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Calcium currents in the A7r5 smooth muscle-derived cell line

Marks, Theodore N., Ph.D.

Case Western Reserve University (Health Sciences), 1990

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**CALCIUM CURRENTS IN THE A7r5
SMOOTH MUSCLE-DERIVED CELL LINE**

by

Theodore N. Marks

Submitted in partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Thesis Advisor: Stephen W. Jones

Department of Physiology and Biophysics
Case Western Reserve University

August 1990

CASE WESTERN RESERVE UNIVERSITY
GRADUATE STUDIES

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**CALCIUM CURRENTS IN THE A7r5
SMOOTH MUSCLE-DERIVED CELL LINE**

Abstract

by

Theodore N. Marks

I have studied voltage dependent calcium channels in the A7r5 smooth muscle cell line using electrophysiological and biochemical techniques. A dihydropyridine (DHP) sensitive inward current resembling the L'-type calcium current was the dominant current in cells under voltage clamp. Inward currents were blocked by extracellular Cd^{2+} (IC_{50} $\sim 1 \mu\text{M}$), and stimulated (in about half of the cells) by isoproterenol ($1 \mu\text{M}$) or forskolin ($10 \mu\text{M}$). A7r5 cells express saturable, high affinity, voltage-sensitive DHP antagonist binding sites. Suspensions of quiescent cells showed changes in resting $[\text{Ca}^{2+}]_i$ in response to dihydropyridine agonists and increased K^+ . Most confluent cell monolayers showed spontaneous transient elevations in $[\text{Ca}^{2+}]_i$, the frequency and magnitude of which were DHP sensitive.

I investigated the gating kinetics of the channels at the level of single channels and whole cell currents, in the absence and presence of DHP calcium channel agonists. Although latencies to

first opening and macroscopic currents are strongly voltage dependent, analysis of amplitude histograms indicates that the primary open-closed transition is voltage-independent. This suggests that the molecular mechanisms for voltage-sensing and channel gating are distinct, but coupled. I propose a modified Monod-Wyman-Changeux (MWC) model for channel activation, where movement of a voltage sensor is analogous to ligand binding, and the closed and open channels correspond to inactive (T) and active (R) states. This model can account for normal gating behavior of the calcium channel, and is consistent with existing information on ion channel structure. DHP agonists lengthen single channel openings, slow deactivation kinetics, shift the activation curve to more negative potentials with an increase in slope, and reduce the latency to first opening. These effects are predicted by the MWC model, if we make the simple assumption that DHP agonists act as allosteric effectors to stabilize the open state of the channel.

PREFACE

I am deeply indebted to Dr. S. W. Jones for his expert guidance and patience throughout my time in his laboratory, and for teaching me to be a discriminating scientist. I also thank Dr. G. R. Dubyak for his unwavering support and excellent direction for many aspects of this project. I thank Dr. T. Rosenberry for introducing me to the MWC model and for his continued interest in its application to problems of ion channel kinetics. I owe a debt to my entire thesis committee for the time and effort taken on my behalf, as well as their many helpful comments. I appreciate Dr. A. Scarpa for providing me with a superior work environment, and for his constant concern for my progress and development as a graduate student and a scientist.

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CHAPTER I

INTRODUCTION

Voltage-dependent ion channels allow for selective passive diffusion of ions down their concentration gradient in response to changes in membrane potential. The redistribution of charge resulting from ion diffusion can lead to further changes in the electrical potential, and thus is key to the expression of electrical excitability (Hodgkin and Huxley, 1952). Calcium channels represent a subtype of voltage-dependent ion channels which are highly selective in permeability for calcium ions. As intracellular calcium ions play an important role in a wide variety of cellular functions including contraction (Fabiato and Fabiato, 1979), secretion (Katz, 1969), and intracellular signalling (Berridge and Irvine, 1984), calcium channel function influences cellular events in addition to those directly related to excitability. The wide ranging physiological implications of calcium channel function makes these channels particularly interesting subjects for study.

