EFFECTS OF CHRONIC VERAPAMIL ADMINISTRATION ON THE BIOCHEMICAL CHARACTERISTICS OF THE L-TYPE CALCIUM CHANNEL

BY

BLAIR BURTON LONSBERRY

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ABSTRACT

The Ca^{2+} channel antagonists are an important group of drugs used in the treatment of a variety of clinical diseases. Patients are often on long-term treatment regimens in order to treat their particular disorder. The purpose of this study was to determine if chronic administration of verapamil (a Ca^{2+} channel antagonist) could alter the biochemical characteristics of the L-type Ca^{2+} channel as determined by [^{3}H]PN 200-110 binding. Various modes of drug administration such as implantable slow-release pellets, s.c. injection and oral dosages were tested as means of raising plasma verapamil levels.

Circulating plasma verapamil levels obtained from rats implanted with slow-release verapamil pellets often reached levels 10 fold higher than s.c. injections and oral dosages. In addition, large quantities of the drug were released within the first 24 hours post-implantation, resulting in a high mortality rate in these animals. It was concluded that implantable slow-release pellets are an unreliable means of verapamil administration. The biochemical characteristics of cardiac, brain and skeletal muscle tissue all appear to be very resistant to alteration by chronic verapamil treatment. Variations in drug dosage by s.c. injection (2.5 to 75 mg/kg/day) and duration of treatment (24 hours to 16 weeks) had little effect on altering the Ca^{2+} channel biochemical characteristics. However, a decrease in B_{max} and K_D was observed in cardiac tissue obtained from rats implanted with a high dosage (50 mg) slow-release pellet after 2 weeks duration. In addition,

a significant increase in B_{max} and K_D was observed in skeletal muscle with increasing verapamil concentration administered by s.c. injection. This increase observed in skeletal muscle may be a consequence of high local verapamil concentrations as a result of the injection protocol.

In summary, our results demonstrate that the implantable pellets are not a reliable administration method for verapamil. Further, cardiac (in addition to brain and skeletal muscle) Ca^{2+} channels are highly resistant to change during chronic verapamil administration. Our data suggest that the beneficial action of Ca^{2+} antagonist therapy in different cardiac pathologies may not involve a change in the biochemical characteristics of the channel. Our data also question the validity of previous studies which have described significant changes in receptor density after Ca^{2+} antagonist therapy.

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Introduction.

Calcium channel antagonists, since their conception some 30 years ago, have received considerable attention both clinically and experimentally. Their unifying characteristic of blocking the L-type Ca^{2+} channel in excitable and non-excitable cells has led to their extensive use in treating a variety of clinical disorders. Research is now being carried out in order to further elucidate their molecular binding properties, mechanism of action and continuing therapeutic considerations.

Four distinct types of Ca^{2+} channels exist in excitable and non-excitable cells.[121] The T-type Ca^{2+} channel has been isolated from heart, skeletal muscle, smooth muscle and neuronal tissue.[123] It is opened by mild depolarization and their ionic currents are fast and transient.[5] T-channels are thought to play an important role in the activity of the sinoatrial node in the heart and are generally thought to be insensitive to Ca^{2+} channel antagonists.[123] The N-type Ca^{2+} channel is located primarily in neuronal tissue and is thought to be responsible for controlling the influx of Ca^{2+} necessary for neurotransmitter release.[130] N-channels are insensitive to Ca^{2+} channel antagonists and are inhibited by a group of snail toxins, ω-conotoxins.[130] A recently discovered Ca^{2+} channel was isolated from cerebellar Purkinje cells and was denoted P-type Ca^{2+} channel.[139] It was found to be insensitive to both Ca^{2+} antagonists and ω-conotoxin but was inhibited by a spider venom toxin.[138] Very little is known about these channels, including whether the P-type Ca^{2+} channel is a distinct channel or a group of

several related channels. The L-type Ca^{2+} channel has been isolated from cardiac, skeletal, smooth and neuronal tissue.[124] It is the primary means of Ca^{2+} influx during the cardiac action potential and provides the necessary Ca^{2+} for the "Ca^{2+} induced Ca^{2+} release" mechanism from the sarcoplasmic reticulum.[161] The organic Ca^{2+} channel antagonists preferentially bind to this channel and block the inward flux of Ca^{2+} across the plasma membrane.[5]

The ability of Ca^{2+} channel antagonists to block the slow inward Ca^{2+} movement was identified by Dr. A. Fleckenstein and his colleagues in the early 1960's. At that time, two prototype Ca^{2+} channel blockers were being studied; prenylamine and verapamil.[209] These two compounds were shown to have a cardiopressant effect similar to that seen by the removal of Ca^{2+}, resulting in the inhibition of excitation-contraction coupling and cardiac contractility.[211] Further research identified the ability of Ca^{2+} channel antagonists to selectively block the L-type Ca^{2+} channel, crucial in the excitation-contraction coupling process in cardiac tissue. In 1966, a new class of drugs was designated, the Calcium Antagonists.[210] At that time, two major groups of Ca^{2+} antagonists existed, the Group A, or highly specific antagonists and Group B, the less specific antagonists.[216] It has been the Group A Ca^{2+} antagonists that have received the majority of clinical and experimental focus. Group A consists of three major subgroups: the dihydropyridines (e.g. nifedipine), the phenylalkylamines (e.g. verapamil) and the benzothiazepines (e.g. diltiazem).[217]

The Ca^{2+} channel antagonists are being used clinically to

treat a variety of disorders. Their primary use has come in the treatment of complications related to the cardiovascular system including arrhythmias, angina, hypertension and cardiomyopathy.[219] Due to the underlying nature of these disorders, treatment regimes incorporating Ca^{2+} antagonists can be administered over prolonged periods of time, from months to even years. This raises the question of what effect these prolonged treatment regimes are having at a cellular level.

Studies looking at the effects of long-term treatments with certain compounds have shown that there is a relationship between alterations at a cellular level and prolonged drug treatment. Certain drugs, like Ca^{2+} channel antagonists, mediate their action by binding to a specific receptor on the tissue plasma membrane. The receptor's activity and density can be regulated by circulating drug concentrations and by particular physiological and pathophysiological states.[187] A classic example of a circulating drug regulating important characteristics of its receptor comes from studies looking at the ß-adrenergic receptor and treatment with ß-receptor agonists and antagonists. Prolonged treatment with the ß-adrenergic blocking agent propanalol may lead to the development of an increase in the number of ß-receptor numbers in certain tissues like the heart.[334] Conversely, prolonged treatment with a ß-adrenergic stimulating agent like epinephrine may lead to a decrease in the number of functional ß-receptors present in a tissue.[266] The resulting increase in functional receptor number following prolonged treatment with an antagonist is referred to as

an "up-regulation", while the decrease following agonist treatment is a "down-regulation". The change in the functional receptor numbers following agonist/antagonist treatment follows this general pattern, but it should be noted that such regulation is not an automatic consequence to drug exposure and in some systems drug application may result in no change or a change in the opposite direction[335].

This study was undertaken to examine the effects on the L-type Ca^{2+} channel after chronic exposure to the Ca^{2+} channel antagonist, verapamil. Previous studies have examined the effects on Ca^{2+} channel density in a variety of tissues using different members of the Ca^{2+} antagonist group. A reduction in the number of mouse brain Ca^{2+} channels was noted after 28 days of oral administration of nifedipine and verapamil.[275] A similar decrease in Ca^{2+} channel number was observed in rat brain and heart after 20 day intravenous nifedipine treatment[276], while no change was observed in rat heart after 14 day oral nifedipine treatment.[277] It is apparent that there are a variety of experimental factors that may play a role in the regulation of channel number. Such factors may include the mode of drug administration (e.g. intravenous, subcutaneous or orally), type of drug (e.g. nifedipine, verapamil or diltiazem), species and tissue specific differences and the duration of drug treatment.

In order to address some of these factors a variety of experimental protocols were introduced. An initial study was undertaken to address whether alterations in Ca^{2+} channel density

were influenced by the duration of drug administration and/or drug concentration. To assess the influence of drug administration duration, a constant drug dosage was administered for varying periods of time (ranging from 1 day to 16 weeks). Concentration dependent changes were assessed by administering a range of verapamil concentrations for a fixed period of time. The mode of drug administration was addressed by utilising subcutaneous (s.c.) implantable slow-release pellets, s.c. injection and oral administration *via* a p.o. intubator. Ca^{2+} channel density and affinity were determined using a radioligand binding assay employing [^{3}H]PN 200-110 as the radioactive ligand. PN 200-110 is a member of the dihydropyridine group and has a high specific binding to the L-type Ca^{2+} channel, allowing for accurate quantitation of density (B_{max}) and activity (K_D).

Presently, it is unclear what the relationship is between Ca^{2+} channel density and circulating levels of Ca^{2+} channel antagonists. The exact circulating concentration of verapamil, for example, which is required to alter Ca^{2+} channel characteristics is unknown. Verapamil is 87-93% protein bound, and has a first pass clearance of approximately 80% by the liver.[219] The elimination half-life of verapamil in the blood is usually between 3-7 hours, but does increase during chronic administration and in conditions where there is liver or renal damage.[219] Therefore, in order to quantitate the amount of verapamil that was actually reaching the different tissues, blood samples were taken at varying times and the plasma verapamil and its metabolites were quantitated by High

Performance Liquid Chromatography (HPLC). Plasma quantitation of verapamil and its metabolites also enabled us to compare the various means of drug administration with respect to circulating plasma levels.

The binding sites for the three major classes of Ca^{2+} channel antagonists (dihydropyridines, phenylalkylamines and benzothiazepines) are located on the α_1 subunit of the Ca^{2+} channel.[164,234] These molecular binding sites are allosterically linked to one another and binding of the antagonists to their respective sites is modified by the presence of other blockers and divalent cations (e.g. Ca^{2+})[338]. In order to assess whether chronic verapamil treatment alters these allosteric interactions or the response to Ca^{2+}, radioligand binding assays were carried out in the presence of varying verapamil concentrations and in the presence/absence of Ca^{2+}.

The purpose of this study was to obtain a better understanding of the molecular consequences of long-term therapy with Ca^{2+} channel antagonists. This information is currently lacking in the literature and vital to the understanding of the long-term effects of prolonged clinical treatments with Ca^{2+} channel antagonists.

REVIEW OF LITERATURE

Excitation-Contraction Coupling in Cardiac Muscle

The E-C coupling process within cardiac muscle can been separated into four stages: (i) action potential depolarization of the sarcolemmal (SL) membrane, (ii) Ca^{2+} release from stores in the sarcoplasmic reticulum (SR) induced by extracellular transsarcolemmal Ca^{2+} movement, (iii) binding of Ca^{2+} to the thin filament troponin C protein and via a series of reactions allowing interaction between actin and myosin to form cross-bridges resulting in muscle shortening (contraction), and (iv) relaxation of the muscle fibres by lowering of the intracellular [Ca^{2+}] via uptake by the SR and Ca^{2+} extrusion through the sarcolemma.

i] Depolarization of the myocardial cell

The resting membrane potential of myocardial cells is approximately -85mV to -95mV. Myocardial depolarization results in a rapid but brief Na^{+} inward movement through tetrodotoxin sensitive sodium channels in the T-tubule/SR junctional space.[3] Further depolarization of the SL membrane results in the opening of voltage dependent Ca^{2+} channels. Two types of Ca^{2+} channels exist in cardiac cells; T channels (transient) and L (long-lasting).[4] T-channels are found primarily in pacemaking cells but are also found in ventricular tissue. These channels are activated rapidly at polarization potentials more negative than -50mV to -60mV, peak at -30mV, and inactivate quickly (5-30 msec). These channels appear to contribute little to the Ca^{2+} current. L-channels activate at -40mV to -30mV, inactivate slower than T-channels and carry

approximately three to fourfold more current. These channels appear to be the major contributor to the formation of the characteristic myocardial action potential plateau and provide the necessary Ca^{2+} flux to initiate contraction. The L-type channels are the main route for transsarcolemmal Ca^{2+} influx.[5]

With the discovery of the SL Na^{+}-Ca^{2+} exchange system, it was thought that this may be another route for Ca^{2+} influx during the E-C coupling process.[6] Much research over the past couple of decades has focused on discovering the role of the Na/Ca exchanger. Electrophysiological and flux measurement studies have shown that the exchanger moves 3 Na^{+} for every 1 Ca^{2+} ions, and is electrogenic.[7,8] The Na/Ca exchanger may contribute little to Ca^{2+} influx during depolarization in cardiac cells, but may act as a high capacity Ca^{2+} efflux mechanism.[9] The Na/Ca exchanger may play a more important role with respect to Ca^{2+} influx in situations where the SL membrane has been depolarized to potentials around 0mV, or when there is an elevation of intracellular [Na^{+}] as would be observed after blocking of the Na^{+}/K^{+} pump (eg. digitalis).[10]

ii] Calcium release from sarcoplasmic reticulum.

The sarcoplasmic reticulum (SR) of cardiac cells is a tubular, lipid bilayer network of membranes analogous to the endoplasmic reticulum of non-contracting cells.[11] The SR is composed of two morphologically distinct components; (i) the junctional SR, and (ii) the longitudinal SR.[12] The junctional SR comes into close apposition to the T-tubule system of the sarcolemma. The junctional SR contains feet that span the gap between the SR and

the T-tubules. These feet are believed to be the mediators of Ca^{2+} release from the junctional SR.[13] The longitudinal SR is responsible for the pumping of Ca^{2+} ions from the cytoplasm into the SR via the ATP dependent Ca^{2+} pump contained within the longitudinal SR membrane.[14] The primary functions of the SR include: (i) release of stored Ca^{2+} via the ryanodine-sensitive Ca^{2+} release channel to provide the final signal in the contractile protein activation, and (ii) relaxation of the muscle by re-accumulation of Ca^{2+} by the calcium pump.[11]

Other sources of Ca^{2+} necessary for activation of the contractile apparatus have been proposed including direct transsarcolemmal Ca^{2+} influx[15] and mitochondria[16] acting as a reversible Ca^{2+} storage site. At present, mitochondria appear unlikely to participate in regulation of cytosolic Ca^{2+} on a beat to beat basis because its affinity for Ca^{2+} and rate of Ca^{2+} uptake are too low.[17] Insufficient data are available presently to adequately answer the question of transsarcolemmal influx of Ca^{2+} directly activating myofilaments. It appears that the relative contribution of direct transsarcolemmal Ca^{2+} influx varies between species. Rat is almost totally dependent upon SR Ca^{2+} release for contraction, while rabbit and guinea pig can develop full contraction (at a slower rate) in the absence of SR Ca^{2+} release and by direct transsarcolemmal influx.[18,19,20] SR is almost nonexistent in frog ventricle, therefore, the frog is completely dependent upon extracellular Ca^{2+} for contraction.[21] It appears that all cardiac muscles are dependent upon some transsarcolemmal

Ca^{2+} influx, if only for activation of Ca^{2+} release from the SR.

The current hypothesis for the activation of Ca^{2+} release from the SR is via Ca^{2+} induced Ca^{2+} release.[22] Transsarcolemmal Ca^{2+} influx results in an increase in the myoplasmic free [Ca^{2+}] on the outer surface of the SR, inducing a release of Ca^{2+} from the SR via the ryanodine sensitive Ca^{2+} release channels. Current experimental data shows that part of the transsarcolemmal influx actually loads the SR with Ca^{2+} available for release during subsequent contractions.[22,24,25] It appears that there is a time dependent and Ca^{2+} dependent component to activation of Ca^{2+} induced Ca^{2+} release. A fast increase of free Ca^{2+} on the outer surface of the SR appears to result in release of Ca^{2+} from stores in the SR, while a slow increase in the same [Ca^{2+}] results in a loading of the SR with Ca^{2+}. This loading of the SR would make that Ca^{2+} available for release on subsequent contractions.[26] Transsarcolemmal Ca^{2+} currents have both a fast and slow component which would appear to confirm the above observations.[25]

Na/Ca exchange may also play a role in influx of SR Ca^{2+}. Recent investigations into Ca^{2+} release from the SR have revealed a possible triggering role by Na^{+}. Na^{+} influx into the T-tubule/SR junctional space during the upstroke of the action potential may result in the activation of the Na/Ca exchanger, extruding Na^{+} while moving Ca^{2+} inward. The resulting inward Ca^{2+} flux via the exchanger results in the triggering the release of Ca^{2+} from the SR.[3,27]

A difficulty raised in the hypothesis of Ca^{2+}-induced Ca^{2+}

release was whether the Ca^{2+} released from the SR was an all-or-none process. If this were the case, then the known gradation of contraction seen in cardiac muscle with varying transsarcolemmal Ca^{2+} flux would not be possible.[28] Activation of SR Ca^{2+} release would result in all of the stored Ca^{2+} being released, or none of it, and graded contractions would not be possible. Experiments performed on skinned cardiac cells demonstrated a negative feedback system operating on the Ca^{2+}-induced Ca^{2+} release from the SR.[22] At high free [Ca^{2+}] there was inhibition of further release from the SR. These skinned cardiac cell experiments demonstrated that the amount of Ca^{2+} released via Ca^{2+}-induced Ca^{2+} release is graded depending upon the [Ca^{2+}] that triggers it.[22,26]

iii] <u>Myofilament interactions</u>

The principle role of Ca^{2+} released from the SR is to initiate the contraction of the muscle. Ca^{2+} initiates the contraction of cardiac muscle at the level of the myofilament, which is the contractile apparatus of the muscle cell. Myofilaments responsible for cardiac contracture include thick and thin filaments which interact with one another resulting in muscle shortening (contracture) and relaxation. There is a parallel arrangement of the thick and thin filaments which are interdigitated throughout the cell. During contraction there is an energy dependent sliding between the two filaments resulting in muscle cell shortening. Relaxation occurs when the sliding motion is passively reversed (i.e., there is no energy expenditure by the filamentous array). The thick/thin filaments are grouped to form fibrils, which when

arranged in parallel, form a sarcomere. Bundles of fibrils cooperate as a functional unit in the whole muscle.[29]

The thick filaments consist of myosin molecules which are specifically arranged to form the filament. The myosin molecule is a dimer of two identical heavy chains arranged in an alpha-helical fashion with each chain ending in a globular head. The heads are arranged so that both heads protrude at the same end. The overall structure of the filament results in the globular heads protruding in a staggered pattern at each end of the filament while the midregion is devoid of heads. Contained within the heads is a myosin ATPase which interacts with the thin filament (specifically the actin component of the thin filament). The myosin ATPase requires magnesium as cofactor and with its association with actin is referred to as actomyosin ATPase. ATP (adenosine triphosphate) is hydrolysed to ADP (adenosine diphosphate) and inorganic phosphate by actomyosin ATPase to provide the biochemical energy required for muscle contraction.[29]

The thin filament is composed of filamentous actin (a constituent of the actomyosin complex), tropomyosin and troponin in a ratio of 7:1:1.[30] The actin is arranged in a double-stranded helix fashion, while tropomyosin is a coiled helical dimer. Tropomyosin appears to associate in a head-to-tail series along the actin molecule within or near the helical groove of the actin double strand. This placement of tropomyosin appears to regulate actin/myosin interaction during contraction.[29] Tropomyosin movement in/out of this groove is regulated by the thin filament

associated troponin, which is a complex of three functionally distinct proteins in a ratio of 1:1:1.[30] Troponin I (TnI) is the subunit that acts to inhibit actomyosin ATPase activity. Troponin C (TnC) mediates calcium sensitivity by binding calcium in a specific manner and acting to decrease the inhibitory action of TnI. TnC will influence TnI only when in the presence of the third troponin subunit, Troponin T (TnT). TnT appears to mediate the inhibitory action of TnC on TnI as well as anchoring the troponin complex to tropomyosin. In order for contraction to proceed, all three components must be present.[29]

Excitation of the myocardial cell and subsequent increases in the free intracellular [Ca^{2+}] reach a threshold point at which time TnC undergoes an interactive change with TnI. Additional changes occur between TnI, TnT and tropomyosin. The result of these alterations in protein-protein interactions is a positional shift of the tropomyosin molecule relative to the actin molecule, removing the inhibition of actin/myosin interaction. Myosin/actin interaction results in activation of actomyosin ATPase, hydrolysation of ATP, and the resultant biochemical energy is transformed into mechanical energy resulting in contraction.[29] Alterations in the levels of cytosolic free Ca^{2+} available for binding will result in differing levels of contractility. For example, increased levels of Ca^{2+} will result in a positive inotropic effect.[31] Adjustments in the activity of the regulatory troponin subunits, such as occurs when magnesium binds to the TnC subunit[34] or when TnI is phosphorylated by cAMP dependent protein

kinase upon ß-adrenergic stimulation[32,33], will also alter the level of contraction.

iv] Relaxation

Relaxation of cardiac muscle occurs upon a decrease in the level of cytosolic free $[Ca^{2+}]$. Decreases in the level of cytosolic free Ca^{2+} causes a dissociation of TnC bound Ca^{2+}, which results in the inhibitory effect of TnI being re-established. Tropomyosin shifts back into the groove on the actin molecule preventing interaction between actin and myosin. Active cross bridge formation is no longer possible and the muscle relaxes. The level of free Ca^{2+} which was critical in the initiation of contraction is also important in the relaxation process.[29]

The reduction in the cytosolic free Ca^{2+} levels occurs by several means and at different levels of the cell. At the level of the sarcolemmal membrane, two extrusion mechanisms are present: (i) Na/Ca exchange[9], and (ii) ATP-dependent Ca^{2+} pump.[36,37] The primary route of efflux of free $[Ca^{2+}]$ at the sarcolemma is via the Na/Ca exchange system. The Na/Ca exchanger's role in the normal influx of Ca^{2+} during E-C coupling is still controversial, but it does have a dominant role in Ca^{2+} extrusion.[9,35] The Na/Ca exchanger moves 3 Na^+ ions into the cytosol for every 1 Ca^{2+} ion extruded.[7] Experimental data in which the SR was inhibited by caffeine so that transsarcolemmal transport was the only means of cell relaxation showed that relaxation was considerably slowed in the absence of external Na^+ which would greatly decrease the effectiveness of the Na/Ca exchanger.[9] It is now apparent that the Na/Ca exchanger and

not the SL Ca^{2+} pump contributes to the beat-to-beat relaxation.

The alternative means of reducing cytosolic free [Ca^{2+}] is via re-uptake by the SR. The longitudinal membrane of the SR contains an ATP-dependent calcium pump, functioning to transport Ca^{2+} from the cytosol back into the SR stores.[14] With each cycle of the Ca^{2+} pump, two Ca^{2+} ions are moved for every ATP hydrolysed to ADP plus inorganic phosphate.[21] The SR Ca^{2+} pump competes with the Na/Ca exchanger as the main relaxing system of myocardium. It is usually the SR Ca^{2+} pump that is the more significant in the movement of Ca^{2+} necessary for relaxation.[38] The Na/Ca exchanger appears to efflux Ca^{2+} equivalent to that which entered via the Ca^{2+} channels during the action potential plateau. Most of the free intracellular Ca^{2+} that was used for activation of the myofilaments during contraction originated from the SR, and it is the SR Ca^{2+} pump that re- accumulates the same quantity of Ca^{2+}.[39]

Excitation-Contraction Coupling in Skeletal Muscle

The E-C coupling process in skeletal muscle is very similar to that of cardiac muscle. Skeletal muscle E-C coupling can also be separated into four stages: (i) action potential depolarization of the sarcolemmal membrane, (ii) Ca^{2+} release from stores in the sarcoplasmic reticulum (SR), (iii) binding of Ca^{2+} to the thin filament troponin C protein and via a series of reactions allowing interaction between actin and myosin to form cross-bridges resulting in muscle shortening (contraction), and (iv) relaxation of the muscle fibres by lowering of the intracellular [Ca^{2+}] via uptake by the SR and Ca^{2+} extrusion through the sarcolemma.

Fundamental differences do, however, exist between skeletal and cardiac tissues. The most evident differences are in the action potential and the mechanism of Ca^{2+} release from the SR. Because the processes are very similar between the two types of tissue, the discussion here will be limited to describing the differences which exist.

The action potential in skeletal muscle is significantly different from that seen in cardiac tissue. The duration of the action potential in skeletal muscle is approximately 1-5 milliseconds, which is a magnitude of 30 to 50 times shorter than the action potential produced in cardiac tissue (250-300 milliseconds).[41] The depolarization upstroke of the skeletal muscle is a result of the opening of tetrodotoxin sensitive sodium channels, resulting in a brief inward movement of Na^+ ions ('fast' sodium current) into the t-tubule/SR junctional space. These sodium channels inactivate and close the channel within a few 10,000ths of a second, not allowing any further Na^+ influx.[42] The same membrane potential change that activated the sodium channels also activates voltage-gated potassium (K^+) channels. These K^+-channels are slower in opening then the Na^+ channels. They open at the same time that the Na^+-channels are inactivating. The K^+-channels permit K^+ ion efflux from the cell, resulting in the downward portion of the action potential (repolarization) towards the resting membrane potential (approximately -90mV).[43,44] Missing in the skeletal muscle action potential that is seen in the cardiac action potential is the plateau phase, resulting from the opening

of voltage-gated Ca^{2+} channels. As described previously, depolarization of the cardiac sarcolemmal membrane results in the activation of voltage-dependent L-type Ca^{2+} channels, which allow transsarcolemmal influx of Ca^{2+} ions.[4] This inward Ca^{2+} current produces the long plateau phase characteristic of the cardiac action potential and necessary for induction of Ca^{2+} release from the SR.[5]

An additional difference in the E-C coupling process between skeletal and cardiac tissues is the means by which Ca^{2+} release from the SR stores is triggered. In cardiac tissue, a Ca^{2+}-induced Ca^{2+} release mechanism resulting from an increase in the free $[Ca^{2+}]$ by transsarcolemmal influx is the current popular hypothesis.[22] The precise means by which an action potential in the t-tubule system of the skeletal muscle produces a release of Ca^{2+} stored in the terminal cisternae of the SR is controversial.[45] Several different hypotheses for this triggering mechanism have been proposed.

