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CALCIUM ANTAGONIST BINDING TO MYOCARDIAL SARCOLEMMA: ADAPTATION TO EXERCISE

by

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B.Sc. (Kines.)(Hons.), Simon Fraser University, 1985

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'Calcium Antagonist Binding to Myocardial Sarcolemma : Adaptation to Exercise

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ABSTRACT

In response to exercise training one generally observes an increase in stroke volume under resting conditions as well as in both submaximal and maximal work. The purpose of this study was to investigate one possible intrinsic mechanism by which stroke volume can be increased with endurance training; increased myocardial contractility as a consequence of increased density of calcium channels in the plasma membrane (sarcolemma).

Female Sprague-Dawley rats were divided randomly into a sedentary-control and an exercise-trained group and the latter was treadmill trained for a minimum of 10 weeks. Upon excision of the hearts, sarcolemma was isolated using differential and sucrose gradient ultracentrifugation. Protein and sarcolemmal enzyme analyses were performed and no statistically significant difference ($p > 0.05$) between the groups was found.

To enumerate calcium channel density, a tritiated dihydropyridine calcium antagonist, [^{3}H]PN200-110 (PN), was employed. The specific binding of PN was found to be maximized in the presence of 2.5 mM $CaCl_2$, 50 nM calmodulin, an extravesicular tonicity of 20-30 mOsm L^{-1} and an incubation time of at least 60 minutes. Calcium addition was shown to increase both ligand binding and receptor site density. Calmodulin antagonists profoundly inhibited PN specific binding to highly-purified sarcolemma. Specific binding of PN to fractions derived from sucrose gradient centrifugation was reasonably well correlated, in both untrained ($R^2 = 0.84$) and trained ($R^2 = 0.85$) groups with the specific activity of the sarcolemmal marker, K^+-stimulated *p*-nitrophenylphosphatase (K^+*p*NPPase). The kinetic 'on' and 'off' rate constants for PN binding were $4.33 * 10^5$ M^{-1} s^{-1} and $3.90 * 10^{-5}$ s^{-1}, respectively.

There was an approximately 50% increase ($p < 0.05$) in PN binding site density in trained as in comparison with untrained in both the crude homogenate and the

subcellular fractions. It was calculated that this is equivalent to an increase from 3.55 to 5.71 sites per um^2 sarcolemma as a result of the training program.

The observed increase in the density of sarcolemmal calcium channels with exercise training, may allow for a greater influx of calcium current during the myocardial action potential. This provides a possible mechanism for the concomitant increase in myocardial contractility.

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ABBREVIATIONS

MOPS: 3-(N-morphilino) propane sulfonic acid

K^+*p*NPPase: K^+-stimulated *p*-nitrophenyl phosphatase

EGTA: Ethyleneglycol-bis-(*B*-aminoethyl ether)-N,N'-tetraacetic acid

CHAPTER I

Introduction

With endurance exercise training one normally observes an increase in maximum cardiac output, a decrease in submaximal heart rate for a given workload and hence an increase in both submaximum and maximum stroke volume. Even though the observation of the latter is well documented in the literature there is little insight to the mechanisms by which this change takes place. Alterations in stroke volume have been attributed to extrinsic (eg. increased venous return) and/or intrinsic myocardial changes (eg. contractility) however many questions about the effects of endurance exercise training on stroke volume still remain unresolved. This can be partially explained by the different training paradigms, species and the experimental parameters that have been proposed and used as indices of contractility. Of particular importance, is the development of techniques to investigate the mechanisms of contractility changes with exercise training. It is the purpose of this study to investigate one possible mechanism by which contractility and hence stroke volume can be increased with endurance training, using isolated and purified sarcolemma (SL).

Stroke Volume Regulation

Stroke volume can be defined as the difference between the volume of blood in the left ventricle just prior to systole (end-diastolic volume) and the volume present at the end of systole (end-systolic volume). Stroke volume is thought to be be under the influence of heterometric and homeometric regulation. Heterometric regulation refers to those adaptive mechanisms that are a consequence of changes in myocardial fiber length. Homeometric regulation refers to those other intrinsic adjustments of cardiac performance that are independent of changes in myocardial fiber length.

Heterometric Regulation

Heterometric regulation is predicated on a change in the end-diastolic volume (EDV) and is affected by the central blood volume and other vascular factors which consequently affect the blood returned to the heart (venous return).

Frank (1895), described the response of the isolated heart of the frog to alterations in the tension of the myocardial fibers just prior to contraction. Initial tension increased with greater degrees of filling and, at each succeeding level, contraction produced a progressively greater peak pressure. Starling (1918) using his famous dog lung-heart preparations, formed the basis of the Frank-Starling law of the heart. He found that the normal heart was distended to a greater EDV in response to a greater venous return and the force of contraction was increased with a greater initial muscle length (Starling, 1918). According to the Frank-Starling law, the energy imparted to the blood by the contraction of the left ventricle, independent of neural or hormonal control, is proportional to the length of the ventricular muscle fibers at the end of the preceding diastole. The results from Starling's studies of the isolated heart were also considered applicable to the intact animal. However, in the intact

animal not only is it difficult to obtain a representative measure of end-diastolic myocardial fiber length but other mechanisms may be superimposed to obtain an increased stroke volume.

The Frank-Starling law of the heart is in agreement with the relationship that has been shown in both skeletal and cardiac muscle fibers in which the tension generating capacity of the fiber is enhanced as sarcomere length is increased, until the optimum length is achieved (Gordon *et al.*, 1966). This length-tension relationship in the myocardium represents a potential control mechanism by which the level of preload (venous return) can influence the tension the muscle generates (Katz, 1977). For the length-tension relationship to mediate the cardiac response to increases in loading it is necessary for the myocardium to be operating on the ascending limb of the sarcomere length-tension curve.

Although the length-tension relationship probably plays a major role in beat-to-beat adjustments of the work of the heart, changes in developed tension arising from changing muscle fiber length appear not to be critical to most long-term circulatory changes. For example, it has been shown, from X-ray studies, that during exercise EDV of the heart was relatively constant (Kjelberg *et al.*, 1949).

The importance of the central blood volume for the stroke volume was demonstrated in 1939 by Asmussen & Christensen. They noted that the cardiac output was approximately 30% higher when the legs were "blood-free" as compared with the experiments with blood pooling in the legs. The high cardiac output was due to a high stroke volume, for the heart rate was actually lower than in exercise with reduced central blood volume and low cardiac output.

Any pooling of blood during exercise is minimized by the skeletal-muscle pump which forces blood from the veins toward the heart because of the intermittent venous compression produced by the contracting muscles and because of the unidirectional orientation of the venous valves. The respiratory pump also greatly enhances venous

return because during inspiration the reduction in intrathoracic pressure is transmitted to the lumen of the blood vessels located within the thoracic cavity. This reduction in central venous pressure during inspiration increases the pressure gradient between extrathoracic and intrathoracic veins (Berne & Levy, 1981). Both of these mechanisms tend to enhance venous return, thereby affecting EDV and potentially increasing stroke volume.

Variations in afterload exert a characteristic effect on the work performance of a muscle. Afterload may be defined as a load that is not apparent to the muscle when it is in a resting state but which is encountered by the muscle when it begins to contract (Katz, 1977). If the left ventricle is faced with an abrupt increase in aortic pressure, less blood is ejected and stroke volume will be reduced. This is not characteristic of endurance exercise where total peripheral resistance (TPR) is decreased due to vasodilation in the vascular bed (Wolthius *et al.*, 1977), although the elevation in cardiac output (CO) causes the mean arterial blood pressure (CO x TPR) to rise (Astrand *et al.*, 1965). Exercise involving isometric or static contractions has been shown to substantially increase systemic pressure, thus placing a larger afterload on the left ventricle (Wolthius *et al.*, 1977).

Homeometric Regulation

Any change in the strength of contraction during a single heartbeat that does not result from a change in initial fiber length can be considered to be the result of a change in contractility as defined by Katz (1977). An increase in contractility (or inotropic state) will augment cardiac performance (a positive inotropic effect) while a depression in contractility will lower cardiac performance (a negative inotropic effect).

Changes in the inotropic state of the myocardium are most readily explained as being due to gradations in the amount of calcium delivered to the contractile proteins for binding to troponin (Solaro *et al.*, 1974). The source of this calcium for activation is, however, controversial.

Several mechanisms have been investigated as being responsible for the rise in intracellular calcium for contraction. These include: (1) extracellular calcium transport through either ionic channels in the SL or (2) through an ion-exchange mechanism or (3) release of calcium from the sarcoplasmic reticulum (SR).

Cardiac muscle contraction has an absolute extracellular calcium (Ca^{2+}) requirement (Ringer, 1883; Niedergerke, 1963). The strength of cardiac contraction is proportional to the external Ca^{2+} concentration up to about 12 mM in most mammalian species (Bers *et al.*, 1981) and 3 mM in rat (Tibbits *et al.*, 1981a). These findings, as well as others (Katz, 1977; Randall, 1962; Reuter, 1979) indicate that there is probably little doubt that transsarcolemmal Ca^{2+} influx plays a critical role in excitation-contraction (E-C) coupling.

Sarcolemmal Calcium Binding

When extracellular calcium is removed force has been noted to decline exponentially with a $t_{1/2}$ of 45 to 50 seconds. The redevelopment of force, upon return of calcium is at least 3 to 5 times faster (Philipson & Langer, 1979). The rate of redevelopment upon replacement of calcium is virtually the same as the rate of vascular exchange for the preparation. This suggests that calcium important for contractile control is bound at a cellular site which is in rapid equlibrium with vascular calcium. The most likely site is the SL. This is supported by the ultrastructural demonstration that almost 40% of the cellular surface is within 0.2 um of a capillary surface (Frank & Langer, 1974).

Further support for significant SL calcium binding comes from experiments in which other di- and tri-valent cations compete with calcium for binding sites (Bers & Langer, 1979; Langer *et al.*, 1974; Philipson & Langer, 1979). These investigators found a high correlation between the ability of certain cations to displace calcium from the SL and their ability to depress contractile function. The divalent cation sequence to displace calcium from intact cultured cells and their ability to uncouple excitation from contraction in neonatal ventricle, relative to lanthanum (La^{3+}) was: cadmium (Cd^{2+}) > manganese (Mn^{2+}) > magnesium (Mg^{2+}) (Langer *et al.*, 1974). Cadmium (0.97 Å) is the ion with its crystal radius the closest to that of calcium (Ca^{2+}) (0.99 Å), Mn^{2+} (0.80 Å) is intermediate and Mg^{2+} (0.66 Å) is the furthest removed (Langer, 1984). This indicates that the size of the non-hydrated ion relative to calcium plays a significant role in determination of its ability to compete with calcium for SL sites (Bers & Langer, 1979; Langer *et al.*, 1984). These observations suggest that these calcium binding sites play an important role in controlling the amount of calcium that is made available to the myofilaments.

Of great importance are recent experiments which demonstrate the critical role that plasma membrane composition and structure play in the regulation of membrane and cell function. Philipson *et al.* (1980) have shown that the SL calcium binding critical for contraction is to phospholipid (PL) moieties and of these the PL that are anionic at physiological pH, phosphatidylserine (PS) and phosphatidylinositol (PI), are most important. The degree of interaction of Ca^{2+} with the PL has been suggested to be governed by the transmembrane electric field and Ca^{2+} can be released from the PL following membrane depolarization (Lullman & Peters, 1977). Phosphatidylserine has also been implicated as being the major contributor of the negative surface charge of SL membranes (Bers *et al.*, 1985) thus attracting calcium ions. The presence of PS is also required for sarcoplasmic reticulum (SR) protein phosphorylation by exogenous Ca^{2+}-activated PL-dependent protein kinase (protein kinase C) (Iwasa & Hosey, 1984).

This evidence implies that PL may play a role in controlling Ca^{2+} movement during the excitation-contraction sequence in cardiac muscle.

Whilst it is widely agreed that E-C coupling in heart muscle requires an inward movement of calcium across the SL (Nayler & Dresel, 1984), controversy exists as to whether sufficient calcium enters on a beat-to-beat basis to account for the activation of contraction. Bers & Langer (1979) claim that the amount of calcium bound to the SL is, by itself, enough to support the interaction of the myofilaments. Others, however, claim that the magnitude of calcium entering the myocardium is insufficient (McDonald, 1982; New & Trautwein, 1972; Reuter, 1979), providing only enough calcium for ~30% activation of the myofilaments (Solaro & Shiner, 1976). However, recent advances in single cell voltage and patch clamp studies (Isenberg, 1977; Lee & Tsein, 1982), radiotracer flux measurements (Lewartowski *et al.*, 1982), and selective microelectrodes (Bers, 1983) have increased estimations in the Ca^{2+} influx (to ~10-183 umol/kg wet tissue) in the range required for half-maximum activation of the myofilaments (15-42 umol/kg wet wt) (Fabiato, 1983; Solaro *et al.*, 1974).

Calcium Channel

One possible route of calcium entry across the SL is best described as a channel or pore through which calcium ions flow and give rise to a current (Beeler & Reuter, 1970). Certain inotropic interventions augment this so-called inward current believed to be largely attributable to calcium. Among these are the catecholamines (New & Trautwein, 1972; Reuter, 1967).

Proposals that cyclic AMP-dependent protein phosphorylation might mediate *B*-adrenergic modulation of cardiac ionic channels were made as early as 1973 (Tsien, 1973a), following the initial evidence for the involvement of cAMP (Tsien, 1973b). Voltage clamp (Reuter, 1974; Reuter, 1979; Reuter & Scholz, 1977; Sperelakis &

Schneider, 1976) and patch clamp experiments have evolved to study the properties of individual channels (Brum *et al.*, 1984; Cachelin *et al.*, 1983; Reuter, 1983; Reuter *et al.*, 1982; Reuter *et al.*, 1983) and whole cell recordings, designed to analyze properties of the entire pool of functional calcium channels (Bean *et al.*, 1984). Increased calcium current is the result of catecholamines exerting their effect by (a) slightly increasing the mean open times of calcium channels, (b) reducing the time intervals between bursts of channel openings, (c) increasing burst length and (d) reducing failures of calcium channels to open upon depolarization (Reuter *et al.*, 1986; Tsien *et al.*, 1986).

In isolated cardiac SL vesicles, stimulation of increased calcium uptake through the calcium channel was induced by the addition of the catalytic subunit of the cyclic AMP-dependent protein kinase, and was paralleled by the phosphorylation of a membrane-bound acidic proteolipid which has been termed calciductin (Rinaldi *et al.*, 1981). Philipson (1983) states that this finding, however, is insufficient evidence for assignment of a role to this protein in the regulation of the voltage-sensitive calcium channel. This was suggested because the putative *in vitro* calcium channel described by Rinaldi *et al.* (1981; 1982) requires a source of energy and requires an outwardly directed sodium gradient. These properties are inconsistent with known attributes of the cardiac calcium channel. This acidic proteolipid, which has a molecular weight of 23,000 (Rinaldi *et al.*, 1981) resembles the acidic proteolipid phospholamban (22,000 daltons (Hicks *et al*, 1979)), which modulates the Ca^{2+}-transporting ATPase of heart SR. Hence, calciductin may represent phospholamban as a consequence of SL contamination by SR.

Na^+/Ca^{2+} Exchanger

Another transsarcolemmal Ca^{2+} transport mechanism is the Na^+/Ca^{2+} exchanger. To what extent the influx of Ca^{2+} on the exchanger contributes to the

enhancement of sarcoplasmic Ca^{2+} required for myofibril activation is not known. The Na^+/Ca^{2+} exchange system has been suggested to be a high-capacity pumping system designed to eject calcium, presumably only when the calcium concentration in the sarcoplasm has increased substantially (ie. at peak activation) (Carafoli, 1984). Under these conditions, the relatively low Ca^{2+} affinity of the exchanger (Bers *et al.*, 1980; Reeves & Sutko, 1979) is probably not a limiting factor, but its high velocity (Caroni & Carafoli, 1981) is particularly useful.

The Na^+/Ca^{2+} exchanger is able to transport at least three Na^+ for every Ca^{2+} ion and is, therefore, electrogenic in nature (Philipson & Nishimoto, 1980; Pitts, 1979). Thus, the magnitude and possibly the direction of the exchanger can be affected by the membrane potential (Philipson & Nishimoto, 1980) and perhaps by the intracellular sodium concentration. There is theoretical evidence to suggest that under depolarizing conditions, the exchanger contributes to calcium influx and therefore tension development and under repolarizing conditions it contributes to calcium efflux (Mullins, 1979). Raising the intracellular sodium concentration in squid axons, or lowering the extracellular sodium concentration has been clearly shown to promote inward calcium transport (Baker *et al.*, 1967; 1969). Since it is well established that intracellular sodium concentration strongly affects contractility, it has been suggested that this is due to the exchanger. Evidence suggests that inhibition of the exchanger may have a negative inotropic effect (Carafoli, 1985) and stimulation of the exchanger may augment contractility (Philipson, 1984).

It has been demonstrated that a phosphorylation-dephosphorylation step catalyzed by a Ca^{2+} plus calmodulin-dependent kinase activates the exchanger, whereas a dephosphorylation reaction catalyzed by either exogenously added phosphatase or by a calmodulin-dependent endogenous phosphatase deactivates it (Caroni & Carafoli, 1983). The concerted operation of the kinase and phosphatase is thought to be made possible by their different affinities for Ca^{2+} and calmodulin

(CaM) , and thus by the levels of Ca^{2+} and CaM in the sarcoplasm (Caroni & Carafoli, 1983).

Sarcoplasmic Reticulum Calcium Transport

It has been clearly demonstrated that the current carried by calcium through the calcium channels is important in the E-C coupling process (Katz, 1977; Randall, 1962; Reuter, 1979), although the magnitude of calcium influx is not by itself enough to account for the large increase in intracellular calcium concentrations (Fozzard & Beeler, 1975; Solaro & Shine, 1976). It may, however, be sufficient to trigger a graded release of calcium from an intracellular store.

Two theories as to the mechanism responsible for SR calcium release in the myocardium have been proposed. The first, which can be looked on as depolarization-induced, has gained support as a mechanism for SR calcium release in skeletal muscle (Ebashi, 1976; Endo, 1977). The second, "calcium-induced calcium release" is less likely to be of significanee in skeletal muscle but may play an important role in the myocardium, in which variations in this calcium release are of major physiological importance.

Bianchi & Bolton (1966) first proposed that calcium itself might trigger a release of calcium from the SR. Since then, evidence has accumulated that a small initial rise in intracellular calcium may indeed be the stimulus for release. Endo *et al.* (1968) demonstrated that an increase in the intracellular free calcium concentration was able to induce a larger release of calcium from the SR.

Skinned cardiac myocytes which contain SR exhibit the phenomenon of calcium-induced calcium release (CICR) (Fabiato & Fabiato, 1978a; Fabiato, 1981; Fabiato, 1983; Fabiato, 1985a). Fabiato (1982) indicated that this CICR from the SR is not an all-or-none process but rather is graded. Since E-C coupling in cardiac muscle is

dependent upon an influx of calcium it is possible that this CICR phenomenon plays a role in mobilizing calcium for interaction with the myofilaments.

The threshold for triggering SR calcium release in the heart occurs around pCa 6.8 (Fabiato, 1982). The magnitude of the SR Ca^{2+} release was presumably thought to be regulated by (1) the rate of Ca^{2+} influx, (2) the level of preload of the SR with calcium and (3) the level of the free calcium concentration that is used as a trigger and not necessarily the total amount of influx (Fabiato, 1983). However, evidence as cited by Langer (1980) indicates that the SR is already saturated with calcium even at low intracellular calcium concentrations. Therefore, any modulation in SR calcium release is probably due to changes in the level or the rate of change of the trigger calcium concentration (Fabiato, 1985a). Experiments with skinned fibers indicate that the maximum amount of calcium released by the SR is sufficient to generate only one-half the tension produced experimentally by decreasing the pCa of the superfusate to 5 or less (Chapman, 1983; Fabiato & Fabiato, 1978; Fabiato, 1983).

The nature of calcium release by the SR, in the intact muscle, still remains poorly understood. While there is general agreement that calcium release from the SR is a passive, downhill process, little is known of the nature of the "channel" that mediates the permeability change that allows calcium to flow out of the SR (Chacko *et al.*, 1977).

A slow calcium efflux from calcium-loaded SR vesicles was shown to be increased by elevation of extracellular calcium (Katz *et al.*, 1977) and it was found that elevation of extracellular calcium could promote a slow CICR when ATP levels outside the vesicles were high and ADP concentrations were low (Katz *et al.*, 1980). This is in accordance with earlier studies conducted by Fabiato & Fabiato (1978) which suggested that CICR from the SR could use part of the same pathway as that for calcium accumulation, or that the dependence on the rate of change of free calcium concentration could be explained partly by a competition between the calcium

pump and the calcium-binding molecule gating the calcium release channel for a time-dependent increase of free calcium concentration that results from the transsarcolemmal calcium influx (Fabiato, 1983). In 1985, Fabiato (1985a) suggested that the mechanism of CICR from the SR is independent of that of calcium accumulation and occurs via a channel with time- and calcium dependent activation and inactivation. Calcium release was found to occur when the channel was already activated by calcium and not yet inactivated by time and the further increase of free calcium concentration at the outer surface of the SR (Fabiato, 1985a).

The calcium pump is a ~90,000 to 100,000 dalton protein that is embedded in and spans the lipid membrane of the SR (Tada *et al.*, 1978). The Ca^{2+}-ATPase binds 1-2 moles of calcium for each mole of ATP; hydrolysis of the latter forms a high energy phosphorylated intermediate with this protein in which the cation is bound to a high affinity calcium binding site (Tada *et al.*, 1978). During the calcium uptake reaction, the phosphorylated calcium pump ATPase protein is converted to a low energy phosphoprotein by a reaction that "raises" the bound calcium to the higher energy level of the high calcium concentration within these membranes. This process allows calcium to be sequestered in the calcium pool within the SR as outlined by Tada *et al.* (1978).

Cystolic calcium concentration has a dual effect on the rate of calcium transport by the SR. Simplest is its role as substrate for the forward reaction of the calcium pump which allows increasing calcium concentration in the micromolar range to accelerate directly the rate of calcium uptake by the SR. More recently, a second, indirect, effect of calcium has been observed that is mediated by phospholamban. It is now clear that this regulatory protein can also be phosphorylated by a calcium (CaM)-dependent protein kinase (Katz & Remtulla, 1978); and that, like cyclic AMP-dependent protein kinase catalyzed phosphorylation (Katz, 1979; Tada & Katz, 1982), the calcium-dependent phosphorylation stimulates the rate of calcium uptake (Kirchberger & Antonatz, 1982; Tada & Katz, 1982). It has recently been demonstrated

that phospholamban is also phosphorylated by protein kinase C (Movsesian *et al.*, 1984). This phosphorylation is associated with a two-fold stimulation of calcium uptake by the SR (Movsesian *et al.*, 1984) similar to that seen following phosphorylation of phospholamban by a CaM-dependent protein kinase or by a cAMP-dependent protein kinase. Thus, these protein kinases appear to stimulate SR calcium uptake in an additive fashion and phosphorylate at different sites.

Exercise-Induced Adaptation of Cardiac Function

Many questions are still unresolved with respect to myocardial changes in response to endurance exercise training, although the effects of training on myocardial and extramyocardial function are known.

