead to the "loss" of physical and chemical integrity. To make this point the example of skinned muscle fibres can be considered, where the skinning procedure causes an increase in the interfilament spacing. It has been suggested (see [130]) that this may be due to a diffusive loss of high molecular weight substances that under ordinary situations exert an osmotic compressive force on the myofibrils. Obviously, such changes may influence the response of the cell. Consequently, a whole series of events may occur due to such possibility. For example there could be a change in the ionic composition and/or the availability

of ions such as $K^+$, $Na^+$, and $Ca^{2+}$. It should also be noted that the argument for a decrease in myofilament calcium sensitivity [16] in the absence of an intact endocardium is not the only possible explanation for an early relaxation, and decrease in time-to-peak force and in magnitude of isometric tension. A possibility is the modulation of the SR calcium uptake which can also lead to an early relaxation and decrease in peak force. Finally, does the endocardial damage affect also the electrical activity of the myocardium; e.g. changing of calcium current which can lead to change in contractile force. In the experimental results described later in this thesis, the above concerns have been addressed. Furthermore, some other experiments have been designed to test some of the possible potentials of the endocardium. Included in these are studies of cardiac action potential which will be seen to also vary with damage to the endocardium.

In light of the fact that the endocardial damage induces changes in both the mechanical and electrical activities of the myocardium, with these two being closely interrelated, it would seem appropriate to review the processes of excitation, contraction, and the coupling process between the two, in the mammalian heart.

## 1.4 Excitation

In order for cardiac contraction to occur, the surface membrane must become depolarized. This takes the form of an "action potential" which is generated by the response of specialized membrane spanning proteins (channels) allowing the passage of various ions (thus electric currents) mainly in one direction. The action potential of the mammalian heart has many phases relating to different time and voltages at which different ions enter or exit the myocardial cell through specific channels.

## 1.5 Excitation-contraction coupling: ECC

In the mammalian heart there have been many models of ECC processes [65]. It should, however, be emphasized that the model of calcium-induced release of calcium from the sarcoplasmic reticulum (SR) [33,88] is the widely accepted model. In this model extracellular calcium causes release of more calcium from the SR. Therefore, in the mammalian heart, both the external and internal calcium pools can contribute to the tension generation. The ratio of these two pools is species-dependent [86] (Figure 1).

The current that represents the link between the surface membrane (sarcolemma) depolarization (i.e., excitation phase) and the contraction of the cardiac muscle is the

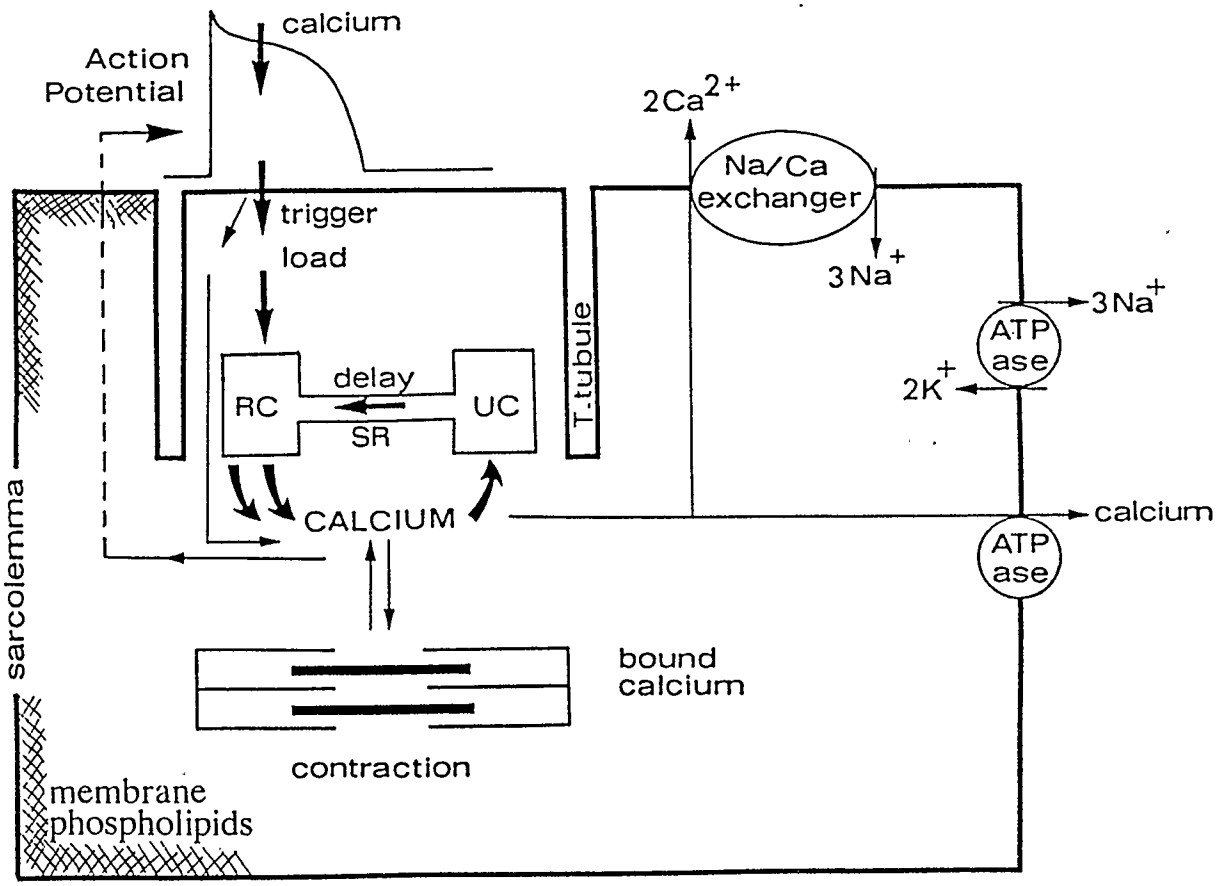



Figure 1 Schematic diagram depicting E-C coupling in mammalian myocardium; calcium-induced calcium release model. Various intracellular calcium transient controlling mechanisms such as calcium current (plateau phase of action potential), sarcoplasmic reticulum (SR), Na/Ca exchanger, Ca-ATPase, myofilaments, and membrane phospholipids are shown. The extent of the contribution of each depends on the species. Note that the SR has been depicted as having a calcium release compartment (RC) and a calcium uptake compartment (UC). The function of the Na/Ca exchanger is coupled to Na/K-ATPase pump. "Delay" indicates a period of time required for the sequestered calcium to become available for release.

calcium current ($I_{Ca}$ or Isi). This current underlies the slow action potential and the plateau depolarization of the fast action potential. Isi causes the release of calcium from the SR, thus amplifying the calcium transient in the myoplasm [33]. Isi is blocked by many inorganic ($Co^{++}$, $Cd^{++}$, $La^{+++}$) and organic compounds (verapamil, nifedipine) [107].

Sarcoplasmic reticulum (SR) which forms a tubular network surrounding the myofibrils constitutes a major site for $[Ca^{2+}]_i$ regulation in mammalian myocardium. Ultrastructural components of cardiac tissue are variable among different species with respect to the diameter of the cells, the extent of the SR presence, as well as the presence of the transverse-tubular system which represents invaginations of sarcolemma [86]. These differences produce variations in the excitation-contraction coupling of the heart [34]. For example rat ventricle has a well-developed SR and T-tubular system as compared with the frog ventricle which has very low SR content and no tubular system [32,86].

## 1.6 Contraction

Ultimately, the calcium ions (from outside and/or from the SR) are made available to the myofilaments, where they bind to troponin, allowing the actin and myosin components of the cardiac myofilaments to interact. This, in the presence of

ATP, causes sarcomere shortening and force production. The properties of the contractile proteins can also be involved in the regulation of contraction. The "sensitivity" of the myofilaments to calcium is the main relevant property. Increasing the length of muscle can apparently increase this sensitivity [129].

## 1.7 Relaxation

Following activation, the myoplasmic calcium concentration falls, due to sequestration by the SR [33], and extrusion from myoplasm to the outside via a sarcolemmal Na/Ca exchanger mechanism and an ATP-dependent calcium pump [24,60,87,119]. It has been observed that the exchanger displays a lower affinity for calcium, but a much higher transport capacity than the ATPase pump [5]. Thus, in the heart,the exchanger can cause a fast reduction in free cytosolic calcium, and the ATP dependent calcium pump probably establishes a somewhat lower free intracellular calcium concentration to complement the exchanger [5]. The combination of these processes leads to dissociation of calcium from troponin and thus muscle relaxation. It should be noted that the activities of ATPase pumps involved in the extrusion and sequestration of calcium are dependent on magnesium ions [24,33]. The proportion of calcium taken up into the SR (recirculated calcium) compared with that

extruded to the outside plays a significant role in the frequency-induced modifications of contractile force in the mammalian heart [86].

The above describes a complete cardiac cycle where calcium acts as the coupling element between the excitation phase and the contraction phase of the excitation-contraction coupling process. By doing so, calcium ions in the mammalian ventricular muscle trigger calcium release as well as modulating the quantity of calcium released from the SR. Moreover, calcium ions replenish the calcium stores in the SR necessary for subsequent contractile cycles, and the activity of the Na/Ca exchanger as well as the time course of Isi can modify this reloading of the SR by changing the amount of calcium available [14,78].

## 1.8 Summary

In heart muscle, where extrinsic factors such as the neurohumoral system and coronary circulation are involved in the control of myocardial function, the endocardium and the subendocardial space may also constitute a regulatory factor modulating the underlying myocardial performance by modifying the ECC processes. This idea is reinforced by the observation that damaging the endocardium induces changes in the ECC phenomenon and isolated muscle contractility. However, since in all cases observed there may be both

denudation of endothelial lining and perforation of the endothelial cells, the role may not necessarily be an active one. Nonetheless, considering the great deal of attention that has been paid to the vascular endothelium, it would seem reasonable to suggest that possible role of the endocardium should be examined since it may be playing a role (at least as a passive barrier) as significant as that of the endothelial lining in the vascular and bronchial tissues, especially since the inside of the heart is continuous with the inside of the vascular system. It is the goal of this thesis to look carefully and objectively at this prospect and provide critical evidence for or against it. This would help determine if the importance of endothelial cells is restricted to the vasculature and bronchial tissues, or if the heart also is benefiting from the action of these complex cells.

# Chapter 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Perfusion salt solutions

The regular physiological salt solution was of composition (mM): NaCl, 145; KCl, 4.5; $CaCl_2$ (concentration as specified in the various experiments), $MgCl_2$, 1.0; $NaH_2PO_4$, 2.4; HEPES, 5.0; and glucose, 11.1. The depolarizing salt solution was of composition (mM): NaCl, 129.5; KCl, 20.0; $CaCl_2$, 1.0; $MgCl_2$, 1.0; $NaH_2PO_4$, 2.4; HEPES, 5.0; and glucose, 11.1.

### 2.1.2 Drugs used

Triton X100, 1% or 0.5% by volume dissolved in salt solution; methylene blue, $10^{-5}$ M; ryanodine, $3.10^{-6}$ M; sodium nitroprusside, $10^{-5}$ M; isoproterenol, $10^{-6}$ M; and indomethacin $10^{-5}$ M. The drugs were introduced dissolved in the regular salt solution.

### 2.1.3 Perfusion and Chamber

The chamber (Figure 2) volume was approximately 3.5 ml. The rate of flow was 20 ml/min and constant volume was maintained by overflow suction. The solution was continuously bubbled with 100% $O_2$ before entering the chamber. All the perfusing salt solutions, with or without drugs, had a pH of 7.45, adjusted with 1M NaOH, and were kept at 33 $^{o}$C.

## 2.2 Methods

### 2.2.1 Muscle preparations and recordings

#### I. Muscle preparation-Cats

Cats of 1.8-3.6 kg were anesthetized intraperitoneally with sodium pentobarbital (30 mg/kg). The chest wall and pericardium were opened, and the heart was quickly excised and placed in the regular salt solution at room temperature. The heart was subsequently perfused retrograde through the aorta by an oxygenated solution (at room temperature) during which the coronary arteries became clear in appearance. This indicated a good perfusion, thus providing good oxygenation of the myocardial tissue during dissection.

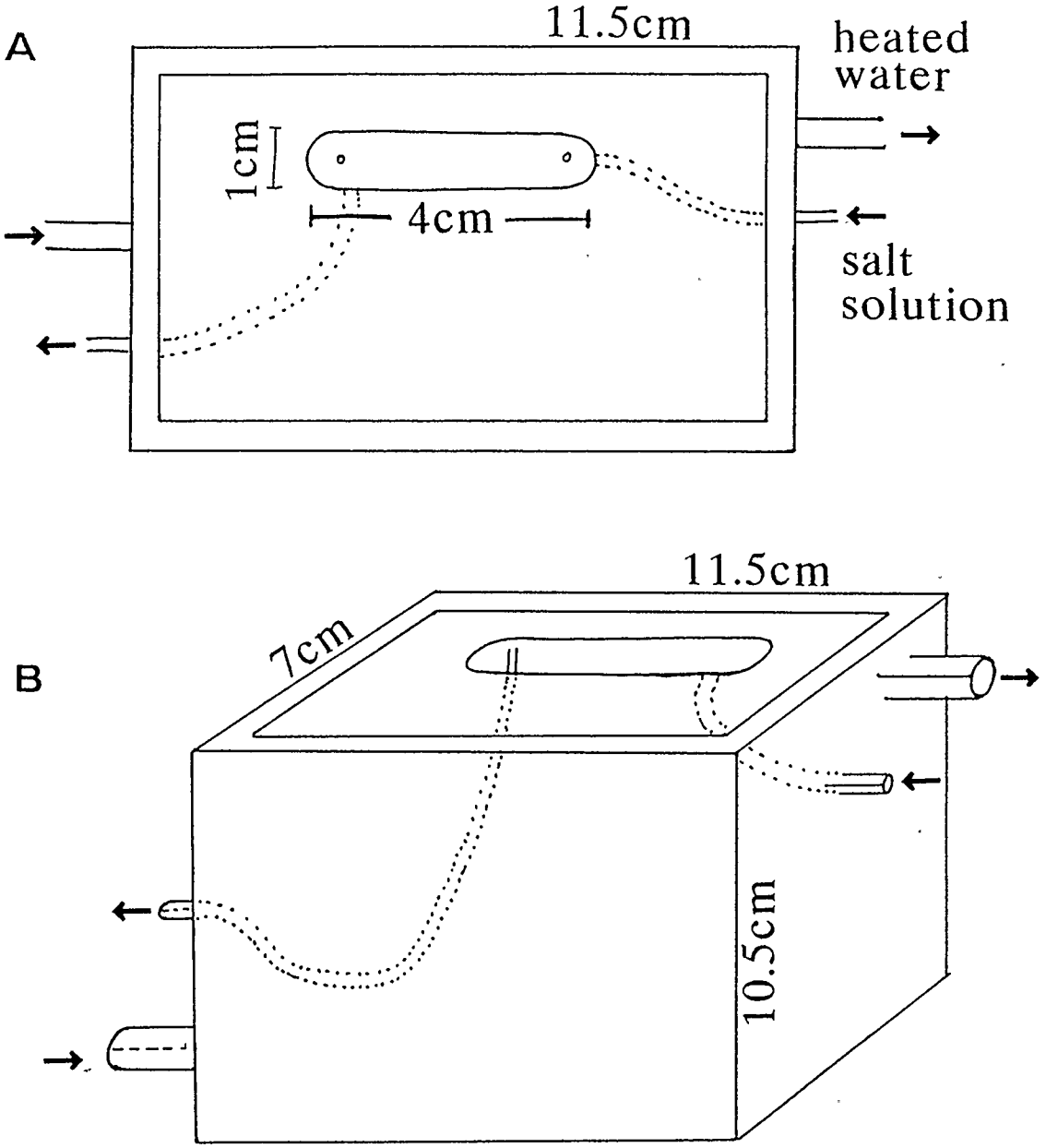



Figure 2 (A) Top view, (B) side view of the perfusion chamber showing the dimensions and the direction of fluid flow. Chamber volume: approximately 3.5 ml. Temperature: 33 $^{o}$C.

## II. Papillary Muscle Isolation

The right ventricular wall was incised and papillary muscle (diameter less than 1.2 mm) carefully dissected. In order not to damage the endocardial surface, papillary muscles were dissected by cutting deeply into the ventricular septum around the non-tendonous end of the muscle. All muscles were mounted horizontally by pinning the ventricular end to the bottom of an organ chamber (described above), and attaching the tendonous end (tied with a thread) to the hook of an electromechanical transducer (Ackers AE801). Through a pair of platinum electrodes the papillary muscles were stimulated at 0.2 Hz and 20% suprathreshold amplitude with 5 ms square wave stimuli from a Grass S88 stimulator, through a stimulus isolation unit. It should be emphasized that in all cases we tried to dissect the non-tendonous end of the muscle in such a way that it allowed stable impalements for simultaneous recording of the action potential and contractile force.

## III. Tension recordings

The tension transducer (Ackers AE801) was a silicon strain gauge tension semiconductor transducer (1 in Figure 3). The silicon beam had one diffused resistor on each surface of the beam. Bending of the beam caused a change in the value

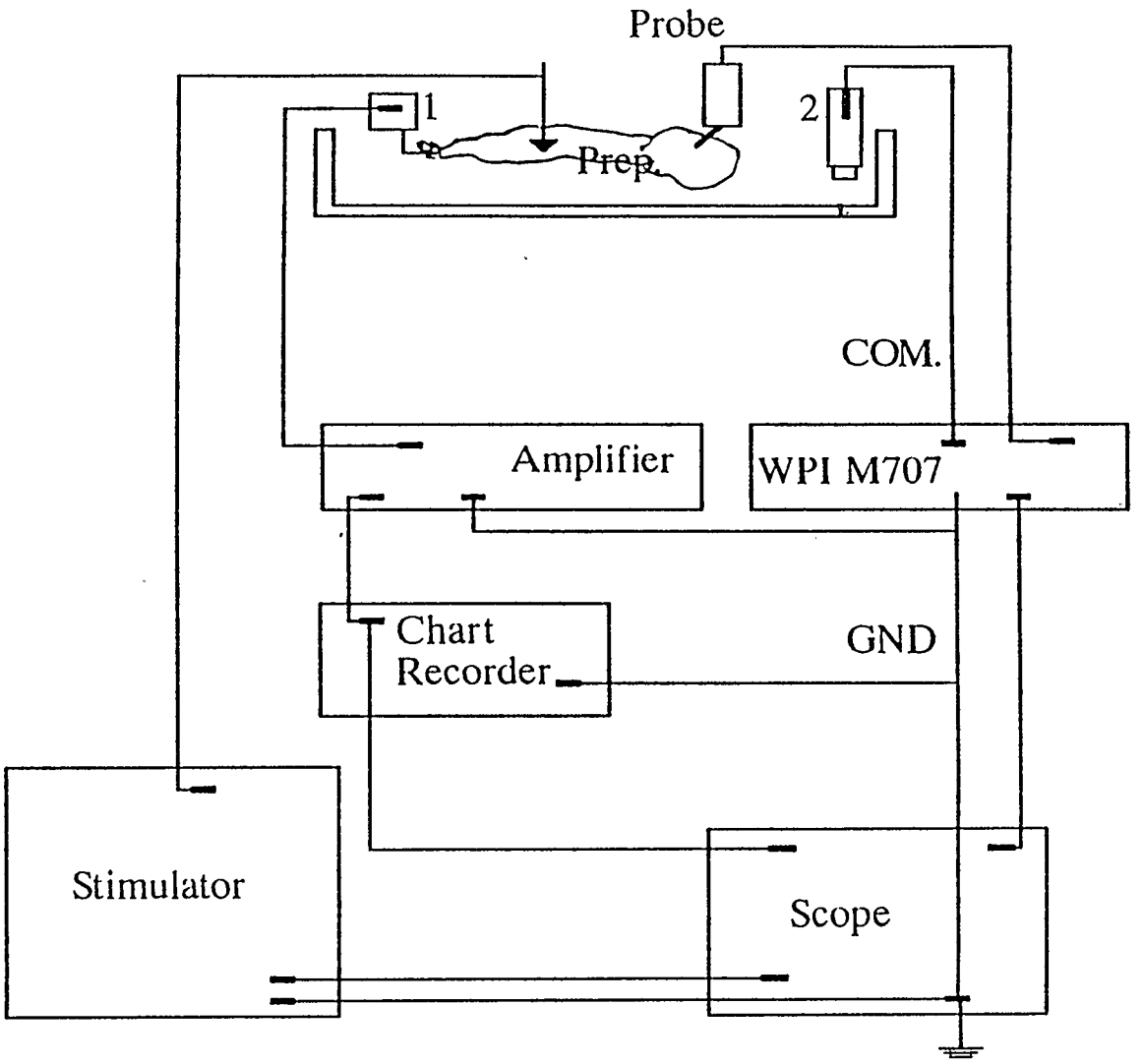



Figure 3 Schematic diagram of the experimental set-up. This arrangement allowed simultaneous contraction and action potential recording. (1) force transducer; (2) reference electrode: Ag/AgCl, Agar (3M KCl). "Probe" refers to microelectrode. WPIM707 is a capacitance-neutralizing amplifier.

of the resistance. The transducer was mounted on a motor which permitted stretching of the muscle as the tension was being measured. The amount of stretch was measured using a calibrated chart recorder. The output of the transducer was fed into a digital oscilloscope (Philips PM3305) and a chart recorder through an operational amplifier (DC-coupled) which amplified the transducer signal. In addition to amplification, the amplifier, having a high input impedance and a low output impedance, acted as an impedance matching device between the transducer and the oscilloscope.