The sub-family of calcium channels can be further divided according to kinetic and pharmacological criteria. Historically, this division has resulted in three primary classes of calcium channels, widely known as 'T', 'L', and 'N' (Nowycky et al, 1985, Fox et al, 1987a,

Fox et al, 1987b). 'T' channels show transient activity on step depolarizations, due to their rapid inactivation kinetics, and their unitary conductance is tiny. 'L' channels are longer lasting, as their inactivation kinetics are relatively slow (with Ba^{2+} as the charge carrier), and their unitary conductance is relatively large. A pharmacological hallmark of the 'L' type channel is its sensitivity to low concentrations of dihydropyridine (DHP) calcium channel agonists and antagonists. Additionally, 'L' channels activate at more positive potentials than 'T'. 'N' channels are found primarily in neuronal cell types, and show properties of activation, inactivation, and pharmacology similar to neither 'T' nor 'L' (Jones and Marks, 1989a; Jones and Marks, 1989b).

'L' channels are perhaps the most well studied of the calcium channels. Their importance to cardiac electrophysiology coupled with the relative ease of obtaining cardiac cells suitable for study with the patch-clamp technique has made for a rich literature on the pharmacology of cardiac 'L' channels. Additionally, the potentiation of cardiac calcium channels by cAMP dependent kinase was among the first investigations into the regulation of ion channel activity by intracellular second messengers (Reuter, 1983; Trautwein and Pelzer, 1988).

'L' type calcium channels are found in a variety of smooth muscle tissues, including vascular smooth muscle, and Ca^{2+} entry through DHP sensitive channels in vascular smooth muscle is thought to be primarily responsible for inducing contraction and

maintaining tone (Nelson and Worley, 1989). The importance of these channels to the pathophysiology of vascular smooth muscle is underscored by the widespread use of calcium channel antagonists in the treatment of hypertension (Wagner et al, 1987).

Most studies of calcium channels in vascular smooth muscle have used freshly isolated cells, since tissue culture of these cells is accompanied by considerable phenotypic modulation (Campbell and Campbell, 1987; Sjolund et al, 1986). These phenotypic changes may involve quantitative or qualitative changes in the expression of voltage-dependent calcium channels. However, the A7r5 cell line, isolated from embryonic rat aorta, has proven useful as a model system for the study of DHP sensitive calcium channels. Besides exhibiting a variety of structural and functional properties of vascular smooth muscle, confluent monolayers of A7r5 cells are electrically coupled, and show evidence of spontaneous electrical activity (Kimes and Brandt 1976; van Renterghem et al. 1988). Additionally, these cells have a voltage- and verapamil-sensitive Ca^{2+} influx pathway (Ruegg et al. 1985). Membranes isolated from A7r5 cells have saturable high affinity dihydropyridine binding sites, and voltage-clamped whole-cell current measurements show the presence of L-type Ca^{2+} currents (Qar et al. 1988; van Renterghem et al. 1988). Whole-cell calcium currents recorded from these cells are often an order of magnitude greater than those recorded from freshly isolated vascular smooth muscle. Additionally, large amounts of cells can be conveniently grown in the laboratory, making it possible to do

biochemical studies in parallel with electrophysiology. As such, the A7r5 cell line provides many advantageous features for exploring properties of calcium currents of vascular smooth muscle.

The remainder of this work serves as an examination of the properties of calcium channels in the A7r5 cell line. Chapter II describes the fundamental electrophysiological properties of the calcium currents at the level of the whole cell and single channel. Basic pharmacology and regulation by neurotransmitters are also discussed. Chapter III examines the channels from a structural perspective using radiolabeled dihydropyridine binding assays. In Chapter IV, effects of calcium influx through the DHP sensitive calcium channel on the intracellular calcium concentration are discussed. In Chapter V I propose and evaluate an allosteric model for calcium channel activation. This model can also explain the actions of DHP calcium channel agonists in a very simple manner.