Oxygen consumption, a function of cardiac output (CO) and arteriovenous oxygen difference ($(A\text{-}V)O_2$), achieved at maximal exercise is uniformly increased (Ekblom *et al.*, 1968; Rowell, 1974; Saltin *et al.*, 1968). Changes in CO are generally proportional to changes in oxygen consumption (Scheuer & Tipton, 1977). Therefore the submaximal CO for a given work output is usually the same in the trained and untrained state (Saltin *et al.*, 1968), but the maximal CO is increased by physical training. Several reports state that the CO at submaximal work and at a given oxygen uptake is either decreased or not changed by training but maximal CO is increased (Bevegard *et al.*, 1963; Rowell, 1974; Saltin *et al.*, 1968). Approximately 50 (Rowell, 1974) to 80% (Blomqvist & Saltin, 1983) of the potential increase in oxygen delivery that results from physical training is due to the augmented stroke volume.

Careful studies concerned with the longitudinal effect of training indicate that the $(A\text{-}V)O_2$ differences at maximal exercise conditions increase with training (Ekblom *et al.*, 1968; Saltin *et al.*, 1968). It is unclear why training enhances $(A\text{-}V)O_2$ difference. Possible mechanisms responsible for the widening of an $(A\text{-}V)O_2$ difference via

training involve and implicate muscle blood flow, number of capillaries, the fiber type of the muscles recruited, alterations in the concentration and activity of aerobic enzymes in cells, plus changes in the number and function of mitochondria. Since fiber types differ in aerobic capacity, their degree of vascularity, and their pattern of recruitment during exercise (Gollnick *et al.*, 1974) training may have more of an effect on local and cellular events than can be detected by regional measurements or by the determination of single $(A\text{-}V)O_2$ values.

It is well documented that the heart rate is lower in trained compared to sedentary subjects for a given CO (Astrand, *et al.*, 1964; Ekblom, 1969; Rowell, 1974) and maximum CO can be increased with training without a concomitant increase in maximum heart rate (Barnard, 1975; Ekblom *et al.*, 1968; Saltin *et al.*, 1968). This clearly illustrates that both submaximum and maximum stroke volume can be enhanced with exercise training. The mechanism for this adaptation, however, remains controversial. Since stroke volume can be affected by extramyocardial and myocardial factors, the effect of exercise training on these variables will be examined.

Extramyocardial Adaptations

Heart rate

For a given submaximal CO heart rate is lower in trained compared to sedentary subjects (Astrand, *et al.*, 1964; Ekblom, 1969; Rowell, 1974). The effect of exercise training on maximal heart rate is variable. Some have observed that the maximal rate is decreased (Barnard, 1975; Ekblom, 1969; Ekblom *et al.*, 1969; Saltin *et al.*, 1968) while others have found it unchanged (Blomqvist & Saltin, 1983).

It has been suggested that training causes an increased centrogenic vagal cholinergic drive combined with a sympathoinhibitory mechanism (Scheuer & Tipton, 1977). There are no significant changes in myocardial tissue concentrations or in the

plasma levels of epinephrine or norepinephrine at rest (Christensen *et al.*, 1979; Cousineau *et al.*, 1977; Peronnet *et al.*, 1981). Plasma concentrations are lower at any absolute submaximal work load after training but there are no differences when comparisons are made on the basis of relative work intensity. The lower post-training plasma levels at any given absolute work load are consistent with the relative bradycardia. This, in turn, could be related to the increased amounts of ACh found in atrial tissue following exercise training (Badeer, 1975) and to the decreased sensitivity of cardiac tissue to catecholamines (Smith & El-Hage, 1978). However, the sensitivity of the S-A (sinoatrial) node to exogenous *B*-adrenergic agonists appears to be unchanged (Williams *et al.*, 1981).

Nonneural mechanisms may contribute to the relative bradycardia after training. Several studies have demonstrated a decrease in the intrinsic rate of the atrial pacemaker or sinoatrial node (Badeer, 1975; Sigvardsson *et al.*, 1977; Smith & El-Hage, 1978). If the intrinsic rate of the pacemaker is decreased with exercise training, then the heart rate would be lower independently of the influences of the autonomic nervous system.

Coronary vasculature

Another potentially important effect of physical training on cardiac function is an increase in myocardial vascularity to meet the increased oxygen demands of the myocardium (Penpargkul & Scheuer, 1970). There is experimental evidence for a training-induced increase in the size of the coronary vascular bed (Hudlicka, 1982; Muntz *et al.*, 1981; Penpargkul & Scheuer, 1970; Scheuer & Tipton, 1977; Wyatt *et al.*, 1978) with changes involving both capillaries and larger vessels. Neogenesis of coronary capillaries is suggested by studies based on light- and electron microscopy and autoradiographic techniques (Leon & Bloor, 1968; Ljungqvist & Unge, 1977) as

well as by data on the rate of incorporation of ^{3}H-thymidine in myocardial capillaries in young rats exercised by swimming (Ljungqvist & Unge, 1977).

Increases in coronary blood flow has also been observed in physically trained rats (Penpargkul & Scheuer, 1970; Scheuer *et al.*, 1974). This greater flow reserve in hearts of conditioned rats is consistent with the evidence showing increased vascularity.

Longitudinal studies (Stone, 1977; 1980) have demonstrated that changes in coronary flow patterns occur very early after the onset of a training program, which suggests significant regulatory adaptations. The extent to which the increase in vascularity exceeds the increase in muscle mass in the normal heart remains to be determined. Training has been implicated to increase the ratio of vascular space to myocardial mass in rats trained by swimming or by running (Denenberg, 1972; Stevenson *et al.*, 1964; Tepperman & Pearlman, 1961). Schiable & Scheuer (1981), however, reported increases in coronary flow proportional to the degree of training-induced increase in heart weight.

Peripheral Vasculature

Preload

An increase in EDV has been proposed as being responsible for the augmented stroke volume. Wolfe & Cunningham (1982) used non-invasive techniques such as echocardiography to determine the effects of chronic exercise on various cardiac functions. Although contractility was not directly, measured the increase in stroke volume found was deduced to be a consequence of an increase in ventricular preload. Another group (Rerych *et al.*, 1980), using radionuclide angiography observed no change in contractility (ejection fraction) but found an augmentation in EDV after a six month training program during submaximal and maximal exercise. Therefore, they

too claimed that the increased stroke volume was due to an increased EDV. The central blood volume was also found to be increased after training. Therefore, it was concluded that this was the mechanism for the increased EDV.

There is evidence to suggest an increased preload during exercise after training. Changes in physical activity and maximal oxygen uptake are paralleled by small but statistically significant changes in total blood volume (Saltin *et al.*, 1968), usually without major changes in hemoglobin concentration or hematocrit. Several studies as outlined by Blomqvist & Saltin (1983) have been conducted involving the reinfusion of blood and indicate a difference between sedentary and well-trained subjects with respect to the cardiovascular response to an acute blood volume increase (increased stroke volume and CO).

Afterload

The increase in stroke volume seen in trained subjects has not been associated with a fall in blood pressure, even though a fall in TPR has been noted (Clausen *et al.*, 1976). This is probably because several authors have found little difference in mean blood pressures before and after training (Hartley *et al.*, 1969; Terjung *et al.*, 1973). Some studies (Ekblom *et al.*, 1968; Saltin *et al.*, 1968) are difficult to interpret because blood pressure measurements were reported for absolute heart rates or specific oxygen consumption rather than for designated work loads.

The increase in the size of the capillary bed of skeletal muscle is a striking feature of the training response, but by far the largest portion of the resistance to systemic blood flow is exerted at the arteriolar level. The primary mechanisms responsible for the reduction in systemic resistance are poorly defined. They are likely to affect the arterioles and to be regulatory rather than anatomical (Blomqvist & Saltin, 1983).

The capacity of the cardiovascular system to deliver oxygenated blood to active tissue exceeds the demand only when a small fraction of the total muscle mass is active. Local vascular conductance is most likely limiting oxygen delivery although blood flow may be quite high (Blomqvist & Saltin, 1983). It has been shown that training causes an important increase in maximal vascular conductance of working skeletal muscle (Clausen, 1976; 1977; Davis & Sargeant, 1975; Gleser, 1973; Saltin *et al.*, 1976).

Myocardial Adaptations

Cardiac Hypertrophy

Cardiac hypertrophy represents a response of the heart to chronically increased hemodynamic demands. Up to a point, the increased mass of cardiac muscle may be beneficial to both the myocardium and the circulation (Katz, 1977). The distribution of the increased demand for mechanical work among a large number of contractile elements reduces the energy expenditure of each, as well as providing for greater strength of cardiac pumping. This condition has been referred to as physiological hypertrophy (Katz, 1977) and has been observed with physical training. However, "pathological hypertrophy" is the consequence of a sustained hemodynamic overloading of the heart resulting in depressed myocardial function (stroke volume) (Katz, 1977). It may be due to systemic or pulmonary hypertension, valvular heart disease and/or loss of functional tissue. These observations are consistent with the findings that contractility may be depressed in the heart that is rendered hypertrophied by a variety of experimental and physiological interventions (Braunwald & Ross, 1979; Spann *et al.*, 1967).

Both swimming and running programs have been shown to produce at least a moderate degree of hypertrophy if defined as an increase in the heart-weight/body-

weight ratio (Fuller & Nutter, 1981; Nutter *et al.*, 1981; Scheuer & Tipton, 1977; Schiable & Scheuer, 1981) or other indices (Crew & Aldinger, 1967; Hepp *et al.*, 1974). This is inconsistent with other investigators (Baldwin *et al.*, 1975; Tibbits *et al.*, 1978; 1981a), who in endurance trained rats (running), did not evidence hypertrophy although cardiac function was enhanced. The discrepancies may possibly be explained by various factors. The extent of the hypertrophy is probably directly related to the intensity and duration of the training program and inversely related to age (Muntz *et al.*, 1981). The mode of exercise (eg. running vs swimming), the index used for assessing hypertrophy and the sex of the experimental species may also be significant determinants of the degree of cardiac hypertrophy, but the findings from several studies conflict (Muntz *et al.*, 1981). The majority of the experimental studies have been performed in rodents, mainly rats. The rodent heart appears to be less responsive to exercise training than other mammalian hearts. Significant changes in cardiac mass after endurance training have been reported in the dog (Wyatt *et al.*, 1974) and the cat (Wyatt *et al.*, 1978) but not in humans (Morganroth *et al.*, 1975; Peronnet *et al.*, 1981), although EDV was increased in the latter studies.

Myocardial Contractility

Introduction

An increase in myocardial contractility has been investigated as another possible intrinsic adaptation to exercise. Many experimental parameters have been proposed and used as indices of contractility. However, indices such as V_{max} (maximum rate of cross-bridge turnover), dP/dt (or dT/dt) (change in left ventricular pressure or tension with respect to time), rate of fiber shortening, dFlow/dt (change in aortic blood flow with respect to time) and peak left ventricular tension all have a length dependence. Therefore, these indices are not valid measurements of

contractility. However, those studies which afford the most control over fiber length (isolated hearts or strips of cardiac tissue) are the most removed from *in vivo* situations.

Crews & Aldinger (1976) measured tension in isolated muscle strips from trained and untrained rats and found that the muscles from the trained hearts had a greater active tension than the untrained. This greater active tension was suggested to be indicative of greater contractility. Bersohn & Scheuer (1976) observed that trained hearts had a greater ejection fraction, greater fiber shortening and increased dP/dt for a given filling pressure. These changes, too, were indicative of greater myocardial contractility. From these findings it could be postulated that the Frank-Starling mechanism of a greater initial fiber length may not account for the greater stroke volume in submaximal exercise. Other investigators using isolated cardiac tissue from trained hearts have also observed an enhanced contractile state (Hepp *et al.*, 1974; Mole, 1978; Penpargkul & Scheuer, 1970; Schiable & Scheuer, 1979; Tibbits *et al.*, 1978; 1981a) while others have found no change (Grim *et al.*, 1963; Williams & Potter, 1976) or even a decrease (Nutter & Fuller, 1977). These differing findings may be due to myriad animal, training and experimental models (Scheuer & Tipton, 1977).

There is some disagreement about the nature of the mechanism responsible for the increase in myocardial contractility. There must either be a change in the number of cross-bridges formed (P_o) or in the rate of turnover of the cross-bridges (V_{max}) or both to account for a change in contractility. Two distinct approaches have been taken to give some insight to the plausible mechanisms. One approach focuses on alterations at the contractile element and the other on changes in the mechanisms of E-C coupling.

Contractile Element Alterations

At the contractile level, some investigators have attributed the enhanced cardiac function, at least in part, to an increase in actomyosin ATPase activity (Bhan & Scheuer, 1972; 1975). Such alterations have been confirmed using rats subjected to an intensive swimming program (Bhan & Scheuer, 1972; Wilkerson & Evonuk, 1971), but they have not been observed on treadmill trained rats (Tibbits *et al.*, 1981a) and dogs (Dowell *et al.*, 1977) despite marked increases in contractile function. The elevation in Ca^{2+}-ATPase and Mg^{2+}-ATPase activity of actomyosin was found to be proportional to the number of hours of swimming (Bhan & Scheuer, 1972).

It was shown that the heavy meromyosin from hearts of conditioned rats had the same types of ATPase changes as those found in myosin (Bhan & Scheuer, 1975). It was suggested that there is a conformational change in the myosin molecule at or near its active site. Thus, increased cardiac contractile protein enzymatic activity (actomyosin, myosin and heavy meromyosin ATPase), which implies alterations in the intrinsic contractile state of the myocardium, appears to be a function of swimming exercise rather than a generalized exercise training response (Flaim *et al.*, 1979; Wilkerson *et al.*, 1971).

Excitation-Contraction Coupling Alterations

Alterations in the mechanism of E-C coupling involve alterations in the delivery of calcium to the contractile element. Both mitochondria and SR are capable of regulating intracellular calcium levels, which in turn, may influence contractile function. Calcium associated with the SR (Fabiato & Fabiato, 1975; 1978a; 1978b) and mitochondria (Lehninger, 1974) have been implicated in E-C coupling.

Mitochondria has been clearly implicated to be more important for long term Ca^{2+} regulation rather than for beat-to-beat control (Scarpa & Graziotti, 1973). Studies on the effects of exercise training on myocardial mitochondria function in both dogs (Sordahl *et al.*, 1977) and rats (Penpargkul *et al.*, 1978) show no improvement in function. Neither the ATP synthesis capability nor the calcium transport properties of isolated mitochondria appear to be enhanced by training.

Penpargkul *et al.* (1977; 1980) have evidence which suggests that training increases calcium uptake by the SR. The increased rate of cardiac relaxation sometimes observed in trained rats may be explained by the increased rate of calcium transport in the SR and the increase in actomyosin ATPase. Sordahl *et al.* (1977), however, found no difference in calcium binding or uptake by the SR from trained and control dogs yet Ca^{2+} release from the SR was found to be affected. Since the techniques used by the former investigators does not allow for the determination of initial calcium transport rates and includes oxalate, the significance of their results is questionable. Although the latter authors used a more sensitive technique, dual-wavelength spectrophotometer, a calcium sensitive dye (murexide), with a relatively low Ca^{2+} affinity, was used and the free calcium concentration was substantially higher than observed under physiological conditions.

Treadmill exercise training of rats has been suggested by Tibbits *et al.* (1981a) to enhance cardiac performance by increasing availability of calcium to the contractile element. They observed a prolonged plateau phase of the action potential in the trained group, consistent with among other things an enhanced inward calcium current. Augmented transsarcolemmal flux has been shown to increase the contractile state of the myocardium (New & Trautwein, 1972) either by triggering a greater release of calcium from the SR (Fabiato & Fabiato, 1975) or perhaps, direct interaction with the myofilaments.

Tibbits *et al.* (1981a) provided further evidence for an increased availability of calcium for the contractile elements. They found a significant difference in the relative number of calcium binding sites between trained and control rats. The trained rats exhibited a 63% increase in the predicted number of binding sites over the control. They proposed that this increase in binding sites, which was suggested to be indicative of an increase in calcium channels, would increase the magnitude of calcium influx and thus make more calcium available for the contractile element.

Tibbits *et al.* (1981b) also clearly showed that treadmill trained rats exhibited substantial alterations in the lipid composition of the plasma membrane. Specific SL content of total PL and PS in the trained group was increased 23 and 50%, respectively. This may be extremely important in the adaptation to exercise, since PS is a major binding moiety of SL calcium (Philipson *et al.*, 1980), has been shown to be involved in protein kinase C activation (Iwasa & Hosey, 1984) and increases the fixed negative charge of the SL (Philipson *et al.*, 1980).

Due to the important role calcium channels apparently play in E-C coupling and may play in the regulation of myocardial contractility further investigation is necessary. Since, calcium antagonists have frequently been used as probes to gain information about calcium channels this section will follow with a discussion on these compounds.

Calcium Antagonists

Introduction

One of the major advances in cardiac electrophysiology has been the discovery that the inward depolarizing current has two components: the fast sodium current which is blocked by tetrodotoxin and local anesthetics and the slow inward current mediated essentially by Ca^{2+} ions and blocked selectively by Mn^{2+} and Co^{2+} ions.

About 10 years ago, it was found that the selective inhibition of the slow channel in either normal tissue or in those solely dependent on the slow channel for depolarization can be achieved by a large number of chemically heterogeneous compounds (Fleckenstein, 1970). Thus, they have come to be known as calcium antagonists or calcium influx blockers.

The term calcium antagonists is generic for a structurally diverse group of agents which are thought to act specifically by inhibiting calcium-mediated E-C coupling in cardiac muscle cells (Fleckenstein, 1970; Triggle & Swamy, 1983). Three major groups of these drugs have been identified: phenylalkylamines (verapamil and the methoxy derivative, D600), benzothiazepines (diltiazem) and dihydropyridines (DHP) which exhibit varying degrees of potency and specificity (Fleckenstein, 1970; Nayler, 1980; Triggle & Swamy, 1983; Opie, 1984).

As shown in a variety of isolated muscle preparations, members of the dihydropyridines (DHP) are typically the most potent inhibitors of E-C coupling and cause an antagonism which is markedly voltage dependent (Triggle & Swamy, 1980; Ehlert *et al.*, 1982; Opie, 1984). The rank order of potency, based on the affinity of the [^{3}H]DHP for cardiac SL (Lee *et al.*, 1984), of the more studied DHP is: PN200-110 (PN) > nitrendipine (NTP) > nicardipine > nifedipine (NF) > Bay K8644.

Dihydropyridines

Effect on Excitation-Contraction Coupling

Dihydropyridines have been demonstrated to exert potent negative inotropic effects (with the exception of Bay K8644 and related compounds; Schramm *et al.*, 1983) in myocardial preparations (Ehlert *et al.*, 1982; Lee *et al.*, 1984; Marsh *et al.*, 1983; Morgan *et al.*, 1983; Ricardo *et al.*, 1983). Both the force and the rate of force development are significantly reduced. This negative inotropic effect has been

attributed to interference with electromechanical coupling and subsequent contractile element shortening.

The absence of inhibitory effects of these agents upon calcium responses in skinned cardiac muscle argues for a SL site of action (Morad *et al.*, 1982), although certain intracellular sites are possible. Morad *et al.* (1983) provided further evidence to substantiate DHP binding to SL. They used rapid photochemical inactivation of DHP to show that the DHP block the pathway of calcium transport which directly activates the myofilaments. Voltage clamp techniques indicate that the blocked transport system was the calcium channel on the surface membrane in the frog heart. Furthermore, these findings are in accordance with Sarmiento *et al.* (1983) and Glossmann *et al.* (1982) who found copurification of the DHP binding site with the SL.

It has been suggested (Morgan *et al.*, 1983) that the effect of a DHP is to decrease the sensitivity of the myofilaments to calcium. Such an action of the calcium channel blocking agents could also account for the abbreviation of the contraction (Morgan *et al.*, 1983).

If a smaller fraction of the calcium released were reaching the contractile apparatus, it would have to be because some intracellular calcium "sinks" were competing more effectively for the released calcium. The most important "sink" likely to alter its rate of calcium sequestration is the SR and indeed it has been shown that interventions known to influence the rate of calcium pumping by the SR do alter the rate of decline of intracellular calcium concentration (Blinks *et al.*, 1982; Morgan & Blinks, 1982). However, Morgan *et al.* (1983) found that the rate of decline of intracellular calcium concentration is not perceptibly changed by the calcium channel blocking agents, even when peak calcium and peak tension are markedly altered. For this reason they do not believe that an increase in the effectiveness with which a calcium "sink" competes with troponin C can readily explain the altered relation between calcium and tension.

Williams & Jones (1983), in canine hearts, found the highest density of NTP binding sites to be a subfraction of SR vesicles, SL vesicles had an intermediate density of binding sites and the mitochondria had the least. The results of their study suggest that NTP binds specifically to macromolecules which may be localized in membranes other than the SL. It is possible that NTP binds to intracellular membrane proteins which regulate calcium mediated phenomena. The small molecular weight and lipophilicity of DHP would probably permit access of these compounds to the intracellular space *in vivo* (Kokubun & Reuter, 1984)). Thus, NTP may in fact bind predominantly to calcium channel molecules but that these channels may be distributed among several types of membranes including the SL and the terminal cisternae of the SR. In the study by Williams & Jones (1983) the microsomal or SR enriched fractions exhibited low specific activities of all SL markers analyzed indicating that NTP binding detected in these fractions cannot be attributed to the presence of contaminating SL. Others have, however, found that DHP binding copurifies with canine (Sarmiento *et al.*, 1983) and bovine (Glossmann *et al.*, 1982) SL. These discrepancies may possibly be attributed to differences in experimental techniques employed.

If in fact it is true that DHP binding sites are localized in membranes other than the SL then the possibility of DHP entering the cell should be reviewed. Nitrendipine and NF have been observed to enter into the muscle cells (Pang & Sperelakis, 1983; 1984) and so have the option of a) exerting a second effect on some intracellular organelle, eg., to depress release of calcium from SR during E-C coupling, or b) exerting their effect on the slow channels from the inner surface of the cell membrane. The order of uptake is NTP >> NF which coincides with their lipid solubilities, as measured from their oil:water partition coefficients, and with their ability to inhibit the inward calcium current (Pang & Sperelakis, 1983).

If DHP depress the tension produced by contractions of cardiac muscle more than what could be accounted for by the depression of maximum rate of rise of the slow action potential and slow inward current then perhaps the drug enters the cell and depresses release of calcium from the SR. Although this has been found not to be the case for NF (Sperelakis, 1984) it may still be the case for NTP.

Studies on CaM and on the regulation of the slow inward current by phosphorylation and dephosphorylation reactions have led to a reappraisal of the mechanism and site of action of DHP. It has been suggested that they might act by inhibiting the phosphorylation responsible for the activation of the slow channel (Henry, 1983). Such an effect might result from an interference with (membrane-bound) CaM involved in the activation of a channel phosphokinase. This would parallel with the finding of Bkaily & Sperelakis (1986) who observed that CaM was required for full activation of the calcium channel. DHP may also act by influencing the synthesis or degradation of cyclic AMP.

Henry and Brum *et al.* (1983) reported inhibition of cardiac cyclic AMP phosphodiesterase activity upon intracellular application of DHP. This, however, is difficult to interpret since decreased breakdown of cyclic AMP may be expected to exert stimulatory rather than inhibitory effects on the heart, thus attenuating their own calcium entry blocking activities. This would be consistent with Cachelin *et al.* (1983) who found that 8-bromocyclic AMP increased the probability of channel opening.