The triggering mechanism for Ca^{2+} release from the SR that is currently favoured in the literature was developed by Frank and Bianchi, and involves the use of "trigger Ca^{2+} ions" to induce Ca^{2+} release from the SR stores.[46,47] A twitch action potential enters the t-tubule system of the skeletal muscle fibre and causes a release of the Ca^{2+} ions that are bound to the t-tubule intracellular surface. These Ca^{2+} ions are released into the t-tubule/SR junctional space and diffuse across to the surface membrane of the terminal cisternae (junctional SR). The released

Ca^{2+} ions are referred to as "trigger Ca^{2+} ions" and they induce Ca^{2+} release from the terminal cisternae of the SR via the ryanodine-sensitive Ca^{2+} release channels. Ca^{2+} from the terminal cisternae is released into the myoplasm of the muscle cell.[48] The increased $[Ca^{2+}]$ produces a mechanical response similar to the process observed in cardiac tissue.

Skeletal muscle and cardiac muscle differ in their requirements for extracellular Ca^{2+} in the E-C- coupling process. In the absence of extracellular Ca^{2+}, cardiac muscle fibres will cease to contract as the necessary Ca^{2+} required for Ca^{2+}-induced Ca^{2+} release from the SR comes from transsarcolemmal influx via the L-type calcium channels.[22] Skeletal muscle fibres that are bathed in Ca^{2+} free solutions will continue to elicit twitch contractions for several minutes. The cessation of twitch contractions appears to be dependent upon the time required for the Ca^{2+} ions bound to the t-tubule intracellular membranes to diffuse out (2-5 minutes).[50] The SR Ca^{2+} stores require several hours to diffuse out a sufficient quantity of Ca^{2+} to interfere with the contraction process and, therefore, are not the reason for termination of contractions.[49] Blockage of the twitch contractions appears to be due to the removal of Ca^{2+} from the t-tubular membranes. Studies using organic channel blocking drugs (verapamil and gallopamil)[51] and blockers of calcium dependent processes (TMB-8)[52] have added support to this hypothesis.

There are two additional proposed mechanisms for E-C-coupling during the twitch contraction. The first proposes that inositol

1,4,5-triphosphate (IP_3) produced by the hydrolysis of membrane bound phosphotidylinositol 4,5-biphosphate is the chemical transmitter in the skeletal muscle.[53,54] Recent investigations into IP_3's action have shown that IP_3 could act as a chemical transmitter in smooth muscle but in skeletal muscle the response to IP_3 is far too slow (a magnitude of 3) and the concentration required far too high for it to play a principle role in the coupling process.[55] IP_3 may play a role in the modulation of cell function rather than a chemical messenger role in the E-C coupling process.[56,57]

The second proposal involves a mechanical link between the t-tubular membrane and the terminal cisternae of the junctional SR. This mechanical connection is proposed to be via the junctional feet. The mechanical link involves the use of voltage-dependent charge movements to activate Ca^{2+} release from the SR. There appears to be far more dihydropyridine binding sites than actual calcium channels in skeletal muscle.[58] If these dihydropyridine receptors are not linked to functional calcium channels, they are thought to possibly act as "voltage sensors"[59] and be associated, anatomically and functionally, with the junctional feet processes that span the t-tubule/SR gap. These dihydropyridine "voltage sensors" are suggested to couple the electrical activity of t-tubule depolarization with Ca^{2+} release from the terminal cisternae at the junctional SR.[60]

Whatever the mechanism of triggering the release of Ca^{2+} from the junctional ryanodine-sensitive Ca^{2+} release channels, the

resultant increase in myoplasmic free [Ca^{2+}] activates the skeletal muscle myofilaments to contract. The process of myofilament activation, cross bridge formation and relaxation are similar to that seen in cardiac tissue.[61] Mechanisms involved in the lowering of intracellular [Ca^{2+}] necessary for relaxation are also similar between the two tissues and include Na/Ca exchange, SR ATP-dependent Ca^{2+} pump, and sarcolemmal ATP-dependent Ca^{2+} pump. The relative importance of each of these mechanisms also appears to be similar to cardiac tissue, with the SR ATP-dependent Ca^{2+} pump contributing the most to the relaxation process.[62]

Excitation-Contraction Coupling in Smooth Muscle

The E-C coupling process in smooth muscle differs significantly from that of either skeletal or cardiac muscle. A major reason for this difference is the structural makeup and functional role of smooth muscle cells. Unlike cardiac and skeletal muscle which are fast contracting fibres, smooth muscle contraction is tonic in nature and may remain in a contracted state for prolonged periods of time.[63] The structural makeup of smooth muscle cells is unlike either cardiac or skeletal muscle. In skeletal muscle the organization of the contractile apparatus is highly ordered with a well defined SR. In smooth muscle cells, the organization is less ordered and ambiguous.

The contractile apparatus in smooth muscle consists of thin (actin), thick (myosin) and intermediate filaments. The thin filaments are compromised primarily of actin and tropomyosin and insert into dense fusiform bodies on the plasma membrane or within

the cytoplasm.[64] These dense bodies are comprised of intermediate filaments which are thought to function in a cytoskeletal role and provide mechanical support for the myofilaments. The intermediate filaments are comprised of vimentin and desmin.[65] Myosin is composed of two heavy chains and two sets of light-chain subunits, one of which (LC_{20}) is 20,000 Da in size and regulatory in function.[66] The ratio of thin:thick filaments differs between smooth muscle types and may range from 5:1 up to 27:1.[67]

The SR of smooth muscle, though present, is not as defineable a structure as it is in skeletal or cardiac tissues. All smooth muscle cells have a system of sarcoplasmic reticulum, though the volume is variable between smooth muscle types and may range from 2 to 7.5% of the cell volume.[68] The SR of smooth muscle cells is often a diffuse structure, with no "defineable" location within the cell. Certain regions of the SR do, however, show structural specialization. For example, the junctional SR is connected via bridging structures to the surface membrane. This structure resembles the triadic formation found in skeletal muscle.[69] The coupling of the surface membrane with the junctional SR allows for the possible transfer of surface electrical activity or the action of drugs on surface receptors to trigger Ca^{2+} release.[70] The longitudinal SR contains an ATP-dependent Ca^{2+} pump which is structurally similar to the Ca^{2+} pump found in both skeletal and cardiac muscle SR.[71] In addition, smooth muscle cells have a similar ryanodine-sensitive Ca^{2+} release channel located in the junctional SR, responsible for release of SR Ca^{2+} stores during

contraction.[72]

The action potential in smooth muscle differs from the action potentials seen in either skeletal or cardiac tissue. The resting membrane potential of vascular smooth muscle cells is approximately -40 to -55 mV.[73] Vascular smooth muscle cells lack tetrodotoxin-sensitive Na^+ channels at the sarcolemmal membrane, and possess a lower permeability to K^+ ions as compared to cardiac tissue.[74] In the absence of Na^+ channels, the inward current is carried by Ca^{2+} ions via voltage-dependent Ca^{2+} channels.[75] The repolarization phase of the smooth muscle cell is carried by K^+ channels. Several types of K^+ channels have been identified, including Ca^{2+}-activated K^+ channels, delayed rectifier K^+ channels, and ATP-sensitive K^+ channels.[76]

There are two E-C coupling mechanisms active in smooth muscle cells. Electrochemical coupling depends upon depolarization of the sarcolemmal membrane by the opening of voltage dependent Ca^{2+} channels, resulting in an inward transsarcolemmal movement of Ca^{2+} ions.[77] Pharmacomechanical coupling results in contractions without polarization of the sarcolemmal membrane and may be a result of the Ca^{2+} released from sequestered stores or Ca^{2+} entry through channels opened by receptor occupation, or both.[77]

Electrochemical coupling is dependent upon depolarization of the sarcolemmal membrane to open voltage-dependent Ca^{2+} channels allowing inward transsarcolemmal flux of Ca^{2+} ions. The opening of voltage-dependent Ca^{2+} channels occurs at varying levels of membrane depolarization in the different smooth muscle tissues.

For example, depolarization above -30mV will result in opening of voltage-dependent Ca^{2+} channels in rabbit intestine.[101] This inward Ca^{2+} flux results in an increase in the myoplasmic free $[Ca^{2+}]$, inducing release of Ca^{2+} from junctional SR stores via the ryanodine-sensitive Ca^{2+} release channels. This is equivalent to the Ca^{2+}-induced Ca^{2+} release observed in cardiac tissue.[72]

Contraction of smooth muscle is also possible without the necessity of sarcolemmal membrane polarization. In addition to those Ca^{2+} channels that are opened upon membrane depolarization, there is a set of Ca^{2+} channels that are not opened by depolarization but opened in response to receptor occupation by an appropriate agonist, for example acetylcholine.[78] This type of smooth muscle contraction is via pharmacomechanical coupling in which binding of an appropriate ligand to the Ca^{2+} channel receptor results in opening of the channel and an inward Ca^{2+} flux. Contraction using this process is dependent upon extracellular Ca^{2+}.[79] Stimulation with certain other compounds (PGE_1-prostaglandin E1, angiotensin II) can elicit contractions in smooth muscle cells without initiating any Ca^{2+} inward current and appear to act in releasing Ca^{2+} from sequestered stores in the SR via a second messenger system (IP_3).[80] The hormone receptors on the sarcolemma are coupled via G-proteins to phosphatidylinositol hydrolysis. The activated G-protein complex is speculated to target phospholipase C which converts phosphatidylinositol-4,5-diphosphate to diacylglycerol and IP_3.[81,83] IP_3 diffuses across to the junctional SR and stimulates IP_3-responsive Ca^{2+} channels, thus

causing release of Ca^{2+} stores and contraction.[82]

It is evident that there are several ways in which to trigger the necessary Ca^{2+} release from the SR stores. The triggered release of Ca^{2+} ions results in an increase in the myoplasmic free $[Ca^{2+}]$. This increase in the $[Ca^{2+}]$ can regulate contraction of smooth muscle in a variety of ways. One of the more important ways involves the phosphorylation of the myosin myofilament. Myosin contains a regulatory light chain subunit denoted LC_{20}. LC_{20} is phosphorylated (at Serine-19) by myosin light chain kinase (MLCK). MLCK is activated by Ca^{2+} and calmodulin (a Ca^{2+}-binding protein).[84] The phosphorylation of LC_{20} by MLCK is the signal that initiates the cycling of cross-bridges necessary for contraction. LC_{20}'s phosphorylation appears to initiate contraction by turning on actomyosin ATPase activity[85] as well as inducing conformational changes within the thick filament facilitating cross-bridge formation.[86]

Additional regulation of smooth muscle contraction has been shown to come from two actin-binding proteins, caldesmon and calponin. These two proteins have been identified in smooth muscle cells and are thought to regulate the thin-filament. Caldesmon from vascular smooth muscle inhibits actomyosin ATPase activity[87] and enhances the binding of myosin to actin.[88] Alterations in actomyosin binding or ATPase activity by caldesmon may slow crossbridge detachment.[88] Calponin binds both myosin and actin and inhibits actomyosin ATPase activity.[89] This inhibition is reversed by phosphorylation by protein kinase C, Ca^{2+} and calmodulin.[90]

Relaxation in smooth muscle appears to be dependent upon the state of phosphorylation of the myosin filament. Phosphorylation of myosin LC_{20} is crucial in initiating contraction, and the reversal of LC_{20} phosphorylation is a prerequisite for relaxation. The state of myosin phosphorylation can be regulated by the opposing actions of myosin phosphatase and by modulation of the activity of MLCK. Myosin phosphatase activity results in dephosphorylation of LC_{20} causing a decrease in actomyosin ATPase activity and reduced cross-bridge cycling. Inhibitors of myosin phosphatase (okaidaic acid toxin[91]) induce contraction or prevent relaxation of fibre preparations[92], highlighting myosin phosphatase's role in the relaxation process. MLCK phosphorylates LC_{20} and requires Ca^{2+} and calmodulin for activation. Phosphorylation of MLCK reduces its affinity for Ca^{2+}, resulting in decreased activity and reduced phosphorylation of LC_{20}.[93] MLCK can be phosphorylated by several protein kinases including cyclic AMP-dependent kinase[94], Ca^{2+}/calmodulin-dependent kinase II[95], and protein kinase C[96].

The relaxation process in smooth muscle is also dependent upon lowering the myoplasmic free $[Ca^{2+}]$ that was the trigger for the contraction process. The principle mode for this reduction is via the SR ATP-dependent Ca^{2+} pump, which is structurally similar to the Ca^{2+} pump of skeletal and cardiac muscle SR.[97] Alterations in the pump's activity are mediated by phospholamban.[98] In addition, Na/Ca exchange[99] and sarcolemmal ATP-dependent Ca^{2+} pump[100] have been identified in smooth muscle and may participate in the

relaxation process by transsarcolemmal efflux of Ca^{2+}.

CALCIUM AND THE CALCIUM CHANNEL

Calcium:

Role of Calcium

Calcium plays an essential role in the normal functioning of the cell, and in certain cases, the abnormal or pathological behaviour of cells. Free calcium is not only involved in the direct regulation of certain cellular functions (E-C coupling) but also acts in maintaining the cell's structural integrity. The following discussion will examine in further detail the role of Ca^{2+} in the cell.

The preceding treatise on excitation-contraction coupling in cardiac, skeletal and smooth muscle described the crucial role of Ca^{2+} in the contraction process. Ca^{2+}'s regulatory role is, however, not limited to the contraction process. The opening and closing of cellular gap junctions is also dependent upon the intracellular $[Ca^{2+}]$. Cardiac gap junctions are connections formed between two opposing sarcolemmal membranes. These connections are hydrophillic protein channels (connexons) insulated from the extracellular space spanning between the cells.[102] Electrical uncoupling between normal cardiac cells occurs upon intracellular injection of Ca^{2+} which is completely reversed when the intracellular $[Ca^{2+}]$ is lowered.[103] The increased $[Ca^{2+}]$ electrically uncouples cell-to-cell interactions by increasing the junctional resistance and abolishing the cell-to-cell movement of molecules. The mechanism by which this happens is not completely understood but two hypotheses have

been proposed: (i) Ca^{2+} ions bind to the gap junction phospholipids resulting in a conformational change and closure of the gate between the cells[104], and (ii) Ca^{2+} triggers an enzymatic reaction that alters the conformation of gap junctional protein and causes blockage of the channel.[105]

The release of neurotransmitters during stimulus-secretion coupling in neurons is also controlled by changing levels of intracellular Ca^{2+}. Douglas and Poisner first demonstrated the necessary role that Ca^{2+} plays in the neurosecretory process.[106] Since then, the mechanism by which Ca^{2+} regulates this process has been further elucidated. Upon excitation, activation of voltage-dependent Ca^{2+} channels embedded in the nerve terminal membrane results in transmembrane influx of Ca^{2+} from the extracellular space.[107] The resulting increase in intracellular [Ca^{2+}] initiates exocytosis, where neurosecretory vesicles fuse with the plasma membrane and release their contents (neuropeptides) into the synaptic junction.[108] In addition, Ca^{2+} not only appears to control the neurosecretory process but may also regulate the development of the neurons themselves. During chick embryonic development, there is a period of naturally occurring motoneuron cell death. It is evident that Ca^{2+} levels preceding this event are critical in the regulation of this process.[109] Therefore, there may be a causal association between the onset of cell death and the expression of the Ca^{2+} channels necessary for Ca^{2+} movement.[110,111]

In a structural capacity, Ca^{2+} has been recognized as necessary for proper cell-to-cell adhesion, as well as for

maintaining cell membrane structural integrity.[112] The exact means by which Ca^{2+} associates with the components of the membrane to establish its structural integrity is not yet known. However, removal of Ca^{2+} from the surrounding medium appears to disrupt Ca^{2+}-dependent connections between the surface coat and the external lamina, resulting in a fluid-filled separation.[113] These connections may be Ca^{2+}-carbohydrate bridges which require Ca^{2+} for stability.[114]

In certain pathological conditions, alterations in Ca^{2+} levels and efflux/influx mechanisms results in irreversible damage and cell death. One such condition is that of the 'Ca^{2+} paradox', observed when Ca^{2+} is removed from the interstitial fluid and then restored. There is a resulting dramatic increase in the intracellular [Ca^{2+}] causing cell damage and eventual cell death. Several routes of Ca^{2+} influx have been examined including Na/Ca exchange, Ca^{2+} channels, K/Ca exchange, and even the passive movement of Ca^{2+} through the damaged cell membrane.[115,116,116a,117]

Irregularities in normal beating of the heart are referred to as cardiac arrhythmias. Cardiac arrhythmias can be separated into two large classes; (i) those due to abnormal initiation of impulses for contraction, and (ii) those caused by abnormal impulse conduction.[118] Ca^{2+} plays an important role in the generation of both types of arrhythmias. Ca^{2+} can affect both the automatic cells required for the initiation of contraction and the conducting fibres required for the spread of the contraction signal.[119] In addition, Ca^{2+} can induce arrhythmias indirectly by influencing the

cardiac oxygen supply and demand. These indirect effects involve Ca^{2+} altering the coronary or peripheral circulation, and changes in the heart rate or myocardial contractility.[120]

Calcium Channels:

Types and Location

There are currently four types of Ca^{2+} channels that have been discovered in both excitable and non-excitable cells. These include: (i) T-channels, (ii) N-channels, (iii) P-channels, and (iv) L-channels. The separation of these Ca^{2+} channels into their respective groupings has come from biophysical, pharmacological, and structural data.[121]

T-type Calcium Channels.

The T-type Ca^{2+} channel was first described in vertebrate sensory neurones by Carbone and Lux.[122] They described this new channel as a fully inactivating, low voltage activated Ca^{2+} channel.[122] Since their discovery, the T-type Ca^{2+} channel has been described in a wide variety of excitable and non-excitable cells including cardiac tissue, skeletal muscle, smooth muscle and neurons.[123]

T-type channels have a low activation voltage (-50 mV to -60 mV), peak at -30 mV and are inactivated quickly (5-30 msec).[5] This quick inactivation results in a small, unitary conductance of Ca^{2+}.[124] In cardiac tissue, the conductance of Ca^{2+} is too brief to be a major contributor to the E-C coupling process.[5] However, this brief conductance may be sufficient to play an important role in the activity of the pacemaking cells in the sinoatrial node where

the majority of the Ca^{2+} channels are of the T-type.[5,126]

Pharmacological data has shown the T-type channels to usually be considered as insensitive to dihydropyridines, and, in sensory neurons, to verapamil (a phenylalkylamine).[123,124] Sensitivity of the T-channels to felodipine (a dihydropyridine) has, however, been demonstrated.[125] The dihydropyridines, phenylalkylamines and benzothiazepines are three groups of a class of organic compounds referred to as calcium channel blockers (antagonists). These compounds will be discussed in detail subsequently. Drugs like tetramethrin[126], diphenylhydantoin[127], and amiloride[128] have all shown to act on the T-type Ca^{2+} channel, although none appear to be selective for the channel. Nickel and cadmium are also capable of blocking the T-channel, with nickel usually more effective.[129]

N-type Calcium Channels

The N-type Ca^{2+} channel was first described by Nowycky in the chick dorsal root ganglion.[130] N-type Ca^{2+} channels appear to be present only in neuronal tissue and do not seem to exist in muscle tissue. N-channels have also been found in certain endocrine cells such as the pancreas, anterior pituitary and the adrenal gland.[130] N-channels are activated by membrane potentials similar to those that activate L-type Ca^{2+} channels (-30 mV to -40 mV) and inactivate within tens to hundreds of milliseconds at potentials similar to L-type channels.[131] The actual differences between N- and L-type Ca^{2+} channels appear to be minimal. They activate and inactivate at similar membrane potentials and their Ca^{2+} conductance also appear to be very similar.[132] The distinguishing

factor between N- and L-type Ca^{2+} channels is their pharmacological properties. N-type Ca^{2+} channels are inhibited by a group of snail toxins, ω-conotoxins, while being insensitive to modulation by dihydropyridines.[130,133] The primary means of modulation of the L-type Ca^{2+} channels is via dihydropyridines (calcium channel blockers) for which they are selective.

In neuronal membranes, both N- and L-type Ca^{2+} channels have been identified.[137] However, there, appears to be a specific distribution of the two channel types. Radioactive antibody binding studies have shown that N-channels are particularly associated with the neuron terminus, probably functioning in neurotransmitter release.[134,135] It appears that the N-type Ca^{2+} channels carry the bulk of the inward current during activation of the neuron, and the L-type channels play a much smaller role in this process.[136]

P-type Calcium Channels

Certain neurons exhibit a Ca^{2+} channel that is activated at high membrane potentials and insensitive to both dihydropyridines and ω-conotoxin.[138,139] This particular channel has characteristics which are dissimilar to other Ca^{2+} channels. These new Ca^{2+} channels are abundant in cerebellar Purkinje cells and virtually absent in other neurons.[139,140] Due to their predominance in Purkinje cells, they were denoted as P-type channels, though it is not known whether it is one type of channel or a group of similar channels. These P-type channels are not blocked by either the ω-conotoxin or DHP, but are blocked by another toxin derived from the venom of the

spider *Agelenopsis aperta*.[139] Little work has been carried out into the biophysical and structural make-up of the P-type channel, but it is suggested from preliminary work completed that the P-channels have a similar subunit structure to that of L-channels.[141]

L-type Calcium Channels

The L-type Ca^{2+} channel has been the most extensively studied of all the Ca^{2+} channels. The L-type Ca^{2+} channel is often referred to as the dihydropyridine-sensitive Ca^{2+} channel, because much of the characterization of the channel has come from the use of dihydropyridine Ca^{2+} channel ligands which specifically bind to this type of Ca^{2+} channel. The L-type Ca^{2+} channel has been identified in all types of cells within the body, including skeletal, cardiac and smooth muscle, and nervous tissue. Within these different tissues, there are certain unifying characteristics among the L-type Ca^{2+} channels found there. These include: (i) sensitivity to inorganic and organic blockers, (ii) stereotypical response to dihydropyridine Ca^{2+} agonists, (iii) activation range (-40 mV to -30 mV)[5], (iv) slow inactivation when Ca^{2+} is not the primary charge carrier, and (v) steady-state inactivation at positive holding potentials.[124] Variations between the various tissue type Ca^{2+} channels exist as well, such as Ca^{2+}-dependent inactivation[142] and the increase in channel activity by cyclic-AMP-dependent kinase activity (seen in cardiac tissue)[143]. The most variable parameter between the tissue L-type Ca^{2+} channels is voltage-dependent inactivation. The most rapidly inactivating channels are from cardiac tissue, while neuronal or secretory L-

type Ca^{2+} channels appear not to have any voltage-dependent inactivation. Variability in the rate of inactivation has also been observed in channels from the same tissue.[144]

To explore the L-type Ca^{2+} channel in further detail, a tissue specific examination of skeletal muscle, cardiac tissue, and smooth muscle follows.

Skeletal Muscle L-type Calcium Channels.

Skeletal muscle has a very high concentration of dihydropyridine-binding sites, and therefore, much of what is known about the dihydropyridine receptor and the L-type Ca^{2+} channel has come from study of skeletal muscle tissue. However, the dihydropyridine receptors found in skeletal muscle tissue are not all functional L-type Ca^{2+} channels. Schwartz determined that the density of dihydropyridine receptors was 35-50 times the number of functional L-channels.[160] Solublization studies of the dihydropyridine-binding site and reconstitution into phospholipid vesicles resulted in an active L-channel. The solublization process yields a multisubunit complex.[145,146] The skeletal L-channel has been shown to be a pentameric formation composed of 4 distinct subunits, α_1 (170 kDa), α_2/δ (175 kDa), ß (52 kDa) and the γ subunit (32 kDa). The δ subunit can be released from the α_2/δ configuration by reduction of a disulfide bond.[147]

The α_1 subunit sequence shows considerable homology to those of other members of a voltage-dependent ion channel superfamily which includes K^+ and Na^+ channels.[145] For example, there is approximately 30% homology in the skeletal muscle α_1 subunit and

the voltage dependent Na^+ channel. The α_1 subunit in skeletal muscle contains four internal repeating units which contain six membrane-spanning helices. The voltage-sensing location of the channel is thought to reside on the fourth of each these helices where there is a positively charged amino acid every three to four residues.[121] The α_1 subunit is also where the organic compounds, the calcium channel antagonists, are thought to bind. The dihydropyridine-binding site is believed to be located on the extracellular surface of the α_1 subunit.[148]

Expression of the α_1 subunit in mouse cells devoid of β,α_2/δ, and γ subunits demonstrated the appearance of dihydropyridine-sensitive L-type Ca^{2+} channels.[149] This observation would suggest that the α_1 subunit itself can form a functional Ca^{2+} channel, and that the additional subunits may provide functional and structural support. The α_1 subunit is also thought to play a distinctive role in skeletal muscle by acting as a voltage sensor in the excitation-contraction coupling process.[146] The previously described E-C coupling process in skeletal muscle indicated that the release of Ca^{2+} from the SR stores may be mediated by a linking of the dihydropyridine voltage sensor to the junctional SR ryanodine-sensitive Ca^{2+} release channel. Depolarizing stimulus is 'sensed' by the dihydropyridine-voltage sensor resulting in a conformational change in the α_1 subunit. This conformational shift is transmitted to the Ca^{2+} release channel causing it to open and release the Ca^{2+} stores.[59] Additional support for this hypothesis comes from the observation that not all the dihydropyridine receptors are actually

linked to functional L-type Ca^{2+} channels and may act as voltage sensors.[160] Therefore, the α_1 subunit of skeletal muscle appears to not only act as the functional unit for the Ca^{2+} channel but may also act as a sensoring mechanism necessary for triggering release of Ca^{2+} from the SR during E-C coupling.