## IV. Action Potential recordings

Transmembrane potentials were recorded using glass microelectrodes (tip diameter of approximately 0.25 um or less, and a resistance of 10-40 Megohm) filled with 3M KCl [90]. The microelectrode was coupled via an Ag/AgCl junction to a high impedance capacitance-neutralizing amplifier (WPI M707), using an Ag/AgCl electrode as reference (2 in Figure 3). The action potentials were recorded using a digital oscilloscope (Philips PM3305). Before penetrating the fibre (using a multidirectional micromanipulator under a direct binocular microscope), DC offset was set to zero. This "bucked out" the potential difference generated between the microelectrode and the reference electrode when they were both in the perfusion solution. The capacitive component in

the circuit was neutralized by capacity compensation and thus the RC-time constant was reduced to help minimize signal distortion. This also helped the rapid transient changes such as phase 0 (the rising phase) of the action potential to be observed.

### 2.2.2 Experiments

#### I. Contraction and Fast Action Potential experiments

A. Triton treatment by immersion:

For one series of experiments, after mounting and establishing the stimulation, papillary muscles were stretched in stepwise fashion to Lmax (the initial muscle length that gives the greatest twitch tension) and following a stabilization period of one hour at Lmax and in 0.5mM $Ca^{2+}$, action potentials were recorded together with the tension. Following control experiments, the muscles were removed from the chamber and immersed for 1-3 seconds in a solution of 1% Triton X100 dissolved in salt solution. This was quickly followed by a thorough wash in salt solution. The action potentials and contractions were subsequently recorded.

B. Bolus Triton treatment:

In another series of experiments, papillary muscles at Lmax were allowed to stabilize in a medium of 1.0 mM $Ca^{2+}$. Once a stable electrode impalement was obtained and action potential equilibrated, the muscle was treated with 100 ul of 1% by volume Triton X100, placed in the chamber containing the muscle. This permitted the observation of the time course of changes in the twitch tension and action potential (shape and dV/dt) following addition of the detergent.

C. Albumin and osmotic pressure:

To examine the possible influence of molecular reorganization and loss in the subendocardial space following endocardial damage, concentration of 4g/100ml of albumin was used on papillary muscle before and after 1% Triton (100 ul) treatment. This procedure increased the osmotic pressure on the preparation and therefore helped examine the suspicions that Triton may be changing the interstitium and the observed effects may be due to reorganization of molecules and change in the osmotic pressure in this milieu following endocardial damage. $[Ca^{2+}]_o$ was 1.0 mM.

## II. Ventricular Wall Muscle preparations-Cats

### A. Right Ventricular Wall:

The right ventricular wall muscle of the cat heart was incised and was separated completely from the heart. It was then divided into smaller square or rectangular pieces of sizes of about 5 mm by 5-8 mm. Each piece was mounted (pinned) in the chamber at slack length or with a very small amount of stretch. Depending on the experiment, either the endocardial side or the epicardial side was at the bottom, thus allowing action potential recording from the other side. Both stable and random (from different parts and depths of the preparations) recording were performed before and after Triton X100 (100 ul 0.5%) in 1.0 mM $[Ca^{2+}]_o$.

### B. Left Ventricular Wall:

The left ventricular wall was cut in a manner so as not to cut into the ventricular chamber. This allowed dissection of the myocardium and the epicardium of the wall (endocardium absent) for action potential recording. These action potentials (from epicardial side) were recorded randomly from different parts and depths of the preparation and averages were taken when stable impalements were not possible. The procedure was repeated after treatment (100 ul 0.5% Triton X100). Experiments were performed in 1.0 mM $[Ca^{2+}]_o$.

III. Contraction and Slow Action Potential experiments

For this series of experiments the papillary muscles were allowed to equilibrate for one hour at Lmax and in a medium containing 1.0 mM $Ca^{2+}$ and 4.5 mM $K^{+}$. Following this stabilization period, stable electrode impalement was obtained and the perfusing solution was changed to one containing 1.0 mM $Ca^{2+}$ and 20 mM $K^{+}$. The muscle became depolarized and the contractions were lost. This perfusion was continued for about 20-25 minutes following which $10^{-6}$ M isoproterenol was introduced into the solution. Once the excitability returned, the contraction and slow action potential were allowed to equilibrate and remain at this equilibrium for about 30 minutes. The effect(s) of Triton on the slow action potential and contraction were subsequently tested. This, as in the case of fast action potential experiments, provided an accurate indication of the time course of the effects on both the electrical and mechanical activities. In some cases a bolus (100 ul of 1% Triton) was directly added to the chamber containing the muscle. Additionally, however, in this series of experiments, attempts were made to determine how low a concentration of Triton provided qualitatively similar results to those obtained using bolus treatment or direct immersion in Triton. This was done by using a syringe (attached to the

perfusing chamber) providing final concentrations of Triton as low as 0.001%.

IV. Ryanodine experiments on Papillary Muscle

$3.10^{-6}$ M ryanodine was added to papillary muscles after having equilibrated at Lmax and in 1.0 mM $[Ca^{2+}]_o$. Stable action potential recording was performed as the effect of ryanodine was taking place. Records of both the isometric contraction and action potential were taken periodically until equilibrium was achieved. The muscle was subsequently treated with 100 ul of 1% Triton, and records from contraction and action potential were taken.

V. Extrasystoles and Post-extrasystolic Potentiation experiments

These experiments were performed on the twitch tension of cat papillary muscles at Lmax and in a medium containing 1.8 mM $Ca^{2+}$ and 4.5 mM $K^{+}$. Following a one hour stabilization period, extrasystoles were elicited at various time intervals (range of which was dictated by the stimulation frequency of 0.2 Hz) giving rise to post-extrasystolic potentiation. This was done in extracellular $Ca^{2+}$ concentration from 1.8 to 8.8 mM (maximum contractile

response). The rate of decay (recirculated amount of calcium into sarcoplasmic reticulum) of potentiated force was examined at each $[Ca^{2+}]_o$. Muscles were then treated by immersion for 1-2 seconds in 1% Triton followed by a thorough wash in salt solution. The above procedure was then repeated.

VI. Ryanodine experiments on Ventricular Wall

These experiments were performed by placing complete pieces of the right ventricular wall in the chamber (epicardial side down) with the salt solution containing $3.10^{-6}$ M ryanodine. Once the muscles equilibrated, action potentials were recorded randomly (various sites and depths) from the endocardial side before and after 100 ul of 1% or 0.5% Triton and averages were calculated. Stable impalements were also possible, and the effect of 100 ul of 0.5% Triton on the action potential was tested by this approach, too. Experiments were performed in 1.0 mM $[Ca^{2+}]_o$.

VII. Influence of $[Ca^{2+}]_o$

The influence of extracellular calcium was examined on the contraction and fast action potential of intact papillary muscle and muscle with damaged endocardium.

As noted, in some of the above experiments 100 uL of 0.5% rather than 1% Triton X100 was used. This was done because it was noticed that a concentration of 0.5% can provide a better safety factor against damage to the underlying myocardium. This was especially true since qualitatively no difference in the effects was observed when either concentration was used.

2.3 Preliminary pharmacological experiments

A. To test the possibility of an endocardium-derived factor similar in nature to the vascular smooth muscle endothelium-derived relaxing factor (EDRF) effects of $10^{-5}$ M sodium nitroprusside (a cGMP enhancer- [12]) and $10^{-5}$ M methylene blue (a cGMP inhibitor- [12]) were individually tested on papillary muscles with intact endocardium. $[Ca^{2+}]_o$ was 1.0 mM.

B. A concentration of $10^{-5}$ M indomethacin (a cyclo-oxygenase inhibitor- [63]) was used on intact papillary muscles to examine if prostacyclin was the controlling factor between endocardial and myocardial cells. This was done on the basis of the observation made recently by Manduteanu et al. [73] that cardiac valvular endothelial cells could produce prostacyclin. $[Ca^{2+}]_o$ was 1.0 mM.

## 2.4 Analysis

### 2.4.1 Contraction analysis

The isometric contractions were analyzed for their peak value, time-to-peak tension, time-to-half relaxation, and twitch duration. These were done using a combination of chart recorder, plotter, and oscilloscope.

### 2.4.2 Action Potential analysis

The action potentials were analyzed for amplitude above zero millivolt, resting potential, threshold potential, excitability, duration at 50% and 80% repolarization, dV/dt, and general shape. A computer wave analyzing programme, a chart recorder and a plotter were used for this. For the statistical analysis of the action potentials two different tests were done: "rank sum test" and "median test". Rank sum test is a nonparametric procedure which tests for differences in the central values of samples from two independent samples. This test was performed in association with "Mann-Whitney U statistics". The use of rank sum test provided a more accurate procedure since it assumes no particular distribution for the data, in contrast to the "two-sample t-test" which may give indications of significant difference if the data compared are not

distributed "normally". The p values given are one-sided tests from Mann-Whitney U statistics. To support the results of the rank sum test, median test was used.

# Chapter 3. Results

## 3.1 Cat Papillary Muscles:

### 3.1.1 Effect of Triton treatment on Isometric Contraction in a normal medium

Whether by immersion of papillary muscle for 1-2 seconds in Triton 1%, or by using a bolus (100 uL) of 1% Triton, as the muscle was stretched to Lmax, there was a consistent earlier onset of isometric relaxation in the treated preparations (Figure 4, upper trace). This was in accordance with Brutsaert et al. observation [16]. In those experiments where a bolus treatment was used, the time course of effect on force was noted to be immediate, that is, the earlier relaxation happened after two beats. Furthermore, there was a consistent decrease in the peak isometric twitch with decrease in time-to-peak and duration of contractile force (Figure 4, upper trace). In no case was there a change in the resting tension (Figure 4, bottom trace). Similar results have been observed in papillary muscles of rabbit and rat (Figure 5).

To examine if the electrical activity of the myocardium had also been changed after endocardial damage, the effects of endocardial damage on the action potential were examined in our laboratory.

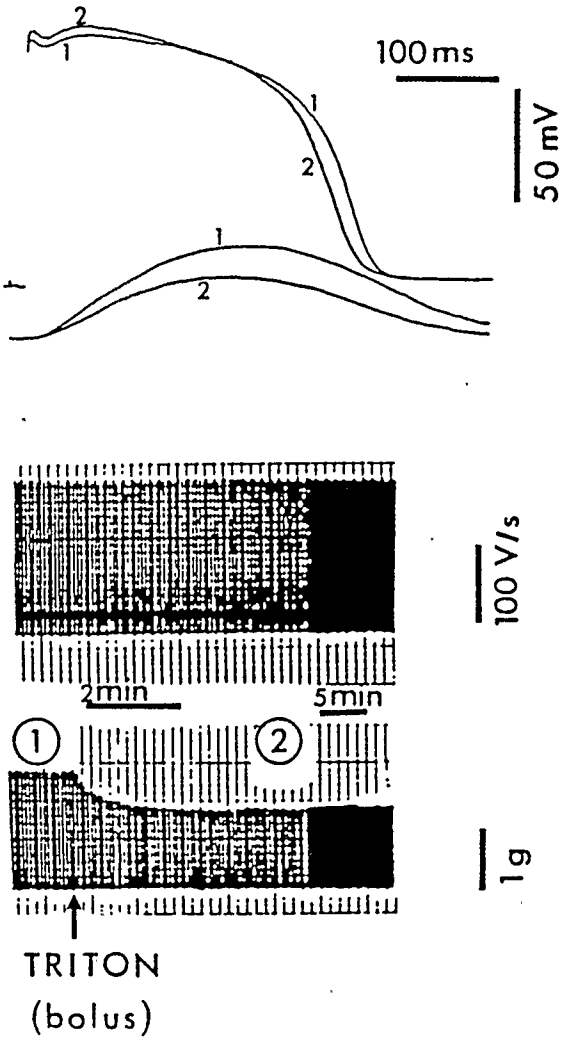



Figure 4 Typical example of the effect of 100 ul of 1% Triton X100 treatment on the mechanical and electrical activities of cat papillary muscle. Muscle at Lmax (initial length at which force is maximal). Treatment was done in a flow of 20ml/min. (1) Untreated muscle, (2) treated muscle. Upper traces: Action potential in 1.8 mM $Ca^{2+}$ in steady state (note shortened duration and increased amplitude); Isometric contraction in steady state (note decrease in time-to-peak and time-to-half relaxation with decrease in force). Middle trace: Upstroke velocity of action potential is unchanged. Lower trace: Note the quick decrease in peak isometric force and the absence of rise in resting tension upon Triton treatment. (cf ref. 12).

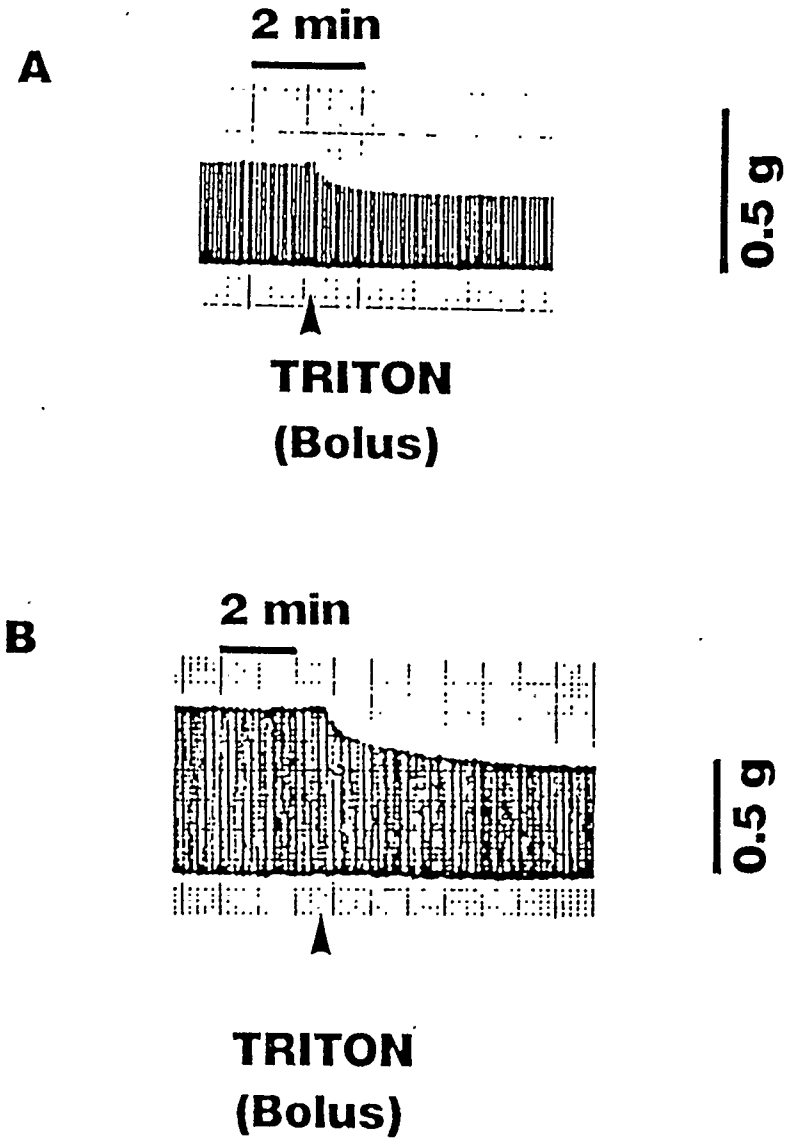



Figure 5 Effect of Triton X100 (100 ul 1%) on contractile force of rabbit (A) and rat (B) papillary muscles. There is a quick decrease in peak isometric force with no increase in resting tension. Note the similarity in the amount of decrease in force in both preparations. $[Ca^{2+}]_o$ 1.0 mM and muscles were at Lmax.

### 3.1.2 Effect of Triton treatment on Fast Action Potential

Triton treatment (immersion or bolus) induced a shortening in the action potential duration (both at 50% and 80% repolarization), and increase in amplitude, but no change in either the resting membrane potential (Figure 4, upper trace) or the upstroke velocity (Figure 4, middle trace). Moreover, the threshold of excitability did not change. When a bolus (100 uL) of 1% Triton was used on a muscle at Lmax, the shortening in the action potential duration and increase in amplitude occurred long after the changes in the contractile force had begun. This delay was not of the order of one or two beats; rather a period of the order of minutes elapsed before any shortening in the action potential duration occurred (Figure 6). This was consistent in all the experiments on the papillary muscles and trabeculae. When studying the time course of changes the recording was stable.