Binding Kinetics

Characterization of the calcium channel has been aided greatly by information about the binding of the calcium antagonists. The simplest model of a ligand-receptor interaction assumes a homogeneous univalent species of ligand and a single non-interacting population of binding sites (receptors). This situation is described by the general scheme:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} LR$$

at equilibrium:

$$[LR] / [L][R] = K_a = 1/K_d$$

where [R] is the concentration of free receptor sites, [L] is the concentration of unbound ligand, and [LR] is the concentration of ligand-receptor complex. The equilibrium and kinetic rate constants for association and dissociation are K_a and k_1 and K_d and k_{-1}, respectively. The ratio of the reaction rate constants gives the dissociation constant (or the affinity of the ligand for the receptor):

$$K_d = k_{-1}/k_1.$$

The two most useful methods of analyzing equilibrium saturation binding data are the Scatchard (Scatchard, 1949) and Hill plot (Dahlquist, 1978). Both are transformations of the data obtained when the binding is determined as a function of the concentration of unbound radioligand. The Scatchard plot is more commonly used than the Hill plot because it provides an estimate of the density of binding sites

without requiring the use of saturating concentrations of ligand. A Hill plot, on the other hand, requires knowledge of the concentration of receptor sites, which is usually obtained from a Scatchard analysis (Weiland & Molinoff, 1981).

For a homologous population of non-interacting binding sites, the equilibrium equation can be rearranged to give the fraction, (Y), of macromolecule sites occupied by the ligand:

$$Y = [DHP{\cdot}R] / ([R] + [DHP{\cdot}R])$$

Combination of this relationship with the equilibrium constant gives

$$Y/L = K(1 - Y).$$

This gives a plot of the fraction of macromolecular receptor sites bound divided by free ligand concentration versus the fraction bound with a straight line of a slope equal to -K and abscissal intercept equal to K. If the absolute concentration of binding sites is known, the fraction bound may be replaced by the concentration of bound sites, [DHP·R], to give the equation below:

$$[DHP{\cdot}R]/[L] = K([DHP_o] - [DHP{\cdot}R])$$

where DHP_o is the concentration of potential binding sites. In this variation, a plot of bound ligand concentration divided by free-ligand concentration versus the concentration of bound ligand gives a slope of -K and the abscissal intercept becomes DHP_o. This transformation is referred to as the Scatchard plot. The total number of binding sites may be extrapolated from data in which complete saturation of the macromolecular binding sites is not observed (Dahlquist, 1978). The binding affinity of the ligand to the receptor site can be obtained by determining the reciprocal (absolute value) of the slope (or the dissociation constant).

Since there are limitations to the Scatchard plot, the Hill plot may sometimes be a more useful means of plotting the data (Dahlquist, 1978). The slope of the plot gives n_H, the Hill coefficient. A linear Scatchard plot implies a Hill coefficient of unity and a Hill coefficient of one is always associated with a linear Scatchard plot. This would be the case for a single noninteracting site where the half-saturating ligand concentration is simply the apparent dissociation constant for the ligand-macromolecular complex.

Mention should be given to the limitations to the use of Scatchard plots. In the case of multiple classes of binding sites or positive or negative cooperativity the plot will be nonlinear and the slope must be extrapolated to determine the dissociation constants. A curvilinear Scatchard plot concave upwards reflects a lower binding affinity at higher ligand concentrations and can be interpreted as negative cooperativity or multiple classes of binding sites (site heterogeneity). A curvilinear Scatchard plot which is concave downwards reflects a lower binding affinity at lower ligand concentrations. This implies positive cooperative site-site interactions. In such cases of more than one binding affinity, the 'Hill plot' is a more useful means of plotting the data (Weiland & Molinoff, 1981).

Sarcolemmal Binding

Binding Characteristics

Nifedipine (Williams *et al.*, 1982), NTP (Rhodes *et al.*, 1985), PN (Hof *et al.*, 1984) and nimodipine (Dompert & Traber, 1984) have been observed to bind with high affinity (K_d= 0.1 - 2 nM) and binding capacities (B_{max}= 0.1 - 1 pmoles/mg protein) to specific binding sites in cardiac membranes. It was also found that binding is reversible, saturable and with Hill coefficients close to unity (Bellemann *et al.*, 1981;

Bolger *et al.*, 1982; Ehlert *et al.*, 1982; Janis *et al.*, 1984; Williams & Tremble, 1982), consistent with interaction at a single set of sites.

The ability of DHP to bind to cardiac and smooth muscle is very similar but there is a major quantitative discrepancy between binding and pharmacological behavior in the heart (Janis & Scrabine, 1983; Janis & Triggle, 1983; Lee & Tsien, 1983; Sarmiento *et al.*, 1983). Lee & Tsien (1983) found a 100-fold difference between NTP binding and its pharmacological effect on isolated heart cells. Consistent with this finding, Sarmiento *et al.* (1983) found that the dissociation constant (K_d) that was obtained from Scatchard analysis for NTP binding was three orders of magnitude lower than the IC_{50} (the concentration required for 50% of the maximal effect) that had been reported for this drug.

In addition to the high affinity binding site a low affinity site has also been reported in some experiments (Bellemann *et al.*, 1981; Glossmann *et al.*, 1984; Marsh *et al.*, 1983). However, this is not in accordance with several previous studies (Colvin *et al.*, 1982; Ehlert *et al.*, 1982; Murphy & Snyder, 1982; Sarmiento *et al.*, 1983; Williams & Tremble, 1982; Yamamura *et al.*, 1982). The discrepancy can be attributed to the fact that these latter authors performed binding studies in homogenates or membrane preparations which may have produced conformational changes in the state of the membrane protein. The former authors used intact, functioning cultured heart cell preparations that obviates limitations inherent in homogenate binding studies and permits direct comparison of binding properties and contractile function under identical conditions. However, intact preparations may introduce other problems of quantification such as non-specific binding of the DHP.

It may be that a reasonable 1:1 correlation exists between binding at the low affinity site and negative inotropic action (Janis *et al.*, 1985). It also may be that this low affinity site is responsible for the calcium antagonistic effects on the heart. Thus,

the discrepancy between binding and the pharmacological effect may possibly be attributed to a loss of the low affinity sites.

Both low and high affinity DHP binding sites were found in intact sartorius muscle (Schwartz *et al.*, 1985). However, there were some 30-50 times more high affinity binding sites than voltage-dependent calcium channels. This suggests that either the DHP binding sites are not necessarily related to functional calcium channels, or that the binding sites represent a large reserve of dormant calcium channels (Schramm & Towart, 1985). The extent to which this finding can be related to cardiac muscle is questionable since not only is the K_d of binding approximately ten-fold higher in skeletal muscle but the transsarcolemmal calcium current is extremely small in comparison to cardiac muscle (Schwartz *et al.*, 1985). Thus, these binding sites may be unrelated to DHP sites associated with functional calcium channels in cardiac muscle.

In cardiac cells it has been shown that estimates of DHP receptor density using radiolabelled ligands (Sarmiento *et al.*, 1984), parallel electrophysiological estimates of functional calcium channels (Bean *et al.*, 1983; Lee and Tsien, 1983). Sanguinetti and Kass (1984) found DHP binding to be ~1000 times stronger to inactivated channels than to resting channels, which could explain the above mentioned discrepancy. These different functional states are not expected to be observed in *in vitro* binding studies. Other explanations for this discrepancy may include the loss of biochemical control, hence, phosphorylation-dephosphorylation (Janis & Triggle, 1984) in *in vitro* preparations although DHP binding to isolated whole rat myocytes is also of extremely high affinity (DePover *et al.*, 1983). Also, there may be an insufficient amount of time for the DHP to bind to the inactivated channels in the normal heart (Bean, 1984).

The question now is how do DHP partition into the membrane to their receptor site. It is not known whether these ligands orient selectively. Certainly, the orientation of these ligands, their position in the bilayer, and the homogeneity of this orientation

will depend on the substitutions present at the 3-or 5 position of the pyridine ring (Rhodes *et al.*, 1985). This fact may well be related to the observation (Triggle & Swamy, 1983) that for very subtle changes in the 3-or 5 substitutions of various DHP, profound differences in rates of binding and activity are observed.

Two models have been proposed (Rhodes *et al.*, 1985) for drug binding to DHP receptors in cardiac SL membrane.

1) In the "aqueous approach", the drug reaches the receptor by diffusion through the bulk aqueous phase.

2) In the "membrane approach", the drug partitions into the membrane bilayer and then diffuses laterally to a specific receptor site.

The calculated diffusion rates for the membrane approach are ~3 orders of magnitude greater that those for the aqueous approach. The membrane approach diffusion-limited rate depends weakly on the sizes of the binding site, the nature of the drug, and the vesicle, but depends strongly on ligand asymmetry (Rhodes *et al.*, 1985).

Although the measured binding rates for several DHP were all slower than the calculated diffusion-limited rates for either model, other experimental data, such as very high partition coefficients and specific positions of these drugs in the membrane bilayer, suggest that the membrane approach is the more likely even though the drug still has to get to the membrane through the aqueous phase. The discrepancy in the measured binding rates and the calculated diffusion limited rates could be due to the presence of unstirred layers on the membrane (Barry & Diamond, 1984). These layers can act as an additional resistance to the permeation of the drug (Barry & Diamond, 1984) under physiological conditions.

Dihydropyridine Receptor Characteristics

Different techniques have been used to estimate the molecular weight of the DHP receptor in various tissues. Reports range from 100,000 to ~300,000 daltons (Borsotto *et al.*, 1984; Curtis & Catterall, 1984; Ferry *et al.*, 1983; Glossmann *et al.*, 1984; Norman *et al.*, 1983; Rengasamy *et al.*, 1985; Rhodes *et al.*, 1985; Towart & Schramm, 1984). Radiolabelled drugs have been used in an attempt to biochemically identify and purify the voltage-sensitive calcium channel. The DHP receptor has been purified from skeletal (Borsotto *et al.*, 1984; Curtis & Catterall, 1984) and cardiac muscle (Rengasamy *et al.*, 1985). It appears to be a noncovalently linked multisubunit protein in both skeletal (subunits: 130-150, 53 and 32-33 kDa) (Borsotto *et al.*, 1984; Curtis & Catterall, 1984) and cardiac (subunits: 60, 54 and 34 kDa) (Rengasamy *et al.*, 1985) muscle. Reconstitution of this protein (derived from skeletal muscle transverse-tubules) into lipid vesicles is associated with reconstitution of voltage-sensitive calcium channel activity (Curtis & Catterall, 1986), however only a small percentage of these channels are functional (Curtis & Catterall, 1986). The apparent low percentage of purified receptors that reconstitute functional channels may result from damage during isolation and reconstitution or may reflect normal physiological and regulatory processes, which would be consistent with Schwartz *et al.* (1985).

The DHP binding site has been characterized to be a glycoprotein macromolecule (Curtis & Catterall, 1984; Ferry & Glossmann, 1983; Towart & Schramm, 1984) which is consistent with its sensitivity to trypsin (Glossmann *et al.*, 1982). The participation of PL in retaining the integrity of the calcium channel is revealed by DHP binding sensitivity to phospholipase A and C treatment (Glossmann *et al.*, 1982). The former enzyme hydrolyses the fatty acid from the 2-position of phosphoglycerides whereas the latter cleaves the bond between phosphoric acid and glycerol. Calcium antagonist binding has been shown to be insensitive to

neuraminidase treatment (Glossmann *et al.*, 1982), indicating the absence of sialic acid associated with the binding site.

Colvin *et al.* (1984;1985) in canine and Tibbits *et al.* (1981) in rat SL have shown that the lipid/protein ratio is ~3.0 and 0.75 *u*mol of lipid/mg of protein, respectively. This ratio suggests that a typical area of membrane would contain large areas of lipid with proteins inserted at widely separated intervals. Most of the SL protein present is Na^+/K^+ ATPase and relatively little represents the specific DHP receptor; the concentration of Na^+/K^+ ATPase binding sites for example is ~400 times that of DHP binding sites in SL (Colvin *et al.*, 1984). This indicates that only 1/35,000 of the total membrane area is receptor (Colvin *et al.*, 1984). The density of calcium channels in cardiac membranes has been estimated at 1,000 - 10,000 channels per cell, or approximately 0.5 - 5 channels per um^{-2} (Bean *et al.*, 1983; Reuter, 1983).

Binding Modulators

Calmodulin Antagonists

Calmodulin is a calcium-binding protein which exhibits an amino acid sequence resembling that of other calcium receptors namely troponin-C (Cheung, 1980). Calmodulin possesses four calcium binding sites with affinities in the micromolar range (Henry, 1983). In eukaryotes, CaM appears to serve as the major intracellular receptor for calcium ions (Cheung, 1980). Binding of calcium ions to CaM produces stable conformers capable of interacting with and activating a number of intracellular enzymes, including cyclic nucleotide phosphodiesterase, Ca(II)-ATPase, myosin light chain kinase, NAD kinase and guanylate kinase (Cheung, 1980).

A group of agents which are known to bind to CaM and inhibit CaM and CaM-dependent processes were found to have an effect on NTP binding at low concentrations (Janis *et al.*, 1984). Therefore, the potency of these agents for inhibition

of NTP binding is very similar to that for inhibition of CaM. The K_I for calmidazolium (R24571) and trifluoperazine (TFP) have been found to be 2-3 nM (Johnson & Wittenauer, 1983) and 5 *u*M (Vincenzi, 1981), respectively, and at a concentration of 1 *u*M, calmidazolium produced 83% inhibition of NTP binding whereas TFP produced only 28% inhibition of binding (Janis *et al.*, 1984). These results suggest that a hydrophobic surface similar to that on CaM and CaM binding proteins (Epstein *et al.*, 1982) may be associated with the DHP binding site (Janis *et al.*, 1984). Recent studies using brain membranes (Gould *et al.*, 1983), indicate that the binding site for these CaM antagonists may be the allosteric site at which non-DHP calcium channel antagonists bind.

Drugs such as TFP bind to CaM in a calcium dependent fashion (Levin & Weiss, 1979). Drug binding is assumed to prevent CaM from binding to the effector, thereby reducing the effectiveness of CaM in the system being tested. Usually this is the only mechanism assumed to apply for CaM binding drugs.

There is evidence for calcium-induced hydrophobicity on the surface of CaM (LaPorte *et al.*, 1980). A simple interpretation (LaPorte *et al.*, 1980) is that hydrophobic site(s) which appear only when calcium binds may bind to drugs or to the CaM receptor or both. That is some CaM binding drugs may not prevent the binding of calcium to CaM ($CaM(Ca^{2+})_n$) but actually accompany the complex to the receptor. Drug binding to $CaM(Ca^{2+})_n$ might alter its affinity for and/or effectiveness in modulating the effector.

Cations

Under physiological conditions the calcium channel is occupied almost continually by one or more calcium ion, which by electrostatic repulsion, guards the channel against permeation by other ions. On the other hand, repulsion between

calcium ions allows high through-put rates and tends to prevent saturation with calcium (Hess & Tsien, 1984).

Elevation of extracellular calcium concentration tends to reverse or antagonize the effects of all of the calcium antagonistic drugs (Triggle & Swamy, 1983; 1984b). This action could include competition between calcium and drug for binding to the outer mouth of the channel and increased electrochemical driving force for calcium influx through the fraction of slow channels not blocked by the drug (Sperelakis, 1984). In all cases, the increase in electrochemical driving force for an inward calcium current with elevated extracellular calcium concentrations would be a factor.

Since the first step in permeation of the calcium ion through the slow channel presumably is binding to the mouth of the channel, inorganic calcium antagonists could act either by displacing calcium from this binding site, which is the putative mechanism of action of Mn^{2+}, Co^{2+} and La^{3+} ions in blocking the slow inward current, or by permeating the membrane, in the case of Mn^{2+} or Co^{2+}, and displacing calcium from an internal obligatory site (Sperelakis, 1983). The inhibition of NTP binding by various di- and tri-valent cations (Co^{2+}, La^{3+}, Mn^{2+}, Ca^{2+}) is consistent with the idea that the binding sites for NTP are associated with the calcium channels (Ehlert *et al.*, 1982; Fosset *et al.*, 1983; Marangos *et al.*, 1982).

The most apparent mechanism of the DHP is a blockade of the membrane calcium channels to inhibit the calcium influx necessary for E-C coupling. Kohlhart & Fleckenstein (1977) found that the effect of 1.4×10^{-6} M nifedipine on E-C coupling in guinea pig papillary muscles could be reversed by doubling the external calcium concentration from 2 to 4 mM. However, McBride *et al.* (1984) found that the negative inotropic effect of 10^{-5} M NTP is not completely reversible by increasing the calcium concentration, suggesting that at high concentrations of NTP, almost all of the SL calcium channels can be blocked.

The inorganic calcium antagonists appear to function as general calcium antagonists (Triggle & Swamy, 1980; Sperelakis, 1983), a property presumably arising from a rather non-selective ability to block calcium binding sites. Since a cation coordination site is a likely feature of all calcium channels, regardless of voltage-sensitivity (Triggle, 1981) and since the organic calcium channel antagonists lack the general inhibitory action of the inorganic cation, it is likely that the inorganic and organic antagonists function differently.

Dihydropyridine binding is divalent cation dependent (Glossmann & Ferry, 1982; Gould *et al.*, 1982). Binding is significantly reduced or abolished in a fully reversible fashion by treatment with chelating agents (Ehlert *et al.*, 1982; Gould *et al.*, 1982). Binding is restored by Ca^{2+} and, to varying degrees, by other di- and trivalent cations in the sequence: Ca^{2+} = strontium (Sr^{2+}) > Mg^{2+} = Mn^{2+} = cobalt (Co^{2+}) > barium (Ba^{2+}) = nickel (Ni^{2+}) > La^{3+} = samarium (Sm^{3+}) (Ehlert *et al.*, 1982; Gould *et al.*, 1982). The relationship of this site to the cation permeating machinery of the channel remains to be established, but one possibility is that the DHP antagonists function by enhancing the binding of a cation, presumably calcium, to the channel and thus effectively blocking permeation (Sperelakis, 1983a). More plausibly, however, the site of cation interaction may be involved in the control of channel activation and inactivation (Sperelakis, 1983a). Although the role of this divalent cation site is not clear, its apparently high affinity (K_d < 1 uM) for calcium and the observation that CaM antagonists inhibit NTP binding suggests the presence of a calcium binding protein regulating calcium channel function, or the interaction of calcium with the channel pore site (Hess & Tsien, 1984).

Sodium and calcium channels seem to be distinct but some authors, namely Langer (1974) suggest that 75% of sodium (Na) entering the cell during depolarization enters through the so called calcium channel. Since the DHP site appears to be associated with the calcium channel (Morad *et al.*, 1983; Curtis & Catterall, 1986)

studies have been conducted to further elucidate calcium channel specificity. Fossett *et al.* (1983) in skeletal muscle, Marangos *et al.* (1982) in rat brain and Ehlert *et al.* (1982) in rat heart found Na^+ to exhibit no influence on NTP binding. The latter finding is inconsistent with Schwartz & Velley (1985) who in guinea pig left ventricle found Na to increase the IC_{50} of NTP binding and increase the number of binding sites. From this finding Schwartz & Velley (1985) postulated that variations in the sodium flow or concentration in the calcium channel could be a regulating factor, with an increase in sodium concentration decreasing the affinity of the protein for calcium.

While both Marangos *et al.* (1982) and Ehlert *et al.* (1982) carried out their experiments in the presence of a low concentration of Na, Schwartz & Velley (1985) used physiological concentrations. With respect to cardiac muscle, Ehlert *et al.* (1982) considered only the maximal specific binding of NTP whereas Schwartz & Velley (1985) performed inhibition curves for NTP binding and equilibrium saturation curves. These methodological differences may account for the discrepancies found.

Divalent cations are able to bind to calcium channels as judged by these ions to facilitate DHP binding. Therefore, these ions may undergo the following sequential reaction scheme with the calcium channel (Ch) as suggested by Glossmann *et al.* (1983):

$$\underset{\text{outside cell}}{Me^{2+} + Ch} \underset{k_{-1}}{\overset{k_1}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} \underset{\text{membrane}}{Me^{2+}Ch} \underset{k_{-2}}{\overset{k_2}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} \underset{\text{inside cell}}{Ch + Me^{2+}}$$

A cation which can bind and dissociate via k_2 will carry current through the channel (eg. Ca^{2+}). A cation which binds but does not dissociate via k_2 will block the channel (eg. Ni^{2+} or La^{3+}) and a cation for which the energy barrier to unbind is intermediate in magnitude between that for ions which are fully permeant and blockers will sometimes pass through the channel (eg. Mn^{2+}) or become adsorbed onto the membrane

structure and thus produce changes in surface charges and ion gating properties (Glossmann *et al.*, 1983).

Glossmann & Ferry (1983) postulated that there was more than one binding site for divalent cations within the calcium channel. This they claimed finds an interesting parallel in the (Ca^{2+}-Mg^{2+})-dependent ATPase which is present in the SR as well as the plasma membrane.

Non-Dihydropyridine Calcium Antagonists

Verapamil incompletely inhibits (+)PN binding in rat myocardial cells (Lee *et al.*, 1984) and completely inhibits (NTP) binding in rat (Ehlert *et al.*, 1982) and rabbit (Janis *et al.*, 1984) SL. The nature of this inhibition is classically referred to as negative heterotropic cooperativity (Ehlert *et al.*, 1982). Ehlert *et al.* (1982) imply, that in the case of NTP binding, verapamil and NTP form a ternary complex with the receptor (calcium channel). Since a verapamil-induced alteration in the kinetics of NTP binding is seen, such an interaction presumably takes place.

A plausible explanation for the negative cooperative effects of verapamil is that verapamil inhibits NTP binding by hindering the isomerization step (decreasing k_2) (Ehlert *et al.*, 1982):

$$D + R \;\text{<----->}\; DR$$

$$D + R^* \;\text{<----->}\; DR^*$$

$$D + R \;\overset{k_1}{\text{<------>}}\; DR \;\overset{k_2}{\text{<------>}}\; DR^*$$

where D = drug, R = low affinity and R* = high affinity receptor. If the isomerization step is slow with respect to the bimolecular association of NTP with its receptor, then Ehlert *et al.* (1982) assume that the following predictions can be made. In the absence

of verapamil, the association of NTP with its receptor should be characterized by a fast molecular association component followed by a slower isomerization or association of NTP with its receptor. In the presence of verapamil, the slow component of association should be reduced such that the approach to equilibrium is faster. Moreover, a fast component of dissociation should be readily apparent when dissociation is mediated in the presence of verapamil since verapamil converts some of the high affinity complex (DR*) into the low affinity state (DR). It is generally thought that voltage-sensitive channels, such as sodium and calcium channels, go through a transition of functional states. Thus, it is possible that the different states of the NTP binding sites postulated above (R and R*) might correspond to the different functional states of the calcium channel (Ehlert *et al.*, 1982).

D-cis diltiazem has been shown to enhance (-)NTP binding in rabbit ventricular cells (Janis *et al.*, 1984), guinea pig heart (Glossmann & Ferry, 1983) and (+)PN binding in rat myocardial cells (Lee *et al.*, 1984), thus exhibiting positive heterotropic cooperativity. In the latter two studies, the enhancement in binding was attributed to a decrease in the K_d value without a significant alteration in B_{max}. The enhancement of binding by d-cis diltiazem may, however, also be due to an increase in B_{max} without a change in the K_d as suggested by Ferry & Glossmann (1982) and Goll *et al.* (1983). The increase in B_{max} may be explained by a mechanism which recruits channels from an intracellular location and incorporates them into the SL or unmasking of pre-existing channels.

It is important to mention that each of these DHP-receptor sites can exist in low-and high-affinity sites, induced by other drugs, temperature, ions or detergents (Glossmann *et al.*, 1984). It is tempting to speculate that these states of the drug-receptor sites in *in vitro* experiments with membrane fragments are analogous (but not necessarily identical) to the different states through which the channel can cycle in the intact cell (open, closed, inactivated).

It is interesting to note that previous studies by Murphy *et al.* (1983) have indicated that non-DHP calcium channel antagonists act at a common allosteric site in brain membranes to modulate DHP binding. Thus, Gould *et al.* (1983) and Janis *et al.* (1984) carried out studies to determine whether similar evidence could be obtained for rabbit heart membranes. They found that NTP binding was regulated by a common allosteric site or, alternatively, by closely linked sites for verapamil and diltiazem.