CHAPTER II.

CHARACTERIZATION AND PHARMACOLOGY OF CALCIUM CURRENTS

INTRODUCTION

Although the A7r5 cell line has been advanced as an *in vitro* system for the study of voltage dependent ion channels in vascular smooth muscle cells (Ruegg et al, 1985), no systematic study of the calcium current in these cells has been reported. It was therefore of interest to examine the calcium currents in the A7r5 cell line in order to compare their fundamental properties and pharmacology those to properties of calcium currents in other cell types. This was accomplished using the voltage clamp technique at the level of whole cell and single channel currents (Hamill et al, 1981).

METHODS

Cells

A7r5 cells were acquired from American Type Cell Culture facilities and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% iron supplemented calf serum (CS) (Hyclone, Logan, UT). Experimental cells were allowed to reach confluence whereupon serum levels were

reduced to 0.5% for 1 - 3 weeks. Confluent cells were trypsinized and replated in 35 mm dishes. Immediately after the cells attached to the dishes, they were treated with cytochalasin-D ($1\text{ }\mu\text{g/ml}$) in order to promote the maintenance of a rounded morphology. All experiments were conducted within two days of replating.

Electrophysiology

For whole cell recording, pipettes with resistances from 10 to 35 M Ω were used to make high resistance seals with the cell membrane. Voltage clamp was established using a List EPC-7 or Axopatch-1B patch clamp amplifier. Voltage commands were given and data were obtained using an AT-type computer, pCLAMP software (CLAMPEX; Axon Instruments, Burlingame, CA) and a Labmaster A-D converter. The incoming signal was filtered at 3 or 5 kHz and digitized at 20 kHz. Leakage current was determined by hyperpolarizing the cell in 2.5 mV increments and was subtracted from depolarizing steps. Series resistance, typically 2-5 M Ω , remained uncompensated. With a series resistance of 5 M Ω and currents smaller than 1 nA we expect a maximum voltage error of 5 mV. The time constant for voltage clamp in the worst case (5 M Ω series resistance, 50 pF capacitance) would be 0.25 ms. Space clamp should present little problem as cells were spherical and less than 50 μm diameter. Pipette solutions consisted of (mM) CsCl or KCl 120, MgCl₂ 4, Na₂ATP 5, Cs₂EGTA 1, and N-methyl-D-glucamine (NMG)

HEPES 2.5, pH 7.2; and bath solutions contained NaCl 112.5, KCl 5, BaCl₂ or CaCl₂ 10, MgCl₂ 1.2, Na-HEPES 2.5, and glucose 10, pH 7.4.

For single channel recording, pipettes of $\sim 5\text{ M}\Omega$ were made from borosilicate glass (World Precision Instruments, New Haven, CT). The pipette contained (mM) BaCl₂ 90, and HEPES 10, pH 7.4. Following seal formation, membrane potential was zeroed with an extracellular solution containing KCl 120, K-EGTA 10, and K-HEPES 10. The analog current signal was filtered at 3 kHz and digitized at 10 kHz. Null sweeps were subtracted from records with openings in order to cancel capacitative transients and leakage currents. On analysis, the data was digitally filtered, usually at 1 kHz. Bathing solutions were superfused by gravity feed and switched between reservoirs containing drugs and drug free solutions. pCLAMP, Lotus 1-2-3, and Micrografx Draw were used for analysis and figure preparation.

cAMP measurements

Intracellular cAMP was assayed after stimulation by various agonists for 5 minutes. The supernatant was aspirated off, and cAMP was extracted into 0.5 ml perchloric acid (33%). The protein was pelleted out by centrifugation, and KOH was added to precipitate the perchlorate. The supernatant was saved for radioimmunoassay (Douglas et al, 1978). Aliquots of samples were suspended in 5 mM Na-acetate buffer, pH 4.75, and triethylamine-acetic anhydride was added to acetylate each sample. Stock antibody was added to each

sample along with ^{125}I -cAMP. Samples were vortexed and incubated at 4°C for 48 to 72 hours.