The α_2/δ subunit (175 kDa) can be reduced to a 150 kDa α_2 subunit and 3 peptides of 25,22, and 17 kDa forming the δ subunit.[146] The separation of the complex occurs when disulfide bonds which hold the subunits together are reduced. The α_2/δ complex is the product of a single gene with the α_2 sequence forming the N-terminal and the δ sequence forming the C-terminal.[151] The α_2/δ complex has a high level of glycosylation with both subunits being glycosylated.[150] The structural arrangement of the α_2/δ complex within the Ca^{2+} channel has not been fully elucidated, but it is thought that the δ portion may act as an anchor for the α_2 portion.[145,150] Functionally, the α_2/δ complex appears to be able to modify the activity of the Ca^{2+} channel. Coexpression studies of α_1 and α_2/δ showed altered Ca^{2+} current, in both magnitude and kinetics, in skeletal muscle preparations.[152,153] In addition, the rate of Ca^{2+} influx through L-channels was enhanced in liposomes reconstituted with α_1 and α_2/δ subunits.[154]

The ß subunit was originally identified as a protein that consistently copurified with the α_1 subunit.[145] The ß subunit contains several phosphorylation sites. Ca^{2+} channel function is known to be modulated by phosphorylation events, and the ß subunit may be the area upon which the enzymes act to alter Ca^{2+} channel

kinetics.

The ß subunit is believed to be associated with the cytoplasmic component of the α_1 subunit.[155] The consistent copurification of the ß subunit with the α_1 subunit suggested that the ß subunit interacted with the α_1 in a functional manner. Coexpression studies of α_1 transfected L-cells along with ß subunits showed a dramatic increase in the number of dihydropyridine binding sites but no effect on current density. There was, however, an increase in the rate of Ca^{2+} current activation.[149,156] Coexpression studies in which the α_2/δ subunit was added ($\alpha_1/\alpha_2\delta$/ß combination) yielded a peak current above that seen with the α_1/ß complex, indicating a close regulatory interaction between the various subunits.[141,157]

The γ subunit is the least characterized of the skeletal muscle L-channel subunits. The γ subunit consistently copurifies with the α_1 subunit, appears to be very hydrophobic and is extensively glycosylated.[145,158,159] Coexpression studies using the skeletal muscle γ subunit with the other subunits has shown little functional significance[141] and requires further research.

Cardiac L-type Calcium Channels.

The cardiac L-type Ca^{2+} channel plays a significantly different functional role than does the skeletal muscle L-channel. In cardiac tissue, the release of SR Ca^{2+} stores is induced by transsarcolemmal influx of Ca^{2+} during the plateau phase of the cardiac action potential. This transsarcolemmal influx of Ca^{2+} is mediated by L-type Ca^{2+} channels.[161] This Ca^{2+}-induced Ca^{2+} release

mechanism in cardiac tissue, as opposed to the possible voltage gated release in skeletal muscle, may account for the observation that the majority of dihydropyridine receptors found in cardiac tissue are functional L-type Ca^{2+} channels.[162]

The L-type Ca^{2+} channel has been characterized, and has been found to consist of four subunits; α_1 (170-190 kDa), α_2 (170 kDa), ß (52 kDa), and δ (28 kDa)[163,164]. The γ subunit found in the skeletal muscle L-channel has not been found in cardiac tissue.[165] The cardiac α_1 subunit shows structural similarities to the skeletal muscle α_1 subunit. The cardiac α_1 subunit contains the four repeating motifs, each consisting of 6 transmembrane domains similar to that of skeletal muscle.[166,167] However, there are several (5) protein kinase A phosphorylation sites identified in the skeletal muscle α_1 subunit which are missing in the cardiac form and are replaced by four new sites.[166] Structural differences have also been noted with respect to the extracellular protein regions, which may account for the lack of cross-reactivity of antibodies raised against the skeletal muscle α_1 subunit.[168] Differences have also been observed in the lengths of the 5' and 3' ends of the cDNA clones that are responsible for encoding the skeletal and cardiac muscle α_1 isoforms.[167] The cardiac isoform of the α_1 subunit appears to have the same functional characteristics as does the skeletal muscle form. The α_1 subunit is capable of functioning as a L-channel and is the site of binding of the calcium channel antagonists.[168]

The ß subunit has been found and cloned in cardiac tissue.

Coexpression of this ß subunit with a cardiac α_1 subunit resulted in altered Ca^{2+} channel kinetics including increased peak currents, accelerated activation kinetics and shifts in the voltage-current relationship to more hyperpolarized potentials. The cardiac ß subunit is similar in structure to the skeletal muscle isoform and is thought to act in a modulatory role.[208] The additional subunits that have been reported likely function in structural support (anchoring) and in regulation of channel kinetics.[168]

Smooth Muscle L-type Calcium Channels

The smooth muscle L-type Ca^{2+} channel is not as well characterized as the cardiac or skeletal muscle channel. There are fewer dihydropyridine binding sites in smooth muscle tissue, as compared to the numerous availability found in skeletal muscle, which has made characterization of the channel difficult. The smooth muscle L-type Ca^{2+} channel is likely very similar to the Ca^{2+} channel found in cardiac muscle. It is proposed to be a quaternary structure, composed of α_1, α_2, ß and δ subunits. The γ subunit which was observed in skeletal muscle but not in cardiac muscle, also appears to be absent in smooth muscle. Using a reverse-transcribed polymerase chain reaction technique, the presence of the γ transcript in RNA isolated from mouse brain, cardiac muscle, spleen, kidney, liver, and stomach as well as from human brain and cardiac muscle was undetectable. The γ subunit was detected in human and mouse skeletal muscle preparations.[165]

A cDNA library isolated from rat aorta has shown the aortic α_1 cDNA to be very similar to α_1 cDNA isolated from cardiac tissue.

The close identity of the two α_1 cDNA libraries has suggested that the α_1 subunit from these two tissues arise from the same gene.[207] Differences that exist in the smooth muscle isoform are thought to arise from alternative splicing that occurs in both the cardiac and smooth muscle α_1 subunits.[207]

Ion Movement Through Calcium Channels

There are currently two popular hypotheses about the method of Ca^{2+} ion movement through Ca^{2+} channels: (i) the allosteric or one-point model[169], and (ii) two-site model.[170,171] The strengths and weaknesses of these two models will be discussed.

The allosteric model (one-site model) predicts that there is a high-affinity Ca^{2+} binding site located on the external surface of the Ca^{2+} channel and has a net charge of -2 e[172]. This Ca^{2+} binding site controls the selectivity of the channel. When the extracellular $[Ca^{2+}]$ is greater than 1 μM, this Ca^{2+} binding site is primarily occupied by Ca^{2+} and there is a resulting conformational change in the channel resulting in only Ca^{2+} ions passing. The ions that are moving through the channel, transiently bind to a site in the pore itself, but only weakly. When the Ca^{2+} binding site on the external surface is not occupied by Ca^{2+}, the channel pore becomes nonselective, allowing passage of monovalent ions.[124]

The weakness of this model is the conformational change that occurs upon binding of Ca^{2+} to the regulatory site, resulting in selective Ca^{2+} ion permeation. The character of this conformational change is difficult to assess and therefore the

precise mechanism by which the channel selects between blocking and permeant ions remains unclear.[124,173]

The two-site model proposes that there are two high-affinity Ca^{2+} binding sites, located at either end of the channel. An ion moving from the outside to inside of the cell has to first bind to the outer site, then inner site, and then finally passing into the cytoplasm.[124] When both sites are occupied, the affinity of each of the binding sites for the Ca^{2+} ion is reduced due to electrostatic repulsions of the two same charged ions. This electrostatic repulsion speeds up the departure of the other ion. The model makes the assumption that an ion can only move into a site that is vacant. Therefore, at high $[Ca^{2+}]$, both binding sites are likely to be occupied. This destabilizes the inner binding site which increases the probability that the binding site will lose its Ca^{2+} ion to the cytoplasm.[174,175] The outer site Ca^{2+} ion can then move to the free inner site. Binding of another Ca^{2+} ion to the outer site, again destabilizes the inner site and that ion then moves into the cytoplasm.[172,174,175] The selectivity of the channel is determined by selectivity sequences contained within the high-affinity binding sites.[124]

The two-site model is able to explain many of the experimental results obtained using dihydropyridine-sensitive Ca^{2+} channels. For example, the two site model can explain monovalent conduction through the channel in the absence of divalent cations, saturation of current as a function of $[Ca^{2+}]$, the ability of very low divalent concentrations to inhibit monovalent conduction, and the

ability of low [Ca^{2+}] to inhibit Ba^{2+} conduction through the channel.[172] The main weakness in this theory is that it assumes symmetry of the channel energy profile, so that the forces at work at the outer binding site are equivalent to those at the inner site. In addition, the model assumes the channel to be a relatively inert structure that does not interact with the ions passing through the channel, except for binding and unbinding reactions. There is, therefore, no conformation change associated with the channel upon ion permeation.[124]

Regulation of Calcium Channel Activity

The Ca^{2+} channel can exist in one of three conformational states at any one time. Those states are: (i) activated or open, (ii) inactivated, or (iii) resting. The activated or open state is evident upon depolarization of the membrane resulting in opening of the Ca^{2+} channel from the resting state. In the activated state, there is conductance of ions across the sarcolemmal membrane into the cytoplasm. Inactivation is the process by which the Ca^{2+} channel enters into; (a) a non-conducting state once it has been activated, or (b) a state where it is not available for activation. The inactivated state is an intermediate stage in which the channel is not conducting any ion current (closed) but it not presently available to be reopened with another depolarizing stimulus. The recovery process moves the Ca^{2+} channel from the inactivated state to the resting state. At this stage, the channel is closed (non-conducting) but is available to be reopened upon being presented with a depolarizing stimulus.[5] The normal progression of the Ca^{2+}

channel upon being presented with a depolarizing stimulus is to move into the open conducting state from the resting state. Following the open state, the channel moves into the inactivated state where there is no further ion movement and the channel is not available to be reopened if another stimulus were to be presented. When the membrane potential reaches a certain point, the channel moves from the inactivated state back to the original resting state. For the channel to be opened it has to first be in the resting state, and for the channel to get back to the resting state it has to go through the inactivated state.

The current flow through the Ca^{2+} channel may be modulated by several enzyme and organic ligand compounds. The resulting current flow may be increased or decreased depending upon the compounds antagonistic or agonistic tendency. One well known modulator of Ca^{2+} current is the stimulatory effect of ß-adrenergic agonists on heart cells. ß-adrenergic stimulation of Ca^{2+} current in heart cells may occur via two means: (i) indirectly via the phosphorylation of the Ca^{2+} channel, and (ii) directly by the stimulation of the Ca^{2+} channel by binding of activated G protein.[176]

The application of a ß-adrenergic agonist (like isoprenaline) to heart cells results in a cascade of events culminating in a stimulation of I_{Ca} (calcium current). ß-adrenergic binding to ß-receptors located on the sarcolemmal membrane stimulates adenylate cyclase activity. Adenylate cyclase activation increases the levels of cyclic adenosine monophosphate (cAMP), an intracellular

second messenger. Increased levels of cAMP activate the enzyme protein kinase A which phosphorylates the α_1 subunit of the Ca^{2+} channel protein complex. Phosphorylation of the α_1 subunit alters the Ca^{2+} channel properties causing an increase of I_{Ca}.[176] The resulting increase in I_{Ca} by cAMP-induced phosphorylation of the Ca^{2+} does not change the rate or the amount of Ca^{2+} that enters via an individual channel, but it does increase the probability that the channel will open. Therefore, there will be an increased number of channels open at any given moment increasing the influx of Ca^{2+}.[180] Experimental evidence supporting this cascade and the resulting increase in I_{Ca} by phosphorylation of the Ca^{2+} channel comes from the use of protein kinase inhibitors[177] and phosphatases.[178] Addition of a heat stable protein kinase inhibitor via internal cell dialysis to inhibit cAMP production resulted in the suppression of the ß-adrenergic agonist enhanced I_{Ca}.[177] In addition, the intracellular application of a protein phosphatase (calcineurin) which would dephosphorylate the Ca^{2+} channel was also able to reverse the increment in I_{Ca} by ß-adrenergic stimulation.[178]

ß-adrenergic stimulation of I_{Ca} may also come from the direct coupling of the activated G_s subunit with the Ca^{2+} channel. When the cAMP-dependent phosphorylation pathway was blocked, addition of a ß-adrenergic agonist (isoprenaline) still evoked an increase in the I_{Ca}.[176] The resulting increase in I_{Ca} by G_s was relatively small compared to that invoked by the cAMP-mediated pathway. It is thought that G_s may act to prime the Ca^{2+} channels for up-regulation by the cAMP-dependent phosphorylation.[176]

Phosphorylation of the Ca^{2+} channel may not be a means of increasing I_{Ca} alone but may also be necessary for the channel to respond to membrane depolarization. Studies in which the α_1 subunit of the Ca^{2+} channel has been used to reconstitute a voltage-activated channel has shown phosphorylation by the cAMP-dependent protein kinase is necessary and sufficient to restore activity of these preparations. Addition of a protein kinase inhibitor to the preparation blocked activity. An endogenous protein kinase A is believed to be associated with the membrane in close proximity to the channel to regulate its gating activity.[179]

ß-adrenergic stimulation also increases the rate of resequestering of Ca^{2+} back into the SR, via the SR Ca^{2+} pump. Phosphorylation of the protein phospholamban by cAMP-induced protein kinases results in increased activity of the SR Ca^{2+} pump.[183] This increased activity of the pump allows for a faster re-accumulation of Ca^{2+}, allowing for the relaxation process to occur at an accelerated rate.[181] Additional proteins like Troponin I, C, and myosin light chain kinase are also phosphorylated by cAMP-dependent kinases upon ß-adrenergic stimulation. The phosphorylation of these protein complexes allows for quicker cycling of the myofilaments during the contraction process.[182] The overall consequence of ß-adrenergic stimulation is an increase in the chronotropic and inotropic aspects of the cardiac cycle.[180,184]

A group of compounds that decrease the conductance of Ca^{2+} via the Ca^{2+} channels are the calcium channel blockers (antagonists). This particular group of compounds will be discussed in detail

later, and it will be sufficient at this time to simply outline their effects on calcium channel kinetics. The calcium channel blockers bind to the α_1 subunit of the L-type Ca^{2+} channel. There are currently, three main classes of calcium channel blockers, the dihydropyridine (dihydropyridines), phenylalkylamines and benzothiazepines.[185] All three classes have their receptor-sites on distinct but closely related sites on the α_1 subunit of the L-type Ca^{2+} channel.[186] It is thought that the calcium channel antagonists preferentially bind to the depolarized or inactivated state of the calcium channel, and in some way decrease the probability of the channel reopening.[185] The process by which the calcium channel antagonists prevent or slow the reopening of the channel is thought to be by slowing channel rephosphorylation.[179] Armstrong proposed that for the Ca^{2+} channel to be opened upon membrane depolarization, it first must be phosphorylated by an associated protein kinase A.[179] If the calcium channel blockers slow the rephosphorylation process, the channels would not be available for opening upon further membrane depolarizations.[179]

The result of binding of the calcium channel blockers to the L-type Ca^{2+} channel is to reduce the influx of Ca^{2+} ions via the channels. This reduction in 'trigger' Ca^{2+} causes a decrease in contractility in cardiac tissue, and relaxation in vascular smooth muscle.[185]

Regulation of Calcium Channel Density

One of the primary means of controlling the Ca^{2+} available for cellular functions is via the activity of the Ca^{2+} channels. The

regulation of the number of Ca^{2+} channels present in a tissue at a particular time is also controlled. It is well known that the number of Ca^{2+} channels present in a specific tissue is influenced by circulating drug (hormone) levels and particular disease states.

The regulation of ion channels and their densities appears to follow a similar pattern to that observed in the regulation of membrane receptors. Receptor regulation can be separated into two main categories: (i) homologous regulation, in which a ligand regulates its own receptor, and (ii) heterologous regulation, where the receptor is regulated by a ligand/process occuring at a discrete receptor system.[187] The exact mechanism by which a ligand will regulate its receptor is different and specific for that particular receptor system.

Receptors can be regulated after either long- or short-term exposure to a ligand. It is usually following a prolonged exposure that changes in receptor regulation are noticed. Short term exposures usually result in temporary modifications to membrane potential, coupling systems, receptor distribution, phosphorylation state and membrane lipid environment causing their change in receptor metabolism[267,268] (i.e. synthesis, membrane insertion, internalization, recycling and degradation[265]). Chronic or long-term ligand exposure usually results in a decrease (down-regulation) of receptors with a agonist ligand and an increase (up-regulation) with an antagonist.[265,269]

The regulation of ion channels (e.g. Ca^{2+} channels) is likely similar to those processes that regulate cell surface receptors as

both are membrane proteins and therefore have similar processing mechanisms. The following discussion will examine the influence on Ca^{2+} channel density of certain disease states and after exposure to chronic circulating drugs (hormones).

Circulating Drugs/Hormones

There are several well characterized responses of cell-surface receptors to circulating hormones. These include down-regulation of ß-adrenergic receptors[266,270], insulin receptors[272] and low-density lipoprotein receptors[274] to high circulating hormone levels. The down-regulation seen in ß-adrenergic receptors occurs after exposure of the ß-receptors to high circulating levels of epinephrine. This exposure of the ß-receptors to high circulating epinephrine results in a nonfunctional binding of ligand to receptor, and effective down-regulation of the ß-receptors. The ß-receptors are still able to bind epinephrine, but the bound epinephrine is not able to activate the adenylate cyclase system necessary for cellular action and this inactivation is thought to be mediated by phosphorylation of the ß-receptor.[266,270,271] The down-regulation seen in insulin and low-density lipoprotein receptors is mediated by a different process. High circulating levels of insulin hormone or low-density lipoproteins results in cell-receptor internalization by endocytosis.[272,273,274] This effectively reduces the number of receptors present on the surface at any one time, resulting in a down-regulation of the receptors.

Exposure to circulating compounds such as ethanol, lead, insulin, thyroid hormone and calcium channel antagonists all have

been implicated in altering the number of Ca^{2+} channels present in certain tissues.[187] For example, animals or cells chronically exposed to ethanol or lead resulted in an increase in the number of 1,4-dihydropyridine-binding sites present in certain brain regions. Lead treatment resulted in an increase (48%) in the B_{max} (nitrendipine binding) in rat striatum and cortex, but was found not to change in the hippocampal region.[188] The development of ethanol dependence is proposed to involve an increase in the number of 1,4-dihydropyridine-binding sites as a result of chronic ethanol administration. Ethanol is known to inhibit Ca^{2+} influx in cells following chronic treatment, resulting in an increase in the density of Ca^{2+} channels.[189,190]

The body's own circulating hormones may also regulate the density of channels. Treatment with the hormone insulin over a 21-day period resulted in an increase in the density of 1,4-dihydropyridine binding sites in cultured human muscle cells.[191] However, an increase in the number of Ca^{2+} channels was also found in cardiac muscle membranes isolated from streptozocin induced diabetic rats.[337] Additional functional alterations may also result from altered hormone levels as was observed in rat ventricular muscle after chronic diabetes mellitus.[192] Chronic thyroid hormone treatment in chick ventricular cells resulted in an increase in the number of 1,4-dihydropyridine binding sites and a concurrent increase in the number of ß receptors present.[193] However, in heart membranes obtained from hyperthyroid rats, a decrease in the number of Ca^{2+} channels present with a concurrent increase in ß-receptor

density was reported. Hypothyroid rats from the same study showed the opposite effect, with an increase in Ca^{2+} channels and a decrease in the number of ß-receptors present.[194] The discrepancy may be due to a species specific difference, but additional work into thyroid hormone regulation is required.

The compounds that would be expected to regulate the density of the Ca^{2+} channels to the greatest degree and with the most specificity would be the calcium channel antagonists. Several studies have been undertaken looking at different types of calcium channel antagonists, modes of administration, species and tissue reactions.[187] In membranes prepared from mouse brain tissue, a 40% reduction in 1,4-dihydropyridine binding sites was noted after oral treatment with either nifedipine or verapamil for 28 days and no change was noted with oral diltiazem over the same period.[275] A 23% decrease in 1,4-dihydropyridine sites in brain and 49% in heart was noted in a rat model after 20 days of intravenous nifedipine.[276] No change was noted in heart tissue prepared from rats treated with a lower dose of oral nifedipine for 14 days[277], indicating that alterations may be time, mode of application and dose dependent. 1,4-dihydropyridine binding sites in PC12 cells were also noted to increase (29%) after 5 days of nifedipine treatment, while a decrease (24%) was noted after 5 days of treatment with Bay K 8644 (Ca^{2+} channel agonist).[279] It is difficult to obtain reliable information on the action of the calcium channel antagonists on Ca^{2+} channel density due to the variability in experimental protocols.

Disease States

Several pathological conditions are associated with an alteration in the number of Ca^{2+} channels present in certain tissues. Studies conducted on hypertensive rats have shown alterations in 1,4-dihydropyridine-binding sites and has led researchers to suggest that these alterations may be a result of some functional change in these animals, playing a role in their hypertensive condition. Several studies were conducted on a strain of rats referred to as SHR (spontaneously hypertensive rats) in which, due to a genetic condition, all develop hypertension. Radioactive nitrendipine binding on heart membranes prepared from 24-week-old SHRs and 9-week old SHRs showed an increase in K_D and B_{max} in the 24-week but not the 9-week group, as related to normotensive controls.[195] This was confirmed later with elevated nitrendipine sites in cardiac tissue isolated from 16-week-old SHRs but not in 10-week-old SHRs.[196] An up-regulation in the number of 1,4-dihydropyridine sites was noted in heart tissue isolated from spontaneously hypertensive rats (33% increase) and salt-sensitive rats (55% increase) after 7-21 day nitrendipine treatment.[278] It has, therefore, been suggested that alterations in the density of Ca^{2+} channels may be related to hypertension and the possible development of the disease.

An increase in the number of Ca^{2+} channels has been reported in hypertrophied cardiac muscle.[197,198,199,200] The Syrian cardiomyopathic hamster serves as a model for human hypertrophy. Radioactive labelling studies have shown increased dihydropyridine

binding sites in heart, brain, skeletal muscle and smooth muscle in the cardiomyopathic hamster.[200] Additional support for the increase in cardiac binding sites has come from hypertrophy induced via aortic stenosis. An increase in the number of dihydropyridine binding sites was noted after 5 days and 3 weeks post surgery.[201] Controversy has been added, however, by the findings of Howlett, *et al*, who have shown that there is no significant alteration in the number of Ca^{2+} channels obtained from cardiomyopathic hamster cardiac tissue.[202] No significant changes in dihydropyridine binding sites were observed from cardiac tissue obtained from 35- to 41-day old myopathic hamsters.[202,203] Further study is required in order to resolve this controversy, and factors like cardiomyopathic strain, age and membrane preparation differences will have to be addressed.[187] Dihydropyridine-binding sites were also increased in human hypertrophied cardiac tissue.[204,205]

Patients suffering from Parkinson's disease have shown a decrease in the number of dihydropyridine-binding sites in various areas of the brain.[206] Nitrendipine binding showed a decrease in B_{max} from the areas of the caudate nucleus, substantia nigra and putamen with no change noted in the affinity of the channels. Parkinson's disease is characterized by a degeneration of substantia nigra dopamine neurons which is suggested to be the cause of the loss of 1,4 dihydropyridine-binding sites.

CALCIUM CHANNEL ANTAGONISTS

History

The calcium channel antagonists are a heterogeneous group of

related compounds, with one common factor linking them together, their action on the voltage-gated Ca^{2+} channel. Research into their structure, mode of action, classification and clinical use has been undertaken for 30 years since their discovery. Recognition for much of the early work related to calcium channel antagonists is attributed to Albrecht Fleckenstein, who in 1963 was approached by two pharmaceutical companies to look into two new compounds that had vasodilatory and unexplained cardiodepressant abilities.[209,215] Dr. Fleckenstein and his colleagues' original work, along with work carried out by Winifred Nayler, utilised one of the originally discovered calcium channel antagonists: prenylamine.[210] This compound was shown to produce coronary vasodilation and had a strong effect on electromechanical uncoupling in cardiac tissue and this effect was similar to that seen by the removal of extracellular Ca^{2+}. The inhibitory effects on contractility could be overcome by approaches that increased cellular Ca^{2+} mobilization.[211,212]

Prenylamine's inhibitory action on myocardial contractility was questioned as to whether it was a result of adrenergic ß-receptor blockage, catecholamine depletion or some other unidentified intervention. At a conference in Capri, Dr. Fleckenstein and Dr. Nayler presented their work and proposed that prenylamine action was mediated by inhibition of calcium permeation across cardiac cellular membranes.[210] This was the starting point for a varied and extensive research history that has spanned several decades.

