IMMUNOLOGICAL CHARACTERIZATION OF VOLTAGE-SENSITIVE CALCIUM CHANNELS

by

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ABBREVIATIONS

ACh	acetylcholine
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
α-BTX	α-bungarotoxin
$[Ca^{2+}]_i$-dependent	calcium-dependent inactivation
$(Ca^{2+}$-$Mg^{2+})$ATPase	Ca^{2+} & Mg^{2+}-activated ATPase (ATP phosphohydrase, EC 3.6.1.3.)
cAMP	adenosine 3',5'-cyclic phosphate
cDNA	complementary deoxyribonucleic acid
ω-CgTx	ω-VIA *Conus geographus* toxin
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate
Con A	Concanavalin A
DAB	3,3'-diaminobenzidine
1,4-DHP	1,4-dihydropyridine
EDTA	ethylenediaminetetraacetic acid
Endoglycosidase F	endo-β-N-glucosaminidase F
Endoglycosidase H	endo-β-N-glucosaminidase H
GABA	gamma-aminobutyric acid
G-protein	guanine nucleotide-binding protein
GDP	guanine diphosphate
GTP	guanine triphosphate
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
Nonidet P-40	octylphenol ethylene oxide condensate

PMSF	phenylmethylsulphonylfluoride
SDS	sodium dodecyl sulphate
SPC	soybean phosphatidylcholine
STX	saxitoxin
TEA	tetraethylammonium
TiTx-γ	*Tityus serrulatus* toxin
TMB	3,3',5,5' tetramethylbenzidine
Triton X-100	iso-octylphenoxypolyethoxyethanol
T-tubule	transverse tubule
TTX	tetrodotoxin
Tween 20	polyoxyethylenesorbitan monolaurate
WGA	Wheat Germ Agglutinin

Calcium Channel Drugs

Azidopine	(-)-2,6-dimethyl-4-(2'-trifluoromethylphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (N-4''-azido)-[3'',5''-^{3}H] benzoylaminoethyl) diester
BAY K 8644	methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate
D888	desmethoxyverapamil
Diltiazem	3-acetoxy-2,3-dihydro-5,2-(diethylamino)ethyl-2-(p-methoxyphenyl)-1,5-benzothiazepine-4-(5H)-one hydrochloride
Flunarizine	trans-1-cinnamyl-4-(4,4'-difluorobenzhydryl) piperazine
LU 49888	(-)-5-[(3-azidophenethyl)[N-methyl-^{3}H]methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile
Nifedipine	dimethyl-1,4-dihydro-2,6-dimethyl-1,4-(2-nitrophenyl)-3,5-pyridine dicarboxylate
Nitrendipine	1,4-dihydro-2,6-dimethyl(3-nitrophenyl)-3,5-pyridinecarboxylic acid, 3-ethyl-5-methy ester
PN200-110	isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate

Verapamil	5-N-(3,4-dimethoxyphenethyl)-N-methylamino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile

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CHAPTER 1 INTRODUCTION

1.1. Regulation of Intracellular Calcium

1.1.1. Introduction

Ca^{2+} is important in the cell as an intracellular messenger regulating many cellular functions, including contraction, secretion, enzyme function and cell proliferation. The messenger function of Ca^{2+} requires the concentration of free Ca^{2+} ions in the cell to be precisely controlled and maintained at very low levels, submicromolar. In this way only small amounts of the cation have to be mobilized, on cell stimulation, to exert an effect. In comparison, the extracellular Ca^{2+} concentration is several orders of magnitude higher, in the millimolar range, resulting in a large electrochemical gradient for Ca^{2+} across the plasma membrane. Following withdrawal of the cell stimulus it is necessary for Ca^{2+} to be rapidly removed to return the cytoplasmic concentration to resting levels. In the case of chemical messengers, such as adenosine 3',5'-cyclic phosphate (cAMP) and inositol 1,4,5-trisphosphate removal of the messenger is achieved by breakdown into inactive compounds. However, removal of free Ca^{2+} ions is achieved by a variety of Ca^{2+} transport mechanisms and Ca^{2+}-binding proteins (Carafoli, 1986).

The precise regulation of intracellular Ca^{2+} is reflected in the number of mechanisms employed by the cell, both to maintain a low intracellular free Ca^{2+} ion concentration, and to elicit small transient localized rises of free Ca^{2+} in the cytoplasm on cell stimulation. The major mechanisms involved in maintaining the low cytoplasmic free Ca^{2+} ion concentration are;

(a) cytoplasmic Ca^{2+}-binding proteins such as calmodulin and troponin C,

(b) intracellular organelles capable of acting as internal Ca^{2+} stores, such as the endoplasmic or sarcoplasmic reticulum and mitochondria and

(c) plasma membrane transport mechanisms, such as the Na^{+}/Ca^{2+} exchanger and the Ca^{2+} & Mg^{2+}-activated ATP phosphohydrase ((Ca^{2+}-Mg^{2+})-ATPase), capable of removing Ca^{2+} from the cell against the electrochemical gradient.

On cell stimulation Ca^{2+} ions are either mobilized from intracellular stores by second messenger stimulated release (Section 1.1.4) or enter the cell from the extracellular space

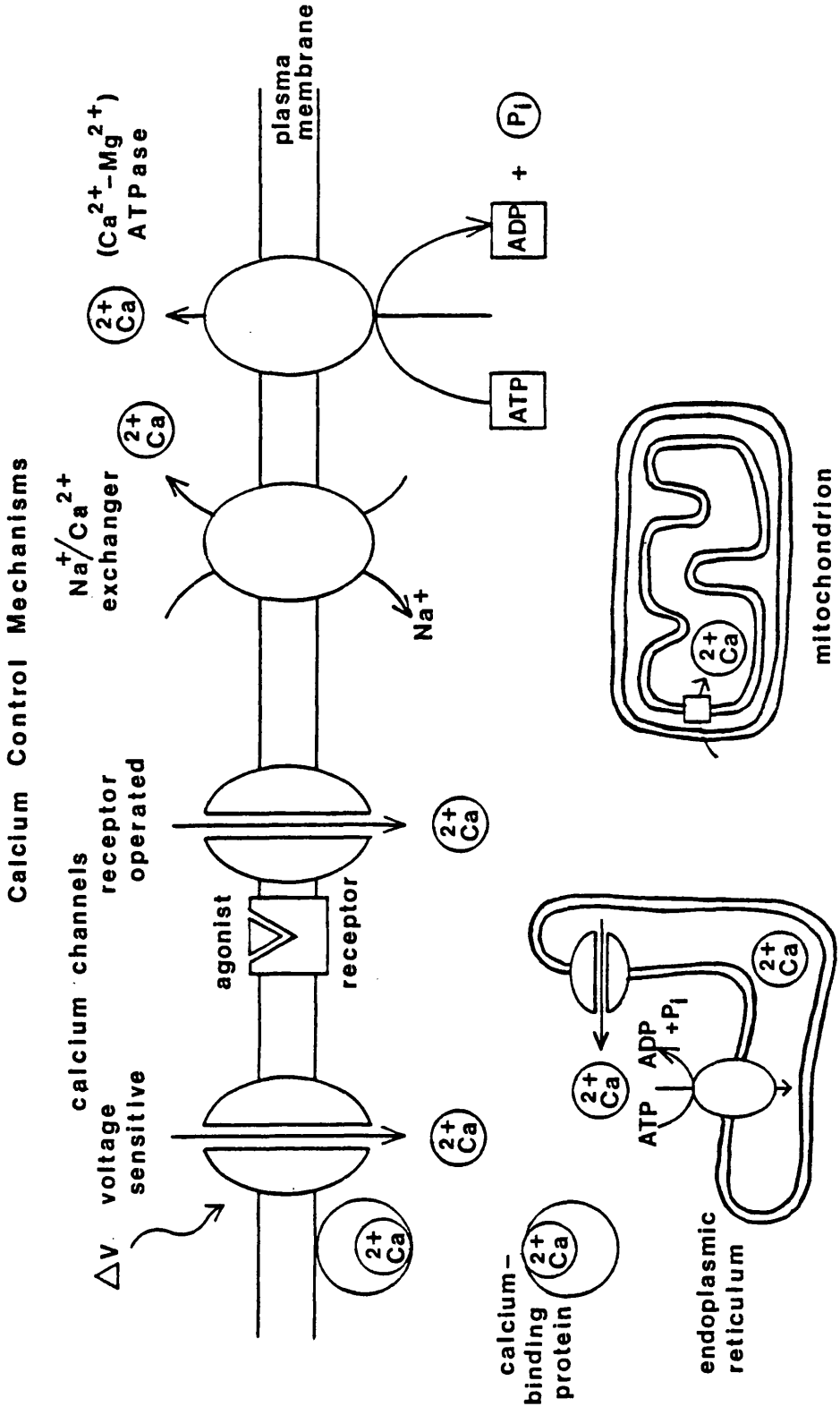


Figure 1

Calcium Control Mechanisms. Mechanisms involved in the regulation of the intracellular free Ca^{2+} ion concentration.

down the electrochemical gradient via plasma membrane Ca^{2+} channels. The major cellular Ca^{2+} control mechanisms are represented schematically in Figure 1.

1.1.2.Calcium Binding Proteins

Cytoplasmic Ca^{2+}-binding proteins are present in all cells (Carafoli, 1987). Calmodulin, is the most important and ubiquitous, and is found both in the cytoplasm and associated with the plasma membrane. Ca^{2+}-binding proteins, such as calmodulin, do not contribute significantly to the transmembrane Ca^{2+} gradient, their major function being the processing of the Ca^{2+} signal. The Ca^{2+}-calmodulin complex regulates the functions of many enzymes including adenylate cyclase, phosphorylase b kinase and the plasma membrane (Ca^{2+}-Mg^{2+})-ATPase (Section 1.1.3.; Carofoli, 1987). Troponin C has similar properties to calmodulin but is only present in muscle cells. The primary sequences of these two proteins show considerable homology, including four homologous repeats in each molecule representing the four Ca^{2+} binding sites. Each Ca^{2+} binding site is composed of a short sequence of amino acids forming a loop, with an α-helical structure on either side (Herzberg & James, 1981). This structure is common to many Ca^{2+}-binding proteins including calmodulin, troponin C and parvalbumin. The binding of Ca^{2+} to the loop region of calmodulin induces a conformational change in the protein structure. The conformation adopted by the calmodulin molecule following the binding of Ca^{2+} allows the calmodulin to interact with various enzymes and modulate their function (Campbell, 1987). Consequently the most important role of calmodulin in the cell is in the control of Ca^{2+}-dependent enzymes.

1.1.3.Na^+/Ca^{2+} Exchange and (Ca^{2+}-Mg^{2+})-ATPase

There are two major mechanisms by which Ca^{2+} is removed from the cell, the Na^+/Ca^{2+} exchange and the (Ca^{2+}-Mg^{2+})-ATPase (Carafoli, 1987). The Na^+/Ca^{2+} exchange system has yet to be fully characterized, however, Na^+/Ca^{2+} exchange activity can be measured following reconstitution of the exchanger protein into phospholipid vesicles from heart cell membranes (Reeves & Sutko, 1979). Studies on the reconstituted protein have demonstrated that 3 Na^+ ions are exchanged for 2 Ca^{2+} ions passing out of

the cell (Reeves & Sutko, 1979). It has been postulated that the energy required to expel Ca^{2+} from the cell against its electrochemical gradient is derived from the passage of Na^{+} ions into the cytoplasm down the large Na^{+} ion concentration gradient. The function of the exchanger is reversible although Ca^{2+} ion influx by this mechanism requires intracellular ATP (Baker, 1986). In addition, the protein can act as a Ca^{2+}/Ca^{2+} exchanger in conditions of high intracellular Ca^{2+}, or as a Na^{+}/Na^{+} exchanger (Baker, 1986). The affinity of the reconstituted exchanger for intracellular Ca^{2+} is low (>micromolar), suggesting that this system may only be important when the cytoplasmic free Ca^{2+} rises to levels greater than micromolar (Reeves & Sutko, 1979).

The plasma membrane $(Ca^{2+}\text{-}Mg^{2+})$-ATPase is the most important mechanism by which Ca^{2+} is removed from the cell. A similar enzyme, in the membrane of the sarcoplasmic reticulum of skeletal muscle, pumps Ca^{2+} ions against an electrochemical gradient into the lumen of the sarcoplasmic reticulum. The sarcoplasmic reticulum $(Ca^{2+}\text{-}Mg^{2+})$-ATPase is better characterized than that of the plasma membrane as it is the major protein component of this membrane system. A model has been proposed for the Ca^{2+} transport mechanism of the $(Ca^{2+}\text{-}Mg^{2+})$-ATPase (de Meis & Vianna, 1979; Inesi, 1985). According to the model, following the binding of two Ca^{2+} ions to high affinity sites exposed to the cytoplasm (E_1-$2Ca^{2+}$), the enzyme is phosphorylated by ATP (ATP~E_1-$2Ca^{2+}$). The phosphorylation of the enzyme is coupled to the trapping of the Ca^{2+} ions to give an ADP-sensitive phosphoenzyme (ADP~E_1P -$2Ca^{2+}$). A change in reactivity of the phosphoenzyme to an ADP-insensitive state (E_1P -$2Ca^{2+}$) is then associated with the translocation of the two Ca^{2+} ions across the membrane. This is followed by the release of the Ca^{2+} ions from the binding sites, now of low affinity (E_2P), into the lumen of the sarcoplasmic reticulum. The hydrolysis of the phosphoenzyme and release of the free phosphate is coupled to the regain of the original conformation of the enzyme (E_1), with the two high affinity Ca^{2+} binding sites exposed to the cytoplasmic side of the membrane. More detailed kinetic models for the reaction mechanism have been proposed involving additional intermediate states for the enzyme (Gould et. al., 1986). All stages of the proposed mechanism are reversible and many depend on the presence of Mg^{2+} ions.

Unlike the sarcoplasmic reticulum enzyme, the plasma membrane (Ca^{2+}-Mg^{2+})-ATPase is modulated by the calmodulin. The binding of Ca^{2+}-calmodulin complex to a site on the (Ca^{2+}-Mg^{2+})-ATPase stimulates the enzyme by increasing the affinity of the Ca^{2+} binding site and by increasing the turnover rate for the enzyme (Carafoli, 1987).

1.1.4.Intracellular Calcium Stores

Two intracellular organelles are known to store Ca^{2+} ions; the endoplasmic reticulum (or the sarcoplasmic reticulum in muscle) and mitochondria. Early investigations demonstrating the large Ca^{2+} storage capacity of mitochondria initially suggested these organelles were important in the regulation of cytoplasmic free Ca^{2+} concentration (Bygrave, 1978). However, later investigations indicated that the endoplasmic reticulum played a more important role in controlling cytoplasmic Ca^{2+} levels, and that mitochondrial Ca^{2+} uptake, via an electrophoretic uniport, served primarily to regulate mitochondrial matrix Ca^{2+} levels (Irvine, 1986). The affinity of the mitochondrial uniport system for Ca^{2+} (μM) suggested that these organelles could only regulate cytoplasmic Ca^{2+} levels in damaged cells, when the intracellular Ca^{2+} concentration increases to high levels (>μM; Irvine, 1986).

Ca^{2+} uptake into the endoplasmic and sarcoplasmic reticulum is mediated by a Ca^{2+}-ATPase system that exhibits similar properties to the plasma membrane (Ca^{2+}-Mg^{2+})-ATPase, however, it is not susceptible to modulation by calmodulin (Carafoli, 1987). Ca^{2+} release from the endoplasmic reticulum is stimulated on cell activation caused by agonist binding to plasma membrane receptors (Irvine, 1986). A model has been proposed for this stimulation. The agonist-receptor complex interacts with a closely associated membrane guanine nucleotide-binding protein (G-protein), termed Gp (p = phospholipid; Taylor & Merritt, 1986; Berridge, 1987; Gilman, 1987).The stimulated Gp binds guanine trisphosphate (GTP) and the G-protein dissociates into two complexes G_{α}-GTP and $G_{\beta\gamma}$. The G_{α}-GTP stimulates a closely associated enzyme, phosphatidylinositol phosphodiesterase, that stimulates the conversion of an intrinsic membrane lipid, phosphatidylinositol 4,5-bisphosphate, into inositol 1,4,5-trisphosphate and diacylglycerol (Taylor & Merritt, 1986; Berridge, 1987). The inositol 1,4,5-trisphosphate released into the cytoplasm acts as a second messenger

triggering the release of Ca^{2+} from the endoplasmic reticulum (Berridge & Irvine, 1984; Berridge, 1986). The pathway by which Ca^{2+} is released from the endoplasmic reticulum is not known, although it has been postulated to be via a channel mechanism rather than by enzymatic means (Smith, J.B. et. al., 1985; Irvine, 1986)

The release of Ca^{2+} ions from the skeletal muscle sarcoplasmic reticulum is essential for muscle contraction. The electrical signal required to stimulate this event is transmitted to the muscle surface membrane at the neuromuscular junction via acetylcholine receptors (Section 1.4.). The excitation is passed deep into the muscle fibre system via the transverse tubule (T-tubule) system. However, the precise mechanism coupling excitation to the Ca^{2+} release that induces muscle contraction has yet to be elucidated. It has been postulated that 1,4-dihydropyridine (1,4-DHP) receptors present in large amounts in the T-tubule system (Section 1.3.4.) are involved in conveying the excitation to the sarcoplasmic reticulum (Rios & Brum, 1987; Agnew, 1987). However, it has also been suggested that a second messenger system, possibly inositol 1,4,5-trisphosphate, may be involved in excitation-contraction coupling (Berridge, 1987). The pathway of Ca^{2+} release from the sarcoplasmic reticulum is not yet fully established. It has been suggested that a large molecular weight protein (Mr 400,000) in the sarcoplasmic reticulum membrane may act as a Ca^{2+} release channel that is activated either by membrane depolarization or by a second messenger system (Smith, J.S. et. al., 1985; Irvine, 1986).

1.1.5.Receptor-Operated and Voltage-Sensitive Calcium Channels

The major mechanism of Ca^{2+} influx into the cell from the extracellular space is via plasma membrane Ca^{2+} channels. Ca^{2+} channels can be broadly divided into two classes;

(a) receptor-operated Ca^{2+} channels and

(b) voltage-sensitive Ca^{2+} channels.

Receptor-operated channels are activated by the binding of an agonist to a specific receptor coupled to the channel protein and their function is independent of membrane potential. Voltage-sensitive Ca^{2+} channels are activated only by membrane

depolarization although their function can be modulated by various hormones and neurotransmitters (Section 1.2.6.). Both these channels, when in the open activated state, form aqueous pores in the membrane allowing a large influx of Ca^{2+} into the cell down its electrochemical gradient.

Receptor-operated channels have been postulated to be present in many cell types (Bolton, 1979), but, as voltage-sensitive channels are also subject to modulation by certain agonists, the existence of purely agonist-activated channels has been difficult to prove. However, more recent experiments on T-lymphocytes (Kuno & Gardner, 1987) and neutrophils (von Tscharner et.al., 1986), have demonstrated transmembrane Ca^{2+} currents that are not dependent on membrane depolarization and are purely agonist-activated. The mechanism by which the agonist-receptor complex activates the channel has not yet been established, although it has been postulated that channel activation is initiated by the stimulation of specific G-proteins (Hofmann, 1987). It is possible that the stimulated G_{α}-GTP complex interacts with the channel protein directly, or second messengers, such as inositol 1,4,5-trisphosphate, may be involved (Kuno & Gardner, 1987; Hofmann, 1987). Kuno & Gardner (1987) have suggested that following stimulation of T-lymphocyte cells the receptor-operated channel is activated by the second messenger inositol 1,4,5-trisphosphate that is also responsible for the release of Ca^{2+} from intracellular stores. In other cells, channels can be activated directly by a rise in cytoplasmic Ca^{2+} released from intracellular stores and not via a direct second messenger interaction (von Tscharner et.al., 1986).

Studies on smooth muscle cells have produced further evidence for receptor-operated channels (Benham & Tsien, 1987). The currents passed by these channels were not dependent on membrane depolarization and were specifically activated by ATP without the involvement of a second messenger (Benham & Tsien, 1987). In this and other studies, receptor-operated channels have been demonstrated to have different ion permeation properties to the voltage-sensitive Ca^{2+} channel (Section 1.2.3.). For example, receptor-operated channels have been shown to be non-selective allowing the passage of other cations in addition to Ca^{2+} (Benham & Tsien, 1987). The mechanisms by which receptor-operated channels are activated have yet to be fully elucidated.

However, these channels are present in many different cell types and are important in cell function particularly in non-excitable cells (Neher, 1987).

Voltage-sensitive Ca^{2+} channels are present in every excitable cell and are important in many cellular functions such as excitation-contraction coupling, secretion and cell proliferation. Voltage-sensitive Ca^{2+} channels are far better characterized than receptor-operated Ca^{2+} channels, in terms of structure, function, modulation and localization. The subsequent parts of this thesis refer only to voltage-sensitive Ca^{2+} channels.

1.2. Calcium Channel Electrophysiology

1.2.1. Introduction

The first action potentials measured due to the flow of Ca^{2+} ions rather than Na^{+} ions, were first identified by Fatt and Ginsborg (1958) working with crayfish muscle. The flow of Ca^{2+} ions generating such action potentials (~million ions per sec) has since been demonstrated to be too fast to be accounted for by a carrier system (~100,000 ions per sec) (Hille, 1984; Tsien, 1983). Hence, it was postulated that the passage of Ca^{2+} ions across the plasma membrane was via a pore or channel forming membrane-bound protein, down the electrochemical gradient that is established for Ca^{2+} across the cell membrane (Hille, 1984; Tsien, 1983). The Ca^{2+} channel is now known to be present in every excitable cell and is essential for a variety of cellular functions, including contraction and secretion (Hagiwara & Byerly, 1983; Tsien, 1983).

Initial investigations measuring Ca^{2+} channel currents were hampered by the co-existence of other channel types in excitable membranes (Reuter, 1979). The currents passed by these other channels, carried by ions such as K^{+} and Na^{+}, are invariably more predominant than Ca+ currents. For this reason experiments have been performed in the presence of tetrodotoxin (TTX), a Na^{+} channel blocker, and tetraethylammonium (TEA), Cs^{2+} or Ba^{2+}, K^{+} channel blockers. Most of the early electrophysiological studies on Ca^{2+} channels were carried out on the large ganglion cells from gastropod molluscs using the whole cell voltage clamp technique. In this technique a glass pipette is pressed against the cell membrane and gentle suction is applied which causes the cell membrane to rupture. This provides a route for current to be passed into the cell and allows the

recording of potential. Initially, single Ca^{2+} channel currents could only be estimated indirectly from whole cell voltage clamp studies of a net Ca^{2+} current passing through a large number of channels (Kostyuk & Krishtal, 1977; Krishtal et. al., 1981). However, the introduction of the patch-clamp technique has since enabled single channel currents to be directly recorded and so advance the understanding of channel kinetics. This technique, pioneered by Neher and Sakmann (1976) initially for the recording of Na^{+} channels, has since been applied to other membrane channels including the Ca^{2+} channel (Neher & Sakmann, 1983; Hagiwara & Byerly, 1981).

1.2.2.The Patch-Clamp Technique

The patch-clamp technique applies the same principle as the voltage clamp method, but allows recordings from small membrane patches. A fine glass pipette is pressed against the cell membrane to form a high resistance seal between the glass pipette and the membrane. The seal formed in this manner isolates the small patch of membrane electrically and chemically from the rest of the cell membrane, so reducing the electrical noise such that single channel conductances as low as 1-2 pAmps can be recorded (Neher & Sakmann, 1976; Hamill et. al., 1981; Sakmann & Neher, 1983). There are four possible cell membrane-pipette configurations (Ruff, 1986). If the cell is large, current flow can be measured from the patch isolated on the intact cell, so called on-cell recording, or if suction is applied the membrane patch is ruptured and whole-cell currents can be measured from small cells (Fenwick et. al., 1982; Ruff, 1986). The on-cell patch can be pulled off the cell to form an inside-out patch, where the solutions on either side of the membrane can be varied. In addition, the whole-cell patch can be pulled from the cell to result in an outside-out patch (Hamill et.al., 1981; Fenwick et. al., 1982). Channel conductance is recorded from the membrane patch after applying controlled voltage steps to the pipette. This allows the voltage dependence of single channel opening and closure (gating) to be investigated by measuring current flow at different applied voltages.

The number of membrane-pipette configurations makes the patch-clamp technique extremely versatile and it has been used, in conjunction with the whole cell voltage

clamp technique, to investigate the effects of various Ca^{2+} channel modulating agents on Ca^{2+} channel behaviour (Section 1.2.3.). The existence of different Ca^{2+} channel subtypes exhibiting different kinetics and voltage dependencies, has been widely studied over the past few years using this technique (Section 1.2.5.; McCleskey et. al., 1986; Miller, 1987). In addition, Ca^{2+} channels from cardiac sarcolemmal vesicles or brain membranes can be incorporated into planar lipid bilayers and studied using the patch-clamp technique (Erlich et. al., 1986; Rosenberg et. al., 1986; Nelson et. al., 1984). Furthermore, purified Ca^{2+} channel protein subunits can be reconstituted into planar lipid bilayers or phospholipid vesicles (Flockerzi, et. al. 1986). Such reconstitution experiments will allow those subunits which are essential for Ca^{2+} channel function and those subunits which have a regulatory role to be distinguished.

1.2.3. Calcium Channel Activation and Open Channel Properties.

Channel activation is defined as the opening of the channel in response to a positive voltage step. Voltage-clamp recordings of whole cells and single channel recordings on membrane patches have been used extensively to investigate Ca^{2+} channel behaviour both in the absence and presence of modulating agents (Tsien, 1983; Reuter, 1983; Stanfield, 1986). These investigations indicate that as the membrane is depolarized, the probability that an individual channel is open (p_o) gradually increases, as does the mean open time for a particular channel (Fenwick et.al., 1982). Increasing the extent of depolarization causes the rate of activation to rise and p_o increases. The value of p_o is never equal to 1.0 as all channels spend some time in the closed state and some may not open at all during a depolarization, as has been established from single channel recordings (Hess et.al., 1984). The time course for channel activation varies with both cell type and channel subtype (Section 1.3.5.).

Single channel recordings have shown that on depolarization there is a slight delay before the channel opens and Ca^{2+} current flow can be recorded (Hagiwara & Ohmori, 1983). This suggests that the kinetics of Ca^{2+} channel opening are too complex to be explained by the first order process initially proposed for the Na^{+} channel by Hodgkin and Huxley (1952). The first order process for channel activation is defined in terms of

one closed channel state and one open channel state:

$$\text{Closed} \underset{k^{-1}}{\overset{k^{1}}{<\!\!\longrightarrow\!\!>}} \text{Open} \qquad \text{Equation 1}$$

k^1=rate constant for opening

k^{-1}=rate constant for closing

Ca^{2+} current measurements in anterior pituitary cells indicated that the channel conducting Ca^{2+} was activated slowly but once in the activated state it flickered rapidly between the open and closed state. This led to the proposal of a three state model for Ca^{2+} channel kinetics, with two closed states and one open state (Hagiwara & Ohmori, 1983). This can be summarized by:

$$C1 \underset{k^{-1}}{\overset{k^{1}}{<\!\!\longrightarrow\!\!>}} C2 \underset{k^{-2}}{\overset{k^{2}}{<\!\!\longrightarrow\!\!>}} O \underset{k^{-3}}{\overset{k^{3}}{<\!\!\longrightarrow\!\!>}} C^* \qquad \text{Equation 2}$$

C1 and C2 are closed states,

O is the open state,

C* is the inactivated state (channel unavailable for opening)

k^1, k^2, k^3 = rate constants for opening

k^{-1}, k^{-2}, k^{-3} = rate constant for closing,

However, it has been postulated that a more complicated model involving several different closed channel states may be required to fully explain Ca^{2+} channel kinetics (Hagiwara & Ohmori, 1983).

The rapid flickering of the activated channel between open and closed states is often termed "gating". Whole cell and single channel recording on cardiac cells have shown that once in the activated state the Ca^{2+} channel exhibits different modes of gating behaviour (Hess et. al., 1984). The most common mode of Ca^{2+} channel behaviour, in the absence of any modulating agents, is the rapid flickering between open and closed states (Mode 1). In addition, two further modes can be detected. At certain times the channel appears to be unavailable for opening (Mode 0), but also the channel can undergo periods of prolonged openings, with very short closed times (Mode 2). In the

absence of modulating agents, the channel spends a certain amount of time in each of these modes, although Mode 0 and Mode 2 behaviour are difficult to detect (Hess et. al., 1984)

The modal nature of Ca^{2+} channel gating mechanisms has provided an explanation for the action of various Ca^{2+} channel agonists and antagonists, drugs that modulate the function of Ca^{2+} channels (discussed in Section 1.3.). In the presence of BAY K 8644, a Ca^{2+} channel agonist, the channel exhibits Mode 2 behaviour, hence, the overall Ca^{2+} conductance is increased. However nitrendipine, a Ca^{2+} channel antagonist, causes the channel to remain closed for relatively long periods of time (Mode 0), hence, greatly reducing the Ca^{2+} conductance (Hess et. al., 1984).

The change in membrane potential on depolarization is thought to cause the movement of charged components within the channel protein, so producing a conformational change that opens the channel (Hille, 1984). The movement of these "gating charges" or "voltage sensors" produces a small current, termed "gating current", that can be recorded slightly preceeding the main ionic current of the channel, under certain experimental conditions (Kostyuk et. al., 1981). Many models have been proposed in an attempt to explain Ca^{2+} channel gating, but the problem will probably remain unresolved until more information on Ca^{2+} channel protein structure is available.

In the open state, all channels exert a certain amount of selectivity on the ions that are allowed to permeate the aqueous pore that forms the open channel (Hille, 1984; Eisenman & Dani, 1987). A particularly narrow region in the channel structure is thought to act as an ion selective pore, only allowing the passage of ions of a certain molecular size. This region of the channel is referred to as the "selectivity filter". However, selectivity in terms of molecular size alone is insufficient to explain this phenomenom, as different ionic species, small enough to enter the channel pore still exhibit different permeabilities through the channel (Eisenman & Dani, 1987). The narrow region of the channel pore brings the permeant ion in close contact with ion specific binding sites on the channel protein enabling ion/protein interaction. The extent of interaction between the ion and the protein determines the permeability of the ion. The situation is further complicated as, in "multi-ion channels" such as the Na^+, K^+, and Ca^{2+} channel, more

than one permeating ion is in the channel at a given time, so there is interaction not only between the ion and the channel protein but also between other permeating ions in the channel (Hille & Schwartz, 1978; Eisenman & Dani, 1987).

In the presence of Ca^{2+} ions the Ca^{2+} channel is highly selective for Ca^{2+} over any other ionic species that may be present. However, in the absence of Ca^{2+} ions the channel becomes relatively non-selective, allowing the passage of certain divalent (Ba^{2+} and Sr^{2+}), and monovalent (Na^{+}) species (Hess & Tsien, 1984). Hess and Tsien (1984) found that the large Na^{+} current through cardiac Ca^{2+} channels, recorded in the absence of external Ca^{2+}, was blocked by over 50% on the application of only 1.3μM Ca^{2+}. Similar block of monovalent ion flux through skeletal muscle Ca^{2+} channels has also been found with the addition of 1μM Ca^{2+} (Almers et. al., 1984). To explain these observations it has been postulated that Ca^{2+} specific binding sites are located within the channel pore (Hess & Tsien, 1984; Almers et. al., 1984; Tsien et. al., 1987). This led to the proposal of a model for the Ca^{2+} channel that is similar to that originally postulated by Urban et.al. (1980) for the pore formed by the antibiotic gramicidin A when incorporated into lipid bilayers.

The proposed model for the Ca^{2+} channel involves two binding sites within the Ca^{2+} channel pore which have a particular high affinity for Ca^{2+} over other ionic species. According to the hypothesis, at physiological levels of Ca^{2+}, at least one of these high affinity binding sites will be occupied by Ca^{2+}. If the outer site is empty any monovalent species binding weakly to this site will be repelled back out of the pore due to the presence of the Ca^{2+} ion bound within the channel pore. However, if a second Ca^{2+} ion were to bind to the outer site, with high affinity, this will repel the Ca^{2+} bound to the inner binding site into the intracellular space (Hess & Tsien, 1984). It has been proposed that the energy required for these ionic transitions could be derived from the electrochemical gradient for Ca^{2+} across the cell membrane. It has also been suggested that this model could also explain the reduction in selectivity of the Ca^{2+} channel found in the absence of Ca^{2+} (Hess & Tsien, 1984). Under these conditions Ca^{2+} binding sites would be empty, hence, the channel would become relatively non-selective allowing the passage of monovalent cations such as Na^{+} (Hess & Tsien, 1984).

Single channel recordings of Ca^{2+} channel currents appear to substantiate this two site model. Lansman et.al., (1986) measured the blocking effect of increasing concentrations of Ca^{2+} on Li^{+} current through single Ca^{2+} channels. The Ca^{2+} channel agonist, BAY K 8644 (Section 1.3.), was present to maintain the channels in the open configuration long enough to measure open channel block. Sustained, uninterrupted Li^{+} conductances could be measured in the absence of Ca^{2+}, however, in the presence of micromolar concentrations of Ca^{2+}, the channel openings were reduced to small bursting events. The rapid channel closures were postulated to correspond to blockade by a single Ca^{2+} ion bound to a channel binding site. The channel open times also decreased with increasing Ca^{2+} concentration (Lansman et. al., 1986).

Although the conductivity of Ca^{2+} channels varies with channel subtype, certain divalent cations, namely Ba^{2+} and Sr^{2+}, are in general more permeant than Ca^{2+} (Hagiwara & Ohmori, 1982). According to the above model, Ca^{2+} ions passing through the channel have a high affinity for the internal binding sites and so will be impeded, reducing the overall Ca^{2+} conductance. The binding affinities of Ba^{2+} and Sr^{2+} for these sites are proposed to be lower, hence, these ions will not be retarded to such an extent on passing through the channel. Other divalent cations such as Cd^{2+} and Co^{2+} block Ca^{2+} channels by reducing the channel open times, with no effect on the single channel conductance (Lansman et.al., 1986). The permeant and blocking divalent cations appear to compete for the channel pore binding sites and this results in both ion/channel and ion/ion interactions, within the pore, that both influence permeability. There is no direct evidence, to date, for the presence of Ca^{2+} specific binding sites within the channel pore, however, more information on the channel protein structure may serve to substantiate the two-binding site model.

1.2.4 Inactivation of Calcium Channels.

The term "inactivation" is used to describe the decline in Ca^{2+} current with prolonged membrane depolarization. "Inactivation" specifically refers to the mechanisms that produce a direct decrease in Ca^{2+} permeability, since a reduction in Ca^{2+} current can also occur due to a depletion in extracellular Ca^{2+}. In general, the rate of Ca^{2+} channel

inactivation is slower than activation, and in some preparations inactivation is so slight as to be almost absent, even after prolonged depolarizations. For example, the decline in Ca^{2+} current in frog skeletal muscle has been demonstrated to be due to Ca^{2+} depletion in the transverse tubule system rather than due to an inactivation of Ca^{2+} channels (Almers et. al., 1981).

Two different inactivation mechanisms have been postulated:

(1) voltage-dependent inactivation, and

(2) Ca^{2+}-dependent ($[Ca^{2+}]_i$-dependent) inactivation due to an increase in intracellular Ca^{2+}.

In certain preparations both mechanisms appear to be important (cardiac cells, Bean, 1985), whereas others exhibit only voltage-dependent (Type 1 channels Neanthes egg cells, Fox et. al., 1981), or $[Ca^{2+}]_i$-dependent inactivation (Aplysia bag neurones, Eckert & Tillotson, 1981). The methods for measuring $[Ca^{2+}]_i$-dependent inactivation have often been indirect, either by replacing external Ca^{2+} with Ba^{2+} or Sr^{2+}, or by injecting the cells with ethylenediaminetetraacetic acid (EDTA) (Eckert & Tillotson, 1981). Consequently, it has been difficult to ascertain whether inactivation was solely $[Ca^{2+}]_i$-dependent, or whether there was always a voltage-dependent component of inactivation. More recent experiments on both cardiac and neuronal cells have further complicated the situation in that the different subtypes of Ca^{2+} channels present in these cells exhibit different rates of inactivation (Bean, 1985; Nowycky et. al., 1985; Fox & Tsien, 1985). The mechanism of $[Ca^{2+}]_i$-dependent inactivation has not yet been elucidated, although it is likely to involve the binding of Ca^{2+} to a site, either within in the channel pore, or on the cytoplasmic side of the channel protein.

Certain Ca^{2+} channel preparations exhibit inactivation irrespective of whether the permeant ion is Ca^{2+}, Ba^{2+} or Sr^{2+} (Type 1 channels, Neanthes egg cells, Fox, 1981). The rate of inactivation is not affected by the internal Ca^{2+} concentration, but rises with increasing membrane depolarization. This is voltage-dependent inactivation and can be demonstrated to a certain extent in most cell types (Fox, 1981; Bean, 1985).

The kinetics of Ca^{2+} channel activation and inactivation appear to be more difficult to assess than those of the Na^+ or K^+ channels. The situation is further complicated in that

single channel recordings have demonstrated the presence of different Ca^{2+} channel subtypes that co-exist in the membranes of most excitable cells (Section 1.2.5.). These subtypes differ in terms of their voltage range of activation, rate of inactivation, selectivity and single channel conductance, and pharmacology.

1.2.5.Different Types of Calcium Channels.

Hagiwara et. al. (1975) found from current recordings from starfish eggs that the Ca^{2+} current consisted of two components, type 1 and type 2 currents. The two current types were initiated following the depolarization of the cell membrane from different holding potentials. Whole cell and single channel recordings have since shown that these two Ca^{2+} current components are passed by different channel subtypes that can be distinguished by differences in; the voltage range of activation, time course of inactivation, selectivity, single channel conductance and sensitivity to different Ca^{2+} channel agonists and antagonists (McCleskey et. al., 1986). Further investigations on a variety of cell types have demonstrated that almost all cell types bearing Ca^{2+} channels possess more than one type of Ca^{2+} channel (McCleskey et. al., 1986). Only few cell types, such as adrenal chromaffin cells, possess only a single type of Ca^{2+} channel (Fenwick et. al., 1982).

At present, the most widely studied Ca^{2+} channel subtype is that responsible for the slowly inactivating component of the Ca^{2+} current recorded from a variety of cell types. The channel responsible for this slowly inactivating Ca^{2+} component, termed I_{slow} by Bean (1985), is the type II channel first described by Hagiwara et. al. (1975), the "High Voltage-Activated" (HVA) channel recorded by Carbone & Lux (1984) from dorsal root ganglion cells, and the L-type (Long-lasting Ca^{2+} current) channel, also recorded from dorsal root ganglion cells, by Nowycky et. al. (1985). The properties of the L-type channel from different cell types are very similar. The channel is activated by depolarizations to positive potentials from a holding potential of -40mV (Table 1). The L-type channel inactivates very slowly, in the order of hundreds of milliseconds, and is the only Ca^{2+} channel type susceptible to modulation by the different classes of Ca^{2+} channel antagonists; the 1,4-dihydropyridines (1,4-DHPs), phenylalkylamines and

benzothiazepines (Table 1; Section 1.3.; Miller, 1987; Nowycky et. al., 1985; McCleskey et. al., 1986; Reuter et. al., 1986). The open times of L-type channels in both cardiac and dorsal root ganglion cell preparations are increased in the presence of the 1,4-DHP agonist, BAY K 8644 (Bean, 1985; Nowycky et. al., 1985; Nilius et. al., 1985), however, the antagonist action of nitrendipine appears to vary with both tissue used and membrane potential (Bean, 1985; McCleskey et. al., 1986). For example, nitrendipine blockade of L-type channels was found to be more potent in cardiac muscle cells than in neuronal cells (Hess et. al., 1984; Lee & Tsien, 1983; McCleskey et. al., 1986).

Recent whole cell recordings on a variety of cell preparations has demonstrated a second Ca^{2+} current component that differs in terms of voltage range of activation and rate of inactivation (Bean, 1985; Fox et. al., 1984; Nilius et. al., 1985). Single channel recording methods enabled this second Ca^{2+} current component to be distinguished from the L-type current component. The channel responsible for this Ca^{2+} current, termed I_{fast} by Bean (1985), was activated by depolarizations from a holding potential of -100mV to less negative potentials, and was found to inactivate very quickly (Table 1; Bean, 1985; Nilius et. al., 1985). This rapidly inactivating channel was referred to as the Type I channel by Hagiwara et. al. (1975), and was termed the "Low Voltage-Activated" (LVA) by Carbone & Lux (1984) in their experiments on dorsal root ganglion cells. Nowycky et. al. (1985), also working on dorsal root ganglion cell preparations have termed this channel the T-type channel (Transient Ca^{2+} current). These channels have also been demonstrated in cardiac preparations (Bean 1985; Nilius et. al., 1985). Investigations have shown that these T-type channels are insensitive to 1,4-DHP agonists and antagonists in both neuronal and cardiac preparations (Nowycky et. al., 1985; Nilius et. al., 1985).

There is some evidence for a third type of Ca^{2+} channel in dorsal root ganglion cell preparations. This channel is similar to the L-type channel in terms of voltage range of activation, but its rate of inactivation is similar to that of the T-type channel, for this reason it has been termed the N-type channel (neither L-type nor T-type; Nowycky et. al., 1985). The presence of N-type Ca^{2+} channels has been further substantiated following the characterization of a toxin from the venom of the fish-hunting snail *Conus*

geographus, ω-VIA *Conus geographus* toxin (ω-CgTx; Olivera et. al., 1984). ω-CgTx has been shown to inhibit the release of neurotransmitter from the presynaptic nerve terminal by the specific block of voltage-sensitive Ca^{2+} channels in the presynaptic membrane (McCleskey et. al., 1987). It has been shown that ω-CgTx distinguishes between different channel subtypes. The toxin blocks N-type and L-type channels in nerve cells, but has no apparent effect on the T-type Ca^{2+} channels (McCleskey et. al., 1987). ω-CgTx was shown to block Ca^{2+} currents of dorsal root ganglion cells to a greater extent than the 1,4-DHP antagonists (McCleskey et. al., 1987; Miller, 1987). This suggests that the toxin blocks other types of Ca^{2+} channels in dorsal root ganglion cells that are 1,4-DHP insensitive. It has been proposed that these ω-CgTx-sensitive, 1,4-DHP-insensitive channels are the N-type Ca^{2+} channels. In addition, block by ω-CgTx has been shown to produce a greater inhibition of transmitter release than block by 1,4-DHP antagonists (Miller, 1987). This suggests that the N-type Ca^{2+} channels are probably primarily responsible for the influx of Ca^{2+} into the presynaptic terminal that induces transmitter release.

Interestingly, the blocking action of the ω-CgTx on the L-type channel is tissue-specific. As previously mentioned, ω-CgTx has been shown to block L-type channels, in addition to N-type channels, in dorsal root ganglion cells, (McCleskey et. al., 1986). In pharmacological experiments, ω-CgTx binds to high affinity receptors in chick brain membrane preparations (Cruz et. al., 1987). However, pharmacological experiments have indicated that there are no high affinity binding sites for the toxin in chick skeletal muscle membrane preparations (Cruz et. al., 1987). In addition, McCleskey et. al. (1987) have shown that the toxin does not block the L-type current component of skeletal muscle. This indicates that the L-type channel is different in these two tissues, at least in terms of ω-CgTx sensitivity, and has led to the suggestion of the existence of four types of Ca^{2+} channel T, N, Ln (neuronal, ω-CgTx-sensitive) and Lm (muscle, ω-CgTx-insensitive) (Table 1; Cruz et. al., 1987; Miller, 1987).

Table 1 summarizes the different electrophysiological and pharmacological properties that have so far been established for the different types of Ca^{2+} channels present in dorsal root ganglion cells. It is noteworthy that nerve cells are the only preparations

Property	Channel Subtype			Ref.
	T	N	L	
Activation range	+ve to -70mV	+ve to -10mV	+ve to -10mV	(1)
Inactivation	20-50msec	20-50msec	>100msec	(1)
Single channel conductance	8-10pS	13pS	25pS	(1)
Cd^{2+} block	weak	strong	strong	(2)
ω-CgTx block	weak	strong	strong*	(1)(2) (3)
1,4-DHP modulation	no	no	yes	(1)(2) (3)

Table 1

Different Properties of Voltage-Sensitive Calcium Channel Subtypes

The pharmacological and electrophysiological properties of different Ca^{2+} channel subtypes in dorsal root ganglion cells.

*ω-CgTx block not detected in L-type channels from skeletal muscle

References:

(1) McCleskey et. al. 1986; (2) Miller 1987; (3) Cruz et. al. 1987

where the N-type Ca^{2+} channels have been demonstrated (Nowycky et. al., 1985). In most other cell types only two Ca^{2+} channel subtypes have been designated (Miller, 1987) and these have the general properties of the L- and T-type channels as described in nerve preparations.

Two recent reports have studied in detail the kinetics and selectivity of the Low Voltage-Activated (LVA) or T-type channel from dorsal root ganglion cell preparations, using cell-attached and outside-out excised patches (Carbone & Lux, 1987a; Carbone & Lux, 1987b). Under these conditions the T-type Ca^{2+} current component could be distinguished from the current component of the L-type channel. Carbone and Lux (1987a,b) demonstrated that the channel kinetics were similar to those previously found for this channel type and that the inactivation of the T-type channels is totally voltage dependent. $[Ca^{2+}]_i$-dependent inactivation was only detected for the L-type channel (Carbone & Lux 1987a,b). In these studies they did not find any evidence for the N-type Ca^{2+} channel in the dorsal root ganglion cell preparation that has been found by other workers (Nowycky et. al., 1985). Hence, Carbone and Lux (1987a,b) suggest that these cells have only two types of Ca^{2+} channels as demonstrated previously in many other cell types (Miller, 1987). Clearly, when more structural information is available for the different channel types it will be of interest to assess any structural differences occuring in the channel proteins and to relate these to differences in channel function.