It has recently been shown that a NF derivative, Bay K8644, may act as a calcium channel agonist (Schramm *et al.*, 1983). The positive inotropic effect of this compound has been shown to be competitively antagonized by NF but not by verapamil or diltiazem (Ishii *et al.*, 1985; Schramm *et al.*, 1983; Uehara & Hume, 1985). Also, Janis *et al.* (1984) found that Bay K8644 did not alter the dissociation rate constant of NTP binding. These observations are in accordance with the findings that the DHP may bind to a common receptor site that differs from the sites at which verapamil and diltiazem bind (DePover *et al.*, 1982; Ferry & Glossmann, 1982; Murphy *et al.*, 1983).

These results suggest that the DHP do not sterically block the calcium channel via either their DHP or phenyl rings (Janis *et al.*, 1984). Recent results (Hess *et al.*, 1984) suggest that NTP promotes a mode of calcium channel gating in which the channels are unavailable for opening, whereas, Bay K8644 promotes a mode of gating where the channels exhibit very long openings. It remains to be determined whether these different gating modes are chemically or just conformationally different.

Although delineation of the structural requirements for antagonistic and activator properties remain to be established, it is likely that there will exist a continuum of properties ranging from pure antagonism to pure activation (Janis *et al.*, 1985).

Membrane Potential

The calcium ion that enters during cardiac excitation is primarily through the voltage- and time- dependent slow channels, and comprise the major portion of the inward slow channel (Sperelakis, 1984b). Generally, calcium current through the calcium channel depends on the net electrical driving force, $V - V_{rev}$ (with the membrane potential, V, and reversal potential, V_{rev}, at which the net current through the calcium channel vanishes), and the single channel conductance, g, which has been shown to be independent of membrane potential (Cavalie *et al.*, 1983). Measurements of single channel currents have shown that the channel opening events are statistically distributed (duration typically 1 ms) and interrupted by variable shut periods of less than 1 to some 100 ms duration (Cavalie *et al.*, 1983; Fenwick *et al.*, 1982; Reuter, 1983). Therefore, for a given membrane potential, V, only a probability that the channel is open, P_o (V), can be determined. If N is the number of available calcium channels, the calcium current, I_{Ca} (V) can be generally calculated as:

$$I_{Ca}(V) = P_o(V) \times N \times g \times (V - V_{rev}).$$

Depending on the experimental conditions the reversal potential is +40 - +80 mV (Lee *et al.*, 1981), whereas the calcium equilibrium potential is greater than 120 mV, indicating that other ions (e.g. potassium, caesium) can pass through the channel, especially at high driving forces.

Recent studies using the 'patch clamp' technique, have shown that the calcium channel opens in bursts; the probability of doing so increases as the transsarcolemmal voltage becomes more positive than -40 mv and then decreases with further depolarization as the driving force for calcium decreases (Reuter, 1984). Present evidence suggests that a 'voltage-sensor' in the membrane (a protein group with dipole

properties, for example, which may be an integral part of an ion channel) reacts to the electric field. Any change in membrane potential will cause a restoration of the charged sensor with the field, hence a change in ion flow through the channel (Reuter, 1983).

The calcium current across SL has been shown to have two major components in heart muscle (Bean, 1985; Nilus *et al.*, 1985) and three in neurons (Nowycky *et al.*, 1985). T-type channels are responsible for a rapidly inactivating, transient calcium current, while the L-type channel openings produce a slowly inactivating, long-lasting calcium current (Armstrong & Matteson, 1985; Bean, 1985; Nilus *et al.*, 1985). The N-type calcium channel requires strong negative potentials for complete removal of inactivation (unlike L) and strong depolarization for activation (unlike T) (Nowycky *et al.*, 1985). T-type calcium channels can be activated by smaller depolarizations (to about -60 mV) than L-type channels (to about -40 mV) in single channel measurements (Reuter *et al.*, 1986). This agrees with calcium current measurements in whole-cell recordings (Bean, 1985). The L-type current is much more common than the T-type in the ventricular myocardium (Nilus *et al.*, 1985). The T-type channel is insensitive to DHP (Bean, 1985; Mitra & Morad, 1985; Nilus *et al.*, 1985) and is more resistant to block by external Cd^{2+} than the L-type channel (Nilus *et al.*, 1985). The Ca^{2+} entering the cell through L-channels is thought to be necessary both for replenishing the Ca^{2+} stores of the SR (Chapman & Niedergerke, 1970) and possibly for triggering intracellular Ca^{2+} release (Fabiato, 1985b). The experiments by Nilus *et al.* (1985) showed that the T-channel current is much smaller and decays much more quickly than the L-channel current, so it probably contributes relatively little to calcium influx during the action potential plateau and contraction. They suggest that T-type channels might have greatest significance for pacemaker depolarization and action potential initiation, electrical phenomena that depend critically on small inward currents at relatively negative potentials.

The potency of calcium antagonistic drugs on calcium current is increased during membrane depolarization as well as during repetitive stimulation (use-dependence) (McDonald *et al.*, 1980; Osterrieder *et al.*, 1981; Trautwein *et al.*, 1981). It is not clear whether the association rate constant of the drug molecules with calcium channels is increased if the channels open during depolarization, or whether the dissociation rate is decreased if the channels are inactivated.

Nitrendipine (McBride *et al.*, 1984) and blocking cations like Mn^{2+} or La^{3+} (Sperelakis, 1984b) do not have the property of frequency-dependence. Since mesudipine, another DHP derivative, NF (Sperelakis, 1984b) and nisoldipine (Kass & Sanquinetti, 1984b) have a significant frequency-dependence the insignificant effect observed with NTP cannot be a property of all DHP derivatives.

The frequency-dependency effect suggests that these drugs do not act as simple plugs for the calcium slow channels, as perhaps Mn^{2+} or La^{3+} might act (Kaufmann & Fleckenstein, 1965). Rather, this property suggests that the drug might act to slow the recovery process of the slow channel from the inactive state back to the resting state. If so, then a slow drive rate or a long quiescent period (ie. 20 - 60 seconds) would allow complete recovery of the drugged slow channel before the next excitation occurred. To exert such an effect on the gate recovery kinetics, the drugs must bind on a channel protein. An alternative possibility is that the drug binds to the channel only in the active state to block it, and then dissociates before conversion of the channel to the sequential states. Another possibility is that any drug which affected the phosphorylation of the slow channels by some direct means would also effectively block the slow channels selectively and could account for frequency-dependence (Sperelakis, 1984b).

The binding affinity for antagonists is greatly increased by membrane depolarization (Reuter *et al.*, 1985a; 1985b). Correspondingly, the blocking potency of antagonistic compounds is also increased (Bean, 1984; Reuter *et al.*, 1985a). It has been

postulated that calcium antagonists reduce calcium current through the channel by facilitating the inactivated state (Reuter *et al.*, 1986).

Potentiation of calcium channel current, by prolonging the open state of the channel, by the DHP agonist Bay K8644 is also voltage-sensitive (Sanquinetti & Kass, 1984) and independent of cyclic AMP-dependent phosphorylation reactions (Reuter *et al.*, 1986). In this case, enhancement of current is observed when pulses are applied from negative holding potentials. But when holding potentials are more positive than -40mv, these effects are less pronounced, and even indicate block (Sanguinetti & Kass, 1984). The positive inotropic action of Bay K8644 appears to depend on the concentration of the drug (dose-dependent). At concentrations higher than 10^{-7} - 10^{-6} mol L^{-1} its effect declines (Schramm *et al.*, 1983). These results show that membrane potential, and perhaps inactivation of the calcium channel is pivotal in regulation (block or enhancement) of calcium channel current by DHP derivatives (Sperelakis, 1984b).

It appears that membrane potential may play an important role in the blockage of calcium channels by DHP. Perhaps future investigations will eventually show that all of the DHP interact with a common voltage-sensitive structure.

Structure/Activity Relationships

Structure activity data for 1,4-DHP are available for isolated cardiac (papillary) muscle (Rodenkirchen, 1979). The general structural requirements for activity are summarized below.

(a) The 1,4-DHP ring is essential, oxidation of the pyridine abolishes activity.

(b) The NH group of the 1,4-DHP ring must be unsubstituted for optimum activity.

(c) The 2,6-substituents of the 1,4-DHP ring should be lower alkyl, although one NH_2 group is tolerated.

(d) Ester substituents in the 3- and 5-positions of the 1,4-DHP ring are optimum. Removal or replacement by COMe or CN greatly reduces activity.

(e) Ester substituents larger than COOMe generally maintain or even increase activity suggesting a region of bulk tolerance in the site of 1,4-DHP interaction.

(f) When the ester substituents at C_3 and C_5 of the DHP ring are different, the C_4 position becomes chiral and stereoselectivity of antagonism is observed.

(g) An aryl substituent, preferably a substituted phenyl group, appears optimum for the 4-position of the 1,4-DHP. The position of the substituent in the phenyl ring is critical: para substitution invariably decreases activity, whereas ortho or meta substituents (ortho > meta >> para) generally increase activity according to steric factors.

A role for steric influences is also suggested from the solid-state to receptor-bound conformations, a correlation has been observed between the extent of 1,4-DHP ring pucker and pharmacological activity. Substituents (ortho or meta) in the phenyl ring influence the 1,4-DHP ring confirmation, activity increasing with increasing ring planarity. The highly detrimental effect of para substitution could arise from hindrance to the actual receptor interaction (Janis & Triggle, 1983).

A further important index of specific drug action is stereoselectivity which has been demonstrated for nicardipine and PN where the positive enantiomer is more potent (Lee *et al.*, 1984; Shibanuma *et al.*, 1980) and NTP where the negative isomer is more potent (Triggle & Swamy, 1981) in cardiac muscle.

As mentioned earlier the 1,4-DHP also show tissue selectivity; being significantly more effective on smooth than on cardiac muscle (Fleckenstein, 1977; Janis & Scriabine, 1983). The virtually 1:1 correlation between binding and K^+-

induced tension in guinea pig ileal longitudinal smooth muscle is clearly suggestive that the high affinity binding sites are those mediating the pharmacologic activity.

Hypothesis

The working hypothesis of this study is that: the well documented increase in stroke volume observed with endurance exercise training is a consequence of increased myocardial contractility due, at least in part, to an increased Ca^{2+} channel density. It has previously been postulated that the enhanced contractility may be attributed to an alteration in E-C coupling, at the level of the SL. Thus, an attempt will be made to determine whether endurance exercise training has any ultimate effect on a specific SL Ca^{2+} transport system, the Ca^{2+} channel. An increase in the density of these channels may contribute to a greater influx of calcium during the myocardial action potential. This could clearly provide a mechanism to explain the increase in stroke volume as a consequence of increased myocardial contractility.

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CHAPTER II

BINDING OF THE DIHYDROPYRIDINE PN200-110 TO HIGHLY-PURIFIED SARCOLEMMA FROM THE RAT HEART

Introduction

Since the DHP have proven to be useful probes in understanding Ca^{2+} channels and their role in skeletal, cardiac and smooth muscle function [1,2] conditions affecting *in vitro* binding of the radiolabelled 1,4-dihydropyridine (DHP), [^{3}H]PN200-110 (PN), to highly-purified sarcolemma were investigated. The relationship between the pharmacological doses required *in vivo* and the *in vitro* binding to sarcolemma (the generally accepted site of action) is, however, poorly understood. The dissociation constant (K_d) of the binding of DHP to sarcolemmal membranes is at least three orders of magnitude lower than the dose required for physiological effects [3,4]. Furthermore, the *in vitro* binding to cardiac plasma membranes is complex. It is influenced by non-DHP Ca^{2+} antagonists such as the phenylalkylamines and the benzothiazipines although they presumably bind at separate sites [5,6], consistent with observations in other tissues such as brain [7]. Additionally, DHP binding is modulated by Ca^{2+} [8] and perhaps membrane potential [9,10]. The *in vitro* binding experiments in cardiac muscle employing subcellular fractionation have yielded conflicting results. Sarmiento *et al.* [11] found that the specific binding of the DHP, nitrendipine (NTP) was significant only in preparations that were enriched in sarcolemma. Williams and Jones [12], on the other hand, found that a microsomal fraction with low sarcolemma marker enzyme activity exhibited considerably higher NTP specific binding than those with high activity. This study was undertaken to 1) clarify the *in vitro* requirements for PN binding to both the crude homogenate and subcellular fractions with varying degrees of sarcolemmal enrichment and 2) determine sarcolemma DHP receptor density.

Methods

Sarcolemmal Isolation. Hearts from Sprague-Dawley rats that were killed by cervical fracture were excised and rinsed with a homogenizing medium which contained 250 mM sucrose and 20 mM MOPS (pH 7.4 @ 37°C). Included in the homogenizing medium in some experiments were the following protease inhibitors: phenylmethanesulfonyl fluoride (1 mM), 1,10 phenanthroline (1 mM), iodoacetamide (1 mM) and antipain (1 mg L^{-1}). The hearts were perfused retrogradely through the aorta with homogenizing medium for about five minutes and then trimmed of connective tissue, fat and atria in homogenizing medium maintained at 4°C. Sarcolemma was isolated from eight to nine pooled hearts (ca 6 g wet wt.) in a manner similar to that described by Bers [13], as modified by Philipson *et al.* [14] and subsequently by Tibbits *et al.* [15]. The pooled hearts were minced in 10 ml of ice-cold homogenizing medium and homogenized with a Tekmar Tissumizer (Cincinnati, OH, Model TCM-1) at setting 45 for 2 bursts of 3 seconds each. The homogenate was filtered through 40 gauge stainless steel mesh and brought up to volume with an ice cold homogenizing medium. An aliquot of 1.5 ml of this crude homogenate was removed and stored at 4°C for future marker enzyme and DHP binding analyses. An aliquot of 5.6 ml of a KCl and sodium pyrophosphate solution was then added (1:10 dilution) so that the final concentrations were 100 and 25 mM, respectively. The suspension was stirred for ten minutes in the cold and then spun at 177,000 xg for one hour. After the supernatants were discarded, the pellets were resuspended in 20 ml of homogenizing medium and incubated with 30,000 Kunitz units of DNase for 45 min. at 30°C. The suspension was rehomogenized at setting 45 for one burst of 4 seconds and then spun at 800 xg for 10 min. The supernatant was carefully removed and then pelleted at 177,000 xg for one hour. The resulting pellet was resuspended in 45% sucrose and steps of 32, 30, 28 and 8% sucrose (each containing 0.4 mM NaCl) were successively layered on top. The gradients were spun for 16 h at 122,000 xg in a swing bucket rotor (SW28). After

fractionation of the gradients, the fractions were diluted with roughly equal volumes of an ice-cold solution containing 560 mM NaCl and 25 mM MOPS and allowed to passively equilibrate for 1.5 h at 4°C. The fractions were gradually diluted with ice-cold loading medium containing 140 mM NaCl and 10 mM MOPS. The fractions were then pelleted at 177,000 xg for 1.5 hours and resuspended in loading medium to yield a final concentration of 1.5-5 mg protein ml^{-1}. The fractions were both frozen and stored in liquid N_2 in cryogenic vials.

Sarcolemmal Characterization. Protein content in all fractions was determined by the method of Bradford [16] using bovine serum albumin as the standard. The activity of the sarcolemmal marker K^+*p*NPPase was assayed with modifications of a procedure we have described previously [15]. In brief, activity was determined in a medium containing 10-20 *u*g of sarcolemmal protein, 50 mM MOPS (pH 7.4 @ 37°C), 5 mM $MgCl_2$, 5 mM EGTA and 5 mM Na^+ *p*-nitrophenylphosphate in a final volume of 1 ml. The difference in activities with and without 50 mM KCl was used to measure activity because of the relative insensitivity of the rat heart to ouabain [17]. The tubes without KCl had 50 mM NaCl substituted. The reaction was initiated by incubation in a water bath at 37°C and then quenched after 20 minutes by reimmersing the tubes in a 4°C bath and adding 2 ml 1N NaOH. The tubes were read at 410 nm. Na-Ca exchange was determined in the sarcolemma fraction in a manner that we reported [18].

[^{3}H]PN200-110 Binding to Membranes. PN was chosen as it was the only radiolabelled DHP available commercially as a pure active enantiomer as opposed to a racemic mixture. [^{3}H]PN binding was measured in both the crude homogenate and subcellular fractions derived from the sucrose gradients. In general, the tubes contained (in a final volume of 5 ml) 50 mM MOPS (pH 7.4 @ 37°C), 10-40 *u*g of protein, 0.41 nM PN and 2.5 mM $CaCl_2$ and were incubated for 90 minutes at 22°C. In separate experiments, however, the time of incubation was varied between 5 and 90

minutes, the MOPS concentration ([MOPS]) was varied between 10 and 100 mM, the PN concentration was varied between 0.1 and 0.5 nM and $CaCl_2$ was varied between 0.002 and 5 mM. Assays were performed in both the presence and the absence of 1 uM unlabelled nifedipine in order to determine specific binding Fig. 1). All binding assays were conducted in dim (10 W) red light in order to prevent photoinactivation of the DHP [19]. Binding was terminated by rapid vacuum filtration through Whatman GF/C filters. The filters were then washed with three 4.5 ml aliquots of ice-cold buffer, placed in a vial and counted by standard liquid scintillation procedures.

Results

The sarcolemmal characteristics of the crude homogenate and the subcellular fractions from the sucrose gradients used in the majority of these experiments exhibited a mean K^+*p*NPPase activity of 25.3 $u$mol mg^{-1} hr^{-1} and a Na/Ca exchange V_{max} of 3.2 nmol mg^{-1} s^{-1}. The inclusion of the protease inhibitors in the homogenizing medium had no apparent effect on either the sarcolemmal characteristics (data not shown) or on the binding of the DHP to the sarcolemma and was, therefore, not routinely employed. The time course of PN binding is shown in Fig. 2. With the DHP and $CaCl_2$ concentrations equal to 0.41 nM and 2.5 mM, respectively, the binding required at least 60 minutes to reach equilibrium at room temperature. Linearization of these data by plotting $\ln[B_e/B_e-B_t]$ (where B_e and B_t are the amounts of binding at equilibrium and at any given time, respectively) as a function of time is shown in the inset of this figure. Because $[B]_e$ was less than 8% of $[L]_T$ for all assays, one can reasonably assume pseudo-first order kinetics [20]. With this assumption, the k_{obs} was calculated from the linear regression of the plot and found to be 0.046 min^{-1}. The 'on' rate constant (k_1) was determined from the equation:

$$k_1 = k_{obs} * [B_e]/([L_T] * [B_{max}])$$

where $[L_T]$ is the total ligand concentration (0.41 nM) and $[B_{max}]$ is the maximum concentration of binding sites. The 'off' rate constant (k_{-1}) was calculated from the equation:

$$k_{-1} = K_d * k_1$$

The dissociation constant (K_d) and the maximum density of binding sites (B_{max}) were derived from Scatchard plot analysis of equilibrium binding data (Fig. 3) and were found to be 0.09 nM and 2.0 pmol mg^{-1} $protein^{-1}$, respectively. From these data, the calculated 'on' and 'off' rate constants were $4.33 * 10^5$ M^{-1} s^{-1} and $3.90 * 10^{-5}$ s^{-1}, respectively.

The effect of varying the [MOPS] is shown in Fig. 4. All solutions used were substantially hypotonic with respect to the intravesicular tonicity (ca 290 mOsm L^{-1}). As illustrated in Fig. 4, peak specific binding is reached at an extravesicular [MOPS] of 20-30 mM which has a tonicity of about 25 mOsm L^{-1}. The effect of varying [MOPS] had a similar impact on total binding (data not shown). Compared to the total and specific binding, non-specific binding exhibited an inverse relationship with respect to MOPS tonicity and reached a minima at a [MOPS] of around 20-30 mM.

Specific PN binding at a ligand concentration of 0.4 nM was determined in all subcellular fractions as well as the crude homogenate. As shown in Fig. 5, specific PN binding to vesicles derived from the sucrose gradient was reasonably-well correlated to the specific activity of the sarcolemmal marker K^+*p*NPPase. Least-squares linear regression of these data (forced to intercept at the origin) yielded a slope of $7.36 * 10^{-3}$ and a correlation coefficient (R^2) of 0.84. Specific binding to the crude homogenate clearly violated this relationship and the goodness of fit described above did not include data from the homogenate, which in general had higher specific binding per unit sarcolemma marker activity than the sucrose gradient derived fractions.

The calcium dependence of PN binding is illustrated in Fig. 6. Specific binding of this ligand was maximized at an extracellular calcium concentration of 2.5 mM (*p*Ca of 2.6).

Discussion

These experiments confirm the complexity of the binding of PN to rat ventricular plasma membranes. With a Hill coefficient close to unity, a linear Scatchard plot (correlation coefficient of 0.95) and a dissociation constant (K_d) of 0.09 nM, the results of this study are consistent with ligand binding to a single population of high affinity sites. The presence of a single class of high-affinity binding sites in isolated heart plasma membranes is consistent with several other studies [11,21]. Both high and low affinity binding sites, however, were observed by Marsh *et al.* [22] in intact chick cardiac myocytes and by de Bruyn Kops *et al.* [23] in isolated rat heart membranes. It has been suggested that intact cells do not exhibit a unique binding affinity for DHP and that the high affinity binding observed in isolated membrane preparations may reflect binding to the depolarized state (inactivated) of the calcium channel [9,10].

The PN binding site appears to be associated with the sarcolemma. Linear regression analysis of sarcolemmal marker enzyme activity, K^+*p*NPPase (represents a partial reaction of Na^+/K^+ ATPase pump [24]), and DHP specific binding in subcellular fractions reveals a relatively strong linear relationship although there is a slight tendency towards a rectangular hyperbolic function (Fig. 4). Linearity of DHP binding and sarcolemma marker activity is in agreement with Grover *et al.* [21] in both cardiac and smooth muscle preparations. This further substantiates copurification of the DHP binding site and the sarcolemma as evidenced by several other investigators [11,25]. Williams & Jones [12], however, found that sarcolemmal marker specific

activity in the subcellular fractions from the myocardium did not correlate with specific DHP binding sites. The highest NTP specific binding was found in a ryanodine-sensitive microsomal fraction with less than 5% of the specific activity of sarcolemmal markers found in the sarcolemmal-enriched fractions. In skeletal muscle, high specific NTP binding has also been reported to be associated with fractions enriched in sarcoplasmic reticulum [26] or T-tubules [27]. While the density of DHP is highest in skeletal muscle T-tubules, the K_d is at least an order of magnitude higher than that observed in both cardiac and smooth muscle sarcolemma. These findings suggest that the DHP binding site is qualitatively different in skeletal muscle and the observation that only a small percentage of these sites are functional Ca^{2+} channels [28] may not be valid in cardiac muscle. Thus, the variations in DHP binding reported in different studies may be a consequence of differences in the techniques, tissues and perhaps to species. It is possible that sarcolemma Ca^{2+} channels may be recruited from some intracellular location or site of synthesis, such as the sarcoplasmic reticulum. This could explain the large reserve of dormant channels apparent in skeletal muscle.

The relatively high specific binding of the ligand to the crude homogenate compared to the subcellular fractions, when normalized per unit sarcolemmal marker enzyme activity, suggests the presence of a binding site regulatory factor which may be lost or altered in the isolation of sarcolemma. This may result in either an underestimation of PN binding sites in the highly-purified sarcolemma fraction or an overestimation in the crude homogenate. Preliminary studies in our laboratory have implicated calmodulin in the modulation of DHP binding [29].