After incubations were complete, 1% gamma globulin and 25% polyethylene glycol were added to each sample. Tubes were vortexed, and centrifuged (1000 x g for 20 min). The supernatant was aspirated from the pellet, tubes were inverted and left to dry, and the dry pellet was counted in a gamma counter.

RESULTS

Whole-cell currents

The magnitude of inward currents was highly variable among preparations of cells as well as between individual cells from the same preparation. Peak currents ranging from < 0.05 to 2 nA were recorded at test potentials between 0 and +20 mV with 10 mM Ba^{2+} as the charge carrier. In most cases currents were characteristic of the L-type calcium channel. In some instances a "shoulder" was noted in current-voltage curves and a rapidly inactivating, low threshold current was obvious when cells indicating the presence of T-type current. Rarely, such a T-current was dominant. A typical family of currents elicited from a holding potential of -60 mV is shown in Fig. II.1A. In most cases the shape of current traces elicited from a holding potential of -80 mV appeared identical to those from a holding potential of -40 mV (Fig. II.1B). With holding potentials more positive than -50 mV proportionately less current could be

activated; at a holding potential of -20 mV all current was inactivated (Fig. II.1C). Currents carried by 10 mM Ba^{2+} could be completely blocked by Cd^{2+} ($\text{IC}_{50} = 1 \mu\text{M}$) (Fig. II.2).

Calcium currents dominated voltage-activated cation fluxes in these cells, and could be well resolved even with near normal intra- and extracellular solutions. Inward currents were similar in terms of their kinetics and amplitudes with either K^{+} or Cs^{+} as the primary cation in the pipette solution. With K^{+} , outward currents were more noticeable but variable at strongly depolarized test potentials (i.e. $> +40$ mV). "Rundown" of the calcium current (Belles et al., 1988) was variable, and often pronounced.

With Ca^{2+} as the charge carrier, the peak currents were smaller, and the currents inactivated over tens of milliseconds. Figure II.3 illustrates Ca^{2+} mediated inactivation as well as the sensitivity of the inward current to DHP agonists and antagonists.

The β -adrenergic agonist isoproterenol ($1 \mu\text{M}$) increased the inward current in approximately half of the cells tested, although responsiveness varied between preparations. When present, this effect was mimicked by forskolin, a direct activator of adenylate cyclase. In a series of obviously responsive cells, isoproterenol ($1 \mu\text{M}$) increased current by $89 \pm 20\%$ (mean $\pm$ SEM, $n = 14$) of control values and forskolin (10 or $30 \mu\text{M}$) increased currents by $69 \pm 37\%$ (mean $\pm$ SEM, $n=7$). Control values are an average of current amplitudes before drug application and after washout of the drug in order to correct for rundown. Cells with a current rundown of greater than

50% between control and washout were discarded from this analysis. Effects of these drugs on a particularly stable cell are shown in figure II.4. Currents were potentiated at all voltages uniformly, and the shape of the current trace was not obviously changed.

In several experiments, muscarine (20 μ M) inhibited the isoproterenol potentiation of the current, with no obvious effects by itself. This was not a consistent finding, but when present it could be quite obvious. Figure II.5 shows this effect on a stable cell which was particularly responsive to isoproterenol.

Single channel currents

Single channels were observed in most cell-attached patches. Openings were short and difficult to resolve in control conditions, but addition of Bay K 8644 or (+) 202-791 led to a characteristic increase in channel open times (see also Chapter V). In the absence of divalent cations, Na^+ could efficiently permeate the single channels (Fig. II.6). Current amplitudes with Na^+ as the charge carrier were greater than with Ba^{2+} , and showed a characteristic pH dependence (Pietrobon et al., 1989).

Channels were usually stable in the cell attached configuration, but activity declined dramatically when patches were excised. In a few cases, long periods of inactivity of channels in excised patches were followed by periods of intense activity (Fig. II.7). The current amplitudes for excised patches were very close to those

at the same potential in the cell attached mode verifying that the high K^+ bath effectively zeroes the membrane potential.