## 3.2 Effect of Triton treatment on the Force-$[Ca^{2+}]_o$ relations

There was a non-parallel shift in the force-$[Ca^{2+}]_o$ curve towards increasing calcium. As evident from Figure 7 at large extracellular $[Ca^{2+}]$ the difference between a control

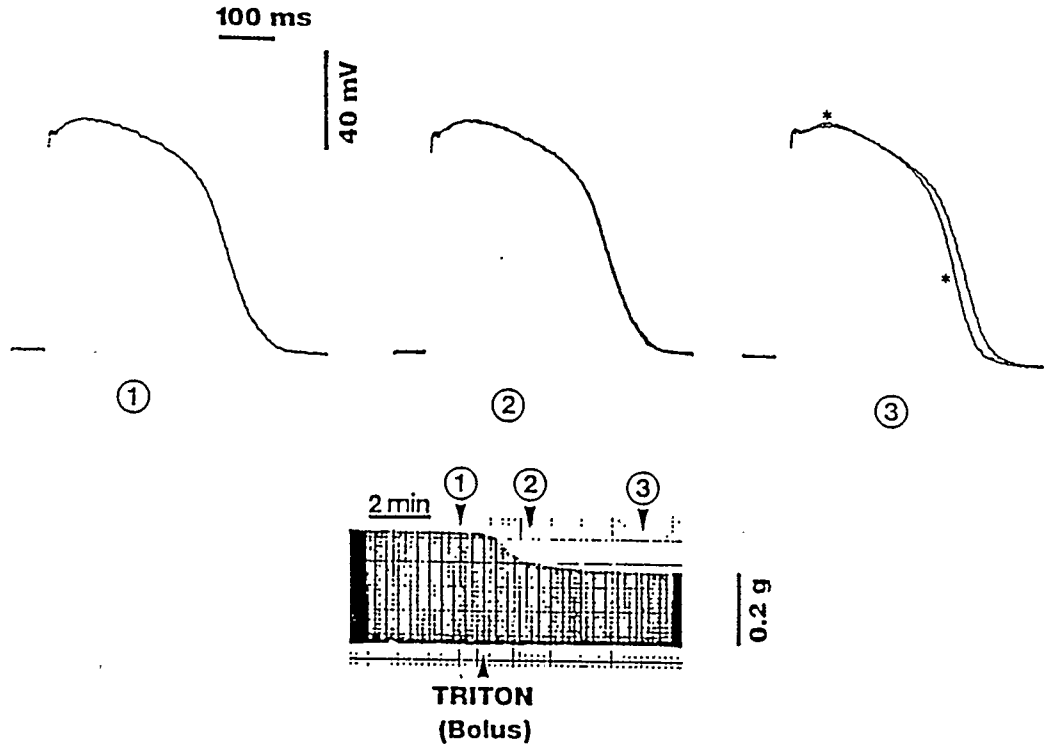



Figure 6 Time course of effect of Triton X100 (100 ul 1%) on the action potential (top) and contraction (bottom) of a cat trabecula muscle. The changes in the action potential do not occur until long time after the contractile force has been altered. At equilibrium (3) the action potential duration is shorter and there is a slight increase in amplitude. The peak contractile force is at a lower level. $[Ca^{2+}]_o$ 1.0 mM and muscle was at Lmax.

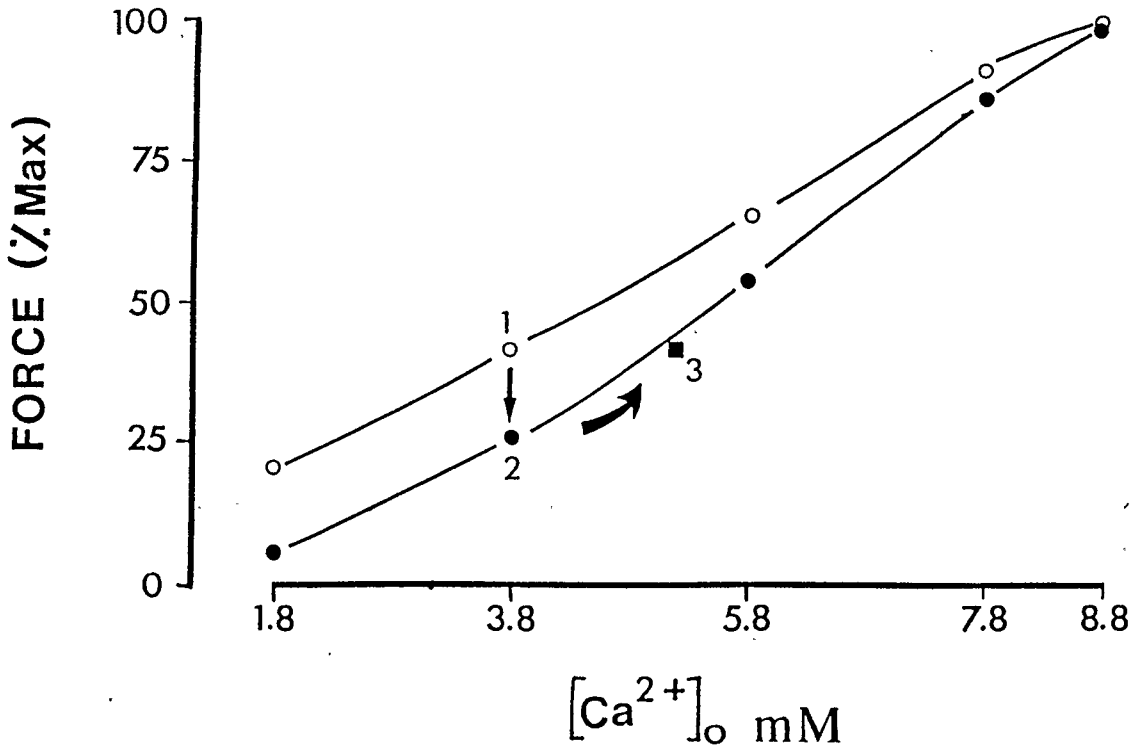



Figure 7 Force-$[Ca^{2+}]_o$ relationships of a cat papillary muscle treated by immersion in 1% Triton X100 for 1-2 seconds. There is a non-parallel shift after Triton treatment (filled circles) with the shift becoming small towards high $[Ca^{2+}]_o$. For a treated muscle (point 2), appropriate increase in $[Ca^{2+}]_o$ can return the peak isometric force to control level (point 1 and point 3).

muscle and a treated one became smaller. The observations of Brutsaert et al. were also similar [16].

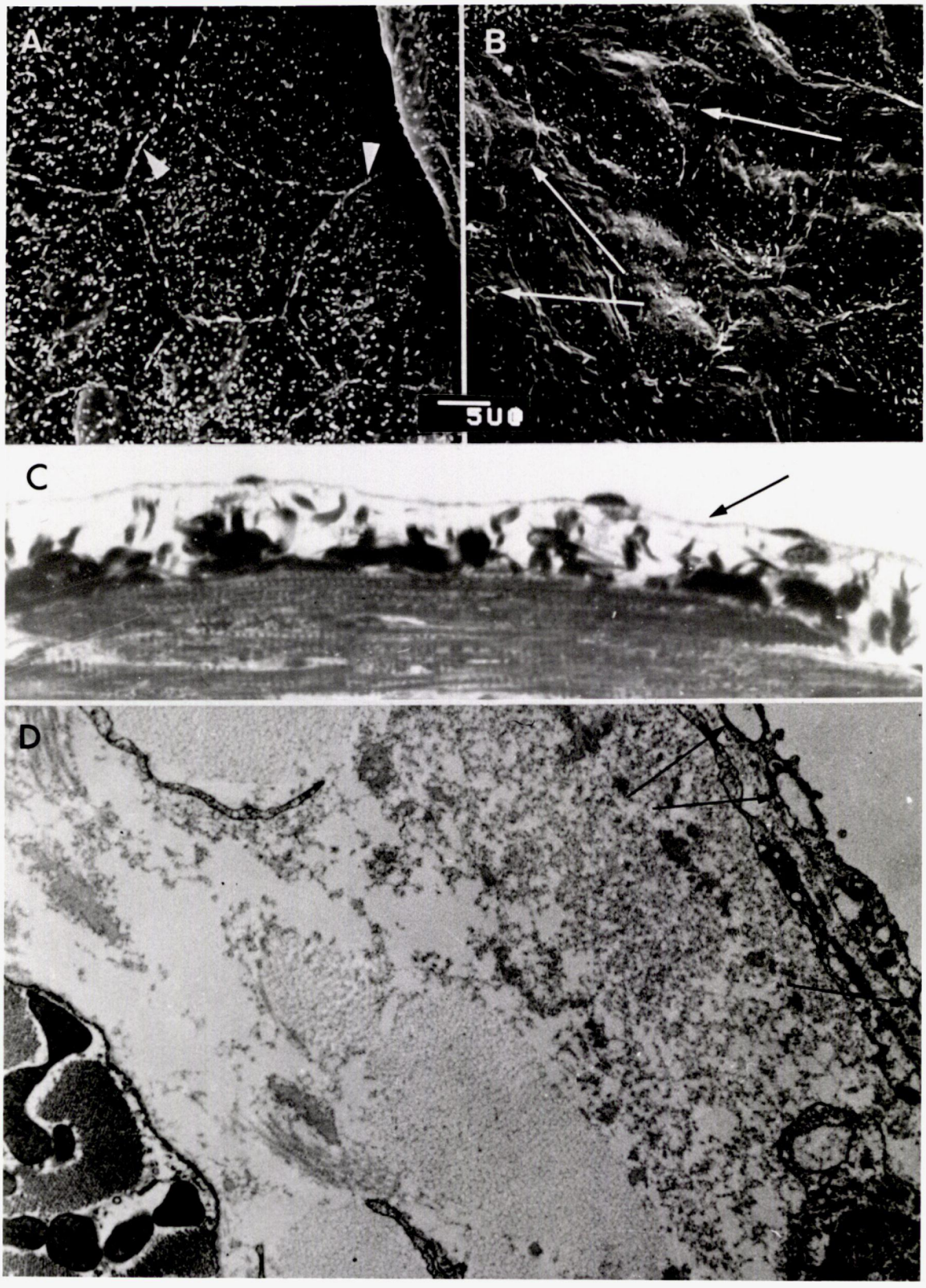
To examine if the changes in contraction and action potential were due to damage to the underlying myocardium, light micrographs and transmission electron micrographs as well as scanning electron micrographs were produced.

### 3.3 Microscopic observations

There was no sign of sarcolemmal or mitochondrial damage following Triton treatment. Furthermore, there was no sign of change in myocardial cell organization and structure as evidenced from transmission electron microscopy. There were, however, vacuoles present in some of the endothelial cells of the endocardium following the Triton treatment (Figure 8D).

The Light micrographs of a Triton-treated muscle showed that there was an apparent continuity in the endothelial cells (Figure 8C). However, by this method it is not possible to conclude anything regarding the absence or presence of denudation in that portion of the muscle which is not shown in the micrographs. Even scanning electron microscopy (Figure 8A, 8B) that clearly demonstrates endothelial cell membrane perforations does not eliminate the possibility

Figure 8 (A,B) Scanning electron micrographs of rabbit papillary muscle. Endothelial cells in control muscle show microvilli and marginal folds (arrow heads). Triton X100 treatment has led to disappearance of most of the microvilli and has caused membrane perforations (arrows) typical for Triton treatment. (C) Light micrograph of rabbit papillary muscle. There is continuity in endocardial endothelial lining (arrow) following treatment thus forming a barrier between the myocardium (lower part) and extracellular space (upper part). The subendocardial space (between endothelial cells and myocardium) contains collagenous material (X240). (D) Transmission electron micrograph (X11500) of rabbit papillary muscle. The only observed difference in this treated muscle is the presence of vacuoles (arrows on right) that are absent in control muscle. The underlying myocardium (bottom left) appears normal. Treatment was done by 15 sec. immersion of muscle in 0.03% Triton X100. (cf ref. 12).


A
B
5U
C
D

that there may have been denudation of endothelial cells in some parts of the preparations. This would imply that there may be discontinuity as well as membrane perforations in some parts.

### 3.4 Effects of increased Osmotic Pressure on Triton effects

With the addition of 4g/100ml albumin to the solution there was a small decrease in the isometric force of an intact muscle (Figure 9A). The muscle was subsequently washed. Upon addition of 100 ul of 1% Triton, the previously observed effects (Figure 9B), (i.e. accelerated relaxation, decrease in peak isometric and time-to-peak force) were immediately observed. The addition of 4g/100ml albumin to the treated muscle did not reverse the effects of endocardial damage. There was only a further small decrease in the peak force (Figure 9C). Clearly, therefore, the effects of endocardial damage were still persistent even when the osmotic pressure on the muscle was increased. It should be noted that negative inotropism due to hyperosmolarity has been observed in cat papillary muscle before [128].

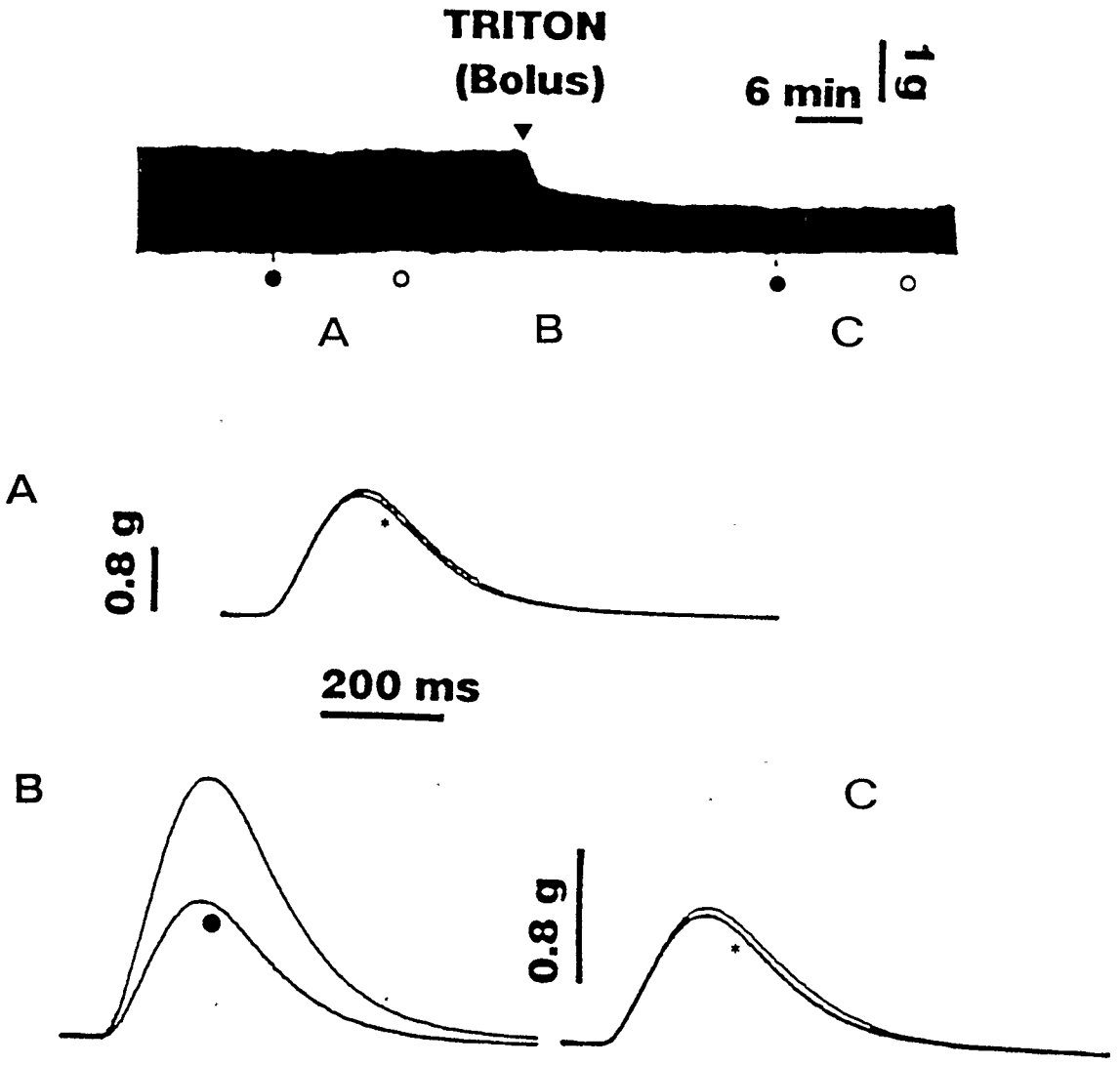



Figure 9 (A) Effect of increasing the osmotic pressure with 4g/100 ml albumin (top trace, first filled circle) on intact cat papillary muscle contractile force (Lmax and 1.0 mM $Ca^{2+}$). There is a small negative inotropism. (B) Note the decreased peak force and time-to-peak force upon addition of Triton X100 (100 ul 1%). (C) Addition of albumin (4g/100ml) (top trace, second filled circle) to the perfusate after Triton did not reverse the effects of endocardial damage. Note a further small negative inotropic effect. (open circle indicates wash).

## 3.5 Ventricular Wall experiments

### 3.5.1 Random Action Potential Recordings

Action potential recordings from various parts of the endocardial side of the complete right ventricular wall (epicardium present) showed statistically significant shortening in duration after Triton treatment (100 uL of 0.5% Triton) (Figure 10B, left columns). On the other hand, there was no statistically significant difference in action potential duration between before and after treatment when random recordings were made from different parts of the epicardial side of the left ventricular wall (endocardium side absent) (Figure 10B, middle columns). Moreover, on average, there was no significant difference in duration between a control muscle and a treated muscle when random action potential recordings were made from the epicardial side of the complete right ventricular wall (with endocardium present) (Figure 10B, right columns).

### 3.5.2 Stable Action Potential Recordings

Where stable impalements were possible the effects of 100 uL of 0.5% Triton X100 on the action potential were examined from sections of ventricular wall similar to those from

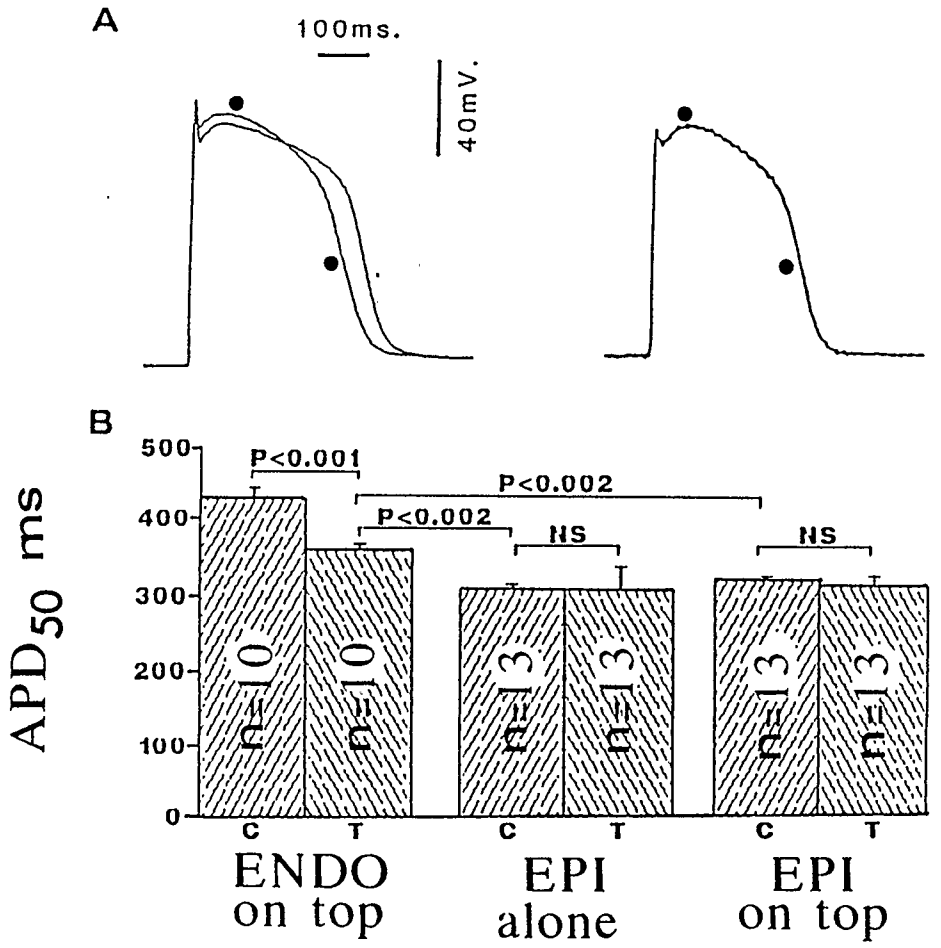



Figure 10(A) Stable action potential recording in 1.0 mM $Ca^{2+}$ from the endocardial (left) and the epicardial (right) side of the cat ventricular wall before and after (filled circles) addition of Triton X100 (100 ul 0.5%). Note the typical alteration of action potential (decreased duration and increased amplitude) following treatment of the endocardial side and the absence of such effects upon treatment of the epicardial side. (B) Statistical analysis of action potential duration at 50% repolarization ($APD_{50}$) before (c) and after (T) Triton X100 (100 ul 0.5%). Action potentials recorded randomly from different locations and depths. Left columns: complete right ventricular wall with endocardium (ENDO) on top, AP recorded from the endocardial side. Middle columns: left ventricular epicardium alone. Right columns: complete right ventricular wall with epicardium (EPI) on top, AP recorded from the epicardial side. Significant difference ($p<0.001$) is observed only when endocardial side is treated. The p values given are one-sided tests from Mann-Whitney U statistics associated with rank sum test. NS:not significant. $APD_{50}$ is given as mean +/- SEM. "n" is number of preparations each one providing about 20 action potentials.

which random recordings were made. Alteration of action potential characteristics occurred only when endocardial side of the right ventricular wall (epicardium present) was treated (Figure 10A, left), and furthermore, the effects were not immediate. It should, however, be noted that in some preparations an action potential duration shortening after Triton treatment was observed in recordings from the epicardial side of the right wall (endocardium present on the bottom). This, however, was not found to occur commonly.