The "Calcium Antagonists" nomenclature was coined by Fleckenstein in 1966, and they were described as a group of compounds that restricted calcium-dependent ATP utilization, contractile energy expenditure and oxygen requirement via an interference with activator calcium in active cardiac tissue.[210,213] Prenylamine was a member of this new group of compounds, but additional compounds with even more specific and more potent antagonistic characteristics were discovered including verapamil (one of the two original compounds)[215], methoxyverapamil (D 600) and nifedipine.[214]

Early experimental studies showed that these compounds (verapamil, D 600 and nifedipine) were able to selectively block the Ca^{2+} influx via the slow-mediated channel in depolarized cardiac membranes without affecting the fast transmembrane inward Na^{+} current that initiates the action potential.[210] It is this ability to interfere with the voltage-gated Ca^{2+} channels in the cardiovascular system that has been the major focus of research with these compounds. Additional research lines have focused on vascular smooth muscle, nonvascular smooth muscle, and neuronal systems.[209]

Classification

Fleckenstein's original work allowed for the classification of calcium channel antagonists into two groups: (i) Group A: Highly specific Ca^{2+} antagonists, and (ii) Group B: Less specific Ca^{2+} antagonists.[216]

Group A, or the highly specific Ca^{2+} channel antagonists,

included one of the originally discovered antagonists, verapamil (phenylalkylamines). Also included in this highly specific group were nifedipine (dihydropyridines) and diltiazem (benzothiazepines).[216] These compounds were able to interfere with the transsarcolemmal influx of Ca^{2+} via the slow-entry channel without major inhibition of the fast inward Na^{+} current. They were also able to depress Ca^{2+}-dependent excitation-contraction coupling in mammalian ventricular myocardium by 90%-100% before the fast Na^{+} current was also affected.[216,217]

The WHO (World Health Organization) has subdivided the Group A calcium antagonists into classes I, II, and III: verapamil-like, nifedipine-like and diltiazem-like compounds.[218]

Verapamil was the prototype Ca^{2+} channel antagonist and a member of the phenylalkylamine group, introduced in Europe in 1963. Verapamil has been one of the most extensively studied experimentally, as well as clinically, of all the antagonists. Verapamil was originally used clinically as an antianginal and antihypertensive agent.[219] However, it soon became evident that verapamil had a much more dramatic effect in treatment of supraventricular arrhythmias.[220] Additional members of the verapamil-like antagonists include gallopamil (D 600), anapamil and tiapamil.[219]

Nifedipine was the prototype of the dihydropyridine group of Ca^{2+} channel antagonists. Nifedipine was discovered by Professor Kroneberg, who in 1969 asked Dr. Fleckenstein to investigate this new substance, denoted as Bay a1040.[215,220] Nifedipine was found to

act at a different site on the slow Ca^{2+} channel than verapamil, and had powerful arterial vasodilatory ability but had little effect at the AV node. Nifedipine was found to be useful in the treatment of all grades of hypertension but had little or no direct effect on supraventricular arrhythmias.[219] The dihydropyridine (nifedipine-like) class of Ca^{2+} channel antagonists has undergone an explosion with respect to new additions to the group. There are currently dozens of members to this group including nitrendipine, felodipine, isradipine, PN-200-110 and amlodipine to mention a few.

Diltiazem is the prototype member of the benzothiazepine group and was initially developed in Japan and is now available worldwide. Diltiazem acts and is used clinically for the same spectrum of disorders that verapamil is used for. This led researchers to think that diltiazem interacted with the same site on the Ca^{2+} channel that verapamil did. It is now known that each of the groups of Ca^{2+} antagonists binds to its own receptor site on the α_1 subunit of the L-type Ca^{2+} channel. Diltiazem is clinically used for conditions such as angina pectoris, hypertension and supraventricular arrhythmias.[219]

The Group B Ca^{2+} channel antagonists are less specific and less potent than those members of Group A. These compounds are still capable of interfering with the excitation-contraction coupling process, resulting in a decreased myocardial contractility. However, due to their less specific nature, they also affect the fast Na^{+} influx during the initial phase of the action potential as well as interfering with Mg (Magnesium)-

dependent phenomena.[215,222] Members of this group include prenylamine, flunarizine, bepridil, caroverine, perhexiline, and cinnarizine.[215]

Flunarizine has been shown to have prominent cerebral vasodilatory effects and is considered a mixed antihistaminic. Clinical uses include migraine, vertigo and transient ischemic attacks. Bepridil is considered a mixed sodium blocker that may have clinical use in angina and arrhythmias, but has been removed from the American market due to prolongation of the QT interval in the cardiac cycle.[219]

Mechanism of Action

The Ca^{2+} channel antagonists are known to bind to the α_1 subunit of the L-type Ca^{2+} channel in order to mediate their Ca^{2+} blocking influences.[164,234] The α_1 subunit of the L-type Ca^{2+} channel is composed of four internal repeating sequences, with each sequence consisting of six membrane spanning helices.[121] Molecular characterization studies have been carried out in order to determine the precise location on the α_1 subunit where each of the Ca^{2+} channel blocking drugs bind.

Modelling studies carried out by Langs, *et al.* proposed that the binding site of the dihydropyridines in skeletal muscle may be located on the S4 helix of the L-channel α_1 subunit.[223] However, studies carried out by Rugella, *et al.*[224], proposed another site of dihydropyridine molecular binding. Employing irradiated [^{3}H]nitrendipine to covalently bind to the purified skeletal muscle α_1 complex, followed by digestion and sequencing of the peptides,

indicated that the nitrendipine bound to the cytosolic tail in the region of the S6 helix of the IV^{th} repeating subunit.[224] Analysis of L-type Ca^{2+} channels from other tissues (cardiac and smooth muscle) showed this particular region to be highly conserved between tissues, suggesting a possible reason as to why dihydropyridines bind with high affinity to Ca^{2+} channels from all tissues.[225] The sequence immediately following this proposed dihydropyridine binding site is thought to be the Ca^{2+} binding domain for the channel, which has also been found to be highly conserved between tissues.[226] Association between these two sites may play an important functional role in regulating channel activity.

The phenylalkylamine binding region also appears to be located on helix 6 of the IV^{th} repeating subunit on the α_1 subunit. Photoaffinity labelling with a phenylalkylamine-receptor-selective verapamil derivative (arylazide) localized the receptor binding site to the intracellular end of the IVS6 helix and adjacent intracellular amino acid residues on the intracellular side of the Ca^{2+} channel.[227] The amino acid sequence of the IVS6 and surrounding C-terminal tail is highly conserved among α_1 subunits isolated from other tissue and species types (rabbit and carp skeletal muscle and rabbit cardiac muscle).[228,230,231] In addition, phenylalkylamine binding properties and modulation of their binding by cations from these different tissues and species are also very similar[232,233] suggesting that this highly conserved area is important in the formation of the phenylalkylamine receptor.

Localization of the receptor binding site to an intracellular domain is consistent with functional studies which have shown that phenylalkylamine Ca^{2+} blocking drugs must be applied to the intracellular surface of the channel before they become active.[228,229]

In identifying the channel drug binding sites, the primary focus has been on the three major Ca^{2+} channel antagonists (dihydropyridines, phenylalkylamines and benzothiazepines). However, evidence has been gathered to suggest that there are five[225] possibly six[234] discrete binding sites associated with the Ca^{2+} channel. On the basis of functional and binding experiments there is evidence for: (i) a dihydropyridine site, (ii) a phenylalkylamine (verapamil) site, (iii) a benzothiazepine (diltiazem) site, (iv) a site for fluspirilene and pimozide (diphenylbutylpiperidines or diphenylalkylamines)[235], and (v) a site for the indolizine SR33557, a novel potent non-dihydropyridine[236]. As new synthetic drugs become available, additional information about the molecular binding sites present on the Ca^{2+} channel will become evident.

Drug interactions with their binding sites on the channels are often dependent upon the state of the channel. As previously discussed, the Ca^{2+} channel may exist in one of three states at any one time; open, closed or inactivated. The interaction of Ca^{2+} channel antagonists with the Ca^{2+} channel are voltage-dependent, with affinity increasing with increasing membrane depolarization. Therefore, the Ca^{2+} channel antagonists preferentially bind to the

inactivated state of the Ca^{2+} channel, which is the state that is favoured by depolarization.[209,237,238] There is conversely, a low affinity for the other two states, closed and open.[225] Ca^{2+} channel activators, such as Bay K 8644, have been shown to preferentially bind to the open state, reducing the deactivation of this state and resulting in increased channel opening times in the presence of this dihydropyridine activator.[225] Conformation of these electrophysiologic observations has come from radioligand binding studies carried out in polarized and depolarized cells.[239,240]

Additional reactions with the channel may be related on a frequency dependent basis, whereby affinity increases with increasing frequency of the depolarizing stimulus. This is apparent for the charged Ca^{2+} channel antagonists, verapamil and diltiazem.[241] Verapamil and diltiazem, being charged and more polar species, have to access the inactivated state of the channel through the open state. Increasing the frequency of the depolarizing stimulus increases the probability that the Ca^{2+} channel will be in an open state. Access to the inactivated state by verapamil and diltiazem through the open configuration is then also increased. The 1,4-dihydropyridines, being nonpolar and noncharged, are able to access their preferred binding site directly through the membrane.[242]

Once the Ca^{2+} channel antagonist has bound to its molecular site on the inactivated state of the channel, the blocker, in some yet unknown fashion, is able to stabilize the inactivated conformation. Stabilization of the inactivated state prevents or

slows the channels transition into the resting or closed state upon repolarization of the membrane to its resting membrane potential. For the channel to be reopened upon further depolarizing stimuli, the channel must first be in the resting or closed state. By maintaining the channel in the inactivated state, the Ca^{2+} channel antagonists reduce the number of Ca^{2+} channels available to be opened upon further depolarizing stimuli. This effectively reduces the I_{Ca} (inward Ca^{2+} current) upon membrane depolarization. In cardiac tissue, this reduction in inward Ca^{2+} movement will reduce the release of Ca^{2+} from the SR stores via the Ca^{2+} induced Ca^{2+} release mechanism, resulting in decreased contractility. In vascular smooth muscle, Ca^{2+} channel blockage results in vasodilation due to reduced inward Ca^{2+} movement.

One theory that has been proposed for how the Ca^{2+} channel antagonists stabilize the inactivated state of the Ca^{2+} channel has come from Armstrong, *et al.*[172] Armstrong and his colleagues suggest that for the Ca^{2+} channel to be opened upon presentation of a depolarizing stimulus, the channel must first be phosphorylated by an endogenously linked protein kinase. The Ca^{2+} channel antagonists bind to the inactivated state of the channel and inhibit or slow the repolarization phase of the channel. This effectively reduces its ability and availability to be reopened upon further stimulation.[172]

Different isomers for each of the Ca^{2+} channel antagonists exist, and these isomers differ in their ability to modulate Ca^{2+} channel kinetics.[245] Verapamil exists in two isomers, the *d* and *l*

form. Clinically, verapamil is administered in a racemic mixture of *d*- and *l*-verapamil, producing a 10-fold greater impairment of atrioventricular conduction than the *d*-isomer.[243,244] Four diltiazem stereoisomers exist including; *d-cis,l-cis,d-trans* and *l-trans*. All four isomers were found to bind to the same benzothiazepine receptor site but had differences with respect to their activity. All four isomers were able to inhibit binding of radioactively labelled diltiazem but the potency of inhibition was different *(d-cis>l-cis>d-trans=l-trans)*. All isomers were also found to modulate [^{3}H]PN200-110 (a dihydropyridine) binding with the *d-cis* isomer stimulating binding and the others showing inhibition.[246] Enantiomers of certain dihydropyridines have differing potencies and also act at different binding sites, with antagonistic or agonistic properties. The dihydropyridine PN 202-791 exists in two enantiomeric formations [S(+) and R(-)]. The S(+)-PN 202-791 isoform is an agonist but its optical isomer R(-)-PN 202-791 is an antagonist.[247] It is generally believed that the antagonistic and agonistic dihydropyridines mediate their action by binding to the same receptor site[248]. The dihydropyridine PN 202-791 appears to bind to two separate sites as determined from competitive binding studies, which assumes if the isomers bind to the same site they should compete for binding to the site. It was found that the concentration-response relationship between the enantiomers showed no competition, suggesting that the action of these two forms involve two separate binding sites.[247]

The three major Ca^{2+} channel antagonist binding sites on the

α_1 subunit are allosterically linked to one another.[209] These allosteric linkages have been determined by the use of radioligand binding studies carried out in membrane preparations from excitable tissues.[249] These allosteric linkages allow for positive and negative heterotropic interactions between the various Ca^{2+} channel antagonists binding sites. Binding of any one of the Ca^{2+} channel antagonist groups to their respective binding site on the α_1 subunit will result in a negative interaction with the Ca^{2+} pore, decreasing I_{Ca}[209,242]. Binding of one of the antagonist groups to their respective site will also have either a positive or negative effect on the binding of the other antagonists to their sites. For example, binding of verapamil (phenylalkylamine) to its receptor site will have a negative allosteric affect on the binding of dihydropyridines to its receptor site. Therefore, phenylalkylamines bound to their receptor site will inhibit or reduce the binding of dihydropyridines. Phenylalkylamines also have a negative influence on the benzothiazepine receptor site. Binding of a dihydropyridine antagonist to its receptor site negatively influences the phenylalkylamine and benzothiazepine sites. However, binding of a benzothiazepine antagonist to its site will positively influence binding at the dihydropyridine receptor site while negatively influencing binding at the phenylalkylamine site.[242] The positive binding influence on dihydropyridine binding showed by diltiazem (a benzothiazepine) is specific to one stereoisomer of the diltiazem antagonist, the *d-cis* isomer. The other three stereoisomers of the diltiazem molecule

showed inhibition of binding to the dihydropyridine receptor site.[246,253] The positive influence that diltiazem and other positive regulators [(+)-tetrandine] have on dihydropyridine binding is postulated by Staudinger, *et al.*, to be an interdependence of the dihydropyridine binding site and the Ca^{2+} binding site on the α_1 subunit.[252] The positive heterotrophic allosteric regulators affect Ca^{2+} rate constants and optimize coordination of Ca^{2+} in the channel pore which in turn increases the affinity of the channel for the dihydropyridines.[252]

The binding of the Ca^{2+} channel antagonists to their receptor sites has been known for some time to be modulated by the presence of divalent cations. Studies have shown that high affinity 1,4-dihydropyridine interactions with the α_1 subunit critically depend on the availability of divalent cations such as Ca^{2+} [250] In the presence of micromolar $[Ca^{2+}]$, the Ca^{2+} channel blocking receptor site was stabilized in a conformation that allowed high-affinity binding of phenylalkylamines. At millimolar Ca^{2+} concentrations, a low-affinity state of binding on the stable Ca^{2+} channel blocking receptor was observed.[251]

The binding of Ca^{2+} antagonists to their tissue receptor sites is also modulated by temperature[254], membrane lipid peroxidation[255], and membrane cholesterol content.[256] An increase in K_D with increasing temperature has been observed and this is thought to be a result of an increase in the dissociation rate of the ligand-receptor complex, with little change with respect to the association rate.[258] The increase in the dissociation rate with

increasing temperature is suggested to be mediated by a configurational change in the dihydropyridine receptor induced by the increased temperature.[257] Lipid peroxidation (an important mechanism in tissue ischemic damage)[259,260] has also been shown to induce a conformational change in the Ca^{2+} channel[264] such that there is an altered binding of radioactively labelled dihydropyridines ([^{3}H]PN-200-110) resulting in altered Ca^{2+} ion fluxes.[255] Alterations in membrane cholesterol content (as seen with age[261], diabetes[262], and atherosclerosis[263]) has been observed to alter binding of Ca^{2+} channel antagonists.[256] An increase in membrane cholesterol content resulted in a decrease in Ca^{2+} channel antagonist binding, which is thought to be a consequence of the increased free cholesterol altering drug partitioning coefficients.[256] Alterations in cardiomyocyte membrane composition by addition of low density lipoproteins[280] and oxidized low density lipoproteins[265] has been shown to increase Ca^{2+} transients by modifying Ca^{2+} transport through L-type channels. Cells treated with oxidized low density lipoprotein were found to be more sensitive to the blocking action of nicardipine (a dihydropyridine) than control cardiomyocytes.[265]

Clinical Application

The capacity of Ca^{2+} channel antagonist to block the slow inward Ca^{2+} current has made these compounds an important addition to the group of drugs used in the treatment of conditions related to the cardiovascular system. Much of the original clinical us of Ca^{2+} channel antagonists were as antianginal and antihypertensive

agents, though their scope of use has now become much more extensive.[219]

The Ca^{2+} channel antagonists tend be lumped together into one large group of compounds because of their unifying blocking action of the L-type Ca^{2+} channel. However, the Ca^{2+} channel antagonists are commonly broken down into three subgroups which have different physiological actions upon administration in an experimental or clinical situation.[281] Verapamil (phenylalkylamine) shows the most significant affect on cardiac (atrioventricular) conduction but has minimal vasodilatory properties. Nifedipine (dihydropyridine) has the most potent vasodilatory effects but has minimal affect on the cardiac conduction system. Diltiazem (benzothiazepine) has intermediate action on cardiac conduction and vasodilation.[281]

Ca^{2+} channel antagonists are currently used to treat a variety of complications within the cardiovascular system. The ability of compounds like verapamil and diltiazem to slow conduction velocity through the atrioventricular node has made these drugs useful in the treatment of cardiac arrhythmias[281,282] such as supraventricular tachycardia.[283] The Ca^{2+} channel antagonists' peripheral vasodilatory and negative inotropic properties have made them useful in the treatment of ischemic heart conditions such as angina pectoris (Prinzmetal's or variant angina and chronic stable angina)[281,284-289]

Ca^{2+} channel antagonists ability to block the slow inward Ca^{2+} current made them attractive prospects in protecting the heart from damage during ischemic events. Post-ischemic infarcts are

generally thought to be a direct result of Ca^{2+} overload, possibly occurring *via* the Ca^{2+} channels.[290] Therefore, the capacity of the Ca^{2+} channel antagonists to block the channel and prevent Ca^{2+} overload coupled with the favourable hemodynamic benefits of vasodilation were believed to be beneficial in heart failure conditions. This has not turned out to be the case. In fact, Ca^{2+} channel antagonist treatment in heart failure has an adverse effect on patient recovery.[281,284,292-297] The negative inotropic properties of Ca^{2+} channel antagonists[293,296] (possibly increased effect in failing heart[292]) and the activation of neurohormonal systems (e.g. renin-angiotensin system)[294] have been proposed to underlie the adverse effects in heart failure. Bersohn and Shine (1983) have shown some protection by verapamil in ischemic rabbit heart.[291] The protection was dependent upon pretreatment of hearts with verapamil prior to the ischemic episode.[291] The second generation Ca^{2+} channel antagonists (verapamil, diltiazem and nifedipine being first generation) like isradipine, appear to have more specific actions on vascular smooth muscle with negligible cardiopressive effects. Isradipine may provide favourable hemodynamic benefits without the detrimental cardiodepression.[295,298,299] The Ca^{2+} channel antagonist verapamil has been shown to beneficial in preventing cardiomyopathy in diabetically induced rats.[300]

Hypertension is a major risk factor for the development of cardiovascular diseases, including coronary artery disease, stroke, left ventricular hypertrophy, congestive heart failure, renal failure and aortic aneurysms.[301,306] Therefore, the control of

elevated blood pressure is an important consideration in order to prevent the above conditions from developing. Ca^{2+} channel antagonists have been used in the treatment of hypertension, almost from their conception some 30 years ago. All three groups of antagonists have some effect on the hypertensive condition, but it is the dihydropyridine (e.g. nifedipine and isradipine) group that has been shown to have the most favourable results.[302-305] One of the characteristics of hypertension is an elevated systemic vascular resistance. Ca^{2+} antagonists reduce the Ca^{2+} influx-dependent component of vascular contraction, therefore, inducing vascular relaxation and a concurrent decrease in blood pressure.[307,308] The Ca^{2+} channel antagonists have been shown to be potent arterial vasodilators that result in reduced blood pressure without activation of sympathetic reflexes that result in sodium and volume retention.[307,308] Treatment with Ca^{2+} antagonists has been shown to prevent and reverse left ventricular hypertrophy induced by the hypertensive condition.[309,310] They have also been shown to slow the progression of renal damage in chronic renal disease and following chronic hypertension.[311,316]

Ca^{2+} has, for a long time, been recognized as an integral part of an atherosclerotic plaque. The process of formation of atherosclerotic plaques includes the accumulation of lipids in the arterial wall, myocyte migration to and proliferation within the intimal layer, and the accumulation of Ca^{2+}.[312,313] The Ca^{2+} antagonists have been observed to retard plaque formation and may affect all levels of the plaque development process.[313] Ca^{2+}

channel antagonists have been shown to enhance cholesterol ester hydrolysis and decrease total cholesterol accumulation in aortic tissue, thereby retarding plaque development.[314] In addition to having anti-atherosclerotic action, Ca^{2+} antagonists are able to provide beneficial hemodynamic effects in animals already having atherosclerosis.[315] Excess Ca^{2+} accumulation in plaque cells is inhibited by Ca^{2+} antagonist blockade of the Ca^{2+} channels.[312]

Ca^{2+} channel antagonists are now being observed to have beneficial actions in cerebrovascular disease.[317-322] Because of their potent dilatory ability, Ca^{2+} antagonists are able to provide systemic arterial dilation that may ameliorate cerebral ischemia by providing increased cerebral blood flow.[317] Isradipine (dihydropyridine) appears to bind preferentially to cerebral Ca^{2+} channels. Application of isradipine to rats experimentally induced to undergo a stroke, showed a 50% reduction in infarct size as compared to 'control' stroke induced animals.[319] This reduction in infarct size may be a result of increased cerebral blood flow (vasodilation) and prevention of neuronal death by reducing the Ca^{2+} influx into the neurons.[319,322] This may reduce the amount of brain damage incurred by stroke victims.[319] Ca^{2+} antagonists also appear to have beneficial results in treating subarachnoid haemorrhages[318]

As research into Ca^{2+} antagonists continues, new clinical uses are being discovered for these compounds. Ca^{2+} channel antagonists have now been linked to inhibition of cancer cell growth[323] and treatment of conditions including migraine[324], vertigo[324] and mood

disorders[325]. As research continues and new derivatives are discovered, additional applications of this diverse group of compounds will become realized.

Methods

Drug treatment protocol. Female Sprague-Dawley rats (Central Animal Care, University of Manitoba) weighing 150-200 grams were given twice daily subcutaneous (s.c.) injections of verapamil in 100 μl of water at varying concentrations: 2.5, 10, 20, 25, 30, 50, 60, and 75 mg/kg/day. The treatment was carried out once in the morning and once in the late afternoon for varying periods of time. Control animals received the vehicle injection. Animal weight was monitored over time in order to ensure that the drug concentration administered was constant.

Verapamil concentrations of 2.5, 10, 20 and 30 mg/kg/day were injected for a period of 8 weeks in order to determine if the channel was sensitive to increasing verapamil concentrations. A standard 10 mg/kg/day verapamil injection was carried out over a span of 2, 4, 8, and 16 weeks in order to assess whether any changes occurred as a function of time with a fixed concentration. In order to ascertain whether changes occurred over a shorter time interval with much higher (near toxic) drug concentrations, doses of 25, 50, 60 and 75 mg/kg/day were injected over a span of 24 hours.

A novel means of drug application, the implantable slow-release pellet was tested. The pellets were designed to release a constant dosage of drug over a 3-week time span. An initial study was undertaken in which a 50 mg verapamil pellet (Innovative Research of America, Toledo, OH) was carefully implanted s.c. in the nape of the neck of separate rats. Care was taken to ensure

that the pellet was not damaged at all and the small incision carefully sutured. The 50 mg pellet size was designed to release a verapamil dose of 11.9 mg/kg/day over a three-week period. Control animals were implanted with a placebo pellet.

Additional verapamil implantable pellet concentrations were tested with varying times of study duration. Verapamil pellets of 0.25 mg (0.06 mg/kg/day), 1.5 mg (0.36 mg/kg/day), 5 mg (1.19 mg/kg/day), 10 mg (2.38 mg/kg/day) and 25 mg (5.95 mg/kg/day) were implanted for a period of 24 hours and 7 days. Twenty-four hour studies were also carried out with 35 mg (8.33 mg/kg/day) and 50 mg (11.9 mg/kg/day) verapamil pellets. Control animals were implanted with placebos of the same size as the corresponding experimental animal pellets.

In order to compare the various means of drug application, a short term (24 hour) study was carried out using 50 mg (11.9 mg/kg/day) implantable verapamil pellets, 11.9 mg/kg/day s.c. injection and 11.9 mg/kg/day oral dosage. The s.c. injections and oral dosages were given twice daily. The oral dosages were administered with a p.o. intubator.

Radioligand binding. Rats were sacrificed after a predetermined time of treatment. In the initial studies which assessed changes occurring after long-term exposure with a constant drug dose and varying the drug concentrations after a fixed time, three different tissue types were examined. These included ventricular tissue, brain (cebrum) and a segment of skeletal muscle (quadriceps). Later studies assessing short term changes were carried out using

ventricular tissue only.

Membranes were prepared from each tissue fraction by the method of Wagner and colleagues.[339] Tissue was scissor minced and then homogenized for 2 X 20 seconds with a Polytron PT-20 on a setting of 5 in a solution containing 50 mM Tris-HCl (pH 7.4) at 4 degrees C. This homogenate was centrifuged at 1000 X *g* for 10 minutes and then the supernatant was recentrifuged at 48,000 X *g* for 25 minutes at 4 degrees C. The resultant pellet was washed twice at this speed in the 50 mM tris-HCl (pH 7.4) and was finally resuspended in the 50 mM Tris-HCl (pH 7.4) medium for protein analysis[340] and radioligand binding.