The DHP antagonist nifedipine blocked DHP agonist stimulated single channel currents in a voltage dependent manner (Fig II.8). With relatively negative holding potentials addition of 300 nM nifedipine resulted in an incomplete block, but as the holding potential was made increasingly positive the block became complete. Complete block was observed at holding potentials where voltage mediated inactivation was observable, but not complete in control conditions, consistent with the inactivated state of the channel having a higher affinity for the DHP antagonist (Bean, 1984; Nelson and Worley, 1989).

In 5 of 8 cell-attached patches, bath application of 10 μ M forskolin led to an increase in channel activity (Fig. II.9). When the experiment lasted long enough to permit removal of the forskolin (3 cases), the potentiation was reversible. Forskolin does not obviously change channel kinetics, but reduced the number of sweeps with no channel openings. Thus, the effects observed here appear similar to those seen for β -adrenergic agonists in cardiac myocytes (Osterrieder et al. 1982; Brum et al. 1984; Kameyama et al. 1985).

cAMP measurements

In order to assess the possible involvement of the cAMP system in the regulation of calcium currents, I examined the effect of isoproterenol and forskolin of intracellular [cAMP]. Isoproterenol

stimulated a modest rise in intracellular cAMP while forskolin was more effective (Table 1). Both agents resulted in greater stimulation when cells were preincubated in the presence of the phosphodiesterase inhibitor, IBMX.

Fig. II.1. Calcium currents under voltage clamp. **A:** Currents were elicited by step depolarizations to indicated test potentials from a holding potential of -60 mV. **B:** Currents during steps to +10 mV from the indicated holding potentials. **C:** Current-voltage relations for peak current from different holding potentials. The cell was clamped at each holding potential for at least one minute prior to recording currents. The charge carrier was 10 mM Ba^{2+} .

Figure II.1.

Fig. II.2. Calcium currents are blocked by micromolar concentrations of Cd^{2+} . **A:** Superimposed currents recorded before, during, and after application of $1\ \mu\text{M}\ \text{Cd}^{2+}$. The holding potential was $-60\ \text{mV}$ and the test potential was $+10\ \text{mV}$. **B:** Current-voltage relationships before, during and after application of Cd^{2+} . **C:** Dose-response curve for Cd^{2+} . Currents during Cd^{2+} application were divided by the average of the control currents immediately before and after Cd^{2+} application in order to correct for "run-down". The curve was drawn according to the law of mass action assuming a single binding site and fit to the data by eye to an IC_{50} of $1\ \mu\text{M}$. All data in this figure are from the same cell. The charge carrier was $10\ \text{mM}\ \text{Ba}^{2+}$.

Figure II.2.

Fig. II.3. Currents were sensitive to nanomolar concentrations of dihydropyridine agonists and antagonists. **A:** This cell was held at -40 mV and stepped to +20 mV. Current traces are shown before and during the application of 300 nM Nifedipine. Note the Ca^{2+} mediated inactivation over the course of the pulse. **B:** Peak current voltage curves for the same cell. **C:** The dihydropyridine agonist (+) 202-791 (300 nM) increases peak and sustained currents. The test potential was +20 mV and 10 mM Ca^{2+} was the charge carrier. **D:** Current-voltage relationship for the same cell. In both cells 10 mM Ca^{2+} was the charge carrier, and the pipette contained K^{+} rather than Cs^{+} .

Figure II.3.

Fig. II.4. Isoproterenol potentiates calcium currents. **A:** Time course of potentiation of Ca^{2+} currents by forskolin and isoproterenol. The cell was held at -60 mV and periodically stepped to $+10$ mV. Peak inward currents are shown. The *horizontal bars* mark the time of drug applications. **B:** Superimposed records of currents at a test potential of $+10$ from a holding potential of -60 mV before, during and after superfusion with forskolin (*left*) and isoproterenol (*right*). **C:** Current-voltage relationships in the same cell. Values are peak currents at indicated test potentials. The charge carrier was 10 mM Ba^{2+} .