## 3.6 Slow Action Potential and Contraction recordings

### 3.6.1 Effect of Triton treatment on Isometric Contraction and Slow Action Potential

In these experiments the effects of endocardial damage on the contraction and slow action potential shape (and thus slow inward current) were examined using 100 uL of 1% Triton X100 (Figure 11), and also the lowest concentration of Triton that could induce the observed changes qualitatively was determined (Figure 12). This was done primarily to confirm the validity of the effects produced by 100 uL 1% Triton. Whether a bolus (100 uL) of 1% Triton or syringe injection was used, it was noticed that the decrease in peak isometric tension, the decrease in time-to-peak and time-to-half relaxation, started before the initiation of slow

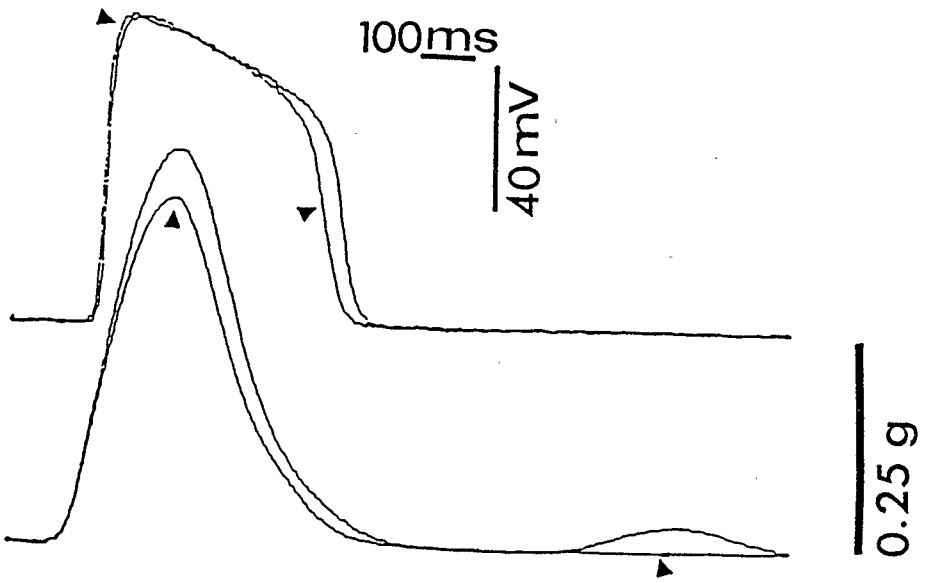



Figure 11 Steady state effect of Triton X100 (100 ul 1%) on contraction and slow action potential of cat papillary muscle (arrow heads). Note the decrease in time-to-peak and time-to-half relaxation accompanying the decrease in peak isometric force, as well as the disappearance of after-contraction following treatment in steady-state. Action potential duration is shorter and amplitude slightly higher. Experiment performed in 20.0 mM K+ and 1.0 mM $Ca^{2+}$. Excitability was restored with $10^{-6}$ M isoproterenol. Muscle was at Lmax.

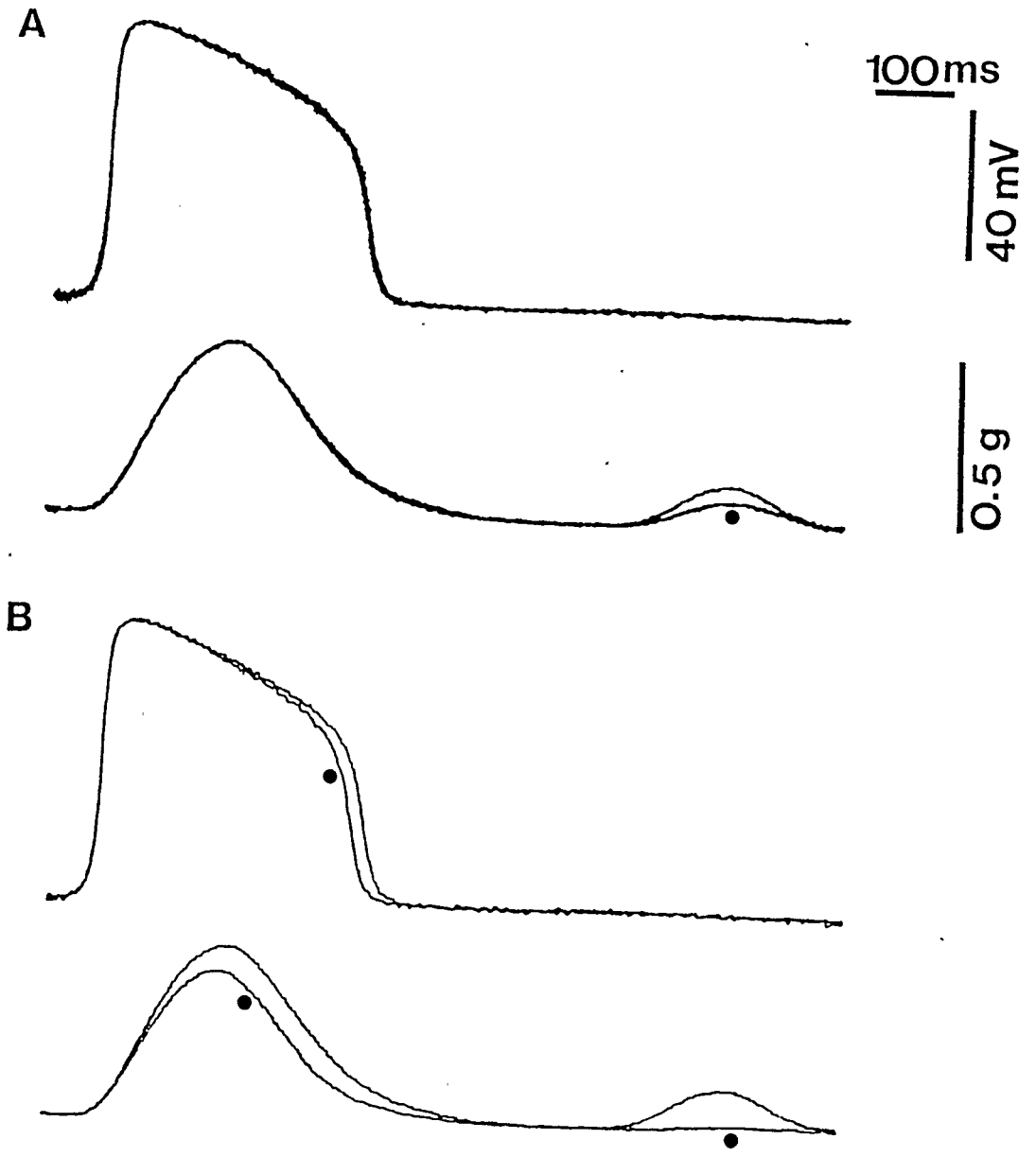



Figure 12 Gradual effect of 0.02% Triton X100 (30 sec syringe injection) on contraction and corresponding slow action potential of cat papillary muscle (Lmax and 1.0 mM $Ca^{2+}$). (A) Upon Triton treatment (filled circles) the after-contraction (lower trace) has immediately (even before the regular contraction) decreased before any change in the action potential shape. (B) Slow action potential (upper trace) and contraction (lower trace) in steady state after Triton (filled circles). The time-to-peak and time-to-half relaxation are shorter and the peak force had diminished. Additionally, the after-contraction has completely disappeared. The action potential duration is shorter. Experiments performed in 20.0 mM K+. Excitability was restored with $10^{-6}$ M isoproterenol.

action potential shortening. Furthermore, there were always after-contractions unaccompanied by any after-potentials in a depolarized medium containing isoproterenol, which upon Triton treatment disappeared completely once steady state had been reached (Figure 12B, lower trace). The decrease in the after-contraction always preceded the decrease in the contraction by one beat. The action potential recording was stable thus permitting the examination of the time course accurately (Figure 12B, upper trace). The lowest concentration of Triton that caused any effects was found to be 0.02% using syringe injection. Since the after-contractions disappeared following Triton treatment, it was apparent that the sarcoplasmic reticulum release characteristic had been affected by the endocardial damage. It was, therefore, necessary to look at this by methods that were designed to help determine modifications of sarcoplasmic reticulum function .

## 3.7 Ryanodine experiments on Papillary Muscle

Papillary muscles with intact endocardium exposed to $3.10^{-6}$M ryanodine demonstrated a gradual reduction in peak isometric force (with increase in time-to-peak force) until a steady state had been reached. For the fast action potential there was prolongation and an increase in the amplitude with time (Figure 13A). Once the steady state for both

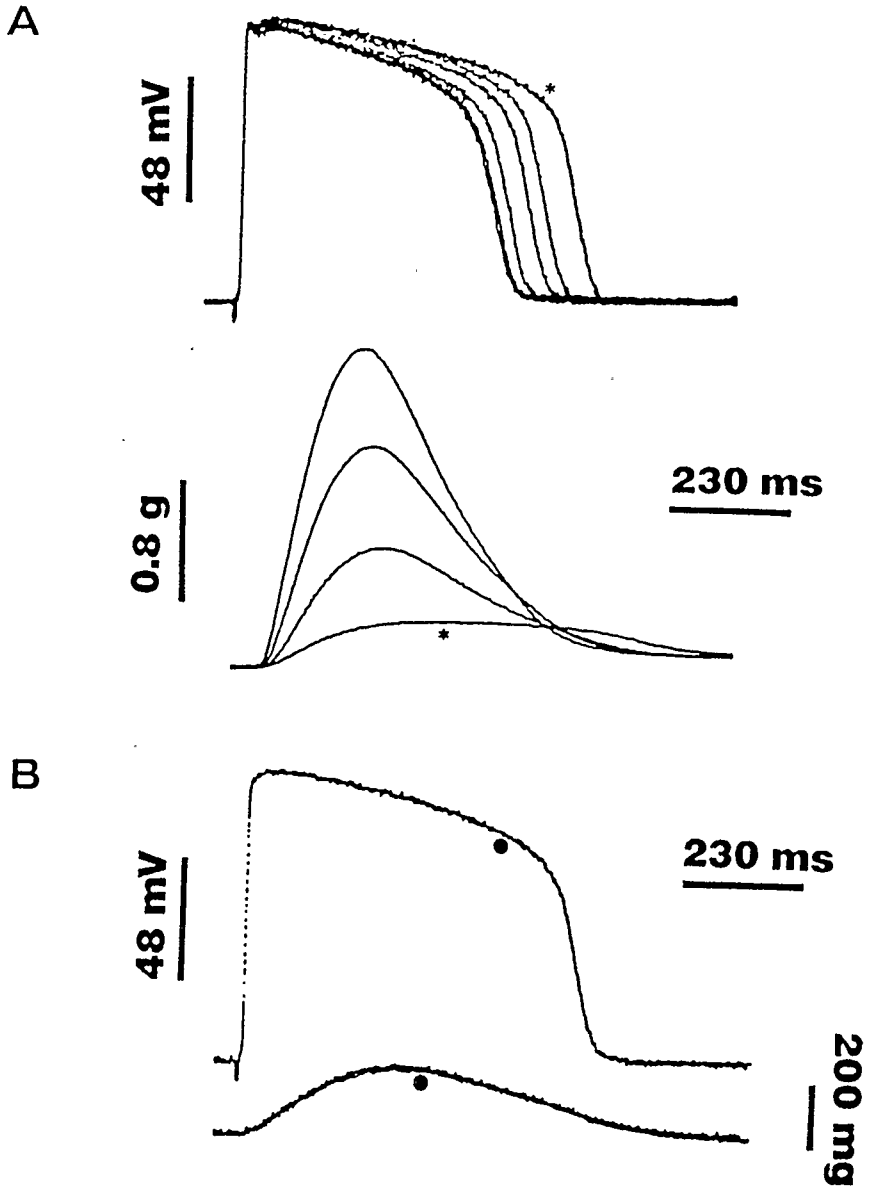



Figure 13 (A) Gradual effect of $3.10^{-6}$ M ryanodine on the action potential (upper trace) and isometric contractile force (lower trace) in cat papillary muscle (Lmax and 1.0 mM $Ca^{2+}$). The duration and amplitude of action potential increase as steady state (*) reaches. Peak isometric force decreases and time-to-peak increases (*). (B) Effect of Triton X100 (100 ul 1%) on the action potential (upper trace) and contraction (lower trace) of cat papillary muscle exposed to $3.10^{-6}$ M ryanodine. Note the absence of any effect on either the action potential or contraction (filled circles).

force and action potential had been reached, a bolus of Triton (100 uL 1%) caused no further reduction in the force and caused no change in the action potential shape with the passage of time (Figure 13B).

### 3.8 Extrasystoles and Post-extrasystolic Potentiation

The observations of the rate of decay of potentiated force indicated that in a muscle with damaged endocardium (by immersion in 1% Triton X100) the amount of recirculated calcium into sarcoplasmic reticulum was larger than in a control muscle. This was evident from the slower decay rate of potentiated force of the treated muscle, which was similar to a situation where extracellular calcium concentration had been raised in an intact preparation (Figure 14). The amount of recirculated calcium also increased when muscle was treated with a 100 ul bolus of 1% Triton X100.

### 3.9 Ryanodine experiments on Ventricular Wall

When action potentials (from the endocardial side of the complete right wall) were recorded randomly in the presence of $3.10^{-6}$ M ryanodine there was statistically no significant difference in the action potential duration before and after

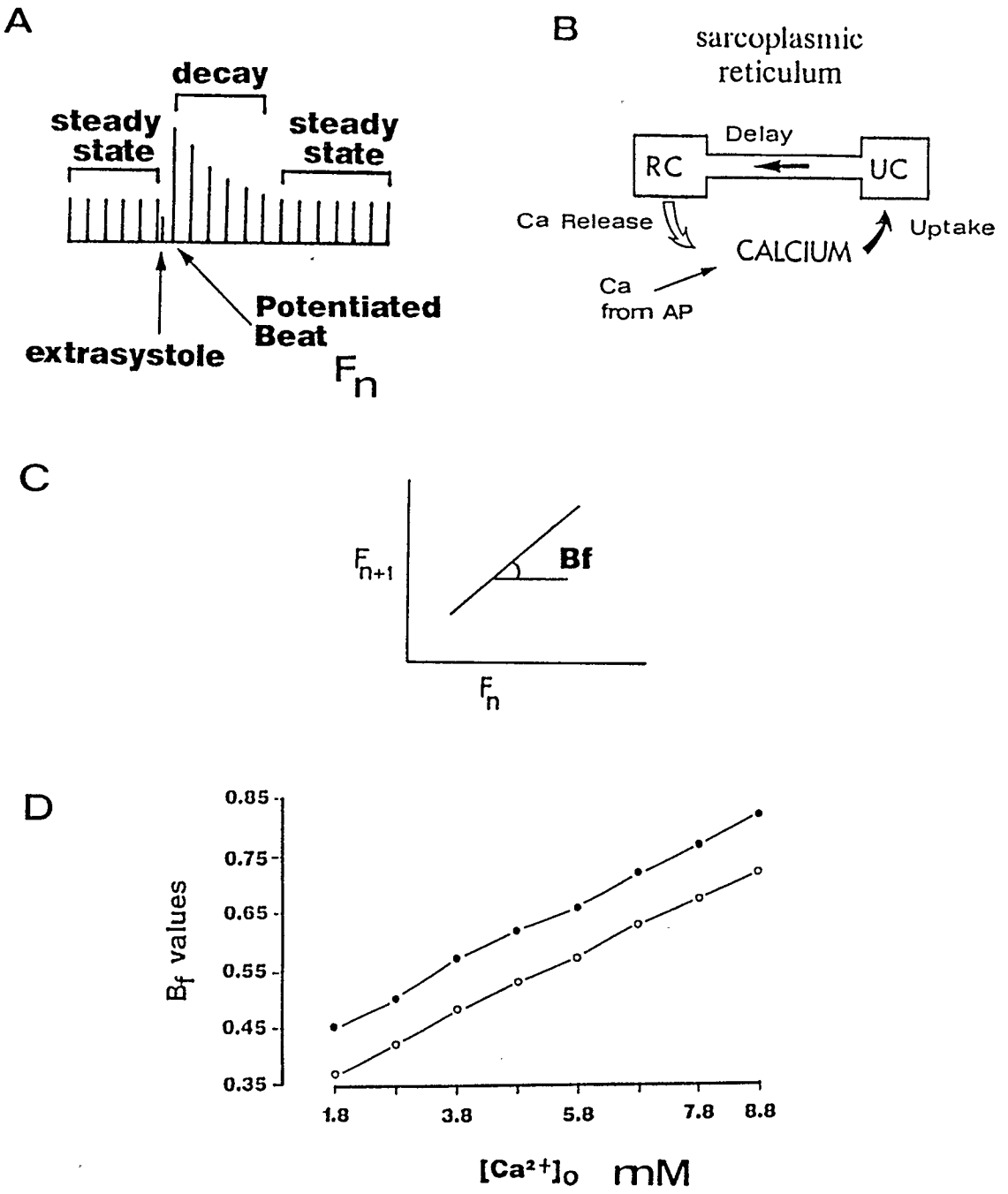



Figure 14 Effect of Triton treatment (by immersion of muscle in 1% Triton X100) and $[Ca^{2+}]_o$ on the recirculated amount of calcium (Bf) in cat papillary muscle (Lmax). (A) Protocol for inducing extrasystoles and calculating Bf. (B) Schematic representation of the sarcoplasmic reticulum indicating the concept of calcium recirculation (see ref. 89 for details). (C) Plot of consecutive contractions to obtain Bf. (D) The Bf value increases with $[Ca^{2+}]_o$ in both control (open circles) and treated (filled circles) muscle. At each $[Ca^{2+}]_o$ the Bf value for the treated muscle is larger than that in control muscle, i.e. treatment effect is similar to effect of raising $[Ca^{2+}]_o$ in a control muscle.

endocardial damage with 100 ul of 0.5% or 1% Triton X100 (Figure 15B). There was, however, significant difference in action potential duration before and after treatment when ryanodine was absent (Figure 15B). With stable recording the addition of 100 uL of 0.5% Triton did not cause any shortening of the action potential duration, or change in the amplitude with time (Figure 15A,right) (as was the case with effect of 100 ul of 1% Triton on action potential and contraction of cat papillary muscle in the presence of ryanodine- Figure 13B).