1.2.6. Modulation of Calcium Channels

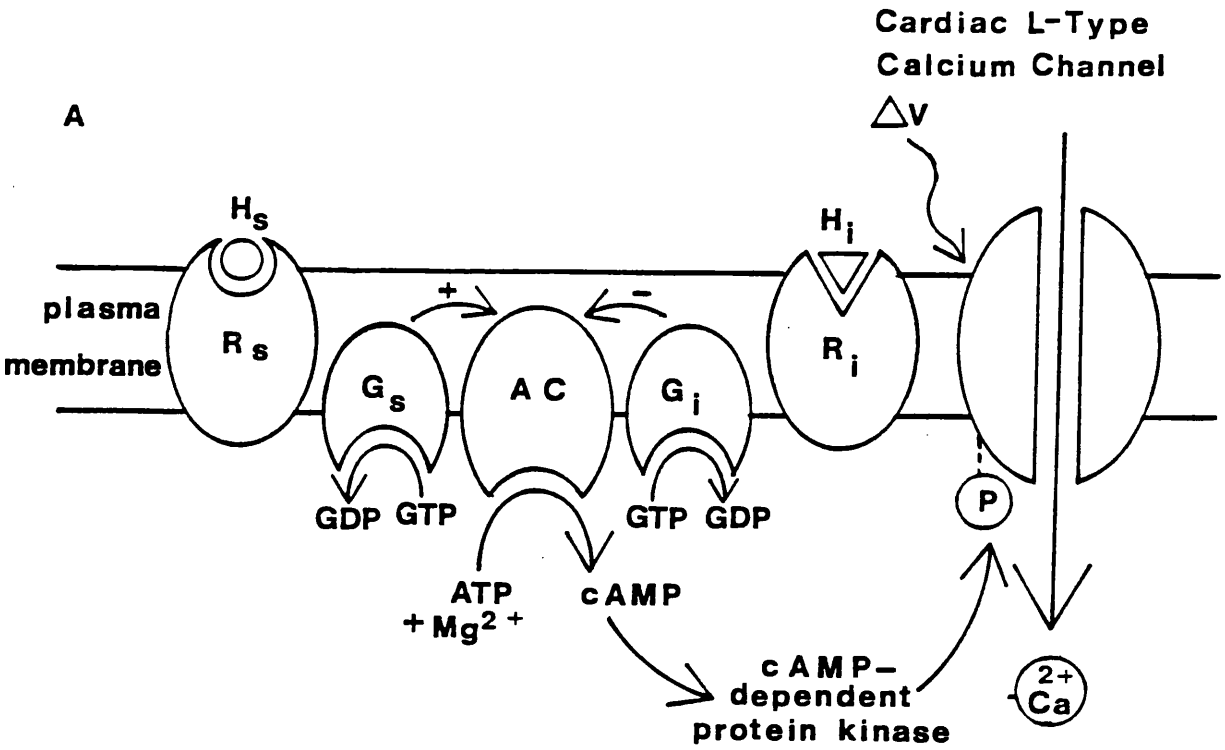
L-type voltage-sensitive Ca^{2+} channels once activated by membrane depolarization are subject to modulation by neurotransmitters and hormones (Reuter, 1983; Hofmann et. al., 1987). The catecholamines have been shown to increase Ca^{2+} current through cardiac L-type channels by increasing the probability of channel opening (p_o) (Reuter 1983). This same effect occurs following the injection of the catalytic subunit of cAMP dependent kinase into cardiac cells (Osterrieder et. al., 1982), suggesting that the phosphorylation of the channel protein or an associated protein causes the increase in p_o (Reuter, 1983; Reuter et. al., 1986). Figure 2A shows the postulated sequence of events resulting in channel phosphorylation. The binding of the catecholamine agonist to the β-

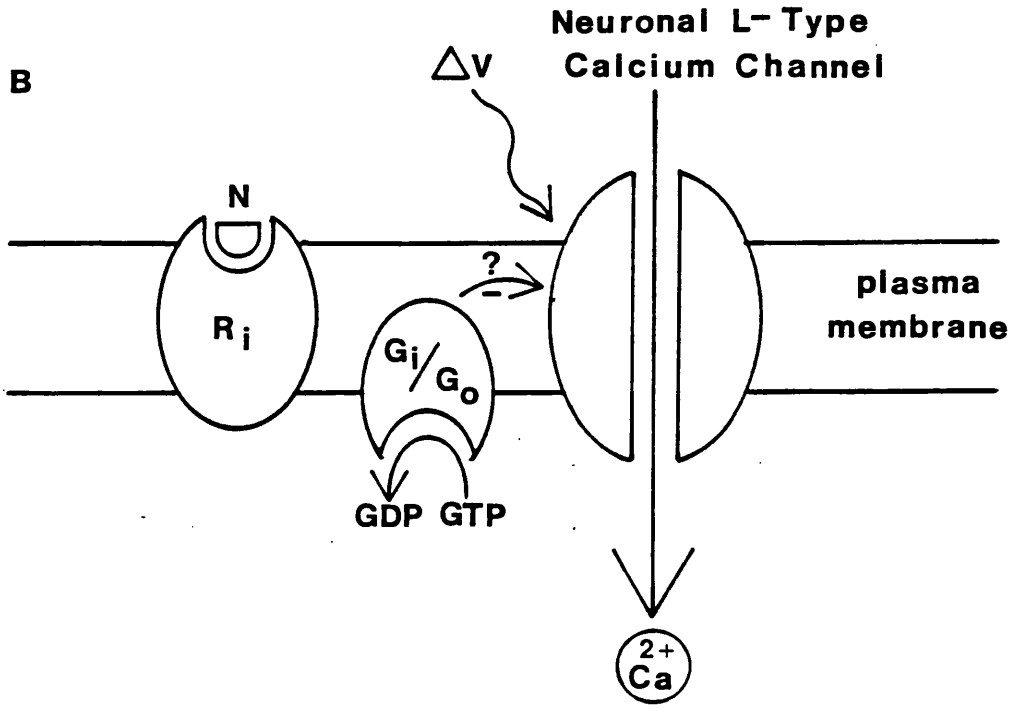
Figure 2

Modulation of L-Type Calcium Channels.

(A) Phosphorylation of cardiac L-type Ca^{2+} channels by cAMP mediated system. The binding of a catecholamine agonist (H_s) to the β-adrenoreceptor (R_s) activates the catalytic subunit of adenylate cyclase (AC) by a coupled stimulatory G-protein (G_s). The increased cAMP levels cause the phosphorylation (P) of the channel protein either directly or indirectly by cAMP-dependent protein kinases. Binding of agonists (H_i) to cardiac muscarinic receptors (R_i) reduces the adenylate cyclase activity through the inhibitory G-protein (G_i) resulting in channel dephosphorylation.

(B) Modulation of neuronal Ca^{2+} channels. Neurotransmitters (N) such as GABA acting via the α_2-receptor (R_i) inhibit neuronal Ca^{2+} channel currents through G-proteins, G_i or G_o.


Cardiac L-Type
Calcium Channel
A
ΔV
H_s
H_i
plasma
membrane
R_s
R_i
+
-
G_s
AC
G_i
GDP GTP
GTP GDP
P
ATP
$+Mg^{2+}$
cAMP
cAMP-
dependent
protein kinase
Ca^{2+}


Neuronal L-Type
Calcium Channel
B
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N
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R_i
G_i/G_o
plasma
membrane
GDP GTP
Ca^{2+}

adrenoreceptor causes an associated G-protein, G_s (s=stimulatory), to bind GTP. This G_s-GTP complex activates adenylate cyclase catalyzing the production of cAMP that stimulates the eventual phosphorylation of the channel protein via cAMP-dependent protein kinases. Stimulation of cardiac muscarinic receptors by agonists reduces the activity of adenylate cyclase through the inhibitory G-protein G_i (Fig. 2A). The resultant decrease in cAMP levels causes the reduction of the cardiac Ca^{2+} current by a reduction in p_o through channel dephosphorylation (Hofmann et. al., 1987).

Noradrenaline, acting via the α2-receptor, inhibits neuronal Ca^{2+} currents (Reuter, 1983). Indeed, other neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin and dopamine have been shown to have an inhibitory effect on Ca^{2+} channels (Hofmann et. al., 1987; Hescheler et. al., 1987). The mechanism of action of these agents is as yet unknown. However, it has been postulated that inhibitory G-proteins (G_i) may be involved (Fig. 2B; Holz et. al., 1986; Miller, 1988). A recent report has suggested that G_i or G_o may be involved in the modulation of Ca^{2+} channels by these agents (Scott & Dolphin, 1987). Protein kinase C, an enzyme activated by diacylglycerol a product of phosphatidylinositol 4,5-biphosphate breakdown (Section 1.1.4.), has also been shown to inhibit Ca^{2+} currents in a neuronal cell line by a mechanism that has yet to be established (Messing et. al., 1986; Rane & Dunlap, 1986; Miller, 1988). It is clear from this evidence that neuronal Ca^{2+} channels are subject to both stimulatory and inhibitory modulation by a variety of agents, reflecting the importance of Ca^{2+} channel control in these tissues. More work is required in this area before these different modulatory mechanisms are fully elucidated.

1.3. Molecular Characteristics of the 1,4-Dihydropyridine Sensitive Calcium Channel

1.3.1. Introduction

Fleckenstein, was the first to recognize the therapeutic importance of certain cardiodepressant and vasodilator drugs. He introduced the principle of Ca^{2+} antagonism, and classified the range of drugs now known as the Ca^{2+} channel antagonists or blockers (Fleckenstein, 1977). Ca^{2+} antagonists are now extensively employed as therapeutic agents for the treatment of various cardiovascular diseases including hypertension,

angina and cardiac arrhythmias. This clinical importance has led to the development of a diverse range of organic compounds that act as Ca^{2+} channel antagonists. Many cellular responses dependent on the influx of extracellular Ca^{2+} are inhibited by the application of these drugs in the nanomolar concentration range (Fleckenstein, 1977). Ca^{2+} channel drugs can be subdivided into four major chemical classes (Fig. 3), the most potent and selective being the 1,4-DHPs (e.g. nifedipine, nitrendipine, PN 200-110; Fig. 3A). Other classes include the phenylalkylamines (e.g. verapamil, D600, D888; Fig. 3B), the benzothiazepines (e.g. diltiazem; Fig. 3C), and the diphenylalkylamines (e.g. flunarizine; Fig. 3D) (Triggle & Janis, 1987).

It has been established that Ca^{2+} channel antagonists exhibit different potencies in different tissues. The 1,4-DHPs are more potent in smooth muscle tissue than in cardiac and skeletal muscle (Henry, 1980). This tissue selectivity can be explained in terms of the different subtypes of Ca^{2+} channels (Section 1.2.5.), only one of which appears to be 1,4-DHP sensitive, and the receptor-ligand interaction which can be dependent on the state of the channel (Section 1.3.3).

Pharmacological investigations using radiolabelled Ca^{2+} channel antagonists have demonstrated the high affinity binding of these drugs to membrane-bound receptors, associated with voltage-sensitive Ca^{2+} channels, in various tissues such as heart, brain, smooth and skeletal muscle. Table 2 shows the results from a few representative studies. Furthermore, competition binding studies involving radiolabelled derivatives, representing the various classes of Ca^{2+} antagonists, have indicated that there are three different allosterically linked high affinity binding sites, each specific for one class of drug (Fig. 4; Ferry & Glossmann, 1982; Glossmann et. al., 1984). The high affinity of the radiolabelled antagonists, in particular the 1,4-DHPs, for specific receptors associated with the channel protein has resulted in their extensive utilization as biochemical probes in studies to characterize the molecular structure of the receptor. These biochemical investigations have led to a recent accumulation of structural data on the receptor protein isolated from various tissues (Section 1.3.4.).

Figure 3

Structures of Calcium Channel Drugs.

(A) 1,4-Dihydropyridines.

(1) Nitrendipine, (2) PN200-110, (3) Nifedipine, (4) BAY K 8644, (5) Azidopine

(B) Phenylalkylamines.

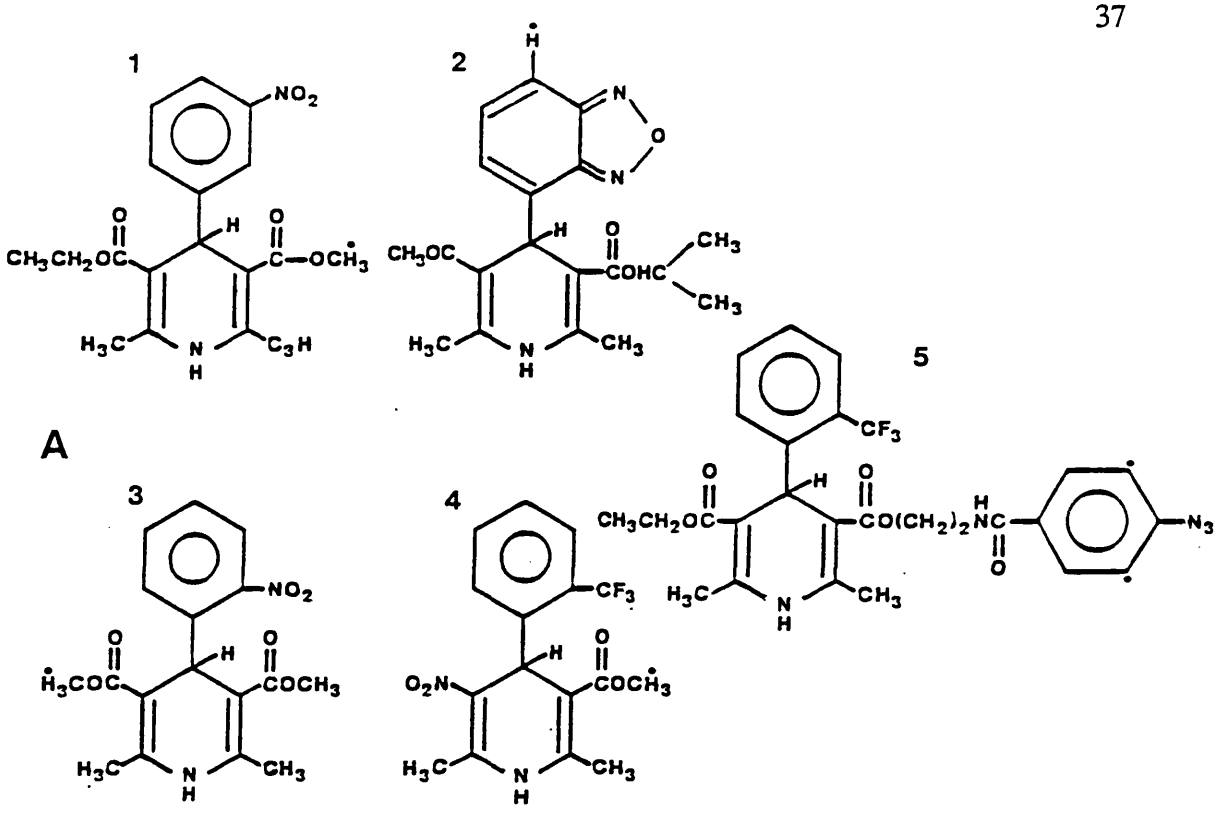
(1) Verapamil, (2) D888 (desmethoxyverapamil)

(C) Benzothiazepines.

Diltiazem

(D) Diphenylalyklamines.

Flunarizine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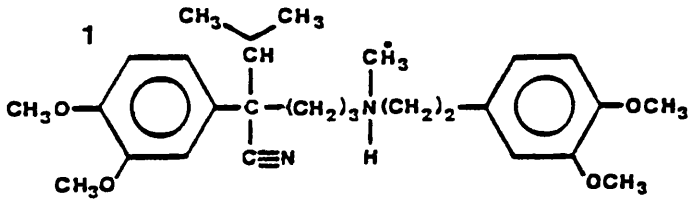
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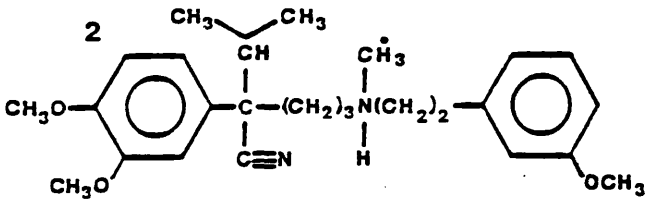
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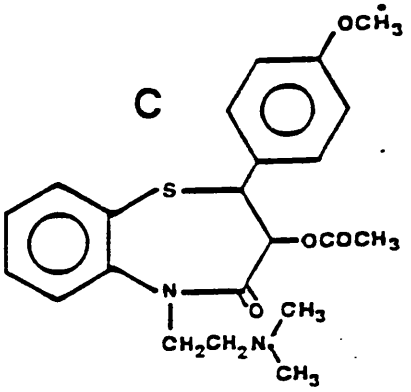
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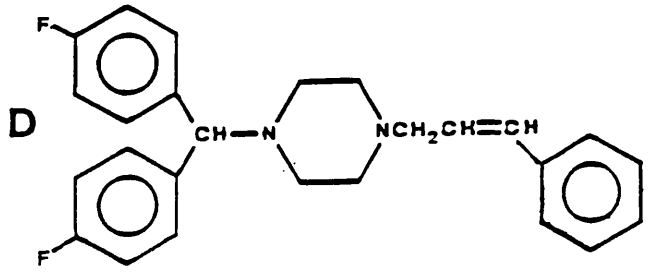

D

Preparation	Antagonist	Conditions	B_{max} pmol/mg	K_d nM	Ref.
Skeletal muscle membranes					
Guinea-pig	[^{3}H]nimodipine	37°C	8.0	3.6	(1)
		+diltiazem	20.6	2.2	(1)
	(+)[^{3}H]PN200-110	37°C	20.6	1.4	(1)
		+diltiazem	25.4	1.5	(1)
Rabbit T-tubule	[^{3}H]nitrendipine	10°C	50.0	1.8	(2)
	[^{3}H]verapamil	10°C	50.0	27.0	(3)
Heart membranes					
Rat	[^{3}H]nimodipine	22°C	0.4	0.24	(4)
Guinea-pig	[^{3}H]nimodipine	37°C	0.35	0.26	(4)
Brain membranes					
Rat	[^{3}H]nimodipine	37°C	0.5	1.11	(5)
Smooth Muscle Membranes					
Guinea-pig ileal	[^{3}H]nitrendipine	25°C	1.13	1.63	(6)

Table 2

Calcium Channel Antagonist Binding to Membranes from Different Tissues

Results from a few representative studies involving the binding of Ca^{2+} channel antagonists to high affinity receptors in different tissues.

References:

(1) Ferry et. al. (1983a); (2) Fosset et. al. (1983); (3) Galizzi et. al. (1984); (4) Glossmann & Ferry (1985); (5) Belleman et. al. (1983); (6) Bolger et. al. (1983).

1.3.2. Calcium Channel Drug Receptors.

Specific inhibition of cell responses by the Ca^{2+} antagonists prompted the use of radiolabelled derivatives of these drugs in ligand binding studies to investigate the presence of membrane-bound receptors in membrane fractions from different tissues. Early investigations identified a high affinity binding site for [^{3}H]nitrendipine in membrane preparations from cardiac tissue (Bellemann et. al., 1981). The specific [^{3}H]nitrendipine binding was saturable and reversible, with an apparent dissociation constant (K_d) of 0.1nM indicative of high affinity binding. Extensive investigations using [^{3}H]nitrendipine, or other radiolabelled 1,4-DHPs (e.g. [^{3}H]nifedipine, [^{3}H]nimodipine, [^{3}H]PN 200-110), phenylalkylamines (e.g. [^{3}H]verapamil, [^{3}H]D600, [^{3}H]D888), or benzothiazepines (e.g. [^{3}H]d-cis-diltiazem), have been carried out on membrane preparations from brain, heart and smooth and skeletal muscle (Table 2). Although the apparent dissociation constants (K_d) and the density of sites (B_{max}) vary with both ligand and tissue, the Ca^{2+} channel antagonists bind with high affinity (K_d in the nanomolar range) to membrane-bound receptors in tissues that are known to contain a high density of Ca^{2+} channels from electrophysiological experiments (Glossmann et. al., 1984; Glossmann & Ferry, 1985).

Many of the Ca^{2+} antagonists are chiral, with the exception of nifedipine which is symmetrically substituted (Fig. 3A). Ligand binding studies have demonstrated that the receptors are stereoselective, discriminating between the (+)- and (-)-enantiomers of a particular antagonist (Belleman et. al., 1981). When an opticially pure antagonist is used in pharmacological investigations, such as (+)- or (-)-PN 200-110, in place of a racemic mixture, only one enantiomer is pharmacologically potent ((+)-PN 200-110 >> (-)-PN 200-110) (Ferry & Glossmann, 1982). This stereospecificity can lead to inaccurate calculation of the K_d when determined with a racemic ligand as only one enantiomer will bind with high affinity. The actions of other classes of Ca^{2+} channel antagonists also appear to be stereospecific. The (-)-enantiomer of verapamil is more effective than the (+)-enantiomer in blocking the Ca^{2+} component of the cardiac action potential (Henry, 1980). Furthermore, only one of the four diastereoisomers of diltiazem, d-cis-

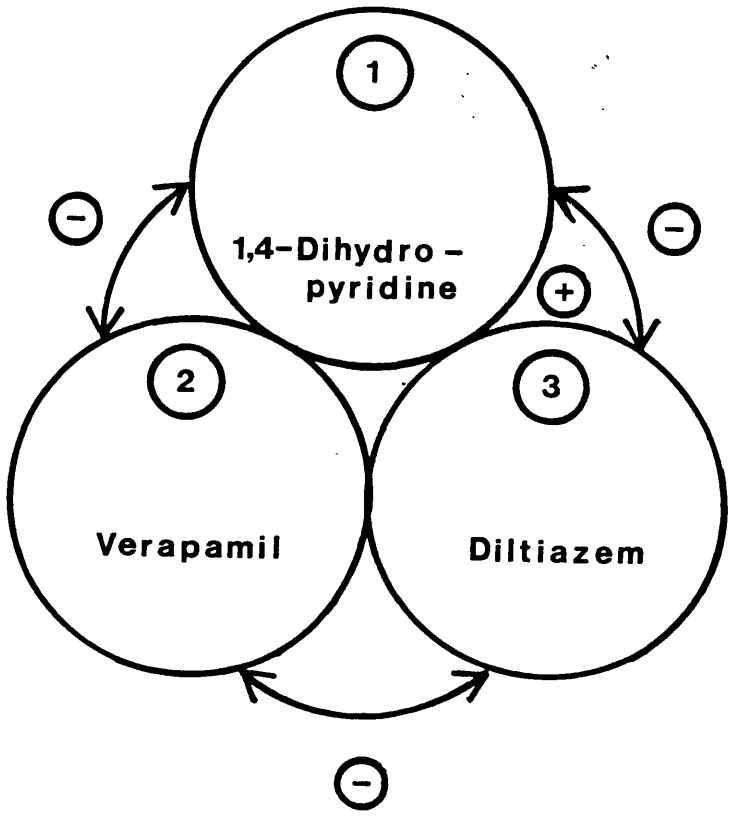


Figure 4

Allosteric Linkage of Calcium Channel Drug Receptor Sites.

Three drug receptor sites; 1,4-DHP, verapamil and diltiazem. Allosteric interactions between the receptor sites are shown by the arrows. Adapted from Glossmann et. al. (1985)

diltiazem, is a potent Ca^{2+} channel antagonist (Glossmann et. al., 1985).

As stated previously, competition binding experiments, performed using the various classes of radiolabelled Ca^{2+} channel antagonists, have implicated the presence of multiple drug receptor sites on the channel protein (Ferry & Glossmann, 1982). All radiolabelled ligands in the 1,4-DHP class compete for a single class of receptor in membrane preparations from different tissues. However, the binding of [^{3}H]nimodipine to receptors is modulated by the presence of d-cis-diltiazem and verapamil. d-cis-Diltiazem increases the binding of [^{3}H]nimodipine, and other 1,4-DHPs (Ferry et. al., 1983a; Glossmann & Ferry, 1985). In skeletal muscle and heart membrane preparations d-cis-diltiazem increases the B_{max} for [^{3}H]nimodipine binding without changing significantly the apparent K_d (Ferry et. al., 1983a; Glossmann & Ferry, 1985; Glossmann et. al., 1985). [^{3}H]nimodipine binding to brain membranes is increased in the presence of d-cis-diltiazem due to a decrease in the apparent K_d with no significant change in the B_{max} (Ferry & Glossmann, 1982; Ferry & Glossmann, 1985). In contrast, the presence of verapamil decreases the binding of both [^{3}H]nimodipine and [^{3}H]diltiazem, although the effects are complicated when racemic radioligands are used (Ferry & Glossmann, 1985).

This evidence suggests the presence of three different stereospecific, allosterically linked receptors on the channel protein, a 1,4-DHP receptor, a verapamil receptor and a diltiazem receptor (Fig. 4). The three receptors must be closely linked within the channel protein in order to exert these allosteric effects, either on the same subunit or on separate, tightly associated subunits. Figure 4 shows a diagrammatical representation of the allosteric linkage between the three drug major receptor sites of the voltage-sensitive Ca^{2+} channel.

1.3.3. Subtype and State-Dependent Binding of Calcium Channel Drugs.

The affinities of the Ca^{2+} channel drugs for respective receptors vary considerably depending on whether the channel is in the open, closed or inactivated state (Hondeghem & Katzung, 1984). The blocking action of the phenylalkylamines increases with increasing frequency of stimulation of the cell. In experiments on single heart cells Lee

and Tsien (1983) demonstrated that the verapamil derivative, D600 (gallopamil), exhibited use-dependent blockade of the Ca^{2+} current. This use-dependency of verapamil and D600 makes these drugs particularly effective in cardiac tissue and this is reflected in their clinical applications. Diltiazem and verapamil have been shown to be more potent under voltage conditions favouring the inactivated channel state (Kanaya et. al., 1983). This suggests that these channel antagonists block the inactivated state of the Ca^{2+} channel (Hondeghem & Katzung, 1984).

The mode of action of the 1,4-DHPs differs in that the efficacy of block appears to be more voltage dependent rather than frequency dependent. Thus, the 1,4-DHPs exhibit different affinities for the open, closed and inactivated states, with the highest affinity being for the inactivated state (Hondeghem & Katzung, 1984; Lee & Tsien, 1983). This state-dependency of Ca^{2+} channel blockade provides an explanation for the different potencies exhibited by the various Ca^{2+} channel drugs in different tissues. Verapamil and its derivatives are effective Ca^{2+} current blockers in both cardiac and smooth muscle, however, the 1,4-DHPs are more potent in smooth muscle tissue having less effect in cardiac tissue (Henry, 1980).

The determination of the mode of action of the 1,4-DHPs was aided by the development of a 1,4-DHP derivative, BAY K 8644, with Ca^{2+} channel activating properties (Schramm et. al., 1983). A comparison of the chemical structures of nifedipine and BAY K 8644 demonstrates only a slight alteration in structure is required to change an antagonist into an agonist (Fig 3A). BAY K 8644 competes directly with 1,4-DHP antagonists and therefore acts at the same site, however, it exerts a completely opposite effect on Ca^{2+} channel function (Schramm et. al., 1983).

In view of this, Hess and coworkers (1984) proposed that the 1,4-DHPs act by modulating the gating behaviour of the Ca^{2+} channel rather than physically plugging the channel pore (Section 1.2.3.). 1,4-DHP Ca^{2+} channel antagonists, such as nimodipine, preferentially stabilize Mode 0, characterized by very few channel openings, so resulting in an overall reduction of the Ca^{2+} current (Hess et. al., 1984). However, the presence of Ca^{2+} agonists, such as BAY K 8644, promote Mode 2 behaviour, characterized by prolonged periods of channel openings, so increasing the overall Ca^{2+} current (Hess et.

Preparation	Ligand	±Diltiazem	Target Size (Mr)	Ref.
Skeletal Muscle Membranes				
Rabbit	[^{3}H]nitrendipine	-	210,000	(1)
Guinea-pig	[^{3}H]nimodipine	-	178,000	(2)
		+	115,000	(2)
Guinea-pig	(+)-[^{3}H]PN200-110	-	136,000	(3)
		+	75,000	(3)
	(-)-[^{3}H]D888	-	110,000	(4)
	[^{3}H]diltiazem		131,000	(4)
Heart membranes				
Guinea-pig	[^{3}H]nimodipine	-	184,000	(5)
		+	106,000	(5)
Brain Membranes				
Guinea-pig	[^{3}H]nimodipine	-	185,000	(6)
		+	111,000	(6)
Smooth Muscle Membranes				
Guinea-pig	[^{3}H]nitrendipine	-	278,000	(7)

Table 3

Radiation Inactivation: Target Size Analysis of Calcium Channel Antagonist Receptors

Membrane protein samples were bombarded with high energy electrons. The loss of functional Ca^{2+} channel antagonist binding sites was measured with increasing radiation dose.

References:

(1) Norman et. al. (1983); (2) Ferry et. al. (1983b); (3) Goll et. al. (1983); (4) Goll et. al. (1984); (5) Glossmann et. al. (1985); (6) Ferry et. al. (1983a); (7) Venter et. al. (1983).

al., 1984). Interestingly, it was found that most of the 1,4-DHPs were both partial antagonists and partial agonists, even the agonist BAY K 8644 exhibited some antagonist behaviour.

Recent electrophysiological investigations have demonstrated the presence of three different subtypes of voltage-sensitive Ca^{2+} channel (McCleskey et. al., 1986; Section 1.2.5.). Only the L-type Ca^{2+} channels appear to be sensitive to block by the 1,4-DHPs and other classes of Ca^{2+} channel drugs (McCleskey et. al., 1986; Miller, 1987). Thus, as the tissue distribution of these subtypes varies, this provides a further explanation of the tissue selectivity of certain Ca^{2+} channel drugs.

1.3.4. Subunit Composition of the 1,4-Dihydropyridine Receptor.

Radiolabelled 1,4-DHPs have been employed extensively as biochemical probes in the characterization of the molecular structure of the receptor associated with voltage-sensitive Ca^{2+} channels. The earliest estimation of molecular size for the 1,4-DHP receptor was derived from radiation inactivation experiments.

Radiation Inactivation

This method involves the bombardment of protein samples with high energy electrons (Levinson & Ellory, 1973; Norman et. al., 1983). The measurement of the loss of functional [^{3}H]nitrendipine binding sites with increasing radiation dose, using this method, indicated an Mr of 210,000 for the 1,4-DHP receptor from both skeletal muscle and brain membranes (Norman et. al., 1983). Table 3 shows the range of target sizes, between 180,000-280,000, produced by other workers employing this same technique, but using different 1,4-DHP derivatives, on different tissue membrane samples. Interestingly, in the presence of d-cis-diltiazem the target size of the 1,4-DHP receptor was consistently reduced by an Mr of approximately 60,000 (Ferry et. al., 1983a; Goll et. al., 1983; Ferry et. al., 1983b; Glossmann et. al., 1985). This suggests the possible dissociation of a component of the Ca^{2+} channel complex on the binding of d-cis-diltiazem.

Tissue	Detergent	Subunit Composition (Mr)		Ref.
		Reducing	Non-reducing	
Skeletal Muscle				
T-Tubules	Digitonin	α 130,000 β 50,000 γ 33,000	160,000 50,000 33,000	(1)
T-Tubules	CHAPS	142,000 33,000 32,000		(2)
T-Tubules	Digitonin	142,000 122,000 56,000 31,000 22-26,000	142,000 56,000 31,000	(3)
T-Tubules	Digitonin	α_1 170,000 α_2 143,000 β 54,000 γ 30,000 δ 24-27,000	170,000 175,000 54,000 30,000	(4)
Triads	Digitonin	170,000 150,000 52,000 32,000	170,000 175,000 52,000 32,000	(5)
Chick Heart	Digitonin	60,000 54,000 34,000		(6)
	Digitonin	140,000 29-32,000		(7)

Table 4

Subunit Composition of the 1,4-Dihydropyridine Sensitive Calcium Channel Determined from Purification Studies

References:

(1) Curtis & Catterall (1984); (2) Borsotto et. al. 1984; (3) Flockerzi et. al. (1986); (4) Takahashi et. al. (1987); (5) Leung et. al. (1987); (6) Rengasamy et. al. (1985); (7) Cooper et. al. (1987)

Solubilization and Purification of the 1,4-DHP Receptor

The solubilization of the 1,4-DHP receptor into detergent or detergent/phospholipid micelles was successfully achieved from rat brain membranes using digitonin as the detergent (Curtis & Catterall, 1983), and from skeletal muscle transverse tubule membranes using 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate (CHAPS) (Borsotto et. al., 1984). Solubilization did not appear to affect the binding characteristics of the receptor, in that the apparent dissociation constant (K_d) for [^{3}H]nitrendipine binding was essentially unchanged from that of the membrane-bound receptor, as were the allosteric effects of diltiazem and verapamil on [^{3}H]nitrendipine binding (Borsotto et. al., 1984).

As the transverse tubule system of skeletal muscle is highly enriched in 1,4-DHP receptors (>50 pmol/mg protein) compared to other tissues (see Table 2), this membrane preparation has been utilized as a starting material in purification studies. (+)-[^{3}H]PN200-110 has been employed extensively in the purification of prelabelled receptors by virtue of the low apparent dissociation constant for the binding of (+)-[^{3}H]PN200-110 to the receptor. Table 4 summarizes the subunit compositions derived for the 1,4-DHP receptor from purification experiments. CHAPS solubilization from rabbit skeletal muscle transverse tubule membranes, followed by purification by gel filtration and Wheat Germ Agglutinin (WGA) affinity chromatography, resulted in a receptor preparation consisting of a multi-subunit complex composed of a large subunit of Mr 142,000, with two small subunits of Mr 32,000 and Mr 33,000 (Borsotto et. al., 1984). The purification of the [^{3}H]nitrendipine labelled receptor initially solubilized using digitonin, also implicated a multisubunit protein complex, but composed of a large subunit of Mr 130,000 (α) and two smaller components of Mr 50,000 (β) and 33,000 (γ) (Curtis & Catterall, 1984). Other purification studies from chick heart membranes only identified low molecular weight subunits of Mr 68,000, 54,000 and 34,000 involved in the receptor complex (Rengasamy et. al., 1985). As will be discussed below, recent investigations have revealed a more complex structure for the 1,4-DHP sensitive Ca^{2+} channel, involving five subunits (Table 4).

Tissue	Ligand	Molecular Size of Receptor		Ref.
		Reducing	Non-reducing	
Skeletal Muscle Membranes				
Rabbit T-tubules	(+)[^{3}H]PN200-110	170,000		(1)
	[^{3}H]Diltiazem	170,000		(1)
	[^{3}H]Bepridil	170,000		(1)
Rabbit T-tubules	[^{3}H]Azidopine	170,000	170,000	(2)
	[^{3}H]LU 49888	155-170,000	155-170,000	(3)
	[^{3}H]LU 49888	165,000	165,000	(4)
Guinea-pig T-tubules	[^{3}H]Azidopine	158,000 99,000	240,000 158,000 99,000	(5)
Guinea-pig T-tubules	[^{3}H]LU 49888	155,000	155,000	(6)
Heart membranes				
Canine	[^{3}H]Nitrendipine	32,000		(7)
Guinea-pig	[^{3}H]Azidopine	165,000	165,000	(8)
Brain Membranes				
Chick Synaptosomes	[^{125}I]ω-Cg-Tx*		135,000	(9)

Table 5

Photoaffinity Labelling of Calcium Channel Antagonist Receptors

* [^{125}I]ω-CgTx crosslinked to receptor protein with disuccinimidylsuberate or ethylene glycol bis(succinimidylsuccinate). In all other cases the receptor proteins were photoaffinity labelled by exposure of the membranes samples to U.V. light following the binding of the Ca^{2+} channel antagonist.

References:

(1) Galizzi et. al. (1986); (2) Sharp et. al. (1987); (3) Vaghy et. al. (1987); (4) Sieber et. al. (1987); (5) Ferry et. al. (1985); (6) Striessnig et. al. (1987); (7) Campbell et. al. (1984) (8) Ferry et. al. (1987); (9) Cruz et. al. (1987).

Photoaffinity Labelling of Ca^{2+} Channel Antagonist Receptors

Photoaffinity labelling studies provided an alternative method for the characterization of the 1,4-DHP sensitive Ca^{2+} channel. These investigations, using high affinity photoactivatable Ca^{2+} channel probes (e.g. (+)-[^{3}H]PN200-110, [^{3}H]azidopine, [^{3}H]LU 49888, d-cis[^{3}H]diltiazem), have also implicated a large subunit of Mr 145,000-170,000 as the receptor for the 1,4-DHP, phenylalkylamine and benzothiazepine drugs (Lazdunski et. al., 1986). Table 5 shows the apparent molecular sizes of the 1,4-DHP, phenylalkylamine and benzothiazepine receptors predicted from these studies in different tissues.

[^{3}H]Azidopine photoaffinity labelled polypeptides of Mr 240,000, 158,000 and 99,000 in partially purified guinea-pig skeletal muscle transverse tubule membranes and a component of Mr 165,000 in guinea-pig heart membranes (Ferry et. al., 1985; Ferry, 1987; Glossmann et. al., 1987). Interestingly, a similar polypeptide of Mr 170,000 was specifically labelled in photoaffinity labelling experiments on transverse tubule membranes using derivatives of all the major classes of Ca^{2+} channel antagonists, [^{3}H]bepridil, d-cis[^{3}H]diltiazem or (+)-[^{3}H]PN200-110 (Galizzi et. al., 1986). This would appear to suggest that the receptor sites for these different drug classes are associated with either the same subunit of the Ca^{2+} channel, or different subunits very similar in molecular size.

Photoaffinity labelling of transverse tubule membranes and purified Ca^{2+} channel preparations using a photoactivatable phenylalkylamine derivative, [^{3}H]LU 49888, specifically labelled a polypeptide of Mr 155,000-170,000 (Table 5; Striessnig et. al., 1987; Vaghy et. al., 1987; Sieber et. al.). These results suggest that the 1,4-DHP, phenylalkylamine and benzothiazepine receptors are all associated with a large subunit of the Ca^{2+} channel.

Reconstitution of the Purified 1,4-DHP Sensitive Ca^{2+} Channel

Pharmacological experiments have been performed following the reconstitution, into phosphatidylcholine vesicles, of purified 1,4-DHP receptor fractions composed of the Mr 140,000 and Mr 32,000 components (Barhanin et. al., 1987). The binding properties of the receptor are unchanged in the reconstituted system from those of the native

membrane-bound receptor. In addition, the binding sites for radiolabelled verapamil derivatives and d-cis-[^{3}H]diltiazem, that appear to be lost on solubilization, are regained in the reconstituted system (Barhanin et. al., 1987).

The 1,4-DHP receptor, purified from skeletal muscle transverse tubule membranes by the method of Curtis & Catterall (1984; Table 4), has been reconstituted into artificial phospholipid bilayer membranes for electrophysiological studies (Flockerzi et. al., 1986). The reconstituted complex exhibited the electrophysiological characteristics of the membrane-bound channel, having a similar single-channel conductance to the L-type channel recorded in cell-attached patches (Section 1.2.5.). Furthermore, the gating behaviour of the reconstituted channel complex was modulated by Ca^{2+} channel agonists and antagonists in a similar way to the native channel. The phenylalkylamine derivative, D600, reduced the open-state probability (p_o) of the reconstituted complex, while the Ca^{2+} channel agonist, BAY K 8644, increased the channel open times (Flockerzi et. al., 1986).

Current Model for 1,4-DHP Sensitive Calcium Channel Structure

The investigations described previously implicate the involvement of three subunits in the structure of the 1,4-DHP sensitive Ca^{2+} channel; Mrs 130,000-170,000, 50,000 and 30,000 (Curtis & Catterall, 1984). Borsotto et. al. (1984) detected a similar high molecular weight subunit (Mr 140,000) and two small molecular weight components (Mr 33,000 and Mr 32,000), but did not detect the Mr 50,000 polypeptide. The use of polyclonal and monoclonal antibodies has enabled the further elucidation of the receptor structure. Experiments involving antibodies raised against purified 1,4-DHP sensitive Ca^{2+} channel preparations suggested the possibility that two of these subunits, Mr 140,000 and Mr 30,000, were linked by disulphide bonds to form a dimer of Mr 170,000 (this study, Section 3.4.1.; Norman et. al., 1987; Schmid et. al., 1986a).

More recent reports involving purification studies and investigations with monoclonal antibodies suggest that the skeletal muscle 1,4-DHP receptor is a more complex oligomer of five different subunits; Mr 175,000 (α_1), Mr 140,000 (α_2), Mr 50,000 (β), Mr 30,000 (γ) and Mr 24,000-27,000 (δ) (Table 4; Fig. 5; Takahashi et. al., 1987; Leung et. al., 1987; Vaghy et. al., 1987; Sieber et. al., 1987). The Mr 140,000, α_2 subunit is a

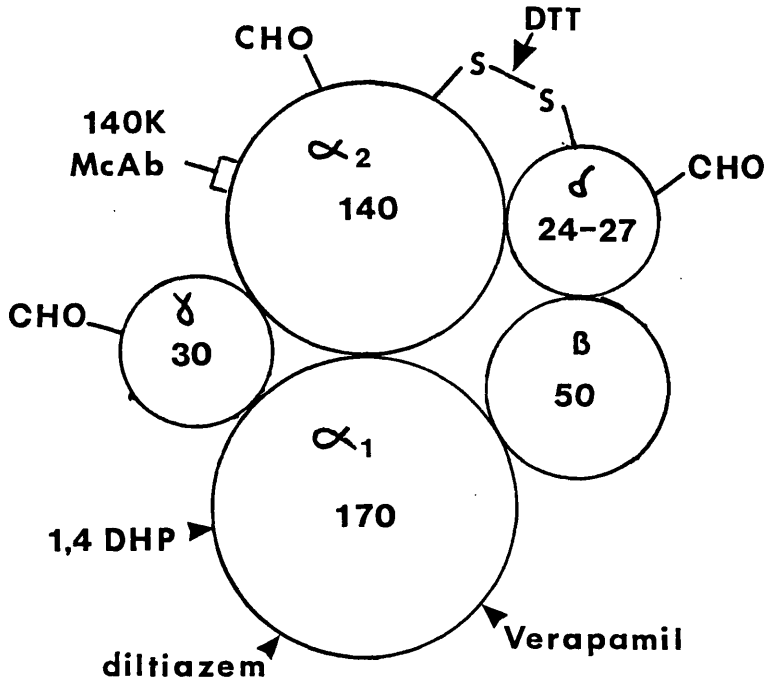


Figure 5

Calcium Channel Subunits.

Schematic representation of the subunits involved in the Ca^{2+} channel complex; showing the apparent molecular weight (x 10^{-3}), glycosylation (-CHO), and Ca^{2+} channel drug receptor sites (▲).

glycoprotein and is linked to the δ subunit by disulphide bonds (this study; Norman et. al., 1987; Schmid et. al., 1987; Takahashi et. al., 1987; Leung et. al., 1987). This α_2 subunit is similar to the receptor component identified by Borsotto et. al. (1984) in their purification studies from skeletal muscle. The second high molecular weight component is a non-glycosylated polypeptide of Mr 175,000 (α_1), the molecular weight of which is unchanged upon the reduction of disulphide bonds (Takahashi et. al., 1987; Leung et. al., 1987).

The photoaffinity labelling studies described earlier (Table 5), suggest that the receptor sites for the 1,4-DHPs, phenylalkylamines and benzothiazepines are associated with a polypeptide of Mr 155,000-170,000. The apparent molecular weight of the labelled component is unchanged on reduction of disulphide bonds (Table 5; Sharp et. al., 1987; Vaghy et. al., 1987; Sieber et. al., 1987). This suggests that the 1,4-DHP binding site is located on the non-glycosylated, subunit designated α_1 (Takahashi et. al., 1987; Leung et. al., 1987; Vaghy et. al., 1987). No incorporation of label was detected for the Mr 140,000, α_2 component in these photoaffinity labelling studies. Consequently, it has been suggested that this component is not involved in the binding of these classes of Ca^{2+} channel antagonists.

In addition to these investigations, the primary structure of the large non-glycosylated α_1 subunit has now been determined from the nucleotide sequence (Section 1.4.5.; Tanabe et. al., 1987). The amino acid sequence of this subunit shows considerable homology with that of the large α subunit of the Na^+ channel (Section 1.4.5.). The large number of hydrophobic amino acid residues arranged in homologous sequence repeats is typical of a transmembrane protein. Hence, it has been suggested that the α_1 subunit is the channel forming component of the Ca^{2+} channel.

From these results Takahashi et. al. (1987) have proposed a model for the 1,4-DHP sensitive Ca^{2+} channel (Fig. 6). According to the model the α_1 subunit is the central channel forming protein and has the binding sites for the major Ca^{2+} channel antagonists. All other subunits involved in the channel structure are associated peripherally with this polypeptide component. In this model the α_2 subunit is only

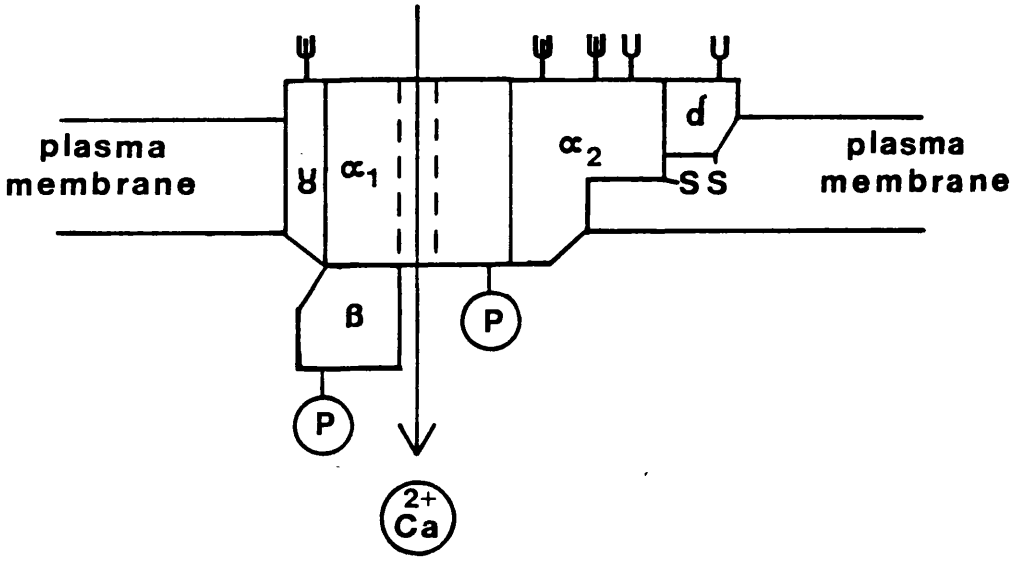


Figure 6

Model of 1,4-Dihydropyridine Sensitive Calcium Channel Structure.

Model postulated by Takahashi et. al. (1987) with the α_1 subunit as the central channel forming component. S-S, disulphide bonds; P, phosphorylation sites; ψψ carbohydrate. Adapted from Takahashi et. al. (1987).

weakly associated with the central α_1 component and is not involved in the 1,4-DHP activity of the Ca^{2+} channel (Takahashi et. al., 1987).

In contrast to this model, other workers have detected 1,4-DHP activity in purified Ca^{2+} channel preparations, apparently lacking the non-glycosylated, α_1 subunit (this study; Norman et. al., 1987; Borsotto et. al., 1984, 1985; Nakayama et. al., 1987). The major polypeptide component of these purified fractions was the Mr 140,000 component analogous to the α_2 subunit of the model of Takahashi et. al. (1987). These investigations suggest that the α_2 subunit may play a role in the 1,4-DHP binding activity of the Ca^{2+} channel. It is noteworthy that Takahashi et. al. (1987) indicated that the 1,4-DHP activity was only detected in purified Ca^{2+} channel preparations when the α_2 component was associated with α_1 subunit. Moreover, it has been reported that functional Ca^{2+} channels cannot be expressed from the cDNA of the α_1 polypeptide alone (Hofmann, 1988; Froehner, 1988; Alsobrook & Stevens, 1988) as has been achieved for the Na^+ channel (Noda et. al., 1986b). These results appear to suggest a more central role for the α_2 subunit in Ca^{2+} channel structure and function. Clearly more work is required to fully elucidate the role of the α_2 subunit in the 1,4-DHP sensitive Ca^{2+} channel complex.

1.4. Characterization of Ion Channels.

1.4.1. Introduction

Certain other ion channels are better characterized than the Ca^{2+} channel both in terms of structure and function. These include the voltage-sensitive Na^+ channel and the ion channel associated with the agonist-activated nicotinic acetylcholine receptor (nAChR). Certain factors have aided the characterization of these channels. Preparations that can be readily investigated using the voltage clamp technique have been available to enable the electrophysiological characterization of these channels. For example, initial electrophysiological investigations on the Na^+ channel were carried out on the squid giant axon under voltage clamp conditions (Hodgkin & Huxley, 1952), while the electrophysiological characteristics of the nAChR were investigated by voltage clamping of the muscle surface membrane, at the neuromuscular junction (Magazanik, 1976).

Secondly, rich sources of both Na^+ channels and nAChR were available as starting material for purification and characterization studies. The electric organ of the electric eel, *Electrophorus electricus* has provided a rich source of both Na^+ channels and nAChR ion channels for these investigations (Catterall, 1980; Popot & Changeux, 1984). In addition, the electric organ of the electric fish *Torpedo californica* is another rich source of nAChR for biochemical investigations (Popot & Changeux, 1984).

A further factor that has led to the successful structural characterization of the Na^+ channel and the nAChR is the availability of naturally occuring toxins that specifically modulate the function of these ion channels (Catterall, 1980; Lazdunski & Renaud, 1982; Popot & Changeux, 1984). Some of these neurotoxins bind with high affinity to receptors associated with the nAChR or the Na^+ channel protein and hence have been useful as biochemical probes in purification studies. These investigations have enabled the subunit composition of both the Na^+ channel (Catterall, 1986) and the nAChR (Popot & Changeux, 1984; Stroud & Finer-Moore, 1985) to be elucidated.