Figure II.4.

Fig. II.5. Muscarine inhibits the isoproterenol stimulation of the calcium current. **A:** Time course of the effects of application of different combinations of isoproterenol (10 μ M) and muscarine (20 μ M) on peak current. The cell was held at -60 mV and periodically stepped to +10 mV. Muscarine had no obvious effects when applied by itself. Isoproterenol increased the current by ~ 2.5 times. When applied together with muscarine, the effects of isoproterenol were greatly attenuated. Another application of isoproterenol by itself evoked a large response. **B:** Records of currents at +10 mV before and after the application of isoproterenol or isoproterenol and muscarine. **C:** Peak current-voltage relationships in the same cell. The charge carrier was 10 mM Ba^{2+} .

Figure II.5.

Fig. II.6. Dihydropyridine sensitive calcium channels can conduct Na^+ in the absence of divalent cations. **A:** Examples of single channel currents from three different patches with different pipette solutions. With 120 mM Na^+ , pH 7.4 the open channel currents were very noisy. At pH 9.0 there was much less open channel noise. The unitary amplitude of the currents with Ba^{2+} (90 mM) as the charge carrier is less than with Na^+ . **B:** Single channel current-voltage plots with different charge carriers. The *filled triangles* are with 90 mM Ba^{2+} . *Open symbols* are with 120 mM Na^+ at pH 7.4 (*triangles*) or 9.0 (*squares*). Conductances were linear in the voltage range tested and were 18 pS (90 mM Ba^{2+} , pH 7.4), 25 pS (120 mM Na^+ , pH 7.4) and 50 pS (120 mM Na^+ , pH 9.0). BAY K 8644 (1 μM) was present in all patches.

Figure 11.6.

Fig. II.7. Excised patches were often silent for long periods of time, but could become active many minutes after excision. **A:** Channel activity in the cell attached configuration is similar to that seen after excision. In this patch the channel was silent for 10 minutes after excision, and then spontaneously became active again. **B:** Each digitized point was binned according to its amplitude in order to construct amplitude histograms. The mean open channel current in the excised configuration was very close to that in the cell attached mode. The pipette contained 90 mM BaCl₂ and 1 μ M BAY K 8644.

Figure II.7.

A.

B.

Fig. II.8. Nifedipine block of BAY K 8644 stimulated currents is voltage dependent. **A:** The probability that a sweep will have at least one opening depends on the holding potential in the presence and absence of nifedipine. Test pulses (135 ms) were to -10 mV. The patch was kept at indicated holding potentials for at least 1 minute before collecting data. **B:** The integrated current shows a similar dependence on holding potential. The idealized channel openings, as detected by Fetchan, were used to determine the integrated current.

Figure II.8.

Fig. II.9. Forskolin potentiates single calcium channel activity in cell-attached patches. **A:** Records illustrating channel activity before, during and after superfusion of 10 μ M forskolin. 1 μ M Bay K 8644 was present throughout. **B:** Point-by-point histograms from records before, during, and after forskolin, in the same patch. At least 60 sweeps were included for each condition. The peak at 0 pA is the closed channel. In forskolin, up to three channel openings could be superimposed.

Figure II.9.

Table II.1. Isoproterenol and forskolin increase intracellular [cAMP].

		Control	Isoproterenol	Forskolin
Exper. 1:	- IBMX	611 $\pm$ 16	832 $\pm$ 29	307 $\pm$ 26
	+ IBMX	106 $\pm$ 3	211 $\pm$ 8	1139 $\pm$ 45
Exper. 2:	- IBMX	663 $\pm$ 12	921 $\pm$ 19	353 $\pm$ 4
	+ IBMX	170 $\pm$ 20	411 $\pm$ 7	1570 $\pm$ 99

Five minute exposure to 1 μ M isoproterenol or 10 μ M forskolin increases intracellular [cAMP], both in the presence and absence of IBMX. cAMP is reported as pM/ 10^6 cells (mean $\pm$ SEM, n = 3).