## 3.10 Influence of $[Ca^{2+}]_o$

### 3.10.1 Effect of Extracellular Calcium on Isometric Contraction and Fast Action Potential in an Intact Papillary Muscle

In the steady state condition in a papillary muscle with intact endocardium, raising the extracellular calcium concentration caused an increase in the peak isometric twitch, and a shortening of the action potential duration (at 50% and 80% repolarization). Furthermore, a consistent increase in the action potential amplitude was always observed (Figure 16,top). The extent of shortening of the action potential duration diminished with increasing $[Ca^{2+}]_o$ (Figure 23, lower panel). The increase in peak isometric

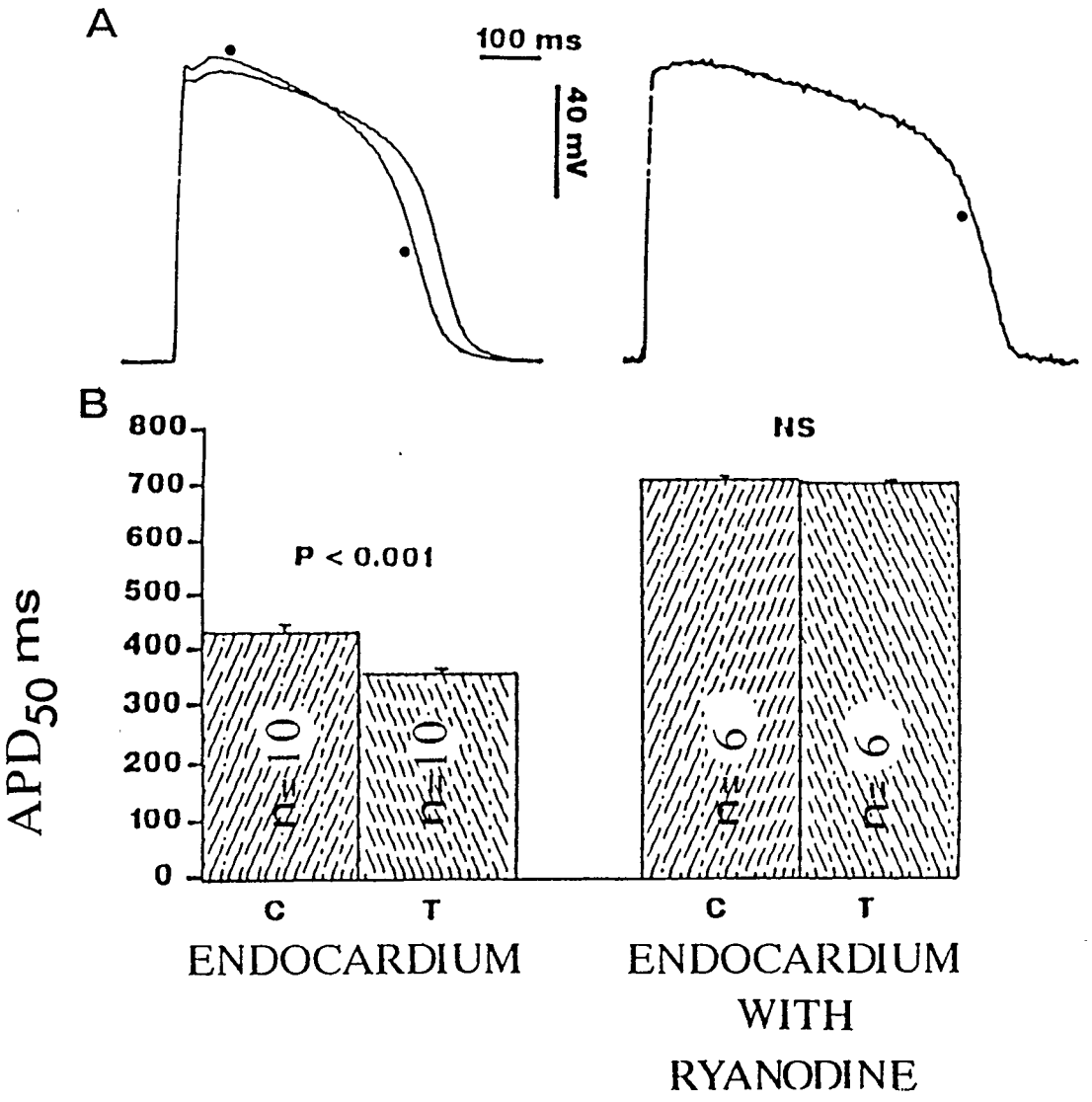



Figure 15(A) Effect of Triton X100 (100 ul 0.5%) on action potential (recorded from one cell) in the absence (left) and presence (right) of 3.10-6 M ryanodine. The shape of action potential alters with endocardial damage (100 ul 0.5% Triton X100) only when ryanodine is absent. (B) Statistical analysis on action potential (recorded randomly from the endocardial side of the complete right ventricular wall at different locations and depths) duration at 50% repolarization ($APD_{50}$) from the endocardial side in the absence and presence of 3.10-6 M ryanodine. Note the presence of statistically significant difference between before (c) and after (T) Triton X100 (100 ul 0.5% or 1%) only when ryanodine is absent. $[Ca^{2+}]_o$ is 1.0 mM. $APD_{50}$ is given as mean +/- SEM. NS:not significant. "n" is number of preparations each one providing about 20 random action potentials.

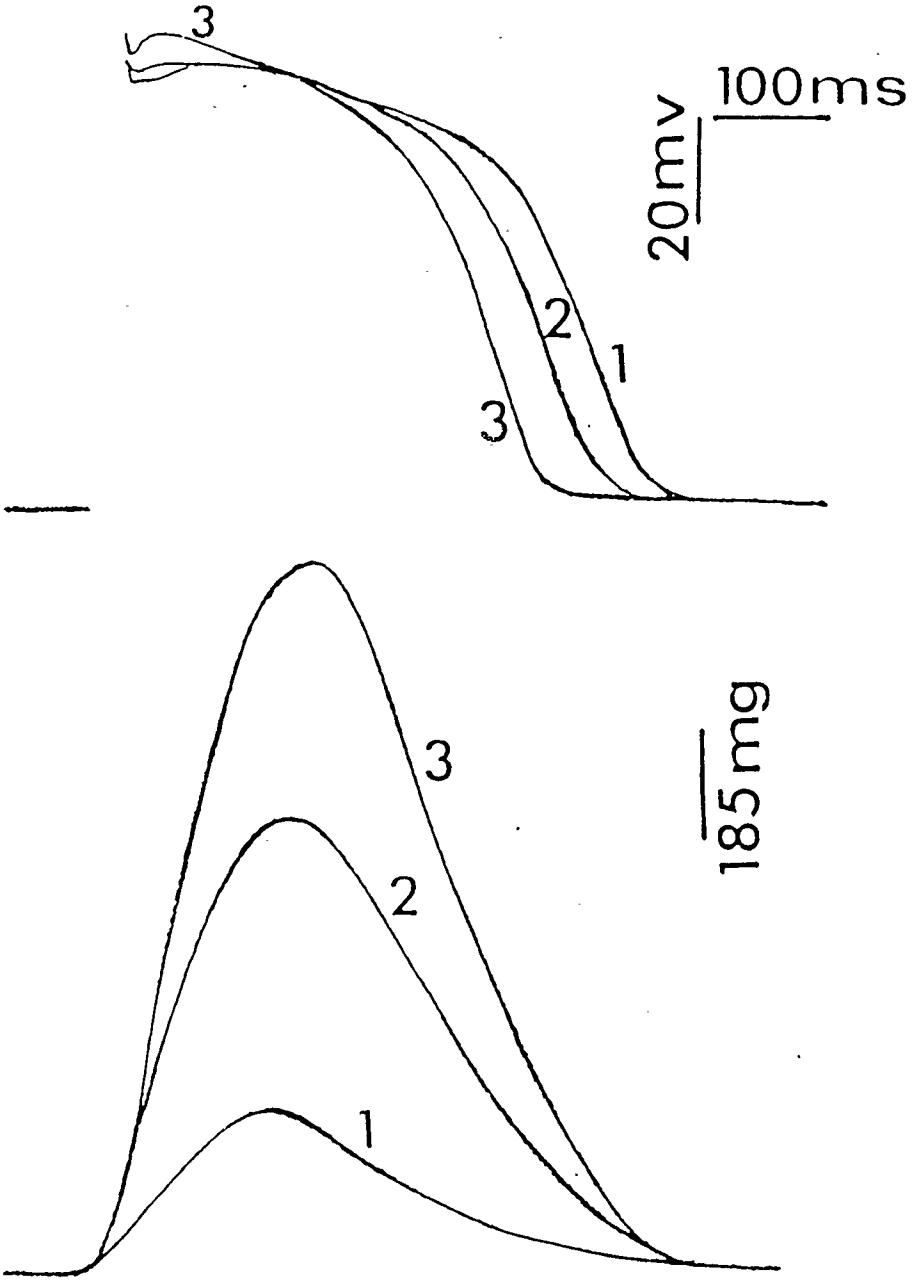



Figure 16 Effect of increasing $[Ca^{2+}]_o$ on contraction (bottom) and associated action potential (top) in intact cat papillary muscle. (1), (2), and (3) are 0.5 mM, 1.0 mM, and 2.0 mM $Ca^{2+}$ respectively. The larger forces are accompanied by shorter action potential durations and higher amplitudes. Muscle was at Lmax. (from Med. Sci. Res. 16:577-578, 1988).

twitch was accompanied by an increase in the rate of tension rise (Figure 16,bottom).

### 3.10.2 Effect of Extracellular Calcium on Isometric Contraction and Fast Action Potential in a Triton-treated Papillary Muscle

Following Triton treatment, there was a decrease in peak isometric force which initiated before a shortening in the action potential duration had begun (Figure 6). In the steady state condition after Triton treatment, raising the extracellular calcium concentration caused increase in force and further shortening in the action potential duration along with increase in amplitude of action potential (Figure 17). The sensitivity of action potential duration to $[Ca^{2+}]_o$ was diminished (Figure 22B,C) and even in some extreme cases (Figure 23, lower panel) was disappeared after Triton treatment. Furthermore, it is evident from Figures 22 and 23 that the difference in the action potential duration between treated and untreated muscle decreases with increasing $[Ca^{2+}]_o$. Although the peak isometric tension could be compensated depending on the amount of increase in extracellular $Ca^{2+}$ concentration, the time-to-peak and time-to-half relaxation remained shorter in a treated muscle (Figure 17,bottom).

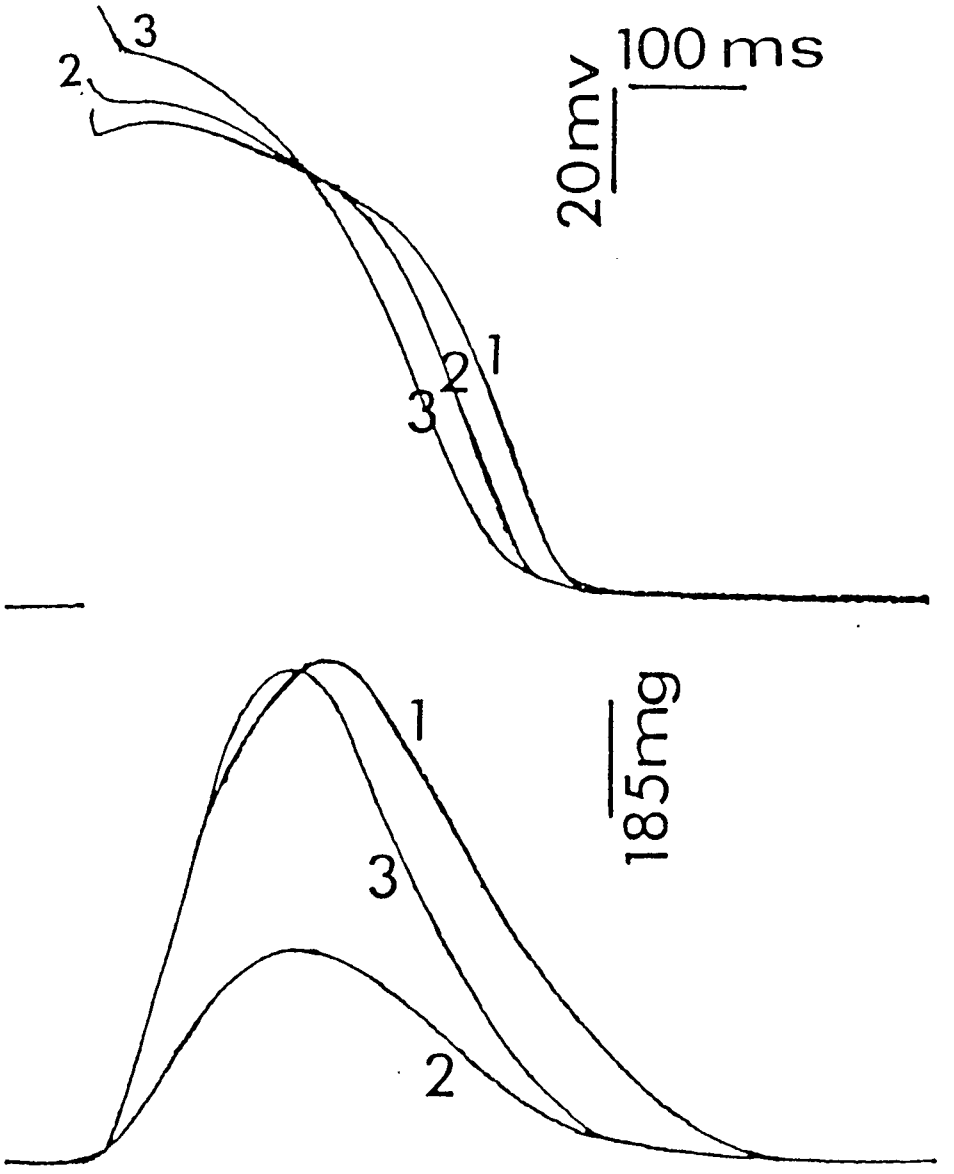



Figure 17 Effect of increasing $[Ca^{2+}]_o$ on contraction (bottom) and associated action potential (top) in a Triton-treated cat papillary muscle. Records show steady state condition. (1) Before Triton treatment, (2) After Triton treatment (1.5 mM $Ca^{2+}$), (3) After Triton (4.0 mM $Ca^{2+}$). Note that the time-to-peak and time-to-half relaxation remain short, despite the restoration of peak isometric force with increased $[Ca^{2+}]_o$ (also see figure 7). Muscle was at Lmax. Treatment done by 1-2 sec immersion in 1% Triton X100. (from Med. Sci. Res. 16:577-578, 1988).

## 3.11 Preliminary pharmacological results

### 3.11.1 Sodium nitroprusside and Methylene blue

To examine the possibility of the release of an endocardium-derived substance similar in nature to the vascular smooth muscle EDRF, which through a cGMP-dependent mechanism modulates smooth muscle tone [47,31], the effect(s) of sodium nitroprusside and methylene blue were examined. Sodium nitroprusside and methylene blue are guanylate cyclase activator and inhibitor respectively. The former induces an increase in cellular cGMP whereas the latter reduces it [12]. Sodium nitroprusside caused early relaxation and decreased peak isometric force; effects similar to those caused by endocardial damage. Meuleman et al. [81] have also observed this. On the other hand, addition of methylene blue to the intact muscle increased the force and prolonged the fast action potential duration; effects opposite to those caused by endocardial damage.

### 3.11.2 Indomethacin effects

It was suggested [73] that cardiac valvular endocardial cells could produce prostacyclin. If other endocardial cells also were to produce this substance one might suspect that

this could be a controlling factor between endocardial and myocardial cells. The use of indomethacin (a cyclo-oxygenase inhibitor) did not, however, cause significant modification of either mechanical or electrical activity of myocardium. Using this method, therefore, prostacyclin did not seem to be the controlling factor.

# Chapter 4. Discussion

The evidence presented in this thesis shows that damaging the endocardial layer is concomitant with an immediate and irreversible decrease in the isometric twitch contraction, the contraction being abbreviated.

## 4.1 Myocardial damage

Since the damage to the endocardium is caused by Triton X100, a chemical detergent usually used to skin muscle preparations, it could be contended that Triton damage is not selective only to endocardium, causing perforations of its plasmalemma and/or denudation of the overall endothelial lining, but that the underlying myocardium is damaged also. The immediate decrease in peak isometric force observed following Triton treatment could be accounted for in this way.

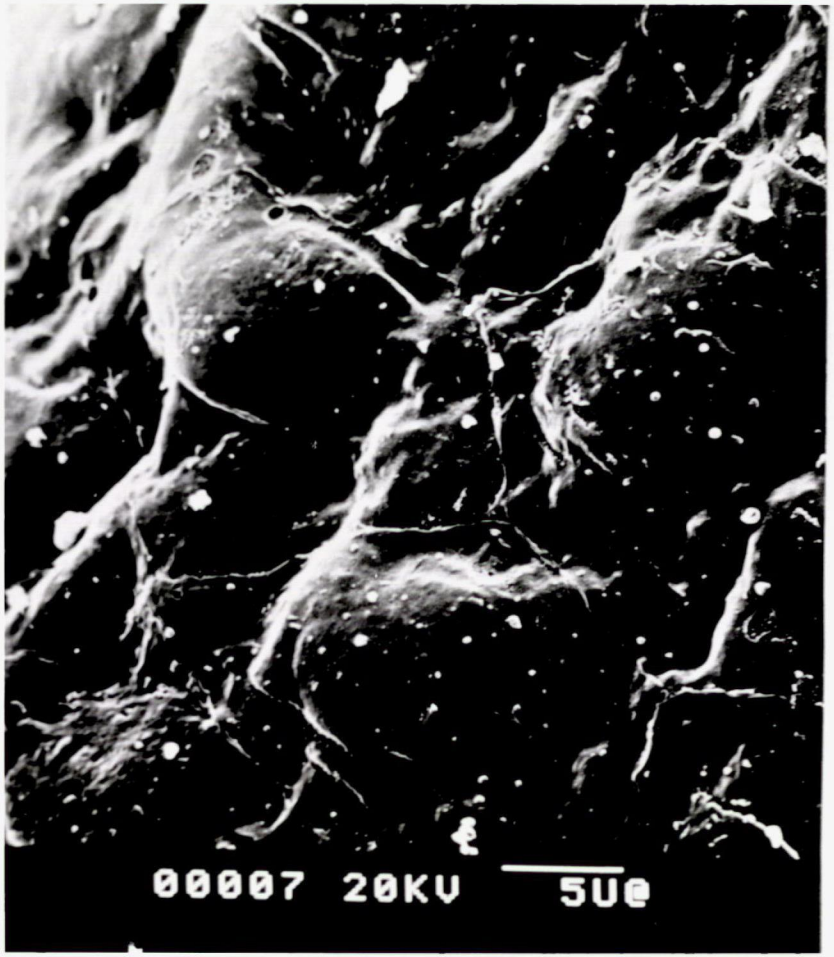
### 4.1.1 Evidence against Myocardial damage

Direct myocardial damage would be expected as a change in the structure and organization of the myocytes. Transmission electron micrographs (Figure 8D) produced following Triton treatment failed to show any damage. Moreover, there was no

evidence of mitochondrial or sarcolemmal damage (Figure 8D). Scanning electron micrographs of cat papillary muscle (Figure 18) and rabbit papillary muscle (Figure 8A, 8B) showed only perforations of the endocardial endothelial plasmalemma with disappearance of the endothelial microvilli. Furthermore, in light micrographs of Triton-treated rabbit papillary muscle (Figure 8C), the myocardium appeared normal, while in transmission electron microscopy, the endothelial lining showed numerous vacuoles which were not present in normal preparations. Microscopic observations by Brutsaert et al. [16] also demonstrated no sign of damage to the myocardium.