Other approaches have been employed in the characterization of these channels. For example, monoclonal antibodies raised against the various subunits have enabled a model to be produced for the structural arrangement of the five subunits of the nAChR (Lindstrom et. al., 1984). In addition, the primary sequences of the subunits involved in the structure of both the nAChR (Noda et. al., 1982, 1983a, 1983b; Sumikawa et. al., 1982), and the Na^+ channel have been determined using recombinant DNA techniques (Noda et. al., 1984, 1986; Dunn et. al., 1985). The topological models predicted from these primary sequences show similarities between the Na^+ channel, the channel associated with the nAChR and the Ca^{2+} channel (Tanabe et. al., 1987), and have led to the hypothesis of a similar core structure for membrane channels (Section 1.4.5.; Unwin, 1986; Alsobrook & Stevens, 1988).

1.4.2. Molecular properties of the Sodium Channel

Early recordings of Na^+ channel currents were carried out on the squid giant axon using the voltage clamp technique (Hodgkin & Huxley, 1952). Two major functions of the Na^+ channel were postulated to describe the biphasic nature of the recorded action

potential. Voltage-dependent activation, the opening of channels in response to depolarization causing the initial increase in Na^+ permeability, and voltage-dependent inactivation, responsible for the subsequent decline in permeability, due to the closing of channels. This led to the proposal of the Hogkin & Huxley model for channel activation, involving one closed state and one open state (Section 1.2.3). However, later investigations suggested that the voltage-dependent activation and inactivation processes were more complicated, probably involving several inactivated states (Bean, 1981).

Hodgkin and Huxley (1952) proposed initially that the voltage-dependent gating was brought about by the movement of charged particles within the membrane as a result of the depolarizing potential. Later experiments confirmed that depolarization induced the movement of charged portions of the channel protein that resulted in channel opening. These "gating currents" were recorded when the main ionic current was reduced to a minimal level. This was achieved by replacing Na^+ and K+ with impermeant ions, and recording in the presence of a neurotoxin channel blocker (Armstrong & Bezanilla, 1973).

The understanding of Na^+ channel function and structure has been greatly advanced by the discovery of several naturally occuring neurotoxins that bind specifically to Na^+ channels and modulate channel function. There are now several groups of neurotoxins classified with respect to their effect on Na^+ channel function (Table 6). Tetrodotoxin (TTX), a potent paralytic poison from the puffer fish, specifically blocked Na^+ currents when applied extracellularly (Catterall, 1980). Saxitoxin (STX) another water-soluble toxin, produced by marine dinoflagellates, exerted similar effects on Na^+ current. Binding studies have demonstrated that the radiolabelled derivatives of these toxins compete for a single class of receptor sites associated with the Na^+ channel protein, now termed receptor site 1 (Catterall, 1980). The block exerted by these toxins does not involve a modification of the voltage-dependent channel gating, consequently it has been suggested that the toxins bind to an external site on the channel and physically block the pore (Catterall, 1980).

The second group of Na^+ channel toxins the lipid-soluble (alkaloid and pyrethroid) toxins were isolated from a variety of sources, including some plant species. The

Neurotoxin	Physiological Effect		
	Activation	Inactivation	Conductance
TTX/STX			block
Lipid soluble toxins Alkaloids Pyrethroids	 increase increase	 slow & inhibit slow & inhibit	 decrease
α Scorpion and sea anemone toxins	no effect	slow & inhibit	
β Scorpion toxins	increase	slow & inhibit	
μ-CgTx			block*

Table 6

Different Classes of Sodium Channel Neurotoxins

The physiological effects exerted on Na^+ channel function by the different classes of neurotoxins.

* μ-CgTx selectively blocks skeletal muscle Na^+ channels, only weakly inhibits Na^+ currents in neuronal and cardiac tissues (Cruz et. al., 1985).

Adapted from: Catterall (1987); Strichartz et. al. (1987)

presence of TTX non-competitively blocks the binding of these toxins suggesting that they act at a different receptor to TTX. The receptor for the lipid-soluble toxins is termed receptor site 2 (Catterall, 1980; Catterall, 1986). The lipid-soluble toxins modulate Na^+ channel gating by shifting the voltage-dependent range of activation, hence, channel activation occurs at the resting potential, and by inhibiting channel inactivation. The hydrophobic properties of these toxins suggest that they act at a site within the membrane, that is associated with the portion of the channel protein involved in the voltage-dependent activation and inactivation mechanism.

Two further groups of toxins, the North African α scorpion toxins and the sea anemone toxins, act at receptor site 3 (Catterall, 1986). These toxins reduce the rate of Na^+ channel inactivation and also enhance the effect of the lipid-soluble toxins. This indicates that there is an allosteric effect between receptor sites 2 and 3. In addition, it has recently been established that a different class of toxins, the American β scorpion toxins, act at a fourth site, receptor site 4 (Barhanin et. al., 1982; Catterall, 1986). The neurotoxins acting at receptor site 4 increase the activation of Na^+ channels and so possibly act at a site involved in voltage-dependent activation. Another toxin, isolated from *Tityus serrulatus* (TiTX-γ), has been shown to compete with the β scorpion toxins for a common site (Barhanin et. al., 1983). Finally, a group of peptide toxins termed μ-conotoxins (μ-CgTxs) have been isolated from the marine snail *Conus geographus* (Cruz et. al., 1985). Interestingly, the effect of the μ-CgTxs appear to be tissue specific in a similar way to ω-CgTx effect on L-Type Ca^{2+} channels (Section 1.2.5.). μ-CgTxs block Na^+ channel currents in skeletal muscle with a similar potency to STX, however, in cardiac and neuronal tissue the blocking effect of μ-CgTx is less potent (Cruz et. al., 1985).

This variety of Na^+ channel specific neurotoxins, acting at different receptor sites, provided a wide range of specific biochemical and functional probes that were invaluable in the elucidation of the molecular properties of the Na^+ channel. The initial target size for the TTX receptor site was determined using the radiation inactivation technique (Section 1.3.4.). A target size of Mr 230,000-260,000 was predicted in this manner for the TTX receptor (Levinson & Ellory, 1973; Barhanin et. al., 1982). Purification and

Tissue	Subunit Composition (Mr)	Ref.
Electric eel electric organ	260,000	(1)
Rat brain	α 260,000 β_1 39,000 β_2 37,000	(2)
Rat skeletal muscle	1 160,000-200,000 2 45,000 3A 38,000 3B 37,000	(3)

Table 7

Subunit composition of the Sodium Channel

Subunit composition of the Na^+ channel from different membrane preparations determined by purification studies.

References:

(1) Miller et. al. (1983); (2) Hartshorne & Catterall (1984); (3) Barchi (1983).

photoaffinity labelling or covalent crosslinking techniques, using the various neurotoxins, have resulted in the characterization of the subunit composition of Na^+ channels from different sources (Table 7; Catterall, 1980; Catterall, 1986).

1.4.3. Molecular Properties of the Nicotinic Acetylcholine Receptor

The most extensively studied nAChR, electrophysiologically, is that of the neuromuscular junction. The nAChR is concentrated at the postsynaptic membrane at the neuromuscular junction, where it conveys electrical excitability from the nerve to the muscle fibre. The neurotransmitter, acetylcholine (ACh), released from the presynaptic nerve terminal binds to the postsynaptic receptor. This ligand-receptor interaction causes a conformational change in the protein, so opening the channel pore. The passage of ions through the channel is sufficient to stimulate the depolarization of the surface membrane of the muscle fibre and initiate an action potential (Popot & Changeux, 1984).

The channel opening event requires the binding of two molecules of ACh, each to a receptor on one of the two α subunits of the nAChR (Cash & Hess, 1980). In the open state the nAChR channel is selective for cations, allowing the passage of Na^+, K^+ and Ca^{2+}. However, Na^+ ions are usually conducted by virtue of the large electrochemical gradient for Na^+ across the postsynaptic membrane (Hille, 1984; Popot & Changeux, 1984). The large influx of Na^+ ions depolarizes the muscle surface membrane sufficiently to initiate the action potential in the muscle fibre (Popot & Changeux, 1984). The ACh present in the synaptic cleft is removed both by diffusion processes and by an enzyme acetylcholinesterase. However, in the prolonged presence of ACh the receptors become "desensitized". This desensitized state is analogous to the inactivated state for Na^+ and Ca^{2+} channels. As with channel inactivation, desensitization involves the transformation through several intermediate states (Hess et. al., 1983; Stroud & Finer-Moore, 1985).

A toxin from the venom of the cobra, *Bungarus multicinctus*, α-bungarotoxin (α-BTX), and cholinergic ligands have been used as probes to purify nAChR from the *Torpedo californica* electric organ using affinity chromatography (Lindstrom et. al., 1979; Conti-Tronconi & Raftery, 1982). The nAChR from Torpedo and skeletal muscle

has been described as a pentamer with four individual subunits; α (Mr 40,000), β (Mr 50,000), γ (Mr 57,000), and δ (Mr 65,000) that form the complex $\alpha_2\beta\gamma\delta$ (Lindstrom et. al., 1979). Furthermore, receptors for both ACh and the neurotoxin α-BTX have been located on each of the α subunits (Conti-Tronconi & Raftery, 1982). Most of the more recent work characterizing the nAChR from Torpedo and other sources has involved monoclonal antibodies raised against the purified subunits. These investigations have led to the prediction of a topological model for the channel associated with the nAChR using a combination of monoclonal antibody methods and recombinant DNA techniques (Unwin, 1986).

1.4.4. Monoclonal Antibodies in Ion Channel Characterization

Sodium Channel

Monoclonal antibodies raised against ion channel subunits can be utilized in the elucidation of channel subunit composition. For the Na^+ channel, monoclonal antibodies were initially raised against the purified TTX binding component from the electric organ of *Electrophorus electricus* (Moore et. al., 1982). These antibodies recognized a polypeptide of Mr 250,000 on SDS polyacrylamide gels, confirmation that this large α subunit is the TTX receptor. Furthermore, the antibodies specifically immunoprecipitated [^{3}H]STX binding activity from crude detergent solubilized membrane fractions (Moore et. al., 1982). Other monoclonal antibodies raised against Na^+ channel protein purified from rat skeletal muscle also identified a large subunit of Mr 260,000 in immunoblot transfers of muscle membrane proteins when care was taken to avoid proteolysis (Casdei et. al., 1984). Casedei et. al. (1984) have also demonstrated that Na^+ channels are concentrated on the surface membrane of the muscle fibres using immunofluorescence localization methods.

The functional properties of ion channels can be investigated using monoclonal antibodies specific for different regions of the channel. For example, in the case of the Na^+ channel, monoclonal antibodies raised against membrane fragments from the eel electroplax were shown to exert an effect on Na^+ currents recorded from intact rat nerve fibres (Meiri et. al., 1986). The antibody appeared to shift the voltage-dependence of

inactivation in voltage clamp experiments (Meiri et. al., 1986). Interestingly, Meiri et. al. (1986) found that only antibodies that modulated channel function crossreacted with Na^+ channels from other species, suggesting the possibility that the functional regions of the channel protein are more likely to be conserved. Another monoclonal antibody was found to change the voltage-dependence of activation, in addition to competing with TiTX-γ for a common binding site (Bahanin et. al., 1985). The identification of the parts of the protein structure that are important for channel function should be possible by the mapping of monoclonal antibodies that modulate the functional properties of channels to specific regions of the channel protein (Meiri et. al., 1987).

Nicotinic Acetylcholine Receptor Channel

The nAChR has been shown to be composed of four subunits (Section 1.4.3.). Although the different subunits are similar in terms of molecular size, amino acid and carbohydrate composition (Lindstrom et. al., 1979), peptide maps have indicated that there are no extensive regions of homology between the different subunits (Lindstrom et. al., 1979; Froehner & Rafto, 1979; Nathenson & Hall, 1979). Monoclonal antibodies raised against the nAChR subunits were used to investigate possible homologies, by the determination of crossreactivity between the different subunits. Iodinated peptide fragments, were produced from the different prelabelled subunits of the eel electroplax nAChR by limited digestion with proteolytic enzymes. These were tested in immunoprecipitation experiments with the different monoclonal antibodies (Güllick et. al., 1981). In this way antibodies were mapped not only to a particular subunit, but to specific regions of the subunit. Many antibodies were found to be specific for a particular subunit, however, a few recognized more than one subunit, providing further evidence for structural homology between subunits (Güllick et. al., 1983). Using these techniques monoclonal antibodies that modulate channel function can be mapped to the specific regions of the channel protein where they exert their effects. Hence, the regions of the channel structure important for channel function, can be traced using monoclonal antibodies as specific probes.

1.4.5. Models for Ion Channel Structure.

The primary sequences of certain ion channel subunits have been determined. The techniques applied to these other channels are now being utilized for the primary sequence determination of the 1,4-DHP sensitive Ca^{2+} channel components. The primary amino acid sequences for all four subunits of the nAChR (Noda et. al., 1982, 1983a, 1983b; Sumikawa et. al., 1982), the α subunit of the Na^+ channel (Noda et. al., 1984, 1986a), the K^+ channel (Papazian et. al., 1987; Tempel et. al., 1987), and the α_1 subunit of the Ca^{2+} channel (Tanabe et. al., 1987) have now been determined. Different strategies have been employed in the deduction of the amino acid sequences of these channel components. In the case of the α_1 subunit of the Ca^{2+} channel, peptide fragments were produced by trypsin treatment of the purified protein, and separated by reverse-phase HPLC. The amino acid sequences of the fragments were determined using a gas-phase sequencer (Tanabe et. al., 1987). The short amino acid sequences, formed in this way, were used to synthesize oligodeoxyribonucleotides. These synthetic oligodeoxyribonucleotides were then used to screen complementary DNA (cDNA) libraries using hyridization techniques (Tanabe et. al., 1987).

Alternatively, short amino acid sequences can be derived from the N-terminal sequence by Edman degradation and used to synthesize oligodeoxyribonucleotides for cDNA screening (nAChR; Raftery et. al., 1980). In a different approach, polyclonal antibodies raised against the purified Na^+ channel protein were used for the screening of cDNA libraries (Noda et. al., 1984). cDNA libraries were formed from mRNA, isolated from *Torpedo californica* (nAChR; Noda et. al., 1983a), or *Electrophorus electricus* (Na^+ channel; Noda et. al., 1984) electric organ, or from rat brain (Na^+ channel; Noda et. al., 1986a), by reverse transcriptase. In all these cases positive clones, detected from the cDNA screening, were sequenced and the amino acid sequence of the channel protein deduced from the nucleotide sequence.

The primary sequences derived in this way for each of the membrane channels displayed certain similarities. Each of the subunit precursors α, β, γ, and δ, of the nAChR from Torpedo electric organ contained four homologous hydrophobic sequences, designated M1, M2, M3, and M4 (Noda et. al., 1983b). These regions were postulated to

be the membrane-spanning regions of the polypeptides, and a considerable degree of homology was found within these regions between the different subunits. Interestingly, analysis of the primary sequence of the α subunit of the Na^+ channel and the α_1 subunit of the Ca^{2+} channel also indicated the presence of four homologous domains in the sequence which were designated I, II, III, and IV (Noda et. al., 1984; Tanabe et. al., 1987). Furthermore, within each of these four repeats were six short sequences of high homology (designated S1-S6; Noda et. al., 1984; Tanabe et. al., 1987). In the case of the Na^+ channel, regions S1, S2, S5 and S6 were composed mainly of hydrophobic amino acid residues, and were postulated as being the regions of the channel protein that spanned the membrane. In comparison the Ca^{2+} channel sequence revealed that all six short regions in each of the four homologous repeats were probably membrane-spanning regions (Tanabe et. al., 1987). Interestingly, the primary sequence of the K^+ channel also has hydrophobic repeats similar to those of the Na^+ and Ca^{2+} channels (Papazian et. al., 1987; Tempel et. al., 1987).

From the initial primary sequence, models that show distinct similarities have been postulated for the nAChR, the Na^+ channel, the K^+ channel and the Ca^{2+} channel (Tanabe et. al., 1987; Unwin, 1986; Alsobrook & Stevens, 1988). It has been proposed that the five subunits of the nAChR are organized in a circular arrangement around a central channel pore (Noda et. al., 1983b). The model suggested that each subunit would traverse the membrane at least four times, consistent with the four hydrophobic regions M1-M4. These hydrophobic regions have been shown to be similar to the membrane-spanning regions of the gap junction channel (Unwin, 1986). X-ray crystallography patterns obtained for the gap junction channel indicated that the membrane-spanning regions formed an α-helical structure (Unwin, 1986;). Hence, it has been postulated that each of these regions, M1-M4 of the different nAChR subunits, forms an α-helix, composed of uncharged amino acids, that traverses the membrane. The model proposes that the α-helical regions surround the central channel pore (Stroud & Finer-Moore, 1985). In the case of the Na^+ and Ca^{2+} channels it has been postulated that the four homologous repeats (I-IV) are arranged around a central channel-forming pore, with, in

the case of the Ca^{2+} channel, each of the six hydrophobic sequences (S1-S6) traversing the membrane.

The models proposed for each of these membrane channels are distinctly similar, particularly with regard to the membrane-spanning regions that form the channel pore. This has initiated speculation regarding a common basic pore structure for the different membrane channels, with the homologous hydrophobic regions forming the channel pore (Unwin, 1986, Alsobrook & Stevens, 1988). The non-homologous regions of the channel protein sequence may well control the different functions of the various channels such as voltage- or agonist-dependent gating and ion selectivities. As previously described, monoclonal antibodies are specific probes directed against specific regions of polypeptide sequence. These antibodies could be used to probe particular portions of the sequence to substantiate proposed topological models of channel structure. This method has already been applied to models proposed for the nAChR (Lindstrom et. al., 1984). In addition, monoclonal antibodies that modulate channel function could be used to probe for the functionally important regions of channel primary structure. These regions could then be used as targets for site-directed mutagenesis experiments.

1.5. Aims of this Study

The objective of this study was to generate a panel of monoclonal antibodies against the 1,4-DHP receptor associated with the skeletal muscle voltage sensitive Ca^{2+} channel. Monoclonal antibodies raised against other ion channel components have proved useful biochemical probes for the characterization of the structure of these channels (Section 1.4.4.). Similarly, antibodies raised against the 1,4-DHP receptor would provide highly specific probes for the structural characterization of the Ca^{2+} channel complex.

CHAPTER 2

PURIFICATION OF THE 1,4-DIHYDROPYRIDINE RECEPTOR ASSOCIATED WITH THE VOLTAGE-SENSITIVE CALCIUM CHANNEL FROM SKELETAL MUSCLE TRANSVERSE TUBULE MEMBRANES

2.1. INTRODUCTION

Radiolabelled derivatives of the antiarrhythmic drugs, the 1,4-DHPs, have been shown to bind with high affinity to receptors associated with the voltage-sensitive Ca^{2+} channel (Section 1.3.). These derivatives have been employed as biochemical probes in studies to elucidate the subunit composition of the 1,4-DHP receptor. In this study the 1,4-DHP receptor was purified from skeletal muscle T-tubule membranes using the high affinity 1,4-DHP, (+)-[^{3}H]PN200-110, as a probe. The purified receptor protein was used to immunize mice for the production of monoclonal antibodies. Furthermore, the purified protein was used as antigen for the screening of the antibody producing cell lines in solid phase immunoassays. The monoclonal antibodies produced in this manner provided novel probes to further characterize the Ca^{2+} channel structure. In addition, the purified receptor protein was utilized for primary sequence analysis in a parallel project.

2.2. MATERIALS

The (+)-[methyl-^{3}H]PN200-110 (82 Ci/mmol) was obtained from Amersham International PLC. The unlabelled nitrendipine was a generous gift from Dr. H. Meyer, Bayer A.G. F.R.G.. Ultrogel A2 and Wheat Germ Agglutinin (WGA) Ultrogel were obtained from LKB and the Sephadex G50 from Pharmacia. The scintillation fluids and the glycerol were from Fisons. Protease inhibitors, molecular weight markers, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate (CHAPS) and Triton X-100 detergents, soybean phosphatidylcholine (SPC), 3-(N-morpholino)propanesulphonic acid (MOPS), N-acetyl-D-glucosamine, Brilliant Blue G250 (C.I. 42655) and R250 (C.I. 42660) and N,N,-methylenebisacrylamide were all obtained from Sigma. The acrylamide

and silver nitrate were from BDH and the GF/B glass microfibre filters were from Whatman. All other chemicals were of analytical grade.

2.3. METHODS

2.3.1. Preparation of Transverse Tubule Membranes

Skeletal muscle tissue dissected from the hind legs and backs of rabbits was vacuum packed before being frozen at -70°C. Tissue was stored in this way for no longer than two weeks before being used for membrane preparation. T-tubule membrane preparation was based on the method of Rosemblatt et. al. (1981), with the exception that 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (40mM, pH 7.5) was used throughout the procedure.

All stages of the membrane preparation were carried out at 4°C. 300g of tissue was thawed and finely chopped before homogenization. Tissue was added to 3.5 volumes of ice-cold 0.3M sucrose, 40mM MOPS, pH 7.5 and homogenized using a Waring blender (4 x 20 sec bursts with 10 sec intervals). The pH of the homogenate was readjusted to pH 7.5 by the addition of 2M KOH before centrifugation at 4,000 x g for 20 min. The supernatant was subjected to further centrifugation at 13,300 x g for 20 min. The pellets were discarded, and the supernatant was adjusted to 0.5M KCl by the addition of the solid salt whilst stirring. The solution was incubated for 15 minutes at 4°C before centrifugation at 22,500 x g for 4 hours. The supernatant was discarded and the pellets were homogenized in 100ml of 0.3M sucrose buffer, using a potter homogenizer with a teflon pestle. Membranes were pelleted by centrifugation at 160,000 x g for 30 min. The pellets were resuspended in 30ml 0.3M sucrose buffer, and were homogenized thoroughly in a potter homogenizer (10 strokes) before layering onto discontinuous sucrose gradients. 5ml aliquots of the homogenate were layered onto 28ml pads of 0.9M sucrose, 40mM MOPS, pH 7.5. The sucrose gradients were subjected to centrifugation at 65,000 x g for 16 hours. T-tubule membranes were collected from the 0.3M-0.9M sucrose interface and were diluted 3 fold with 20mM Tris/HCl, pH 7.5 before centrifugation at 160,000 x g for 30 minutes. The pellets were resuspended in 20mM Tris/HCl, pH 7.5 to achieve a final protein concentration of 6-10mg/ml.

Protein concentration was estimated routinely by measuring the absorbance of an aliquot of the membrane fraction, in 2% (w/v) sodium dodecyl sulphate (SDS), at 260nm and 280nm. Accurate protein concentrations were determined by a modification of the

method of Peterson (1977, Section 3.3.12.). T-tubule membranes were assayed for 1,4-DHP binding sites as described in Section 2.3.2. Membranes were frozen in 50µl aliquots in liquid nitrogen and stored at -70°C.

2.3.2. Equilibrium Binding of (+)-[^{3}H]PN200-110 to Membrane-Bound and Detergent Solubilized 1,4-Dihydropyridine Receptors.

Assay for Membrane-Bound 1,4-Dihydropyridine Receptors

All binding experiments were carried out in the absence of direct light due to the light sensitivity of the 1,4-DHP derivatives. Routine assays for membrane-bound 1,4-DHP receptors were based on the method of Fosset et. al. (1983). T-tubule membranes (15µg protein) were incubated in 1ml of assay mixture containing 50mM Tris/HCl, pH 7.5, with varying concentrations of (+)-[^{3}H]PN200-110 up to ~3nM. Non-specific binding was determined in parallel incubations in the presence of excess (1µM) unlabelled nitrendipine. Assays were incubated for 60 minutes at 4°C. Duplicate 400µl aliquots of the incubation mixture were filtered through Whatman GF/B microfibre glass filters, under reduced pressure. Filters were immediately washed twice with 5ml 20mM Tris/HCl, pH 7.5. The filters were counted in 4ml Optiscint T scintillation fluid on a Packard B2450 Beta-counter. The total concentration of (+)-[^{3}H]PN200-110 present in each incubation was determined by counting a 100µl aliquot of assay mixture in 4ml Optiphase T. Specific (+)-[^{3}H]PN200-110 binding was calculated by subtracting the non-specific binding from the total binding found in the absence of unlabelled nitrendipine.

Assay for Detergent Solubilized 1,4-Dihydropyridine Receptors

Routine assays for detergent solubilized 1,4-DHP receptors were based on the method of Borsotto et. al. (1984a). T-Tubule membranes (10-15µg protein) solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate (CHAPS) detergent (Section 2.3.3.) were diluted 1/20 in medium A (0.1% (w/v) CHAPS, 0.5% (w/v) glycerol, 0.02% (w/v) soybean phosphatidylcholine (SPC), 140mM NaCl, 20mM Tris/HCl, pH 7.5) and assayed for 1,4-DHP binding activity in a total volume of 0.5ml. The incubations contained 0.1% CHAPS, 50mM Tris/HCl, pH 7.5 and varying

concentrations of (+)-[^{3}H]PN200-110. Assay tubes were incubated for 60 min at 4°C. Duplicate 200µl aliquots of the incubation mixture were subjected to rapid gel filtration on 1.6ml minicolumns of Sephadex G50, pre-equilibrated with 0.05% CHAPS, 20mM Tris/HCl, pH 7.5. Filtration was facilitated by the centrifugation of the minicolumns at 1,000 x g for 1 min. Excluded volumes were added to 3ml Optiphase X and counted in a Packard B2450 Beta-Counter. The total concentration of (+)-[^{3}H]PN200-110 present in each incubation was determined by counting a 50µl aliquot of the assay mixture in 4ml Optiphase T. Non-specific binding was determined in parallel incubations in the presence of excess (1µM) unlabelled nitrendipine.

2.3.3. Purification of the (+)-[^{3}H]PN200-110 Receptor

The (+)-[^{3}H]PN200-110 receptor was purified from rabbit skeletal muscle T-tubule membranes using a modification of the method of Borsotto et. al. (1984b). All stages were carried out at 4°C. The purification was monitored by pre-labelling a proportion of the receptors with (+)-[^{3}H]PN200-110, consequently all procedures were carried out in the absence of direct light due to the light sensitivity of this 1,4-DHP derivative.

Solubilization of the (+)-[^{3}H]PN200-110 Receptor

T-Tubule membranes (1ml, 6-10mg protein), prepared as described previously (Section 2.3.1.), were diluted to a volume of 1.5ml with 20mM Tris/HCl, pH 7.5 and adjusted to contain the following protease inhibitors; iodoacetamide (2mM); phenylmethylsulphonylfluoride (PMSF) (0.2mM); pepstatin A (2µM). Following the addition of 1.5ml 2% CHAPS, 20% glycerol, 20mM Tris/HCl, pH 7.5 the mixture was incubated for 30 min at 4°C. The suspension was subjected to centrifugation at 160,000 x g for 30 min at 4°C. The supernatant was retained and aliquots were assayed for protein concentration (Section 2.3.4.) and diluted 1/20 in medium A for assay of solubilized 1,4-DHP binding sites as previously described (Section 2.3.2.).

Gel Filtration on Ultrogel A2

The CHAPS extract was incubated in the presence of 4nM (+)-[^{3}H]PN200-110 for 30 min at 4°C. The mixture was loaded onto a 0.9 x 50cm Ultrogel A2 column, pre-equilibrated with 0.1% CHAPS, 5% glycerol, 0.02% SPC, 140mM NaCl, 20mM Tris/HCl, pH 7.5 (medium A). The column was eluted overnight with medium A and 1ml fractions were collected. 50µl aliquots from each fraction were filtered on 1.6ml minicolumns of Sephadex G50 as described in Section 2.3.2. Excluded volumes were counted in Optiphase X to locate the eluted peak of receptor-bound (+)-[^{3}H]PN200-110. A small aliquot from each fraction was retained for protein assay (Section 2.3.4.). Fractions containing the peak of (+)-[^{3}H]PN200-110 activity were pooled for subsequent affinity chromatography. A small proportion of this material was assayed for 1,4-DHP binding sites as previously described for the solubilized 1,4-DHP receptor (50µl pooled fraction per assay tube).

Affinity Chromatography on Wheat Germ Agglutinin Ultrogel

10% (w/v) Triton X-100, 20mM Tris/HCl, pH 7.5 was added to the pooled Ultrogel A2 fractions to result in a final concentration of 1% Triton X-100. After gentle mixing the mixture was incubated at 4°C for 15 min. The mixture was loaded onto a 1.8ml column of WGA-Ultrogel, pre-equilibrated with 0.1% Triton X-100, 5% glycerol, 0.02% SPC, 140mM NaCl, 20 mM Tris/HCl, pH 7.5 (medium T). The breakthrough was reapplied to the column to ensure interaction with the soluble receptor. The breakthrough from the repeated applications was collected and the column was washed sequentially with 10ml medium T, followed by 10ml 1% CHAPS, 5% glycerol, 0.02% SPC, 140mM NaCl, 20mM Tris/HCl, pH 7.5 (medium B) and 5ml medium A. The 1,4-DHP receptor was eluted with 10ml 200mM N-acetyl-D-glucosamine in medium A. Fractions (1ml) were collected throughout the washing and elution procedure. A 50µl aliquot of each fraction of the 200mM N-acetyl-D-glucosamine elution was incubated in the presence of 1nM (+)-[^{3}H] PN 200-110 for 30 min at 4°C, followed by rapid gel filtration on Sephadex G50 minicolumns as previously described (Section 2.3.2.) to determine the eluted peak of (+)-[^{3}H]PN200-110 receptor. Full Scatchard analysis of (+)-[^{3}H]PN200-110 binding

was not routinely performed on the purified fractions in order to conserve purified protein for subsequent experiments. A small aliquot from each fraction was retained for protein assay (Section 2.3.4.). Purified fractions containing the peak of (+)-[^{3}H]PN200-110 activity were frozen and stored at -20°C.

2.3.4. Determination of Protein Concentration

Protein concentration was determined using the Coomassie Blue technique of Bradford (1976), using bovine serum albumin as a standard. Protein assays were performed on diluted CHAPS extract and 50µl aliquots of column fractions. All test and standard samples were adjusted to 200µl in volume with deionized water before the addition of 1ml of protein assay reagent (0.1% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 4.25% (w/v) phosphoric acid or Pierce Protein Assay Reagent). After mixing well and incubating for 5 min at room temperature, the absorbance at 595nm was measured against a water/reagent blank. Every sample was read within an hour of the addition of protein reagent. Any absorbance due to detergent was corrected for by the inclusion of appropriate buffer blanks in the assay. A standard curve was plotted of bovine serum albumin protein (µg) vs corresponding absorbance and used to determine protein concentrations of the test fractions.

2.3.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

A modification of the method of Laemmli (1970) was used to analyse protein profiles of column fractions by SDS gel electrophoresis on linear polyacrylamide gradients. Samples, containing 2-6µg of protein were denatured by incubation for 3 min at 95°C in 2% (w/v) SDS, 9% (w/v) glycerol, 20% (w/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue, 75mM Tris/HCl, pH 6.8. The gel system used consisted of a stacking gel containing 4% (w/v) acrylamide, and a resolving gel containing a 4-12% (w/v) linear acrylamide gradient (see Appendix 1). Electrophoresis was carried out at a constant current of 50mAmps per gel, with cooling of the lower tank buffer (see Appendix 1).

The molecular weight markers used were Sigma High Molecular Weight and Low Molecular Weight standard kits containing; myosin (205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (21,100), α-lactoglobulin (14,200).

2.3.6. Silver Staining of Polyacrylamide Gels

Polypeptides separated by SDS polyacrylamide gel electrophoresis were stained using a modification of the method of Merril et. al. (1981). Proteins were fixed by soaking the gel overnight in 200ml 50% methanol, 12% acetic acid. The gel was then washed for three periods of 10 min in 200ml of a solution containing 10% ethanol, 5% acetic acid. The proteins in the gel were oxidized by incubation with 200ml 0.1% (w/v) potassium dichromate, 0.05% nitric acid, for 5 min with continual rocking. This was followed by four 30 sec washes in deionized water. After washing the gel was soaked for 30 min in 200ml 0.2% (w/v) silver nitrate, with illumination for the first 5 minutes only, and gentle rocking for the remaining 25 min. The silver nitrate solution was discarded and the gel was washed in 300ml 3% (w/v) sodium carbonate, 0.4% formaldehyde, until silver precipitate formed on the protein bands. The gel was allowed to stand in fresh sodium carbonate solution until the required intensity of staining was achieved. Photographs of the developing gel were taken to allow the overdevelopment of staining to detect the minor protein components. Staining was terminated by the addition of 1% acetic acid after first discarding the sodium carbonate solution. For storage, gels were normally washed well with water before drying using an LKB slab gel dryer.

2.4. RESULTS

2.4.1. Transverse Tubule Membrane Preparation

T-Tubule membranes were prepared from rabbit skeletal muscle tissue as described in Section 2.3.1. Typical yields of T-tubule membrane protein using this preparation procedure were 20-25mg protein from 300g of tissue. The quality of the T-tubule membrane preparation was routinely assessed by equilibrium (+)-[^{3}H]PN200-110

binding assays (Section 2.3.2.). The membrane fractions, stored at -70 C, were generally used for further study within a month of freezing. It was determined that the inclusion of protease inhibitors throughout the preparation procedure did not improve the 1,4-DHP binding activity of the T-tubule membranes, consequently protease inhibitors were not routinely included in the preparation.

2.4.2. Equilibrium Binding of (+)-[^{3}H]PN200-110 to Transverse Tubule Membranes

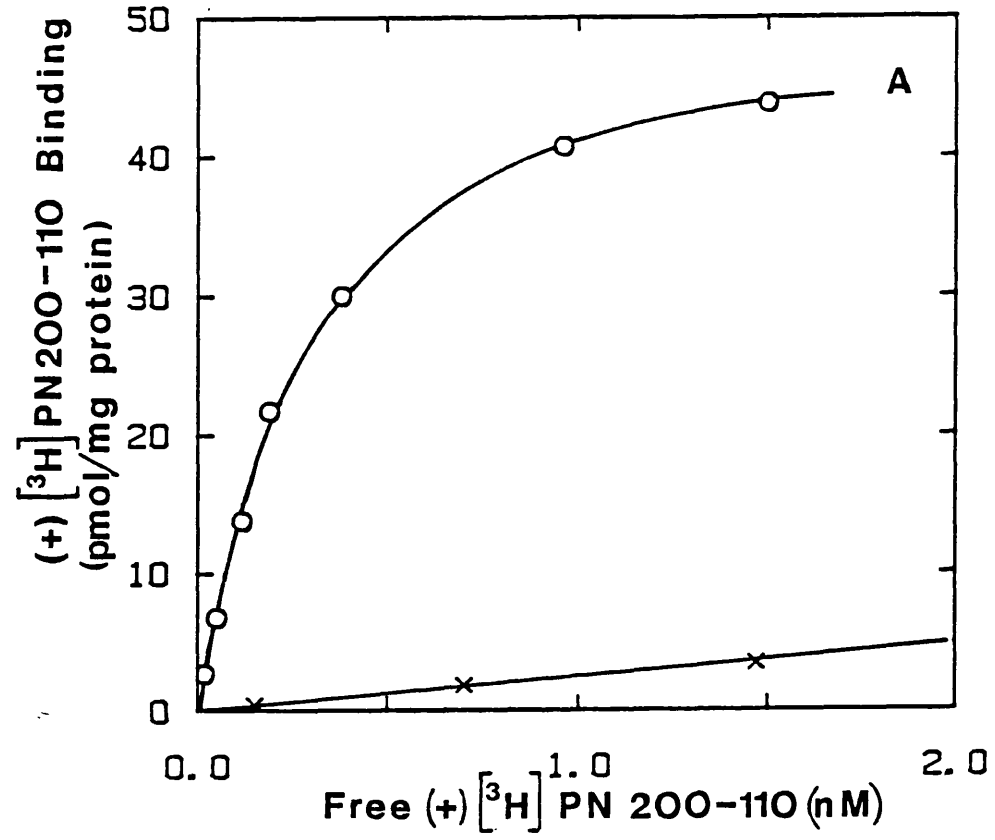
Figure 7A shows the equilibrium binding of the 1,4-DHP derivative, (+)-[^{3}H]PN200-110, to T-tubule membranes prepared from rabbit skeletal muscle. Non-specific binding, measured in the presence of excess unlabelled 1,4-DHP (1μM nitrendipine), is non-saturable and increases linearly with increasing tritiated ligand concentration. Specific binding, determined as the difference between the total (+)-[^{3}H]PN200-110 binding and the non-specific binding, is a saturable function of (+)-[^{3}H]PN200-110 concentration. The Scatchard plot derived from these data gives a linear plot indicating the presence of a single class of non-interacting (+)-[^{3}H]PN200-110 binding sites (Fig. 7B). The apparent dissociation constant (K_d) for (+)-[^{3}H]PN200-110 binding to T-tubule membranes, evaluated from the slope of the plot is; K_d= 0.48 ± 0.07 nM (n=10) (Fig. 7B). This low K_d indicates the high affinity of this ligand for the membrane-bound 1,4-DHP receptors. For this reason, (+)-[^{3}H]PN200-110 was used in this study to prelabel receptors for purification studies. The maximal (+)-[^{3}H]PN200-110 binding capacity (B_{max}) for T-tubule membranes, defined by the x-axis intercept, is; B_{max}=47.6 ± 6.0 pmol/mg protein (n=10) (Fig. 7B). This is high compared to the B_{max} for 1,4-DHP binding to membranes prepared from other tissues; 0.5-2.0 pmol/mg protein for cardiac, brain and smooth muscle tissue (Section 1.3.1. Table 2). Such an enriched source of 1,4-DHP receptor activity make T-tubule membranes a good starting material for 1,4-DHP receptor purification.

Equilibrium (+)-[^{3}H]PN200-110 binding assays were performed to assess the quality of the T-tubule membrane preparation. The normal range of 1,4-DHP binding activities determined in this way were; B_{max}=30-60 pmol/mg protein. On occasions, low binding activities of 20-30 pmol/mg protein were detected. In general, these low activities were

Figure 7

Equilibrium binding of (+)-[^{3}H]PN200-110 to transverse tubule membranes.

T-Tubule membranes (15μg protein/ml) were incubated for 60 min. at 4°C as described in Section 2.3.2 with the indicated concentrations of (+)-[^{3}H]PN200-110. (A) (o) Total binding to T-tubule membranes; (x) Non-specific binding measured in the presence of 1μM nitrendipine. (B) Scatchard plot of the specific binding.


A
(+) [3H] PN200-110 Binding
(pmol/mg protein)
50
40
30
20
10
0
0.0
1.0
2.0
Free (+) [3H] PN 200-110 (nM)

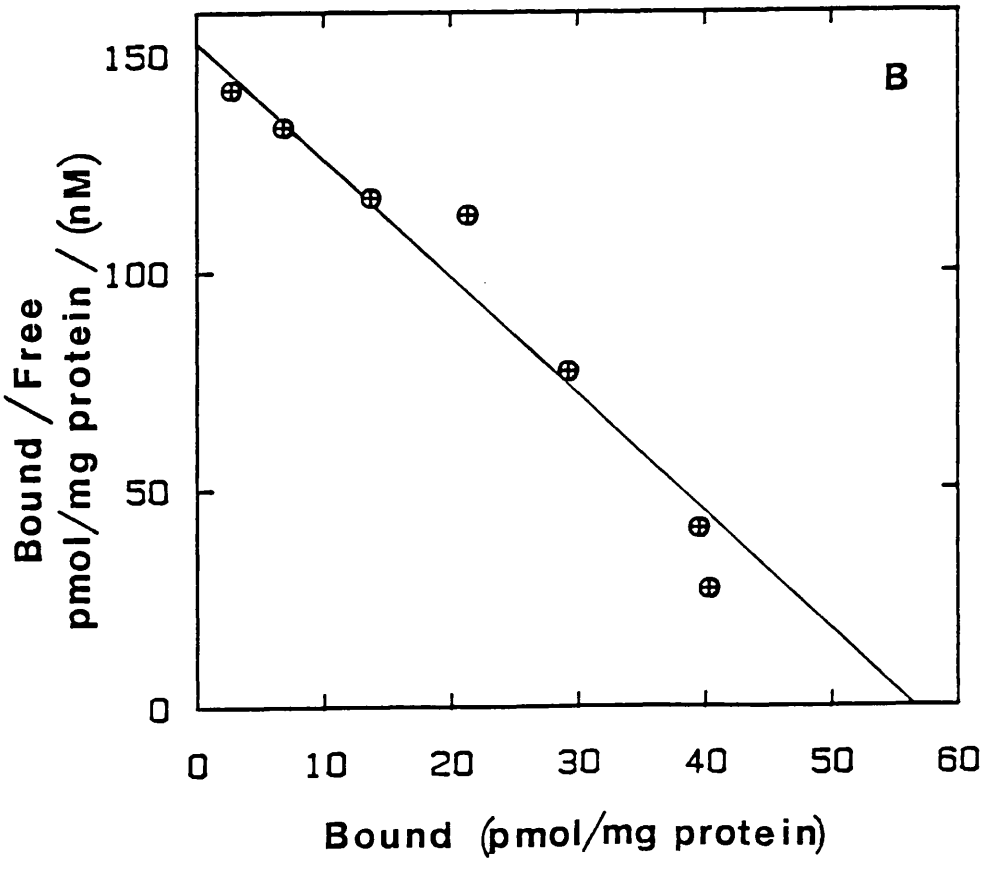
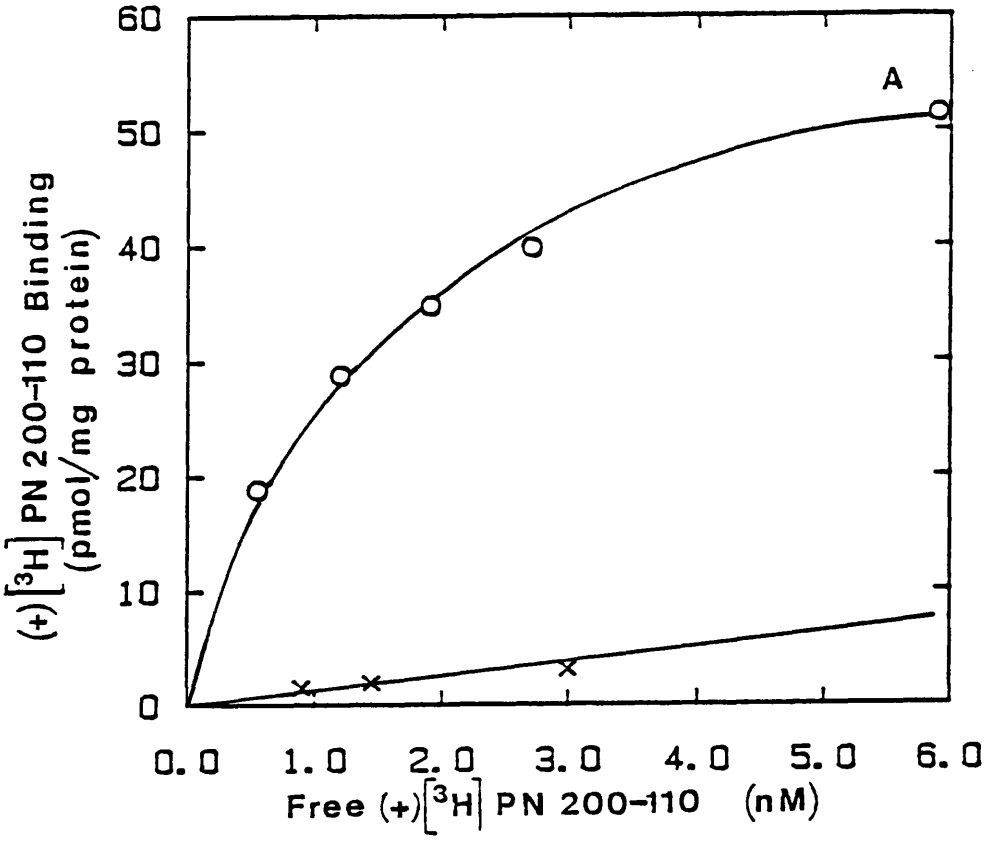
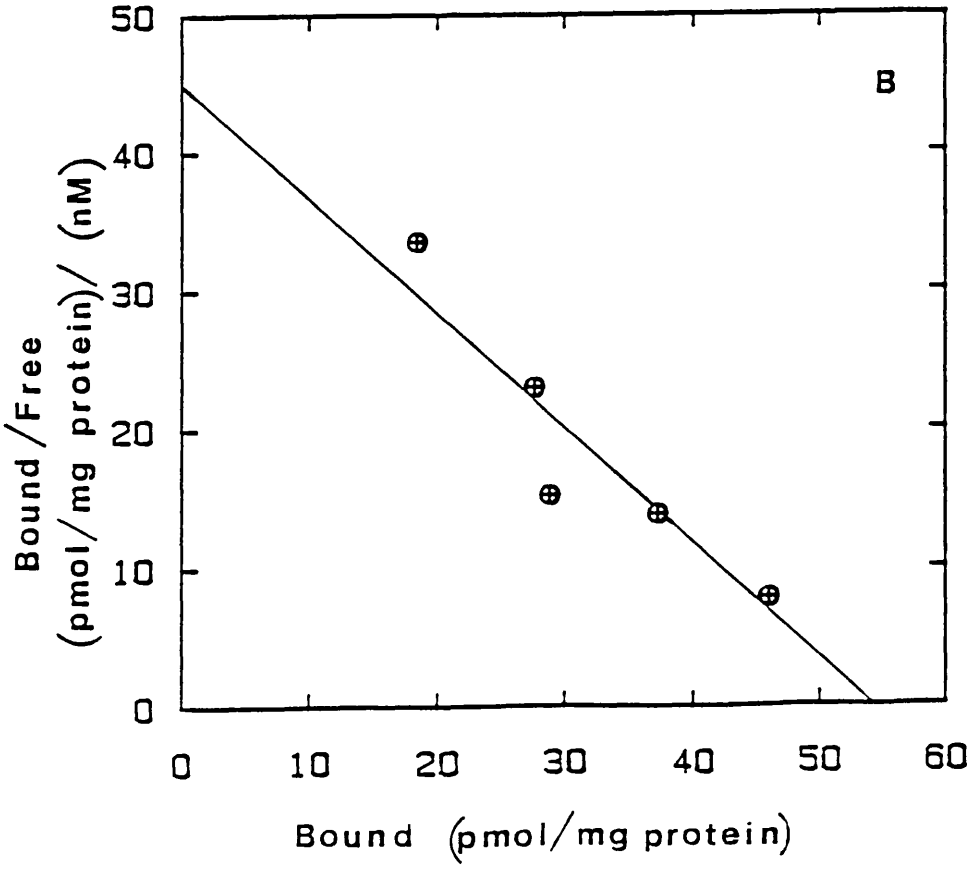

B
Bound / Free
pmol/mg protein / (nM)
150
100
50
0
0
10
20
30
40
50
60
Bound (pmol/mg protein)

Figure 8

Equilibrium binding of (+)-[^{3}H]PN200-110 to CHAPS solubilized transverse tubule membranes. CHAPS solubilized T-tubule membranes diluted 1/20 in medium A (14μg protein/0.5ml) were incubated as described in Section 2.3.2. with the indicated concentrations of (+)-[^{3}H]PN200-110. (A) ⊙ Total binding to CHAPS solubilized T-tubule membranes; ⊗ Non-specific binding measured in the presence of 1μM nitrendipine.

(B) Scatchard plot of the specific binding.


A
(+)[3H] PN 200-110 Binding
(pmol/mg protein)
0
10
20
30
40
50
60
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Free (+)[3H] PN 200-110 (nM)


B
Bound /Free
(pmol/mg protein)/ (nM)
0
10
20
30
40
50
0
10
20
30
40
50
60
Bound (pmol/mg protein)

due to long periods of storage of the frozen tissue (>2 weeks) before T-tubule membrane preparation.