DISCUSSION

Most currents observed showed kinetics and voltage dependence characteristic of 'L'-type calcium channels found in cardiac and smooth muscle tissues (Fig. II.1)(Hess et al., 1986). More rarely, a 'T'-type current was observed, characterized by rapid inactivation with Ba^{2+} as the charge carrier and a distinct shoulder on current voltage curves. 'T' currents have been reported in these cells by some (Fish et al., 1988, McCarthy and Cohen, 1989) but not by others (van Renterghem et al., 1988).

The block of the current by Cd^{2+} with an $\text{EC}_{50} \sim 1 \mu\text{M}$ in 10 mM Ba^{2+} (Fig. II.2), Ca^{2+} mediated inactivation and sensitivity to nM concentrations of DHP agonists and antagonists (Fig. II.3) further supports the similarity between the currents observed in these cells and other reported 'L'-type currents.

Properties of single channels, including DHP agonist sensitivity and permeability were also similar to those reported for cardiac channels (Figs. II.6 and II.8). The high degree of DHP antagonist induced block at relatively negative holding potentials is less consistent with cardiac calcium channels than those from smooth muscle (Bean et al., 1986; Bean, 1984). However, I do not believe that my data addressing this point is compelling enough to make firm conclusions. These channels can be clearly distinguished from DHP

sensitive calcium channels found in skeletal myocytes on account of their relatively rapid activation kinetics.

Because the cardiac 'L' channel can be potentiated by β -adrenergic stimulation, I asked whether or not the calcium current in the A7r5 cells could also be regulated in such a manner. Although not all cells were responsive to the β -agonist isoproterenol, when present the increase in calcium current was clear. In particular, the effect was reversible, with a slow time course consistent with a second messenger-mediated action (Figs. II.4 and II.5). Isoproterenol and forskolin also increased cAMP levels measured by radioimmunoassay, suggesting involvement of the cAMP cascade, as is the case for β -adrenergic potentiation of Ca^{2+} currents in cardiac cells (Kameyama et al. 1985). The inhibition of isoproterenol stimulation by muscarine noted in some cases further argues for the involvement of the cAMP cascade (Hartzell and Fischmeister, 1987). Unfortunately, the effects of elements of intra-cellular signalling pathways (i.e. kinases and phosphatases) could not be assessed directly by applying them to the single channels, as channels were very unstable in excised patches (Fig. II.7).

Potentiation of calcium currents in vascular smooth muscle by β -adrenergic agonists was not expected, since β -agonists are vasorelaxants, and Ca^{2+} mediates muscle contraction. However, the relationship between $[\text{Ca}^{2+}]_i$ and contraction of smooth muscle is complex. Recent studies with tracheal smooth muscle suggest that increases in intracellular $[\text{Ca}^{2+}]_i$, resulting from Ca^{2+} influx, can be

concomitant with relaxation (Takuwa et al. 1988). Alternatively, it has been proposed that phosphorylation by cAMP dependent protein kinase is a requirement for a functioning calcium channel (Armstrong and Eckert, 1987).

Adrenergic agonists previously have been reported to increase or decrease smooth muscle calcium currents, acting through α , β , or γ adrenergic receptors (Droogmans et al. 1987; Pacaud et al. 1987; Nelson et al. 1988; Benham and Tsien 1988). A similar discrepancy exists for activators of protein kinase C, which have been reported to enhance (Sperti et al. 1987; Fish et al. 1988) or inhibit (Galizzi et al. 1987) calcium currents in A7r5 cells. In our hands, neither phorbol esters (PMA and TPA, 0.1 and 1.0 μ M) nor the Ca^{2+} mobilizing hormones AVP (100 nM) and endothelin (100 nM) had any effect on calcium currents in A7r5 cells. The explanation of this variability is not clear, but may be related to phenotypic instability of this cell line. Such instability may also be responsible for the varying expression of T currents, the wide range of current amplitudes seen under voltage clamp, as well as the inconsistent responses to β -adrenergic stimulation.