Calcium channel density was assessed *via* specific binding at the dihydropyridine receptor with the radioligand [^{3}H]PN 200-110.[338] Approximately 50 μg of skeletal muscle membrane protein, 150-300 μg cardiac membrane protein and 250 μg of brain membrane protein were incubated for 1 hour at 25 degrees C in 0.5 ml of a medium containing 0.025 to 4.0 nM [^{3}H]PN 200-110 and 50 mM Tris-HCl (pH 7.4). Nonspecific binding was assessed in the presence of 2.5 μM nifedipine. The reaction was terminated by filtration through Gelman Type A/E glass fiber filters (which had been presoaked in 0.3% polyethylenimine to reduce background activity). The filters were washed twice with 2 ml of ice-cold 50 mM Tris-HCl (ph 7.4) buffer, dried and then the radioactivity measured by standard liquid scintillation spectrophotometric techniques. In some cases, the reaction medium contained 0.1 to 100 μM verapamil $\pm$ 1.0 mM Ca^{2+} or 10μM verapamil to determine the allosteric interactions between

the verapamil binding site and the [^{3}H]PN 200-110 binding. All reactions were carried out under dimly lit conditions. Saturation binding data were analyzed using the nonlinear least-squares curve-fitting program LIGAND[329].

Verapamil quantitation. Plasma was prepared from blood samples collected from the tail several times during the treatment regimes. The plasma was stored at -85 degrees C until the time of extraction. Verapamil was extracted into a heptane organic phase as described by Kapur *et al.*[333], with back extraction into 0.1 M H_2SO_4. A 10μl extraction sample was run on a Waters High Performance Liquid Chromatography (HPLC) system with a Waters C_{18} μBondapak (10-μm particle size) reversed-phase column (30 cm X 3.9 mm) and eluted with acetonitrile-KH_2PO_4 (0.1 M; pH 3.0) (34:66) at a flow rate of 1 ml/min. Detection was carried out on a Waters 470 Fluorescence detector with 203 and 320 nm wavelengths for the excitation and emission bands, respectively. Standards were prepared by spiking control plasma samples with known quantities of verapamil and its metabolites (D-617, D-620 and norverapamil).

Statistical analysis. Statistical significance was determined by one-way analysis of variance test and Duncan's multiple range test.[341] Significance was arbitrarily set at a 0.05 level.

Materials. All chemicals were of standard reagent quality except for the chromatographic solutions which were HPLC grade. Verapamil was obtained from Sigma Chemical Co. (St. Loius, MO). Nor-methyl verapamil hydrochloride (norverapamil) was purchased from Research Biochemicals Inc. (Natick, MA). The verapamil metabolites 5-

methylamino-2-(3,4-dimethyloxyphenyl)-2-isopropylvaleronitrile(D-617) and 5-amino-2-(3,4-dimethyophenyl)-2-isopropylvaleronitrile (D-620) were kindly donated to us by Knoll Pharmaceuticals Canada (Markham, Ontario, Canada).

Results

The first series of experiments in this investigation addressed the issue of optimizing the mode of verapamil delivery to the rats. In our initial trials utilizing the 50 mg (calculated daily release of 11.9 mg/kg/day) implantable slow-release verapamil pellets, a 63% mortality rate was observed in the first 18 hours post-implantation. There were no deaths in animals implanted with the placebo pellet or those given injections of an identical dosage (11.9 mg/kg/day) of verapamil. Later studies utilizing different sizes of implantable pellets demonstrated a dose dependent mortality rate (Figure 1). A very different dose dependent mortality rate was observed at increasing doses of verapamil injected subcutaneously (Figure 1). The dose at which mortality first occurred with the slow-release implantable pellet was considerably lower than for the injected group. The verapamil pellets were designed to release the drug continuously over a 3-week period. This was obviously not the case and the striking differences in mortality between the initial two modes of drug delivery (s.c. implant and s.c. injection) suggested that the circulating verapamil concentration may not be similar in the two models.

Another set of animals was implanted with the verapamil pellets (50 mg) and blood samples were collected regularly in order to carefully monitor the release of the drug from the pellet into the bloodstream. The concentration of verapamil metabolites was also monitored to determine if the mode of administration may have

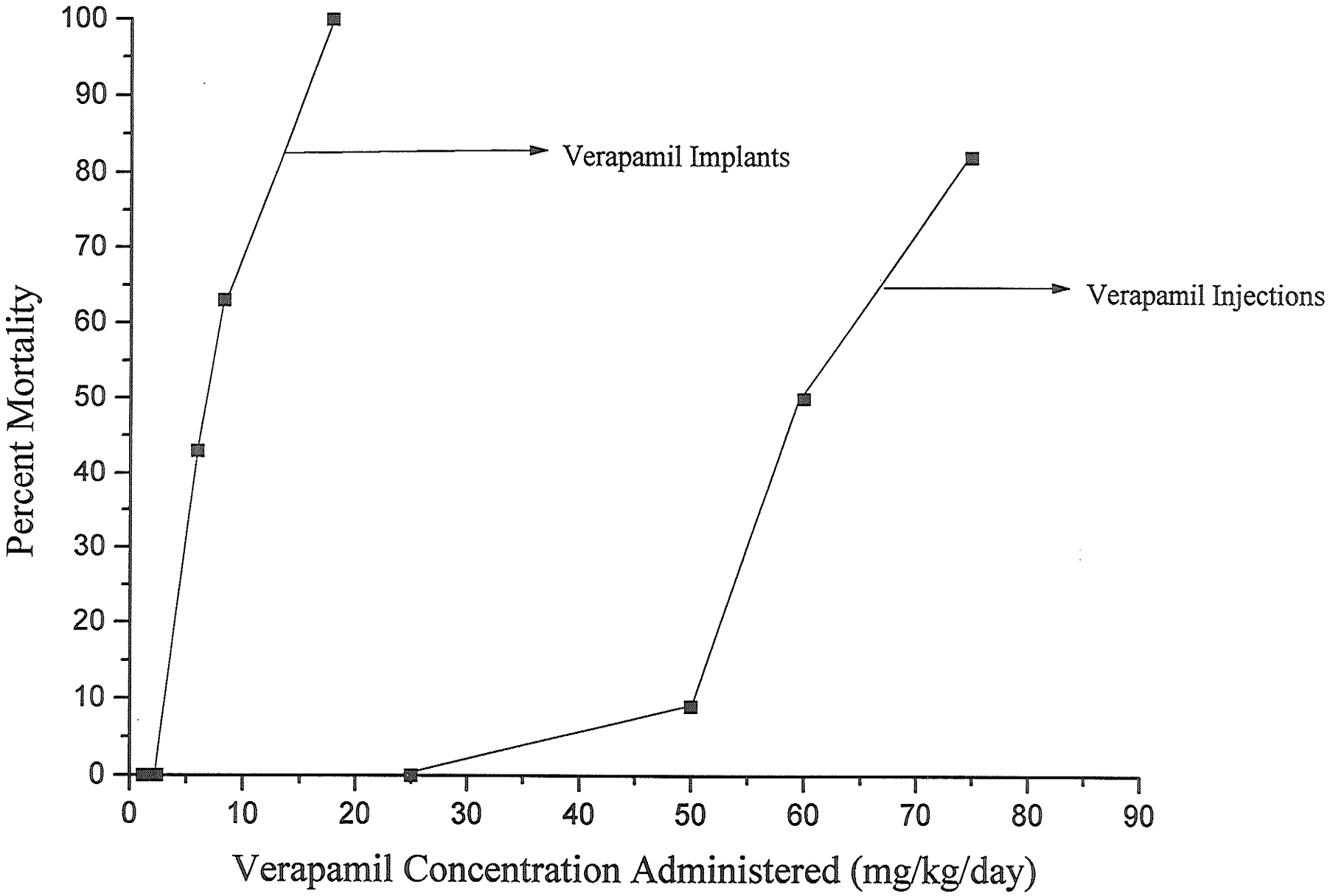

Figure 1. Percent mortality as a function of verapamil concentration administered (mg/kg/day) via slow-release implant pellets or subcutaneous injection. The theoretical values for daily release of verapamil presented above were calculated for the 5, 10, 25, 50 and 75 mg pellets.

altered the metabolism of the drug.

A representative tracing of a typical chromatogram produced by the HPLC after injection of a plasma sample spiked with a 100 ng standard of verapamil and its metabolites is seen in Figure 2A. The primary verapamil metabolites D-620 and D-617 are the first to appear. These two metabolites appear around the 5-7 minute mark after injection of the sample into the HPLC column. D-620 appears first followed very closely by D-617. At the 17 to 21 minute interval, the metabolite norverapamil and the parent drug verapamil peaks appear. The norverapamil peak immediately precedes the appearance of the verapamil peak. Using the absorbance peaks produced by this spiked plasma sample, a standard curve was produced. Comparing our unknown plasma samples absorbance to the standard curve, the concentrations of verapamil and its metabolites was able to be determined. A representative tracing of an unknown plasma sample obtained from a verapamil implanted rat is presented in Figure 2B. The metabolites D-617 and D-620 were usually not detectable or detected in very small quantities.

For comparative purposes, verapamil was administered to separate rats via s.c. injection or p.o. intubator and blood was collected in an identical fashion. As shown in Figure 3, plasma verapamil concentration was significantly higher in the pellet implanted rats than in the two other groups. The verapamil concentration rose over time and reached its peak within 8 hours after implantation. Thereafter, it remained at a level ~ 10-fold higher than the other treatment regimes. Verapamil was not

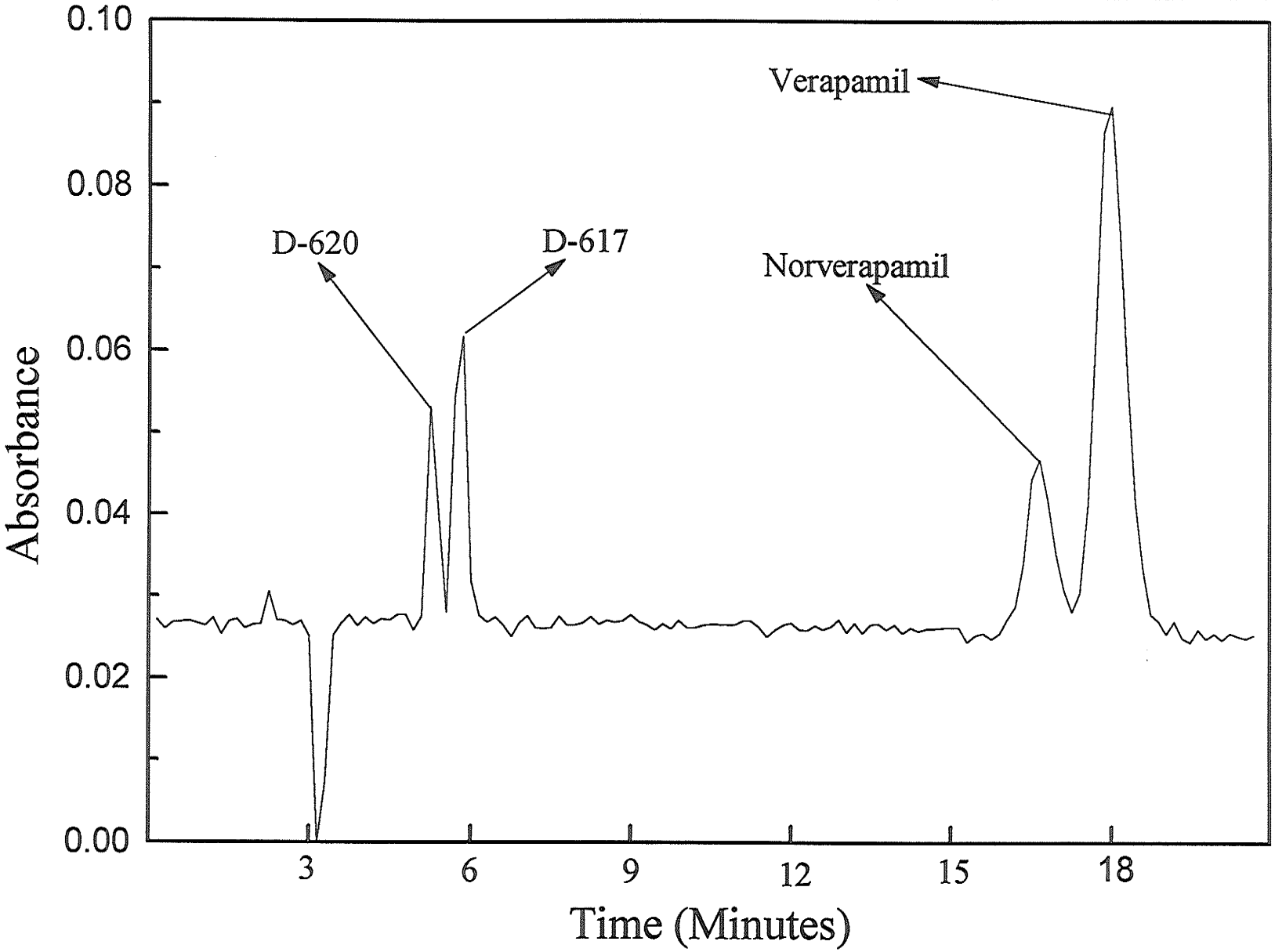

Figure 2A. Representative tracing of a High Performance Liquid Chromatography (HPLC) chromatogram showing verapamil and its metabolites (norverapamil, D-617 and D-620) as a function of time. The tracing is from a control plasma sample spiked with 100 ng each of verapamil, norverapamil, D-617 and D-620 standards.

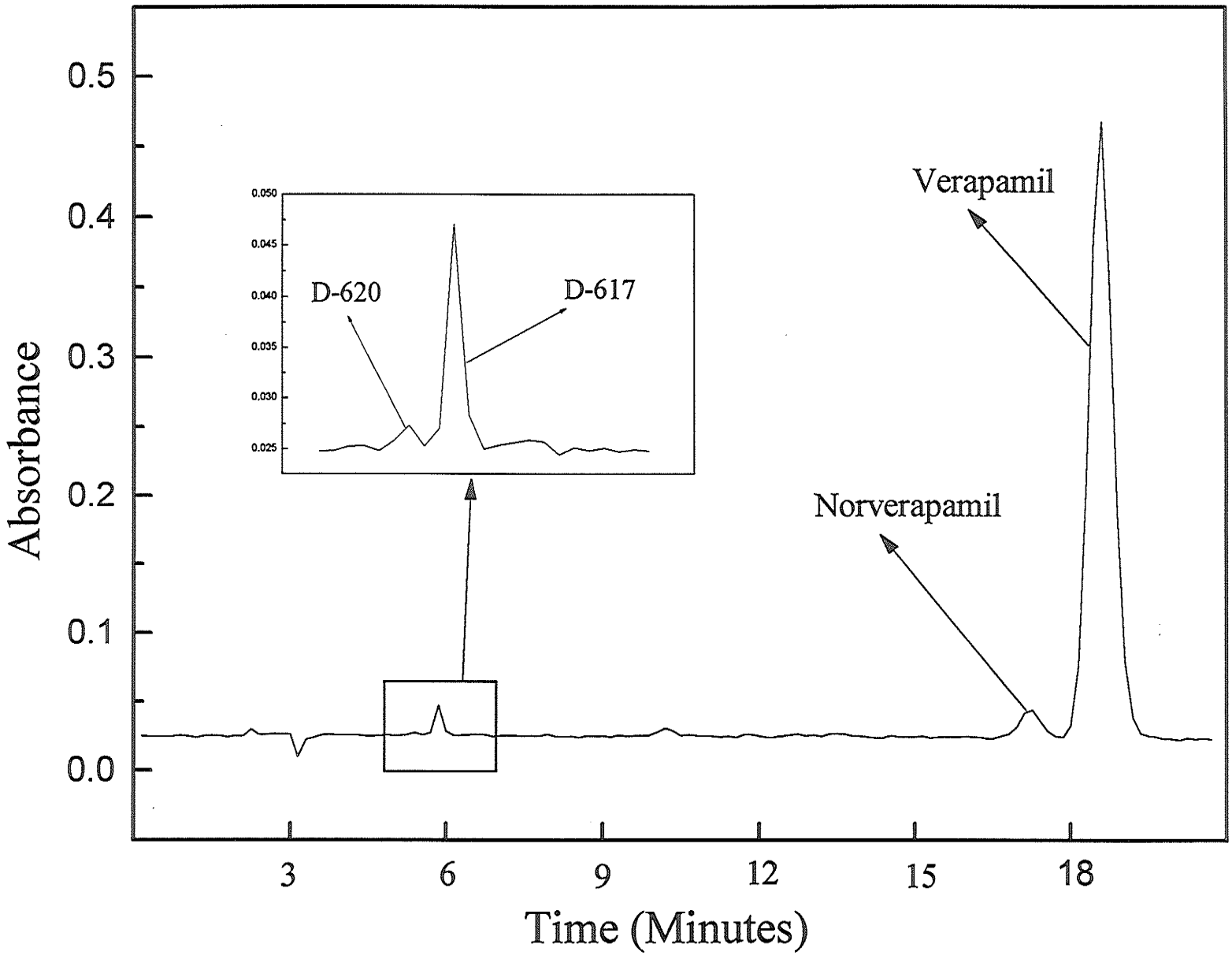

Figure 2A. Representative tracing of a High Performance Liquid Chromatography (HPLC) chromatogram showing verapamil and its metabolites (norverapamil, D-617 and D-620) as a function of time. The tracing is from a blood sample obtained from a verapamil implanted rat (50 mg) 8 hours post-implantation.

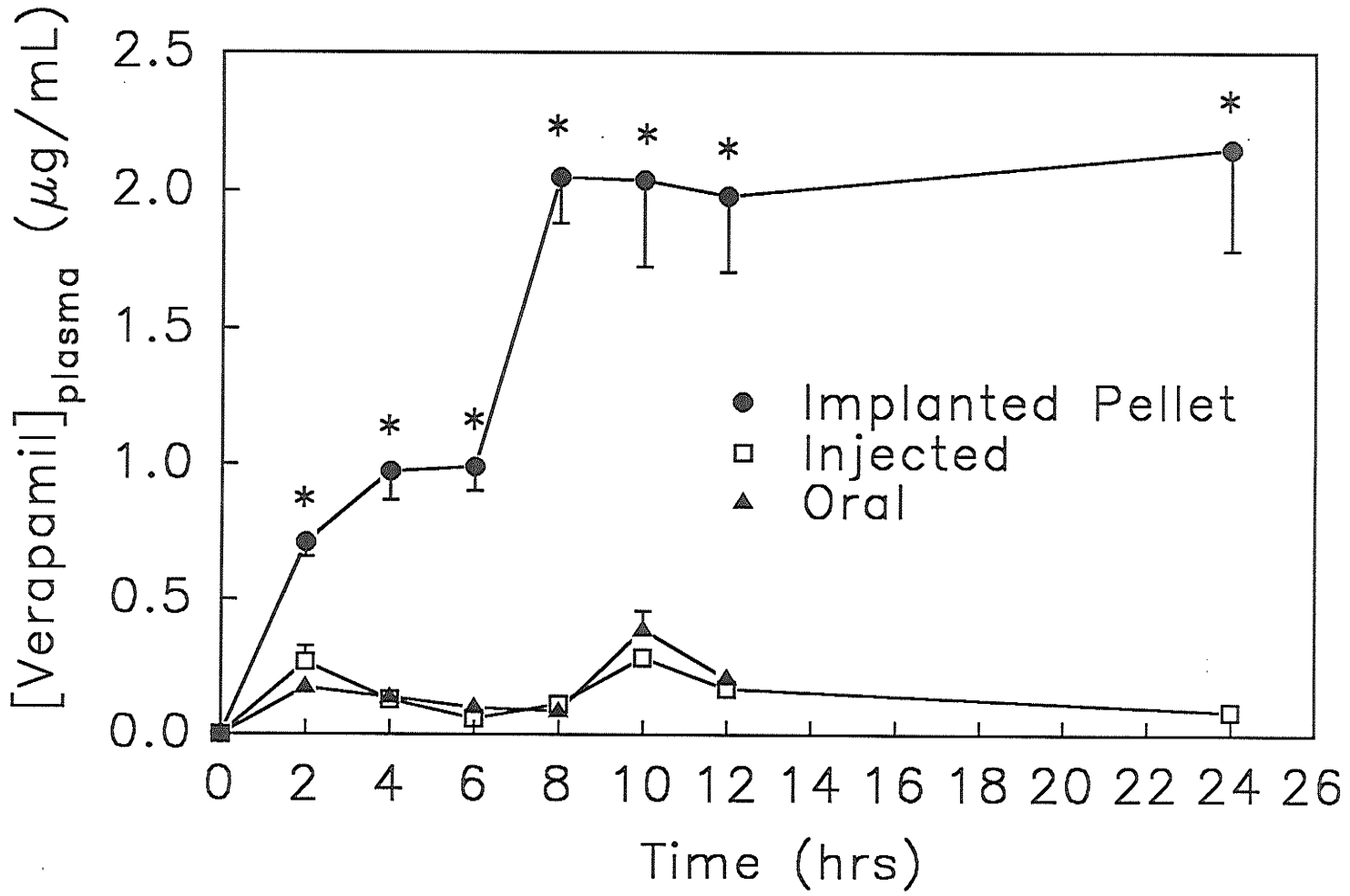

Figure 3. Plasma verapamil concentrations during the first day of treatment with different modes of verapamil administration (11.9 mg/kg/day). Values represent the mean ± S.E.M. for four to eight separate animals. *P<.05 vs. other treatment modalities.

detectable in plasma from control animals.

The concentration of the primary verapamil metabolites norverapamil (Figure 4), D-617 (Figure 5), and D-620 (Figure 6) were all increased in the plasma from verapamil pellet-implanted rats. In general, the metabolites appeared later in the treatment regime than when verapamil was detected. The quantity of each of the metabolites was an order of magnitude higher 1 day after implantation in plasma samples from the pellet-implanted rats as compared to the other modes of treatment. The metabolites D-617 and D-620 were either not detectable or measured in extremely low quantities in the rats given verapamil orally or by injection.

The drug was not released in a continuous, even fashion over the two-week period that it was monitored. At both 1 and 2 weeks after implantation, the drug levels were lower or similar to the concentrations observed with the injection protocol (Figure 7, upper graph). The norverapamil concentration also dropped precipitously from 1 to 7 days after implantation (Figure 7, lower graph). The other metabolites, D-617 and D-620, were not detectable at 1 to 2 weeks (data not shown).

The analysis of the binding characteristics of [^{3}H]PN 200-110 in these experiments was carried out using the LIGAND program. This program analyzes the specific and non-specific binding characteristics of the radioactive ligand (PN 200-110) and allows for a Scatchard plot analysis of the data. A representative tracing of a typical Scatchard plot of binding data from a control heart is presented in Figure 8. Scatchard plot analysis allows for

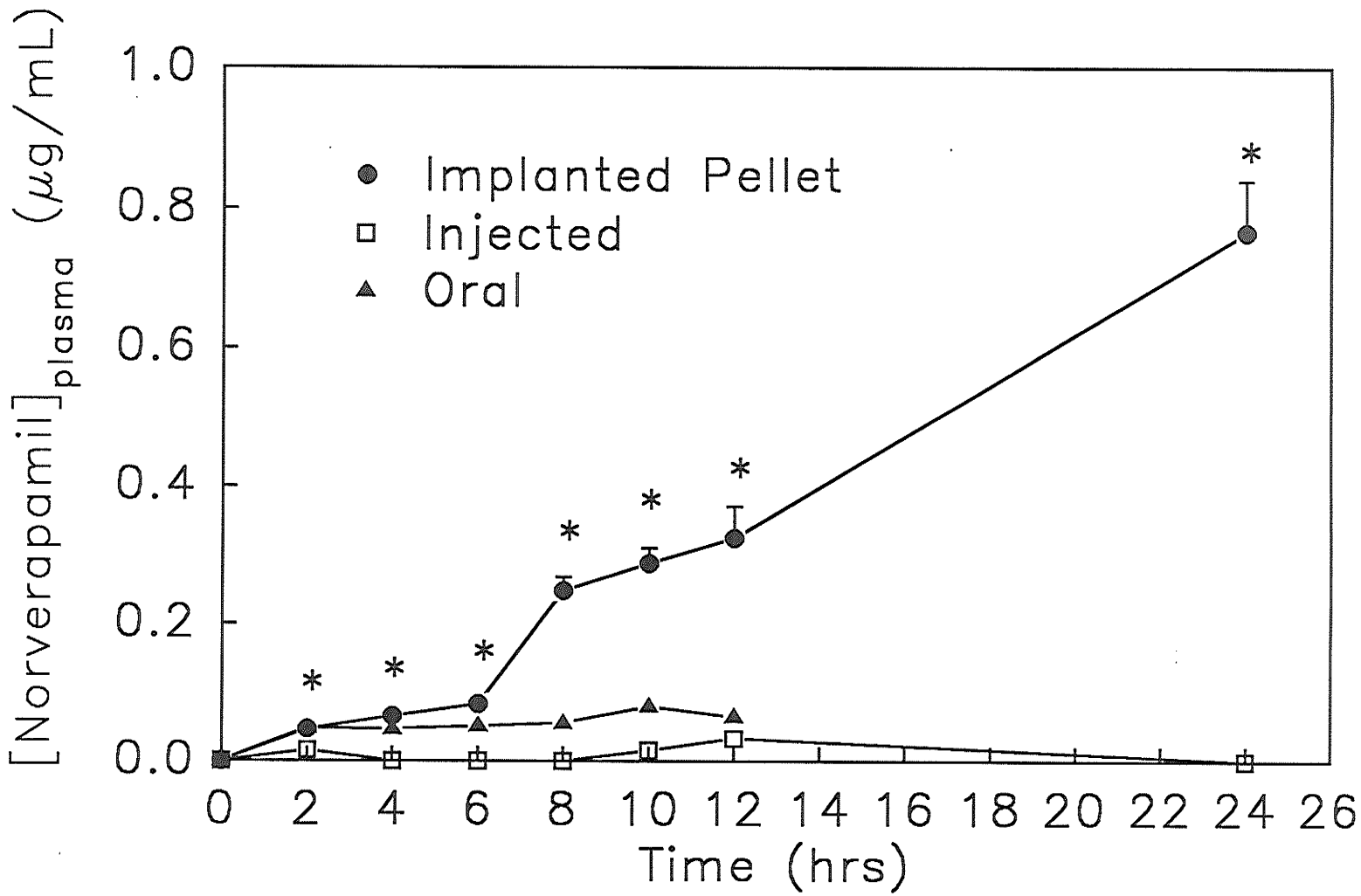

Figure 4. Plasma norverapamil concentrations during the first 24 hr after treatment with different types of verapamil administration (11.9 mg/kg/day). Values represent the mean ± S.E.M. for four to eight rats. *P<.05 vs. other treatment modalities.