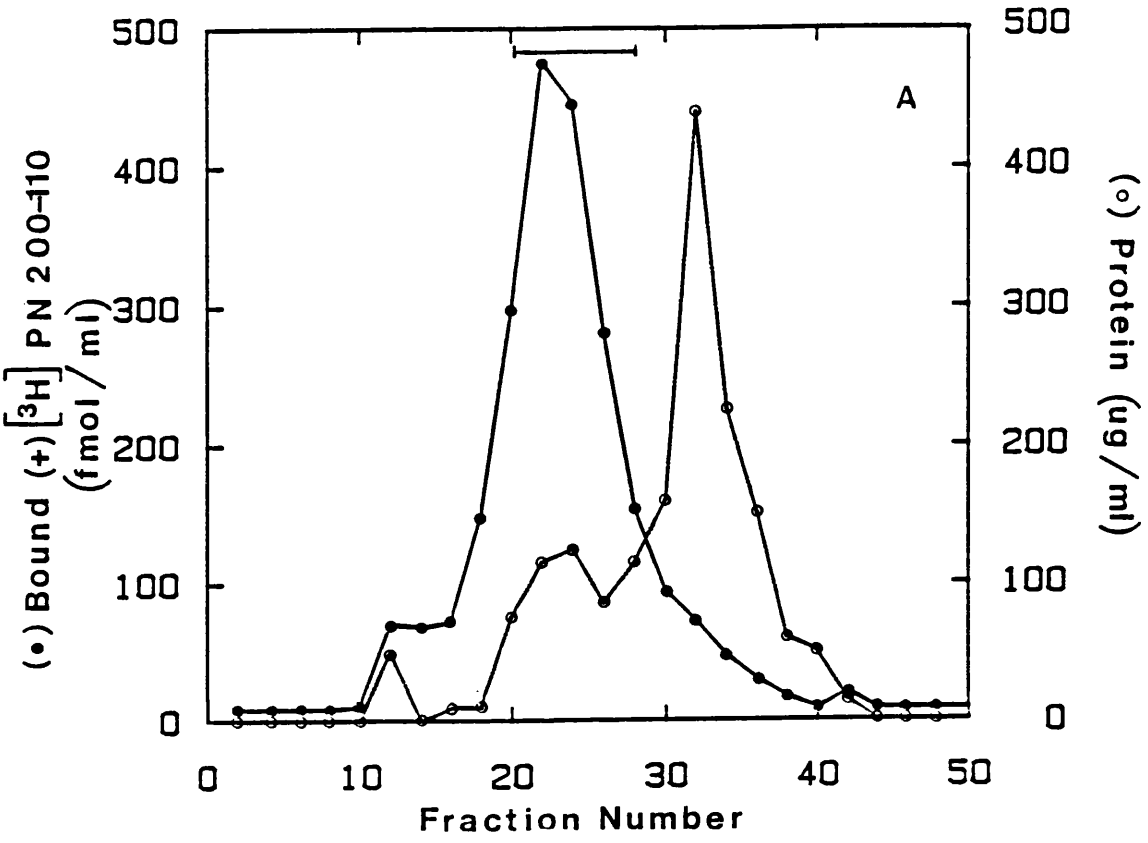
2.4.3. Solubilization of the (+)-[^{3}H]PN200-110 Receptor from Transverse Tubule Membranes

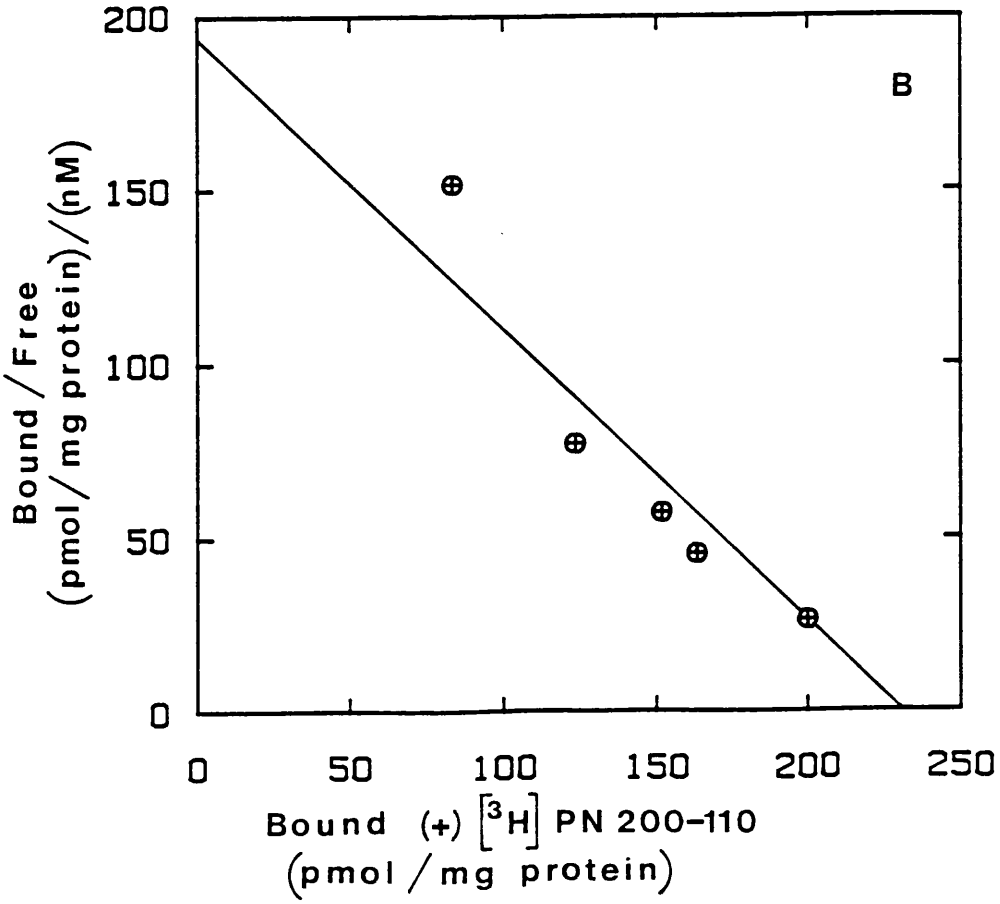
(+)-[^{3}H]PN200-110 receptors were solubilized as described in Section 2.3.3. in the presence of 1% (w/v) CHAPS and 10% (w/v) glycerol. CHAPS detergent with glycerol present as a stabilizing agent has been shown to solubilize active receptors with very similar 1,4-DHP binding properties to the membrane-bound form (Borsotto et. al. 1984a). Using this method 75.0% ± 10.0% of the (+)-[^{3}H]PN200-110 binding activity measured by equilibrium binding (Section 2.3.2.) could be recovered routinely in the CHAPS extract for subsequent purifications. Figure 8 shows the equilibrium binding of (+)-[^{3}H]PN200-110 to the crude CHAPS extract. The Scatchard plot of the binding data (Fig. 8B) is linear indicating that (+)-[^{3}H]PN200-110 binding is to a single class of non-interacting solubilized receptor sites. The apparent dissociation constant (K_d) is 1.2 ± 0.48 (n=10). nM^This is slightly higher than that obtained for the membrane-bound receptor, indicating that the affinity of the detergent solubilized receptor for (+)-[^{3}H]PN200-110 is lower than that in the membrane-bound state. The B_{max} is 5 1.1 ± 10 pmol/mg protein (n=10), essentially unchanged from that for T-tubule membranes.

2.4.4. Purification of the (+)-[^{3}H]PN200-110 Receptor from Transverse Tubule Membranes

Before application to the Ultrogel A2 column, the CHAPS extract was incubated with (+)-[^{3}H]PN200-110 (4nM) as described in Section 2.3.3. Under these conditions there was a large excess of available 1,4-DHP receptor sites over ligand (see Table 8 legend). Therefore, the radiolabelled ligand would bind stoichiometrically to the receptor prelabelling a small proportion of the available sites (4-8%) and hence enabling the monitoring of the purification procedure by rapid gel filtration of aliquots of the column fractions.

The CHAPS extract was loaded onto an Ultrogel A2 gel filtration column and eluted with medium A as described in Section 2.3.3. Figure 9A shows a typical elution profile for the gel filtration procedure. Receptor-bound (+)-[^{3}H]PN200-110 was monitored by


A
(•) Bound (+) [3H] PN 200-110 (fmol / ml)
(○) Protein (ug / ml)
Fraction Number
0
100
200
300
400
500
0
10
20
30
40
50


B
Bound / Free (pmol / mg protein) / (nM)
Bound (+) [3H] PN 200-110 (pmol / mg protein)
0
50
100
150
200
250

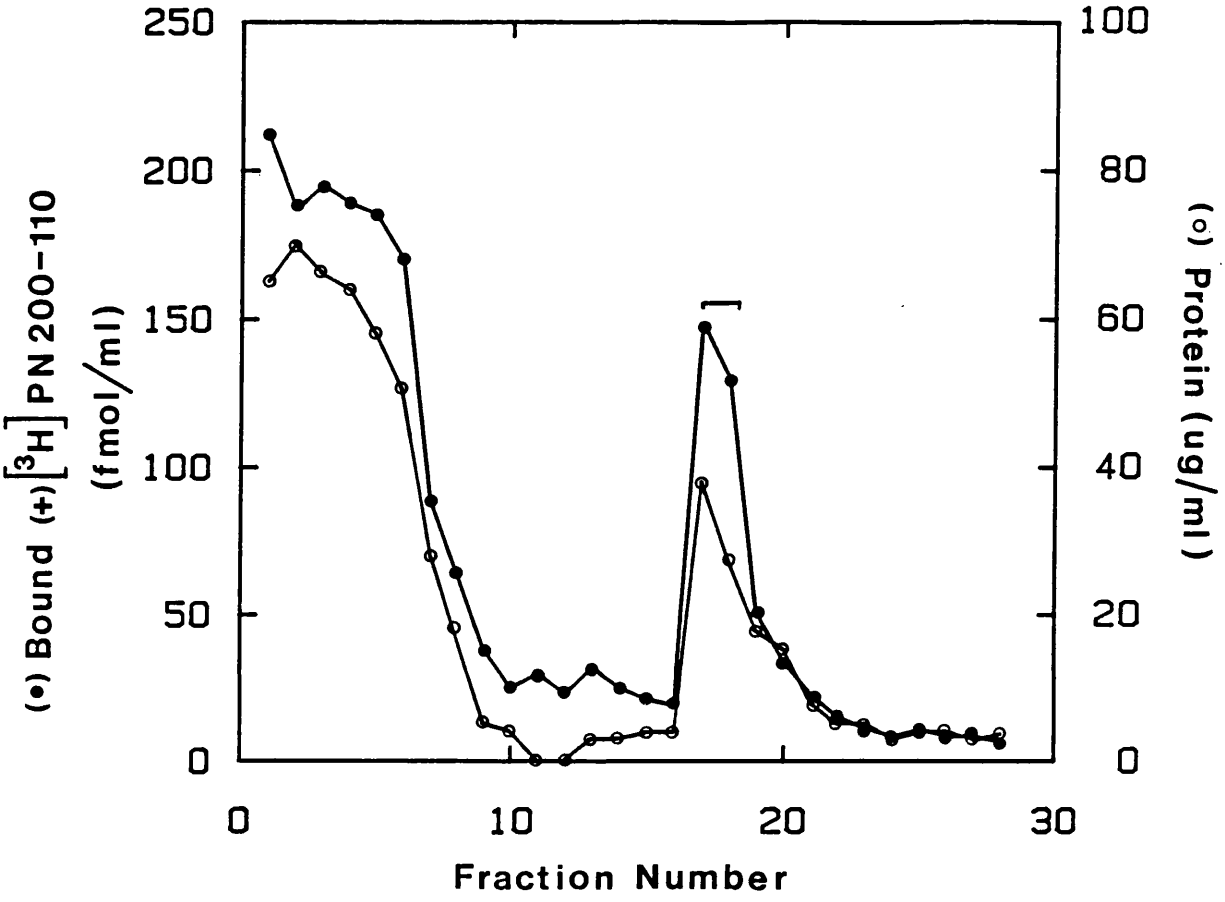


Figure 10

Elution profile of detergent solubilized (+)-[^{3}H]PN200-110 receptor from Wheat Germ Agglutinin. Receptor-bound (+)-[^{3}H]PN200-110 was determined by the incubation of 50µl aliquots of column fractions for 30 min at 4°C with 1nM (+)-[^{3}H]PN200-110. Following rapid filtration on G50 minicolumns the excluded volumes were counted as described in Section 2.3.3. (•) Bound (+)-[^{3}H]PN200-110 (fmol/ml); (o) Protein (µg/ml) determined as described in Section 2.3.4. Pooled fractions indicated by bar.

counting the excluded volumes of Sephadex G50 mini columns following rapid gel filtration of aliquots from the eluted fractions as described previously (Section 2.3.2). Receptor-bound (+)-[^{3}H]PN200-110 was eluted as a single peak between fractions 20-29. The major peak of protein (fractions 30-40) contained very little (+)-[^{3}H]PN200-110 binding activity. Free (+)-[^{3}H]PN200-110 eluted from the column in later fractions (data not shown). Fractions containing the peak of bound (+)-[^{3}H]PN200-110 activity were pooled. Figure 9B shows the Scatchard plot for equilibrium binding of (+)-[^{3}H]PN200-110 performed on the pooled fractions. The assay was performed as described in Section 2.3.2. for the crude CHAPS extract, with 50µl Ultrogel A2 pooled fractions per assay tube. The apparent dissociation constant (K_d) determined for this partially purified material (1.23 ± 0.25 nM, $n=6$) was very similar to that for the crude CHAPS extract.

In initial experiments the Ultrogel A2 peak fractions were loaded immediately onto the WGA Ultrogel column without any pretreatment. However, it was found that the presence of contaminating peptides was reduced if an additional detergent treatment with 1% Triton X-100 (Section 2.3.3.), was performed before the lectin gel affinity chromatography step. The mixture was loaded onto the WGA Ultrogel column, with a second pass to ensure efficient adsorbtion of the material onto the column. Following extensive washing to remove any material bound non-specifically to the column, the specifically bound material was eluted in the presence of 200mM N-acetyl-D-glucosamine. Elution with this competing sugar resulted in a single peak of protein containing the (+)-[^{3}H]PN200-110 binding activity. Figure 10 shows a typical elution profile for the WGA Ultrogel. The large amount of protein in the early wash fractions was due to the high concentration of Triton X-100 present which interferes with the Bradford protein assay. Table 8 shows the yields and specific activity of the purified fractions.

2.4.5. Subunit Composition of the 1,4-Dihydropyridine Receptor

The polypeptide profiles of purified fractions eluted from the WGA Ultrogel column were analysed by SDS polyacrylamide gel electrophoresis under reducing conditions as described in Section 2.3.5. and were visualized by silver staining, (Section 2.3.6.; Fig.

Step	Receptor-Bound [^{3}H]PN200-110 pmol	%	Protein mg	%	Specific Activity pmol/mg protein
T-Tubule membranes	325*	100.0	7.39	100.0	44
CHAPS extract	284*•	74.0	5.9	80.0	48
Ultrogel A2	122*	37.5	0.52	7.0	235
WGA Ultrogel	18"	5.5	0.065	0.88	554°

Table 8

Purification of the (+)-[^{3}H]PN200-110 Receptor

Table of the typical yields and specific activities of the Ultrogel A2 and WGA Ultrogel purified (+)-[^{3}H]PN200-110 receptor fractions.

* determined from Scatchard analysis; " determined from one point binding assays.

o The lack of substantial increase in the specific activity of the WGA Ultrogel purified fractions was a consequence of receptor inactivation during the purification procedure.

• Total 1,4-DHP receptor sites, determined from Scatchard analysis, in 3ml CHAPS extract = 284 pmol. Concentration of available sites in CHAPS extract = 95 pmol/ml.

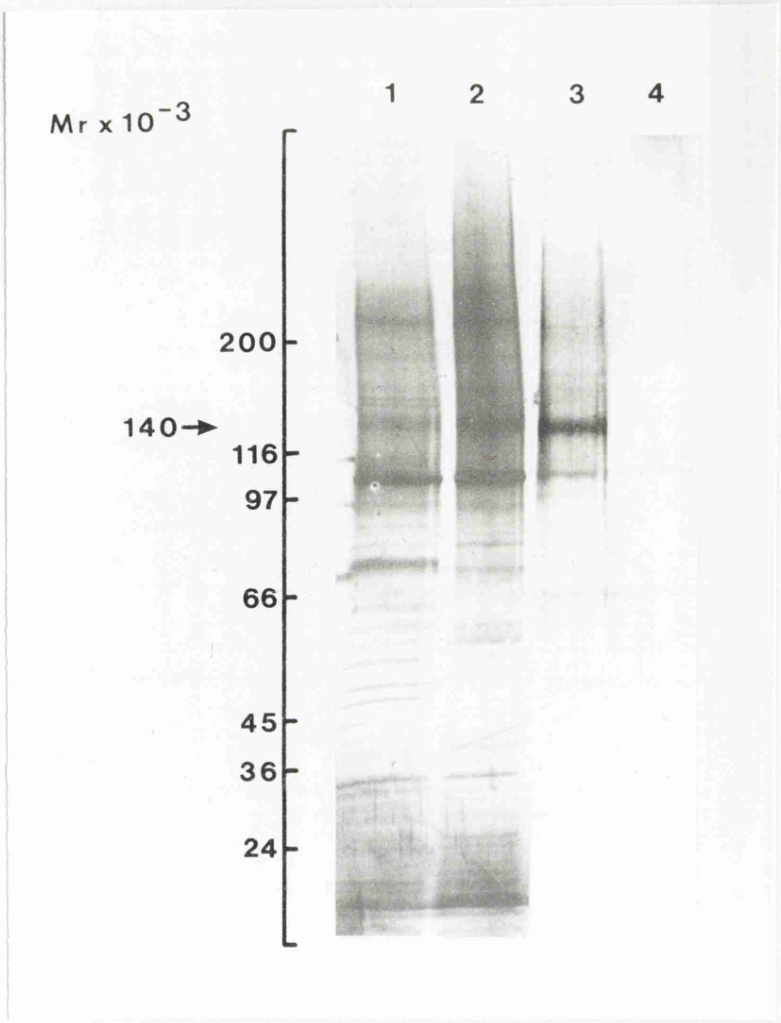


Figure 11

Subunit composition of the 1,4-dihydropyridine receptor analysed by SDS polyacrylamide gel electrophoresis. Electrophoresis of column fractions, denatured under reducing conditions, on 4-12% polyacrylamide gel as described in Section 2.3.5. Silver stained as described in Section 2.3.6. Lane 1, crude CHAPS extract (6μg); lane 2, pooled Ultrogel A2 column fractions (6μg); lane 3, eluted peak fraction from WGA-Ultrogel (2μg); lane 4, medium A (50μl). Mr markers: myosin (200,000); β-galactosidase (116,000); phosphorylase b (97,000); bovine serum albumin (66,000); ovalbumin (45,000); glyceraldehyde-3-phosphate dehydrogenase (36,000); carbonic anhydrase (29,000). Specific activities; CHAPS extract, 42 pmol/mg protein; pooled Ultrogel A2 fractions, 90 pmol/mg protein; WGA-Ultrogel peak fraction, 517 pmol/mg protein.

11). Fractions containing the eluted peak of (+)-[^{3}H]PN200-110 activity were composed of a major polypeptide component of apparent molecular weight (Mr) 140,000 (Fig. 11, lane 3). In addition to this component, a small polypeptide of Mr~30,000 was occasionally detected in silver stained polyacrylamide gels of the WGA affinity chromatography purified fractions. Another minor polypeptide component occasionally co-purified with the Mr 140,000 polypeptide and was detected in silver stained polyacrylamide gels as a sharp band of Mr 105,000 (Fig. 11, lane 3). As these components were not consistently detected it is possible that they were degradation products of the large Mr 140,000 polypeptide. However other workers consistently detect a small molecular weight polypeptide of Mr~30,000 indicating that this may be a further component of the 1,4-DHP receptor (Borsotto et. al., 1984b). It is likely that the Mr 140,000 component is a glycoprotein since it is purified by lectin affinity chromatography. The diffuse staining of this component in silver stained polyacrylamide gels is also characteristic of a glycosylated polypeptide.

Using the purification procedure described in this study the Mr 140,000 glycoprotein subunit was the only polypeptide component consistently detected in WGA affinity purified fractions containing the eluted peak of receptor-bound (+)-[^{3}H]PN200-110 activity. This indicates that this glycoprotein component is involved in the 1,4-DHP receptor structure. The other minor components of Mr 30,000 and Mr 100,000 may be contaminating polypeptides that co-purify with the Mr 140,000 component. Alternatively, it is possible that they may be other subunits involved in the receptor complex. The purified 1,4-DHP receptor protein was used to immunize mice for the production of monoclonal antibodies. These antibodies were used as specific probes to further characterize this glycoprotein and to resolve the possible role of the minor components detected in the purified fractions in the voltage-sensitive Ca^{2+} channel structure.

2.5. SUMMARY

The T-tubule membrane system of skeletal muscle provides a rich source of 1,4-DHP receptors, typical values 30-60 pmol/mg protein, and consequently is an ideal starting material for the purification of these receptors. The purification is monitored by prelabelling a small proportion of the 1,4-DHP receptors with the high affinity radiolabelled 1,4-DHP, (+)-[^{3}H]PN200-110. The purification procedure is a three stage process involving; the detergent solubilization of the (+)-[^{3}H]PN200-110 receptor in an active form, the separation of the receptor activity from the major peak of contaminating protein by gel filtration, and finally an affinity chromatography step on WGA Ultrogel. The major component of the purified fractions is a glycoprotein of Mr 140,000, however in some experiments minor components of Mr 105,000 and Mr 30,000 are detected. Fractions purified in this manner were used to immunize mice for the production of monoclonal antibodies.

CHAPTER 3

CHARACTERIZATION OF THE 1,4-DIHYDROPYRIDINE SENSITIVE CALCIUM CHANNEL USING MONOCLONAL ANTIBODIES

3.1. INTRODUCTION

Monoclonal antibodies provide highly specific probes for the characterization of channel protein structure and have proved useful in the elucidation of the subunit comoposition of the Na^+ channel and the nAchR (Section 1.4.4.). Immunocrossreactivity studies can be performed to demonstrate possible homologies between protein from different sources or between different subunits of a particular protein complex. Topological models for the protein structure can be produced by mapping the monoclonal antibody binding sites to particular regions (epitopes) of the polypeptide sequence under investigation. Epitope mapping of the binding sites of antibodies that affect function of the channel protein can also be utilized to relate the protein function to particular regions of the channel protein structure.

Monoclonal antibodies can be covalently linked to fluorescent markers. In this form antibodies can provide useful probes for the localization of channel protein components in different tissues. In addition, more rapid protein purification procedures can be facilitated by the coupling of the monoclonal antibodies to affinity gel supports to form specific immunoaffinity columns. The amino acid sequences of some channel proteins have now been determined (Section 1.4.5.). Monoclonal antibodies have also proved useful in these studies for the identification of fusion proteins containing amino acid sequences encoded by channel mRNA. This is one approach by which cDNA libraries can be screened for the eventual elucidation of the amino acid sequences of channel proteins.

In this study the purified 1,4-DHP receptor was used to immunize mice for the

production of monoclonal antibodies. These provided specific probes for the further characterization of the 1,4-DHP sensitive Ca^{2+} channel.

3.2. MATERIALS

Two different types of nitrocellulose paper were routinely used; Sartorius nitrocellulose and Hybond C. Hybond C, biotinylated anti-mouse IgG, and the [^{125}I]Streptavidin (0.74MBq/μg) were from Amersham International PLC. Rabbit anti-mouse IgG, 3,3',5,5' tetramethylbenzidine (TMB), Freunds Incomplete and Complete Adjuvant were obtained from Miles. Goat anti-rabbit peroxidase, swine anti-rabbit IgG, rabbit peroxidase anti-peroxidase were all from Dakopatts. The microtitre plates were obtained from Flow Labs. Endo-β-N-glucosaminidase (Endoglycosidase F) came from Boehringer. The X-ray film was obtained from Figi and Napthol Blue Black (C.I. 20470), trichloracetic acid (TCA) and Folin-Ciocalteu's phenol reagent were from BDH. All the following were obtained from Sigma; dithiothreitol (DTT), Concanavalin A-peroxidase and Wheat Germ Agglutinin-peroxidase, neuraminidase, α- and β-glucosidase, β-galactosidase, endo-β-N-glucosaminidase (Endoglycosidase H), trypsin, soybean trypsin inhibitor, N-acetyl-D-glucosamine, α-methyl-D-mannoside, 1-0-methyl-α-D-glucopyranoside, 3,3' diaminobenzidine (DAB), Nonidet P40, sodium lauryl sarcosine, trifluoromethanesulphonic acid (TFMS), pyridine. All other chemicals were of analytical grade.

ENZYMES Trypsin (EC 3.4.2.1.4.), neuraminidase (EC 3.2.1.18), α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.103), endo-β-N-glucosaminidase F (EC 3.2.1.96) and endo-β-N-glucosaminidase (EC 3.2.1.96).

3.3. METHODS

3.3.1. Preparation of Monoclonal Antibodies to the 1,4-Dihydropyridine Receptor

Monoclonal antibodies were prepared in collaboration with Dr T.M. Harrison. Alum precipitates were prepared containing 1,4-DHP receptor protein, purified as described in Section 2.3.3. 10μg purified receptor protein in 200μl were mixed with an equal volume of 10% (w/v) aluminium potassium sulphate ($AlK(SO_4)_2.12H_2O$), pH 6.0. The mixture was incubated for 30 min at room temperature and the precipitate pelleted by centrifugation (300 x g, 15 min). The precipitate was washed 3 times in phosphate buffered saline (PBS; 50mM sodium phosphate, pH 8.0, 0.9% (w/v) NaCl) and

resuspended in 0.4ml PBS, pH 8.0. The alum precipitate (0.2ml, 5.0μg protein) was used to immunize BALB/C mice by intraperitoneal injection, followed 6 weeks later by the injection of a similar amount of protein (5.0μg) in saline. One week before culling the animal received about 5.0μg purified receptor protein in Freund's Incomplete Adjuvant, followed by intraperitoneal injections of similar amounts of purified receptor in saline on each of the next six days. One day later the animal was culled and the spleen was removed. The spleen cells were fused with 10^7 NSO/1 myeloma cells by the method of Galfrè and Milstein (1981) with the exception that 50% (w/v) polyethylene glycol 4000 was used.

The hybrid-myeloma (hybridoma) cells were selected from parental myeloma and spleen cells by growing the fusion products in HAT medium (hypoxanthine (6-hydroxypurine), 1.0×10^{-4}M; aminopterin (4-amino-folic acid), 4.0×10^{-7}; thymidine, 1.6×10^{-5}M). In this medium only the hyridoma cells were able to actively multiply (Milstein, 1980; Galfrè & Milstein, 1981). Hybridoma cell lines were selected for replating by solid phase immunoassay (Section 3.3.2.). The supernatants from the antibody secreting cells were tested in the solid phase immunoassay using the purified receptor as the antigen. Cell supernatants containing the secreted antibody of interest were detected by visual screening of the immunoassay wells. The positive antibody producing cells were plated out to limiting dilution to ensure the monoclonal nature of the cell lines. About 10^7 monoclonal hybridoma cells were injected into pristane-primed BALB/C mice for the production of ascites fluids. Routinely, fluid was tapped from one animal up to three times before the animal was sacrificed. The different ascitic fluid taps from any one animal were not pooled but were stored separately at 4°C in the presence of sodium azide (0.02%). The solid phase immunoassay was performed on the ascitic fluid taps to enable the antibody titre to be assessed.

3.3.2. Solid Phase Immunoassay

Positive hybrid clones were selected for replating by a solid phase antibody assay (ELISA; enzyme-linked immunosorbant assay) in flat-bottomed microtitre plate wells (Flow Laboratories). Wells were coated for 16 hours at 4°C with 50ng purified 1,4-DHP

receptor protein in 50µl PBS, pH 8.0. The final concentration of CHAPS detergent applied to the microtitre plate wells was less than 0.003%. Wells were incubated for 1 hour at room temperature with 0.5% (w/v) casein, 10% calf serum (w/v), PBS, pH 8.0, to block any remaining protein binding sites, followed by a 2 hour incubation with 50µl of the hybridoma cell supernatants to be tested. Control wells, incubated with either non-immune serum or PBS, pH 8.0, were included to monitor the background signal. Subsequent incubations were each for 1 hour with the following antibodies: rabbit anti-mouse IgG (1:1000 dilution); goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 dilution). The antibodies were diluted in 1% (w/v) calf serum, 0.05% (w/v) Tween 20, PBS, pH8.0. Between each incubation the wells were washed 3 times with 0.05% Tween 20, PBS, pH 8.0. 50µl of 10mg/ml 3,3',5,5'tetramethylbenzidine in 0.1M sodium acetate/citrate, pH 6.0, 0.0045% hydrogen peroxide (H_2O_2) was added to each well as substrate for the IgG-linked peroxidase enzyme. The development of a blue colouration in wells was monitored visually and signified the presence of positive hybridoma supernatants.

3.3.3.Membrane Preparations

Transverse Tubule Membranes

T-Tubule membranes were prepared as described in Section 2.3.1.

Skeletal Muscle Microsomal Membranes

Microsomal membranes were prepared from skeletal muscle by the method of Fosset et. al. (1983). All stages were carried out at 4°C. Muscle tissue was homogenized in 10 volumes cold 0.3M sucrose, 40mM MOPS, pH 7.5. A Waring blender was used for rabbit and frog tissue and an omnimixer for tissue from rat and mouse. Following homogenization (4 x 20 sec with 10 sec intervals) the homogenate was subjected to centrifugation at 3,300 x g for 20 min at 4°C. The pellets were discarded and the supernatant was centrifuged at 13,200 x g for 20 min. Microsomal membranes were pelleted by the centrifugation of the resulting supernatant at 160,000 x g for 30 min. Pellets were washed twice in 0.3M sucrose, 40mM MOPS, pH 7.5 and twice in 20mM

Tris/HCl, pH 7.5 and resuspended in the latter buffer. Protein concentrations were monitored routinely by measuring the absorbance of an aliquot of the membrane fractions in 2% SDS at 260nm and 280nm. Accurate protein determinations were carried out using a modification of the method of Peterson (1977) (Section 3.3.8.).

Microsomal Membranes from Different Tissues

Microsomal membranes were prepared from tissues other than skeletal muscle according to the method of Ferry & Glossmann (1985). All stages were carried out at 4°C. Tissues, dissected from rabbit, were homogenized in 10 volumes cold 0.3M sucrose, 40mM MOPS, pH 7.5 in the presence of 1mM PMSF. A Waring blender was used for heart and liver tissue and an omnimixer for brain. With the exception of brain, the microsomal membranes were prepared from the tissue homogenates as previously described for skeletal muscle microsomal membranes. Pelleted microsomal membranes were resuspended in 20mM Tris/HCl, pH7.5. Following the homogenization of brain tissue, microsmal membranes were pelleted directly from the homogenate by centrifugation at 160,000 x g. The membranes were washed twice in 20mM Tris/HCl, pH 7.5 and resuspended as above. Protein concentrations were determined as for skeletal muscle microsomal membranes.

3.3.4. Transblotting

T-Tubule membranes, prepared as described in Section 3.3.1., were denatured in 2% SDS, 9% glycerol, 75mM Tris/HCl, pH 6.8 in the presence of either 0.6% (w/v) dithiothreitol (DTT) (disulphide-bond reducing conditions) or 8mM iodoacetamide (non-reducing conditions). Denatured membrane fractions (10-100µg protein per track, see figure legends) were subjected to electrophoresis on 4-12% linear polyacrylamide gradient gels (Appendix 1). Electrophoresis was carried out under conditions described in Section 2.3.4.

Microsomal membrane fractions, prepared from skeletal muscle from different species and from different rabbit tissues, were denatured as described for T-tubule membranes. In the case of denatured skeletal muscle microsomal membrane fractions (0.25-0.75mg

protein per lane, see figure legends) electrophoresis was carried out on 4-12% linear polyacrylamide gradients as described in Section 2.3.4. For microsomal membranes prepared from different tissues (1.85-3.0mg protein per track, see figure legends) electrophoresis was carried out on 4-12% linear polyacrylamide gradients. However, due to the large amounts of protein loaded on the gel, electrophoresis was performed at a low constant voltage of 30V for 16 hours.

T-Tubule membranes or skeletal muscle microsomal membranes, treated with trypsin (Section 3.3.6.), deglycosylated enzymatically (Section 3.3.7.) or chemically (Section 3.3.9.), were denatured as described above. Electrophoresis was carried out on 8% polyacrylamide gels (Appendix 1) at a constant current of 30mA per gel for 4 hours.

The resolved polypeptides were transferred onto nitrocellulose paper by a modification of the method of Gershoni and Palade (1982). Transfer was carried out for 2.5 hours at 80V using a Biorad Transblot cell with cooling of the tank buffer (25mM Tris, 192mM glycine, pH 8.4, 10% methanol). Resolved molecular weight markers were either stained in the gel using Fairbanks 1 solution (see Appendix 2), or transferred onto nitrocellulose and stained with amido black stain (see Appendix 2).

3.3.5. Antibody Overlay Protocol

Nitrocellulose bearing the electrophoretically resolved membrane proteins was incubated for 16 hours at 4°C with 200ml 1% (w/v) bovine serum albumin, 0.5% (w/v) casein, 0.05% (w/v) Tween 20, 0.9% (w/v) NaCl, 10mM Tris/HCl, pH 7.5 to block excess protein binding sites. All subsequent antibody incubations on the nitrocellulose were performed in a Deca-Probe (Höefner Scientific Instruments), which isolates individual tracks obviating the need to cut the paper. In all immunoblot analyses a control ascitic fluid, α-gyrase B (Harrison, T.M., unpublished results), raised against a bacterial protein was included to confirm specific staining by the αDHP-R antibodies.

Antibody binding to reduced membrane components was assayed by incubating the nitrocellulose bearing the resolved proteins for 2 hours at room temperature with the diluted ascitic fluid (1:500 dilution) in 0.05% Tween 20, 0.9% NaCl, 10mM Tris/HCl, pH 7.5. This was followed by sequential 1 hour incubations with; rabbit anti-mouse IgG

(1:500 dilution), swine anti-rabbit IgG (1:500 dilution) and finally rabbit peroxidase anti-peroxidase complex (1:500 dilution). All antibodies were diluted in 0.05% Tween 20, 0.9% NaCl, 10mM Tris/HCl, pH 7.5.

For antibody binding to resolved non-reduced membrane components the rabbit anti-mouse IgG incubation was followed by incubation with goat anti-rabbit IgG coupled to peroxidase (1:500 dilution). This overlay protocol reduced the non-specific staining due to endogenous immunoglobulin (Section 3.4.4.).

Immunoblots were then developed using 0.2mg/ml 3,3'diaminobenzidine (DAB), 0.9% NaCl, 20mM Tris/HCl, pH 7.5, 0.003% H_2O_2. Between each step the nitrocellulose was washed for 10 min with two changes of 0.25%(w/v) sodium lauryl sarcosine, 0.25%(w/v) Nonidet P-40, 1M NaCl, 10mM Tris/HCl, pH 7.5. Developed immunoblots were washed in deionized H_2O and dried between 3mm Whatman paper.

3.3.6. Lectin-Linked Peroxidase Overlay Protocol

Nitrocellulose paper bearing the electrophoretically resolved membrane proteins was incubated for 16 hours at 4°C with 200ml 1% bovine serum albumin, 0.5% Tween 20, 0.9% NaCl, 10mM Tris/HCl, pH 7.5. The nitrocellulose was then incubated for 3 hours with lectin-linked peroxidase in either the presence or absence of the competing sugar. Concanavalin A peroxidase (Con A peroxidase) was used at a concentration of 51µg/ml and the competing sugars, when required were α-methyl-D-mannoside (0.5M) and 1-0-methyl-α-D-glucopyranoside (0.5M). Wheat Germ Agglutinin peroxidase (WGA peroxidase) was used at a concentration of 36µg/ml and N-acetyl-D-glucosamine (0.5M) was the competing sugar. Following incubation the nitrocellulose was washed for 3 x 10 min with 0.25% sodium lauryl sarcosine, 0.25% Nonidet P-40, 1M NaCl, 10mM Tris/HCl, pH 7.5. Blots were developed with DAB solution as described in Section 3.3.5.

3.3.7. Mapping of Immunoreactive Peptides of Skeletal Muscle Microsomes from Different Species Following Trypsin Digestion

Skeletal muscle microsomal membranes, prepared from different species as described in Section 3.3.3, were diluted with 20mM Tris/HCl, pH7.5 to achieve a final protein

concentration of 4mg/ml in the final CHAPS extract (assuming 80% solubilization). Equal volumes of 2% CHAPS, 20% glycerol, 20mM Tris/HCl, pH 7.5 were added to the membrane fractions. Following a 30 min incubation at 4°C the mixtures were centrifuged for 30 min at 160,000 x g at 4°C to remove particulate material. The CHAPS extracts were subjected to limited digestion with trypsin based on the method of Cleveland et. al. (1977). Fractions were incubated in the presence of 1μg/ml or 2μg/ml trypsin for 30 min at 37°C. Reactions were terminated by the addition of soybean trypsin inhibitor (2μg/ml final), followed by immediate denaturation by incubation for 3 min at 95°C in the presence of 2% SDS, 9% glycerol, 75mM Tris/HCl, pH 7.5, 0.6% DTT, 0.001% bromophenol blue.

Trypsin treated samples were loaded on 8% polyacrylamide gels (see Appendix 1) and subjected to electrophoresis at a constant current of 30mA per gel. Resolved tryptic fragments were transferred to nitrocellulose as previously described (Section 3.3.4.). Tryptic peptides of the Mr 140,000 (α_2) subunit were stained by incubating the nitrocellulose bearing the resolved tryptic fragments with αDHP-R 13 (1:500 dilution) followed by sequential antibody incubations and staining with DAB solution as described in Section 3.3.5.

3.3.8. Enzymatic Deglycosylation of Skeletal Muscle Transverse Tubule Membranes

T-Tubule membranes (6-10mg/ml protein) were adjusted to contain 2mM PMSF, 0.2mM iodoacetamide and 2μM pepstatin A. Detergent solubilization was performed by the addition of an equal volume of 2% CHAPS, 20% glycerol, 20mM Tris/HCl, pH 7.5. After incubation for 30 min at 4°C and centrifugation to remove particulate material the CHAPS extract was incubated with glycosidases. The incubation was performed in a total volume of 200μl containing 1.75mg/ml protein, 50mM Na acetate/citrate, pH 5.5, 0.1% SDS, 10mM EDTA and the enzymes at the required concentration (see Figure 16B legend). After incubation for 16 hours at 37°C the reactions were terminated by denaturation for 3 min at 95°C in the presence of 2% SDS, 9% glycerol, 75mM Tris/HCl, pH 6.8, 0.6% DTT, 0.001% bromophenol blue. Following the electrophoresis of samples on 8% SDS polyacrylamide gels (Section 3.3.7.), resolved enzymatically treated

components were transferred to nitrocellulose (Section 3.3.4.). Reaction products of the α_2 subunit were stained by the incubation of the nitrocellulose with αDHP-R 13 (1:500 dilution) followed by sequential antibody incubations as described in Section 3.3.5. Immunoblots were developed with DAB solution as above.

Enzymatic deglycosylation was also carried out on SDS denatured CHAPS solubilized T-tubule membrane samples. 10% SDS was added to the CHAPS extract (3.5mg protein/ml) to result in a final concentration of 1% SDS and the mixture was incubated for 3 min at 95°C. The concentration of SDS was reduced to 0.2% by dilution of the denatured sample into the assay buffer (50mM Na acetate/citrate, pH 5.5, 10mM EDTA final). Enzymes were then added at the required concentration and the fractions were incubated for 16 hours at 37°C. Reactions were terminated by denaturation for 3 min at 95°C in the presence of 2 % SDS, 9% glycerol, 75mM Tris/HCl, pH 6.8, 0.6% DTT, 0.001% bromophenol blue. After electrophoresis on 8% polyacrylamide gels, resolved components were transferred to nitrocellulose (Section 3.3.4.). The reaction products of the α_2 subunit were detected as described above.

Enzymatic deglycosylation was also performed on native T-tubule membrane fractions for subsequent analysis in equilibrium binding assays with (+)-[^{3}H]PN200-110. In this case T-tubule membrane samples (1mg protein/ml final concentration) were incubated in 20mM Tris/HCl, pH 7.5 containing protease inhibitors (1mM PMSF, 0.1mM iodoacetamide, 1μM pepstatin A), in the presence of glycosidases at the required concentration for 3 hours at 37°C. Treated samples were then cooled to 4°C for subsequent analysis of 1,4-DHP binding sites by the equilibrium binding assay as described in Section 2.3.2. (15μg T-tubule protein per tube). Treated membrane samples were also subjected to analysis by immunoblot assay with αDHP-R 13 as described above.

3.3.9. Chemical Deglycosylation of Transverse Tubule and Microsomal Membranes

Chemical deglycosylation was performed by the method of Edge et. al. (1981), as modified by Kalyan and Bahl (1981), using trifluoromethanesulphonic acid (TFMS).

T-Tubule or microsomal membrane fractions were initially adjusted to contain protease inhibitors (final concentrations 1mM PMSF; 0,1mM iodoacetamide; 1μM

pepsatin A) and 10% Triton X-100 was added to give a final concentration of 2%. Following incubation for 30 min at 4°C particulate material was removed by centrifugation for 30 min at 160,000 x g. Triton X-100 solubilized membrane fractions were lyophilized and treated with 2ml TFMS/Anisole (3:2 v/v) for 5 hours at 4°C under nitrogen. Reactions were terminated by the addition of 3.2ml ice-cold pyridine/water (3:2 v/v) with agitation. Treated samples were dialysed for 24 hours against several changes of deionized water and were then lyophilized. The TFMS treatment was repeated routinely for each sample to ensure complete deglycosylation. Samples were again dialysed versus deionized water and, following lyophilization, were resuspended in 20mM Tris/HCl, pH7.5. Protein content of the treated fractions were determined by a modification of the method of Peterson (1977) (Section 3.3.12.) prior to denaturation and immunoblot analysis.

TFMS treated samples were denatured by incubation for 3 min at 95°C in the presence of 2% SDS, 9% glycerol, 75mM Tris/HCl, pH 6.8, 0.6% DTT, 0.001% bromophenol blue. Following SDS electrophoresis on 8% SDS polyacrylamide gels (48-100µg protein per track, see figure legends) resolved proteins were transferred to nitrocellulose (Section 3.3.4.) The non-glycosylated product of the α_2 subunit was detected by incubation of the nitrocellulose with monoclonal antibody αDHP-R 13 as described in Section 3.3.5. To test the extent of deglycosylation blots of treated membrane proteins were incubated with lectin-linked peroxidases as described in Chapter 3.3.6.

3.3.10. Development of the α_2 Subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel

Skeletal muscle microsomes were prepared as described in Section 3.3.2 from the hind legs of foetal and post-partum Wistar-Kyoto rats from 19 days gestation to 45 days post-partum. Protein concentrations of the membrane fractions were determined accurately by the method described in Section 3.3.12. A standard amount of skeletal muscle microsomal membrane protein (150µg) was analysed in the immunoblot assay with αDHP-R 13 following electrophoresis on 4-12% gradient polyacrylamide gels. Resolved membrane polypeptides were transferred to nitrocellulose as described previously

(Section 3.3.4.). The nitrocellulose paper bearing the proteins was incubated for 16 hours at 4°C with 200ml 1% bovine serum albumin, 0.5% casein, 0.05% Tween 20, 0.9% NaCl, 10mM Tris/HCl, pH 7.5. This was followed by a 2 hour incubation at room temperature with αDHP-R 13 (1:500 dilution) in 0.05% Tween 20, 0.9% NaCl, 10mM Tris/HCl, pH 7.5. αDHP-R 13 binding to the α_2 subunit was quantified by sequential incubations with biotinylated anti-mouse IgG (1:500 dilution in 1% bovine serum albumin, PBS, pH 8.0; 2 hours), followed by [^{125}I]Streptavidin (0.5μCi/ml in 1% bovine serum albumin, PBS, pH 8.0; 30 min). Between each incubation the nitrocellulose was washed for 10 min in with two changes of 0.25% sodium lauryl sarcosine, 0.25% Nonidet P-40, 1M NaCl, 10mM Tris/HCl, pH 7.5. After the final incubation the nitrocellulose was washed extensively in this buffer and rinsed in deionized water before drying for autoradiography. The nitrocellulose paper was exposed to Figi film for 6 hours to determine the bands specifically labelled and the levels of background staining. The appropriate region of the paper (1cm^2) was counted for bound [^{125}I] in a Packard Auto-Gamma 5650 Counter. For each gel an internal control (150μg protein, skeletal muscle microsomal membranes prepared from 28 day old rats) was included to assess any difference in the efficiency of transfer in the transblotting procedure.

3.3.11. Development of the 1,4-Dihydropyridine Receptor Characterized by the Equilibrium Binding of (+)-[^{3}H]PN200-110

Skeletal muscle microsomes prepared from the hind legs of rats of various ages were assayed for membrane-bound (+)-[^{3}H]PN200-110 binding as described in Section 2.3.2. Microsomal membrane fractions were diluted to 1mg protein/ml in 20mM Tris/HCl, pH 7.5 before further dilution into the assay mixture (15μg protein per assay tube). Parallel incubations were carried out in the presence of excess (1μM) unlabelled nitrendipine to determine the non-specific binding.

3.3.12. Determination of Protein Concentration

Protein concentrations of membrane and CHAPS solubilized fractions were determined by a modification of the method of Peterson (1977) using bovine serum

albumin as a standard. Fractions containing between 5-100μg protein were adjusted to 1ml with deionized water. 0.1ml ice-cold 72% (w/v) trichloroacetic acid (TCA) was added to each fraction and incubated for 15 min at 4°C. After centrifugation for 15 min at 3000 x g in an eppendorf centrifuge the supernatants were discarded and the pellets resuspended in 0.2ml 7.2% TCA. The samples were subjected to centrifugation as before and the supernatant was discarded. The pellets were resuspended in 25μl 2%(w/v) SDS. Following incubation for 15 min at room temperature, 0.9ml Reagent B (see Appendix 3) was added and the tubes were mixed well. Following a further incubation for 10 min at room temperature, 0.45ml Reagent A (see Appendix 3) was added and mixed. The assay mixtures were incubated for 30 min at room temperature and the absorbance at 750 nm was then measured. A standard curve between 2.0-50.0μg protein/ml was generated using bovine serum albumin as standard.

3.4. RESULTS

3.4.1. Monoclonal Antibodies

All aspects of monoclonal antibody preparation involving injections and tissue culture were carried out by Dr. T. M. Harrison in the Department of Biochemistry, University of Leicester. I was involved in the preparation of the purified protein for injection and in the screening of hybrid clones by enzyme-linked immunosorbant assay (ELISA) of the hybridoma cell supernatants.

Initial attempts to raise monoclonal antibodies to the 1,4-DHP receptor involved the immunization of BALB/C mice with T-tubule protein (1.2mg protein/injection) in Freunds Complete Adjuvant. These preliminary experiments were unsuccessful possibly due to the low abundance of Ca^{2+} channel protein amongst other protein components with higher immunogenicity in the T-tubule membrane fractions. After these initial experiments, the purification procedure for the 1,4-DHP receptor was established resulting in a homogenous preparation, that retained 1,4-DHP binding activity, containing a polypeptide of Mr 140,000. The immunization of BALB/C mice with this

purified preparation, in the form of an alum precipitate, eventually resulted in the successful production of a panel of monoclonal antibodies.