To characterize PN binding further, several methodological procedures were examined more closely. The equilibration of PN binding to isolated sarcolemma was found to require at least 60 minutes (Fig. 2) at 23°C. The time course for DHP binding to highly-purified sarcolemma is comparable to that in previous experiments [5,8] using cruder preparations and illustrate the slow kinetics of DHP binding *in vitro*.

The kinetic 'on' ($4.33 * 10^5$ M^{-1} s^{-1}) and 'off' ($3.90 * 10^{-5}$ s^{-1}) rate constants of PN binding were approximately one-order of magnitude lower than previously found for NTP [5], although the association rate constant was comparable to that found by Glossmann & Ferry [8]. The discrepancy may be due to differences in the ligands being used, preparation purity or more likely to the techniques used to analyze binding kinetics.

Use of a hypotonic buffer precludes differential binding of the ligand to various subcellular fractions, resulting from differential vesicular orientation. An extravesicular MOPS concentration of 20-30 mM maximized PN binding (Fig. 4), a consequence of both increased total and decreased non-specific ligand binding. While the increased total binding is consistent with vesicular rupturing due to hypotonic shock, the explanation of a decrease in non-specific binding is unknown but may be associated with the low ionic strength.

PN binding was maximal in the presence of an extravesicular calcium concentration of 2.5 mM (Fig. 6). This finding corroborates with the results of several other studies that demonstrate an extracellular cation requirement for DHP binding [8,30,31] and adds support to the notion that DHP binds to the Ca^{2+} channel or a protein associated with the channel [32].

Following an analysis similar to that of Colvin *et al.* [33], an estimate of receptor site density was obtained from a knowledge of the specific phospholipid content of the sarcolemmal vesicles and the B_{max} derived from Scatchard plots. In the calculations, it is assumed that the ligand binds to functional Ca^{2+} channels in the sarcolemma, the binding sites are distributed randomly on the sarcolemmal surface, one-half of the phospholipid molecules contribute to the surface area of each monolayer of the membrane bilayer and 80% [14] of the membrane is composed of phospholipids. Given that the specific phospholipid content of sarcolemma from the rat heart is 0.75 umol mg^{-1} $protein^{-1}$ [15], the average surface area occupied by each

phospholipid molecule is 60 A^2 in the liquid-crystalline state [34] and the B_{max} is 2.0 pmol mg^{-1} $protein^{-1}$, it is estimated that there are approximately 3.6 PN binding sites um^{-2} sarcolemma. From whole cell electrophysiological measurements of membrane capacitance in rat cardiac myocytes by Bean [35] and assuming a specific membrane capacitance of 1 uF cm^{-2}, myocyte surface area is estimated to be 3.1 * 10^3 um^2. The derived receptor number per myocyte is, therefore, 11.2 * 10^3. This is within the range of functional Ca^{2+} channel density (1.7 to 7.5 * 10^3) estimates from patch clamp analysis of tissue cultured rat heart [36] although it is an order of magnitude higher than previously found in isolated sarcolemma [33]. The latter could be explained by the higher specific phospholipid content apparent in canine sarcolemma and the use of a different ligand (NTP). The similarity found in DHP binding site density estimations and functional Ca^{2+} channel density determination [36] provides stronger evidence for the association of the myocardial sarcolemma DHP site with the Ca^{2+} channel.

The current results clarify the requirements for *in vitro* binding of the DHP PN to highly-purified sarcolemma. While the results illustrate the complexity of the binding process, the experiments also offer support for the hypothesis that the DHP receptor site in the myocardium represents the calcium channel or a protein closely associated with it.

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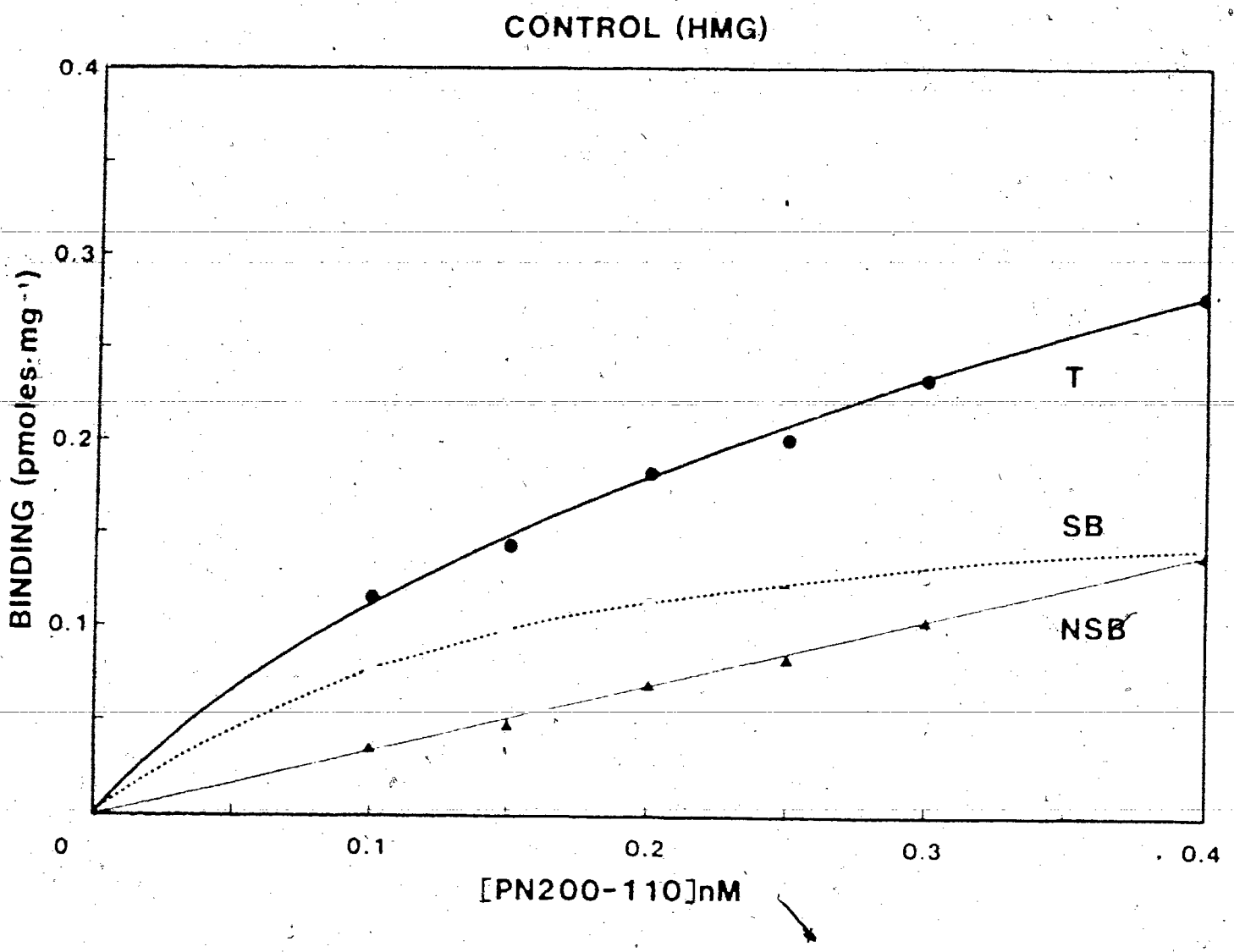
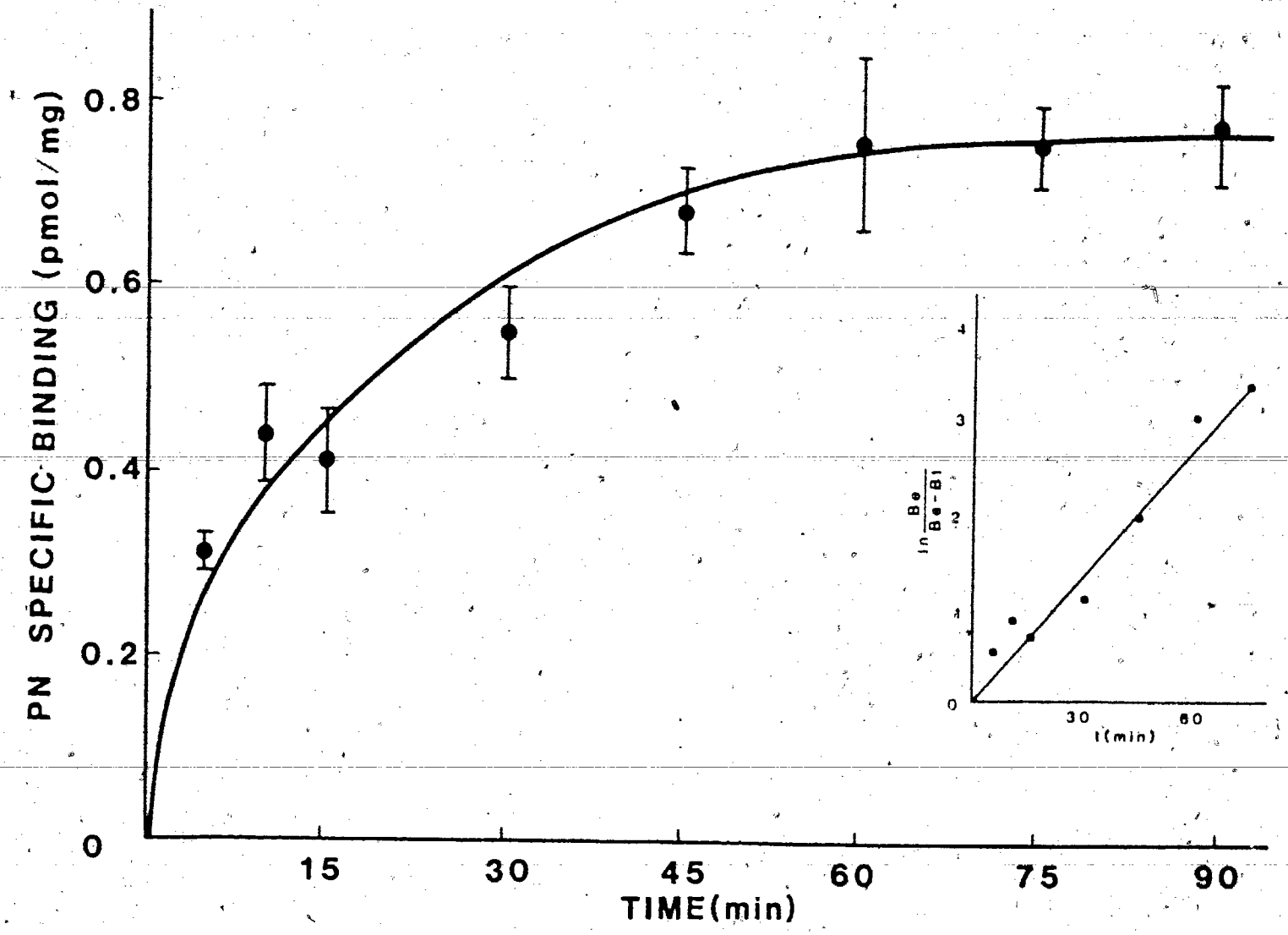


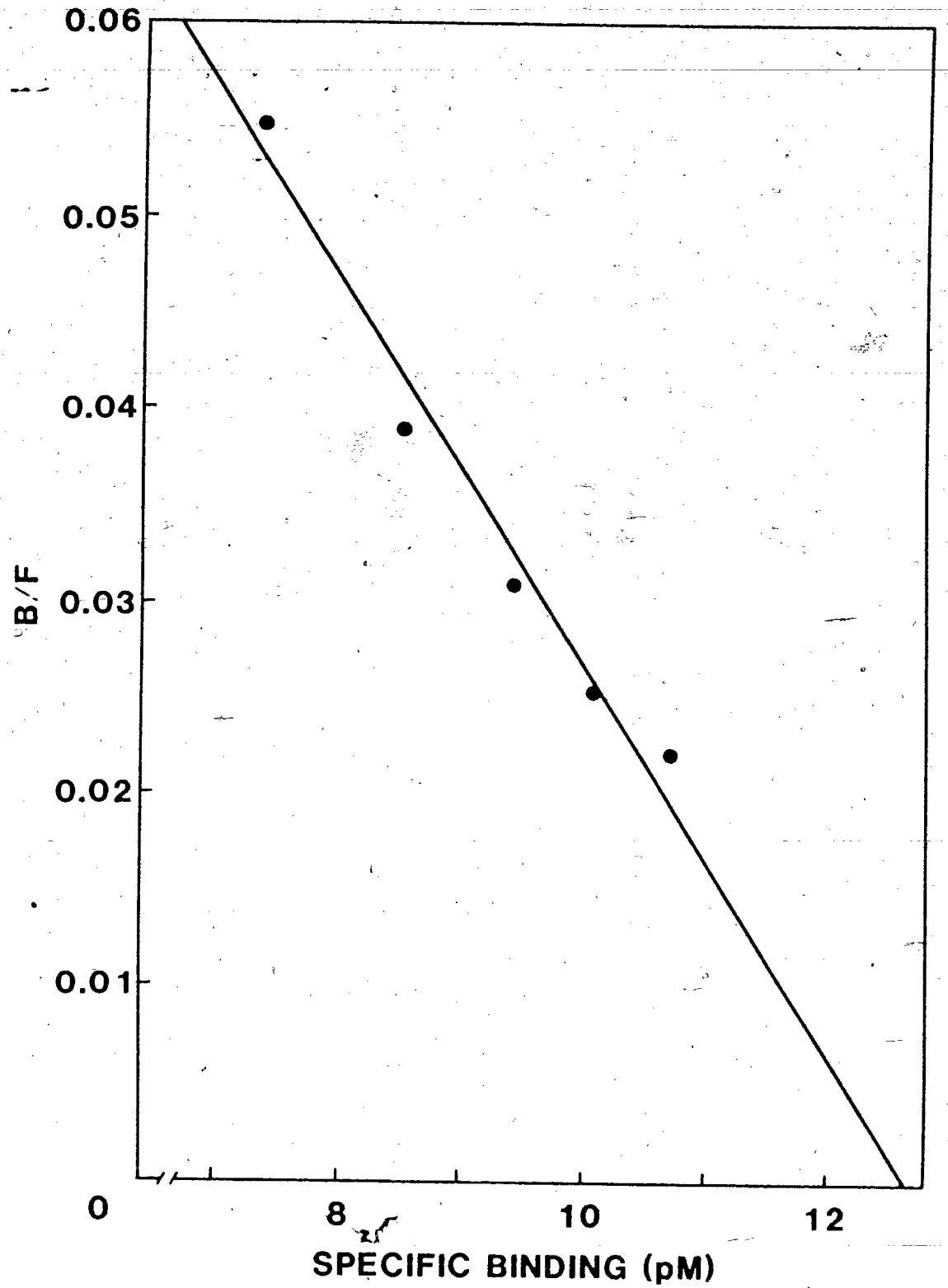
PN200-110 binding (pmoles/mg protein) to crude homogenate as a function of PN200-110 concentration

Fig. 1. Specfic binding (SB) is defined as the difference between total (T), and non-specific binding (NSB), in the absence and presence of nifedipine, respectively. Buffer and $CaCl_2$ concentrations were 25 and 2.5 mM, respectively and the incubation time was 90 min. at 23°C.



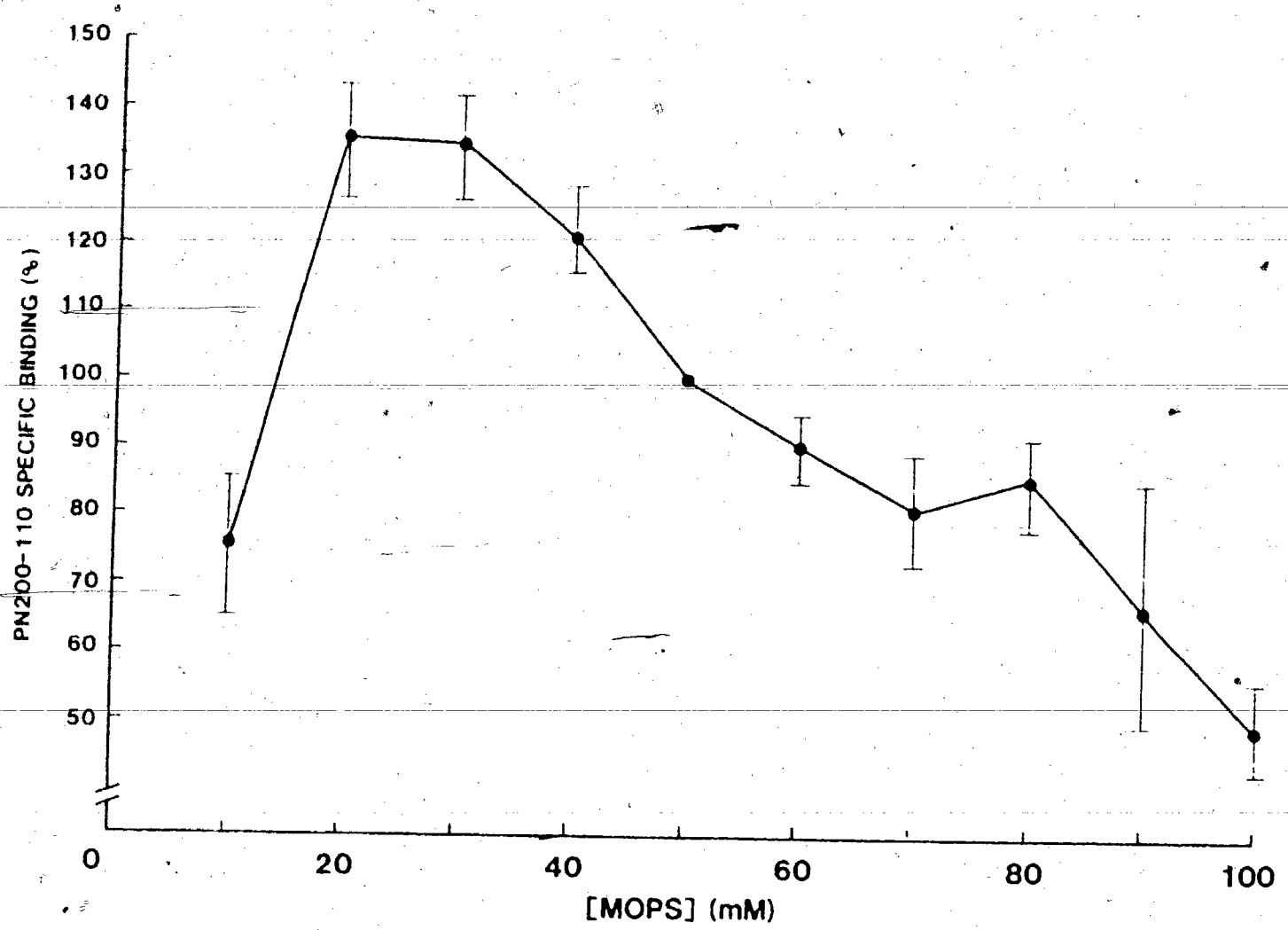
Time course of PN200-110 specific binding to highly-purified sarcolemma

Fig. 2. PN200-110, buffer and $CaCl_2$ concentrations were 0.41 nM, 50 mM and 2.5 mM, respectively, temperature was 23°C and number of different preparations was 4. Bars represent ± SEM. The inset shows a linearization of these data in which $\ln(B_e/[B_e-B_t])$ was plotted as a function of time in order to generate K_{obs} (where B_e is the specific binding at equilibrium and B_t is the specific binding at time t).



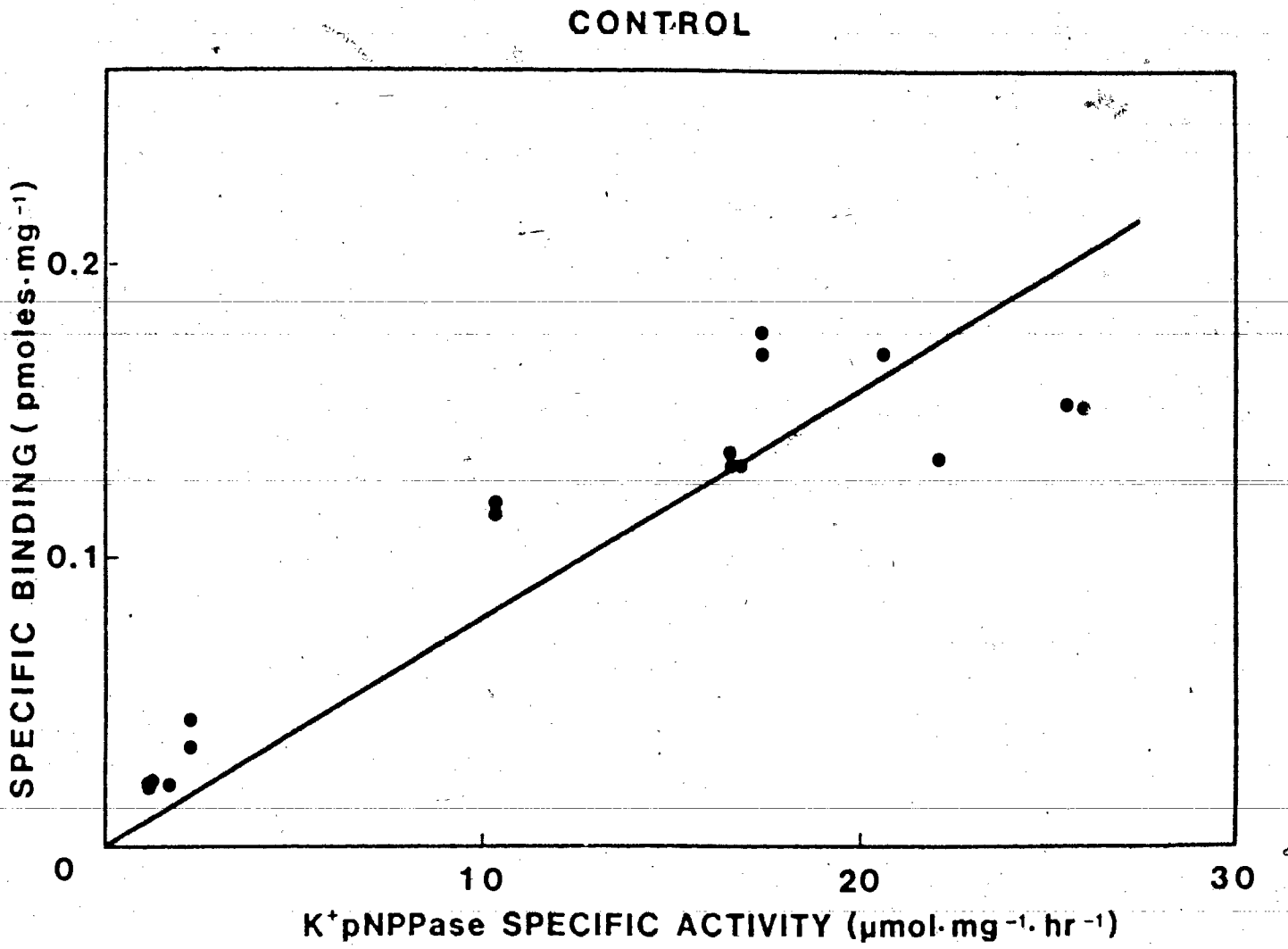
Scatchard analysis of PN200-110 binding to highly-purified sarcolemma

Fig. 3. $CaCl_2$ and buffer concentrations were 2.5 and 50 mM, respectively and the incubation time was 90 min. at 23°C.