The results of fast action potential studies before and after Triton treatment offer support to an absence of damage to the myocardial cells. As noted previously, there was a shortening of the action potential duration and an increase in the amplitude which occurred after the changes in the contractile force had been observed and almost become stabilized (Figure 6). There was no depolarization of the resting membrane potential upon and following Triton treatment (Figure 19A). This, plus the persistence of a well-developed plateau phase (Figure 19A), (i.e. the absence of decrease in the action potential amplitude) were factors against damage. The stability of action potential characteristics after Triton was also noteworthy; a characteristic that is not necessarily true for a damaged

Figure 18 Scanning electron micrographs of the endocardial surface of cat papillary muscle. (left) Untreated muscle with layer of endothelial cells and marginal folds (arrow). (right) Following 3 seconds immersion of muscle in 1% Triton X100. There are perforations of plasmalemma of endothelial cells. (from Med. Sci. Res. 16:577-578, 1988).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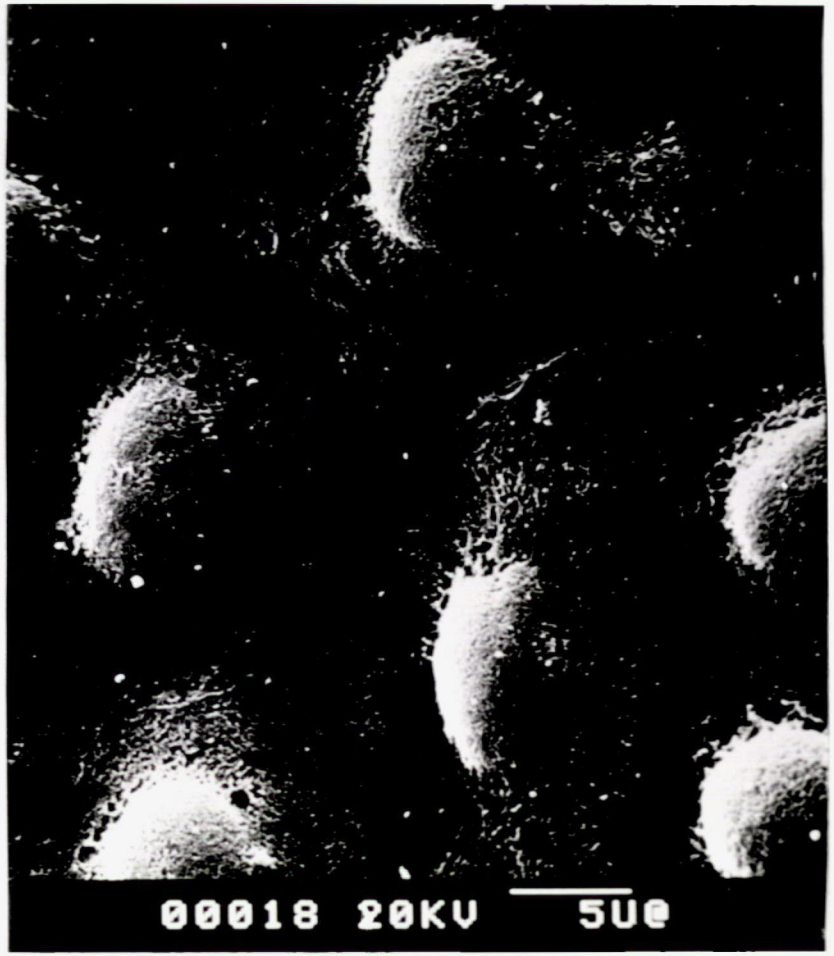
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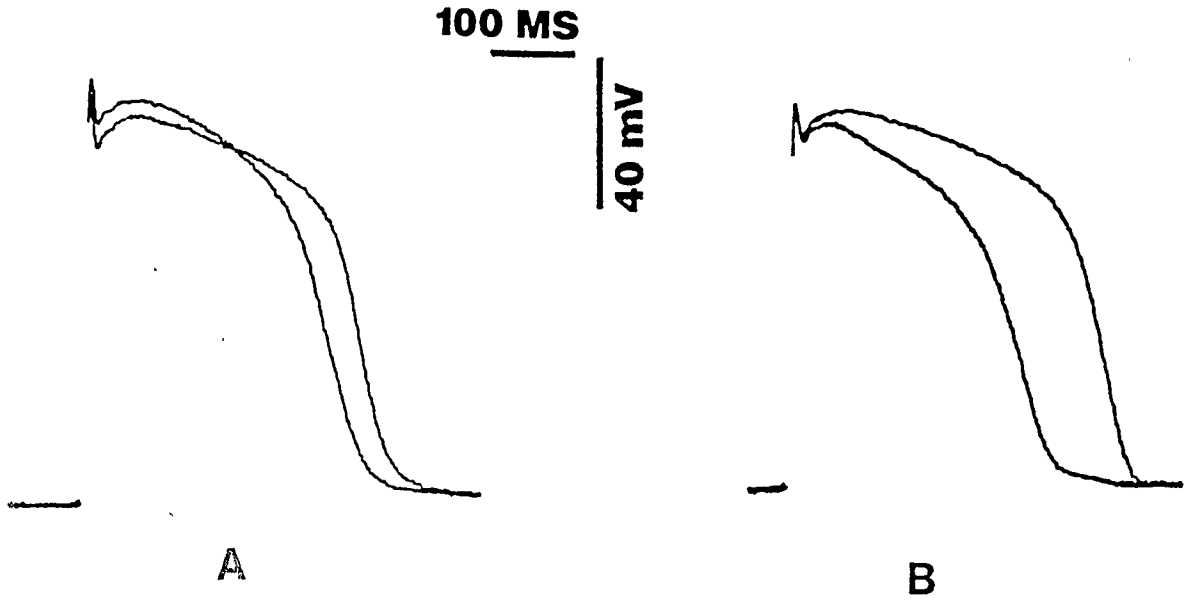


Figure 19 (A) Typical effect of Triton X100 (100 ul 1%) on the action potential of cat papillary muscle (Lmax and 1.0 mM $Ca^{2+}$). (B) A damaged cat papillary muscle. Note the lower and less developed plateau as well as the exaggerated shortened duration with damage. These effects were immediate.

muscle (Figure 20). Since there was no change in the maximum rate of rise of the action potential (Figure 4, middle trace) or the threshold value for the excitability, the fast sodium current did not seem to have been modified. Such observations on the action potential represent evidence against damage, especially since the action potentials were recorded from the outer layer cells. If the Triton treatment had not been selective to the endocardium the effect of its damage would have first been observed as rapid changes in the action potential of these cells, before changes at the contractile level were to occur. This clearly is not the case.

In addition to the absence of microscopic evidence of damage, the use of cat papillary muscles & trabeculae (Figure 4,6) and rat and rabbit papillary muscles (Figure 5) showed similar amounts of decrease in contractile force. There was, therefore, no correlation between the muscle thickness and Triton effect. Such a correlation exists when damage is present [16]. This was congruent with the observations of Brutsaert et al. [16], who also pointed out that true myocardial damage would lead to the muscle's inability to produce maximum force at the highest extracellular calcium concentration. They also suggested that the absence of a lessening in the maximum velocity of shortening at all calcium concentrations (1.25-7.5 mM) was evidence against damage. The Force-$[Ca^{2+}]_o$ relationship in this thesis showed that the maximum peak isometric force was

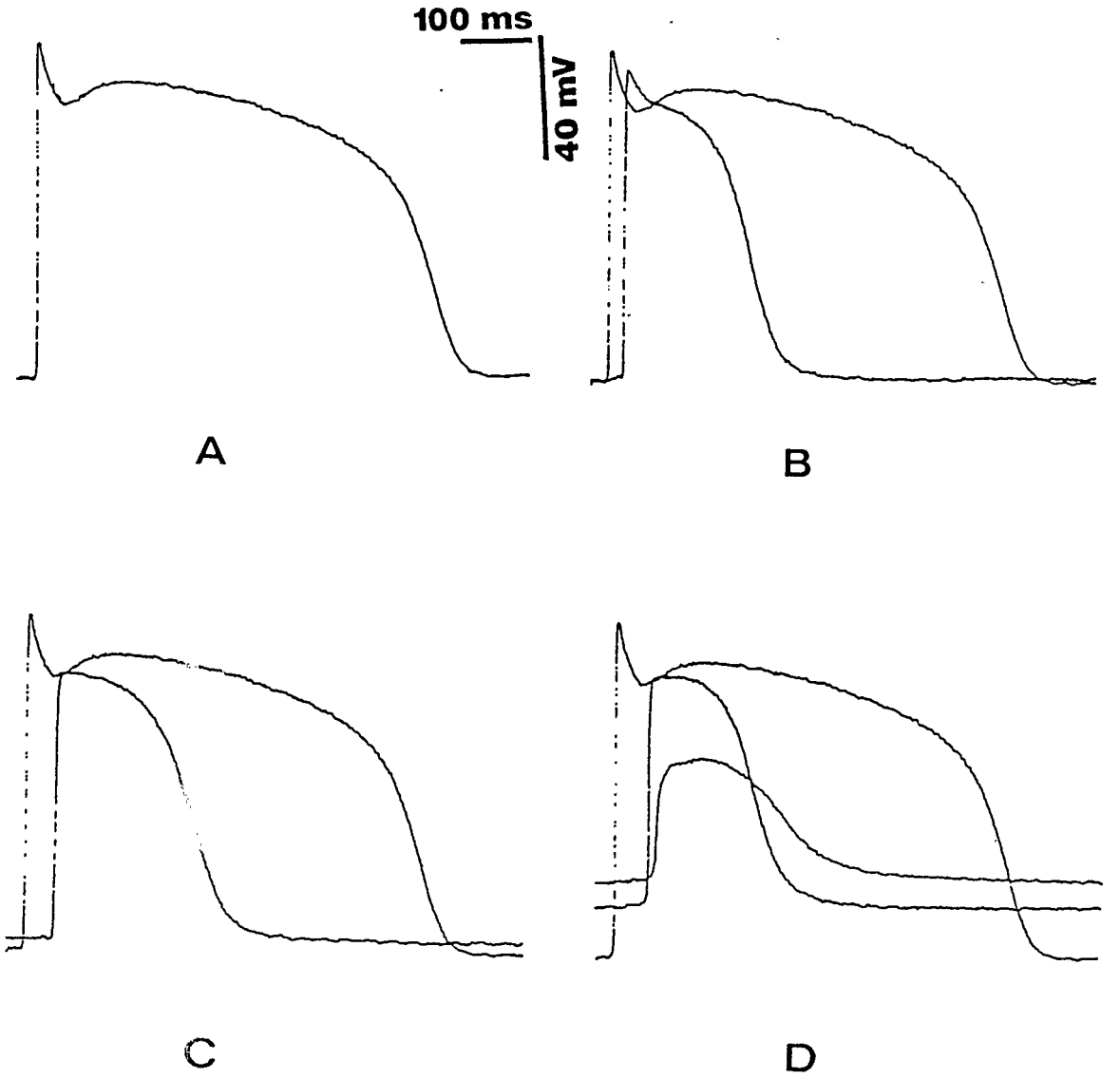


Figure 20 (A) Action potential recorded from untreated cat papillary muscle (Lmax) (0.5 mM $Ca^{2+}$). (B,C,D) Deterioration with time of the action potential characteristics after Triton treatment. The amount of shortening in duration is large and there are both a depolarization and a decrease in amplitude.

not compromised at highest $[Ca^{2+}]_o$ (Figure 7). As is shown (points 1 and 3 in Figure 7), upon appropriate increase in the extracellular calcium concentration the force increased to the levels of tension in the intact muscle. In experiments where papillary muscles were damaged due to prolonged immersion in Triton it was observed that raising extracellular $Ca^{2+}$ concentration failed to increase the force to the pre-treatment levels.

### 4.1.2 Evidence against Calcium Overload

An important factor against calcium overload is the disappearance of the after-contractions that are observed in a $K^+$-depolarized medium. When the after-contraction was regular, it was not accompanied by any after potential. This may have been because the cell from which the action potential was recorded did not experience any after potential. However, this is unlikely because with regular after-contractions no after potentials were observed in any of the experiments performed. Therefore, this observation implied that the calcium release giving rise to the after-contraction was probably not triggered by a change in membrane potential (and thus a calcium current) but rather it was due to a spontaneous release from the sarcoplasmic reticulum (SR). If Triton had caused a calcium overload by producing holes in the myocyte sarcolemma, one would expect

the after-contraction would not disappear (Figure 12B, lower trace) but rather become larger by enhanced "overload-induced" calcium release. Indeed, upon increasing extracellular $Ca^{2+}$ concentration after endocardial damage, the after-contractions returned, indicating enhancement of the SR spontaneous calcium release because the calcium current not only triggered calcium release, but also reloaded the SR. Moreover, it must be re-emphasized that the pattern of variation in the shape of fast and slow action potentials were not indicative of a cellular damage and calcium overload [52]. A relatively quick rise in the resting tension is also a clear sign of calcium overload [79]. This is in light of the fact that in cases where there is inhomogeneity of the diastolic SR calcium loading (as well as that myoplasmic free calcium) not only is the twitch force compromised, but there is usually a resting "tone" present [110]. No such observation has been made following Triton treatment (Figure 4, lower trace; Figure 5; Figure 6, lower trace). This agrees with the observation of Brutsaert et al. [16]. Our length-tension graph of cat papillary muscle also shows this (Figure 21). One case was noticed when a papillary muscle had been exposed to saponin (a chemical detergent) for a long period of time. The resting tension increased both when the muscle was and was not being stimulated. In this case, a long exposure time to saponin caused holes in the muscle and calcium ions crossed the membrane indiscriminately.

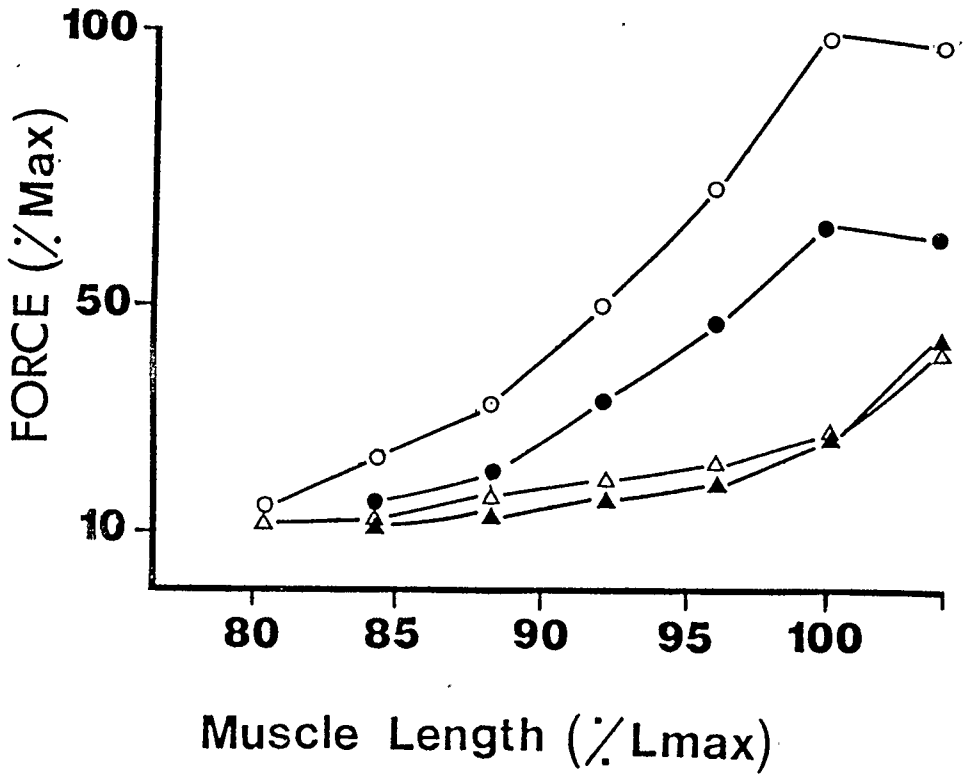


Figure 21 Effect of 1% Triton X100 (1-2 seconds immersion) on length-tension relations of cat papillary muscle. There is no increase in the resting tension of the muscle following treatment (filled triangles). There is, however, a parallel downward shift of active tension after Triton treatment (filled circles).

One more point deserves mentioning. The addition of a large bolus of Triton (in a low solution flow) to the chamber containing a muscle caused not only a decrease in the amplitude of the action potential, but also a shortening of the action potential duration that was almost immediate (Figure 19B); an effect which was not the case with smaller amounts of Triton.

It should be noted that the experiments on the effects of endocardial damage with lower concentrations of Triton (0.02%) on slow action potential and its corresponding contraction (Figure 12) demonstrated qualitatively similar results to those when a bolus of 1% Triton had been used. This similarity supported the idea that 1% Triton was not damaging the underlying myocardium.

## 4.2 Ventricular Wall experiments

Studies of the effects of endocardial damage on the fast action potential of the ventricular wall raised two significant points. First, the results provided further support against damage by Triton. This became clear when the shape of the action potential did not resemble that of a damaged preparation (depolarization and decrease in amplitude). Action potential duration shortened and amplitude increased (Figure 10A, left). Moreover, in stable recordings it was noticed that the effect of endocardial

damage was not immediate, but rather a period of the order of minutes elapsed before any variations became evident. This was in contrast with the immediate action potential duration shortening sometimes accompanied by a combination of decreases in amplitude and decreases in resting potential in a truly damaged myocardium. The stability of action potential shape for the duration of an experiment, once it had equilibrated after Triton treatment, was also a factor against damage. It was observed that in some muscles, with ventricular wall or papillary muscle, which were believed to be damaged because of the rapidity of the effect of Triton on action potential and less developed plateau (perhaps a shunted calcium current), the shape of the action potential deteriorated continuously as a function of time (Figure 20B,C,D), although sometimes it stabilized (Figure 19B). A second outcome of the results of action potential studies from the three types of myocardial samples: endocardial side, epicardial side (endocardium present), and epicardial side (endocardium absent), was support for a significant endocardial role. Statistical tests were performed on the effect of Triton on duration of action potential recorded from the endocardial side and that recorded from the epicardial side of a complete right ventricular wall. Using random action potential recording from different parts of preparations it became apparent that endocardial damage led to a significant action potential duration shortening (Figure 10B, left columns). However, with a similar

procedure no significant changes were observed when Triton was used on the epicardial side of preparations whether endocardium was present at the bottom or not (Figure 10B, middle and right columns). So only damage to the endocardium and not to the epicardium was causing modification of electrical activity, implying a role for the endocardium. Moreover, after Triton, the absence of any effect on action potential measured from the epicardial side of the complete right ventricular wall (endocardium present) perhaps suggests that the envisaged regulatory influence of the endocardium does not reach the epicardial side of the ventricular wall. It should be noted that the action potential durations of the control epicardial side of the left and right ventricular wall were similar. Similar results were obtained when action potentials were recorded with stable impalements, i.e. only when the endocardial side was damaged by Triton was there an effect on action potential characteristics (Figure 10A, left).