Table 9

Ascitic Fluid Titres Determined by Solid Phase Immunoassay

Assays were performed as described in the text (Section 3.3.2.). Development of blue colouration in microtitre plate wells was monitiored visually following the addition of TMB substrate;

++++ = very strong

+++ = strong

++ = medium

+ = weak

- = negative

Table 9

Ascitic Fluid Titres Determined by Solid Phase Immunoassay (ELISA)

Ascitic fluid αDHP-R	Tap	Dilution 10^{-2}	10^{-3}	10^{-4}
1	1	++++	++++	++
	2		++++	++++
	3	++++	+++	++
2	1	++++	++	+
	2	++++	+	
	3	++++	++	+
	4	++++	++	+
3	1	++++	++	+
	2	+++	+++	+++
4	1	++	+	
	2	++	+	
	3	+++	+	
5	1	-		
	2	++	+	
	3	+++	++	
6	1	++		
	2	++++	+++	+
	3	++++	++	+
7	1	++++	++++	+++
8	1	++	+	
	2	+++	++	
	3	+++	++	
9	1	+		
10	1	++++	+++	+++
	2	++++	++++	+++
	3	++++	++++	+++
11	1	++++	+++	++
12	1	++++	++++	++
	2	++++	++++	++
13	1	+++	++	+
	2	++++	+++	++
14	1	++	++	+
	2	+++	++	+
15	1	++	+	
	2		++	+

Table 9 (continued)

Ascitic fluid αDHP-R	Tap	Dilution 10^{-2}	10^{-3}	10^{-4}
17	1	++	+	
	2	++++	++++	++
18	1	+	+	
	2	++	+	
19	1	+++	++	+
20	1	+		
	2	++	+	
22	1	+		
23	1	++		
24	1	+++	++	+
	2	++	+	+
	3	++	++	+
25	1	+		
	2	++	+	
26	1	+++	+	
	2	+++	++	
	3	+++	++	+
27	1	++	++	+
	2	-		
	3	+		
28	1	++	+	
	2	+++	+++	+
29	1	++	++	+
	2	++	+	
	3	++	+	
	4	++	+	
30	1	+++	+	
	2	+++	+	
31	1	++	+	
32	1	++	++	
33	1	+++	++	
	2	+++	++	

After fusion of the spleen of a hyperimmune mouse with NSO/1 myeloma cells the hybridoma cells were selected from the parental myeloma and spleen cells by growing the fusion products in HAT medium. Only hybridoma cells have the required enzymes actively multiply in this medium (Milstein, 1980; Galfrè & Milstein, 1981). The hybridoma cell supernatants were assayed for the antibody of interest by solid phase immunoassay as described in Section 3.3.2. Thirty three of the 360 hybridoma cell supernatants tested in the solid-phase assay were positive and were consequently selected for replating. Thirty one individual clones were isolated by plating out to limiting dilution. These monoclonal hybridoma cell lines were injected (10^7 cells/injection) into pristane-primed mice for the subsequent production of ascitic fluids. Table 9 shows the titres of the ascites fluids screened in the solid-phase antibody assay. Ascitic fluids produced from these positive clones were tested in immunoblot assays.

3.4.2. Immunoblot Analysis of Transverse Tubule Membranes

Four of the panel of thirty one monoclonal antibodies produced a detectable reaction in the immunoblot assay on denatured rabbit T-tubule membranes. The four antibodies (αDHP-R 11, 13, 14 and 15) recognized specifically a polypeptide of Mr 140,000 in immunoblots of reduced T-tubule membrane proteins (Fig. 12A). This component co-migrated with the large glycoprotein subunit detected in silver-stained SDS polyacrylamide gels of purified 1,4-DHP receptor fractions (Section 2.4.5., Fig. 11). No staining was detected with a control anti-bacterial antibody, α-gyrase B, indicating the staining with the αDHP-R antibodies was specific (Fig.12A, lane 5).

In immunoblots of T-tubule membrane proteins electrophoresed under non-reducing conditions all four antibodies recognized a component of Mr 170,000 (Fig 12B). In addition, αDHP-Rs 13, 14 and 15 specifically detected higher molecular weight components of approximately Mr 310,000 and 330,000 under these conditions (Fig. 12B; lanes 2, 3 & 4). Diffuse weak staining in the Mr 130,000-140,000 region was detected with all four antibodies in immunoblots of non-reducing gels (Fig. 12B). This staining, however, was also present in the control lane with α-gyrase B (Fig. 12B, lane 5), indicating that the staining was non-specific. This non-specific staining has been

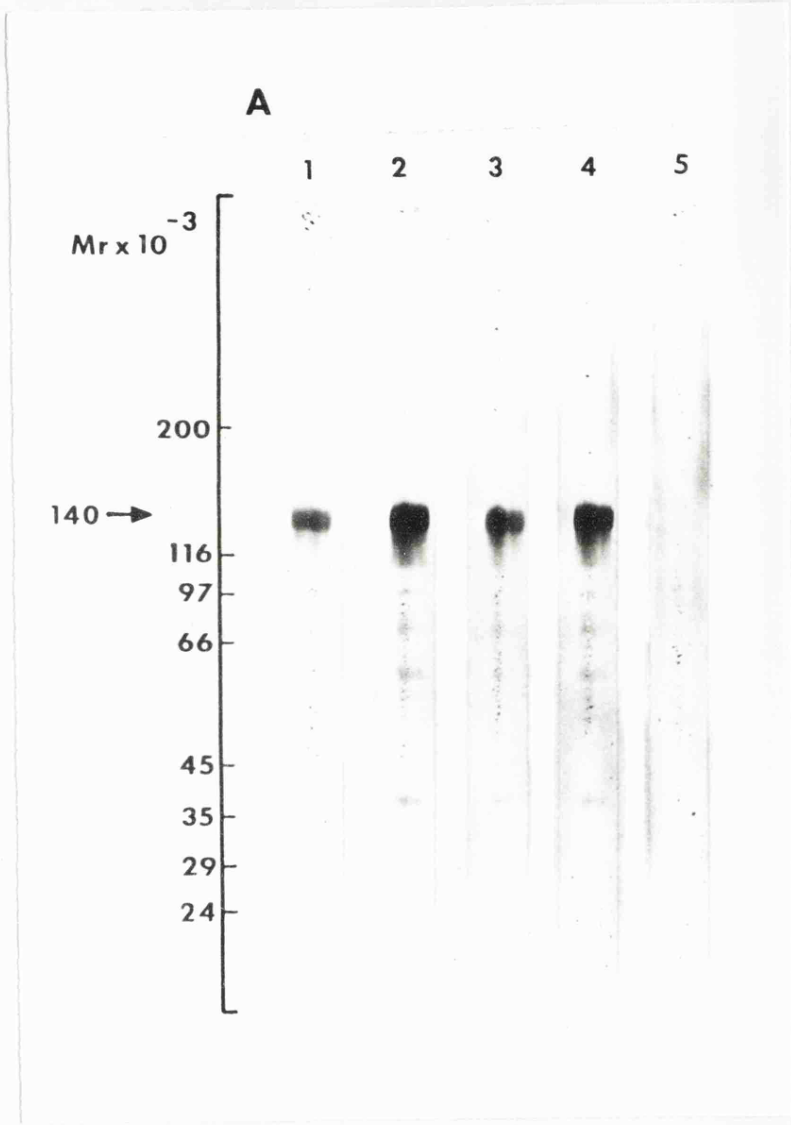


Figure 12

Immunoblots of rabbit muscle transverse tubule membranes after electrophoresis under (A) reducing conditions and (B) non-reducing conditions. Lane 1, αDHP-R 11; lane 2, αDHP-R 13; lane 3, αDHP-R 14; lane 4, αDHP-R 15; lane 5, α-gyrase B. Mr markers as in Fig. 11 except for pepsin (35,000); trypsinogen (24,000).

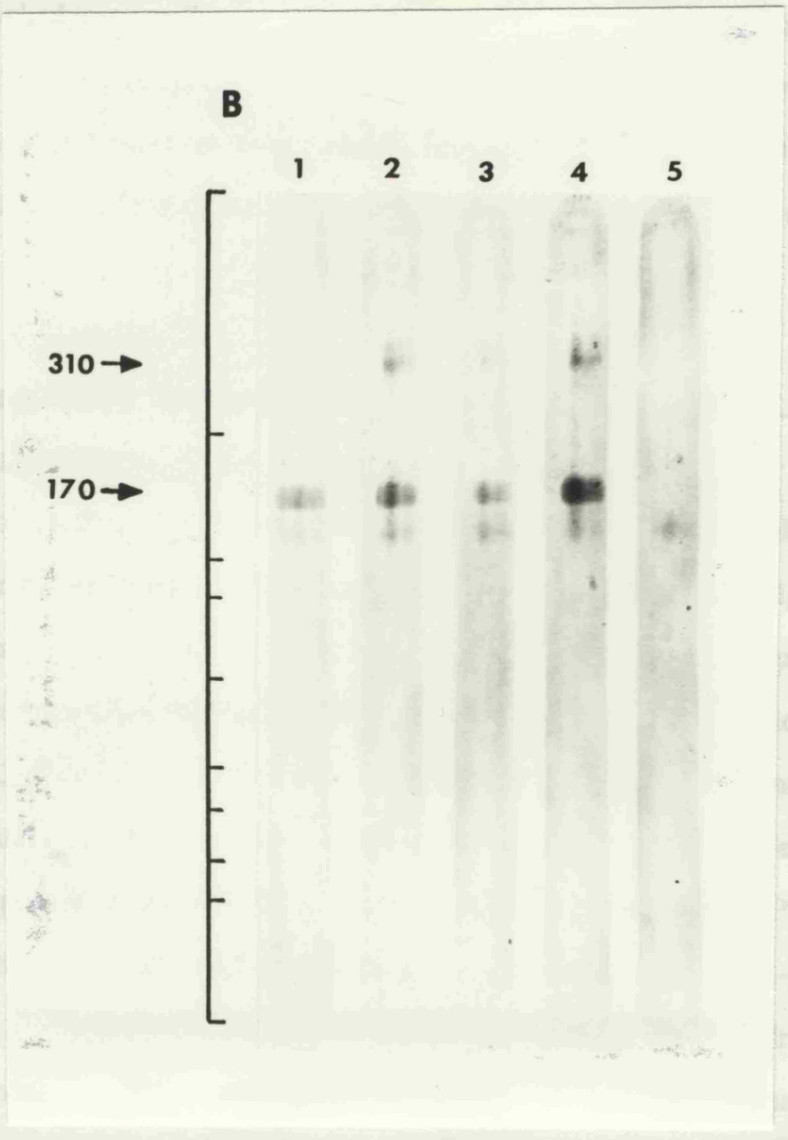


Figure 12

detected by other workers (Schmid et. al., 1986), and it is possible that it was due to endogenous immunoglobulin associated with the membrane preparations.

This change in the migration of the Mr 140,000 component to that of 170,000 under non-reducing conditions suggests the possibility that the Mr 140,000 subunit of the 1,4-DHP receptor is linked to a smaller component of Mr~30,000 by disulphide bonds. This is consistent with the results of other workers (Section 1.3.4.; Section 4.2.1.). In addition, the detection of the high molecular weight components by the 3 antibodies, αDHP-R 13, 14 and 15, raises the possibility of the involvement of other components in the Ca^{2+} channel structure.

3.4.3. Immunocrossreactivity Between 1,4-Dihydropyridine Sensitive Calcium Channels from Different Tissues

Possible immunological similarities between the rabbit skeletal muscle 1,4-DHP sensitive Ca^{2+} channels and putative Ca^{2+} channels in other tissues were investigated. Microsomal membranes, prepared from various rabbit tissues (Section 3.3.3.), were tested in the immunoblot assay with αDHP-R 13 as described previously (Section 3.3.4. & 3.3.5.). Figure 13 shows immunoblots of brain, heart and liver microsomal membranes with αDHP-R 13 and control antibody α-gyrase B. In lanes of both brain and heart microsomes a component of Mr~140,000 was specifically recognized by αDHP-R 13. Staining in this region was specific as not detected with the control antibody, α-gyrase B (Fig. 13, lanes 2 & 4). High levels of microsomal membrane protein were required for a positive signal with αDHP-R 13 in brain and heart (3.0mg brain and 1.85mg heart microsomes). This could reflect the lower densities of 1,4-DHP binding sites in these tissues (0.5-2.0 pmol/mg protein), as compared to the density of binding sites in skeletal muscle T-tubules (50 pmol/mg protein). In lanes of brain microsomal membranes αDHP-R 13 detected two smaller molecular weight components (Mr 65,000-70,000). However, this staining was shown to be non-specific as it was also present in lanes incubated with the control antibody α-gyrase B (Fig. 13, lanes 1 & 2).

Interestingly, no specific staining was detected with αDHP-R 13 with liver microsomal membranes (Fig. 13, lane 5) and membranes prepared from several other

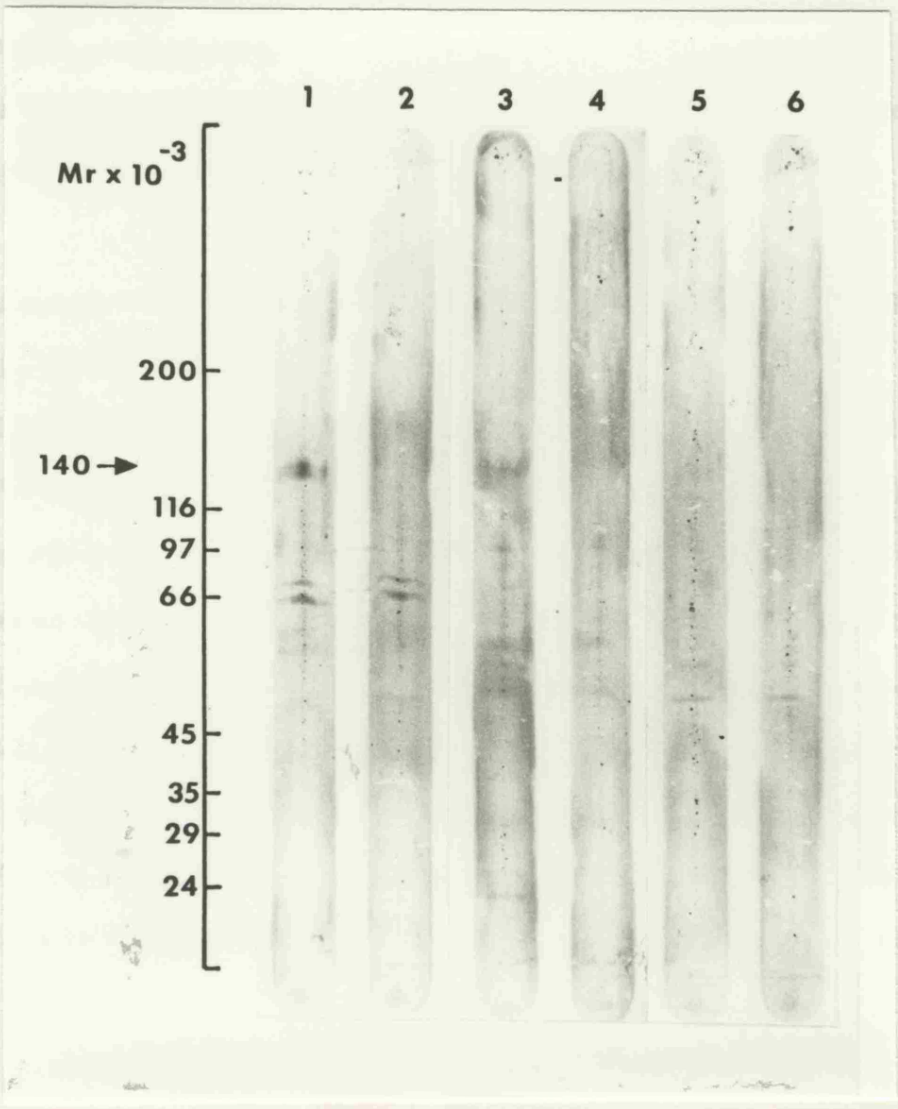


Figure 13

Immunoblots of rabbit brain, heart and liver microsomes after electrophoresis under reducing conditions. Lanes 1 & 2, brain (3.0mg); lanes 3 & 4, heart (1.85mg); lanes 5 & 6, liver (3.0mg). Lanes 1, 3 & 5 with αDHP-R 13; lanes 2, 4 & 6 with α-gyrase B. Mr markers as in Fig. 12.

rabbit tissues; kidney, small intestine, lung, aorta, and spleen (data not shown). The identification of the Mr 140,000 subunit of the 1,4-DHP sensitive Ca^{2+} channel in brain, cardiac and skeletal muscle tissue, but not in other tissues, is consistent with data obtained by pharmacological methods. Extensive ligand binding studies have established the presence of 1,4-DHP receptors in both brain and cardiac membranes but only very low levels of high affinity 1,4-DHP binding sites have been detected in membranes prepared from liver tissue and the other tissues studied (Glossmann et. al., 1985).

3.4.4. Immunocrossreactivity Between Skeletal Muscle 1,4-Dihydropyridine Sensitive Calcium Channels from Different Species

αDHP-R 13 was also used to investigate possible immunological similarities between the 1,4-DHP receptor from rabbit skeletal muscle and that from the skeletal muscle of other species. Microsomal membranes were prepared from skeletal muscle tissue from a variety of species (Section 3.3.3.), and were tested in the immunoblot assay with αDHP-R 13 as described previously (Section 3.3.4. & 3.3.5.). Fig. 14A shows immunoblots of skeletal muscle microsomal membranes from rabbit, rat, mouse and frog, stained with αDHP-R 13 and control antibody α-gyrase B, following electrophoresis under reducing conditions. As seen for rabbit T-tubule membranes, αDHP-R 13 recognized specifically a polypeptide of Mr 140,000-145,000 (Fig. 14A, lanes 1, 3, 5 & 7; α-gyrase B, lanes 2, 4, 6 & 8).

Moreover, under non-reducing conditions a component of Mr 170,000-180,000 was recognized by the αDHP-R 13 (Fig. 14B). In addition, a high molecular weight component of approximately Mr 310,000 was detected in the case of rabbit, rat and mouse, but not frog microsomal membranes, under non-reducing conditions (Fig. 14B). Interestingly, the apparent molecular weight of the frog muscle component, detected by αDHP-R 13, was slightly larger than that of the other species tested (reducing conditions, Mr 145,000; non-reducing conditions, Mr 180,000). Despite minor differences in molecular size of the skeletal muscle Mr 140,000 component from these different sources (Fig. 14A & B), the similarities in the immunoblot profiles suggest that the α_2 component is similar in the different species investigated. Under non-reducing

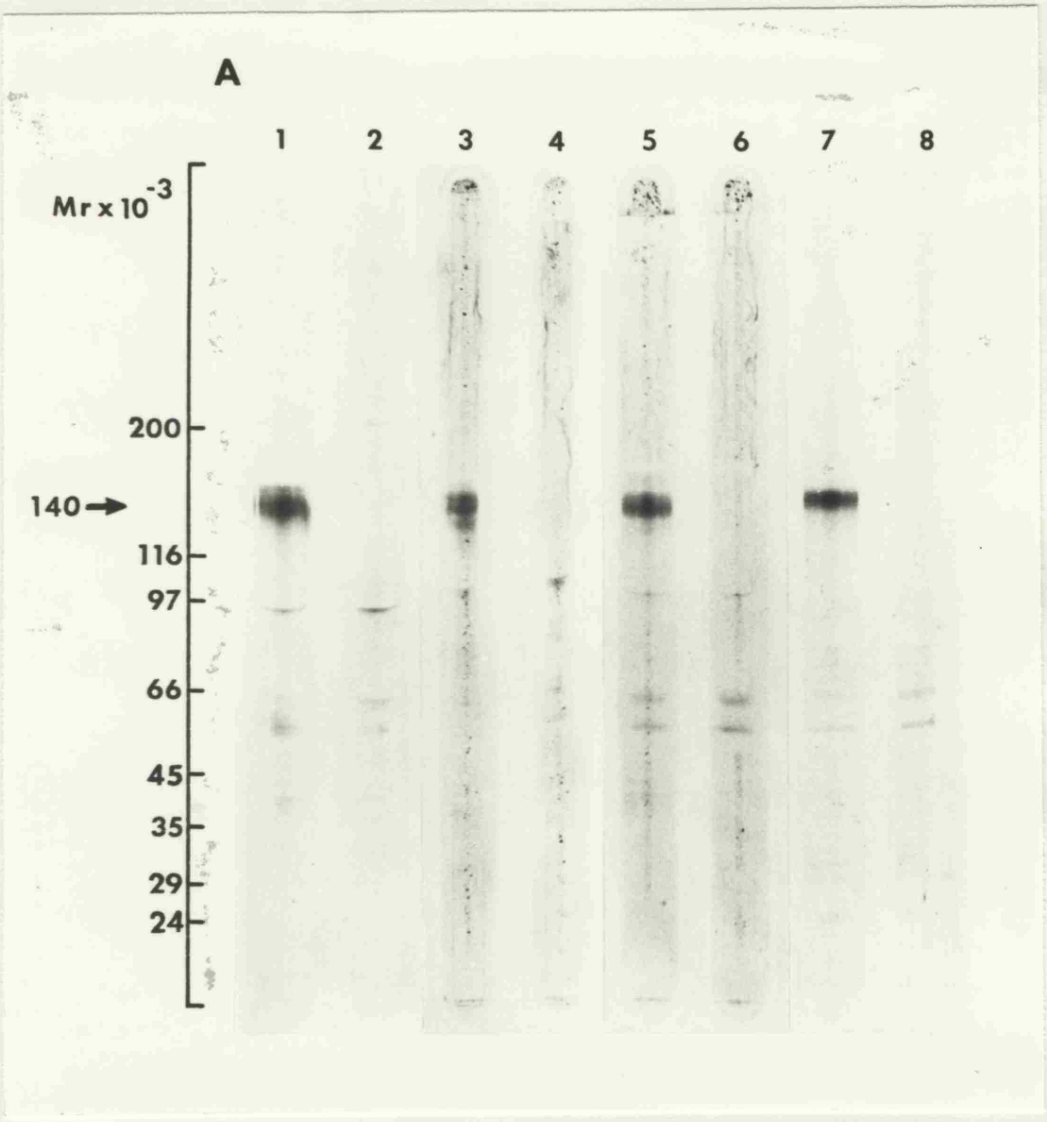


Figure 14

Immunoblots of skeletal muscle microsomes prepared from different species under (A) reducing conditions and (B) non-reducing conditions. Lanes 1 & 2, rabbit (0.25mg); lanes 3 & 4, rat (0.75mg); lanes 5 & 6, mouse (0.3mg); lanes 7 & 8, frog (0.33mg). Lanes 1, 3, 5 & 7 with αDHP-R 13; lanes 2, 4, 6 & 8 with α-gyrase B. Mr markers as in Fig. 12.

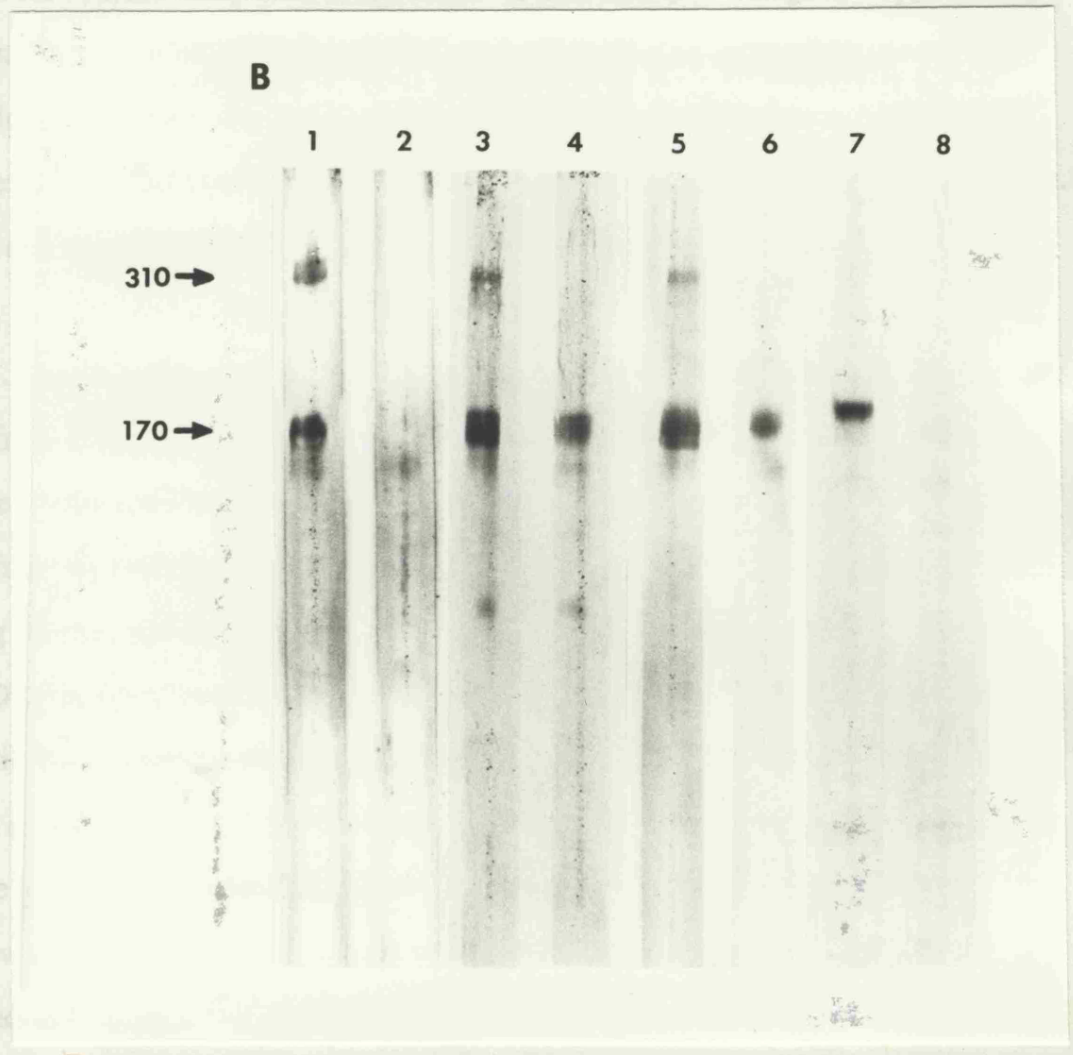


Figure 14

conditions a diffuse region of non-specific staining in the region of Mr 140,000 was evident with rabbit, rat and mouse but not frog microsomal membranes (Fig. 14B), as previously found in immunoblots of rabbit T-tubule membranes (Fig. 12B). However, in the case of rat and mouse this non-specific staining partially masked the specific staining of the Mr 170,000 component by the αDHP-R 13 antibody. Although less apparent than the staining of the Mr 170,000 component of rabbit and frog membranes, specific staining was reproducibly detected with αDHP-R 13 in the rat and mouse samples as darker and broader bands in the Mr 170,000 region as compared to the control lanes (Fig. 14B, odd numbered lanes).

3.4.5. Mapping of Immunoreactive Peptides of Skeletal Muscle Microsomal Membranes from Different Species Following Trypsin Digestion

The similarities illustrated between the rabbit skeletal muscle 1,4-DHP receptor and that from skeletal muscle membranes of rat, mouse and frog were further investigated using the immunoblot technique. Possible homologies in the primary sequences of the 1,4-DHP receptor from the different species were investigated following the partial trypsin digestion of the skeletal muscle membrane samples. Microsomal membranes were solubilized using CHAPS detergent before partial digestion with trypsin according to the method of Cleveland et. al. (1977) (Section 3.3.7.). Tryptic fragments were resolved by SDS polyacrylamide gel electrophoresis under reducing conditions and subjected to immunoblot analysis with αDHP-R 13 (Section 3.3.5.). An initial experiment was performed with CHAPS solubilized T-tubule membranes to determine the amount of enzyme required in the incubation to produce tryptic peptides of the α_2 subunit that were recognized by the αDHP-R 13 antibody (Fig 15).

In immunoblots of CHAPS solubilized skeletal muscle microsomal membranes following incubation in the absence of trypsin (Fig. 16A, lanes 1-4), αDHP-R 13 specifically recognized a polypeptide of Mr 140,000-145,000, as in immunoblots of non-solubilized membrane fractions (Fig. 14A). When trypsin treated solubilized membrane fractions were analysed in this way with αDHP-R 13 the intensity of staining of the Mr 140,000 component was substantially reduced (Fig. 16A, lanes 5-8). In addition, the

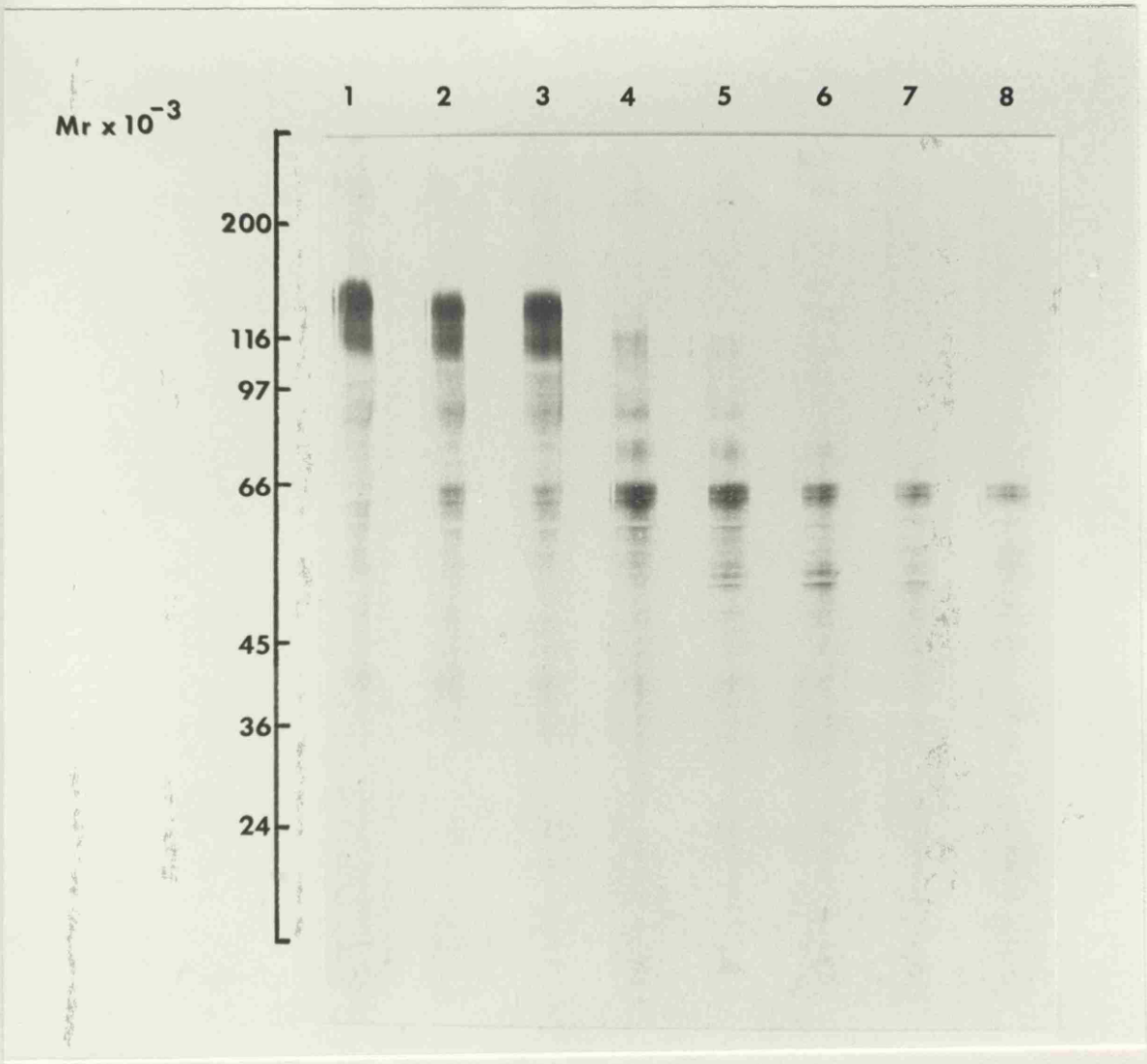


Figure 15

Immunoblots of CHAPS solubilized transverse tubule membranes after digestion with varying concentrations of trypsin stained with αDHP-R 13. Trypsin concentrations: Lane 1, 0.05μg/ml; lane 2, 0.25μg/ml; lane 3, 2.5μg/ml; lane 4, 5.0μg/ml; lane 5, 10.0μg/ml; lane 6, 25.0μg/ml; lane 7, 50.0μg/ml; lane 8, 100.0μg/ml. 100μg T-tubule protein loaded for each lane. Mr markers as in Fig. 11.

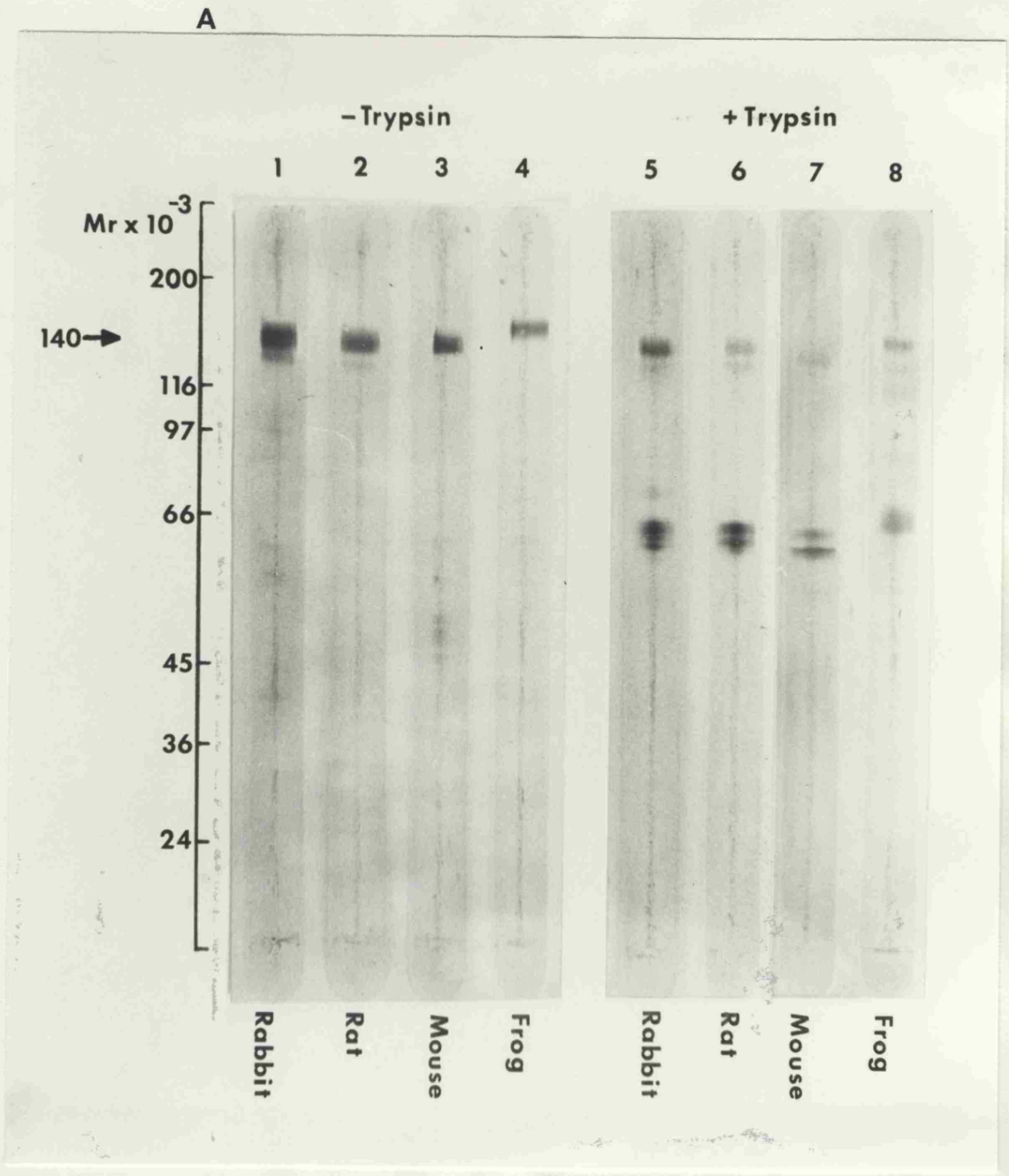


Figure 16

Immunoblots of CHAPS solubilized skeletal muscle microsomes after limited trypsin digestion stained (A) with αDHP-R 13 and (B) with α-gyrase B. Lanes 1-4, after incubation without enzyme; lanes 5-7, after incubation with 2μg/ml trypsin; lane 8, after incubation with 1μg/ml trypsin. Lanes 1 & 5, rabbit (160μg); lanes 2 & 6, rat (100μg); lanes 3 & 7, mouse (150μg); lanes 4 & 8, frog (180μg). Mr markers as in Fig. 11.

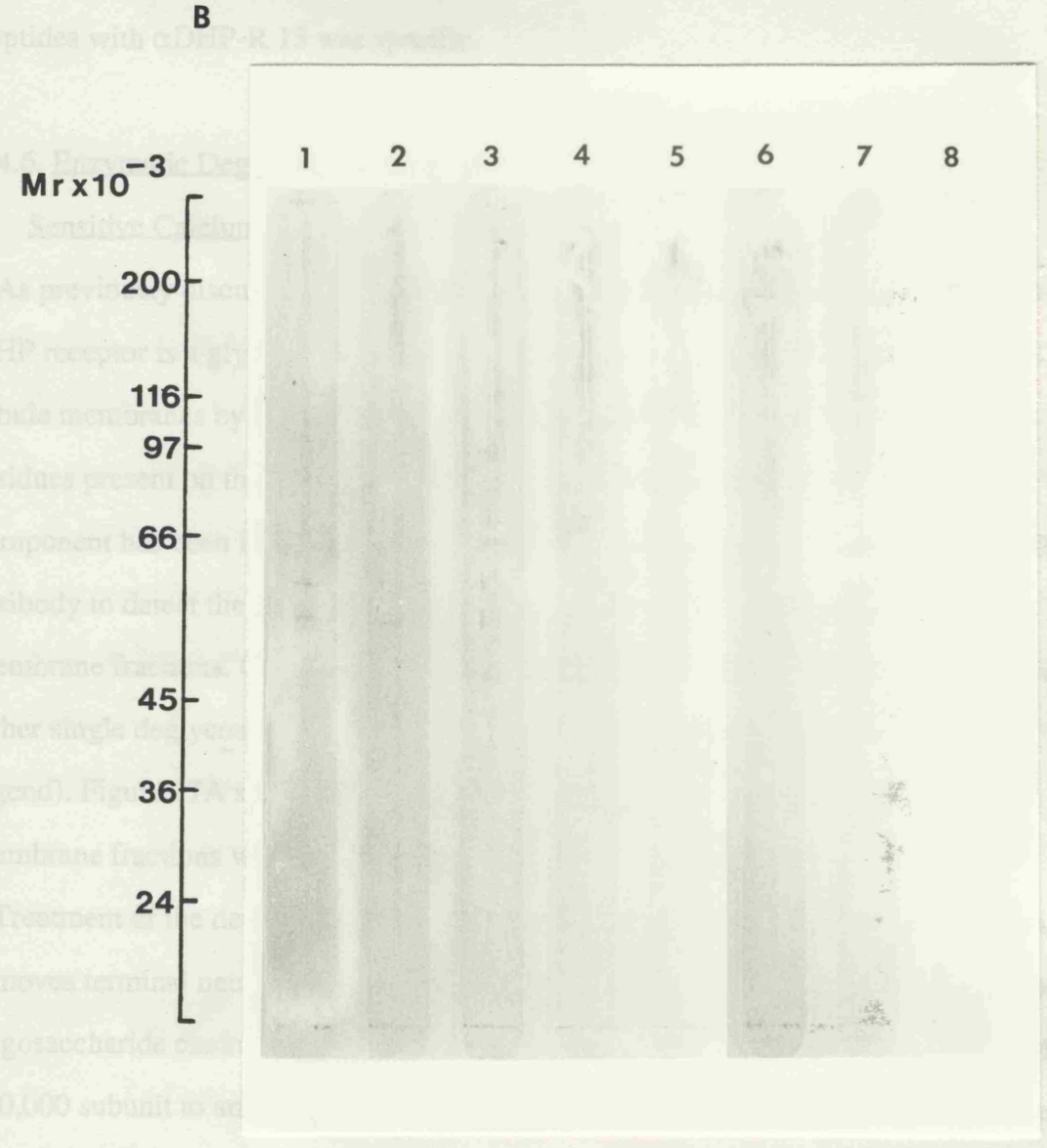


Figure 16

appearance of specifically stained smaller molecular weight components of Mr 60,000-70,000 was observed (Fig. 16A, lanes 5-8). A similar pattern of immunoreactive tryptic products of the Mr 140,000-145,000 component was detected for rabbit, rat, mouse and frog with αDHP-R 13 (Fig. 16A, lanes 5-8). No staining was detected in the control lanes with the α-gyrase B antibody (Fig. 16B), indicating that the staining of the tryptic peptides with αDHP-R 13 was specific.

3.4.6. Enzymatic Deglycosylation of the α_2 Subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel: Immunoblot Analysis

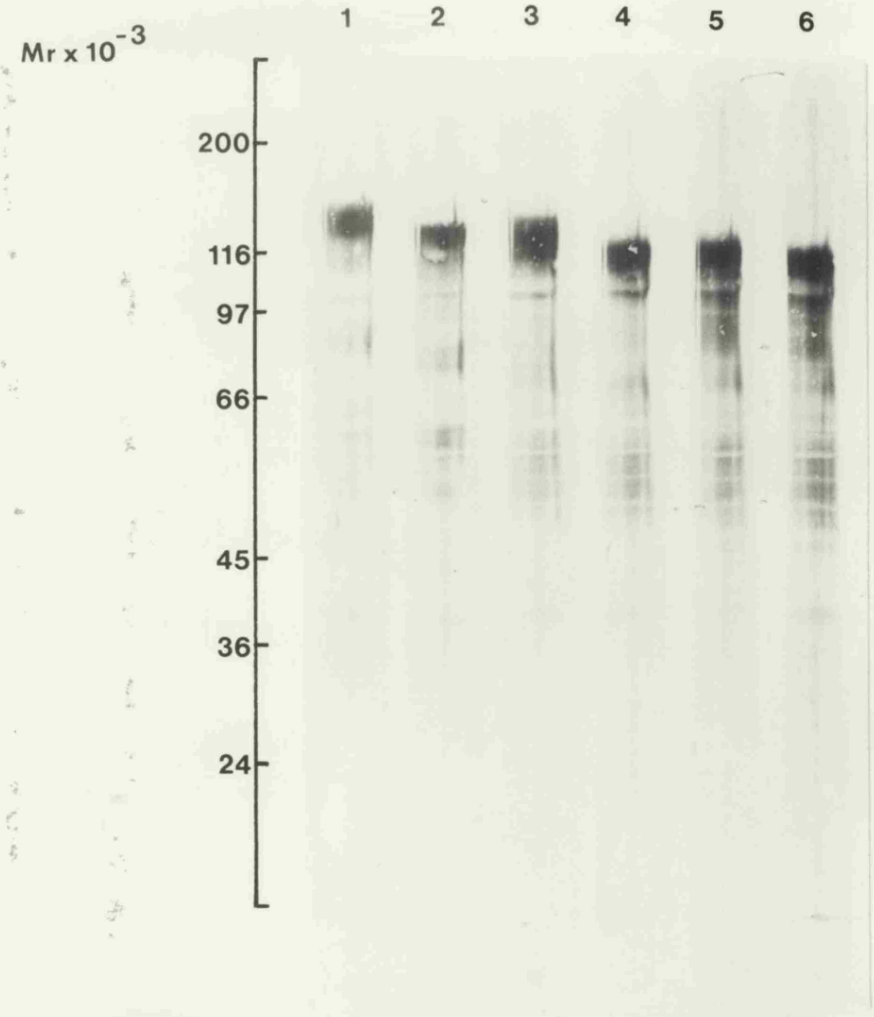
As previously discussed in Chapters 1 & 2 the Mr 140,000 (α_2) subunit of the 1,4-DHP receptor is a glycoprotein. The receptor is purified from CHAPS solubilized T-tubule membranes by lectin affinity chromatography by virtue of the carbohydrate residues present on the Mr 140,000 subunit. The extent of glycosylation of this component has been investigated, initially by enzymatic methods, using the αDHP-R antibody to detect the deglycosylated species in immunoblots of treated T-tubule membrane fractions. CHAPS solubilized T-tubule membrane fractions were treated with either single deglycosylating enzymes or with complex enzyme mixtures (Fig. 17, legend). Figure 17A shows the immunoblot analysis of the enzymatically treated membrane fractions with αDHP-R 13.

Treatment of the detergent solubilized membranes with neuraminidase only, which removes terminal neuraminic acid (sialic acid) and N-acetylglucosamine residues from oligosaccharide chains, resulted in a reduction in apparent molecular weight of the Mr 140,000 subunit to an Mr of 133,000 (Fig. 17A, lane 2). When the CHAPS solubilized T-tubule membrane fractions were incubated in the presence of both neuraminidase and Endoglycosidase F (endo-β-N-glucosaminidase F) the apparent molecular weight of this component was further reduced to Mr~115,000 (Fig. 17A, lane 4). Endoglycosidase F catalyses the hydrolysis of accessible terminal glycosidic bonds in both complex and high mannose oligosaccharide chains leaving only the N-linked N-acetylglucosamine residue attached to the polypeptide chain (Edge et. al. 1981). Other glycosylating enzymes, α- and β-glucosidase and β-galactosidase, which remove terminal glucose,

Figure 17

Immunoblots of CHAPS solubilized transverse tubule membrane protein following enzymatic deglycosylation stained (A) with αDHP-R 13 and (B) with α-gyrase B. Lane 1, no enzyme; lane 2, neuraminidase; lane 3, Endoglycosidase F; lane 4, neuraminidase and Endoglycosidase F; lane 5, neuraminidase, α-glucosidase, β-glucosidase, β-galactosidase and Endoglycosidase H; lane 6, neuraminidase, α-glucosidase, β-glucosidase, β-galactosidase and Endoglycosidases F and H. Enzyme concentrations used were: neuraminidase, α- and β-glucosidase and β-galactosidase, 0.75 units/ml; Endoglycosidase H, 0.075 units/ml; Endoglycosidase F, 2.5 units/ml. 0.35mg T-tubule protein loaded for each lane. Mr markers as in Fig. 11.