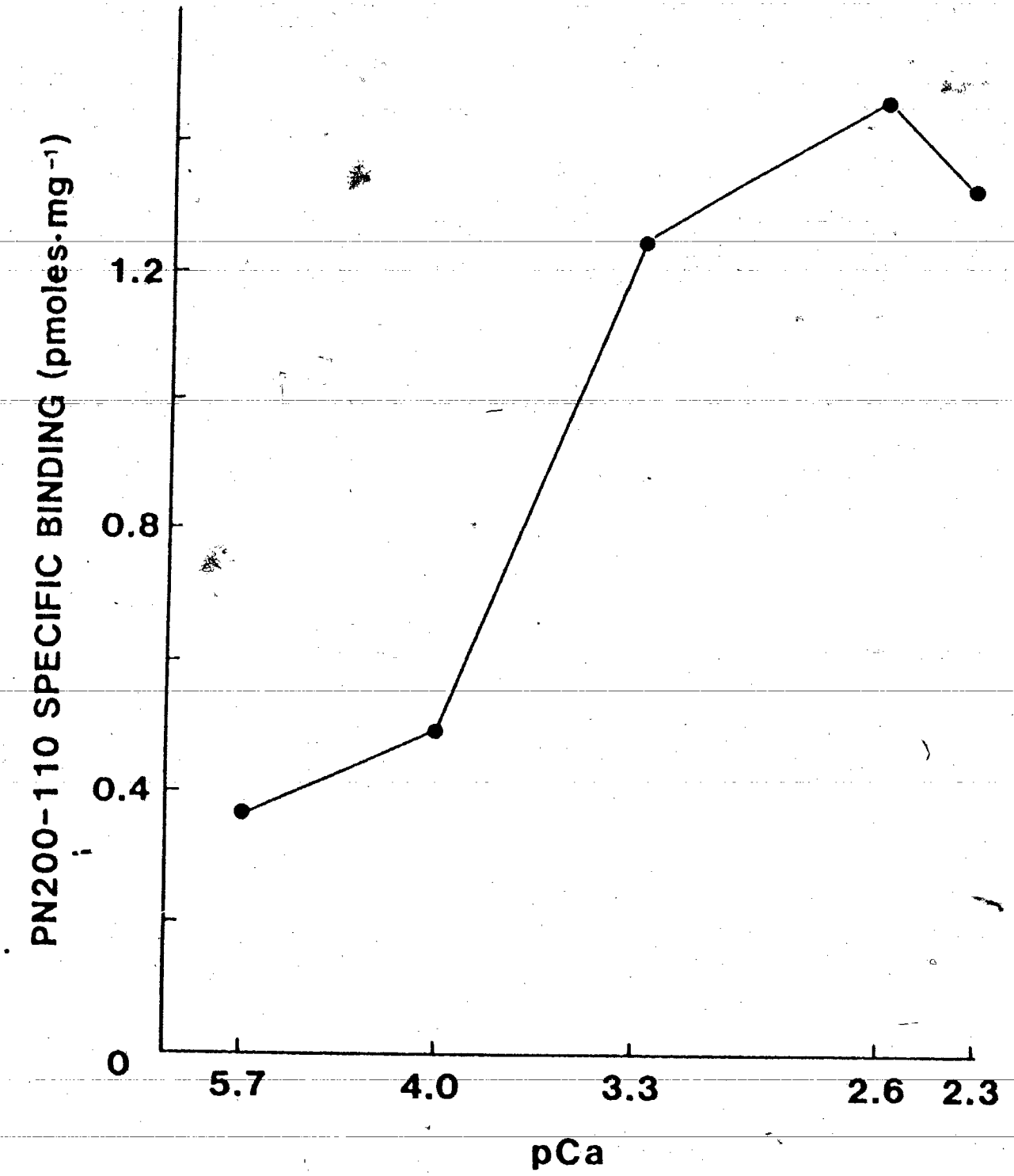
PN200-110 specific binding to highly-purified sarcolemma as a function of buffer concentration

Fig. 4. PN200-110 and $CaCl_2$ concentrations were 0.40 nM and 2.5 mM, respectively, the incubation time was 90 min. at 23°C and n= 5. Buffer concentration was varied between 10 and 100 mM and specific PN200-110 binding was expressed as a percentage of binding at a buffer concentration of 50 mM. Bars represent ± S.E.M.



Specific PN200-110 binding vs sarcolemma marker (K^+*p*NPPase) activity

Fig. 5. Specific binding of PN200-110 at equilibrium to subcellular fractions as a function of the specific activity of the sarcolemmal marker, K^+*p*NPPase. PN200-110 and $CaCl_2$ concentrations were 0.40 nM and 2.5 mM, respectively and the incubation time was 90 min. at 23°C. Enzyme assay was performed as described in Methods.



Ca^{2+}-dependence of PN200-110 specific binding to highly-purified sarcolemma

Fig. 6. PN200-110 concentration was 0.41 nM and incubation time was 90 min. at 23°C. $CaCl_2$ concentration was varied between 0.002 and 5 mM where pCa is -log $[Ca^{2+}]$ M.

CHAPTER III

Ca^{2+}-DEPENDENCE OF PN200-110 BINDING TO HIGHLY-PURIFIED SARCOLEMMA OF THE RAT HEART

Introduction

The present study was undertaken to characterize further the calcium dependence of PN200-110 binding to highly-purified myocardial sarcolemma. Radiolabelled calcium (Ca^{2+}) antagonists have become an important tool for gaining information about calcium channels found in the plasma membranes of various tissues. The 1,4-dihydropyridines (DHP) in general and PN200-110 specifically, are most useful because of their apparent high binding specificity and potency (1,2). It has been demonstrated that high-affinity radiolabelled DHP binding is dependent upon the presence of divalent cations, especially the alkaline earth metals (3,4,5,6,7) including Ca^{2+} or Mg^{2+}. In both smooth and cardiac muscle binding is lost upon removal of divalent cations and is restored by the addition of low concentrations of Ca^{2+} (7). The role of this divalent cation site, however, is not known. The apparently high affinity of the divalent cation site for Ca^{2+} (8) and the observation that calmodulin antagonists inhibit DHP binding (7) suggests the presence of a Ca^{2+} binding protein regulating DHP binding and perhaps Ca^{2+} channel function. This may give some insight to a previous observation (9) that specific PN200-110 binding (pmol·mg^{-1} protein^{-1}) *in vitro* was comparable in a fraction in which sarcolemmal marker activity was increased about 25-fold to that in the crude homogenate. It was suggested that a Ca^{2+} binding protein was altered or lost during the isolation procedure. The purpose of this study was to determine the extent of Ca^{2+} and calmodulin involvement in PN200-110 binding allowing for further characterization of PN200-110 binding to highly-purified myocardial sarcolemma.

Methods

Sarcolemmal isolation and characterization. The sarcolemmal isolation technique, described in detail previously (9), follows that of Bers (10) as modified by Philipson *et al.* (11) and subsequently by Tibbits *et al.* (12). Protein content in the subcellular fractions was determined by the method of Bradford (13), using bovine serum albumin as the standard. Sarcolemmal marker enzyme activity, K^+*p*NPPase, was measured as described previously (9).

[^{3}H]PN200-110 binding. [^{3}H]PN200-110 (PN) binding was measured in the highly-purified sarcolemmal fraction harvested from a sucrose gradient and follows the procedure outlined previously by Tibbits *et al.* (9). In general, for Scatchard analyses, tubes were prepared with PN (78 Ci/mmol, New England Nuclear) concentrations ranging from 0.15 to 0.5 nM, 2.5 mM $CaCl_2$, 50 mM MOPS (pH 7.4 @ 37°C), 0.5% v/v absolute ethanol (95-100%), 10-40 ug protein and distilled/deionized water to bring the tubes to a final volume of 5 ml. In separate experiments, $CaCl_2$ was varied between 0.002 - 5.0 mM and calmodulin was varied between 10 - 1000 nM. The concentrations of the putative calmodulin antagonists were varied between 0.05 to 2.0 *u*M and 5 to 200 *u*M for calmidazolium (R24571) and trifluoperazine (TFP), respectively. All assay tubes were incubated for 90 minutes at room temperature.

Results

The sarcolemmal characteristics included a specific K^+*p*NPPase activity of 25.3 umol mg^{-1} hr^{-1} and a Na/Ca exchange V_{max} of 3.2 nmol mg^{-1} s^{-1}.

Specific binding of PN as a function of ligand concentration was maximized in the presence of 2.5 mM Ca^{2+} (Fig. 1 & 2). A sigmoidal relationship between Ca^{2+} concentration ($[Ca^{2+}]$) and PN specific binding was found as illustrated in Fig. 2. Scatchard analysis of equilibrium PN binding for any given $[Ca^{2+}]$ (Fig. 3) produced linear plots. At a Ca^{2+} concentration of 2.5 mM, enhanced ligand binding appears to

be associated with an increase in maximum receptor density (B_{max}) (Fig. 3; Fig. 4) and binding affinity (decreased K_d) (Fig. 4). Hill plot analysis of equilibrium PN binding at varying [Ca^{2+}] is shown in Table 1, where n and r^2 are the Hill and correlation coefficients, respectively. With increasing [Ca^{2+}] there was a decrease in non-specific ligand binding (results not shown) although it did not appear to account for the several fold increase in specific binding observed at higher [Ca^{2+}].

The calmodulin antagonists, calmidazolium and TFP, inhibited PN specific binding in a dose-dependent manner, as shown in Fig. 5. The presence of the calmodulin antagonists appeared to have little effect on non-specific ligand binding (results not shown) although specific binding was considerably decreased.

Specific PN binding (0.40 nM) was enhanced by approximately two-fold in the presence of 50 nM calmodulin (Fig. 6). This was associated with an increase in total and a decrease in non-specific ligand binding (results not shown).

Illustrated in Fig. 7 is the Ca^{2+}-dependence of calmodulin (moles of Ca^{2+} bound per mole of calmodulin) and PN specific binding (at 0.5 nM). Ca^{2+} concentration is expressed as pCa^{2+} (-log[Ca^{2+}]).

Discussion

The nature of the Ca^{2+}-dependence of PN binding to highly-purified sarcolemma from the rat heart has been investigated. The equilibrium binding of PN to sarcolemma was associated with a linear Scatchard plot (Fig. 4) and a Hill coefficient close to unity (Table 1), for any given [Ca^{2+}]. These findings confirm previous observations of binding to a single non-interacting set of sarcolemmal PN receptor sites.

Ca^{2+}-dependence of PN binding was clearly illustrated in this study and is in accordance with various other studies (3,4,5,6) which indicated that alkaline earth metals were critical for DHP binding. A Hill coefficient close to unity was obtained

suggesting the absence of cooperativity between the DHP receptor/Ca^{2+}/PN interaction. Glossmann *et al.* (3), however, observed positive cooperativity, as assessed by Hill plot analyses, between receptor/Ca^{2+}/nitrendipine (NTP) interaction when the [Ca^{2+}] was varied between 0.1 and 1000 *u*M. The inconsistent findings may be accounted for by the different ligands or the [Ca^{2+}] ranges (0.1 to 1000 *u*M and 2 to 5000 *u*M by Glossmann *et al.* (3) and in the present study, respectively) employed.

Increasing extravesicular [Ca^{2+}], to an optimum of 2.5 mM (Fig. 4), was associated with an increase in ligand binding affinity (decrease K_d). Whether the increase in affinity was a consequence of an altered kinetic association (k_1) or dissociation (k_{-1}) constant was not established. The increase in binding affinity was associated with an increase in PN receptor site density (B_{max}), suggesting a binding site alteration that may allow for a more accurate enumeration of PN sites. The higher K_d and lower receptor site density evidenced at micromolar [Ca^{2+}] are consistent with unsaturated ligand binding (Fig. 1 & 3). DHP binding to the sarcolemmal Ca^{2+} channel is suggested by these observations and the finding that the affinity of the Ca^{2+} channel for Ca^{2+} is < 1 *u*M (8) similar to that for the cation restoration of DHP binding (7), upon cation-depletion.

The apparent inhibition of PN binding in the presence of calmodulin antagonists, calmidazolium and TFP, is in accordance with Janis *et al.* (7) and suggests an involvement of calmodulin in the binding of DHP to the sarcolemma. At a calmidazolium concentration of 1 *u*M and TFP concentration of 10 *u*M, inhibition of PN binding was approximately 75 and 25%, respectively. The former is comparable to that found for inhibition of the DHP, NTP binding (83%) (7) although the latter is lower by about 30% (7). As shown in Table 2, the IC_{50} (concentration at which 50% of the activity is inhibited) values obtained for calmidazolium (0.28 uM) and TFP (28 uM) are in the same range as those found for other processes thought to be calmodulin-mediated, such as Ca^{2+}-ATPase (14, 15, 16) and phosphodiesterase (17). The

inhibition of PN binding by calmodulin antagonists, however, may be a non-specific effect related to the presence of a hydrophobic surface associated with the DHP binding site (7), similar to that on calmodulin and calmodulin-binding proteins (18).

An involvement of calmodulin in the binding of PN was substantiated by the finding that the addition of 50 nM calmodulin enhanced PN specific binding by greater than 2-fold (Fig. 6). Preliminary evidence suggests that the addition of calmodulin increases the B_{max} with no apparent effect on the binding affinity (data not shown). Glossmann & Ferry (3), however, found that the addition of purified calmodulin to membranes had no apparent effect on NTP binding and NTP did not bind to calmodulin in a direct centrifugation binding assay. The differences may be a consequence of different ligands employed or more likely to the sarcolemmal isolation technique used. If the sarcolemma in the above mentioned study, were not calmodulin depleted then the further addition of calmodulin would probably not enhance NTP binding. Clearly, further experimentation (including extraction and quantification of membrane bound calmodulin) is required to determine if the isolation procedures can account for the inconsistent findings.

Glossmann & Ferry (4) also found that DHP receptor sites were heat sensitive in contrast to the finding that calmodulin is a heat stable protein (19). The inclusion of 1 mM Ca^{2+}, however, protected the drug receptor against heat inactivation (4). This observation and the finding that the binding of the DHP, NTP, was actually heat stable (4), suggests a possible Ca^{2+}-dependent role of calmodulin in NTP binding. The former observation is in accordance with the Ca^{2+}-dependence of calmodulin binding observed in rat brain microsomal fractions (20). Ca^{2+}-dependence of calmodulin and PN binding was apparent but their Ca^{2+}-sensitivities differ by several fold (Fig. 7), questioning the extent of calmodulin involvement in PN binding. The presence of either an optimal extravesicular Ca^{2+} or calmodulin concentration was associated with an increase in total and a decrease in non-specific PN binding. The similar effects of

Ca^{2+} and calmodulin on PN binding implicate an involvement of Ca^{2+}-dependent calmodulin in PN binding and would parallel the observation that calmodulin is required for full activation of the Ca^{2+} channel (21).

If in fact calmodulin is involved in the binding of PN to myocardial sarcolemma, it can be postulated that a loss or alteration in calmodulin during the experimental procedure would decrease PN binding. This would provide an explanation for the comparable specific binding of PN in the crude homogenate and highly-purified sarcolemmal fraction found previously (9). The addition of Ca^{2+} to the isolation process may circumvent the loss of calmodulin.

The results of this study provide further clarification of the *in vitro* sarcolemmal DHP receptor site and requirements for PN binding. Ca^{2+}-dependence of ligand binding and the possible involvement of calmodulin in association to the receptor site are consistent with the postulation that binding of PN is to the sarcolemmal Ca^{2+} channel.

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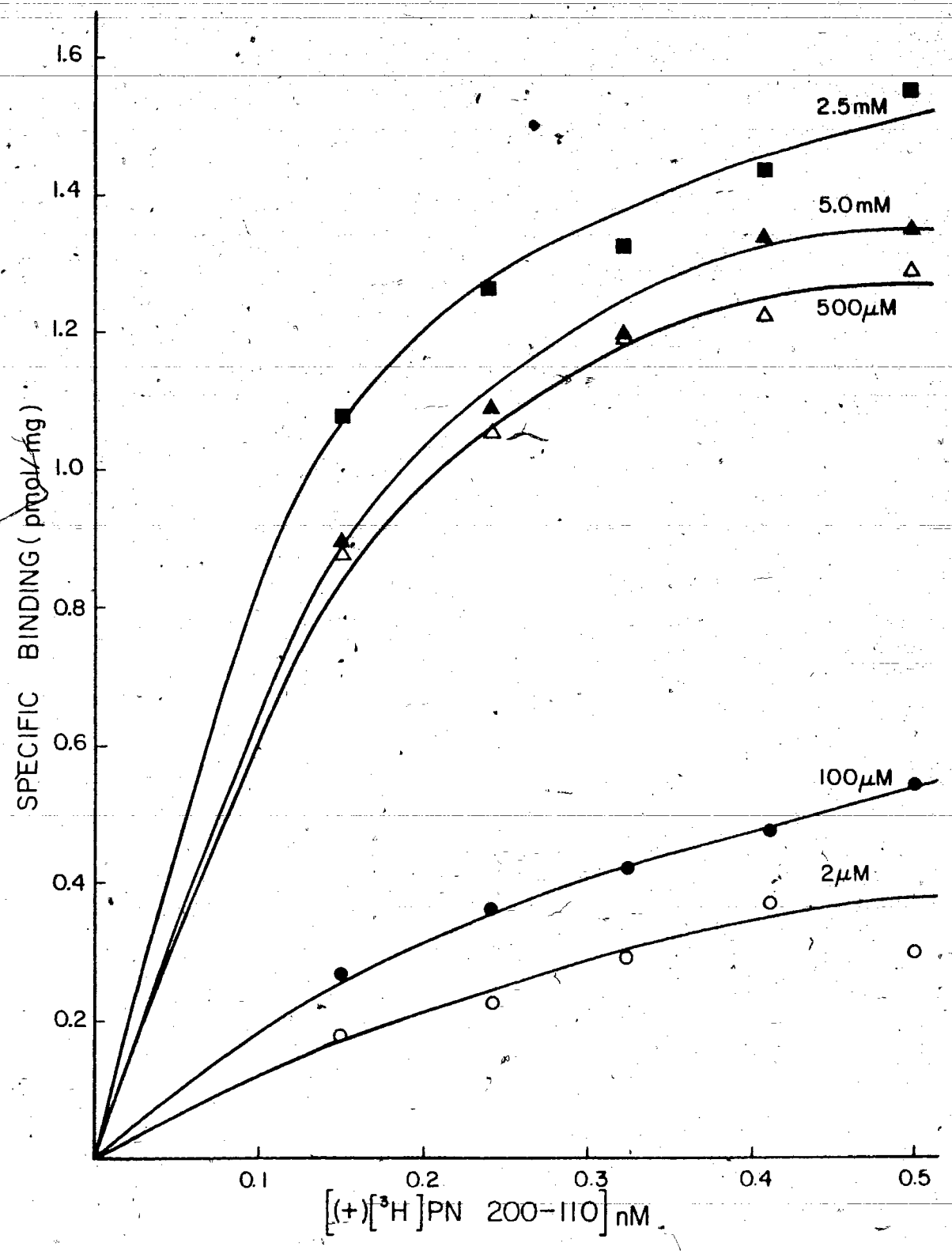
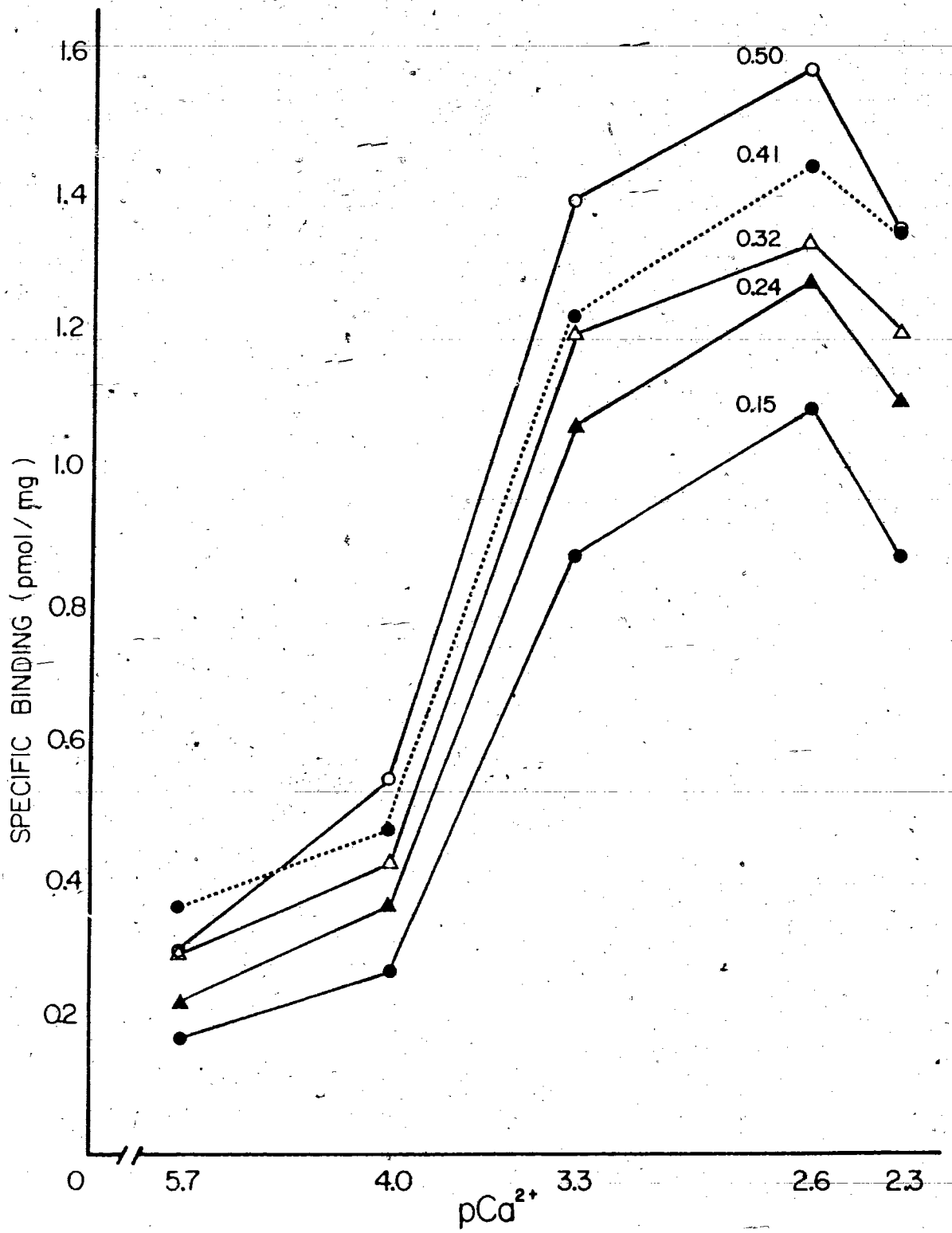


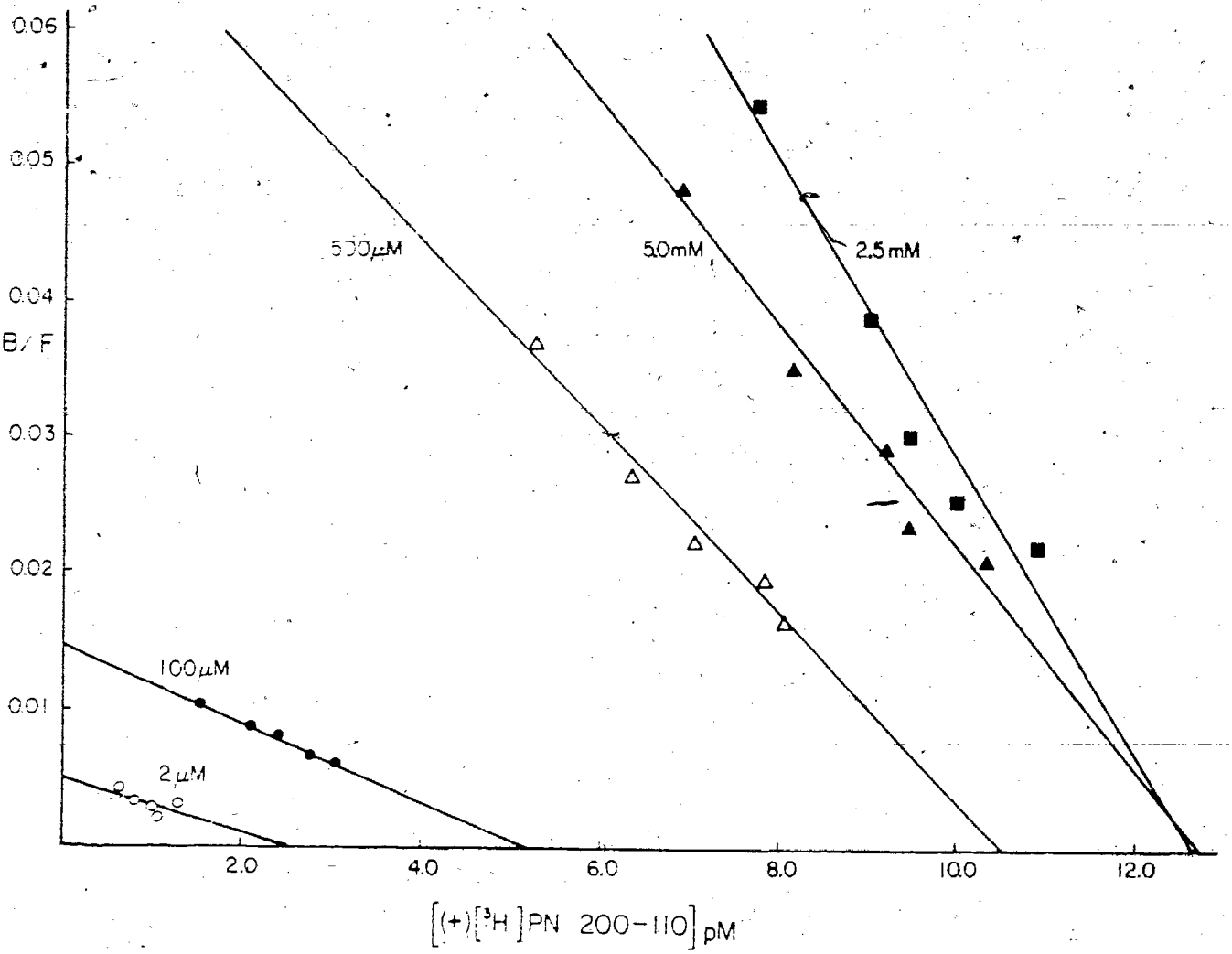
Ca^{2+}-sensitivity curves of PN200-110 binding

Fig. 1. Ca^{2+}-sensitivity curves of PN200-110 specific binding as a function of ligand concentration in highly-purified sarcolemma. $CaCl_2$ and PN200-110 concentrations were varied between 0.002 to 5 mM and 0.15 to 0.5 nM, respectively. Buffer concentration was 50 mM and the incubation time was 90 min. at 23°C.