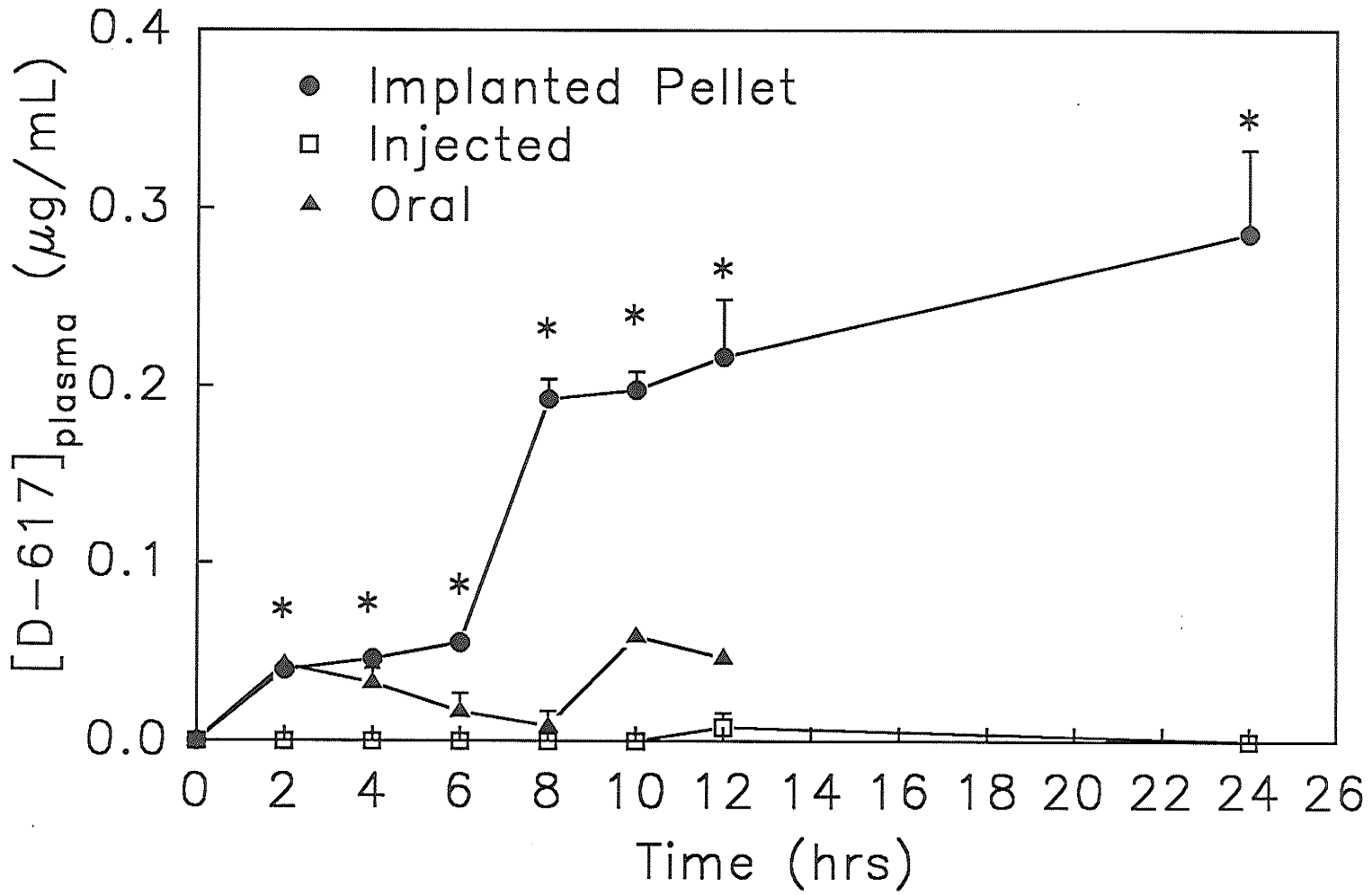

Figure 5. D-617 concentrations over time in plasma from rats treated with different types of verapamil administration (11.9 mg/kg/day). Values represent the mean ± S.E.M. for four to eight rats. *P<.05 vs. other treatment modalities.

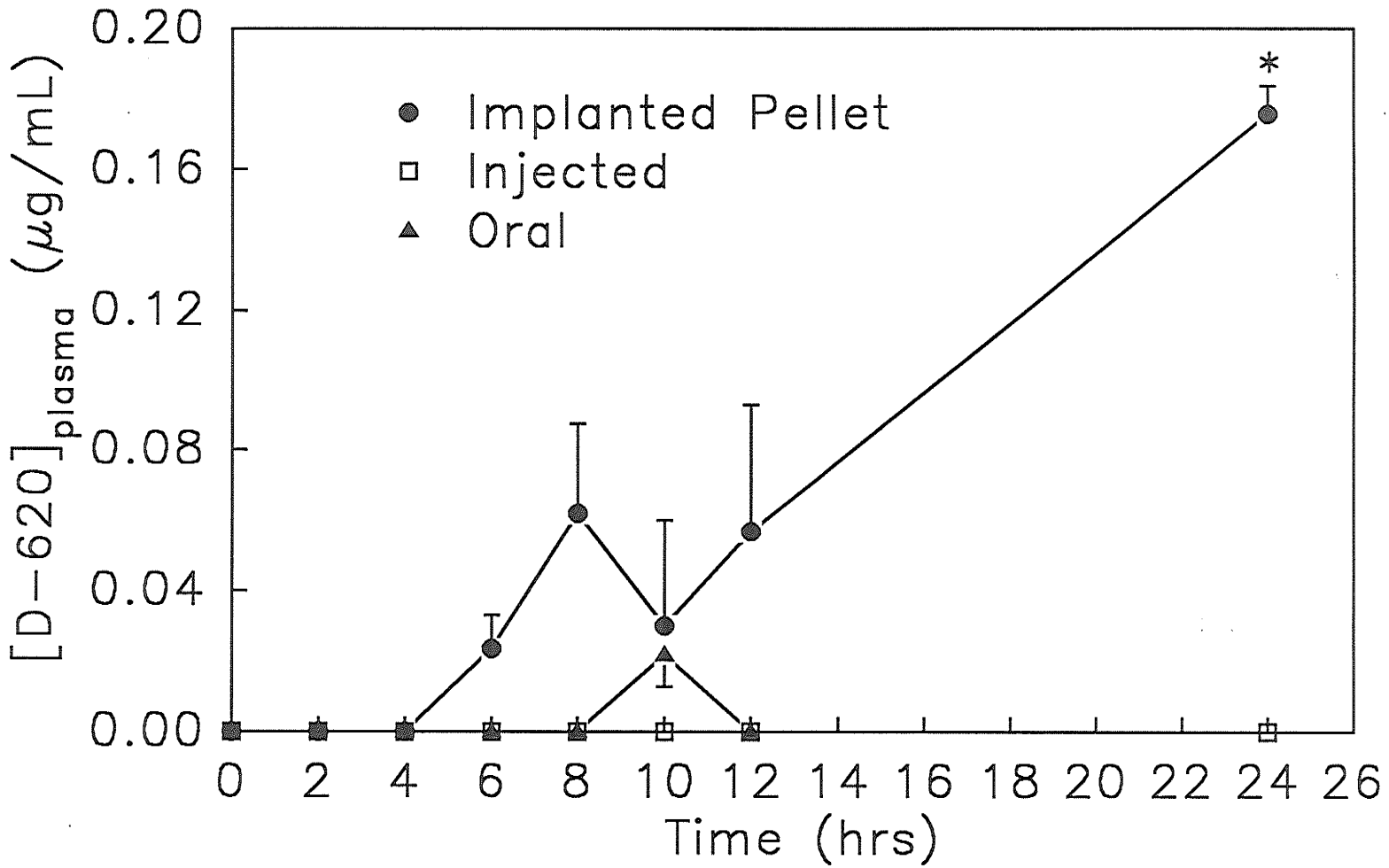

Figure 6. Concentrations of the verapamil metabolite D-620 over the course of 1 day in plasma from rats treated with different modes of verapamil administration (11.9 mg/kg/day). Values represent the mean ± S.E.M. for four to eight rats. *P<.05 vs. other treatment modalities.

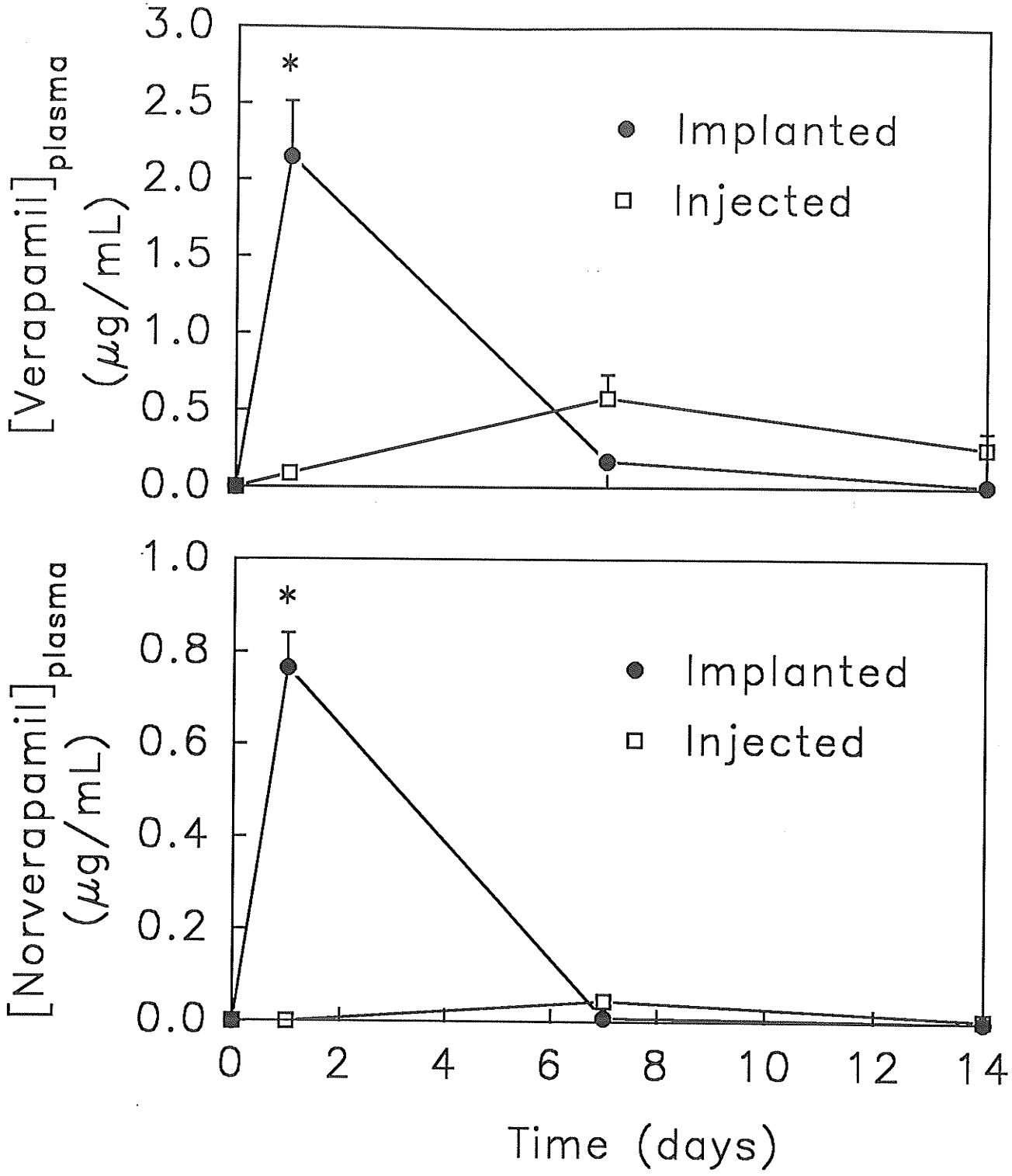

Figure 7. Plasma concentrations of verapamil (upper graph) and norverapamil (lower graph) after 1, 7 and 14 days of treatment with verapamil injections (11.9 mg/kg/day) or the implanted pellet (calculated to release 11.9 mg/kg/day). Values represent the mean ± S.E.M. for three to six rats. *P<.05 vs injected group.

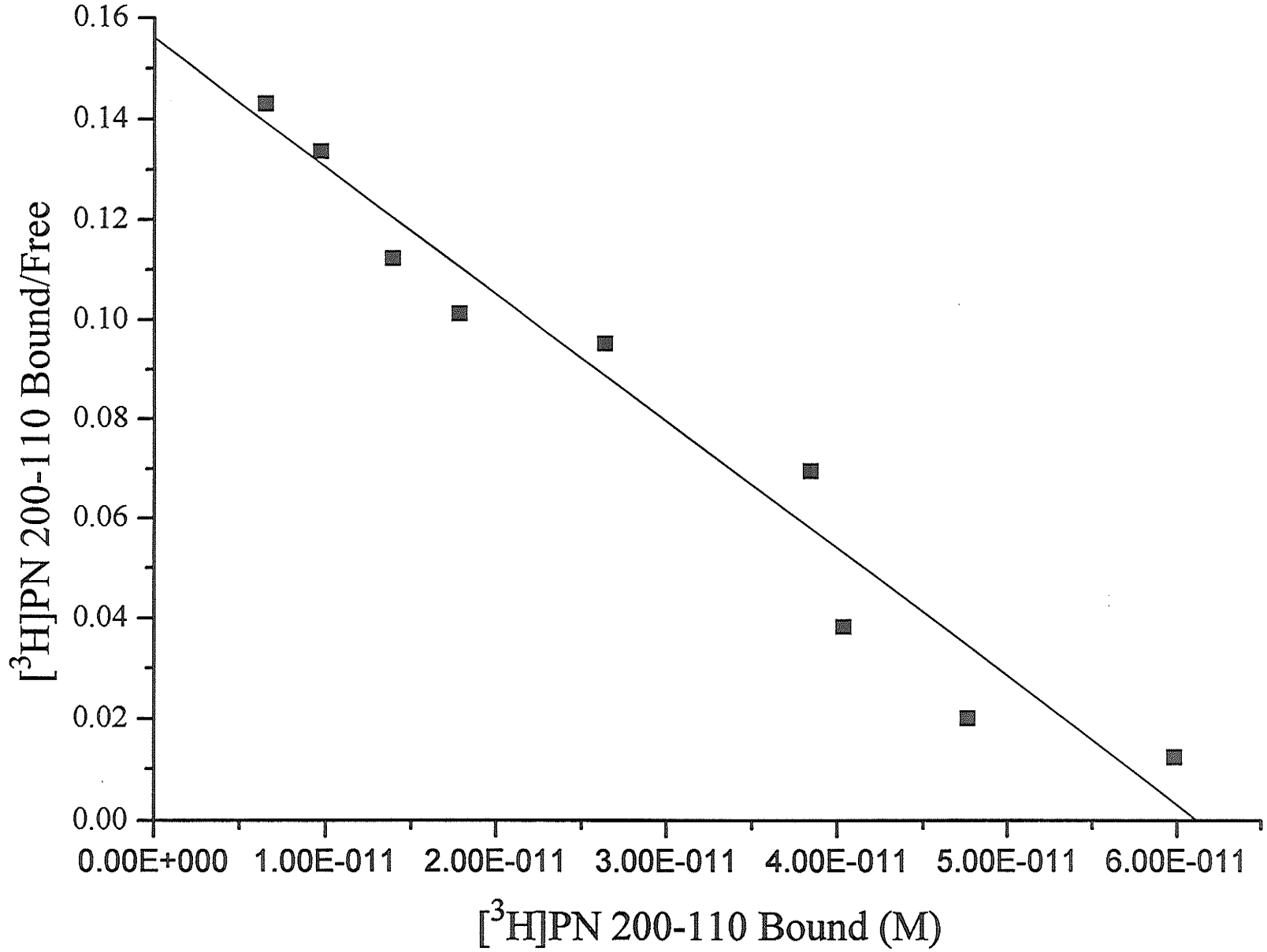

Figure 8. Representative data demonstrating Scatchard plot analysis of [^{3}H]PN 200-110 binding to a crude membrane fraction of control ventricular tissue. B_{max}: 6.067 x 10^{-11} M, K_D: 3.941 x 10^{-10} M and protein concentration: 0.184 mg/ml.

the determination of B_{max} and K_D. In order to ensure that there was saturation of binding of the radioactively labelled ligand (PN 200-110) to the membrane bound Ca^{2+} channels, a saturation curve was produced (Figure 9). The saturation curve demonstrates that saturation of [^{3}H]PN 200-110 binding occurred at the higher PN 200-110 concentrations (2-4 nM) which is in agreement with others[338].

The rats that survived the initial verapamil implant (50 mg) drug treatment were examined for specific binding of [^{3}H]PN 200-110 binding to cardiac and brain membranes, two weeks post-implantation. The results are presented in Table 1. Both the B_{max} and K_D for PN 200-110 binding to cardiac membranes were significantly depressed in rats administered the verapamil *via* the pellets. Rats injected with an equivalent dose of verapamil (11.9 mg/kg/day) did not demonstrate any significant alterations in these parameters. Binding to the brain membranes was unaffected by either of the drug treatment protocols.

The unreliability of the implantable slow-release verapamil pellets to release a constant non-toxic dose of the drug persuaded us to discontinue their use at this stage of the study. Instead, we decided to continue our initial experiments on the effects of dose and time on the Ca^{2+} channel biochemistry *via* s.c. injection as a mode of drug administration. Implantable slow-release verapamil pellets were used in later experiments to examine the effects of short-term exposure. Results from Table 1 demonstrated that there were no significant effects on Ca^{2+} characteristics after 2 weeks of s.c. injection at 11.9 mg/kg/day. It is possible

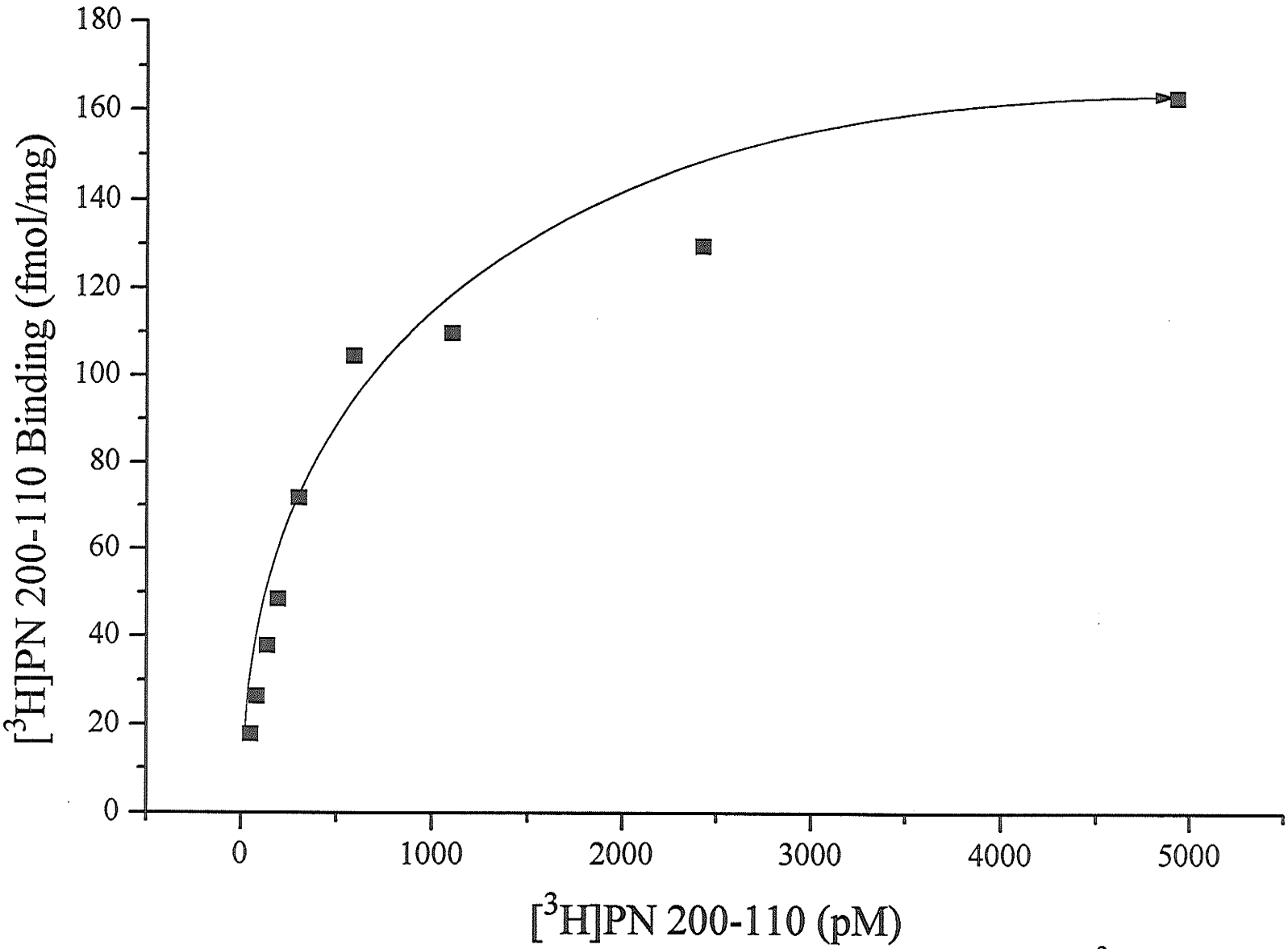

Figure 9. Representative data demonstrating saturation of binding of [^{3}H]PN 200-100 to a crude membrane fraction of control ventricular tissue. The specific binding is a function of different concentrations of [^{3}H]PN 200-110. B_{max}: 1.648 x 10^{-13} (fmol/mg), K_D: 3.941 x 10^{-10} M and protein concentration: 0.184 mg/ml.

Table 1. Specific binding characteristics of [^{3}H] PN 200-110 to crude membrane fractions of heart and brain tissue in control, verapamil injected and verapamil implanted (2 weeks) rats.

	K_d (nM)			B_{max} (fmol/mg)		
Tissue	Control	Injection	Implant	Control	Injection	Implant
Ventricle	0.25 ± 0.05	0.15 ± 0.02	0.10 ± 0.01*	263 ± 55	191 ± 16	120 ± 9*
Brain	0.14 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	192 ± 25	211 ± 18	173 ± 16

Data are expressed as mean ± S.E.M. of 5 - 8. K_d: dissociation constant; B_{max}: maximal density. * $P < 0.05$.

that longer treatment periods are necessary to induce changes in receptor characteristics. Therefore, rats were injected with 10 mg verapamil/kg/day for 2 to 16 weeks and PN 200-110 binding was assessed in membrane fractions from three tissues: ventricular, brain and skeletal muscle. The results from these experiments are shown in Table 2. There were no significant differences in B_{max} or K_D values for [^{3}H]PN 200-110 binding in any of the tissues examined.

The concentration of verapamil injected may be an important factor in inducing changes in Ca^{2+} channel receptor characteristics. Therefore, the verapamil concentration injected daily over an 8-week period was varied from 2.5 to 30 mg/kg. Plasma samples were taken at the end of the 8 week administration period and measured for verapamil and norverapamil content. These results are shown in Table 3. There was a concentration dependent rise in the circulating levels of both the parent drug (verapamil) and its primary metabolite, norverapamil. Consistent with previous results, the other verapamil metabolites, D-617 and D-620, were not detectable.

Specific binding of PN 200-110 was measured in cardiac, brain and skeletal muscle membranes isolated from control animals and those treated with varying verapamil concentrations. There were no changes detected in cardiac or brain fractions (Table 4), but there was a trend for increasing B_{max} and K_D in skeletal muscle membranes with increasing verapamil concentrations. This attained statistical significance at the highest dose (30 mg/kg/day) used

Table 2. Specific binding characteristics of [^{3}H]-PN200-110 to cardiac, brain and skeletal muscle membranes of rats treated for varying times with verapamil injections.

	2 weeks		4 weeks		8 weeks		16 weeks	
	C	VI	C	VI	C	VI	C	VI
1. Cardiac								
K_d (nM)	0.25 ± 0.05	0.15 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.17 ± 0.04	0.20 ± 0.03	0.22 ± 0.04	0.22 ± 0.04
B_{max} (fmol/mg)	263 ± 55	191 ± 16	222 ± 20	260 ± 26	315 ± 53	296 ± 48	341 ± 50	480 ± 90
2. Brain								
K_d (nM)	0.14 ± 0.02	0.12 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	0.13 ± 0.02	0.13 ± 0.04	0.21 ± 0.06
B_{max} (fmol/mg)	192 ± 25	211 ± 18	281 ± 25	288 ± 26	337 ± 48	317 ± 38	520 ± 120	500 ± 110
3. Skeletal Muscle								
K_d (nM)	ND	ND	0.35 ± 0.02	0.40 ± 0.05	0.35 ± 0.07	0.44 ± 0.05	0.73 ± 0.12	0.40 ± 0.03
B_{max} (pmol/mg)	ND	ND	4.02 ± 0.28	4.20 ± 0.52	4.19 ± 0.53	4.80 ± 0.48	4.00 ± 1.10	2.80 ± 0.40

Values represent the mean ± S.E. (n=8-10).