A



Neuraminidase	–	+	–	+	+	+
Exoglycosidases	–	–	–	–	+	+
Endoglycosidase F	–	–	+	+	–	+
Endoglycosidase H	–	–	–	–	+	+

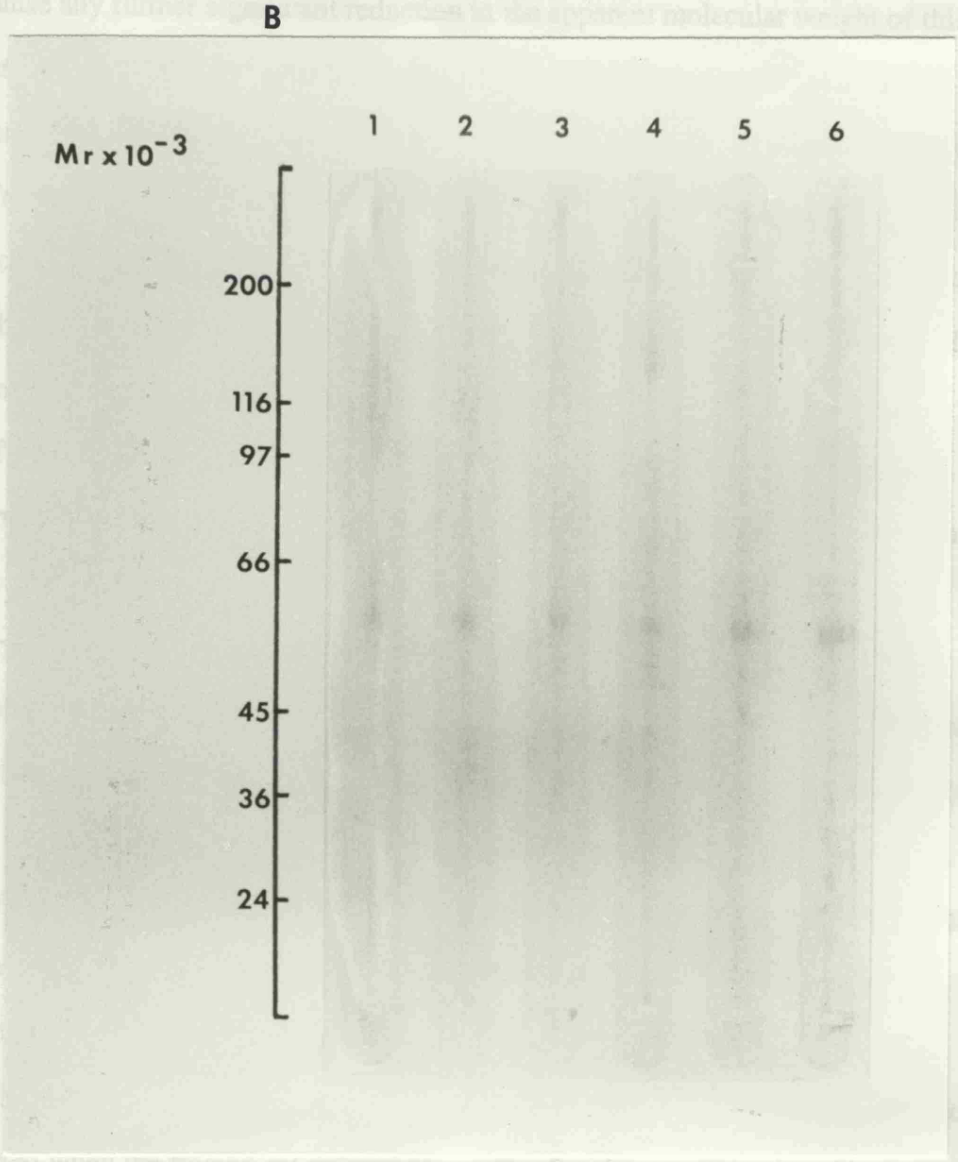


Figure 17

mannose and galactose residues from oligosaccharide chains, and Endoglycosidase H (endo-β-N-glucosaminidase H) were added to the incubation in addition to neuraminidase and Endoglycosidase F. The inclusion of Endoglycosidase H and the various glycosidases in the reaction mixture with neuraminidase and Endoglycosidase F did not cause any further significant reduction in the apparent molecular weight of this component from an Mr of 115,000 (Fig 17A, lane 6). Endoglycosidase H catalyses a similar reaction to Endoglycosidase F but is specific for high mannose oligosaccharide chains (Trimble & Maley, 1977). No reaction products were detected when the enzymatically treated solubilized membrane fractions were analysed in the immunoblot assay with the control antibody, α-gyrase B3 (Fig. 17B), indicating that the staining of the enzyme treated products with αDHP-R 13 was specific.

The diffuse area of staining in the region of Mr 115,000 with αDHP-R 13 suggests that the enzymatic treatment was insufficient to fully deglycosylate the component. This suggested either the possibility of the presence of O-linked carbohydrate or that the carbohydrate residues were buried within the folded protein structure, resulting in the inaccessibility of glycosidic bonds to glycosidic attack. To investigate the possibility of buried carbohydrate residues the CHAPS solubilized membrane fractions were initially denatured with SDS prior to treatment with neuraminidase and Endoglycosidase F. Figure 18A shows the immunoblot of SDS denatured and enzymatically treated T-tubule membranes with αDHP-R 13. Although the products of the enzymatic treatment were more defined, the SDS pretreatment did not result in any further reduction in the apparent molecular weight of the component. (Fig. 18A). As before, no reaction products were detected when the treated solubilized membrane fractions were analysed in the immunoblot assay with the control antibody α-gyrase B (Fig. 18B). In view of the apparent difficulty in removing the carbohydrate residues from the α_2 subunit by enzymatic methods, a chemical technique was employed to remove essentially all the carbohydrate from the α_2 component.

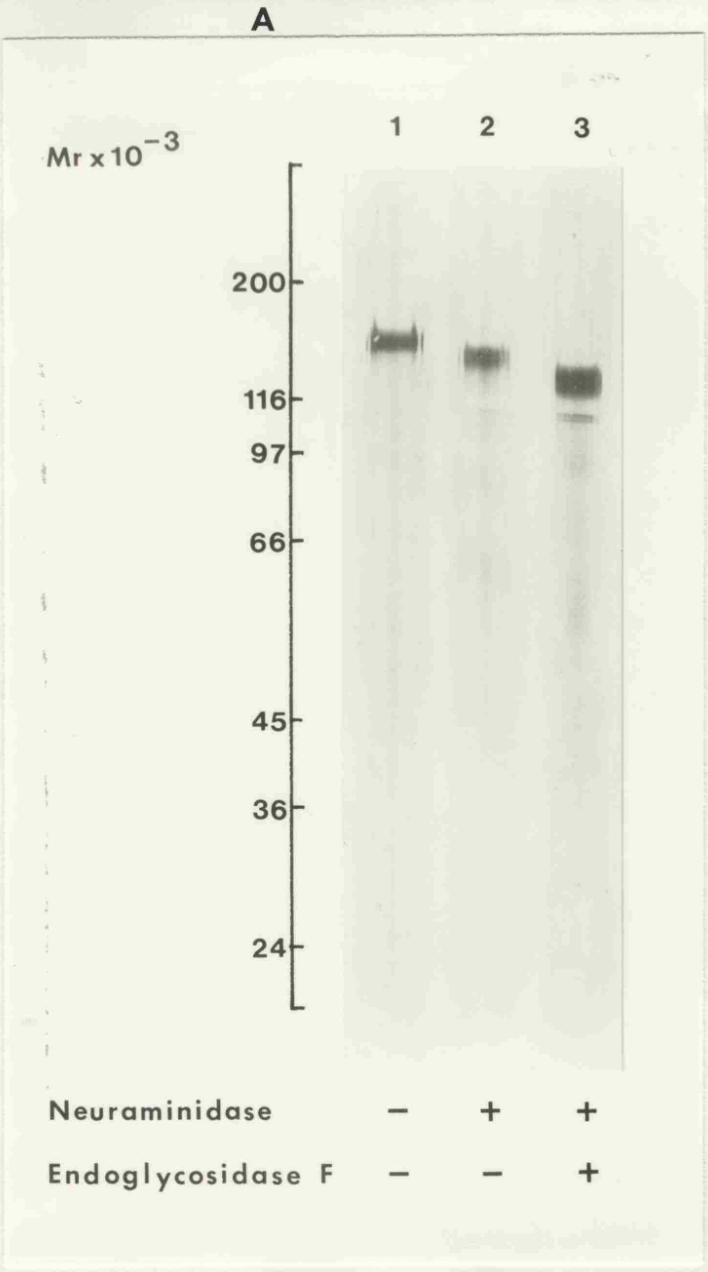


Figure 18

Immunoblots of CHAPS solubilized transverse tubule membrane proteins subjected to SDS denaturation prior to enzymatic deglycosylation stained (A) with αDHP-R 13 and (B) with α-gyrase B. Lane 1, no enzyme; lane 2, neuraminidase; lane 3, neuraminidase and Endoglycosidase F. Enzyme concentrations as used in Fig. 17. 0.7mg T-tubule protein loaded for each lane. Mr markers as in Fig. 11.

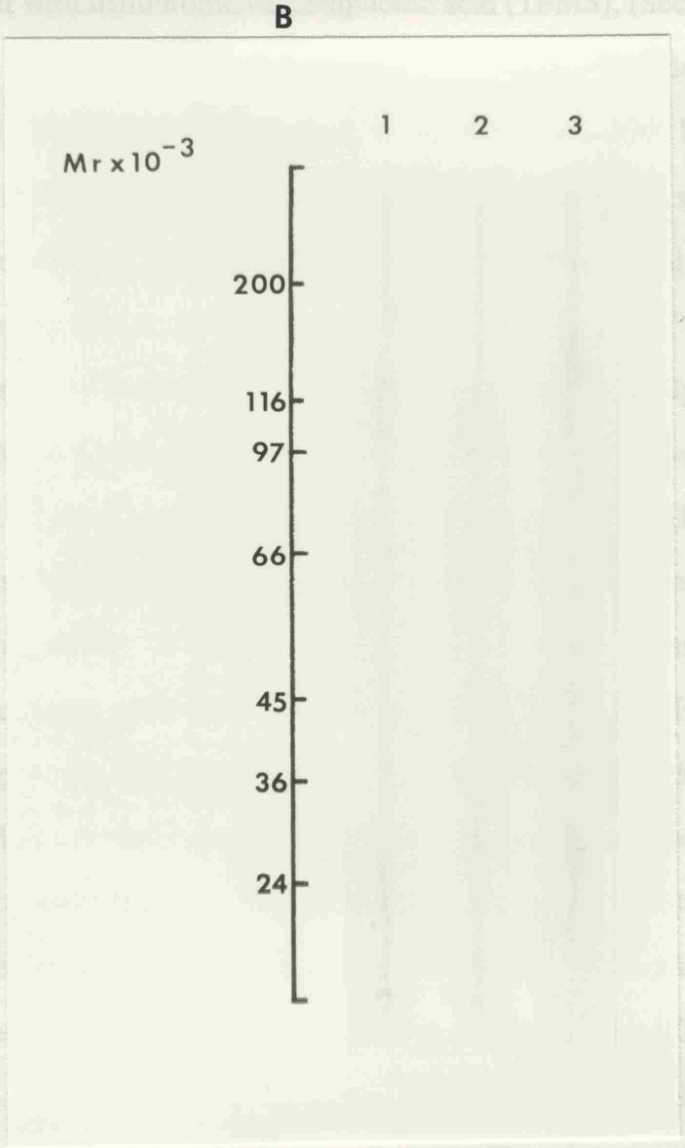


Figure 18

3.4.7. Chemical Deglycosylation of the α_2 Subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel from Rabbit Transverse Tubule Membranes

T-Tubule membrane fractions were solubilized using Triton X-100 before being subjected to treatment with trifluoromethanesulphonic acid (TFMS), (Section 3.3.10.). TFMS hydrolyses all glycosidic bonds of glycoproteins but leaves peptide bonds intact (Kalyan & Bahl 1981). Following this treatment, samples were analysed by immunoblot analysis with αDHP-R 13 to detect the deglycosylated product of the α_2 subunit. After only a single treatment with the TFMS, the αDHP-R 13 antibody recognized a diffuse stained band in the region Mr 115,000 suggesting incomplete removal of the carbohydrate residues from the component (Fig. 19, lane 2). Consequently, the treated proteins were subjected to a second treatment with the TFMS. In the immunoblot analysis the αDHP-R 13 antibody detected a sharply stained band of Mr 105,000 in the doubly treated T-tubule membrane fraction, (Fig. 19, lane 3). This component was not detected in similar immunoblot analysis with the control antibody α-gyrase B3 (Fig. 19, lane 4). Other bands of lower molecular weight were detected by the αDHP-R 13 antibody. These components were probably proteolytic fragments of the Mr 105,000 polypeptide produced as a result of the stringent deglycosylating conditions.

To confirm the complete removal of carbohydrate residues from the T-tubule membrane glycoproteins, blots of treated proteins were incubated with lectin-linked peroxidase and stained with DAB as described in Section 3.3.6. Figure 20 shows a blot of untreated (lanes 1 & 2) and TFMS treated (lanes 3 & 4) T-tubule membrane proteins following incubation with Concanavalin A (Con A) conjugated to horseradish peroxidase in both the absence and presence of competing sugars (see Fig. 20 legend). Con A binds specifically to both mannose and glucose residues and many membrane glycoproteins in blots of the untreated membranes were detected by this method (Fig. 20, lane 1). This staining was specific as no staining was observed with the untreated membranes when excess competing sugars were included in the incubations (Fig. 20, lane 2). When blots

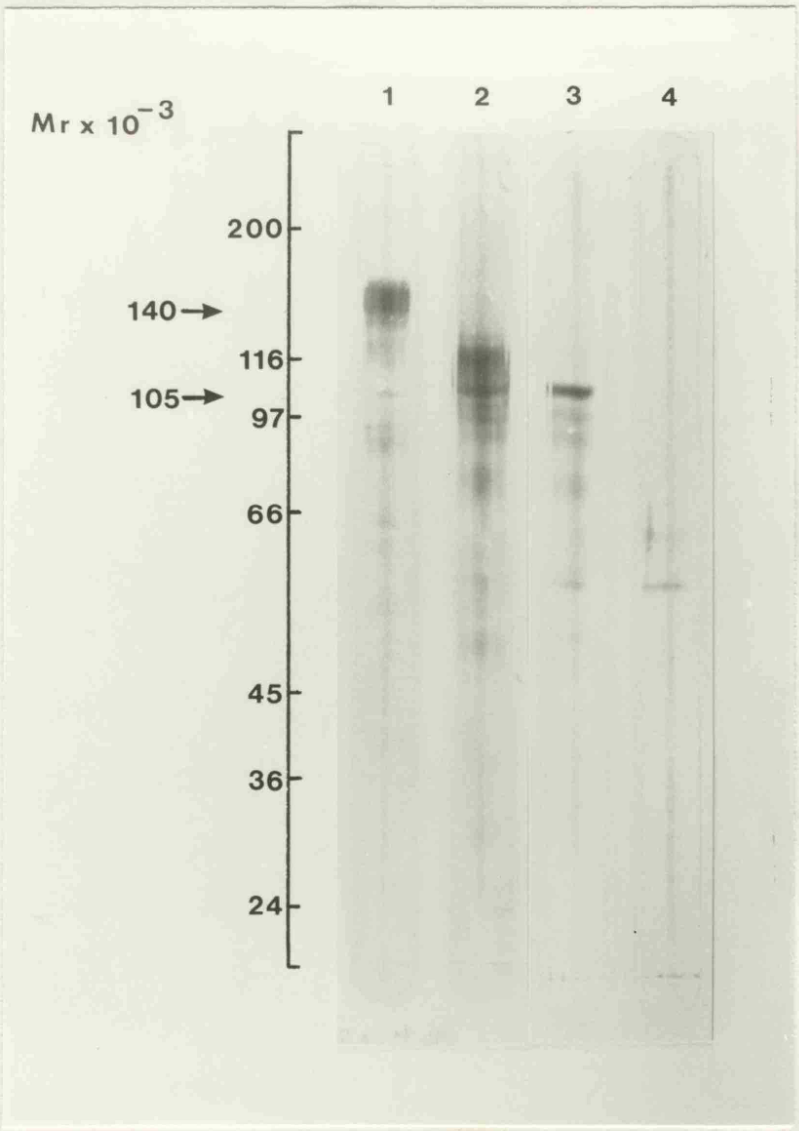


Figure 19

Immunoblot of solubilized transverse tubule membrane proteins following deglycosylation with TFMS. αDHP-R 13 staining of untreated (lane 1), 1 x treated with TFMS (lane 2) and 2 x treated with TFMS (lane 3). α-gyrase B staining of 2 x treated with TFMS (lane 4). 0.1mg T-tubule protein loaded for each lane. Mr markers as in Fig. 11.

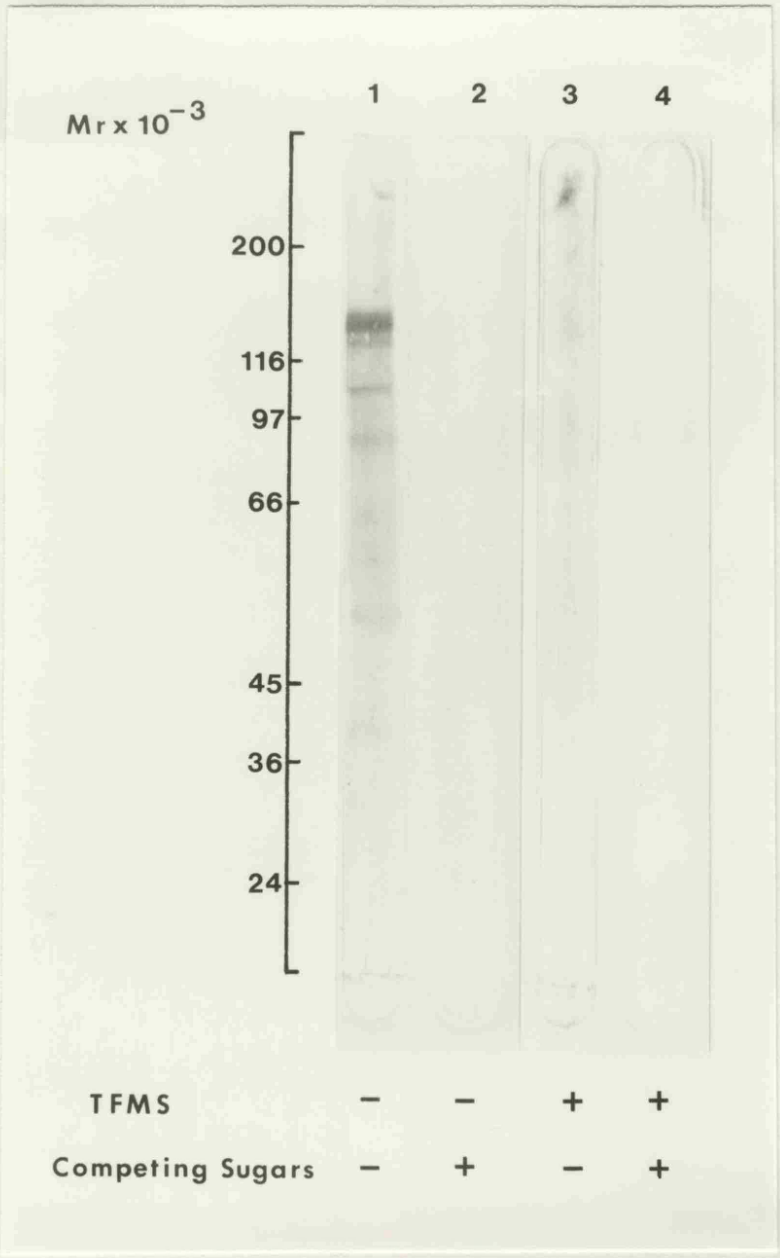


Figure 20

Blots of solubilized transverse tubule membrane proteins following deglycosylation with TFMS, stained with Concanavalin A peroxidase. Lanes 1 & 2, untreated membrane proteins; lanes 3 & 4, 2 x treated with TFMS. Con A peroxidase staining in the absence (lanes 1 & 3) and the presence (lanes 2 & 4) of competing sugars; 0.5M α-methyl-D-mannoside, 0.5M 1-0-methyl-α-D-glycopyranoside. 26μg T-tubule membrane protein loaded for each lane. Mr markers as in Fig. 11.

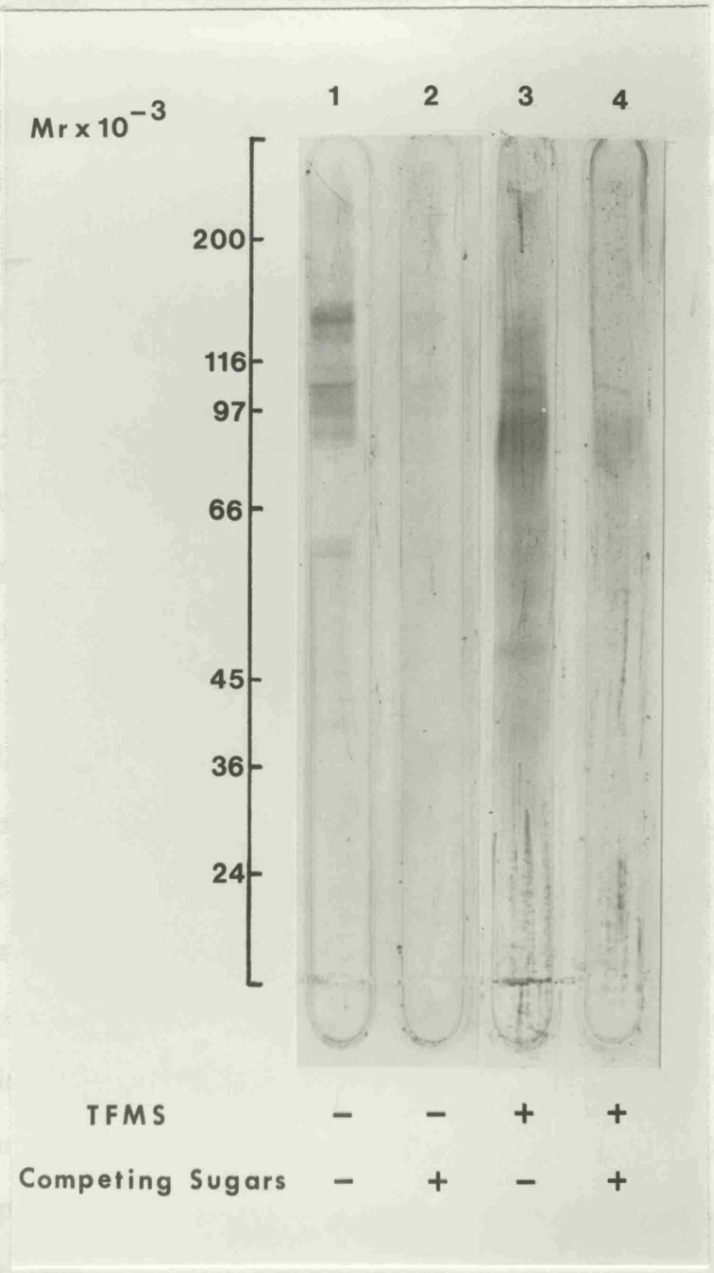


Figure 21

Blots of solubilized transverse tubule membrane proteins following deglycosylation with TFMS, stained with Wheat Germ Agglutinin peroxidase. Lanes 1 & 2, untreated membrane proteins; lanes 3 & 4, 2 x treated with TFMS. WGA peroxidase staining in the absence (lanes 1 & 3) and presence (lanes 2 & 4) of competing sugar 0.5M N-acetyl-D-glucosamine. 26μg T-tubule membrane protein loaded for each lane. Mr markers as in Fig. 11.

of TFMS treated membrane fractions were tested with Con A peroxidase no staining was observed with indicating complete removal of mannose and glucose residues from all T-tubule membrane glycoproteins (Fig. 20, lane 3).

This same procedure was repeated for both untreated and TFMS treated T-tubule membrane fractions using Wheat Germ Agglutinin-linked peroxidase in place of Con A to further confirm the extent of deglycosylation. WGA specifically recognizes neuraminic acid and N-acetylglucosamine residues. N-acetylglucosamine was included in the control lanes to check for specific staining (Fig. 21, lanes 2 & 4). The WGA peroxidase detected fewer membrane glycoproteins, in blots of untreated membrane fractions (Fig. 21, lane 1) than the Con A peroxidase (Fig. 20, lane 1). Interestingly, compared to other proteins, the Mr 140,000 component was stained relatively strongly following incubation with WGA peroxidase (Fig. 21, lane 1). This apparent selectivity for the Mr 140,000 component from many other T-tubule membrane proteins by the WGA peroxidase illustrates the reason for the efficacy of the WGA affinity chromatography step in the purification of the 1,4-DHP receptor. When blots of TFMS treated T-tubule membrane proteins were tested with WGA peroxidase, very little specific staining was observed indicating essentially complete deglycosylation. However, a faintly stained band of Mr 105,000 could be observed (Fig. 21, lane 3).

Essentially complete deglycosylation of the α_2 subunit achieved using the TFMS technique resulted in a reduction in apparent molecular weight 140,000 to 105,000. This is consistent with approximately 25% of the apparent molecular mass of this subunit being due to carbohydrate.

3.4.8. Chemical Deglycosylation of the α_2 Subunit of the Skeletal Muscle 1,4-Dihydropyridine Sensitive Calcium Channel from Different Species

Microsomal membranes, prepared from skeletal muscle tissue from different species (Section 3.3.3.), were subjected to a double treatment with TFMS following solubilization with Triton X-100 (Section 3.3.10.). The Triton X-100 solubilized untreated membranes were analysed in the immunoblot assay with αDHP-R 13. The

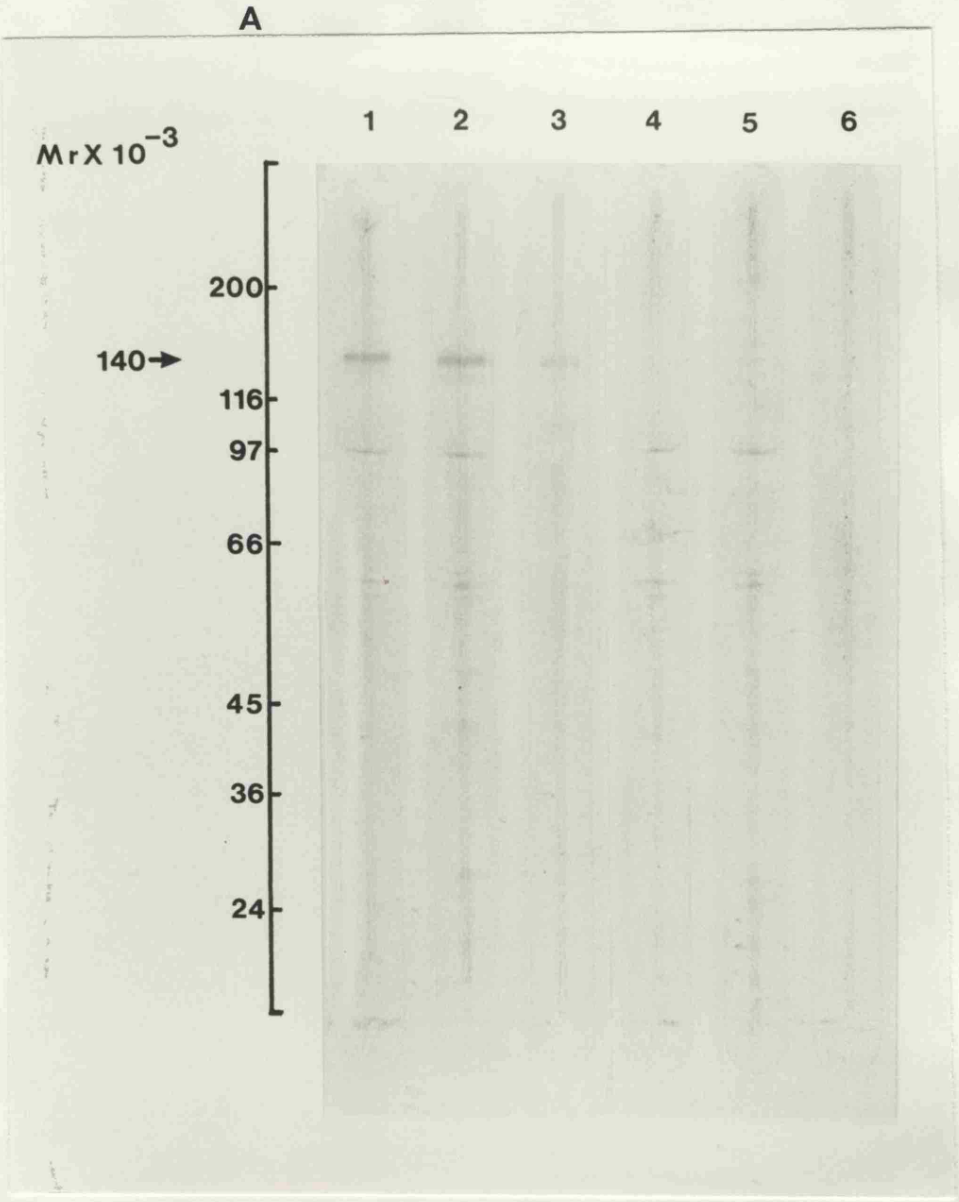


Figure 22

Immunoblots of solubilized skeletal muscle membrane proteins (A) untreated and (B) following deglycosylation with TFMS. Lanes 1-3 staining with αDHP-R 13; lanes 4-6 staining with α-gyrase B. Lanes 1 & 4, rabbit (57μg); lanes 2 & 5, rat (100μg); lanes 3 & 6, mouse (48μg). Mr markers as in Fig. 11.

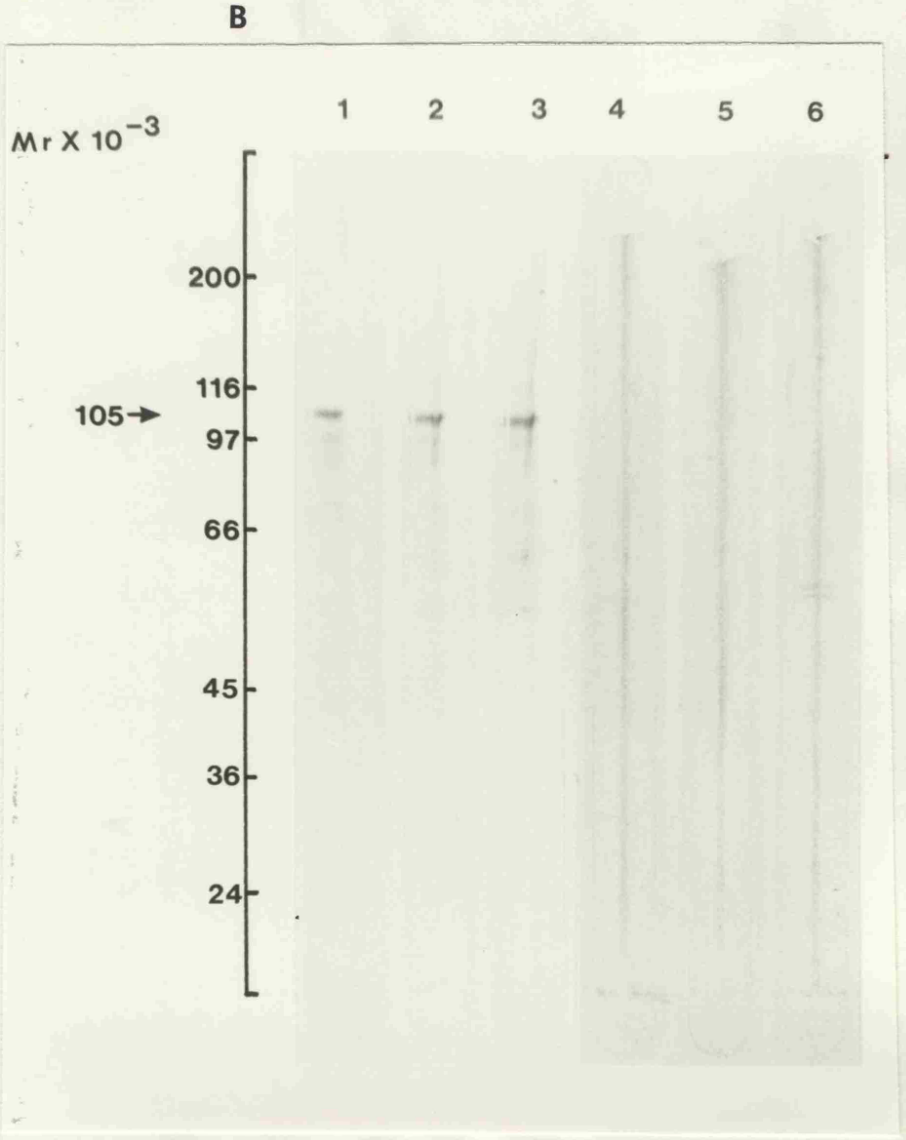


Figure 22

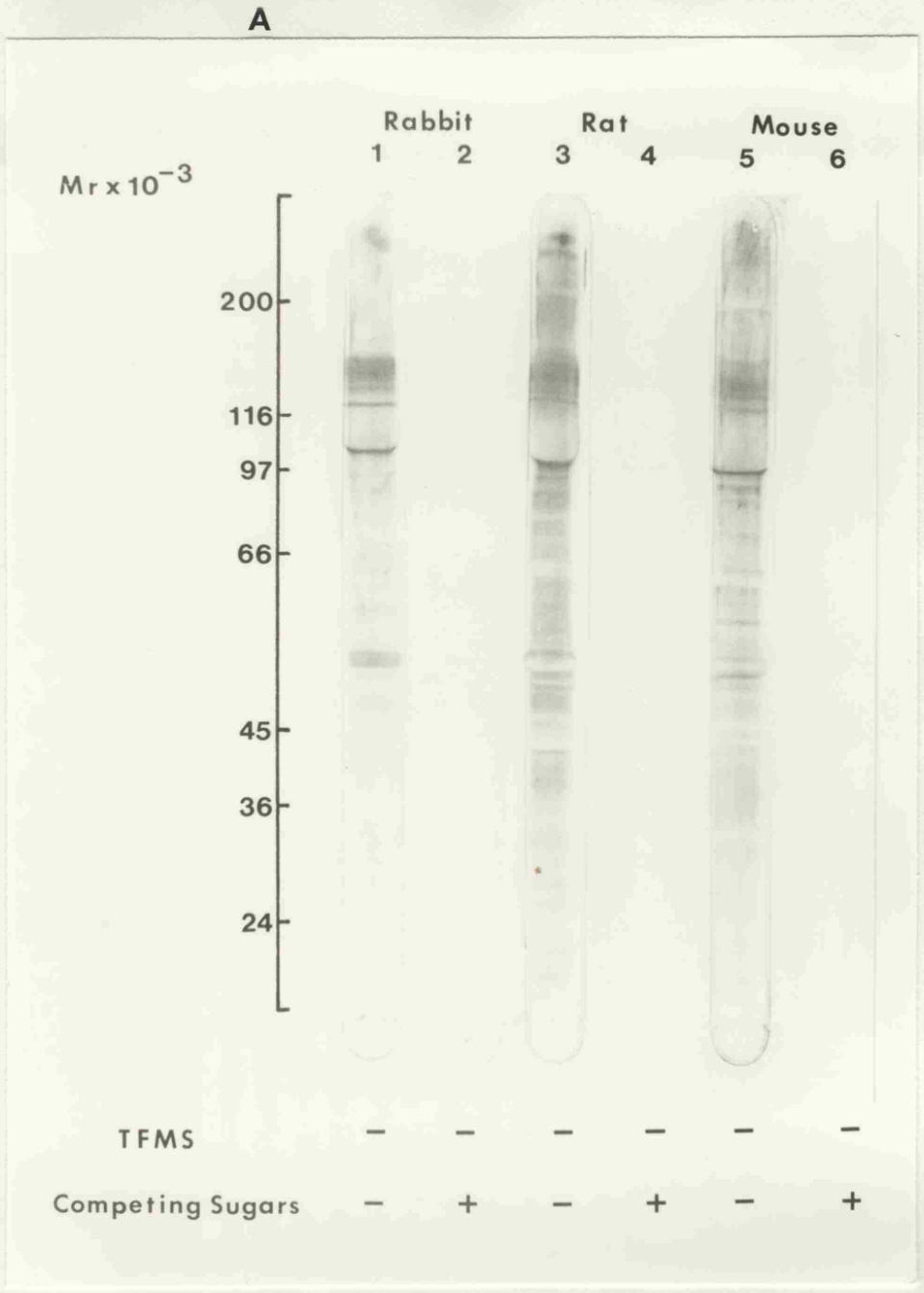


Figure 23

Blots of solubilized skeletal muscle membrane proteins stained with Concanavalin A peroxidase. (A) untreated membrane proteins and (B) following deglycosylation with TFMS. Staining in the absence (lanes 1, 3 & 5) and presence (lanes 2, 4 & 6) of competing sugars as in Fig. 20. Lanes 1 & 2, rabbit (57μg); lanes 3 & 4, rat (100μg); lanes 5 & 6, mouse (95μg). Mr markers as in Fig. 11.

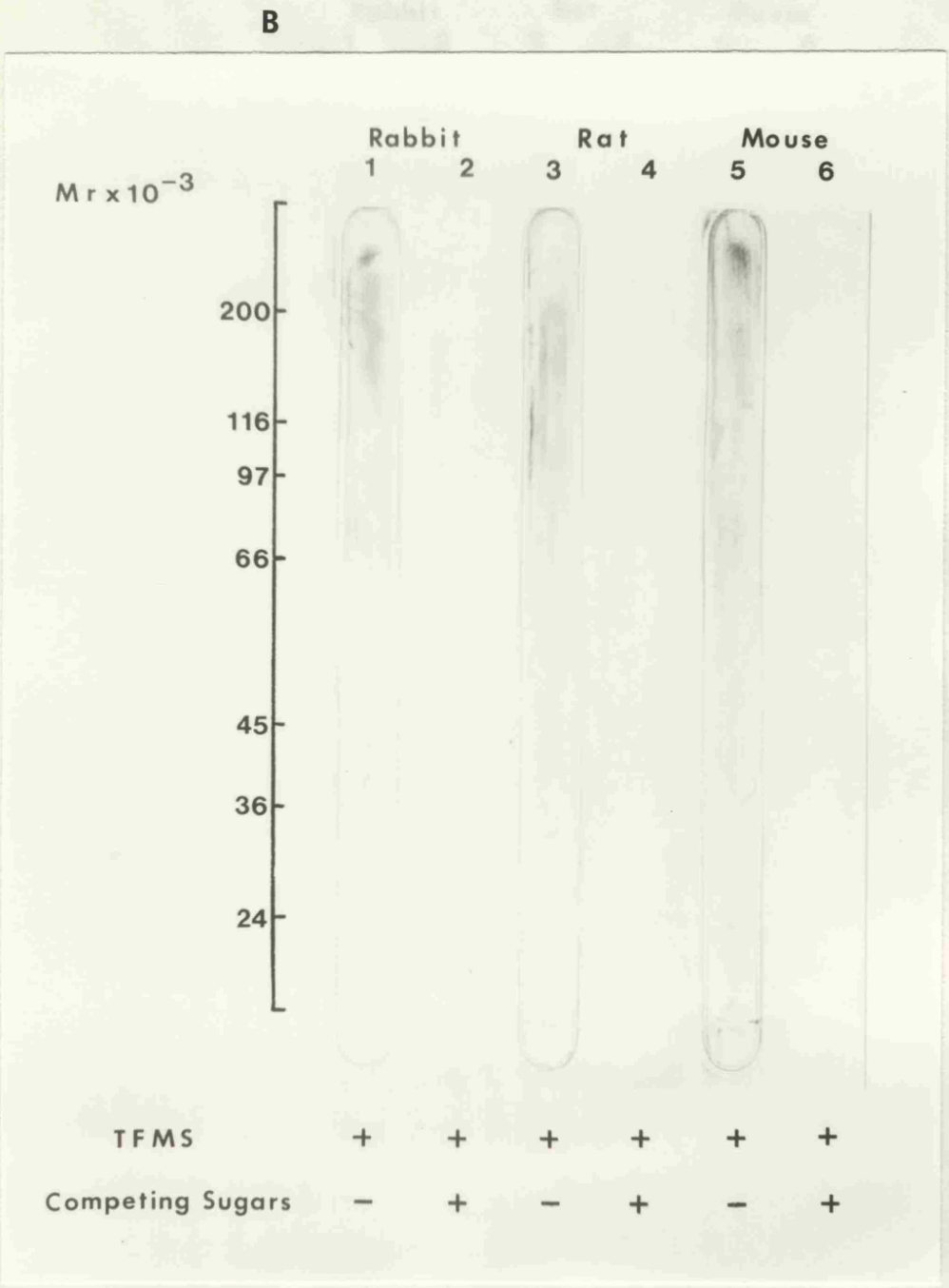

B
Rabbit
Rat
Mouse
1
2
3
4
5
6
Mr x10⁻³
200
116
97
66
45
36
24
TFMS + + + + + +
Competing Sugars − + − + − +

Figure 23

Figure 24

Blots of solubilized skeletal muscle membrane proteins stained with Wheat Germ Agglutinin peroxidase. (A) untreated membrane proteins and (B) following deglycosylation with TFMS. Staining in the absence (lanes 1, 3 & 5) and presence (lanes 2, 4 & 6) of competing sugar as in Fig. 21. Lanes 1 & 2, rabbit (57µg); lanes 3 & 4, rat (100µg); lanes 5 & 6, mouse (95µg). Mr markers as in Fig. 11.

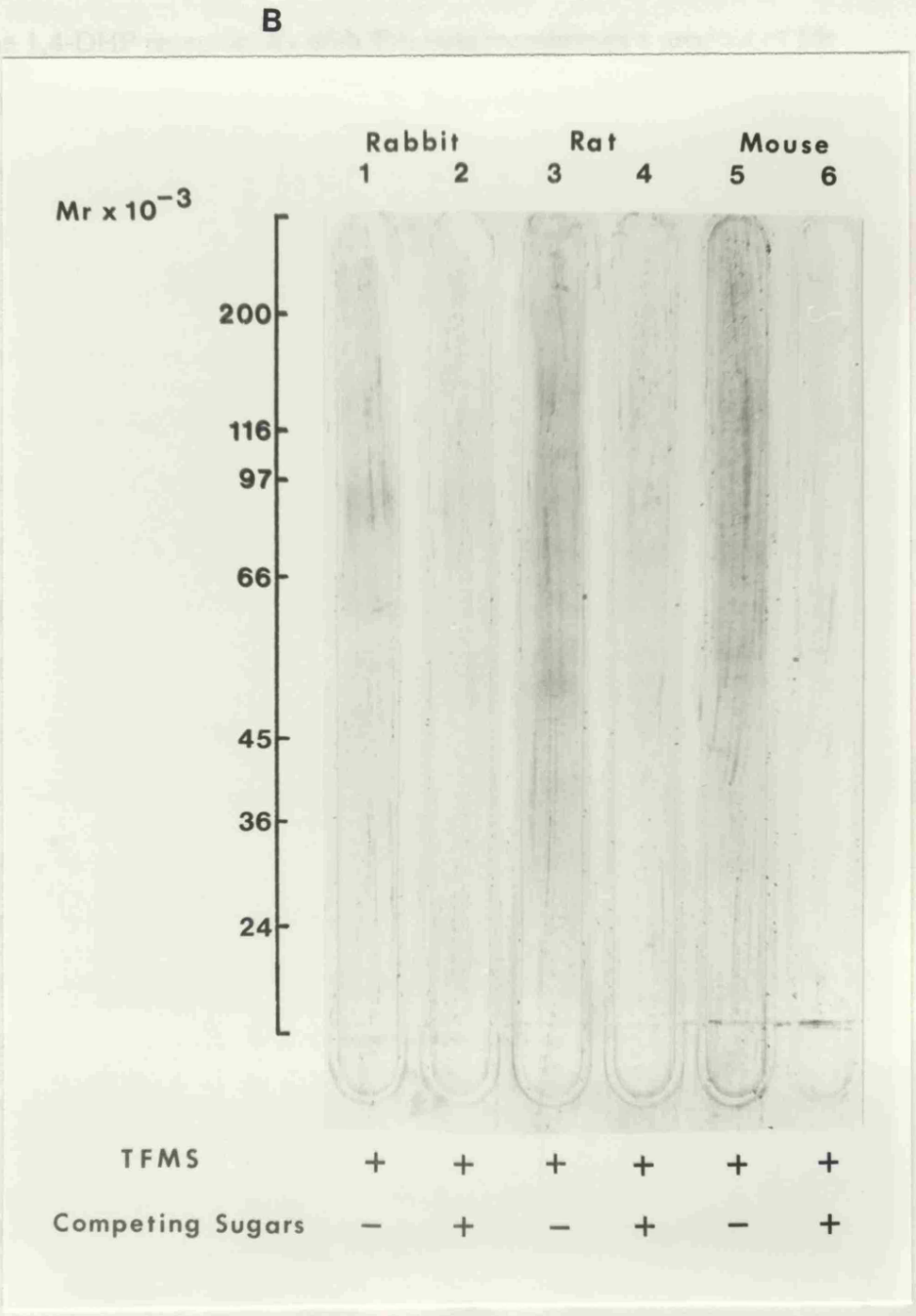

B
Rabbit
Rat
Mouse
1 2 3 4 5 6
Mr x 10⁻³
200
116
97
66
45
36
24
TFMS + + + + + +
Competing Sugars − + − + − +

Figure 24

antibody detected specifically components of Mr 140,000-145,000 (Fig. 22A, lanes 1-3) as described previously (Section 3.4.4., Fig. 14A). The TFMS treated membranes were similarly analysed in the immunoblot assay to detect the non-glycosylated product of the α_2 subunit of the 1,4-DHP receptor. As with T-tubule membranes a product of Mr 100,000-110,000 was specifically recognized by the αDHP-R 13 antibody (Fig. 22B, lanes 1-3). No staining was detected in immunoblots with the control antibody α-gyrase B (Fig. 22B, lanes 4-6).

Blots of both untreated and TFMS treated skeletal muscle membranes were tested with lectin-linked peroxidases in a similar manner to the T-tubule membranes. Figure 23A shows the blot of untreated microsomal membranes with Con A peroxidase in the absence and the presence of competing sugars (see Fig. 23 legend). When the treated membranes were tested with Con A peroxidase no specific staining was observed (Fig. 23B), indicating essentially complete deglycosylation. Blots of treated proteins were tested with WGA peroxidase to confirm the extent of deglycosylation (Fig. 24B). As seen with T-tubule membranes slight staining in the region Mr 105,000 was observed with the rat microsomal membranes (Fig. 24B, lane 3), possibly due to residual N-linked N-acetylglucosamine.

The use of the lectin-linked peroxidases in the blot analyses confirmed the removal of essentially all carbohydrate residues from all skeletal muscle membrane glycoproteins using the double TFMS treatment. The results from the immunoblot assays with αDHP-R 13 indicate that the fully deglycosylated peptide of the α_2 subunit of the 1,4-DHP receptor has an apparent molecular weight of 105,000. The α_2 subunits of the 1,4-DHP receptor from the skeletal muscle of different species have previously been shown to be similar in terms of molecular size (Section 3.4.4., Fig. 14). It is now clear that the α_2 subunits of the skeletal muscle 1,4-DHP receptors from different species are also similar in terms of the extent of glycosylation.

3.4.9. The Effect of Enzymatic Deglycosylation on the Equilibrium Binding of (+)-[^{3}H]PN200-110 to the Transverse Tubule 1,4-Dihydropyridine Receptor

A number of recent reports have postulated the involvement of a number of subunits in the 1,4-DHP receptor complex (Section 1.3.4.). Photoaffinity labelling studies have suggested that the binding sites for the 1,4-DHPs and other Ca^{2+} channel drugs are located on the Mr 170,000, non-glycosylated, α_1 subunit of the receptor complex (Galizzi et. al. 1986; Tanabe et. al. 1987). To investigate the possible involvement of the Mr 140,000, glycosylated, α_2 subunit in the binding site for the 1,4-DHPs, the effect of enzymatic deglycosylation on (+)-[^{3}H]PN200-110 binding was investigated.

Native T-tubule membranes were treated with various deglycosylating enzymes (Section 3.3.8.), prior to assay for membrane-bound (+)-[^{3}H]PN200-110 binding sites as described in Section 2.3.2. Treated membranes were also analysed in the immunoblot assay with αDHP-R 13 to assess the extent of deglycosylation (Section 3.3.4. & 3.3.5.). The incubation conditions were different to the conditions in Section 3.4.6. to enable binding assays to be performed. However, the immunoblot assay with αDHP-R 13 (Figure 25A) shows that the 3 hour incubation with the deglycosylating enzymes produced similar reductions in the apparent molecular weight of the α_2 subunit in native membranes as previously seen in the CHAPS solubilized samples (Section 3.4.6., Fig. 17A). No staining was detected with the control antibody α-gyrase B (Fig. 25B) indicating that the staining with αDHP-R 13 was specific.