Recently, it has been shown [53] that the action potential duration of the endocardial side was longer than that of epicardial side. The authors have proposed that the difference was at least partially due to a higher number of the Na/K-ATPase pumps in the muscle on the epicardial side and thus a larger contribution of this pump to outward current in this region of the muscle. As will be discussed, if Triton treatment were causing a decrease in the activity

of the Na/K-ATPase pump by endothelial cells losing their ability to accumulate $K^+$, then a change in the action potential configuration should be observed which would actually be simultaneous with alteration of the contractile force. This has repeatedly been seen not to be the case. This observation implies that the decrease in duration following Triton cannot be attributed to variation in the activity of the Na/K-ATPase pump. Moreover, the persistence of a statistically significant difference between the action potential duration in a Triton-treated endocardium and untreated epicardium (Figure 10B, left, middle and right columns) may be due to a difference in the Na/K-ATPase pump activity (and even partly due to some residual endocardial influence). Nonetheless, at least part of the difference in the action potential duration between endocardial and epicardial side seems to be a consequence of an endocardial influence.

## 4.3 An Endocardial Role

Having established (using a combination of observations) that myocardial damage cannot (on average) explain the immediate decrease in force and the shortening in its duration, and that the difference in the action potential duration of muscle as measured from the epicardial side and endocardial side may be at least partially due to an

endocardial role, the prospect of such a role becomes more exciting in that one then proceeds to envisage how the absence of an intact endocardium could lead to a modulation of myocardial performance, because evidently normal functioning depends on the presence of an intact endocardium.

In its simplest definition the endocardium may be considered as a protein barrier keeping the microenvironment surrounding the myocardial cells in a certain order. Clearly, once these endothelial cells of cardiac muscle have been damaged, there would be a change in the interstitial organization which could somehow influence the myocardial cells. The proper functioning of cells depends on the homeostasis that exists between the intracellular and extracellular (particularly interstitial) partitions. Any disorder in this microenvironment acts on the myocardial sarcolemma first, which could lead to a chain of events leading to the modification of contraction. Of course, such an hypothesis is based on the assumption that the endocardial role is a passive one but nonetheless an important one. On the other hand, in addition to, or instead of, the aforementioned possibility, the endocardium could be an active component. This may be a secretory role by means of which a "receptor" at the level of sarcolemma would be activated which in turn could initiate a chain of events. The absence of an active endocardium would lead to the lack of such a substance and consequently lead to cessation of

the normally occurring sequence of events, and thus to the observed effect on contractile force.

The significant aspect with regard to all of this is that as far as the envisaged endocardial role is concerned, the primary event in what perhaps leads to a series of events is some type of alteration at the sarcolemmal level of myocardial cells. Two potential variations at the sarcolemmal level deserve consideration. One is a change in the ionic availability to the sarcolemma, which may cause a difference in ionic gradient across the membrane, which would then show itself as a modification of electrical activity of the cell. The implications of such a possibility would be an immediate change in the action potential shape following endocardial damage. Another possible alteration at the level of the sarcolemma is a chemical change which could initiate a sequence of reactions resulting in a decrease in contractile force. The observations made by examining the fast and slow action potentials and their corresponding contractile forces have consistently pointed out that upon endocardial damage the first parameter that immediately undergoes a change is the contractile force, and this is subsequently followed (in a period of the order of minutes) by a shortening in the action potential duration. Given these observations, the idea of changes at the sarcolemmal level following endocardial damage becomes more specific in that it does not appear to involve initial changes in the

ionic arrangements across the membrane. An immediate change in the action potential would be expected otherwise.

## 4.4 Chemical integrity

To consider in greater detail why a modification of availability (chemical integrity) of ions such as $K^+$, $Na^+$, and $Ca^{2+}$ would result in an initial change in action potential, each will be analyzed separately.

### 4.4.1 Potassium ions

The possibility of a sudden increase in extracellular potassium concentration upon endothelial damage should be considered. Such an increase would primarily be transient since the accumulation of $K^+$ is actually hindered by endothelial barrier being less effective in maintaining homeostasis. Nonetheless, such a transient increase in $[K^+]_o$ would lead to an increased Na/K-ATPase pump [74] (Figure 1) activity and thus transiently enhance the Na/Ca exchanger (a calcium extrusion mechanism [5,87]) activity, which would lead to an increased relaxation rate and a decreased peak isometric tension. In this case, however, since there is a new $E_K$ one would expect to see a transient decrease in the resting potential as well as a transient shortening of the

action potential duration. Furthermore, these changes in action potential should occur simultaneously with the changes in mechanical activity. Later, due to subsequent less pronounced $K^+$ accumulation, the effects on action potential would have to reverse and change in the opposite direction. The results of the action potential studies did not demonstrate a transient membrane depolarization, nor did they show a transient action potential shortening occurring with decrease in isometric force. The significant point, therefore, is that a change in $K^+$ availability cannot explain the sustained effects that are consistently observed at the level of both contraction and action potential, with the former preceding the latter.

### 4.4.2 Sodium ions

The contribution of the Na/Ca exchanger to relaxation (Figure 1) which can be altered by a direct change in sodium concentration gradient [5], also deserves attention. In this regard the following scheme could exist: damage to the endocardium acting as a diffusion barrier would allow a more rapid sodium entry into the interstitial space and thus accelerate the relaxation phase by enhancement of Na/Ca exchange activity. Since this process would involve changes of ions across the membrane, an alteration of action potential would have to precede the force diminution. The

expected action potential alterations would be a prolongation of duration owing to the fact that the Na/Ca exchanger constitutes an electrogenic mechanism [5,87] and an enhanced sodium ion entry would favour a net inward current. Moreover, an increased Ca efflux/Na influx having a higher reversal potential value than the resting potential [87] would tend to make the membrane potential more positive. Such a phenomenon has been observed in cat papillary muscle upon muscle shortening [64]. The results on papillary muscle and ventricular wall did not show any of these modifications. Thus a modification of Na/Ca exchange function, at least by these means, does not appear to be occurring.

The argument against enhanced Na/Ca exchange activity being responsible for the early relaxation following endocardial damage can also be supported by the consistent observation that increasing the extracellular $Ca^{2+}$ concentration only partially reverses the effects of endocardial damage (Figure 17). Furthermore, if the Na/Ca exchanger is involved, one would expect to observe a Triton effect on action potential when recorded from the epicardial side also which was not found to be the case (Figure 10A, right).

### 4.4.3 Calcium ions

Along a similar line of reasoning, from our observations the fact that the time-to-peak and duration of force still remained shorter even upon raising the extracellular $Ca^{2+}$ concentration (Figure 17) is an argument emphasizing that a decrease in the interstitial calcium activity did not lead to the obtained results. This was found to be the case by Brutsaert et al. also [16]. Such a decrease would lead to a decreased calcium influx (seen first as a change in the action potential shape), and (perhaps through a diminished level of the SR calcium release and a subsequent decreased SR calcium reload) it would cause a decrease in peak contractile force. Furthermore, if a decreased calcium current was the cause of decreased force, then one would expect to see similar effects when action potentials were recorded from the epicardial side of the ventricular wall. Moreover, the observation that the after-contractions were unaccompanied by any after potentials and yet they were influenced before the slow action potentials were changed offers further evidence suggesting that the decrease in the force of contraction after Triton treatment is not due to a decreased inward calcium current.

### 4.4.4 Other potential alterations

One other mechanism that could be initiated by changes in the microenvironment of the interstitial medium (or simply by Triton itself, assuming it does reach the sarcolemmal level) is an alteration of the activity of transmembrane ionic channels (such as the calcium channel) leading to changes in this current. Change in the action potential duration (shortening) would, via activity of the Na/Ca exchanger, also reduce the amount of calcium for internal storage by the SR, and therefore cause a diminution of the subsequent peak isometric force [14]. In any of these cases a change in action potential characteristics would precede alteration of the contractile force.

Obviously, therefore, such aforementioned models are not justified by our observations due to the fact that changes in the action potential seem to be subsequent to changes at the level of contraction. A criticism that may be made with regard to this is that because the action potentials of the working muscles were recorded from the cells located at the end of the preparation where motion was not significant, these electrical recordings may not have correlated with the mechanical recordings made from the cells that are more involved in the production of force. The following observations are believed to provide evidence against such criticism. First, it is important to note that in

trabeculae, as well as in papillary muscles, the change at the level of action potential followed that at the level of contraction. This should be noted in light of the fact that trabeculae are thin preparations and the recording of action potential was done from parts of the muscle that were very close to the cells producing most of the force. Furthermore, when papillary muscles were being used, it was attempted to record the action potentials from parts that are believed to be part of the papillary muscle. Even though these cells may not have made a major contribution to the production of force, there does not seem to be any reason to suspect that their electrical responses were significantly different from those producing most of the contractile force. Secondly, when recording from the ventricular wall, here also there was always a delay before any changes in the action potential characteristics were observed.

### 4.4.5 Alterations of Membrane Phospholipids

The loss of chemical integrity that has been considered so far can cause another modification. It is known that phospholipids provide most of the sarcolemmal calcium binding sites [21,66,93]. An alteration of availability of these could diminish calcium binding and cause a subsequent decrease in force. This would be similar to the effects of cationic amphiphiles. The results of recording action

potential from the epicardial side of the ventricular wall (Figure 10B) does not support such a possibility, since statistically no significant action potential duration shortening occurred when compared with action potential recordings from the endocardial side before and after Triton treatment, where there was significant shortening (Figure 10B).

## 4.5 Physical integrity

The loss of endothelial barrier cannot only lead to the loss of chemical integrity (as discussed above), but also the loss of physical integrity. In the introductory section we considered the example of muscles that are skinned and thus experience less external compressive pressure. These preparations have large interfilament spacings that have been attributed primarily to a diffusive loss of high molecular weight substances that usually exert a compressive force on the myofibrils (see [130]). If, in fact, the loss of large molecules following endocardial endothelial damage is causing the observed changes, then increasing the osmotic pressure by introduction of albumin should at least partially have reversed the effects. The experiments on the contractile force of papillary muscle exposed to 4g/100ml albumin (normally found in blood plasma and constituting about 68% of total osmotic pressure) [70] after Triton

treatment showed that the effects of endocardial damage persisted and the albumin in fact caused a further minor decrease in peak force after treatment (Figure 9C). Exposure of an untreated muscle to albumin also caused a small decrease in peak isometric force (Figure 9A). This negative inotropic effect of a hyperosmotic medium on the muscle has been previously observed [128]. Thus experimental evidence suggests that if there is loss of physical integrity after Triton treatment of the preparation, the modification of the force after endocardial damage is not necessarily due to this. Moreover, the lack of effects of Triton on the shape of the action potential recorded from the epicardial side of a ventricular wall (Figure 10) is also against the hypothesis that the loss of physical integrity is the reason for the effects observed following endocardial damage.

## 4.6 An active Endocardium

Up to this point the consequences of loss of chemical and physical integrity have been considered. What this has led to is the conclusion that the endocardium does not appear to act as an electrochemical barrier, because in such a case the onset of change in contraction would not precede the onset of change in action potential characteristics. The changes would be either simultaneous or the changes at the contractile level would lag behind those at the action

potential level. There was another observation that was also against the possibility of an electrochemical barrier. As noted in the experiments on ventricular walls, the walls were cut on the sides during dissection. This should have caused a disturbance of the electrochemical barrier and have given rise to the effects, since the endocardium is damaged at the incision sites. Yet it was only upon Triton addition, and thus damage to the intact endocardium, that the aforementioned results were obtained.

Let us now consider the possibility of a chemical change at the sarcolemmal level being caused by absence of a putative substance usually secreted by the endothelial lining. Such a process can have two main implications. Firstly, as is the case with the well-known beta-adrenoceptor inactivation [91,106], there may be a decrease in the calcium current and this would quickly affect the slow action potential shape; an effect that would occur just before the decrease in force of contraction. Secondly there may be an internal modification of organelles involved in regulation of contraction on a beat-to-beat basis. Obviously a combination of these two processes does not seem to be the case considering that under such circumstances also, a change at the level of action potential would occur before the decrease in the force of contraction.

Clearly, therefore, the fact that the modification of contractile force after endocardial damage occurs before changes in the action potential offers strong support for a mechanism (under the control of the endocardium) that influences the contractility of the myocardium by influencing (directly or indirectly) some cellular organelle whose function is contributing to the regulation of contraction. The modification at the contractile level would then modify the electrical activity, perhaps by some feedback loop.

### 4.6.1 A model for the modulation of contractile force by endocardium -Importance of the Sarcoplasmic Reticulum

In the introductory chapter the widely accepted calcium-induced calcium release model for mammalian cardiac excitation-contraction coupling was outlined briefly, and in the context of this model the mechanisms that control the calcium transient and thus contractile force on a beat-to-beat basis were described. These included the inward calcium current, the outward $K^+$ current (rapid activation of this allows less time for calcium entry), the SR, myofilament calcium sensitivity, the Na/Ca exchanger activity, and membrane phospholipids. In their recent publication [16] Brutsaert et al. have stated that increased peak tension with increased length (at any $[Ca^{2+}]_o$), resulted from both

an increase in the velocity of tension development and a delay in the onset of early tension decline. From an analytical viewpoint this will lead to an increase in time-to-peak tension and time-to-half relaxation and thus an increase in duration. In other words, the time course of twitches varies with muscle length [2,3]. This can be explained by an increase in myofilament calcium sensitivity [11,59]. Because of similarity of endocardial damage effect and effect of decreasing length on contraction, Brutsaert et al. [16] have proposed a decrease in the myofilament calcium sensitivity as the cause for force diminution following endocardial damage. This implies that the endocardium exerts its influence by modulating the myofilament calcium sensitivity. The experimental evidence described in this thesis does not eliminate this as a possibility. However, it introduces a different potential mechanism, one involving the SR, as the primary and more significant factor for the modulation of myocardial performance by endocardium.

One of the functions of the sarcoplasmic reticulum (complementary to the Na/Ca exchanger mechanism [5,14,60,87] and the Ca-ATPase pump [24,119]), is to remove calcium from the cytoplasm, thus initiating relaxation (Figure 1). Indeed, since calcium sensitivity may be considered as a balance between the rates of binding and unbinding of calcium and the troponin molecule, if the SR calcium uptake (which can be a determinant of the time course of

contraction and relaxation) [33,78,89] is increased in the absence of an intact endocardium, the unbinding rate can actually be enhanced and thus lead to an early relaxation. In such a case a decrease in myofilament calcium sensitivity would in fact be unnecessary. Changes in the SR calcium uptake have also been suggested as altering tension duration in senescence [39].

### 4.6.2 Proposed sequence of events following endocardial damage

The experimental evidence described in this thesis has shown that the proportion of calcium recirculated back into the SR was larger in a muscle with damaged endocardium. Following endocardial damage the decrement of force with each beat became less until a steady level was attained (Figure 6, bottom). Such a pattern of change in force suggests a gradual increase in calcium uptake (Figure 14D) (accelerated relaxation) to a new level. The pattern of decrease in force also suggests that the SR calcium release has been reduced. This is also evident from the disappearance of after-contraction in a depolarized medium within one beat following endocardial damage (Figure 12, filled circles). This combination of increased SR calcium uptake and decreased calcium release would lead to an early relaxation and a gradual decrease in time-to-peak and duration of

force as well as a gradual decrease in peak contractile force until a new steady state has been reached (Figure 4, lower trace).

The absence of an intact endocardium would set the function of the SR to a new level, i.e. on a beat-to-beat basis the SR would sequester more calcium and release less. It is the transition of SR function to this new level of activity that takes time, and this is observed as a gradual change in force characteristics until equilibrium is reached. Accordingly, such a new level of SR activity would dictate a change in the SR passive calcium leak. That is, the passive leak would increase to a new background level since the enhanced sequestration and decreased release of calcium would otherwise cause SR calcium overload. Our observations, as mentioned previously, indicate that no calcium overload of the SR and thus no overload-induced calcium release leading to spontaneous contractions occurs following endocardial damage.

In a well balanced system such as the mammalian cardiac myocytes, other calcium controlling mechanisms will, under the circumstances of increased the SR passive calcium leak, attempt to compensate and thus prevent the diastolic free calcium concentration from rising more than perhaps transiently. Because of the enhanced activity of these other calcium buffering mechanisms a new equilibrium would be reached to control the diastolic free calcium levels. Among these buffers are Na/Ca exchange, Ca-ATPase pump, membrane

phospholipids, and the myofilaments themselves. For myofilament activity to become amplified with this enhanced leak, the short transient rise in calcium would have to last long enough (as well as be large enough) to reach a threshold for such myofilament activity enhancement, and indeed if other buffering mechanisms act quickly they can prevent such conditions from developing. Of these, membrane phospholipids seem to be important as their uptake of calcium would lead to a decreased calcium gradient and enhanced Isi inactivation and thus a shortening in the action potential duration, and also a rise in amplitude similar to the effects on action potential induced following increase in extracellular calcium concentration. The involvement of these phospholipids has been observed to influence the action potential duration in cat papillary muscles by others [7].

This model would thus explain why the change at the level of action potential happens following that at the contractile level.

The Na/Ca exchanger activity in this process does not appear to have been modified significantly, since one would expect this to cause a shift in resting membrane potential towards more positive values, as well as prolongation of action potential duration [5,64,87]. The Na/Ca exchanger has a low calcium affinity and thus presumably exports calcium efficiently only when myoplasmic calcium concentrations have increased substantially. This has been shown in isotonic

contractions also, where muscle shortening led to a significant increase in intracellular calcium concentration (due to decreased myofilament calcium sensitivity) and an enhanced Na/Ca exchange activity [64]. Therefore, we can envisage that the influence of increased passive calcium leak from the SR may not be large enough to modify Na/Ca exchange activity sufficiently to be detected. Moreover, the activity of the high affinity Ca-ATPase pump may also have increased accordingly, as this pump would respond to smaller changes in calcium concentration.

One can, therefore, envisage that once the SR calcium leak has become large relative to that before endocardial damage, the activity of other calcium buffers change accordingly, thus reaching a new equilibrium point. But the change in one of the calcium buffering systems (i.e. increase in calcium content of membrane phospholipids) in the cell causes action potential duration shortening in the process. If this is the case, then one would predict that the action potential duration should become less sensitive to a rise in $[Ca^{2+}]_o$ as the membrane calcium content is now at a higher level. This is addressed below by a comparison of the results of a series of experiments on the effect of $[Ca^{2+}]_o$ on action potential duration before and after Triton treatment. At this stage it should be noted that in this context, a decrease in myofilament calcium sensitivity (as suggested by Brutsaert et al. [16]) may not be justified because this

would cause a rapid increase in free intracellular calcium concentration which would immediately be compensated for by, among other buffers, the Na/Ca exchanger in which case we should see rapid changes at the level of the action potential [64].