ND: not determined; C: control group; VI: verapamil injected (10 mg verapamil/kg/day).

Table 3. Plasma verapamil and norverapamil concentrations in rats injected for 8 weeks with varying concentrations of verapamil.

Experimental Group	Verapamil (ng/ml)	Norverapamil (ng/ml)
Control	ND	ND
2.5 mg verapamil/kg	23.8 ± 15.8	6.9 ± 6.4
10 mg verapamil/kg	51.5 ± 16.5	17.3 ± 7.7
20 mg verapamil/kg	95.4 ± 34.1	25.2 ± 8.5
30 mg verapamil/kg	111.1 ± 24.6	57.4 ± 13.2

Values represent mean ± S.E. of 5 - 9 separate determinations. ND: not detectable.

Table 4. Specific binding characteristics of [^{3}H]-PN200-110 to cardiac, brain and skeletal muscle membranes of rats treated for 8 weeks with varying concentrations of verapamil.

	0 mg verapamil/kg	2.5 mg verapamil/kg	10 mg verapamil/kg	20 mg verapamil/kg	30 mg verapamil/kg
1. Cardiac					
K_d (nM)	0.17 ± 0.04	0.15 ± 0.03	0.20 ± 0.03	0.24 ± 0.03	0.18 ± 0.03
B_{max} (fmol/mg)	315 ± 53	249 ± 39	296 ± 48	324 ± 42	357 ± 38
2. Brain					
K_d (nM)	0.12 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	0.11 ± 0.02	0.14 ± 0.02
B_{max} (fmol/mg)	337 ± 48	302 ± 40	317 ± 38	264 ± 38	305 ± 38
3. Skeletal Muscle					
K_d (nM)	0.35 ± 0.07	0.41 ± 0.06	0.44 ± 0.05	0.48 ± 0.06	0.54 ± 0.05*
B_{max} (pmol/mg)	4.19 ± 0.53	4.39 ± 0.39	4.80 ± 0.48	4.82 ± 0.45	5.94 ± 0.37*

Values represent the mean ± S.E. of 8-10 independent observations.

* $P < 0.05$ vs. control values (0 mg verapamil/kg).

(Table 4).

The above studies demonstrated that there were no significant alterations in the cardiac Ca^{2+} channel receptor characteristics with either long-term drug dosage or varying drug dosage *via* s.c. injection, though a change was noticed in skeletal muscle (Table 4). The serum plasma verapamil concentrations (~ 2.5 to 250 ng/ml) for our injected doses (2.5 to 30 mg/kg/day respectively) were in the range of the reported therapeutic concentrations of verapamil in the serum (80 to 400 ng/ml)[219]. It is apparent, therefore, that in the therapeutic drug dosage range of verapamil, there was no significant alteration in the receptor characteristics of the cardiac Ca^{2+} channels. A depression in cardiac binding sites was noted, however, in the 50 mg verapamil implanted animals (Table 1). The plasma verapamil levels attained in these animals reached a level of ~2.2 ng/ml by the 8^{th} hour post-implantation (Figure 3). This plasma verapamil concentration is higher than the therapeutic level by ~10-fold. It is possible that there may be an alteration in the Ca^{2+} channel binding characteristics at near toxic doses of verapamil. In order to test this hypothesis, a group of rats were implanted with a range of concentrations of slow-release verapamil pellets. The decision to use the implantable pellets again was taken because they had shown an alteration in the cardiac Ca^{2+} channel binding characteristics previously, and they released massive, potentially toxic concentrations of verapamil. Contrary to their design, the slow-release pellets released these toxic (or near toxic) doses of verapamil in a short time period post-

implantation, and were able to maintain that level for at least 24 hours (Figure 3). A range of pellet sizes were tested (0.25, 1.5, 5, 10, 25, 35, 50, and 75 mg) for varying periods of time (24 hours and 7 days). The 75 mg pellet implant group had a 100% mortality rate, and the 50 mg implant group had only 2 out of 5 animals survive, too small a number in order to get an accurate result. The B_{max} results for the remaining groups are presented in Figure 10 (24 hours) and Figure 11 (7 days). No significant differences were noticed between the various implant groups with respect to [^{3}H]PN 200-110 binding to cardiac membranes. The K_D values for the 24 hour study ranged from $0.9 \pm 0.1 \times 10^{-10}$ nM (control) to $1.1 \pm 0.2 \times 10^{-10}$ nM (25 mg). The 7 day treatment group had a K_D range from $0.9 \pm 0.1 \times 10^{-10}$ nM (control) to $1.1 \pm 0.2 \times 10^{-10}$ nM (25 mg). No significant differences were noted in K_D amongst any of the groups.

Blood samples taken from the above verapamil pellet implanted groups demonstrated a similar variability in the release of verapamil that the original 50 mg verapamil pellet implant study demonstrated (Figure 12). There was a dramatic rise in plasma verapamil concentration in the first 24 hours post-implantation which dropped significantly by 3 days and remained at a relatively constant and low level for the remainder of sampling. The concentration of verapamil present in the plasma was increased with increasing pellet size. It is evident that the unreliability of these slow-release verapamil pellets is not restricted to only the 50 mg pellet size.

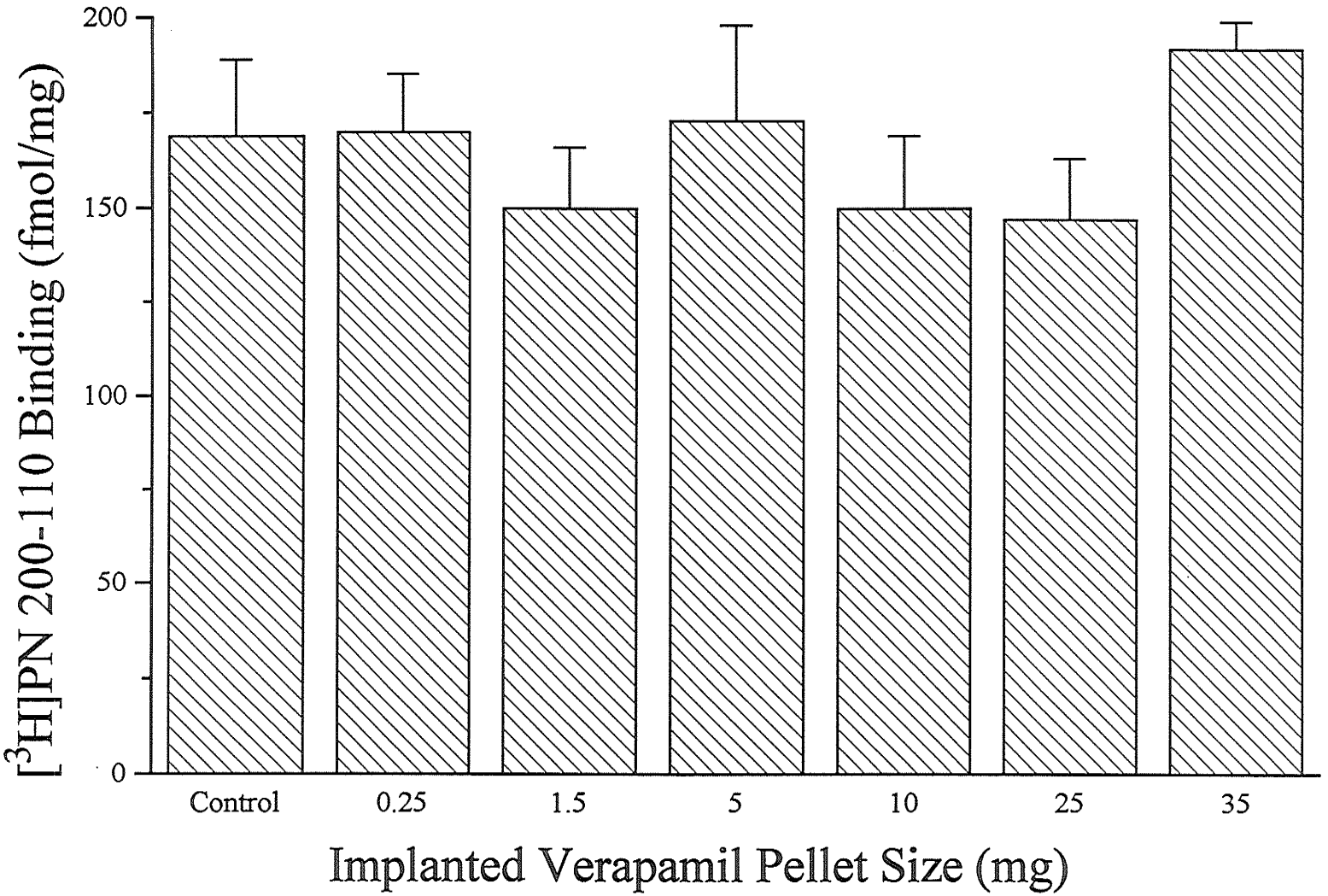

Figure 10. Specific binding characteristics of [^{3}H]PN 200-110 to crude membrane fractions of heart tissue from control and verapamil implanted (24 hours) rats. Verapamil pellet size is in mg and implanted into 200 gram female Sprague-Dawley rats. Data are expressed as mean ± S.E.M of five to nine animals

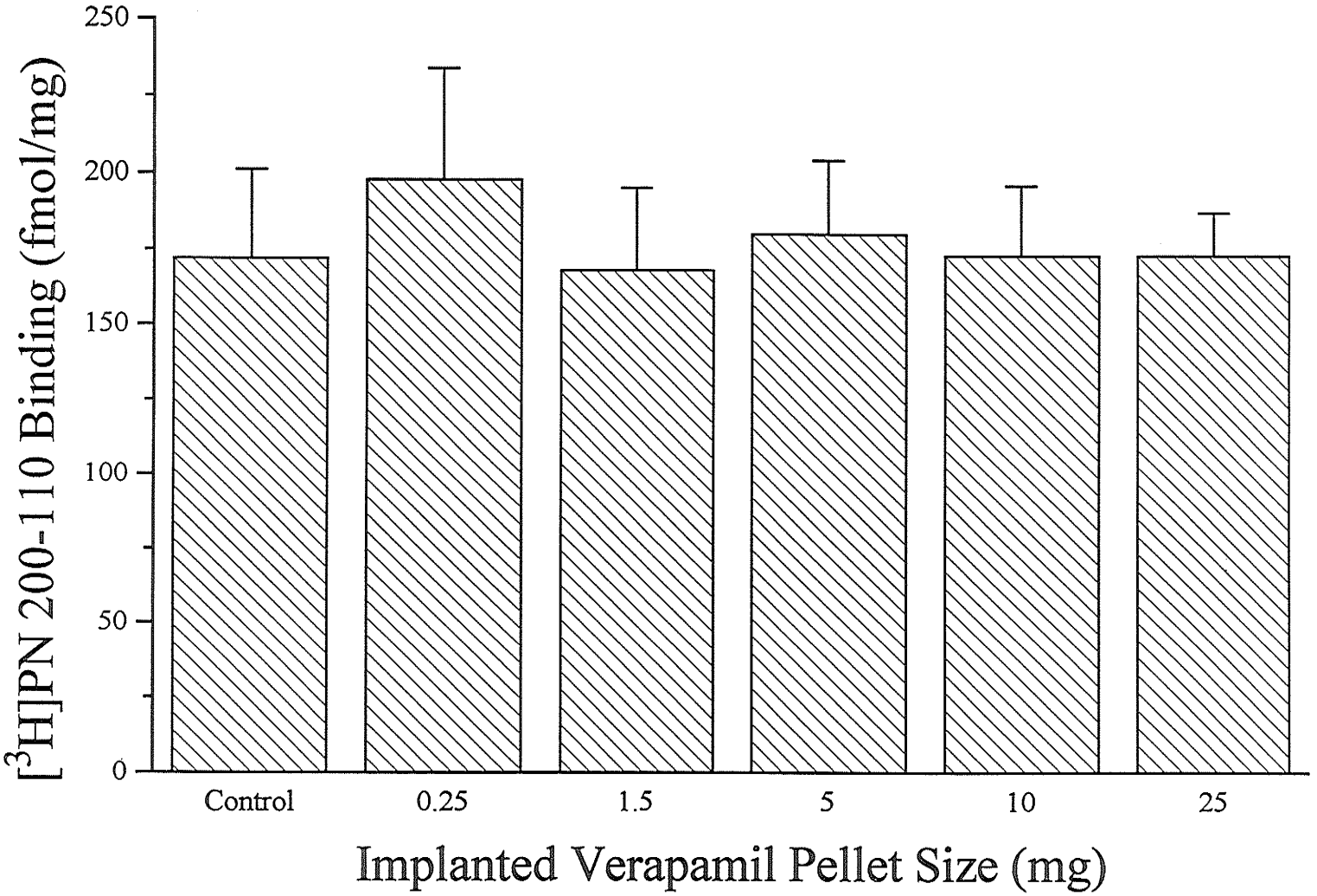

Figure 11. Specific binding characteristics of [^{3}H]PN 200-110 to crude membrane fractions of heart tissue from control and verapamil implanted (7 days) rats. Verapamil pellet size is in mg and implanted into 200 gram female Sprague-Dawley rats. Data are expressed as mean ± S.E.M. of five animals.

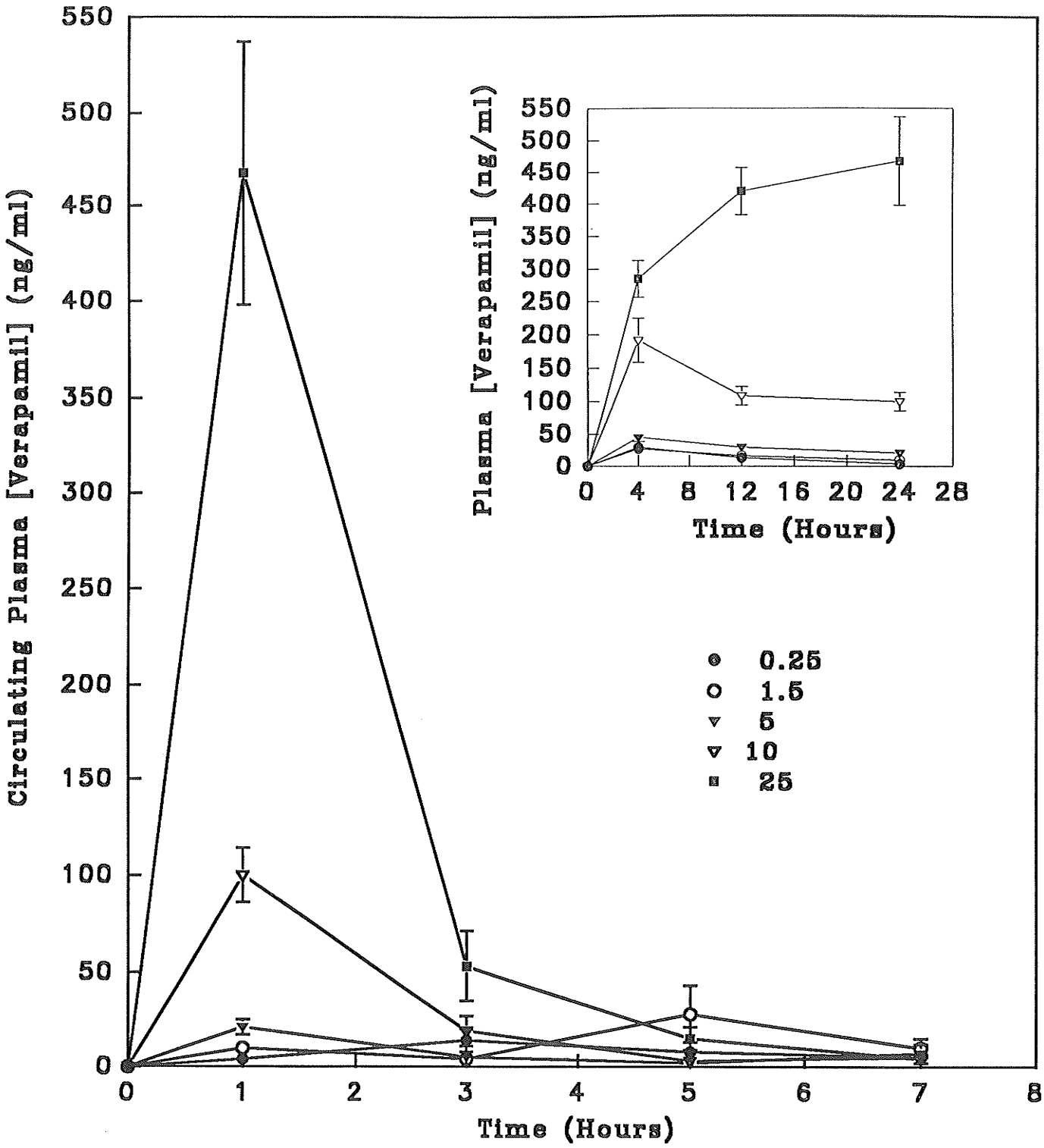

Figure 12. Plasma verapamil concentrations (ng/ml) over time from rats implanted with different verapamil pellet sizes (mg). Data represent mean ± S.E.M. of five to eight animals.

A final [^{3}H]PN 200-110 binding study was carried out on cardiac tissue isolated from rats that had received near toxic doses of verapamil *via* s.c. injection over a 48 hour time span. The injection groups consisted of 25, 50, 60, and 75 mg/kg/day. The results for the B_{max} data obtained from these experiments are presented in Figure 13. Due to the high mortality rate in the 75 mg/kg/day group (82%), no reliable binding data were obtained. No significant difference was noted between the other groups of injected animals with respect to controls. The K_D values ranged from $1.7 \pm 0.3 \times 10^{-10}$ nM (60 mg/kg/day) to $1.9 \pm 0.3 \times 10^{-10}$ nM (control). No significant differences were detected in these K_D values amongst the groups.

Blood samples were obtained during the drug treatment regimen at 4 hours and 48 hours post first injection. The analysis of the verapamil concentrations obtained from these samples are presented in Figure 14. The plasma verapamil concentration increases with the increased drug treatment group and is higher at 4 hours than at 48 hours in all respective groups. It is significant to note that the plasma verapamil concentrations observed in the 48 hours group were obtained approximately twelve hours after the last injection.

The potential for allosteric interactions between the dihydropyridine (PN 200-110) binding site and the phenylalkylamine (verapamil) binding site were monitored throughout all the [^{3}H]PN 200-110 binding experiments. It is known that allosteric interactions exist between the various binding sites located on the Ca^{2+} channel and these interactions are modified by the presence of

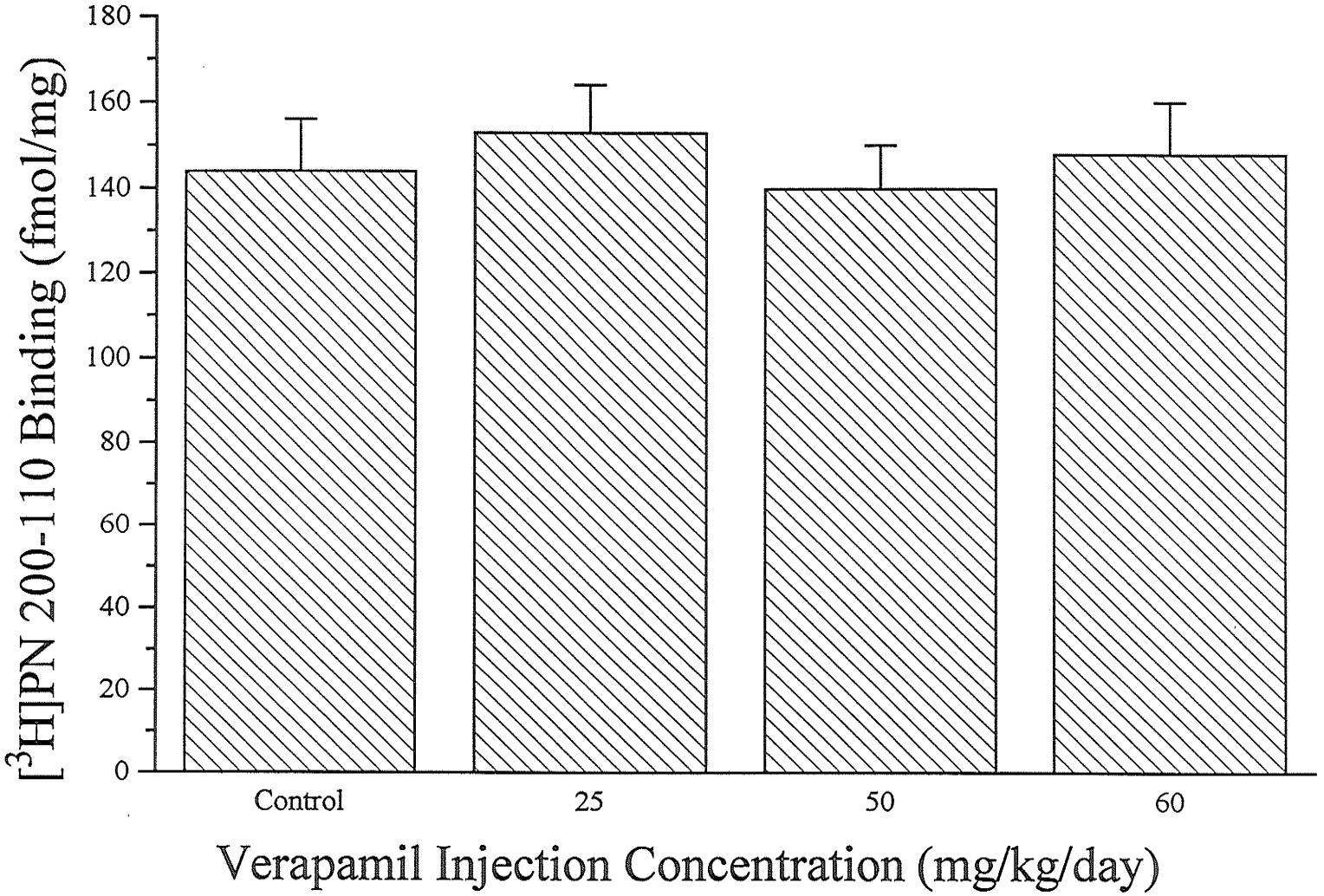

Figure 13. Specific binding characteristics of [^{3}H]PN 200-110 to crude membrane fractions of heart tissue from control and verapamil injected (48 hours) rats. Verapamil injections were in mg/kg/day and injected into 200 gram female Sprague-Dawley rats. Data are expressed as mean ± S.E.M. of six to twelve animals.

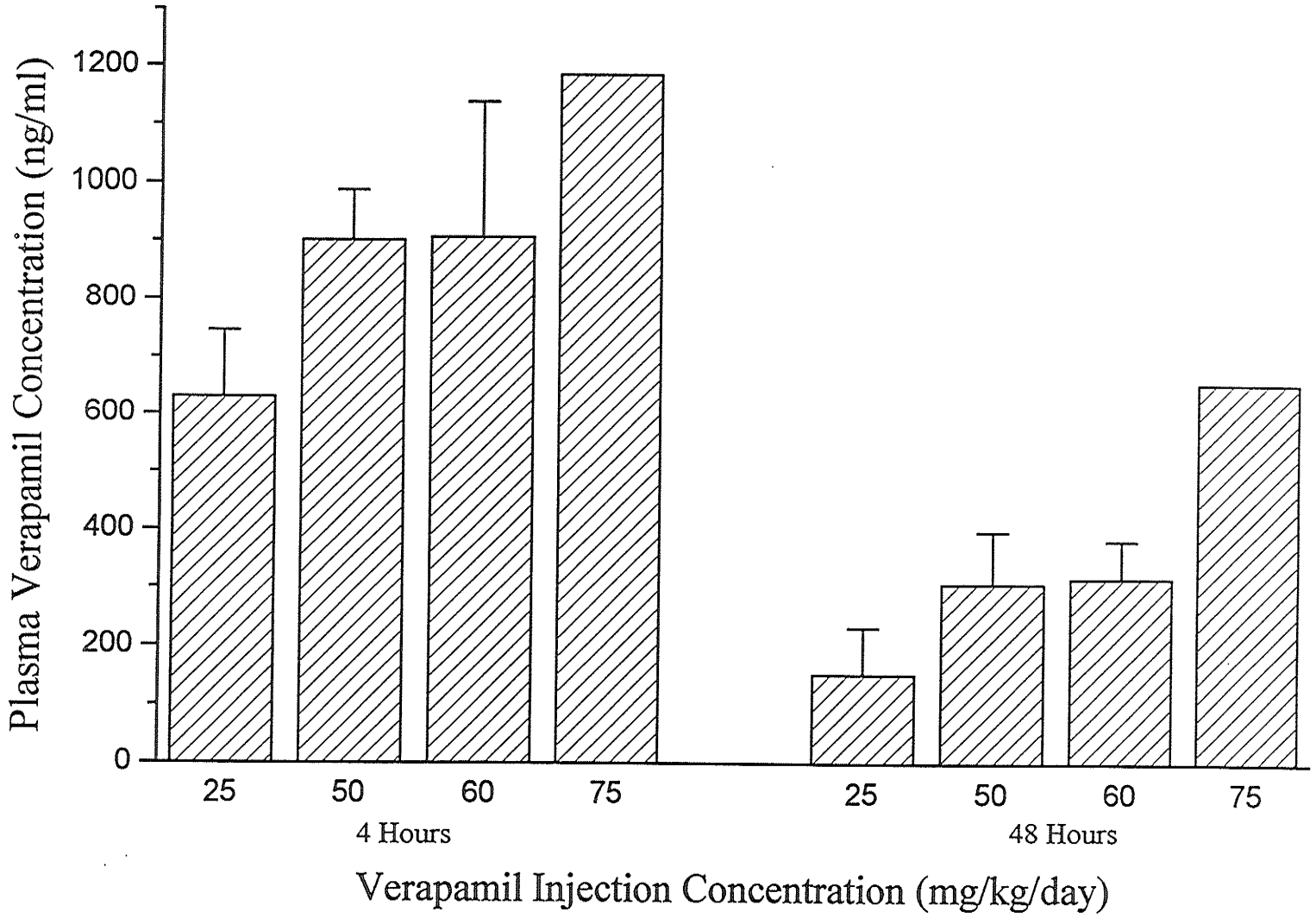

Figure 14. Analysis of blood samples obtained from verapamil injected animals at 4 and 48 hours after the initial injection. Plasma verapamil concentration is in ng/ml. Data are expressed as mean ± S.E.M. of two to twelve animals.

cations such as Ca^{2+}.[338] In order to ensure that there was no alteration in the normal interaction between the dihydropyridine and the phenylalkylamine binding sites due to the verapamil treatment regime, allosteric studies were performed. To test if verapamil had altered this allosteric interaction, cardiac membranes were isolated from control and verapamil-treated rats and PN 200-110 binding was determined in the presence or absence of verapamil and also in the presence or absence of Ca^{2+}. Verapamil inhibited [^{3}H]PN 200-110 binding and this effect was enhanced if calcium was omitted from the reaction medium (Figures 15 and 16). However, there was no change in the percentage of inhibition by verapamil *in vitro* as a function of verapamil treatment of the rats (see Figures 15 and 16 and Table 5).