Figure 26 shows the effect of the various enzymatic treatments on the equilibrium binding of (+)-[^{3}H]PN200-110 to native T-tubule membrane 1,4-DHP receptors. Incubation with neuraminidase, which removes the terminal neuraminic acid residues from oligosaccharide chains, only produced a small reduction in the apparent molecular weight of the α_2 subunit and resulted in complete conversion to a defined reaction product of Mr of 133,000 (Fig. 25A, lane 2). However the neuraminidase treatment (10U/ml) resulted in a large decrease of 73% ± 2% in the maximum number of available (+)-[^{3}H]PN200-110 binding sites with no significant change in the apparent dissociation constant (K_d) of the remaining binding sites (0.2-0.83nM, Fig. 26). The inclusion of

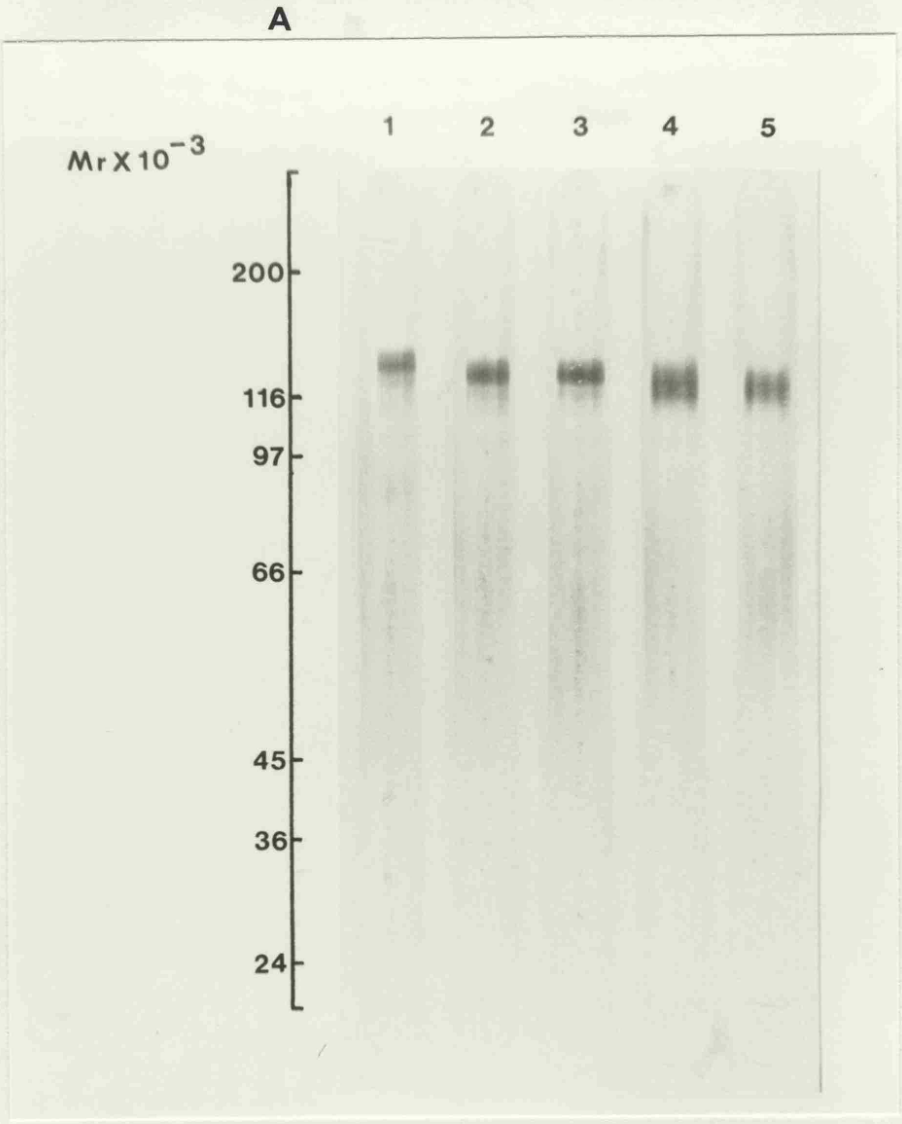


Figure 25

Immunoblots of native transverse tubule membrane proteins following enzymatic deglycosylation stained (A) with αDHP-R 13 and (B) with α-gyrase B. Lane 1, no enzyme; lane 2, neuraminidase; lane 3, neuraminidase, α-glucosidase, β-glucosidase and β-galactosidase; lane 4, neuraminidase and Endoglycosidase F; lane 5, neuraminidase, α- and β-glucosidase, β-galactosidase, Endoglycosidases F & H. Enzyme concentrations were as in Fig. 17 except for neuraminidase 10 units/ml. 50μg T-tubule membrane protein loaded for each lane. Mr markers as in Fig. 11.

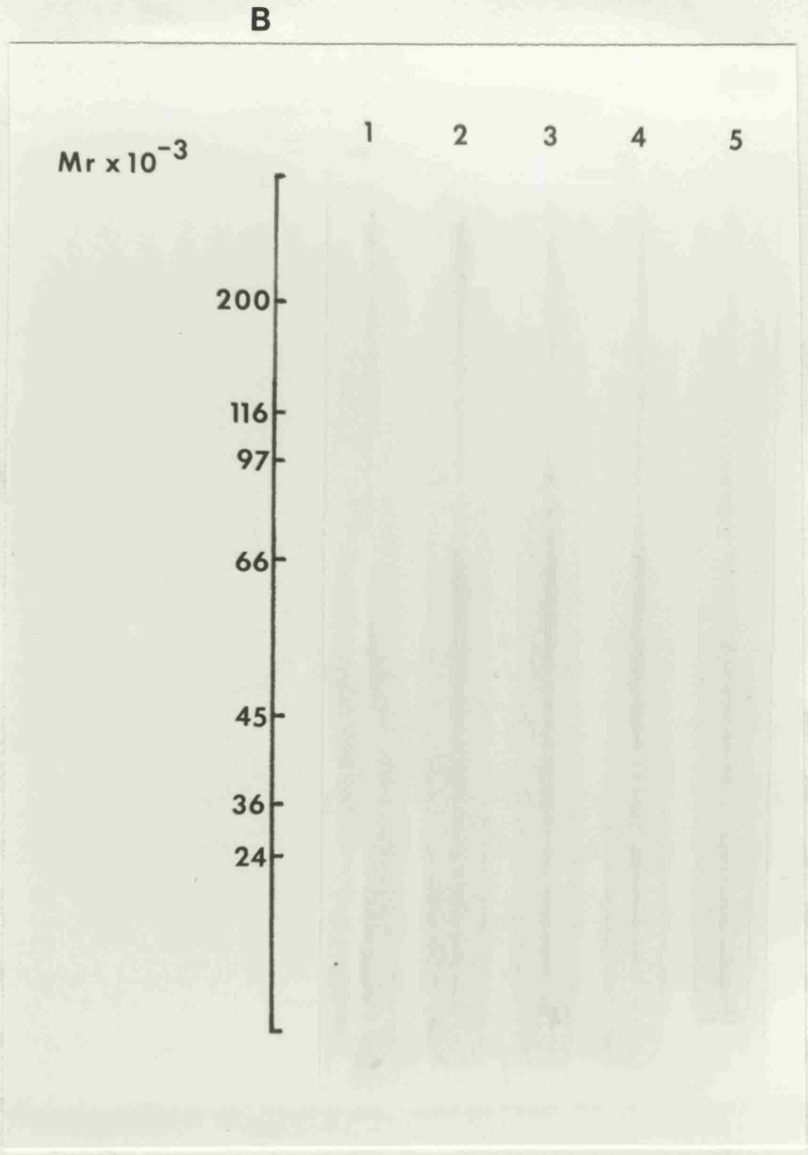
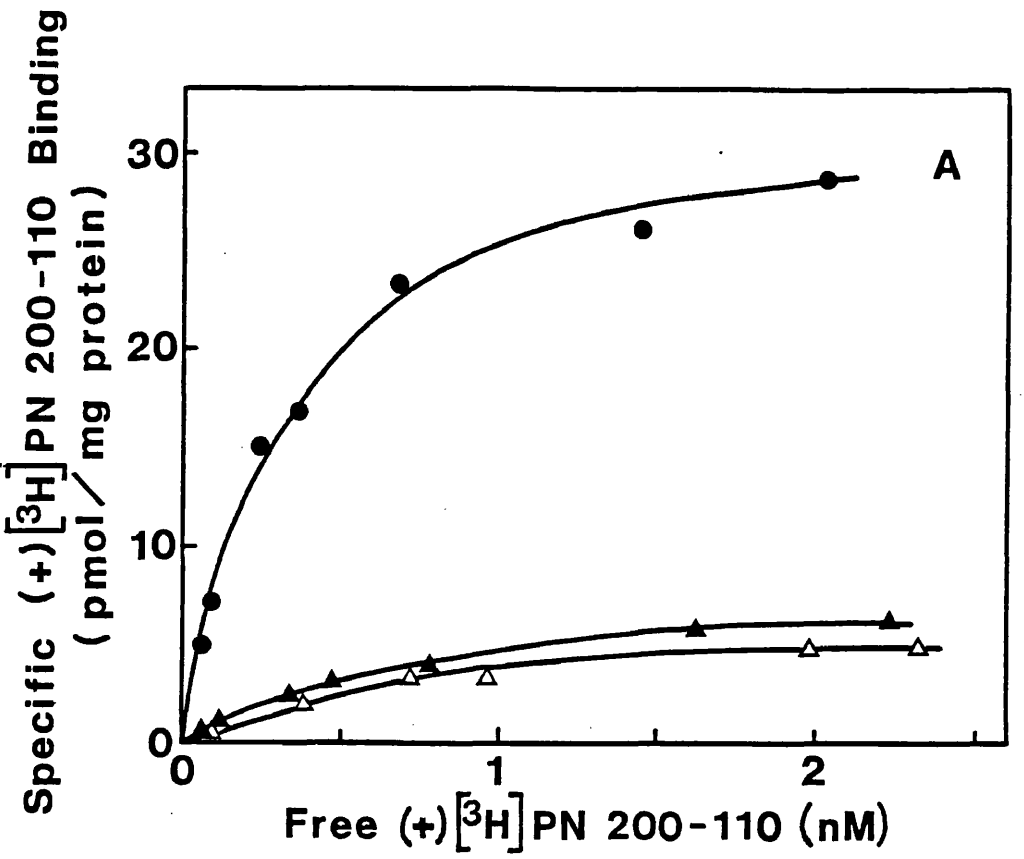


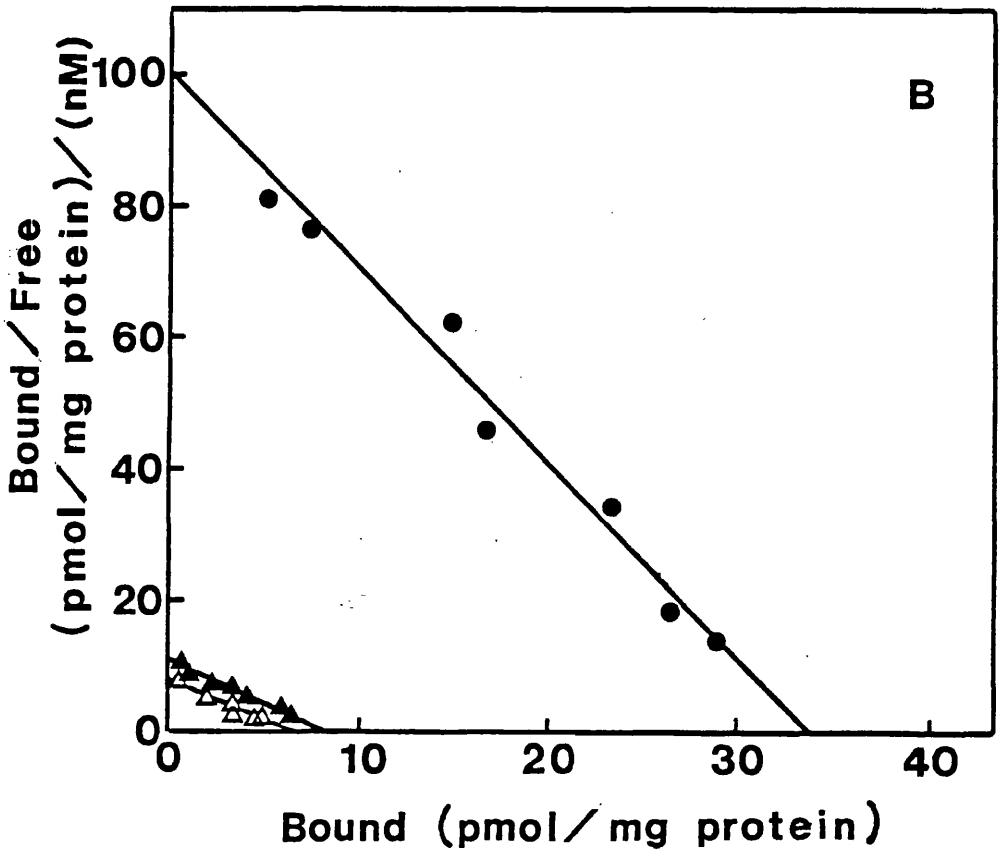
Figure 25

Figure 26

Equilibrium binding of (+)-[^{3}H]PN200-110 to transverse tubule membranes before and after enzymatic deglycosylation. Membranes were assayed for binding activity as described in Section 2.3.2. at the indicated concentrations of (+)-[^{3}H]PN200-110 (0.5mg protein/ml). (A) Specific binding of (+)-[^{3}H]PN200-110 prior to (●) and following treatment with neuraminidase (▲) or neuraminidase and Endoglycosidase F (△). Non-specific binding was determined in the presence of 1μM nitrendipine.

(B) Scatchard plots of the specific binding.


Specific (+)[3H]PN 200-110 Binding
(pmol/mg protein)
30
20
10
0
0
1
2
A
Free (+)[3H]PN 200-110 (nM)


Bound/Free
(pmol/mg protein)/(nM)
100
80
60
40
20
0
0
10
20
30
40
B
Bound (pmol/mg protein)

Treatment	B_{max} pmol/mg protein (%)	K_d nM
No enzyme	33.0 (100)	0.33
Neuraminidase	9.0 (28)	0.82
Neuraminidase & Endoglycosidase F	7.6 (23)	0.89

Table 10

Effect of Enzymatic Deglycosylation on (+)-[^{3}H] PN 200-110 Binding to Transverse Tubule Membranes

Treatment of native T-tubule membranes with neuraminidase causes a reduction of 72 ± 3% in the maxim$\hat{}^{um}$ (+)-[^{3}H]PN200-110 binding sites. Treatment with neuraminidase and Endoglycosidase F resulted in only a slight further reduction in the (+)-[^{3}H]PN200-110 binding to 77 ± 5%. Figures in parentheses represent the proportion of (+)-[^{3}H]PN200-110 binding sites remaining.

Endoglycosidase F with neuraminidase in the incubation medium resulted in a further reduction in the apparent molecular weight of the α_2 subunit (Fig. 25A, lane 4) but there was only a slight further reduction in the number of available [^{3}H]PN200-110 binding sites giving a total loss of 77% ± 5% (Fig. 26). Table 10 summarizes the effects of the various enzymes on the α_2 subunit and on (+)-[^{3}H]PN200-110 binding.

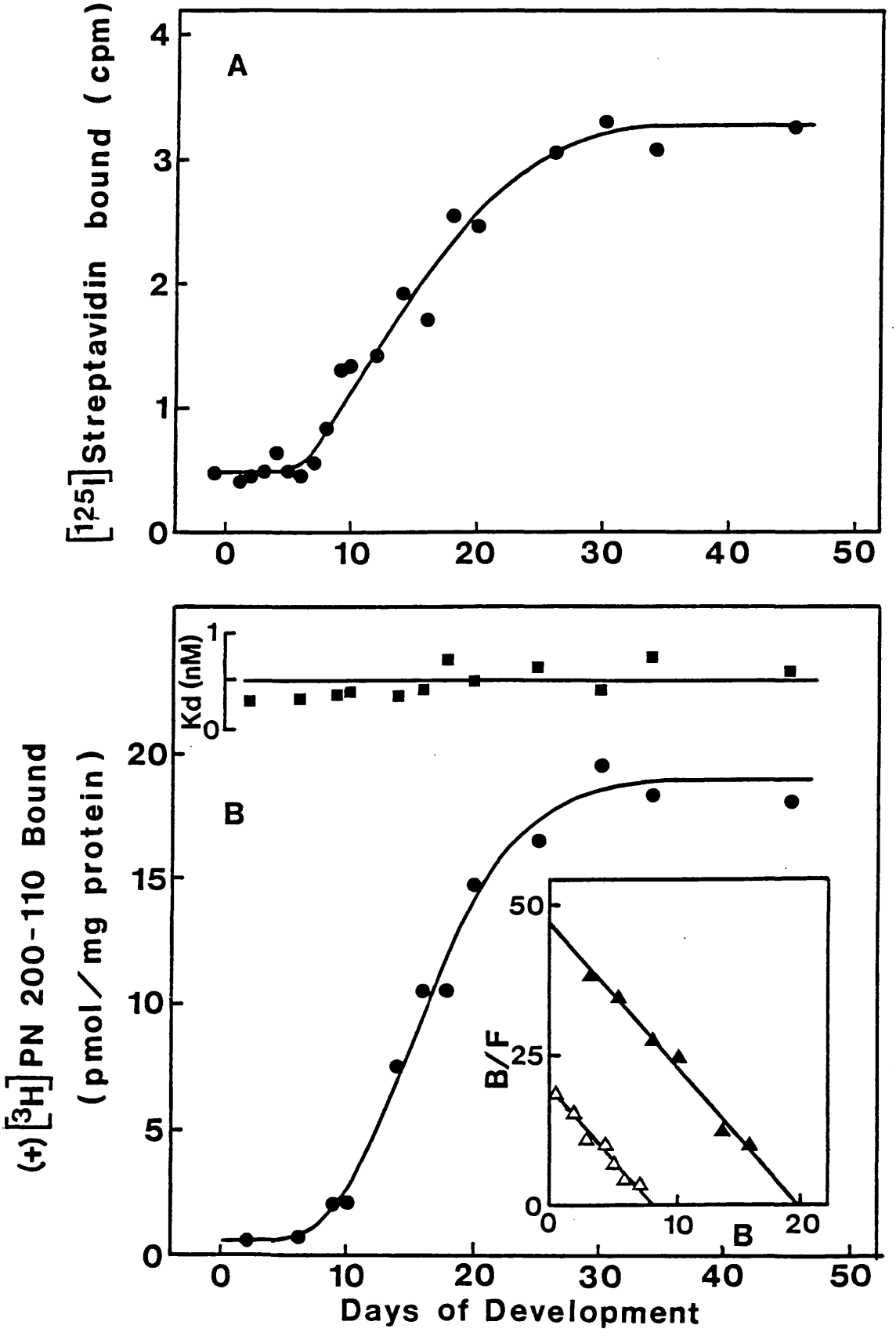
3.4.10. Co-Development of the α_2 Subunit and 1,4-Dihydropyridine Binding Activity

It has been shown previously that 1,4-DHP binding to T-tubule membranes is sensitive to the extent of glycosylation (Section 3.4.9., Fig. 26). This suggests that the glycosylated, Mr 140,000 component is involved in the 1,4-DHP sensitive Ca^{2+} channel complex. To substantiate this result the development of the α_2 component was analysed by semi-quantitative immunoblot (Section 3.3.10.) and correlated to the development of the receptor as measured by 1,4-DHP binding (Section 3.3.11.).

Figure 27 (A) shows the development of the Mr 140,000 component with age, quantified in immunoblots using [^{125}I]Streptavidin. The Mr 140,000 component in skeletal muscle membranes remains at a steady low level over the first few days development from 19 days gestation to 8 days post-partum. The levels of this component then increase rapidly to reach a new high plateau level at 30 days post-partum. Interestingly, this development of the α_2 subunit parallels the appearance of the 1,4-DHP receptor as measured by the equilibrium binding of (+)-[^{3}H]PN200-110 to muscle membranes prepared from rats of different ages (Fig. 27 (B)). This co-development of the α_2 subunit and the 1,4-DHP binding site suggests strongly that the large glycoprotein subunit is involved in the structure of the 1,4-DHP-R sensitive Ca^{2+} channel throughout development.

Figure 27

Development of the α2 glycoprotein subunit and (+)-[^{3}H]PN200-110 binding component in rat skeletal muscle. Development of (A) α2 subunit determined as described in Section 3.3.10. and (B) (+)-[^{3}H]PN200-110 binding activity vs time in days. K_d values (■) obtained at each stage of development from Scatchard analysis. Specific binding compared to total binding was between 50-90%. Inset Scatchard plots of specific (+)-[^{3}H]PN200-110 binding to homogenates of skeletal muscle tissue of 14 day (△) and 30 day (▲) rats. B is bound (+)-[^{3}H]PN200-110 expressed in fmol bound/mg protein and B/F is Bound/Free (+)-[^{3}H]PN200-110 expressed in nM.


A
[125I]Streptavidin bound (cpm)
0
1
2
3
4
0
10
20
30
40
50
B
Kd (nM)
0
1
(+)[3H]PN 200-110 Bound
(pmol/mg protein)
0
5
10
15
20
B/F
0
25
50
0
10
20
B
Days of Development

3.5. SUMMARY

The 1,4-DHP receptor associated with the skeletal muscle voltage-dependent Ca^{2+} channel involves a large glycoprotein subunit (α_2) of Mr 140,000. The immunoblot assays carried out under non-reducing conditions indicate that this component is disulphide-linked to a smaller component of Mr 30,000. Similar components of Mr 140,000 were detected in tissues known from ligand binding studies to contain 1,4-DHP receptors. The α_2 subunits of the 1,4-DHP receptor in skeletal muscle membranes prepared from different species are similar in molecular size and there is a certain amount of sequence homology in terms of antibody binding sites and trypsin cleavage sites. On deglycosylation the apparent molecular weight of the α_2 subunit is reduced to an Mr of 105,000. This indicates that approximately 25% of the molecular weight of this glycoprotein component is carbohydrate. This extent of glycosylation is very similar to that of the α subunit of the Na^+ channel. The removal of the terminal neuraminic acid residues from the α_2 subunit of the 1,4-DHP receptor resulted in a large decrease of 73% in the number of available 1,4-DHP binding sites, suggesting that the glycosylated, α_2 subunit is involved in the 1,4-DHP sensitive Ca^{2+} channel structure. Additionally, the co-development of the α_2 subunit with the appearance of 1,4-DHP binding suggests that this component is an integral part of the 1,4-DHP sensitive Ca^{2+} channel throughout development.

CHAPTER 4 DISCUSSION

4.1. Purification of the α_2 Subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel

The 1,4-DHP receptor associated with the voltage-sensitive Ca^{2+} channel was purified from skeletal muscle T-tubule membranes using the high affinity 1,4-DHP, (+)-[^{3}H]PN200-110, as a probe. Receptor protein purified in this manner was used as antigen for the production of monoclonal antibodies. These antibodies provided specific probes for the further characterization of the molecular structure of the 1,4-DHP receptor. The purified receptor protein was used in a parallel project concerned with the analysis of the primary sequence of the 1,4-DHP receptor.

The purification procedure was based on that of Borsotto et. al. (1984b), however, an additional detergent treatment step was included to reduce the presence of contaminating polypeptides in the purified fractions when analysed by SDS polyacrylamide gel electrophoresis. With the inclusion of this extraction step, the major component consistently detected in fractions containing the 1,4-DHP receptor activity was a polypeptide of Mr 140,000 (Section 2.4.5., Fig. 11). This result suggested that a polypeptide of Mr 140,000 (later designated α_2 subunit by Takahashi et. al., 1987) was involved in the molecular structure of the voltage-sensitive Ca^{2+} channel. Reports by other workers using different purification procedures have shown similar results (Borsotto et. al., 1984b, 1985; Curtis and Catterall, 1984) In these studies, when the purification was performed on CHAPS solubilized T-tubule membranes, a polypeptide of Mr 142,000 was identified in fractions containing the 1,4-DHP receptor activity. This component was very similar to the subunit (Mr 130,000) detected in receptor preparations purified from digitonin solubilized T-tubule membranes by Curtis and Catterall (1984). Furthermore, in a recent publication Nakayama et. al. (1987), using a large-scale purification procedure, also detected a polypeptide with similar properties to this subunit, in samples containing 1,4-DHP receptor activity.

In addition to the major component of Mr 140,000, two other minor components were detected in silver stained polyacrylamide gels of samples containing the 1,4-DHP

receptor activity. A small molecular weight component of Mr~30,000 was occasionally found to co-purify with the Mr 140,000 subunit. It is possible that this peptide, not consistently detected in stained SDS polyacrylamide gels of our purified fractions, is a proteolytic product of the Mr 140,000 subunit. However, results from the immunoblotting experiments in this study, using monoclonal antibodies raised against the 1,4-DHP receptor, suggest that a component of similar molecular weight (~30,000) is disulphide-linked to the Mr 140,000 subunit and hence is involved as a distinct component of the voltage-sensitive Ca^{2+} channel complex (Section 3.4.2., Fig. 12B). Furthermore, other workers using different purification protocols (Borsotto et. al., 1984b, 1985; Curtis and Catterall, 1984) have demonstrated that a polypeptide of Mr~30,000 co-purifies consistently with the Mr 140,000 subunit. These workers have also suggested that this small component is an additional subunit involved in the molecular structure of the voltage-sensitive Ca^{2+} channel.

Another polypeptide of Mr~100,000 was occasionally detected as a minor component in silver stained SDS polyacrylamide gels of 1,4-DHP fractions purified as described in this study (Section 2.4.5., Fig. 11). This component could be a contaminating protein in the receptor preparation. Vaghy et. al. (1987) have suggested that a similar component, detected in 1,4-DHP receptor preparations purified using a different purification protocol, is due to contamination by the skeletal muscle (Ca^{2+}-Mg^{2+})-ATPase which has an Mr 105,000. Alternatively, the Mr 100,000 component could be a proteolytically derived fragment of the Mr 140,000 polypeptide. It is noteworthy that, the deglycosylation experiments described earlier in this study (Section 3.4.7.) indicate that the non-glycosylated Mr 140,000 polypeptide has an Mr of 105,000 (Fig. 19). It is therefore a possibility that this component, occasionally detected in WGA affinity gel purified fractions, is the non-glycosylated moiety of the Mr 140,000 subunit. However, this component was not detected in immunoblots of the purified receptor (data not shown) or of T-tubule membrane fractions (Section 3.4.2., Fig. 12).

It is clear that, using the purification procedure described in this study, the Mr 140,000 subunit is the major polypeptide detected consistently in WGA affinity gel purified fractions containing 1,4-DHP receptor activity. Consequently, it is probable that this

component is involved in the molecular structure of the 1,4-DHP sensitive Ca^{2+} channel.

The purified 1,4-DHP receptor preparations were used to immunize mice for the production of monoclonal antibodies. Hybridoma cell lines secreting the antibodies of interest were selected by ELISA assay of the cell supernatants using the purified receptor as antigen. Monoclonal antibodies produced against this channel component were used to further characterize the 1,4-DHP sensitive Ca^{2+} channel structure.

4.2. Properties of the α_2 Subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel

4.2.1. Evidence for Disulphide Linkage to a Mr 30,000 Polypeptide Subunit

The properties of the α_2 subunit of the voltage-sensitive Ca^{2+} channel were investigated using monoclonal antibodies, raised against the 1,4-DHP receptor, as specific probes. Diluted ascitic fluids were tested in immunoblot assays on T-tubule membranes denatured in the presence of a disulphide reducing agent (DTT). The antibodies that specifically recognized the Mr 140,000, α_2 subunit in immunoblots under these conditions (αDHP-Rs 11, 13, 14 and 15; Section 3.4.2., Fig. 12A) were further assayed in immunoblots of T-tubule membranes denatured in the absence of the reducing agent. Under these conditions the antibodies detected a component that migrated more slowly in SDS gels with an Mr of 170,000 (Section 3.4.2., Fig. 12B).

It is possible from the change in migration of the Mr 140,000 (α_2) subunit under non-reducing conditions that this Ca^{2+} channel component is disulphide-linked to a small polypeptide of Mr 30,000. Another possibility is that the observed change in migration of the Mr 140,000, α_2 subunit under these conditions is a consequence of an alteration in the shape of the protein component, due to internal disulphide bonds that remain intact in the absence of the reducing agent. Results published by other workers have also identified a small component of Mr 30,000 linked by disulphide bonds to the Mr 140,000 polypeptide (Schmid et. al., 1986a; Schmid et. al., 1986b). Schmid et. al. (1986a) prepared antiserum directed against the Mr 30,000 polypeptide of purified Ca^{2+} channel preparations. In immunoblots of rabbit skeletal muscle microsomes under reducing conditions the antiserum detected components of Mr 30,000. Under non-reducing

conditions the Mr 30,000 antiserum specifically recognized a polypeptide of Mr 170,000 indicating that the Mr 30,000 component was disulphide-linked to a large polypeptide of Mr 140,000 (Schmid et. al., 1986a). They confirmed this result in immunoblot studies using antiserum directed against the Mr 140,000 Ca^{2+} channel component (Schmid et. al., 1986a). This result is consistent with the findings in this study (Section 3.4.2., Fig 12B) suggesting that the Mr 170,000 polypeptide detected in non-reducing gels is composed of two smaller components of Mr 140,000 and Mr 30,000 linked by disulpide bonds.

The presence of a similar polypeptide of Mr 170,000 consisting of a disulphide-linked dimer of the Mr 140,000 and Mr 30,000 components has been demonstrated in other tissues containing 1,4-DHP sensitive Ca^2 channels. An Mr 140,000 component has been identified in purification studies from cardiac tissue (Cooper et. al., 1987). In immunoblots of smooth muscle and heart microsomes under non-reducing conditions antiserum directed against the Mr 30,000 component detected polypeptides of Mr 170,000 and Mr 176,000 respectively (Schmid et. al., 1986a). Furthermore, antiserum directed against the Mr 170,000 glycoprotein component of purified skeletal muscle Ca^{2+} channel preparations recognized both the Mr 140,000 and Mr 30,000 polypeptides in immunoblots of both skeletal muscle membranes and brain synaptosomes under reducing conditions (Schmid et. al., 1986b). Takahashi and Catterall (1987a & b) have detected a similar component in cardiac and brain membranes using polyclonal serum. These findings using polyclonal sera confirm the immunoblot results of this study with the αDHP-R 13 monoclonal antibody. Taken together these investigations provide substantial evidence for the involvement of an Mr 170,000 component, composed of a disulphide-linked dimer (Mr 140,000-Mr 30,000), in the 1,4-DHP sensitive Ca^{2+} channel structure.

An alternative explanation was suggested for similar results derived from immunoprecipitation experiments with a monoclonal antibody raised against the 1,4-DHP receptor (Vandaele et. al., 1987). Under non-reducing conditions, the antibody precipitated a polypeptide of Mr 170,000 from both CHAPS and digitonin solubilized T-tubule membranes. However, different profiles were obtained following

immunoprecipitation under reducing conditions. From the membranes solubilized in CHAPS detergent, polypeptides of Mr 140,000, 30,000 and 26,000 were immunoprecipitated, whereas polypeptides of Mr 170,000, 140,000, 30,000 and 26,000 were precipitated from digitonin solubilized membranes by the antibody. Vandaele et. al. (1987) suggested that the Mr 140,000 and 30,000 polypeptides were components of an Mr 170,000 subunit that possessed an internal disulphide bond and was susceptible to proteolysis. They proposed that the Mr 170,000 polypeptide was proteolytically nicked into the two components of Mr 140,000 and 30,000, that remained associated by the internal disulphide bond in the absence of a reducing agent. However, under reducing conditions, disruption of the internal disulphide bond resulted in the dissociation of the two proteolytic fragments (Vandaele et. al., 1987). They suggested that in CHAPS detergent the proteolysis was complete, whereas in digitonin only partial proteolysis occurred giving rise to polypeptides of Mr 170,000, 140,000 and 30,000 in the presence of reducing agent. It was proposed that the Mr 26,000 polypeptide was a proteolytic product of the Mr 30,000 component. Recent reports now suggest that the Mr 170,000 polypeptide detected by Vandaele et. al. (1987), the molecular weight of which is unchanged on reduction, is a distinct subunit of the Ca^{2+} channel complex (Takahashi et. al., 1987; Vaghy et. al., 1987).

Certain antibodies raised in this study (αDHP-Rs 13, 14 and 15), identified high molecular weight components of Mr 310,000-330,000 (Fig. 12B), in addition to the Mr 170,000 polypeptide in immunoblots of T-tubule membranes under non-reducing conditions. This result suggests the possibility that a higher order structure is involved consisting either of a dimer of the Mr 170,000 polypeptide or indicating that other polypeptide components are involved in the Ca^{2+} channel structure.

4.2.2. Immunocrossreactivity of the α_2 Subunit

One of the monoclonal antibodies, αDHP-R 13, that recognized specifically the α_2 subunit in immunoblot analyses, was used to investigate possible immunological similarities between this subunit of the Ca^{2+} channel from different tissues and species.

Components of Mr 140,000 were specifically detected by αDHP-R 13 in immunoblots of both rabbit brain and heart microsomal membranes (Section 3.4.3., Fig. 13). Takahashi and Catterall (1987a & b) have also detected a similar component in cardiac and brain membranes with polyclonal antibodies raised against the skeletal muscle Ca^{2+} channel. The high levels of microsomal membrane protein required for detectable staining with αDHP-R 13 in these tissues (Section 3.4.3., Fig. 13) could possibly be a reflection of the low levels of high affinity 1,4-DHP binding sites measured in these tissues (0.5-2.0 pmol/mg protein; Section 1.3.2., Table 2). Interestingly, the Mr 140,000 subunit was only detected in those tissues where the presence of voltage-sensitive Ca^{2+} channels has been established by 1,4-DHP ligand binding studies (Glossmann et. al., 1985). αDHP-R 13 did not produce detectable staining in similar immunoblot assays on other rabbit tissues such as liver (Section 3.4.3., Fig. 13, lane 5), kidney, lung, spleen, aorta and small intestine (data not shown). It is possible that 1,4-DHP sensitive Ca^{2+} channels are present in these tissues but not detectable under the conditions used in this study. Indeed, other workers using antisera directed against purified skeletal muscle Ca^{2+} channel preparations have identified similar α_2 components in immunoblots of rabbit smooth muscle microsomes (Schmid et. al., 1986a). αDHP-R 13 did not stain immunoblots of pancreatic Islets of Langerhans (data not shown) although 1,4-DHP sensitive Ca^{2+} channels are known to have a functional role in this tissue. Ca^{2+} channels in these cells are postulated be responsible for the increase in the internal free Ca^{2+} concentration that triggers the secretion of insulin from these cells (Hedeskov, 1980). The immunocrossreactivity demonstrated between the Mr 140,000, α_2 subunit of skeletal muscle Ca^{2+} channels and that from heart and brain indicates that these components are similar both in terms of molecular size and in the conservation of structural determinants (Section 3.4.3., Fig. 13).

The α_2 subunits of Ca^{2+} channels from the skeletal muscle of different species were also shown to be similar in terms of molecular size. αDHP-R 13 detected a component of Mr 140,000-145,000 in immunoblots of membranes prepared from skeletal muscle tissue of rabbit, rat, mouse and frog, denatured in the presence of a reducing agent (Section 3.4.4., Fig. 14A). In addition, the α_2 subunit, from the different species tested, appears to

be disulphide-linked to a smaller component of Mr~30,000 as previously demonstrated in immunoblot analyses of rabbit T-tubule membranes (Section 3.4.2., Fig. 12B). Similar components of Mr 170,000- 175,000 were detected with αDHP-R 13 when the muscle membranes from these species were tested in the immunoblot assay following denaturation under non-reducing conditions (Section 3.4.4., Fig. 14B). The detection of the high molecular weight components, of Mr 310,000-330,000, by αDHP-R 13 under non-reducing conditions with rabbit, rat and mouse muscle membranes suggests the possibility that skeletal muscle voltage-sensitive Ca^{2+} channels from different sources could also be similar in terms of the oligomeric structure of the channel (Section 3.4.5., Fig. 14B).

Mapping of immunoreactive peptides following tryptic digestion further demonstrated the immunological similarities between α_2 subunits from the different species (Section 3.4.5., Fig. 16A). Conditions which produced limited tryptic digestion of the T-tubule membrane proteins were chosen, consequently, αDHP-R 13 recognized fragments of the α_2 subunit (Section 3.4.5., Fig. 15). The patterns of immunoreactive fragments produced by this treatment, recognized by αDHP-R 13, in the different species, were very similar (Section 3.4.5., Fig. 16A). The results from this experiment suggest that the α_2 components in skeletal muscle from different species contain regions of primary sequence homology, not only within the antibody binding site, but also with respect to retention of similarly positioned tryptic cleavage sites.

4.2.3. Glycoprotein Nature of the α_2 Subunit

Lectins, such as WGA, have the ability to bind carbohydrate residues and hence are often used in affinity chromatography to purify glycoproteins by virtue of their oligosaccharide side chains. The 1,4-DHP receptor associated with voltage-sensitive Ca^{2+} channels is purified from detergent solubilized T-tubule membranes by WGA affinity chromatography which suggests that the receptor component is a glycoprotein. Glycosylated polypeptides are often identified on stained SDS polyacrylamide gels as broadly stained bands, as the attached carbohydrate residues interfere with the mobility of the polypeptide on electrophoresis. The glycoprotein nature of the Mr 140,000, α_2

subunit of the 1,4-DHP receptor is suggested by the diffuse staining of this component in silver stained SDS polyacrylamide gels. The extent of glycosylation of this Ca^{2+} channel subunit was investigated using both enzymatic and chemical deglycosylation techniques (Sections 3.4.6., 3.4.7.). To obviate the need for prior purification, the αDHP-R 13 antibody was used to detect the deglycosylated product(s) of the α_2 subunit. The extent of glycosylation was determined by the reduction in apparent molecular weight on SDS electrophoresis of the immunoreactive product following the deglycosylation treatment.

Enzymatic methods were employed initially in an attempt to fully deglycosylate the α_2 subunit. The enzymes produced a slight decrease in the apparent molecular weight of the α_2 subunit indicating the removal of some carbohydrate residues (Section 3.4.6., Fig. 17A). However, the most complex mixture of enzymes tested was insufficient to completely remove the carbohydrate residues from the α_2 subunit even after prior denaturation of the T-tubule membranes in SDS (Section 3.4.6., Fig. 18A). The greatest reduction in apparent molecular weight of the α_2 subunit was achieved with neuraminidase and Endoglycosidase F. However, the Mr 115,000 component detected by αDHP-R 13 in immunoblots of treated membranes stained as a diffuse band indicating incomplete removal of the carbohydrate residues (Section 3.4.6., Fig 18A). Other workers have also been unable to completely deglycosylate this component with Endoglycosidase F (Marlene Hosey et. al., 1987). The difficulty in removing carbohydrate residues from the α_2 component using the Endoglycosidase F treatment could be explained by the presence of O-linked carbohydrate. However, deglycosylation of the α_2 subunit of iodinated purified receptor has been achieved by other workers using Endoglycosidase F (Takahashi et. al., 1987), suggesting that all the carbohydrate is in fact asparagine-linked. The lack of susceptibility of the α_2 subunit to Endoglycosidase F cleavage found in this study and by other workers (Marlene Hosey, et. al., 1987) could be explained if the oligosaccharide residues were buried within the folded protein structure, even under denaturing, non-reducing conditions, resulting in the inaccessibility of the glycosidic bonds to enzymatic attack.

Essentially complete deglycosylation of the α_2 subunit was achieved by the chemical (TFMS) treatment of detergent solubilized T-tubule and muscle microsomal membranes.

The fully deglycosylated product of the α_2 subunit stained as a sharp band of Mr 105,000 with αDHP-R 13 on immunoblots of treated membranes (Section 3.4.7., Fig. 19; Section 3.4.8., Fig. 22B). No staining was seen on blots of treated membranes with Con A peroxidase confirming the complete removal of mannose and glucose residues from the membrane glycoproteins (Section 3.4.7., Fig. 20; Section 3.4.8., Fig. 23B). However, with WGA peroxidase faintly stained bands at Mr 105,000 were detected in blots of treated T-tubule and rat microsomal membranes (Section 3.4.7., Fig. 21; Section 3.4.8., Fig. 24B). The chemical treatment used hydrolyses glycosidic bonds leaving peptide bonds and N-glycosyl bonds between N-acetylglucosamine residues and asparagine intact (Edge, et. al., 1981). It is therefore possible that this faint staining at Mr 105,000 with αDHP-R 13 was due to residual N-linked N-acetylglucosamine attached to the polypeptide.

The TFMS treated skeletal muscle membranes prepared from different species were analysed with αDHP-R 13 on immunoblots. The non-glycosylated moieties of the α_2 subunits of skeletal muscle Ca^{2+} channels from the different species tested were very similar in terms of molecular size (Mr 100,000-110,000, Section 3.4.8., Fig. 22B). From these results the similarities illustrated previously between these components from the different species (Section 4.2.2.) can be extended to include the extent of glycosylation.

The reduction in apparent molecular weight of the α_2 subunit of the voltage- sensitive Ca^{2+} channel to Mr 105,000 on essentially complete deglycosylation is indicative of a total carbohydrate content of approximately 25%. This result is confirmed in a recent report by Takahashi et. al. (1987) using enzymatic methods. Similar high levels of glycosylation have been reported for the α subunit, the channel forming component of the Na^+ channel (29%, Miller et. al., 1983).

4.3. Oligomeric Structure of the 1,4-Dihydropyridine Sensitive Calcium Channel

4.3.1. Multisubunit Structure of the Calcium Channel

The Mr 140,000 glycoprotein has been characterized in this study using monoclonal antibodies raised against this component as highly specific structural probes. The results from the immunoblotting studies on T-tubule membranes, denatured in the absence of a

reducing agent, indicate that the Mr 140,000 polypeptide is disulphide-linked to a small component of Mr 30,000. Moreover, the antibody also detected high molecular weight immunoreactive species of Mr 310,000-330,000 under these conditions. These results implicate the involvement of other subunits in the molecular structure of the Ca^{2+} channel.

As described previously, results from early purification studies implicated a trimeric structure for the 1,4-DHP sensitive Ca^{2+} channel involving polypeptides of Mr 142,000, 33,000 and 32,000 (Section 1.3.4.; Borsotto et. al., 1984b, 1985) or of Mr 130,000-150,000 (α), 50,000-65,000 (β) and 31,000-33,000 (γ) (Section 1.3.4.; Curtis and Catterall, 1984, 1985; Flockerzi et. al., 1986; Striessnig et. al., 1987). More recently, investigations involving purification, photoaffinity labelling and studies with polyclonal antibodies have revealed a multisubunit complex for the 1,4-DHP sensitive Ca^{2+} channel most likely involving five subunits.

Purification experiments from digitonin solubilized skeletal muscle membranes have suggested the presence of two high molecular weight components of Mr 175,000 and 170,000 in purified Ca^{2+} channel preparations (Leung et. al., 1987; Takahashi et. al., 1987). The resolution of these two components by SDS polyacrylamide gel electrophoresis in the absence of a reducing agent proved difficult due to the similarity in apparent molecular weight. However, these components can be distinguished by other properties (Leung et. al., 1987; Takahashi et. al., 1987). Under reducing conditions, the Mr 170,000 polypeptide dissociated into two components of Mr 140,000-150,000 (α_2) and Mr 27,000-30,000 (δ). This distinguished the Mr 170,000 ($\alpha_2\delta$) component from the Mr 175,000 (α_1) subunit, the molecular weight of which was unchanged on reduction. The dissociation of the Mr 170,000, $\alpha_2\delta$ complex under reducing conditions has also been shown by other workers using monoclonal and polyclonal antibodies (Section 3.4.2., Fig. 12B; Norman et. al., 1987; Schmid et. al., 1986a, 1986b; Vandaele et. al., 1987).

Results from WGA peroxidase staining experiments also distinguished between the two large molecular weight, α_1 and α_2 subunits. The α_2 subunit is stained by WGA-linked peroxidase (Section 3.4.7., Fig. 21; Leung et. al., 1987) and [^{125}I]WGA

Takahashi et. al., 1987) suggesting that this polypeptide component is glycosylated. The α_1 subunit is non-glycosylated as indicated by the lack of staining of this channel component by lectin-linked peroxidase (Leung et. al., 1987; Takahashi et. al., 1987). Furthermore, the peptide maps of the α_1 and α_2 subunits produced after treatment with various proteases are different (Marlene Hosey et. al., 1987; Sieber et. al., 1987). This suggests differences in the primary sequences of these two components, an indication that they are non-homologous. Moreover, monoclonal antibodies raised against the α_2 subunit of purified Ca^{2+} channels do not recognize the α_1 subunit suggesting that the two high molecular weight subunits are distinct (Section 3.4.2., Fig. 12A). Similarly, antibodies raised against the α_1 subunit do not detect the α_2 component (Leung et. al., 1987; Takahashi et. al., 1987). The detection of the α_1 subunit in these investigations would appear to suggest that Vandaele et. al. (1987) mis-interpreted the results of their immunoprecipitation experiments (Section 4.2.1.), in describing the Mr 140,000 component as a proteolytic cleavage product of an Mr 170,000 polypeptide.

The α_1 subunit has only been detected in 1,4-DHP receptor preparations purified from digitonin solubilized membranes (Takahashi et. al., 1987; Vaghy et. al., 1987; Sieber et. al., 1987) and not in fractions purified using different detergents (Section 2.4.5., Fig. 11; Borsotto et. al., 1984b, 1985). One explanation for this could be susceptibility of the α_1 to proteolytic cleavage by endogenous proteases in the muscle membrane preparations, despite precautions taken against such degradation (Section 4.3.2.; Vaghy et. al., 1987). The α_1 protoelytic fragments may not be detectable on silver stained polyacrylamide gels. Alternatively, it is possible that the α_1 subunit dissociates from the α_2 Ca^{2+} channel component in certain detergents. Takahashi et. al. (1987) purified the 1,4-DHP receptor from digitonin solubilized T-tubule membranes. In addition to the α_1, non-glycosylated component they identified a component of Mr 24,000-27,000 (δ) that was disulphide-linked to the α_2 subunit, and two further components of Mr 50,000 (β), and Mr 30,000 (γ) (Takahashi et. al., 1987). Results from immunoprecipitation experiments indicated that this multisubunit complex of the voltage-sensitive Ca^{2+} channel was susceptible to dissociation in different detergents (Takahashi et. al., 1987). They utilized monoclonal antibodies specific for the α_1 subunit to immunoprecipitate iodinated

receptor components from detergent solubilized receptor preparations. All five subunits remained associated when solubilized in 0.5% digitonin or low concentrations of CHAPS (0.1%). However, solubilization in higher concentrations of CHAPS (1%) or in Triton X-100 resulted in the dissociation of the α_2 subunit from the α_1 subunit. When purified 1,4-DHP receptor preparations, initially immunoprecipitated in the presence of digitonin, were resuspended in Triton X-100 the multisubunit complex dissociated into $\alpha_2\delta$ and $\alpha_1\beta\gamma$ complexes. It is noteworthy that these studies indicated that the functional properties of the 1,4-DHP receptor were preserved only in those detergents that allowed the association of the α_1 and α_2 subunits (Takahashi et. al.,, 1987).

The α_1, β and γ subunits were not detected in 1,4-DHP receptor fractions purified using our protocol. It is possible that the inclusion of the Triton X-100 extraction step in our purification procedure disrupts the multisubunit complex. Consequently, the WGA affinity chromatography results in the purification of the α_2 glycoprotein and δ subunits by virtue of the carbohydrate residues. It is noteworthy that purified 1,4-DHP receptor fractions consisting primarily of the α_2 subunit and apparently lacking in the α_1 subunit still retain receptor activity, which suggests that the α_2 subunit is involved in receptor function (Section 2.4.5.).