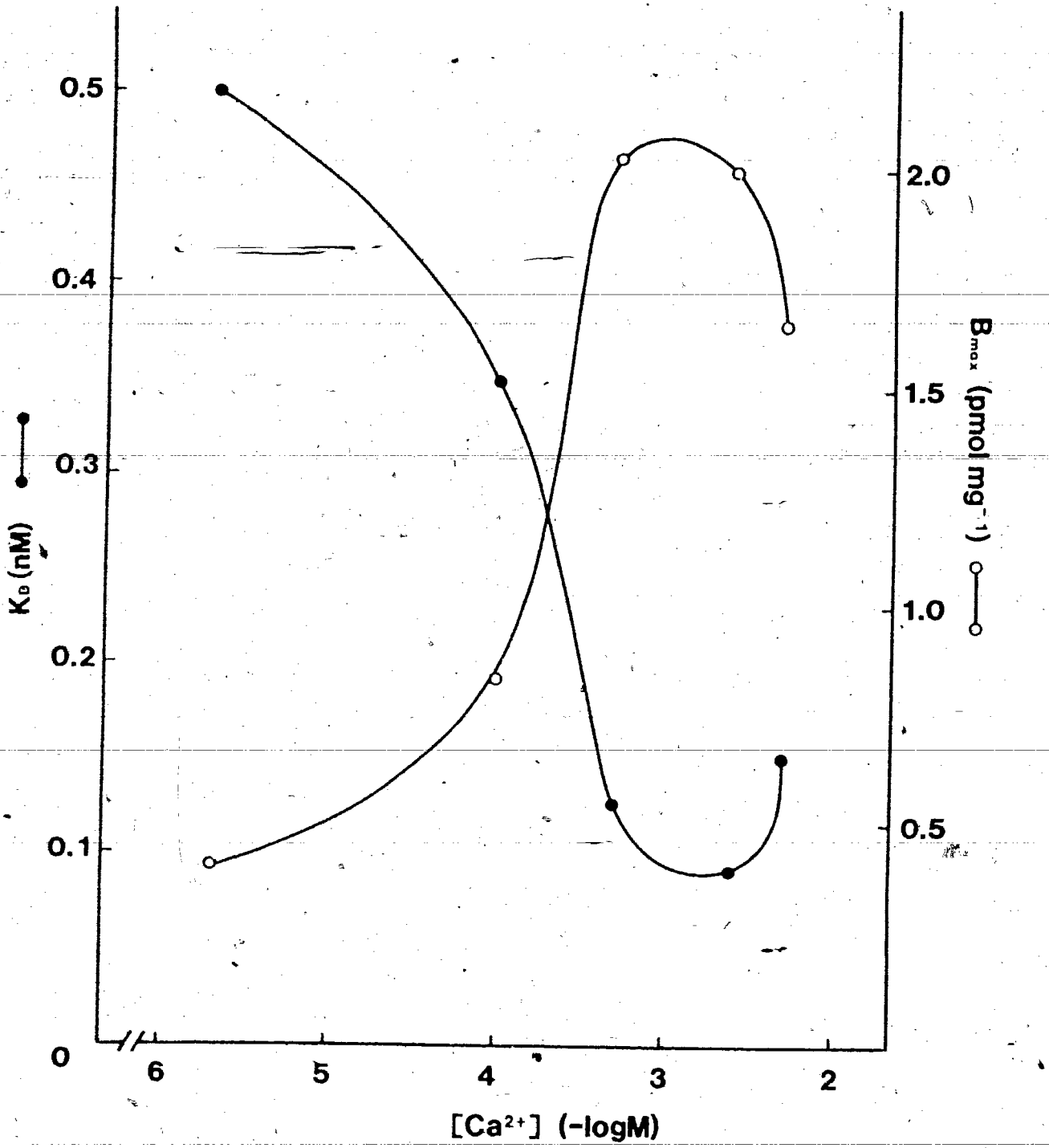
Specific PN200-110 binding as a function of Ca^{2+} concentration

Fig. 2. Specific PN200-110 binding as a function of Ca^{2+} concentration (expressed as *p*Ca (-log[Ca^{2+}])) and ligand concentration in highly-purified sarcolemma. $CaCl_2$ and PN200-110 concentrations were varied between 0.002 to 5.0 mM and 0.15 to 0.50 nM, respectively. Extravesicular buffer concentration was 50 mM and the incubation time was 90 min. at 23°C.



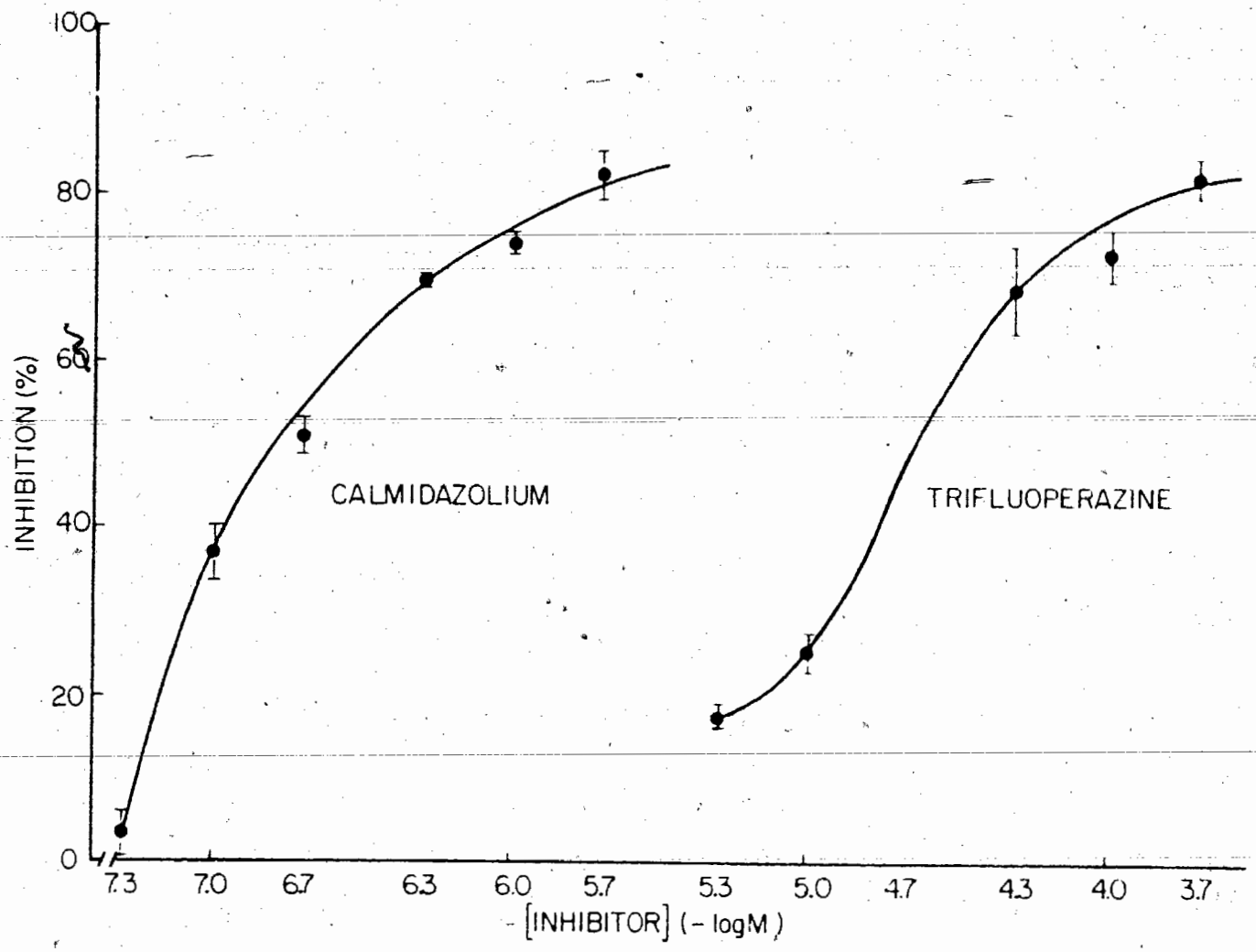
Scatchard analysis of PN200-110 binding at varying Ca^{2+} concentrations

Fig. 3. Scatchard analysis of equilibrium PN200-110 binding to highly-purified sarcolemmal preparations where Ca^{2+} concentration was varied between 0.002 to 5 mM. For Scatchard plot analysis (B/F (specifically bound PN200-110/free PN200-110) vs specific PN200-110 binding) where PN200-110 concentration was varied between 0.15 to 0.5 nM. Extravesicular buffer concentration was 50 mM and the incubation time was 90 min. at 23°C.



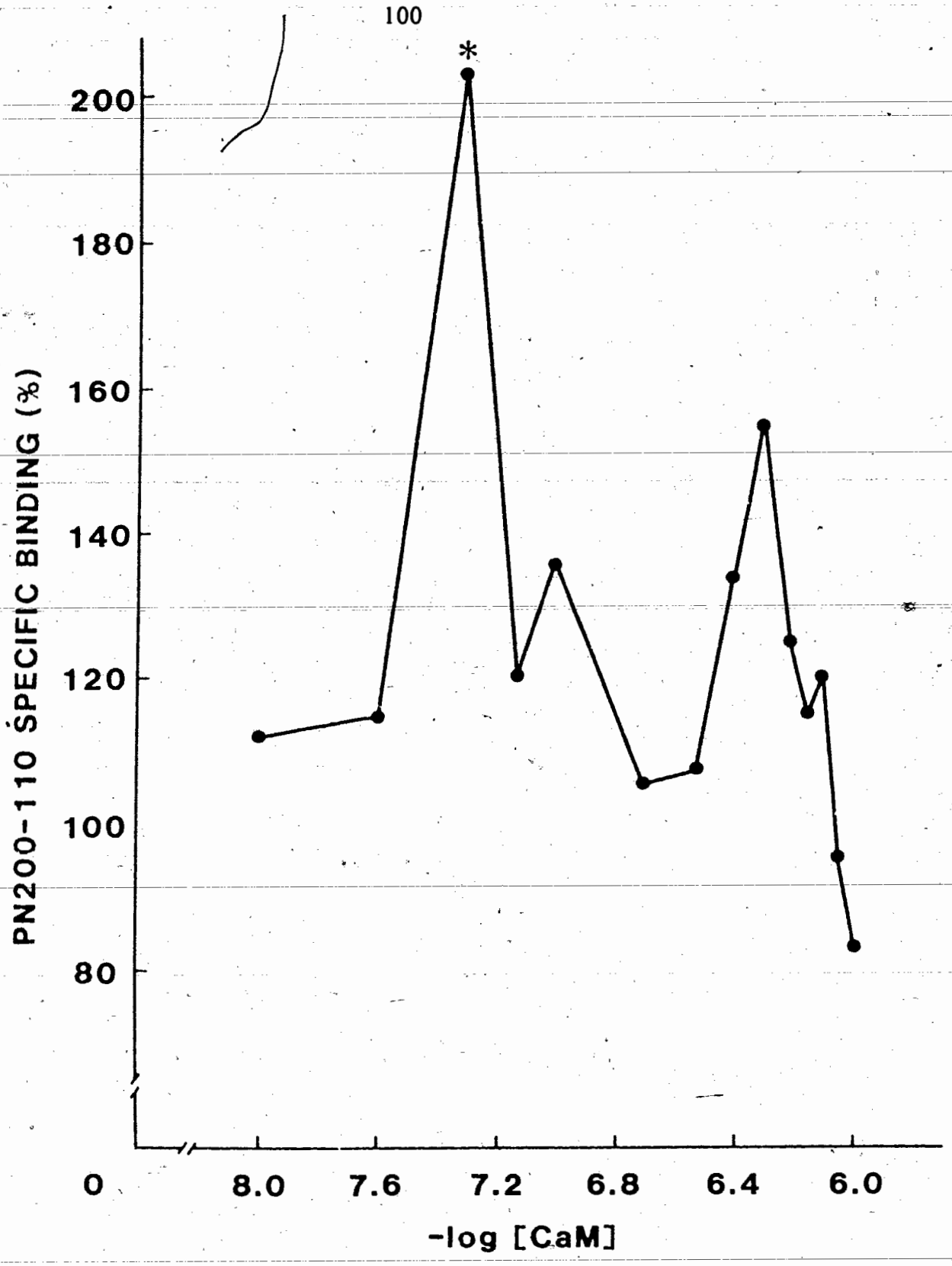
The effect of varying Ca^{2+} concentrations on the binding affinity and sarcolemmal receptor density of PN200-110

Fig. 4. PN200-110 binding affinity (K_d) (●) and maximum PN200-110 receptor density (B_{max}) (○) in highly-purified sarcolemma as a function of Ca^{2+} concentration (expressed as *p*Ca). Extravesicular buffer concentration was 50 mM and the incubation time was 90 min. at 23°C.



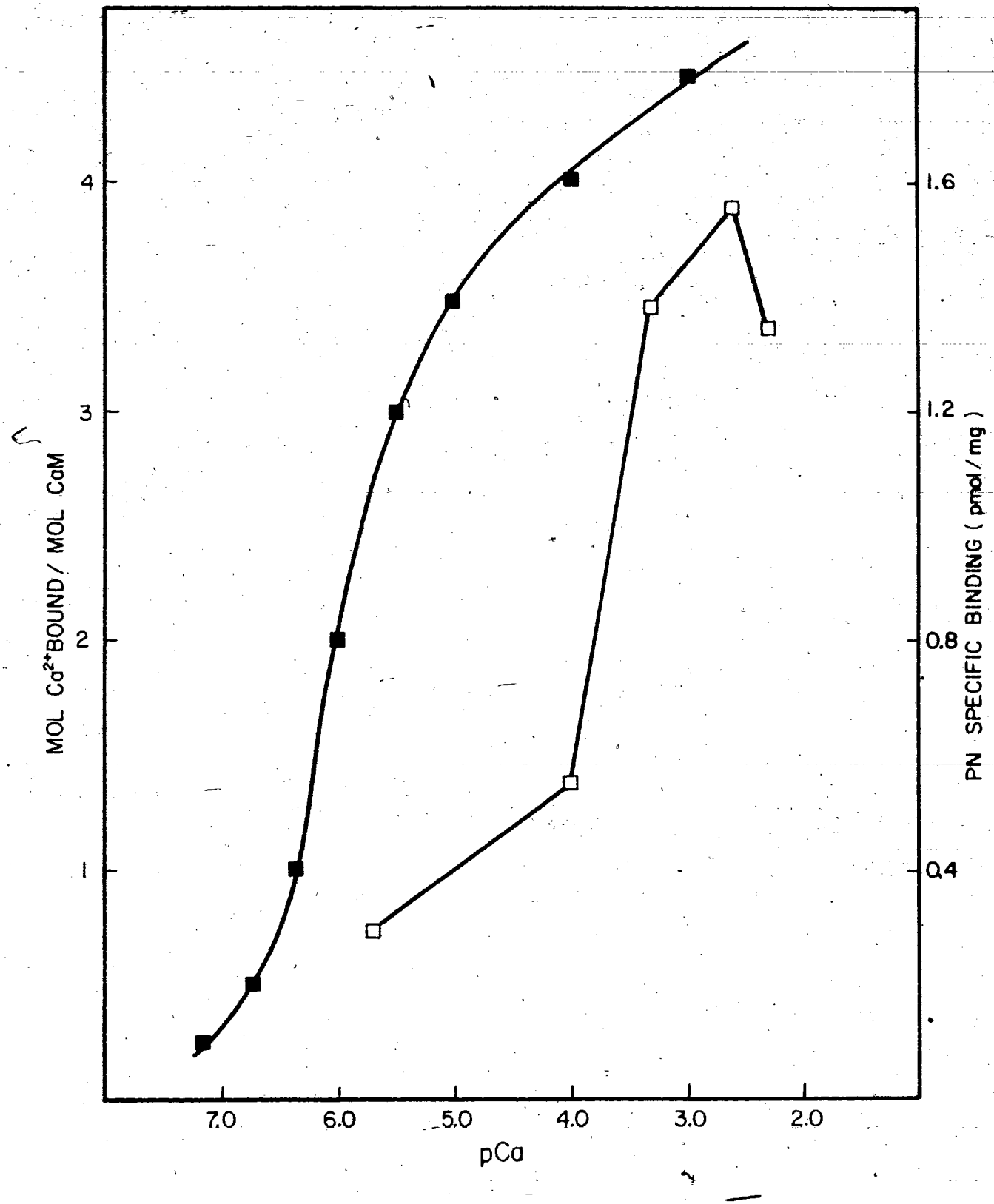
Inhibition of PN200-110 binding by calmodulin antagonists

Fig. 5. Relative (%) inhibition of PN200-110 specific binding as a function of calmidazolium and trifluoperazine concentration (-log[M]) in highly-purified sarcolemma at a PN200-110 concentration of 0.50 nM. Calmidazolium and trifluoperazine concentrations were varied between 0.05 to 2.0 μM and 5 to 200 μM, respectively. Extravesicular buffer concentration was 50 mM and the incubation time was 90 min. at 23°C. Bars represent $\pm$ S.E.M.



PN200-110 specific binding as a function of calmodulin concentration

Fig. 6. Relative (%) PN200-110 specific binding to highly-purified sarcolemma as a function of exogenous calmodulin addition, where calmodulin concentration (-log[calmodulin]) was varied between 10 to 1000 nM, PN200-110, buffer and $CaCl_2$ concentrations were 0.4 nM, 25 mM and 2.5 mM, respectively. The symbol, *, refers to a significant difference ($p < 0.05$) as compared to specific PN200-110 binding in the absence of calmodulin addition.



Ca^{2+}-dependence of PN200-110 and calmodulin binding

Fig. 7. Ca^{2+}-dependence of PN200-110 (at 0.50 nM) specific binding and Ca^{2+} binding to calmodulin (mol Ca^{2+} bound/mol calmodulin) in highly-purified sarcolemma, where Ca^{2+} concentration is expressed as *p*Ca.

$[Ca^{2+}]\mu M$	n	r^2
2	1.12	0.92
100	1.14	0.99
500	0.82	0.96
2500	0.84	0.97
5000	1.15	0.98

Hill analysis of PN200-110 binding at varying Ca^{2+} concentrations

Table 1. Hill plot analysis of PN200-110 equilibrium binding at varying Ca^{2+} concentrations, where $\log[B/B_{max}\text{-}B]$ was plotted against $-\log[PN200\text{-}110]_{free}$. The Hill coefficient (n) and the coefficient of correlation (r^2) are given.

TISSUE	REACTION	CALMID IC_{50} (μM)	TFP IC_{50} (μM)
RBC (14)	Ca/Mg ATPase		30
RBC (15)	Ca/Mg ATPase	0.35	
"	Ca Transport	2.00	
RABBIT/SKEL (16)	SR Ca Uptake		20-40
BOVINE BRAIN (17)	Phosphodiesterase		10
DANSYL BOVINE (22)	CaM (1 mM Ca)	1.50	10
CARDIAC SL (present study)	DHP Binding	0.28	28

The inhibitory capacity of calmodulin antagonists for calmodulin mediated processes

Table 2. The IC_{50} (concentration at which specific binding inhibited by 50% at equilibrium), in various tissues, of the calmodulin antagonists, calmidazolium (CALMID) and trifloperazine (TFP), on calmodulin-mediated processes. Abbreviations: RBC (red blood cell); SKEL (skeletal muscle); SL (sarcolemma); SR (sarcoplasmic reticulum); CaM (calmodulin).

CHAPTER IV

CALCIUM ANTAGONIST BINDING TO MYOCARDIAL SARCOLEMMA: ADAPTATION TO EXERCISE

Introduction

The regulatory role of cardiac plasma membrane (sarcolemma) in myocardial function is well documented. This study was conducted to test the hypothesis that endurance exercise training induces adaptations in myocardial sarcolemma which may account for the augmentation in contractility observed with training.

In the myocardium, transsarcolemmal calcium (Ca^{2+}) influx plays a key role in excitation-contraction (E-C) coupling (1). This is consistent with the findings that cardiac muscle contraction has an absolute extracellular Ca^{2+} requirement (2) and contractility varies as a function of extracellular Ca^{2+} concentration (3).