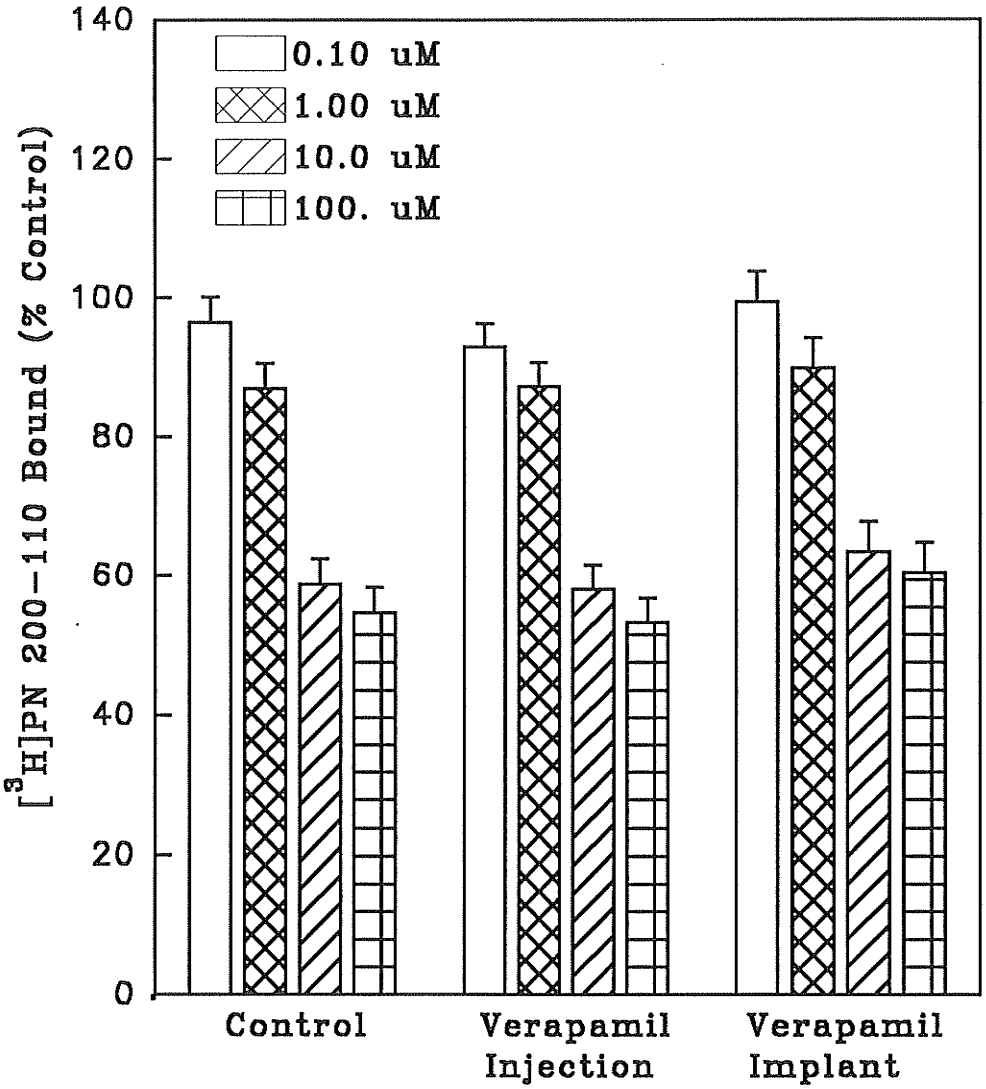

Figure 15. Inhibition of [^{3}H]PN 200-110 binding to crude membrane preparations of ventricular tissue by verapamil. Ventricular membranes were incubated with 0.25 nM [^{3}H]PN 200-110 with various concentrations of unlabeled verapamil in the presence of 1 mM Ca^{2+}. Values represent mean ± S.E.M. of eight experiments.

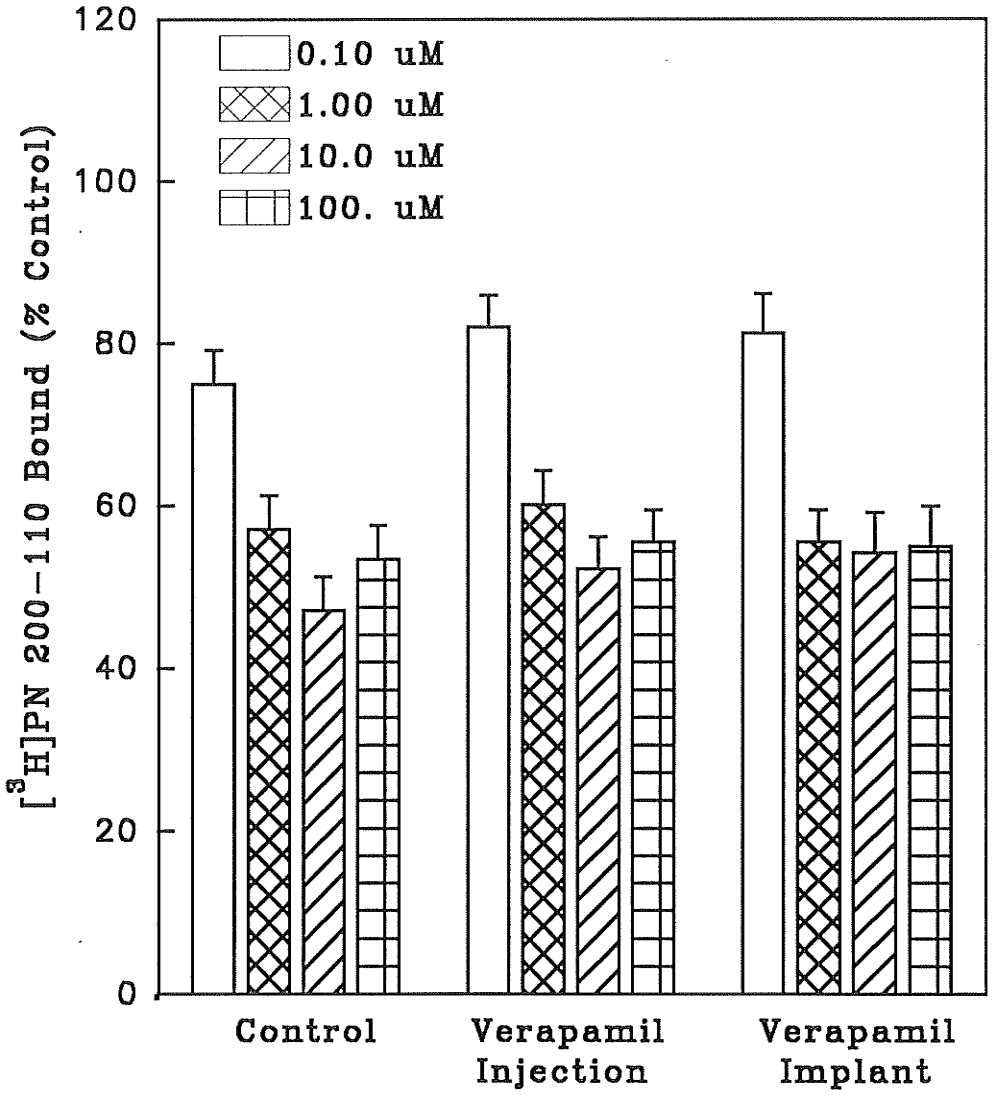

Figure 16. Inhibition of [^{3}H]PN 200-110 binding to crude membrane preparations of ventricular tissue by verapamil. Ventricular membranes were incubated with 0.25 nM [^{3}H]PN 200-110 with various concentrations of unlabeled verapamil in the absence of Ca^{2+}. Values represent mean ± S.E.M. of eight experiments.

Table 5. Inhibition of 0.25 nM [^{3}H] PN 200-110 binding by 10 μM verapamil. Inhibition is a percent of control, where 100% is binding of [^{3}H]PN 200-110 in the absence of verapamil.

Verapamil Implants							
	Control	0.25 mg	1.5 mg	5 mg	10 mg	25 mg	35 mg
24 Hours	68.9± 3.3	68.7 ± 2.9	71.9 ± 1.7	69.9 ± 3.9	65.1 ± 2.1	70.5 ± 2.9	67.8 ± 2.3
7 Days	67.4 ± 1.1	69.3 ± 2.8	72.2 ± 3.9	75.3 ± 5.4	71.3 ± 4.7	74.9 ±4.1	

Verapamil Injections				
	Control	25 mg/kg/day	50 mg/kg/day	60 mg/kg/day
48 Hours	71.0 ± 2.7	65.8 ± 2.4	66.8 ± 2.7	67.2 ±4.3

Values represent mean ± S.E.M. of 5 to 10 separate experiments.

Discussion

In order to determine the biochemical status of the Ca^{2+} channel in the present study, a radioactive ligand binding assay was employed. [^{3}H]PN 200-110, a dihydropyridine, was used as the radioactively labelled ligand because of its high binding specificity for the L-type Ca^{2+} channel. A possible functional problem arises in using a dihydropyridine ligand when the drug we are testing is from another group, a phenylalkylamine. However, the choice to use a highly specific dihydropyridine in assessing changes in the Ca^{2+} channel after phenylalkylamine treatment is justified in two ways. First, although radioactively labelled verapamil compounds are available (e.g. [^{3}H]verapamil), their specificity for the Ca^{2+} channel is low and, therefore, it would have been impossible to accurately determine any subtle changes occurring in the channels. Second, it is highly improbable that there would be an alteration in the binding site of one of the compounds without the concurrent alteration in the other. The binding sites for the Ca^{2+} channel antagonists are all located on the α_1 subunit of the Ca^{2+} channel[164,234]. Molecular characterization studies have isolated the proposed binding sites for the dihydropyridines and the phenylalkylamines to helix 6 of the IVth repeating subunit of the α_1 subunit.[224,227] It is, therefore, likely that if any change were to occur at the verapamil binding site, a concurrent change would also occur at the PN 200-110 binding site.

Currently, it is not completely understood what effects

chronic Ca^{2+} channel antagonist treatment has on the biochemical characteristics of the Ca^{2+} channel. Panza, *et al.*[275], showed a down regulation in the number of [^{3}H]nitrendipine binding sites in membranes prepared from mouse brain tissue after long-term treatment (28 days) with nifedipine (280 mg/kg/day) and verapamil (270 mg/kg/day) but not with diltiazem (380 mg/kg/day).[275] The drugs were administered to the experimental mice by feeding them powdered food containing the drugs. A down-regulation in the number of neuronal (brain) and cardiac Ca^{2+} channels was shown by Gengo, *et al.*[276], in rats receiving chronic intravenous administration of nifedipine (0.864 and 8.640 mg/kg/day) for 20 days.[276] A concurrent down-regulation in the number of ß-adrenoreceptors was also noted. Le Grand, *et al.*, observed that there was a depression in Ca^{2+} current in human atrial myocytes after chronic oral treatment with Ca^{2+} antagonists nifedipine (80-120 mg/day), nicardipine (60-80 mg/day) and diltiazem (120-180 mg/day).[326] They attributed this Ca^{2+} current depression to a down-regulation in the Ca^{2+} channels. In opposition to these studies, Nishiyama, *et al.*[277], showed that there was no change in the Ca^{2+} channel density (or ß-adrenergic receptor density) after chronic treatment with oral nifedipine in rats. They administered 100 mg/kg/day of nifedipine *via* oral stomach tube for a period of two weeks.

Much of the controversy, with respect to these drugs' actions on Ca^{2+} channel biochemistry following chronic administration comes from a variety of factors: animals, duration, type of drug and

concentration, tissues and modes of drug administration. For example, in order for the Ca^{2+} antagonist to mediate its blocking effects, it has to reach the specific tissue *via* the circulation. Differential plasma antagonist concentrations might be attained using the various modes of drug administration available and, therefore, could account for the variability in results noted above. In order to correlate any differences we may have observed in Ca^{2+} channel biochemistry in our experiments, we felt it important to monitor the circulating plasma verapamil concentrations at various points in the treatment regimens. Three modes of drug administration were tested with respect to their ability to raise the circulating verapamil concentrations. These included an implantable slow-release verapamil pellet, subcutaneous (s.c.) injection and oral administration (*via* p.o. intubator).

Slow-release pellets used to administer drugs have become more prevalent in both experimental and clinical situations. The use of an implantable slow-release pellet is a very convenient means of administering a constant drug dosage over a period of time, thus eliminating twice or thrice daily injections or oral dosages. In certain situations these slow-release pellets have proven to be an effective means of administration.[327] For example, a slow-release calcitriol pellet has been used in avian embryos and showed no difference in mortality (with respect to controls) at lower concentrations, while there was an elevation above control mortalities with higher dosages.[327] Therefore, the availability of an implantable slow-release verapamil pellet presented a very

attractive means of administering the drug as it eliminated the need for a twice daily injection, 7 days a week. In our initial studies utilizing a 50 mg implantable pellet, the pellet was to deliver a constant verapamil dose of 11.9 mg/kg/day for a period of three weeks. In the first 18 hours post-implantation, we observed a high mortality rate (63%) in the animals. As there were no deaths in our placebo implanted group, and care was taken not to damage the pellet during implantation, we hypothesized that the pellets were not releasing even doses of the drug, but instead were releasing toxic dosages of verapamil. In order to test our hypothesis, we directly examined the plasma concentrations of verapamil and its primary metabolites (norverapamil, D-617 and D-620) from implanted animals, and animals that had received a similar dose of verapamil (11.9 mg/kg/day) *via* s.c. injection and orally (*via* p.o. intubator). The plasma verapamil concentration was significantly higher in the pellet implanted rats than in the other two treatment groups (Figure 3). Verapamil concentration rose over time and peaked at approximately 8 hours, where it stayed at a level ~ 10-fold higher than the other treatment modes. There was then a dramatic drop in the plasma verapamil concentration between 1 to 7 days (Figure 7, upper graph), where it remained at this lower level for the remainder of the monitoring period. The primary verapamil metabolites norverapamil (Figure 4, Figure 7 [lower graph]), D-617 (Figure 5), and D-620 (Figure 6) all showed elevated levels in the plasma from verapamil-implanted animals compared to the other two treatment modes. D-617 and D-620 were,

in fact, very difficult to measure (not detectable) in rats administered verapamil by injection or oral means.

Additional verapamil pellet implant experiments using a variety of pellet sizes (0.25 mg to 75 mg) confirmed our observations that drug release was indeed unpredictable and uneven. Furthermore, there was a dramatic increase in the number of deaths in the implanted groups with increasing pellet size (> 10 mg). Standardization of the theoretical daily drug dose between the verapamil implanted and s.c. injected animals, indicated that the dose at which death was occurring was much lower for the implanted group than for the injected group. Therefore, the verapamil implanted rats must have been receiving a toxic dose of the drug within a short time after implantation. Death always occurred within 18 hours post-implantation (data not shown). It is evident from these data that the implantable slow-release verapamil pellets were not delivering a low, even dose of verapamil over the prescribed 3-week period. They were, in fact, releasing large quantities (often toxic dosages) of the drug within a short time after implantation, peaking within 24 hours then falling to lower levels for the remainder of time. Therefore, although the use of implantable slow-release pellets could have important advantages as a means of drug administration, these experiments do not support its use as a reliable method of verapamil administration.

In those animals that did survive the verapamil implantation, [^{3}H]PN 200-110 binding was carried out in membranes isolated from a variety of tissues. Analysis of PN 200-110 binding data was

carried out using a Scatchard plot from which receptor characteristics such as B_{max} and K_D can be determined. B_{max} refers to the maximum binding capacity of the ligand to the membrane fraction. K_D is referred to as the dissociation constant and is a measure of the affinity of the particular receptor for the ligand. PN 200-110 binding demonstrated saturation at the higher PN 200-110 concentrations (2-4 nM). This value is in agreement with other reported studies of PN 200-110 binding[338]. A decrease in the B_{max} and K_D values for PN 200-110 binding to membranes obtained from animals which survived the 50 mg verapamil pellet implant was noted after two weeks duration with respect to controls. No significant alterations in Ca^{2+} channel characteristics were observed in the s.c. injected or orally administered groups (equivalent 11.9 mg/kg/day for 2 weeks). The unreliability of the implantable slow-release pellet forced us to continue our experiments utilizing s.c. injection as the means for drug administration instead of the pellet implants. Since there were no changes noticed after 2 weeks at 11.9 mg/kg/day, it was hypothesized that a higher drug dosage or possibly longer duration of treatment was required in order to observe a significant change in channel characteristics. There is a precedent that increased levels of the Ca^{2+} channel antagonist may alter channel characteristics. Gengo and his colleagues demonstrated a reduction in the number of [^{3}H]nitrendipine binding sites after 20 days of intravenous treatment with 0.864 mg/kg/day nifedipine. There was a further reduction in binding sites in animals treated with a higher dose of nifedipine (8.640 mg/kg/day

i.v.).[276] Therefore, a verapamil concentration range of 2.5 to 30 mg/kg/day was chosen to be administered by s.c. injection into rats over an 8-week period. This particular range was also chosen because of other studies demonstrating this drug concentration to be effective in the treatment of conditions such as diabetic cardiomyopathy in rats[300,330], genetic cardiomyopathy in hamsters[331] and hypertrophic cardiomyopathy in humans[332]. Cardiac, brain and skeletal muscle tissue were examined and no significant changes in receptor characteristics were noted at any of the concentrations for either brain or cardiac tissue (Table 4). However, a trend was observed for increasing B_{max} and K_D with increasing verapamil concentration for skeletal muscle which reached significance at the highest concentration (30 mg/kg/day) (see Table 4). The increase in B_{max} and K_D observed in skeletal muscle may have been a consequence of the high local verapamil concentrations resulting from the s.c. injections. The s.c. injections were administered in the hind region which might have resulted in a high local verapamil concentration in the area from which we obtained our muscle samples (quadriceps).

In order to determine whether the duration of the drug treatment had any effect on channel characteristics, a constant dose of 10 mg/kg/day was administered *via* s.c. injection for up to 16 weeks. Cardiac, brain and skeletal muscle were again excised and PN 200-110 binding was carried out on the crude membrane fractions. No significant changes were noted in the Ca^{2+} channel characteristics (Table 2).

The circulating verapamil concentrations observed in the present study have clinical relevance. Therapeutic concentrations of verapamil in the serum range from 80 to 400 ng/ml.[219] The verapamil injections in this study resulted in plasma verapamil concentrations of ~ 25 to 250 ng/ml. Verapamil is approximately 90% bound to serum protein [219], therefore, the effective circulating verapamil concentration range was 2.5 to 25 ng/ml. This range of circulating verapamil concentrations may display some pharmacological action on contractile function of the heart. Verapamil at 2.5, 25 and 250 ng/ml can depress developed tension by 16 ± 2, 49 ± 6 and 76 ± 4 %, respectively, in cardiac muscle.[116] Therefore, the circulating verapamil concentrations determined in the present study would be expected to have had some pharmacological effects on cardiac performance. However, no change was noticed in cardiac Ca^{2+} channel characteristics in the therapeutically relevant verapamil range. A change was noticed in the toxic dosage range after 50 mg verapamil pellets implantation. At this point in our work, therefore, we considered it possible that in order to see alterations in channel characteristics, a higher (near toxic) dosage of verapamil was required.

To determine what the threshold verapamil dosage (and the threshold circulating verapamil concentration) was which is required to elicit a change in the biochemical characteristics of the channel, experiments were performed utilizing the implantable slow-release verapamil pellets and s.c. injections. The verapamil pellets (pellet size range from 0.25 mg to 75 mg) were utilized in

this case to take advantage of their capacity to release high levels of verapamil and maintain them for at least 24 hours. From our previous results it was known that these pellets delivered a high (near toxic) dose of verapamil. In addition, s.c. verapamil injections at a concentration of 25 to 75 mg/kg/day were also administered. Mortality data (Figure 1) indicated that the levels of verapamil being administered by these treatment protocols were potentially toxic (up to 80 to 100% mortality). Plasma verapamil concentrations were determined at various points in the drug treatment regimen and found to be elevated. However, no significant changes were noted in B_{max} or K_D in cardiac membranes prepared from either the verapamil implanted rats (Figures 10 and 11) or the verapamil injected rats (Figure 13). It is apparent that even at high doses of the drug, the Ca^{2+} channels appear to be highly resistant to alteration.

There are currently five[225] (possibly six[226]) identified molecular binding sites on the α_1 subunit for the various Ca^{2+} channel antagonists and novel derivatives, all allosterically linked to one another.[209,243,245] These allosteric interactions allow for increased or decreased binding of one Ca^{2+} antagonist in the presence of another bound antagonist. For example, the binding of dihydropyridines is inhibited in the presence of verapamil[209], while stimulated in the presence of the *d-cis* isomer of diltiazem[246]. Alterations in the allosteric interaction between the two receptor subclasses as a function of verapamil treatment would be expected to be a sensitive index of change in one or the other

receptor. Radioligand binding studies were carried out in which allosteric changes between these two receptor subclasses were examined as a function of verapamil treatment. Verapamil inhibited the binding of PN 200-110 as expected[242,250a]. However, addition of 0.1 to 100 μM verapamil into the binding reaction medium *in vitro* showed a similar inhibition of [^{3}H]PN 200-110 binding in both verapamil treated animals and control animals (Table 5). This would strongly suggest that verapamil treatment of the animals did not alter allosteric interactions between the two binding sites for phenylalkylamines and dihydropyridines. This conclusion was further supported by data obtained with varying the [Ca^{2+}] in the binding reaction media. The cation Ca^{2+} is an important modulator of Ca^{2+} antagonist binding.[250,250a] In the presence of 1 mM Ca^{2+}, the ability of verapamil to inhibit PN 200-110 binding was reduced (Figure 15), as expected[338]. The effects of verapamil on PN 200-110 binding were not different between the two groups in the absence or presence of Ca^{2+}. The above interventions would be expected to detect changes in allosteric interactions between the receptor sites had they existed. No differences were noted with respect to allosteric interaction or modulation by cations, therefore, it is unlikely that verapamil treatment caused even subtle changes in the biochemical characteristics of the Ca^{2+} channel in the heart under the experimental conditions used in the present investigation.

CONCLUSIONS

1. The biochemical characteristics of the cardiac L-type Ca^{2+} channel appears to be very resistant to alteration during chronic administration of the Ca^{2+} channel antagonist, verapamil to rats. No alterations in channel biochemical characteristics were observed in either a therapeutically relevant verapamil range or in toxic dosages. In addition, there was no change observed after varying durations of treatment. The one exception to this was noted when a decrease in B_{max} and K_D was observed in the 50 mg implanted animals.
2. Brain and skeletal muscle L-type channels also appear to be resistant to alteration by verapamil. No changes in channel biochemical characteristics in brain were observed. The trend of increasing B_{max} and K_D noted in skeletal muscle may be a consequence of high localized verapamil concentrations.
3. As no changes in the cardiac Ca^{2+} channel biochemical characteristics were noted after long-term usage of therapeutic dosages of verapamil, it is unlikely that verapamil treatment would significantly alter these characteristics when used clinically in the treatment of certain disease states like diabetes and cardiomyopathy. However, it should be noted that the disease states may respond to the drug with a different sensitivity and, therefore, this would have to experimentally tested before definitive conclusions may be attained.

4. Theoretically, implantable slow-release pellets are a very attractive means of drug administration. However, our studies demonstrate that verapamil pellets are unreliable in their release of the drug, and often release toxic doses within a short time post-implantation. Quantitation of circulating plasma verapamil concentrations from the larger pellet sizes resulted in levels (~2.2 μg/ml) similar to clinical cases of verapamil toxicity where serum concentrations of 1.5 to 5.3 μg/ml have been reported.[336] These implantable pellets are not a practical alternative for verapamil drug administration.

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