4.3.2. The α_1 subunit of the 1,4-Dihydropyridine Sensitive Calcium Channel

Early photoaffinity labelling experiments have shown that the 1,4-DHP, phenylalkylamine and benzothiazepine derivatives specifically photolabel polypeptides of Mr 140,000-170,000 in T-tubule membranes (Galizzi et. al., 1986; Ferry et. al., 1984) and in purified 1,4-DHP sensitive Ca^{2+} channel preparations (Striessnig et. al., 1986). The labelled component could correspond to either of the α subunits since identified by purification studies (Takahashi et. al., 1987; Leung et. al., 1987). More recent photoaffinity labelling investigations have indicated that the binding sites for these Ca^{2+} channel drug derivatives are associated with the α_1, non-glycosylated subunit (Takahashi et. al., 1987; Tanabe et. al., 1987; Sharp et. al., 1987; Vaghy et. al., 1987; Sieber et. al., 1987; Marlene Hosey et. al., 1987). In these studies no incorporation on to the α_2 subunit was detected with the photoactivatable 1,4-DHP and phenylalkylamine

derivatives [^{3}H]Azidopine, (+)-[^{3}H]PN200-110 and [^{3}H]LU 49888, [^{3}H]LU 47781. It now seems likely that the Mr 158,000 polypeptide labelled with [^{3}H]Azidopine (Ferry et. al., 1984; Streissnig et. al., 1986) and the Mr 170,000 polypeptide (Galizzi et. al., 1986) labelled with d-cis-[^{3}H]Diltiazem, [^{3}H]Bepridil and (+)-[^{3}H]PN200-110 are analogous to the α_1 subunit of the Ca^{2+} channel. This being the case it implies that the binding sites for these classes of Ca^{2+} channel drugs are associated with the α_1, non-glycosylated subunit.

Recent phosphorylation studies have implicated the α_1 subunit as the site of phosphorylation for cAMP-dependent protein kinase (Imagawa et. al., 1987). Early studies suggested that the β subunit of the 1,4-DHP receptor was the only detectable substrate for phosphorylation in purified preparations (Curtis & Catterall, 1985). However, recent investigations have indicated that both the α_1 and β subunit are phosphorylated by protein kinase C (Hofmann et. al., 1987) and cAMP-dependent protein kinase (Takahashi et. al., 1987; Hofmann et. al., 1987). These photoaffinity labelling and phosphorylation investigations suggest that the α_1 subunit has all the sites necessary for the modulation of voltage-sensitive Ca^{2+} channel function.

All evidence from photoaffinity labelling experiments indicates that the 1,4-DHP binding site is associated with the α_1 subunit. However, certain workers have published purification studies in which the purified 1,4-DHP sensitive Ca^{2+} channel preparations, apparently lacking in the α_1 subunit, retain 1,4-DHP activity (Section 2.4.5.; Norman et. al., 1987; Borsotto et. al., 1984b, 1985; Barhanin et. al., 1987; Curtis and Catterall, 1984)

To investigate this discrepancy Vaghy et. al. (1987) purified voltage-sensitive Ca^{2+} channels using different published protocols (Striessnig et. al., 1987; Curtis and Catterall, 1984) from both fresh and frozen skeletal muscle tissue. They found that a polypeptide of Mr 155,000-170,000, corresponding to the α_1 subunit, was specifically labelled by the photoactivatable drug derivatives, provided that the starting tissue had not been previously frozen and thawed. The photoaffinity labelling was repeated on purified Ca^{2+} channel samples prepared from previously frozen and thawed tissue, that did not appear to contain the α_1 subunit, but did contain 1,4-DHP receptor activity. Small peptides of Mr 60,000-90,000 were specifically labelled by the drug derivatives (Vaghy et. al.,

1987). The α_1 subunit is susceptible to proteolytic degradation (Vaghy et. al., 1987), therefore it is possible that the freezing and thawing of the skeletal muscle tissue resulted in the proteolytic cleavage of this component by endogenous proteases. It was suggested that these smaller components (Mr 60,000-90,000) were fragments of the α_1 subunit (Vaghy et. al., 1987).

It is possible that this proteolysis could explain the lack of detection of the α_1 subunit in purified Ca^{2+} channel preparations in this study, as the skeletal muscle tissue was invariably frozen and stored at -70°C for up to two weeks, before thawing for the preparation of T-tubule membranes for subsequent Ca^{2+} channel purification. The detection of 1,4-DHP receptor activity detected in the purified fractions could be due to the presence of proteolytic fragments of the α_1 subunit, that retained structural integrity under non-reducing conditions, but were not visible on silver stained polyacrylamide gels. However, the purification procedure utilized in this study was different from any protocol used by Vaghy et. al. (1987) in that an additional detergent treatment was included in the protocol. It is likely that the treatment with Triton X-100 caused the dissociation of the α_1 fragments from the α_2 subunit, as was suggested for the intact α_1 subunit by Takahashi et. al. (1987) (Section 4.3.1.). The possible dissociation of the α_1 fragments from the α_2 component in Triton X-100 would indicate that the 1,4-DHP receptor activity detected in purified Ca^{2+} channel fractions is in this study associated with the α_2 subunit.

In contrast, the involvement of the α_1 subunit as the major functional element of the 1,4-DHP sensitive Ca^{2+} channel has been highlighted by the publication of the complete primary sequence of this component (Tanabe et. al., 1987). The amino acid sequence was deduced from analysis of the cDNA sequence (Section 1.4.5.). It was found that considerable homology existed between the primary sequence of the Ca^{2+} channel α_1 subunit and that of the α subunit of the Na^+ channel (Tanabe et. al., 1987; Noda et. al., 1986a). Moreover, the transmembrane topology of the α_1 subunit predicted from the sequence data was also found to similar to that modelled for the channel-forming, α subunit of the Na^+ channel. The sequence data has led to the suggestion that the α_1 subunit is the transmembrane ion conducting pore of the channel, in addition to the

binding site for Ca^{2+} channel modulators (Takahashi et. al., 1987, Tanabe et. al., 1987).

This raises questions regarding the functional significance of the other putative subunits of the 1,4-DHP sensitive Ca^{2+} channel. Indeed, the ease of dissociation of the multisubunit complex in different detergents has led to the suggestion that the $\alpha_2\delta$ complex is only weakly associated with the postulated channel core subunit, α_1 (Takahashi et. al., 1987). In addition, hydrophobic labelling studies have indicated that the α_2 subunit is not a membrane-spanning polypeptide (Takahashi et. al., 1987). This led to the proposal by Takahashi et. al., (1987) of a model for the voltage-sensitive Ca^{2+} channel, with the α_1 subunit forming the central ion conducting pore and only peripheral association of the α_2 and other putative subunits (Section 1.3.4., Fig. 6).

In contrast to this postulation, certain studies have indicated the possibility that other subunits may be required in addition to the α_1 subunit for full 1,4-DHP receptor activity and channel function. As discussed previously, 1,4-DHP activity has been detected in purified Ca^{2+} channel preparations apparently lacking in the α_1 subunit (Section 2.4.5.; Norman et. al., 1987; Borsotto et. al., 1984b, 1985; Barhanin et. al., 1987). The major component of these purified preparations was the glycosylated α_2 subunit. It has been shown that such preparations reconstituted into phospholipid vesicles have binding sites for the three main classes of Ca^{2+} channel modulating drugs (Barhanin et. al., 1987). Moreover, functional Ca^{2+} channels have been identified following incorporation of this type of purified preparation into artificial phospholipid bilayers (McKenna et. al., 1987; Wray, D.W., unpublished results). In addition, Takahashi et. al. (1987) suggested that 1,4-DHP activity was only retained when the α_2 subunit was associated with the α_1 subunit in receptor preparations. Finally, it has not been possible to induce the expression of functional voltage-sensitive Ca^{2+} channels from the mRNA produced from the cDNA of the α_1 subunit (Hofmann et. al., 1987; Froehner, 1988; Alsobrook & Stevens, 1988). All these studies suggest that the α_1 subunit alone is not sufficient to account for full 1,4-DHP receptor activity and Ca^{2+} channel function. To investigate the possible role of the α_2 subunit in 1,4-DHP receptor activity further experiments were carried out in this study using the monoclonal antibodies and 1,4-DHP binding studies.

4.3.3. Functional Significance of the α_2 subunit

To investigate the possible involvement of the α_2 subunit in the binding site of the 1,4-DHPs, the effect of enzymatic deglycosylation on the binding of the 1,4-DHP, (+)-[^{3}H]PN200-110, to native T-tubule membranes was studied. Immunoblot assays with the antibody αDHP-R 13 were performed on the enzymatically treated native T-tubule membranes, in parallel experiments, to enable the extent of deglycosylation to be estimated by the decrease in apparent molecular weight of the α_2 subunit on SDS polyacrylamide gel analysis.

A substantial proportion of available (+)-[^{3}H]PN200-110 binding sites were sensitive to the state of glycosylation. Treatment with neuraminidase only, which caused the complete conversion of the Mr 140,000, α_2 subunit to a component of Mr 130,000 (Section 3.4.9., Fig. 25A), reproducibly reduced the number of available (+)-[^{3}H]PN200-110 binding sites by 72 ± 3% (Section 3.4.9., Fig. 26). The inclusion of Endoglycosidase F with neuraminidase resulted in further deglycosylation to a defined product Mr 115,000 (Section 3.4.9., Fig. 25A). However, only slight further reduction in the binding of (+)-[^{3}H]PN200-110 to 77 ± 5% was observed (Section 3.4.9., Fig. 26). These results implicate a role for the α_2, glycosylated subunit in the binding of this class of Ca^{2+} channel drugs.

It is possible that the binding of the 1,4-DHPs is to a site on the α_1 subunit but requires the association of the α_2, glycosylated Ca^{2+} channel component. This is in agreement with the result of Takahashi et. al. (1987) which suggested that the association of these two subunits was required for 1,4-DHP activity. If this were the case, 1,4-DHP activity in purified Ca^{2+} channel preparations apparently lacking the α_1 subunit could be explained by the presence of proteolytically derived fragments of the α_1 subunit, that retained structural integrity under non-reducing conditions but are not detectable on silver stained SDS polyacrylamide gels. As discussed above, Vaghy et. al. (1987) have detected proteolytic fragments of the α_1 component in purified 1,4-DHP receptor fractions, prepared from frozen tissue, by photoaffinity labelling techniques.

After treatment with both neuraminidase and Endoglycosidase F a substantial proportion of (+)-[^{3}H]PN200-110 sites (23%) remained unaffected by the deglycosylation procedure (Section 3.4.9., Fig. 26). This result raises the possibility of

two classes of receptor sites for 1,4-DHPs in the T-tubule membrane; one sensitive to the extent of glycosylation, one insensitive to deglycosylation. This possibility could explain the presence of 1,4-DHP activity in purified Ca^{2+} channel preparations containing α_2 and δ subunits, but lacking in α_1.

To further define a role for the α_2 subunit in 1,4-DHP receptor activity, the development of the α_2 Ca^{2+} channel component of rat skeletal muscle was investigated and compared to the appearance of 1,4-DHP receptor activity in the same tissue. The development of 1,4-DHP receptor activity has been investigated previously by ligand binding studies (Kazazoglou et. al., 1983). It was shown that the number of available 1,4-DHP binding sites increased rapidly over the first 10-15 days of post-natal development to a constant maximum level at 20 days (Kazazoglou et. al., 1983). In this study a similar developmental profile for (+)-[^{3}H]PN200-110 binding was found, only the rapid increase in available binding sites occurred between 8-20 days post-natal development (Section 3.4.10., Fig. 27). Interestingly, the development of the α_2 subunit detected in immunoblot assays with αDHP-R 13 parallels the appearance of the 1,4-DHP receptor as measured by the equilibrium binding of (+)-[^{3}H]PN200-110 (Section 3.4.10., Fig. 27). This co-development suggests that the α_2 subunit is an integral structural component of the 1,4-DHP receptor associated with the voltage-sensitive Ca^{2+} channel at all stages of development.

Both the deglycosylation and development studies appear to indicate that the α_2 subunit is likely to have a more central role in 1,4-DHP sensitive Ca^{2+} channel structure and function than has previously been suggested. Clearly more work is required to fully elucidate the roles played by the different subunits in Ca^{2+} channel function.

4.4. Implications of this Study

The α_1 subunit has been proposed as the main channel-forming component of the 1,4-DHP sensitive Ca^{2+} channel by primary sequence analysis (Tanabe et. al., 1987). In addition, results from photoaffinity labelling studies have indicated that the binding sites for the main classes of Ca^{2+} channel drugs are associated with the α_1 component (Tanabe et. al., 1987; Takahashi et. al., 1987; Sharp et. al., 1987; Vaghy et. al., 1987; Sieber et. al., 1987, Marlene Hosey et. al., 1987). This work led to the proposal of a model for the Ca^{2+} channel by Takahashi et. al. (1987) (Section 1.3.4., Fig. 6). In this model the α_1 is described as the central ion-conducting component with the binding sites for Ca^{2+} channel drugs and a site susceptible to phosphorylation by cAMP-dependent protein kinases. The β subunit, which also has a phosphorylation site, is shown to be associated with the cytoplasmic face of the α_1 subunit. The γ subunit, a glycosylated subunit of Mr 30,000, is also associated with the α_1 component and is postulated to be similar to the β_1 subunit of mammalian Na^+ channels (Takahashi et. al., 1987; Catterall, 1986). The model proposed by Takahashi et. al. (1987) suggests that the $\alpha_2\delta$ complex is more weakly associated with the α_1 subunit than the β and γ subunits. This model implicates that the α_2 subunit is only peripherally associated with the α_1 component and no function is defined for the α_2 subunit.

In contrast to this model, the results of this study imply that the α_2 subunit may play a more central role in 1,4-DHP sensitive Ca^{2+} channel structure and function. It has been shown that a large proportion of 1,4-DHP binding sites are sensitive to the state of glycosylation of the α_2 component. Two possible explanations have been suggested in this study for the effect of α_2 subunit deglycosylation on the binding of (+)-[^{3}H]PN200-110 to T-tubule membranes. Either binding to the α_1 subunit requires the association of the glycosylated, α_2 component or it is possible that two similar receptor populations exist; one sensitive to deglycosylation and one insensitive to the extent of glycosylation.

Clearly, further studies are required to fully elucidate the stucture and possible functions of the α_2 subunit of the 1,4-DHP sensitive Ca^{2+} channel. The monoclonal antibodies raised in this study are likely to prove useful specific probes for the α_2

component for use in further studies. The screening of cDNA libraries using these antibodies is currently underway for the selection of positive clones encoding for the α_2 polypeptide (Harrison T.M. unpublished results). The amino acid sequence of this component can then be deduced from the nucleotide sequence. It may then be possible to express the α_2 component from the encoding cDNA, as has been successfully achieved for the α subunit of the Na^+ channel (Noda et. al., 1986b).

In addition, the monoclonal antibody binding sites can be mapped to regions of the primary structure, using methods involving partial proteolytic digestion of the α_2 polypeptide and sequencing of the immunoreactive fragments. In preliminary experiments, certain antibodies have been shown to modulate the 1,4-DHP binding to native T-tubule membranes (unpublished results). The binding sites for these antibodies could be mapped to identify regions of the α_2 polypeptide associated with 1,4-DHP receptor activity. Preliminary electrophysiological investigations have shown that Ca^{2+} channel activity can be recorded from 1,4-DHP sensitive Ca^{2+} channel preparations purified as described in this study and reconstituted into artificial phospholipid bilayers (Wray D.W., unpublished results). It is possible that some of the antibodies may modulate the function of the reconstituted Ca^{2+} channel preparation. This being the case, the mapping of the antibody binding sites on the polypeptide, in conjuction with the electrophysiological studies may serve to relate structure of the α_2 subunit to function of this Ca^{2+} channel component.

The importance of the different Ca^{2+} channel subunits in channel structure and function could be investigated by reconstitution studies. The functional reconstitution of the channel subunits into artificial phospholipid vesicles or bilayers for pharmacological and electrophysiological experiments would enable any subunits essential for channel function to be distinguished from those components in channel modulation.

In addition to the investigations described previously, other preliminary experiments were performed using the antibodies raised in this study. Immunoaffinity columns were formed by coupling αDHP-R antibodies to cyanogen bromide activated Sepharose. However, such columns were not successful in the purification 1,4-DHP sensitive Ca^{2+} channels from CHAPS solubilized T-tubule membranes. The antibodies were also employed in preliminary experiments involving the immunoprecipitation of 1,4-DHP activity from detergent solubilized membrane fractions. *Staphylococcus aureus* cells were used to precipitate the antigen/antibody complex. It was not possible to immunoprecipitate 1,4-DHP binding activity with any of the antibodies from CHAPS solubilized T-tubule membranes using this method. However, more recent experiments in Dr Norman's laboratory have shown that 1,4-DHP activity can be immunoprecipitated from digitonin solubilized membranes using certain αDHP-R antibodies. Furthermore, some of the antibodies have been shown to modulate 1,4 DHP binding activity to native T-tubule membranes. The antibody αDHP-R3 had a similar effect to d-cis-diltiazem (Section 1.3.2.) on 1,4-DHP binding. In preliminary experiments specific binding of (+)-[^{3}H]PN200-110 to native T-tubule membranes was increased in the presence of αDHP-R3.

I was also involved in the reconstitution of T-tubule Ca^{2+} channels into phospholipid vesicles. The reconstituted channels were incorporated into artificial lipid bilayers in a collaborating laboratory for electrophysiological measurement. Ca^{2+} channel currents, that had similar characteristics to L-type Ca^{2+} channel currents detected by other workers, were recorded from this preparation (Wray, D.W., unpublished results). Certain antibodies raised in this study significantly decreased the Ca^{2+} channel current when applied to this preparation (Wray, D.W., unpublished results). However, none of the antibodies had any effect on Ca^{2+} channel currents recorded from cultured neuronal NG 108 cells.

The monoclonal antibodies produced in this study may find applications in other areas. For example, the antibodies that have been shown to crossreact with similar polypeptide components in different tissues. These antibodies could be fluorescently labelled and utilized in immunofluorescence studies to localize the more functionally important voltage-sensitive Ca^{2+} channels in other tissues such as brain and heart.

As discussed in Section 1.2., three different subtypes of voltage-sensitive Ca^{2+} channel have been identified by electrophysiological studies. To date, only the L-type, 1,4-DHP sensitive Ca^{2+} channel has been structurally characterized. It is possible that the antibodies, utilized in this study primarily to characterize the α_2 subunit of the 1,4-DHP sensitive Ca^{2+} channel, may prove useful probes for the elucidation of the oligomeric structure of the other types of voltage-sensitive Ca^{2+} channels. Hence, it may eventually be possible to relate functional differences between the various channel subtypes identified by electrophysiological investigations (Section 1.2.), to any differences found in the structure of these types of voltage-sensitive Ca^{2+} channels.

4.5. Conclusions

The monoclonal antibodies raised against the purified 1,4-DHP receptor have been utilized primarily in this study to characterize structurally the α_2 subunit of the 1,4-DHP sensitive Ca^{2+} channel. Other workers have suggested that the α_1 subunit of the 1,4-DHP sensitive Ca^{2+} channel is the central ion conducting polypeptide of the channel and has the binding sites for the Ca^{2+} channel modulators (Section 4.3.2.). However, this investigation suggests that the α_2 subunit may be required for functional 1,4-DHP receptor activity. The effect of deglycosylation on the binding of 1,4-DHPs to native T-tubule membranes indicated that a large proportion of the available binding sites were sensitive to the state of glycosylation of the α_2 subunit. It is possible that the association of the glycosylated α_2 component with the α_1 subunit is required for full 1,4-DHP receptor activity. A proportion of the 1,4-DHP binding sites appear to be insensitive to the extent of glycosylation. This allows a second possibility to be raised, the presence of two similar receptor populations distinguished by their different sensitivities to the deglycosylation of the α_2 subunit. The involvement of the α_2 subunit in the binding of

the 1,4-DHPs was further highlighted by the co-development of the α_2 subunit with (+)-[^{3}H]PN200-110 binding activity. This co-development suggests that the α_2 component is an integral part of the 1,4-DHP sensitive Ca^{2+} channel at all stages of development. The investigations described in this study indicate a more central role for the α_2 subunit in the 1,4-DHP sensitive Ca^{2+} channel structure and function than has been suggested by other workers to date.

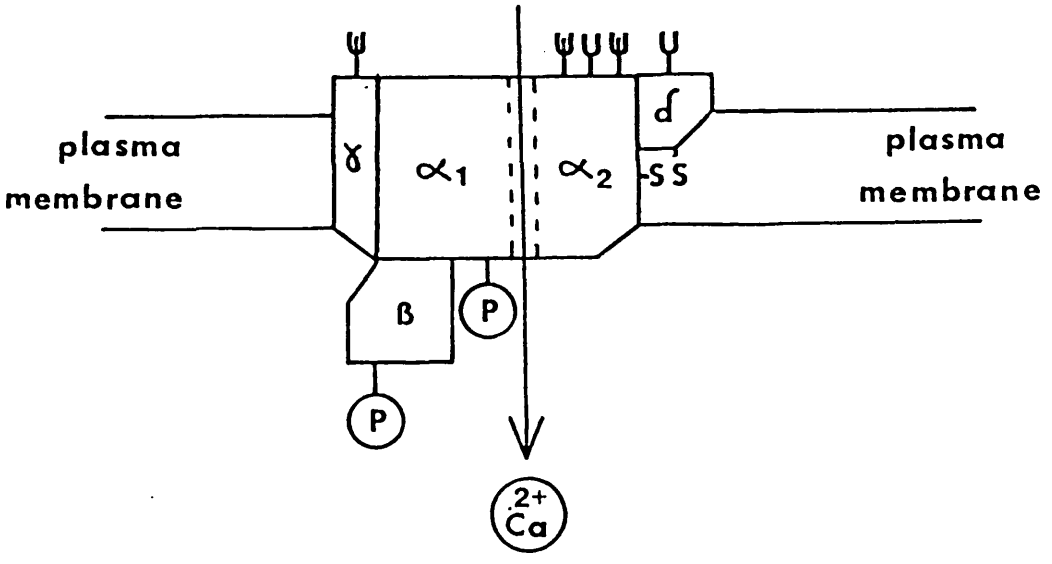


Figure 28

Second Model of 1,4-Dihydropyridine Sensitive Calcium Channel Structure.

Possible model for 1,4-DHP sensitive Ca^{2+} channel structure derived from the results described in this study. S-S, disulphide bonds; P, phosphorylation sites; Ψ Ψ carbohydrate.

The model for Ca^{2+} channel structure proposed by Takahashi et. al. (1987) described previously (Sections 1.3.4. and 4.3.), involved the α_1, non-glycosylated subunit as the major transmembrane, channel forming component and suggested that all three drug binding sites were located on this subunit. This model raises questions as to the functional significance of the other components involved in the multisubunit channel structure.

The investigations described in this study enable the proposal of another model for the channel structure, with the α_2 subunit playing a more central role in channel structure and function (Fig. 7). The large decrease in available 1,4-DHP binding sites on enzymatic deglycosylation (Section 3.4.9., Fig. 26) indicates that the glycosylated α_2 subunit is required for the fully active 1,4-DHP receptor. Furthermore, the co-development of the α_2 subunit and 1,4-DHP binding activity (Section 3.4.10., Fig. 27) suggests that the α_2 component is involved in the 1,4-DHP sensitive Ca^{2+} channel complex at all stages of development.

The model proposed in this study suggests that the association of the α_1 and α_2 components is necessary for the fully functional 1,4-DHP sensitive Ca^{2+} channel. The lack of expression of functional voltage-sensitive Ca^{2+} channels from the mRNA produced from the cDNA of the α_1 subunit alone suggests strongly that that other channel subunits may be required for channel function (Froehner, 1988). It is possible that the membrane-spanning Ca^{2+} channel pore may arise by the association of the large α_1 and α_2 subunits and the smaller subunits serve a modulatory function. Clearly more work is necessary before the correct model of the voltage-sensitive Ca^{2+} channel structure can be established and functions assigned to the different subunits. The primary sequence of the α_2 glycosylated subunit is essential for the determination of the transmembrane topology of this component, and hence in establishing the functional significance of this component in the channel structure.

APPENDICES

Appendix 1

SDS Polyacrylamide gel Electrophoresis

Gel Tank Buffer;

B 250mM Tris, 192mM glycine, pH 8.3, 0.1% (w/v) SDS

Gel Stock Solutions:

30A 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide

45A 45% (w/v) acrylamide, 1.2% (w/v) bisacrylamide

C 0.25M Tris/HCl, pH 6.8

D 0.75M Tris/HCl, pH 8.8

E 1% (w/v) SDS

F 0.1% (w/v) ammonium persulphate

N,N,N',N'-tetramethylethylenediamine (TEMED)

Resolving Gel Compositions:

8% polyacrylamide gels;

solution	volume
30A	8.0ml
D	15.0ml
E	3.0ml
F	1.0ml
H_2O	3.0ml
TEMED	20µl

4-12% linear polyacrylamide gradient gels;

4% acrylamide solution		12% acrylamide solution	
solution	volume	solution	volume
30A	2.0ml	45A	4.0ml
D	7.5ml	D	7.5ml
E	1.5ml	E	1.5ml
F	0.75ml	F	0.75ml
H_2O	3.3ml	H_2O	1.26ml
TEMED	15µl	TEMED	15µl

4% polyacrylamide stacking gel composition;

solution	volume
30A	1.33ml
C	5.0ml
E	1.0ml
F	0.5ml
H_2O	2.17ml
TEMED	50µl

Appendix 2

Protein Stains

Fairbanks 1; SDS polyacrylamide gels

140mg Brilliant Blue G250 (C.I. 42655)

140mg Brilliant Blue R250 (C.I. 42660)

1 litre 25% propan-2-ol, 10% acetic acid

Destain;

10% acetic acid

Amido Black; nitrocellulose

0.1mg Naphthol Blue Black (C.I. 20470)

100ml 45% methanol, 10% acetic acid

Destain;

90% methanol, 2% acetic acid

Appendix 3

Peterson Protein Assay Reagent

Stock Solutions:

CTC, 0.1% (w/v) copper sulphate, 0.2% (w/v) potassium tartrate, 10% (w/v) sodium carbonate,

0.8N NaOH

10% (w/v) SDS

Folin-Ciocalteu's phenol reagent (2N)

Working Solutions:

Reagent A: equal volumes of; CTC, 0.8N NaOH, 10% SDS and H_2O,

Reagent B: 1 volume Folin-Ciocalteu's phenol reagent and 5 volumes H_20.

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Monoclonal antibodies against the 1,4-dihydropyridine receptor associated with voltage-sensitive Ca^{2+} channels detect similar polypeptides from a variety of tissues and species

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Four monoclonal antibodies have been raised against voltage-sensitive Ca^{2+} channel dihydropyridine receptors from rabbit skeletal muscle. When tested by immunoblot assay of denatured transverse tubule membranes in reducing polyacrylamide gels, each recognised a single polypeptide of $M_r \sim 140000$ that co-migrated with the large glycoprotein subunit of the purified receptor. In blots of nonreducing gels, a larger protein of $M_r \sim 170000$ was seen and three of the antibodies recognised additional components at $M_r \sim 310000$ and ~ 330000. Crossreactive material of similar molecular mass was also seen in rabbit heart and brain, and in the skeletal muscle of other species.

Dihydropyridine; Dihydropyridine receptor; Ca^{2+} channel; Monoclonal antibody

1. INTRODUCTION

Due to their high-affinity binding, the 1,4-dihydropyridine (DHP) Ca^{2+} channel antagonists have proved most useful as biochemical probes for the voltage-sensitive Ca^{2+} channel [1,2]. To date, most studies of the molecular properties of the DHP receptor have concentrated on the relatively abundant receptor of the skeletal muscle transverse tubule (T-tubule) membrane [3].

There is general agreement, both from purification [4–6] and affinity labelling studies [7,8], that a large glycoprotein of M_r 130000–150000 is implicated in the skeletal muscle DHP receptor structure and several laboratories have provided evidence for additional components of M_r 50000 and 32000–36000 [4–7,9]. Subunit analysis of pharmacologically more interesting DHP receptors such as those from heart [10] and smooth muscle has proved more difficult.

Using monoclonal antibodies raised against the skeletal muscle DHP receptor from rabbit, we demonstrate in this report that antigenic determinants on the skeletal muscle DHP-sensitive Ca^{2+} channel are shared between putative Ca^{2+} channel components in various tissues and species.

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2. MATERIALS AND METHODS

2.1. *Membrane preparations*

Skeletal muscle microsomal membrane fractions were prepared according to Fosset et al. [3]. Microsomes from other tissues were prepared according to Glossmann and Ferry [11] in 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Mops, pH 7.5. T-tubule membranes were prepared from rabbit white skeletal muscle as described by Rosemblatt et al. [12].

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2.2. *1,4-Dihydropyridine receptor purification*

Purification of the DHP receptor from T-tubule membranes was performed as described by Borsotto et al. [4] in medium A (0.1% CHAPS, 5% glycerol, 0.02% phosphatidylcholine, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM iodoacetamide, 0.1 mM PMSF, 1 μM pepstatin A, 20 mM Tris, pH 7.5) with a modification following the gel filtration step. Ultrogel A2 fractions containing DHP receptor activity were pooled and adjusted to contain 1% Triton X-100. Following incubation at 4°C for 15 min the mixture was applied to a 1.8 ml wheat germ agglutinin-Ultrogel (WGA-Ultrogel, LKB) column. The breakthrough was reapplied and the column washed sequentially with 10 ml medium A containing 0.1% Triton X-100 in place of CHAPS, 10 ml medium A containing 1% CHAPS and finally 5 ml medium A. The receptor was eluted with 200 mM *N*-acetyl-D-glucosamine in medium A.

2.3. *Preparation of monoclonal antibodies*

BALB/C mice were immunized by intraperitoneal injections of extensively purified DHP receptor (0.8–1.0 nmol [^{3}H]PN 200-110-binding sites per mg of protein; 10 μg protein per animal) prepared as an alum precipitate, followed after 6 weeks by a similar amount of protein injected in saline. 7 days before being killed one animal received about 5 μg purified receptor in Freund's incomplete adjuvant followed on each of the next 6 days by an intraperitoneal injection of a similar amount of receptor in saline. The spleen was excised 1 day later and the cells fused with 10^7 NSO/1 myeloma cells as described by Galfre and Milstein [13] except that 50% polyethylene glycol 4000 (Merck, Cat. no.9727) was used. After selection of hybridoma cells, positive clones were replated at limiting dilution to ensure the monoclonal nature of the cell lines. Ascitic fluids were produced by injecting about 10^7 monoclonal hybridoma cells into pristane-primed BALB/C mice.

2.4. *Solid-phase antibody assay*

Microtitre plate wells were coated for 16 h at 4°C with 50 ng of purified DHP receptor in 50 μl of phosphate buffered saline (PBS; 50 mM sodium phosphate, pH 8.0, 0.9% NaCl). The CHAPS concentration in the diluted receptor was <0.003%. Each well was washed for 1 h with 0.5% casein, 10% calf serum in PBS. Wells were incubated for 2 h at room temperature with the hybridoma supernatant being tested, then sequentially with 50 μl of rabbit anti-mouse IgG (Miles; 1:1000 dilution) and goat anti-rabbit immunoglobulin coupled to peroxidase (Miles; 1:1000 dilution) in 1% calf serum, 0.05% Tween 20, PBS, each for 1 h at room temperature. Positive wells were identified by the development of blue colouration following the application of 50 μl of 10 mg/ml 3,3',5,5'-tetramethylbenzidine (Miles) in 0.1 M sodium acetate/citrate, pH 6.0, 0.0045% H_2O_2. Between steps, wells were washed three times with 0.05% Tween 20 in PBS.

2.5. *Immunoblots*

Samples of T-tubule (60 μg) or microsomal membranes (0.25–3 mg protein) were denatured in 2% SDS, 9% glycerol, 75 mM Tris-HCl, pH 6.8, and either 0.6% dithiothreitol (disulphide reducing conditions) or 8 mM iodoacetamide (nonreducing conditions) and loaded on 4–12% linear polyacrylamide gradients according to Laemmli [14]. Following transfer of resolved proteins to nitrocellulose [15], the paper was incubated for 16 h at 4°C with 1% bovine serum albumin, 0.5% casein, 0.05% Tween 20, 0.9% NaCl, 10 mM Tris-HCl, pH 7.5. Antibody binding to nonreduced membrane components was assayed by incubating the nitrocellulose paper with diluted ascitic fluids (1:500 in 0.05% Tween 20, 0.9% NaCl, 10 mM Tris, pH 7.5) for 2 h at room temperature followed by sequential incubations with rabbit anti-mouse IgG (1:500 dilution, 1 h) and goat anti-rabbit IgG coupled to peroxidase (Miles; 1:500 dilution, 2 h). For reduced membrane samples, the rabbit anti-mouse IgG incubation was followed by sequential incubations with swine anti-rabbit IgG (Dakopatts; 1:500 dilution, 1 h) and rabbit peroxidase anti-peroxidase complex (Dakopatts; 1:500 dilution, 1 h). Immunoblots were then developed using 0.2 mg/ml diaminobenzidine (Sigma), 0.9% NaCl, 20 mM Tris-HCl, pH 7.5, 0.003% H_2O_2. Between steps the nitrocellulose paper was washed for 10 min in 0.25% sodium lauryl sarcosine, 0.25% Nonidet P-40, 1 M NaCl, 10 mM Tris-HCl, pH 7.5.

3. RESULTS AND DISCUSSION

The spleen of a hyperimmune BALB/C mouse was removed and monoclonal antibody producing hybridoma cells isolated as described in section 2. Cell supernatant from monoclonal cell lines was assayed in the solid-phase assay against extensively purified DHP receptor (0.8–1.0 nmol (+)-[^{3}H]PN 200-110-binding sites per mg of protein). From the receptor molecular size of M_r ~210000 determined by radiation inactivation [16] and assuming one DHP binding site per receptor molecule, the specific activity of a homogeneous preparation would be 4.76 nmol/mg. The minimum purity of the preparations used for assay was estimated at 17% but it is likely that the degree of purity was substantially underestimated as a result of activity loss due to the half-life of the DHP receptor in CHAPS solution [17] and to the high detergent concentrations used during the WGA-Ultrogel purification step [4]. When analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions, these receptor preparations consisted of a major component of M_r ~140000 with minor bands at M_r ~100000 and 33000 (not shown). Out of 360 hybridoma supernatants tested, 33 were positive in the solid-phase assay against this extensively purified DHP receptor and 31 individual clones were isolated by plating at limiting dilution.

Both purification [4–6] and affinity labelling studies [7,8] have identified a polypeptide chain of M_r 140000 as a component of the DHP receptor of the skeletal muscle voltage-sensitive Ca^{2+} channel. Four antibodies (αDHP-R 11, 13, 14 and 15), out of the panel of 31, produced detectable reaction on immunoblots of skeletal muscle T-tubule membranes. Under reducing conditions all four an-

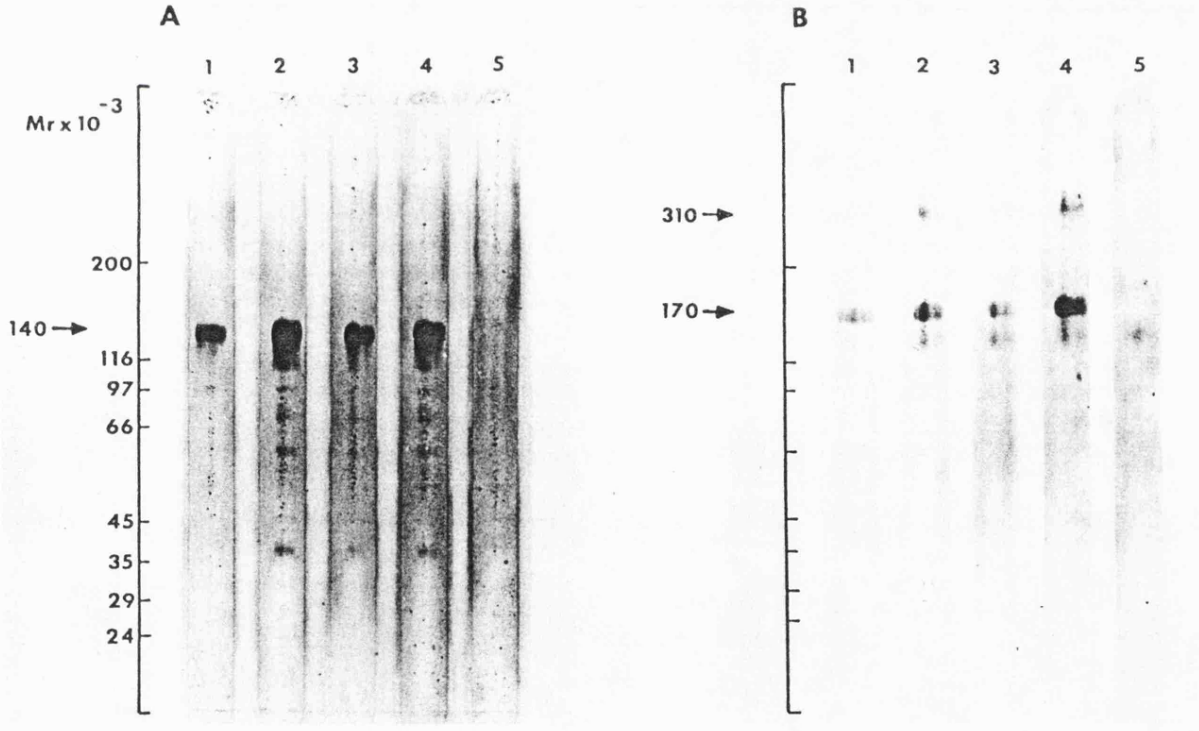

Fig.1. Immunoblots of rabbit skeletal muscle transverse tubule membranes after electrophoresis under reducing (A) and nonreducing conditions (B). Lanes: 1, αDHP-R 11; 2, αDHP-R 13; 3, αDHP-R 14; 4, αDHP-R 15; 5, anti-gyrase B control ascitic fluid (Harrison, T.M., unpublished). M_r markers are from Sigma: myosin (200000), β-galactosidase (116000), phosphorylase b (97000), bovine serum albumin (66000), ovalbumin (45000), pepsin (35000), carbonic anhydrase (29000) and trypsinogen (24000).

tibodies bound specifically to a single polypeptide of M_r ~140000 (fig.1A). This polypeptide co-migrated with the 140 kDa polypeptide present in the extensively purified DHP receptor preparations (not shown) indicating that these monoclonal antibodies detect specifically the large component of the Ca^{2+} channel protein.

Under nonreducing conditions the four antibodies detected larger protein bands (fig.1B). All four recognized a protein of M_r 165000–170000 and αDHP-Rs 13, 14 and 15 detected two larger proteins of M_r 310000 and 330000. It has been suggested that the transition between M_r 170000 and M_r 140000 on disulphide reduction is due to the release of a small disulphide linked component of M_r ~32000 [9]. Specific labelling of a 140 kDa polypeptide could not be detected under nonreducing conditions. A diffuse region of nonspecific staining in the 130–140 kDa region was also present in the control track of nonreduced material in which the αDHP-R antibody was replaced by a monoclonal anti-bacterial Gyrase B (see fig.1B). It is possible that this region of nonspecific staining, which has been observed previously in immunoblot studies on rabbit skeletal muscle membranes [9], was due to endogenous immunoglobulin molecules associated with the membrane preparation.

The observation of specific anti-DHP receptor antibody binding to large protein bands of M_r >300000 indicates that additional polypeptides may be required for the complete native structure of the DHP-receptor Ca^{2+} channel protein. Although there are no indications of their identity in this study, possible polypeptide candidates of M_r 50000–55000 and ~99000 have been implicated in other studies of the DHP receptor protein [6,7].

Possible immunochemical similarities between putative DHP-sensitive Ca^{2+} channels in different species and tissues were investigated by immunoblot assay using αDHP-R 13. Immunocrossreactivity was detected between the rabbit skeletal muscle DHP receptor and a component in skeletal muscle microsome preparations from mouse, rat and frog. In each case a single polypeptide of M_r 140000–145000 was detected by αDHP-R 13 under reducing conditions (fig.2A), whereas under nonreducing conditions the antibody identified a protein of M_r 170000–180000 (fig.2B). Although not observed in frog microsomes, a large component of M_r ~310000

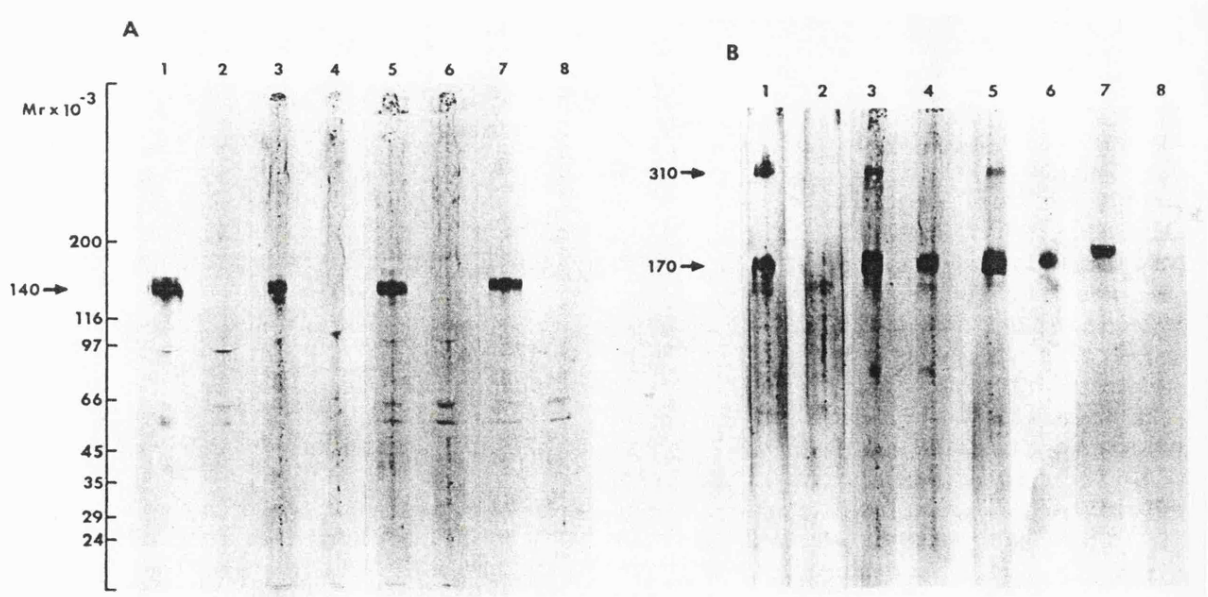

Fig.2. Immunoblots of skeletal muscle microsomes after electrophoresis under reducing (A) and nonreducing conditions (B). Lanes: 1 and 2, rabbit (0.25 mg); 3 and 4, rat (0.75 mg); 5 and 6, mouse (0.3 mg); 7 and 8, frog (0.33 mg). Lanes 1, 3, 5 and 7 with αDHP-R 13, lanes 2, 4, 6 and 8 with anti-gyrase B control ascitic fluid. M_r markers as in fig.1.

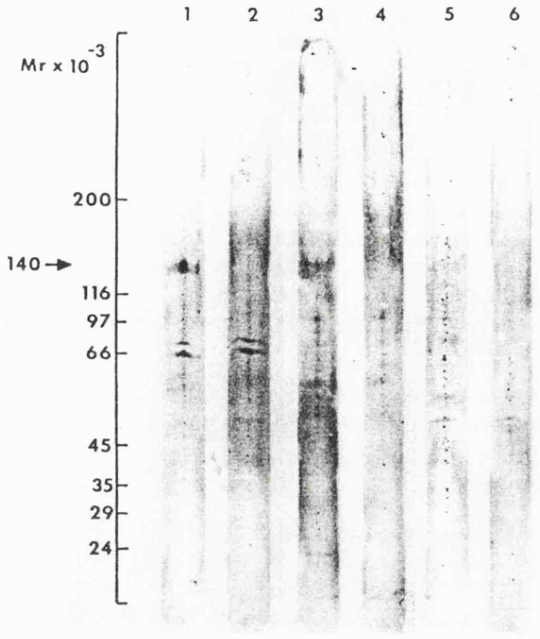

Fig.3. Immunoblots of brain, heart and liver microsomes after electrophoresis under reducing conditions. Lanes: 1 and 2, brain (3.0 mg); 3 and 4, heart (1.85 mg); 5 and 6, liver (3.0 mg). Lanes 1, 3 and 5 with αDHP-R 13, lanes 2, 4 and 6 with anti-gyrase B control ascitic fluid. M_r markers as in fig.1.

was recognised by αDHP-R 13 in both mouse and rat preparations as well as rabbit under nonreducing conditions. This similarity in reducing/nonreducing immunoblot profiles indicates that, although minor differences in subunit molecular mass exist (fig.2 and [5]), the structure of the skeletal muscle DHP receptor is very similar in each of the species investigated. As with the immunoblots of rabbit T-tubule membranes (fig.1) a diffuse region of nonspecific staining was present under nonreducing conditions on blots of all three mammalian microsomal preparations but absent from those of frog (see fig.2B). Due to this nonspecific staining, the presence of specific staining at M_r 170000 in mouse and rat samples was not as clearly identified as in rabbit and frog samples. In these cases specific staining appeared as darker and broader bands in the 170 kDa region compared to control tracks (fig.2B).

Immunocrossreactivity between the rabbit skeletal muscle DHP receptor and microsomal components from a number of other rabbit tissues was also investigated. Heart and brain microsomes both contained a component of M_r ~140000 under reducing conditions which was recognised specifically by αDHP-R 13 (fig.3). It is noteworthy that in these two tissues the presence of DHP-sensitive Ca^{2+} channels has been established by extensive ligand binding studies [1,2]. We were unable to detect this component in any other tissue examined, including liver (fig.3), kidney, lung and small intestine, which is not surprising in view of the low levels of specific DHP binding which have been reported in these cases [2].

In conclusion, this work provides evidence that the structures of the voltage-sensitive Ca^{2+} channel DHP receptors, both in different species and in different tissues, are immunologically related. Our results are consistent with the hypothesis that the large 140 kDa polypeptide of the native DHP receptor is disulphide linked to a second small polypeptide component [9]. Furthermore, data obtained under nonreducing conditions indicate that additional components of the Ca^{2+} channel protein remain to be resolved unequivocably.

The series of monoclonal antibodies described in this paper will provide useful tools, not only for future immunological studies of the DHP-sensitive Ca^{2+} channel but also in purification studies of pharmacologically important Ca^{2+} channels from heart and smooth muscle and for the isolation of Ca^{2+} channel cDNA for primary sequence determination.

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Immunological Characterization of Voltage-Sensitive Calcium Channels

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Abstract

A panel of monoclonal antibodies were raised against the 1,4-dihydropyridine sensitive Ca^{2+} channel of rabbit skeletal muscle. When tested on immunoblot assay of denatured and reduced transverse tubule membranes, four of the antibodies specifically recognized a polypeptide of Mr 140,000. This component co-migrated with the large glycoprotein α_2 subunit of purified Ca^{2+} channel preparations. On immunoblots of non-reducing gels the antibodies detected a component that migrated more slowly in the gel, with a Mr of 170,000, consistent with the disulphide-linkage of the α_2 subunit to a small component of Mr 30,000. Additionally, three of the antibodies also recognized high molecular weight components of Mr 310,000-330,000 under these conditions. Crossreactive polypeptides of similar apparent molecular weight were detected in immunoblot assays of rabbit heart and brain membranes and of skeletal muscle membranes from different species.

Further similarities between the α_2 components of Ca^{2+} channels from different species were investigated by immunoblot assay, following the limited tryptic digestion of the skeletal muscle membranes. A similar pattern of immunoreactive peptides were detected in each case, suggesting that the α_2 subunits of Ca^{2+} channels from different species are similar, not only in terms of antibody binding sites but also with respect to similarly positioned trypsin cleavage sites.

The extent of glycosylation of the α_2 component was investigated using enzymatic and chemical deglycosylation techniques. Chemical deglycosylation resulted in a core polypeptide of Mr 105,000, consistent with a carbohydrate content of approximately 25%. Enzymatic treatments, although insufficient to completely deglycosylate the α_2 component, reduced the maximal 1,4-dihydropyridine binding capacity of transverse tubule membranes by 73-77%. The co-development of the α_2 subunit with 1,4-dihydropyridine binding activity was shown in rat skeletal muscle. These results indicate that the '$_2$ subunit is an integral structural component of the 1,4-dihydropyridine sensitive Ca^{2+} channel.