Although endurance exercise training has been shown to enhance cardiac performance (4), the mechanism of this adaptation is not fully understood. We have previously postulated that it may be attributed to an alteration in myocardial contractility as a consequence of sarcolemmal adaptation(s). Hearts from trained animals have exhibited a) lowered sensitivity to La^{3+} (5) (an agent that acts on the sarcolemma to uncouple excitation from contraction) (6) b) an increase in the predicted number of sarcolemmal calcium binding sites (7), c) prolongation of the action potential (7) and d) substantial alterations in the lipid composition of the sarcolemma (8). In the latter study the specific sarcolemmal content of phospholipids and phosphatidylserine were increased 23 and 50%, respectively. These findings are consistent with an increase in transsarcolemmal calcium influx that results from training producing an increase in myocardial contractility. Na^{+}/Ca^{2+} exchange is

enhanced with exercise training, likely as a result of an increase in the affinity (lowered K_m) of the exchanger for Ca^{2+} (9). The purpose of this study was to investigate the hypothesis that myocardial sarcolemmal Ca^{2+} channel density is modified in response to exercise training. Calcium antagonists, in particular the 1,4-dihydropyridines (DHP), are thought to bind specifically with the sarcolemmal Ca^{2+} channel (10). A new labelled DHP, [^{3}H]PN200-110 (PN), has been employed in this study since it is available in its active enantiomer form and binds with a high degree of specificity and potency (10).

The Ca^{2+} current in heart is regulated by two distinct channel types, L and T, which give rise to slow and fast Ca^{2+} currents, respectively (11). DHP block only the L-type channel (12) and can completely inhibit tension production (13). Electrophysiological studies illustrate that the L-type channel is the major contributor of Ca^{2+} influx during the ventricular action potential (11) and thereby is thought to play a crucial role in the regulation of contractility.

Methods and Results

Animal training and body characteristics. Sixty-six female Sprague-Dawley rats were randomly divided into exercise-trained and sedentary control groups. Rats were housed in cages of four, in a temperature and light controlled room and were fed *ad libitum*.

At the completion of the 10 week treadmill regimen, the animals were sacrificed by decapitation at the same time daily to circumvent possible circadian variations (14). At the time of sacrifice, the thorax was opened quickly and the heart was excised and perfused retrogradely through the aorta with homogenizing medium (250 mM sucrose and 20 mM MOPS to maintain pH 7.4 @ 37°C). The hearts were then trimmed of connective tissue, blotted dry and weighed (14).

Sarcolemmal isolation and characterization. The sarcolemmal isolation technique (15) follows that of Bers (16) as modified by Philipson *et al.* (17) and subsequently by Tibbits *et al.* (18). Protein content in all of the fractions and crude homogenate was determined by the method of Bradford (19) using bovine serum albumin (Sigma) as a standard. The activity of the sarcolemmal marker enzyme, K^+*p*NPPase was measured as we have described previously (20).

[^{3}H]PN200-110 binding. Binding of PN was measured (23) in the crude homogenate and the subcellular fractions (F2, F3 and F4) in trained and control. Linear regression analysis of PN specific binding (at 0.4 nM) vs. K^+*p*NPPase activity was performed for the trained (R^2 = 0.85) and control (R^2 = 0.84) (Fig. 1) group. A significant ($p < 0.05$) difference in the slope was found between the regression line for the trained vs untrained, illustrating an increase in specific binding in the trained in the absence of increased sarcolemmal purity. Specific binding (homogenate, F2 and F3) was statistically ($p < 0.05$) greater in the trained than the control (Fig. 2; F2) Scatchard (fit by least-squares linear regression) plots (Fig. 3) of equilibrium binding for the trained and control group provided values for the maximum binding site density (B_{max}) and the binding dissociation constant (K_d) (Table 1). The trained rats exhibited significantly ($p < 0.05$) greater (~ 50%) B_{max} values than the controls and lower ($p > 0.05$) binding affinities for any given preparation, with the exception of F3.

Discussion

Sarcolemmal adaptation. It is clear from this study that endurance exercise training induces adaptations of the sarcolemma. In preliminary studies (5,7), using the same training protocol, an augmentation in myocardial contractility was found independent of hypertrophy and alterations in myofibrillar ATPase activity.

An increase in the density of sarcolemmal calcium channels is strongly suggested ($p < 0.05$) in the Scatchard analysis (Fig. 3) of PN binding which revealed an approximately 50% increase in the maximum density of DHP binding sites in the trained rats. The increase is in agreement with a previous investigation in our laboratory using another DHP, nitrendipine (23), and was found in the absence of a significant change in the activity of the sarcolemmal marker, K^+*p*NPPase, or protein yield. Differential vesicular sidedness does not account for the difference because the vesicles were loaded with an isotonic medium and then diluted into an optimally hypotonic solution (20) causing the vesicles to rupture and allowing access to both sides of the vesicle.

The sarcolemmal adaptation to exercise training is apparently quantitative rather than qualitative although the binding affinity of PN in the trained was generally lower than in the untrained as evidenced by an increase in the K_d. This finding may be a consequence of an alteration in the DHP binding site with training. Phospholipids have been shown to be an important component of the binding site (24) and exercise training has been shown to alter the membrane phospholipid composition (8). An alteration in the composition of phospholipids associated with the PN binding site may ultimately affect the affinity of the receptor for the DHP molecule. The lowered binding affinity, evidenced in the untrained, would serve to underestimate the difference between the trained and untrained, shown in Fig. 2 & 3, at a ligand concentration of 0.4 nM.

Evidence is available to suggest that there may be some parallel in the nature of a physiological stressor, such as exercise, and pathological stressors. Wagner *et al.* (25), in cardiomyopathic hamsters, found a substantial increase in the number of cardiac DHP binding sites. Furthermore, Lossnitzer *et al.* (26) found the concentration of Ca^{2+} in cardiac myocytes of cardiomyopathic hamsters to be elevated and a prolonged action potential was found by Rossner and Sachs (27). The latter has also

been detected in hearts of trained rats (7). Although the physiological effects of these stressors are substantially different (5, 25) there may be some similarity in the signals that induce the adaptation and/or the strategies of myocardial adaptation.

The adaptations observed in this study may still be a consequence of other sarcolemmal component alterations. Evidence suggests that DHP bind to the calcium channel or to a protein closely associated with the channel, as suggested by Towart and Schramm (28). Calmodulin has been shown to modify DHP binding (29) and is required for full activation of the Ca^{2+} channel (30).

Sarcolemmal Ca^{2+} channel density is modified in response to exercise training, thus, further substantiating the important role of the sarcolemma in myocardial function and the process of adaptation. The evidence presented is consistent with the notion that exercise training increases Ca^{2+} channel density (approximately 5.71 channels um^{-2} (21) vs 3.55 channels um^{-2} (20) in trained vs control). This increase in Ca^{2+} channel density is in accordance with enhanced myocardial contractility (5,7).

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14. The treadmill workload for the trained group was increased from ~1.0 km/hr, 8% grade, 15 min/day to 1.8 km/hr, 8% grade and 1 hr/day. The mean body

weight of the trained group was 294.0 ± 5.4 g (S.E.M., n=33) as compared to 275.3 ± 7.0 g (S.E.M., n=33) for the control group. The mean heart weights were 817.8 ± 12.3 mg (S.E.M., n=33) and 772.3 ± 16.8 mg (S.E.M., n=33) for the trained and control group, respectively. The absence of myocardial hypertrophy was illustrated by the heart/body weight ratio which was 2.78 ± 0.02 (mean ± S.E.M.) in the trained group as compared to 2.82 ± 0.11 (mean ± S.E.M.) ($p > 0.05$) for the control groupC. Typically, this training protocol increases the aerobic capacity of skeletal muscle by about 35% (5,7).

15. The sarcolemmal isolation technique has been described in detail (20). Briefly, eight or nine hearts from each group were pooled and minced in 10-15 ml homogenizing medium and homogenized with a Tekmar Tissumizer (Cincinnati, OH, Model TCM-1) at setting 40 for 2 bursts of 3 seconds each. Sarcolemmal isolation involved differential and sucrose gradient ultracentrifugation. The procedure included actomyosin extraction with KCl (100 mM) and sodium pyrophosphate (25 mM) and an incubation period of 60 minutes at 30°C with 30,000 Kunitz units (~20 mg) of DNase to increase both sarcolemmal purity and yield (17).

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21. In the estimation of the calcium channel density it is assumed that the ligand binding site density is analagous to functional Ca^{2+} channel density, exercise training increases the number of functional Ca^{2+} channels and that the surface area of the myocyte remains relatively constant.

22. The [^{3}H]PN binding technique follows the procedure outlined previously (20). Briefly, for Scatchard analysis, tubes were prepared with [^{3}H]PN (78 Ci/mmol, New England Nuclear) concentrations ranging from 0.1 to 0.4 nM. Tubes also contained 2.5 mM $CaCl_2$, 25 mM MOPS (pH 7.4 @ 37°C), 0.5% v/v absolute ethanol (95-100%), 10-30 ug protein (crude homogenate 150-200 ug) and HPLC (high-performance liquid chromatography)-grade water to bring the tubes to a final volume of 5 ml. Assays were performed in both the presence and absence of 1 *u*M nifedipine. Specific binding was defined as that displaced by unlabelled nifedipine.

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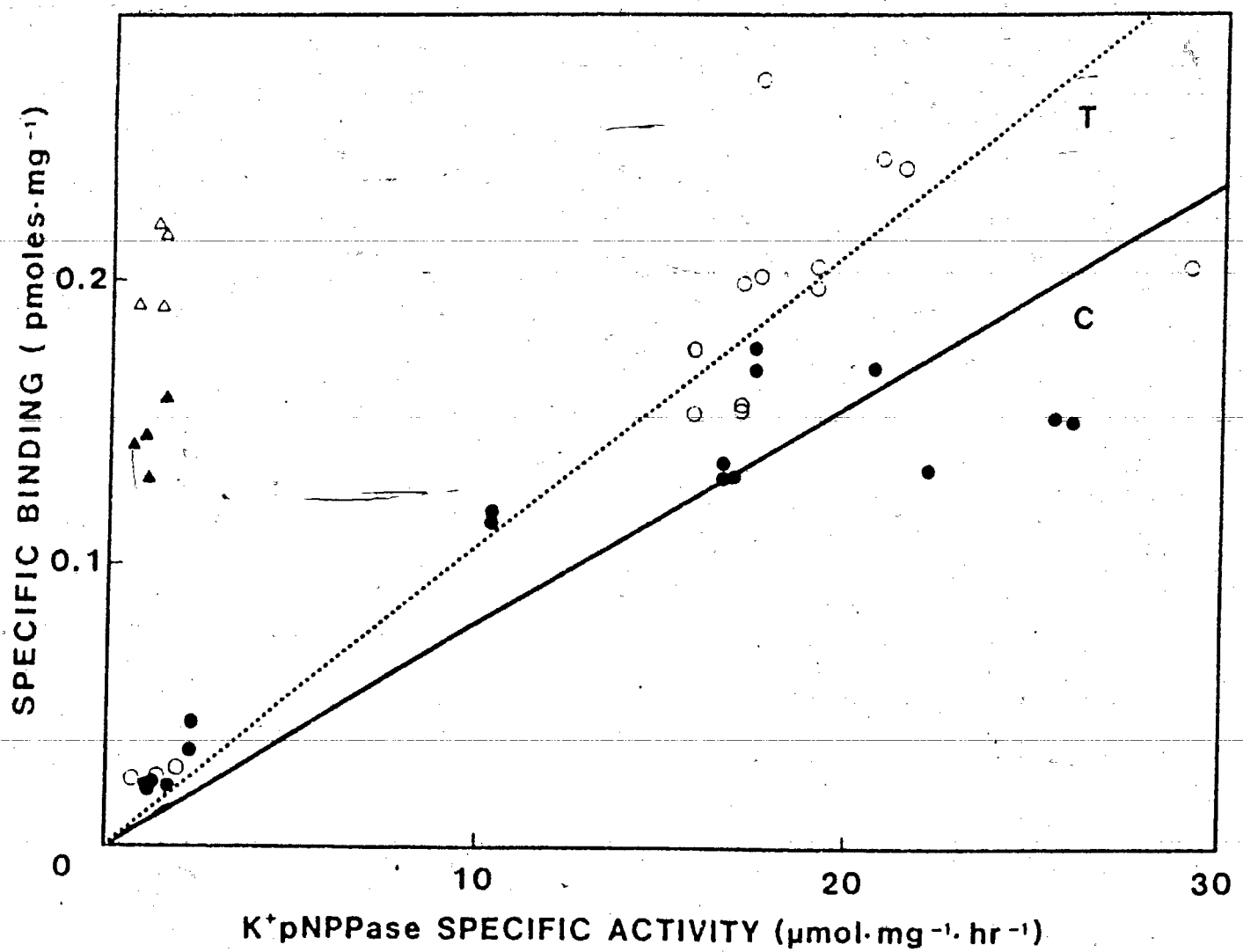
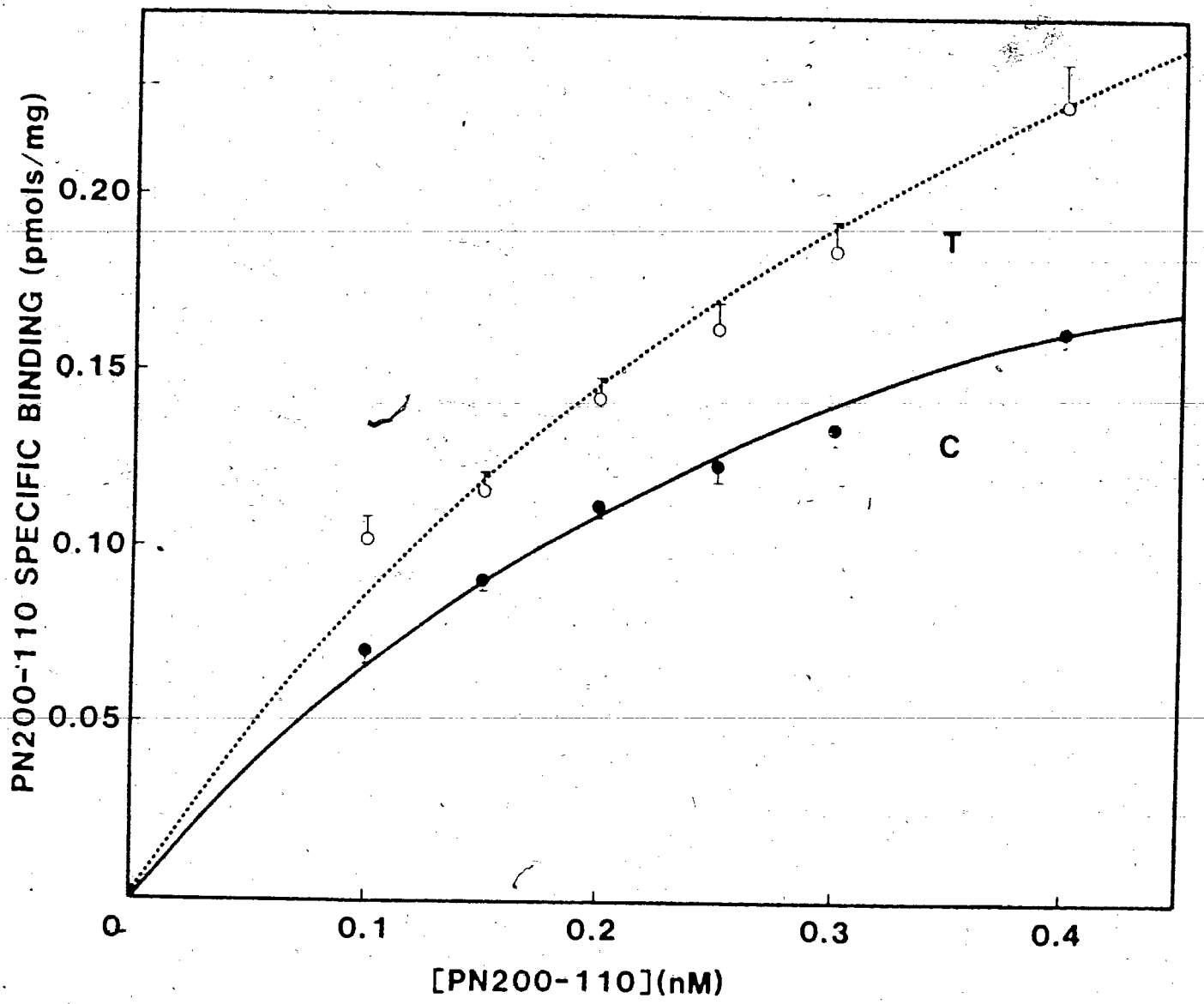


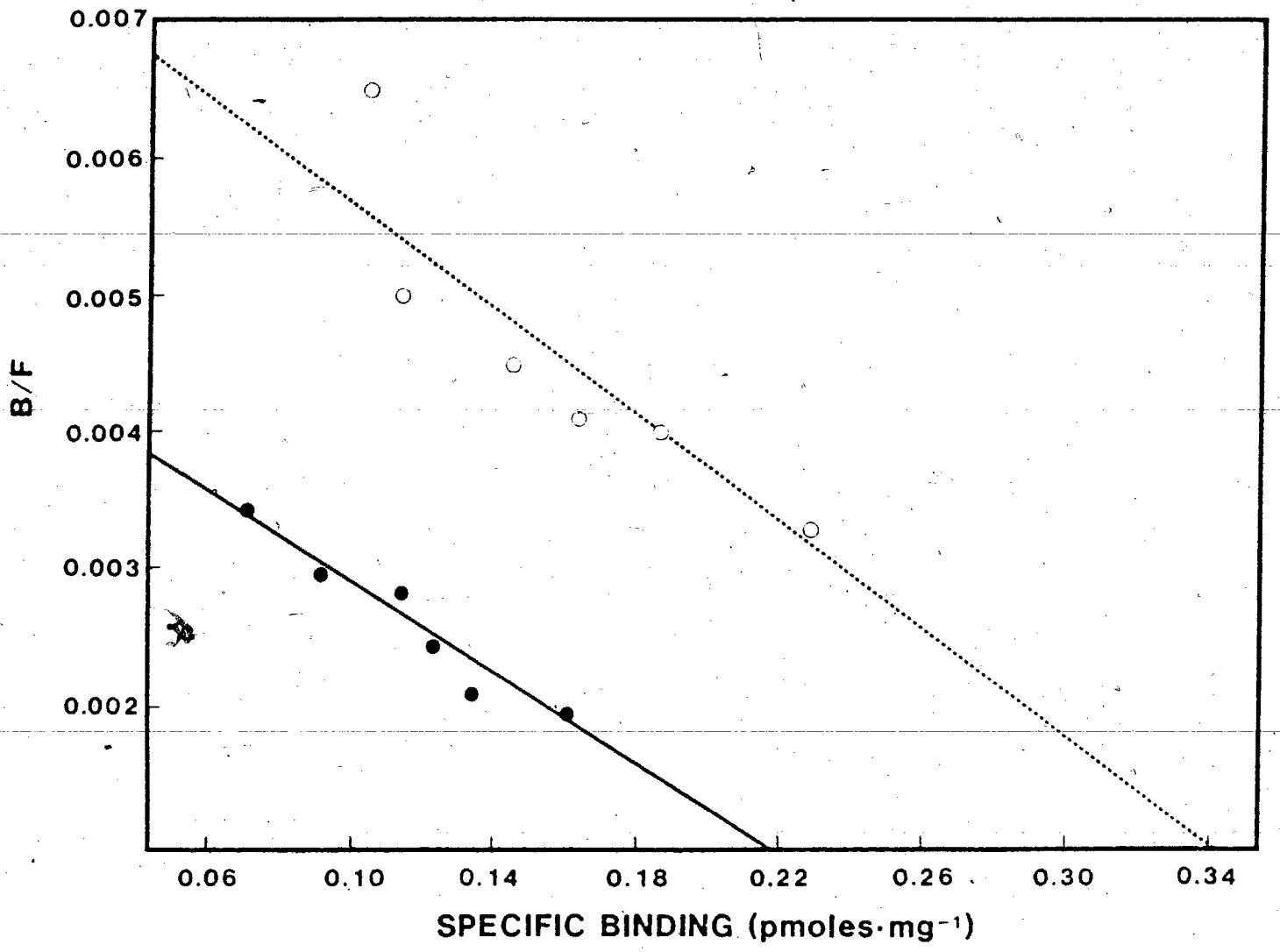
PN200-110 binding vs K^+*p*NPPase activity in the trained and untrained

Fig. 1. Relationship between PN200-110 specific binding (pmoles/mg protein) (PN200-110 concentration = 0.4 nM) at equilibrium and specific activity of the sarcolemmal marker enzyme, K^+*p*NPPase, in trained (T) crude homogenate (△ ; n=8) and subcellular fractions (○ ; n=20) and control (C) crude homogenate (▲ ; n=8) and subcellular fractions (● ; n=20). Buffer and $CaCl_2$ concentrations were 25 and 2.5 mM, respectively and the incubation time was 90 min. at 23°C.



PN200-110 binding in trained vs control as a function of PN200-110 concentration

Fig. 2. PN200-110 specific binding (pmoles/mg protein) in trained (T) vs control (C) as a function of PN200-110 concentration (nM). PN200-110 concentration was varied between 0.1 to 0.4 nM, buffer and $CaCl_2$ concentrations were 25 and 2.5 mM, respectively, and the incubation time was 90 min. at 23°C. Bars represent ± S.E.M.



Scatchard plot of PN200-110 binding in the trained and untrained

Fig. 3. Scatchard plot of PN200-110 specific binding to a highly enriched sarcolemmal fraction (F2) in the trained (○; n=6) and control (● ; n=6) group.

	TRAINED		CONTROL	
	B_{max} (pmoles/mg)	K_d (nM)	B_{max} (pmoles/mg)	K_d (nM)
HMG	0.288 (±) 0.015	0.160 (±) 0.011	0.186 (±) 0.006	0.138 (±) 0.023
F2	0.418 (±) 0.025	0.360 (±) 0.021	0.282 (±) 0.011	0.305 (±) 0.020
F3	0.285 (±) 0.016	0.280 (±) 0.029	0.210 (±) 0.006	0.298 (±) 0.015
F4	0.047 (±) 0.002	0.264 (±) 0.014	0.033 (±) 0.005	0.208 (±) 0.012

Table 1. Maximum binding site density (B_{max}) and dissociation constant (K_d) determination from Scatchard analysis of linear plots of PN200-110 binding to homogenate (HMG) (n=8) and subcellular fractions, F2 (n=6), F3 (n=6) and F4 (n=8) for the trained and control groups.

CHAPTER V

Conclusion

It has been demonstrated that exercise training can induce a number of adaptations in the myocardium. Many of these alterations lead to an increased stroke volume and therefore an improved oxygen delivering ability of the cardiovascular system. These adaptations include homeometric changes that enhance the contractile ability of the myocardial fiber. Among the mechanisms postulated for this enhancement is an increase in calcium transport across the sarcolemma, particularily through the calcium channel.

Dihydropyridine binding sites were enumerated using a radiolabelled calcium antagonist, PN200-110. Binding of PN200-110, to highly-purified sarcolemma, was characterized and provided evidence to suggest that this ligand binds to the calcium channel. Therefore, PN200-110 was used as a tool to gain information about the density of the calcium channels in response to exercise training.

There was a substantial increase in receptor site density with exercise training, equivalent to an increase from 3.55 to 5.71 sites per um^2 sarcolemma. With the assumption that PN200-110 binding sites reflected the density of functional calcium channels, the apparent increase with exercise training supports the hypothesis. The increase in contractility could be a consequence, therefore, of enhanced calcium current density during the plateau phase of the myocardial action potential. This inturn would increase stroke volume and account for enhanced cardiac performance with training. To substantiate that this increase in binding site density parallels an increase in functional calcium channels an electrophysiological analysis should be undertaken.

Another finding of this study, that deserves further investigation, was the apparent involvement of calmodulin in the binding of PN200-110 to sarcolemma.

Evidence gathered suggests that calmodulin may be lost or altered in the isolation and purification procedure employed in this study. Further experimentation is required to explain the role of endogenous calmodulin in the regulation of the sarcolemmal calcium channel and the effect of endurance training on this protein.