### 4.6.3 Calcium ions and Action Potential Characteristics

Evidence has been presented [1,7] that demonstrates the dependance of the action potential shape on the extracellular $Ca^{2+}$ concentration. Our results also showed that in a control muscle the action potential duration decreased and the action potential amplitude increased upon increase in extracellular calcium concentration (Figure 22). Different mechanisms have been presented for this. Among these is one involving a calcium-dependent $K^+$ outward current which is activated when the intracellular calcium concentration reaches a certain level [8], although this has not yet been reported in isolated mammalian ventricular myocytes in patch clamp experiments. Another suggestion is one that proposes that an increase in the intracellular $Ca^{2+}$ concentration (following an increase in $[Ca^{2+}]_o$) would reduce the driving force for calcium current and speed up the inactivation of Isi [15,57,58,69,75], and this would cause not only an increase in the contractile force but also a shortening of the action potential duration [1]. It has

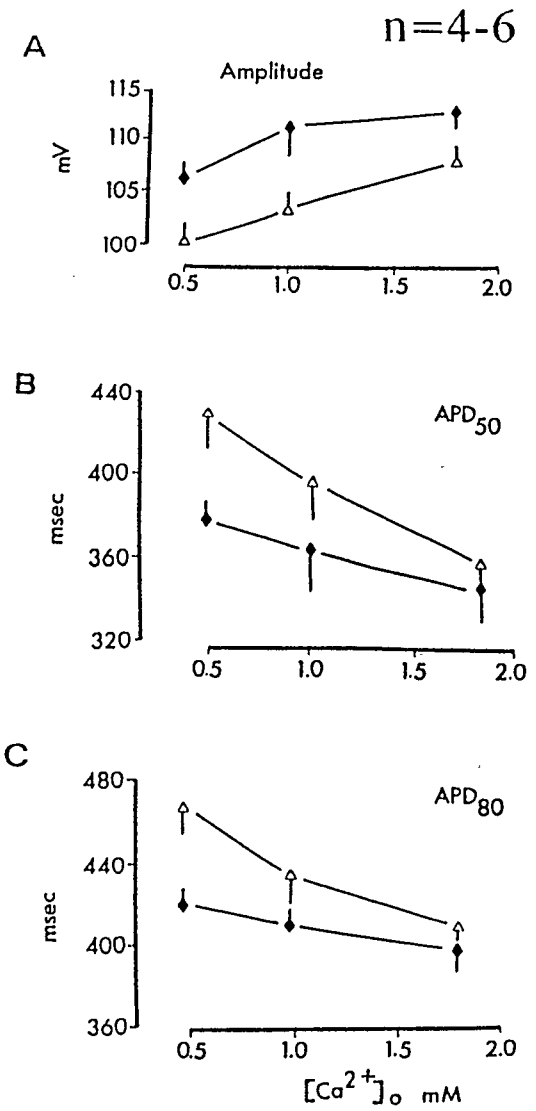


Figure 22 A: Variation of the action potential amplitude with $[Ca^{2+}]_o$ in control (open triangles) and treated (closed triangles) papillary muscles. The amplitudes of action potential in treated muscles are higher than in control muscles. B and C: Differential $[Ca^{2+}]_o$-dependance of control (open triangles) and treated (filled triangles) muscle action potential duration at 50% and 80% repolarization. Note the diminished responsiveness of treated muscles to $[Ca^{2+}]_o$ as compared with high sensitivity of control muscles action potential to $[Ca^{2+}]_o$. Also, note that the difference in APD between treated and untreated muscle diminishes with increasing $[Ca^{2+}]_o$. Muscles were at Lmax. Muscles were treated by 1-2 seconds immersion in 1% Triton X100. Effect of raising $[Ca^{2+}]_o$ on a control muscle resembles Triton effect. APD is mean +/- SEM. "n" is number of preparations.

been proposed that membrane calcium content is elevated in such a case and that this may be the cause of these effects [7].

The extent of change in action potential shape becomes smaller with increasing $[Ca^{2+}]_o$ (Figure 22B,C, open triangles; Figure 23, lower panel), thus suggesting the presence of a saturation point for this feedback mechanism. The observations reported in this thesis suggest that the effect of endocardial damage on the action potential resembles the effects of raising the extracellular $Ca^{2+}$ concentration on the action potential of a muscle with intact endocardium (Figure 24). Furthermore, there is a reduced sensitivity (Figure 22B, C) and in some cases almost no sensitivity (Figure 23, lower panel) of action potential shape to $[Ca^{2+}]_o$, in a Triton-treated muscle. Moreover, at higher $[Ca^{2+}]_o$ the extent of action potential duration shortening following endocardial damage is smaller (Figure 23, lower panel; Figure 22B,C). It would thus appear that the membrane does indeed undergo an increase in its calcium content following endocardial damage, and this feedback mechanism (membrane phospholipids) becomes enhanced so that it can no longer contribute to action potential duration shortening efficiently with increase in $[Ca^{2+}]_o$; an effect similar to that observed in a control muscle when $[Ca^{2+}]_o$ is raised beyond a certain level.

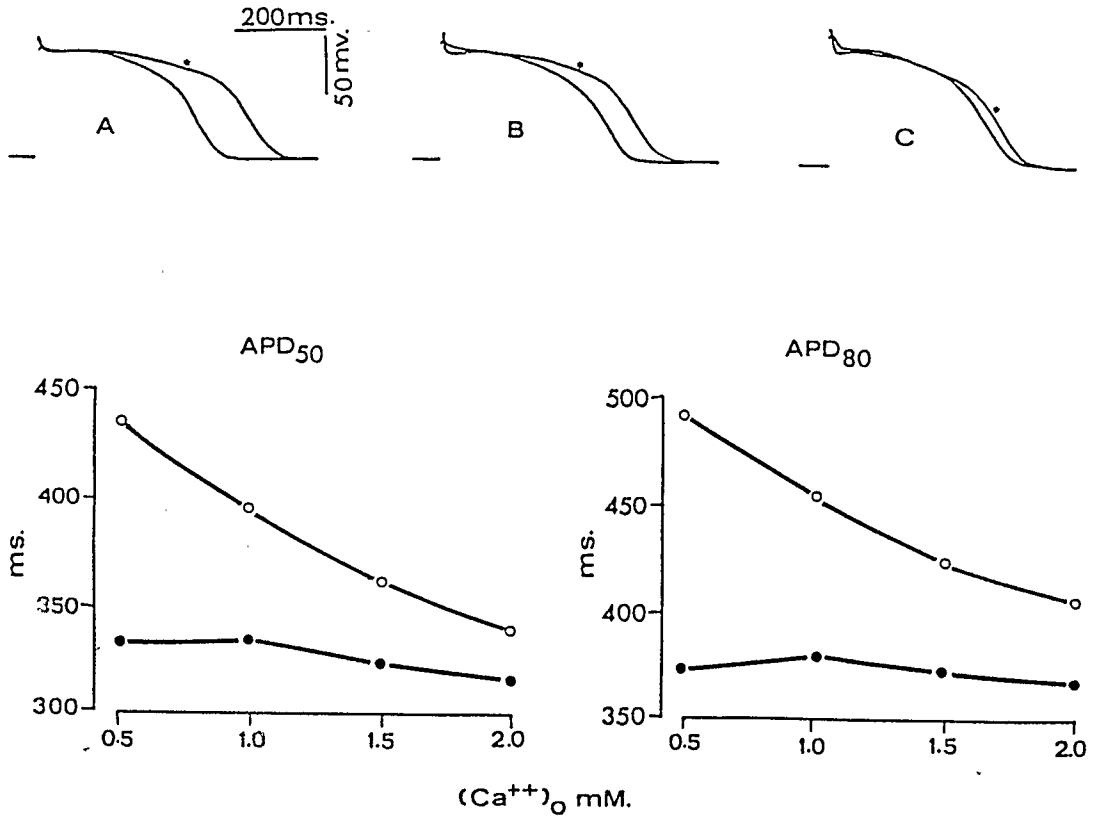


Figure 23 Effect of $[Ca^{2+}]_o$ on the shape of action potential . Cat papillary muscle treated by 1-2 seconds immersion in 1% Triton X100. Upper panel: (A) 0.5 mM $Ca^{2+}$, (B) 1.0 mM $Ca^{2+}$, (C) 2.0 mM $Ca^{2+}$, (*) before treatment. Note smaller difference in the action potential duration (APD) between untreated and treated muscle at high $[Ca^{2+}]_o$ (C). Lower panel: Action potential duration at 50% and 80% repolarization ($APD_{50}$ and $APD_{80}$) as a function of $[Ca^{2+}]_o$. Untreated (open circles) treated (filled circles). Both $APD_{50}$ and $APD_{80}$ decrease with increasing $[Ca^{2+}]_o$ in the untreated muscle but not in the treated one. Note that in the control muscle the extent of decrease in APD with increasing calcium concentration diminishes. Also, the difference in APD between treated and untreated muscle diminishes with increasing $[Ca^{2+}]_o$. Muscle was at Lmax. (from Med. Sci. Res. 16:577-578, 1988).

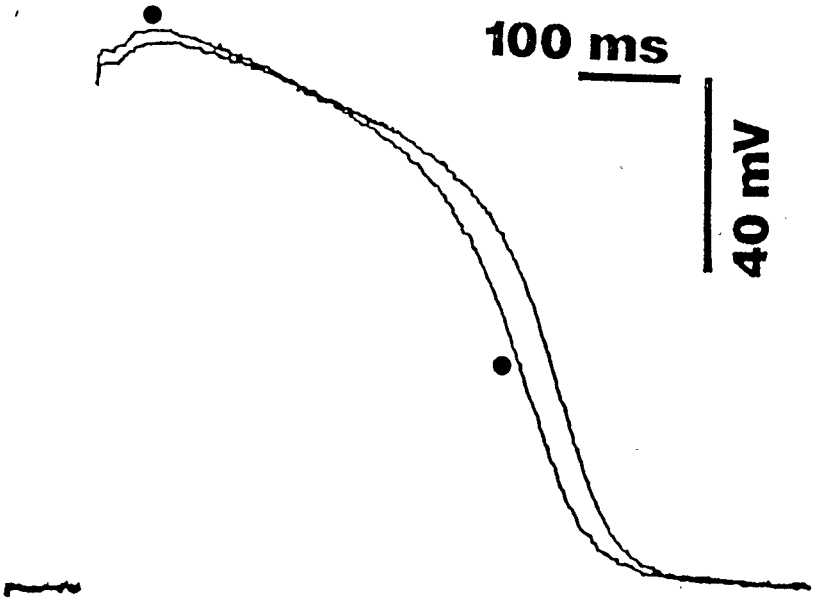


Figure 24 Effect of raising $[Ca^{2+}]_o$ (filled circles) from 1.0 mM to 1.8 mM on action potential characteristics of an intact cat papillary muscle. Note similarity to the effect of endocardial damage (top trace of figure 4).

It should be emphasized that this is not a case of calcium overload because of the reasons mentioned previously in this discussion.

## 4.7 Endocardial damage effect on SR function

The evidence gathered in this thesis suggests a fundamental change in SR function which leads to diminution of force and decrease in time-to-peak and duration. Subsequently the enhanced calcium leak from the SR would, via a negative feedback, modify action potential characteristics in an attempt to equilibrate diastolic calcium. The validity of a model of the SR involvement in the mechanism of myocardial performance modulation by the endocardium was examined using ryanodine in the perfusion medium.

### 4.7.1 Ryanodine experiments

The proposal was that if in fact SR function is modulated following endocardial damage, then removing or minimizing its contribution to ECC (excitation-contraction coupling) using ryanodine should minimize or eliminate the effects of endocardial damage, both at the level of contraction and action potential. Evidence has been presented that ryanodine ($C_{25}H_{35}NO_9$ - isolated from Ryania speciosa), when used at

high concentration, will diminish and even block the SR calcium release and also diminish SR calcium uptake after binding to low affinity sites on the SR [9,35,112,113]. Indeed, it has been shown that in cat papillary muscle ryanodine decreases contractile force greatly (the amount of this decrease depends on the species) and eliminates after-contractions [113]. The observations in this thesis indicated that in cat papillary muscle and ventricular wall, ryanodine prolonged the action potential duration and increased the amplitude of the action potential (Figure 13A, upper trace; Figure 15A, right). At the contractile level it decreased force and increased time-to-peak force (Figure 13A, lower trace). In a medium containing ryanodine the effects of endocardial damage were abolished. There was no action potential shortening or reduction in contractile force (Figure 13B). In few preparations a slight decrease in force and a shortening in action potential duration occurred, but this may have been due to residual functional SR present in these preparations, or perhaps due to some degree of damage. In the case of randomly recorded action potentials from the ventricular wall, no statistically significant difference was observed in duration following endocardial damage in the presence of ryanodine (Figure 15B). These results lead to another implication. If endocardial damage were to cause a decrease in calcium sensitivity of myofilaments, a persistent Triton effect should have been observed even in the presence of ryanodine.

Or, if the endocardial damage results were caused by both modifications of myofilament calcium sensitivity and SR function, the effect of Triton should have become less pronounced on average in the presence of ryanodine. Furthermore, if the decrease in the contractile force was caused by a decrease in calcium current, then one would expect such a change of force even in the presence of ryanodine. Therefore, it would seem reasonable to suggest that the typical modifications of contractile force following endocardial damage are primarily caused by modification of SR function. At this point, however, one can only speculate on how this modification may occur, i.e. what the actual link between endothelial cells and the SR might be. A possibility is that damage to the endocardial endothelial lining may cause a decrease in, or absence of, a putative chemical factor, which would lead to modification of SR function.

## 4.8 Physiological Significance of the Endocardium

Clearly as a passive barrier endocardial endothelial lining maintains the homeostasis of the microenvironment surrounding the myocardial cells. This by itself is very important. However, an active endocardium (as well as passive), as Brutsaert et al. and we have proposed (although through different mechanism) could via secretion of a

chemical factor help regulate myocardial performance, in addition to any extrinsic regulatory factors such as the nervous system and the coronary circulation. For example, an enhanced endocardial secretion of an as yet unknown substance would obviously have a positive inotropic effect on the cardiac muscle, and if a stimulator for the secretion of such a chemical is discovered it could be of considerable significance in a clinical setting. For a complete dynamic regulatory mechanism the secretion of such a substance would have to be under the influence of other factors such as stretch and/or hormones in the blood.

### 4.8.1 Putative factor

One of the possibilities that was mentioned above was the release of a chemical factor that would affect the SR. Damage to the endocardium would diminish this substance and thus immediately modulate SR function without directly affecting the passage of ions across the membrane. Manduteanu et al. [73] have shown that valvular endocardial cells can produce prostacyclin. If such a substance were the controlling factor between the endocardial endothelial cells and myocardial cells, then it would be expected that indomethacin (a cyclo-oxygenase inhibitor- [63]) would mimic the effects induced by endocardial damage. However, preliminary experiments showed no significant modifications

of myocardial activity, either mechanical or electrical following introduction of indomethacin. Moreover, Couttenye et al. [29] have shown that $PGI_2$ had no direct influence on myocardial contractility, in either mammalian papillary muscles or single cardiac cells. The lack of effect, therefore, seems to eliminate the possibility of prostacyclin acting as the mediator of this endocardium-dependent reaction.

Following the failure of indomethacin to cause any effect we proceeded to examine a hypothesis that the putative factor may be similar to vascular EDRF (endothelium-derived relaxing factor). The experimental results imply that the function of an endocardium-derived factor does not resemble the function of vascular EDRF at the level of cGMP, if in fact a cGMP process is present. This was found to be the case because methylene blue (a guanylate cyclase inhibitor) [12] did not cause effects similar to those induced by endocardial damage. Interestingly this drug caused opposite effects. Sodium nitroprusside (a guanylate cyclase stimulator) [12], on the other hand, caused effects similar in pattern to the endocardial damage effects

### 4.8.2 Other possibilities

Meulemans et al. [81] have recently shown that in isolated rat and cat papillary muscles the relaxant effects of atrial

natriuretic factor (ANF) were abolished after endocardial endothelial damage. They concluded that the effect of ANF on cardiac muscle was modulated through an endocardium-dependent process. This is interesting in light of the report by Bianchi et al. [10] that rat endocardium possesses receptors for ANF. Therefore, the endocardium may be acting as a sensor for some chemicals in the blood.

## 4.9 Future prospects

Perhaps of all possible experiments that can be performed to further document the endocardial role two deserve immediate attention. Firstly, it should be determined if the suggested diminished calcium release from the SR is via a modification of the availability of calcium to the release compartment. This is perhaps best done by studies of mechanical restitution and force-interval relations before and after endocardial damage. Secondly, the link between endothelial lining and the myocardial sarcolemma, and in the process the sequence of events leading to altered SR activity should be determined. It would be interesting to discover a chemical that can fully reverse the endocardial damage effects and thus shed some light on the nature of the putative chemical that cardiac endothelial cells may be producing. It would also be interesting to determine what factor(s) may regulate the release of such a chemical, especially since endocardium

may sense other chemicals. Although the involvement of a chemical modulator is an attractive theory, bioassay experiments using cultured endothelial cells are needed to support this theory.

Brutsaert et al. [16] have reported that ATP reversed the abbreviation induced in the contractile force following endocardial damage. It therefore seems that ATP acts perhaps directly, on the myocardium to offset the effects induced by endocardial damage. It would be interesting to see if the effects on action potential can also be reversed.

## Chapter 5. Conclusion

In this thesis we have examined a possible role for mammalian endocardium in modulating the contractile performance of myocardium. We have incorporated a study of action potential simultaneous with measurements of contractile force. To examine the time course of effects of endocardial damage on both the action potential and contraction, Triton detergent was added as a bolus to the chamber containing the working muscle. Our experimental evidences (electron micrographs, action potential characteristics, absence of rise in resting tension, time course of effects) suggest that the diminished myocardial contractile performance is not due to damage to underlying myocardium, but rather due to the absence of an intact, possibly active, endocardium. The Triton treatment procedure used may not only damage the endocardial endothelial plasmalemma, but it may also cause denudation of endothelial lining, although in the light micrographs the muscle appeared to be covered by endothelial lining after treatment. The examination of the time course of the effects of Triton on contraction followed by changes in action potential, the studies of the effect of Triton on the action potential on the endocardial and epicardial sides of the ventricular wall muscle, and the effect of albumin on contraction, indicate that it is not the loss of chemical and physical integrity that leads to the variation of

contractile force, and furthermore, it is not the changes in action potential currents that alter contractile state. The contractile changes precede action potential changes. From the balance of evidence obtained by our work it is believed that endocardium could act as an active (as well as a passive) barrier, and its damage causes the cardiac muscle to operate at a new level of performance. We propose that endocardial modulation of contractile force is through modulation of SR function, and that perhaps this happens by means of variation in a chemical factor acting on the myocardial sarcolemma. A hypothesis would be that the secretion of this chemical is under the influence of other chemicals in the blood, or elements such as muscle stretch. The altered shape of the action potential following damage to endocardium is believed to be a consequence of change at the contractile level due to the altered SR activity.

Indeed, if an active (as well passive) role for the endocardium can be seen in vivo, then we may be able to extend our knowledge of cardiac regulatory mechanisms to include the endothelial lining of the heart and in the process discover that the beneficial effects of endothelial cells are not just restricted to the vasculature. We mention this in light of the fact that the inside of the heart is continuous with the inside of the vascular system, and moreover, the endothelial monolayer is present from the very early stages of development, giving rise to the vascular system [123]. It should, however, be added that at this

stage we cannot be sure of the extent of endocardial involvement in an in vivo situation and indeed in the presence of well-developed regulatory mechanisms such as coronary circulation the endocardium may play only a background role. Consequently, the extent of endocardial contribution to the underlying myocardial functioning in vivo remains to be seen.

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