CHAPTER III.

RADIOLABELED DIHYDROPYRIDINE

BINDING STUDIES

INTRODUCTION

The interaction of DHP's with the calcium channel can be assessed by radioligand binding studies, in addition to electrophysiological means applied in chapters II and V. Binding studies are directed towards an understanding and comparison of the structural aspects of the calcium channel and its pharmacology. As the DHP antagonists are very potent they are expected to bind to their sites of activity with a very high affinity. This specific binding can be exploited in order to better understand the features of the calcium channel.

The use of intact cells for binding studies offers several advantages over studies involving membrane fragments. The intact cells maintain a voltage gradient across the plasma membrane, allowing for investigation of the voltage dependence of DHP binding. Also, because no fractionation of the cells is involved, a quantitative assessment of the number of binding sites per cell can be made.

METHODS

Cells were grown as described above in 12-well cluster dishes and the experiments were done with the cells attached to the dishes. Assays were conducted according to a modification of the methods of Greenberg et al. (1986) which allowed us to conduct incubations with substrate-attached cells. The cells were rinsed with a balanced salt solution (BSS) containing (mM) NaCl 125, KCl 5, MgCl₂ 15, Na-HEPES 25, and glucose 10 (pH 7.4) and incubated in 2 ml of buffer in the cluster dishes. The "depolarizing" or "high K⁺" buffer contained (mM) NaCl 85, KCl 50, MgCl₂ 1, CaCl₂ 2, Na-HEPES 50, and glucose 5 (pH 7.4); and the "polarizing" or "low K⁺, low Ca²⁺" buffer contained (mM) NaCl 85, KCl 5, MgCl₂ 1, CaCl₂ 0.5, choline-Cl 46.5, Na-HEPES 50, and glucose 5 (pH 7.4). (+)³H-PN-200-110 and nifedipine dissolved in ethanol were added in 2 μ l aliquots. Cells were incubated in the dark (90 minutes for equilibrium binding) at room temperature after which they were quickly rinsed with ice-cold buffer. NaOH was added to each well to extract all protein, followed by a rinse with H₂O. The cell extract was neutralized with HCl, scintillation cocktail was added, and the mixture was counted in a scintillation counter.

Samples consisting of $\sim 10^5$ cells provided enough material to make reliable measurements. Nonspecific binding was assumed to be that (+)³H-PN-200-110 bound in the presence of 1 μ M nifedipine, and has been subtracted from all data presented. Specific binding was

-50% of total binding at 50 pM and -15% at 600 pM (+)³H-PN-200-110. Values for K_d and B_{max} were derived by weighted regression according to the method of Wilkinson (1961).

RESULTS

(+)³H-PN-200-110 binds to intact A7r5 with a high affinity in a time dependent manner (Fig. III.1). With 50 pM (+)³H-PN-200-110 in depolarizing conditions, equilibrium was reached at - 90 min.

A7r5 cells have saturable voltage-sensitive (+)³H-PN-200-110 binding sites (Fig. III.2). In a high K⁺ (50 mM) buffer the saturation isotherm was fit assuming a single high affinity site, $K_d = 54 \pm 9$ pM (mean $\pm$ SE, n = 3). When the assay was conducted in a buffer low in Ca²⁺ and K⁺ (in order to maintain cell membrane potential), then the data were fit assuming a single site with a considerably lower affinity, $K_d = 167 \pm 37$ pM (n = 3). Maximum binding was 47 ± 2 and 47 ± 4 fmoles/10⁶ cells under depolarized and polarized conditions respectively, or about 30,000 sites per cell. When binding sites in proliferative cells were probed in a high K⁺ buffer, neither the affinity or the number of binding sites was found not to differ significantly from the quiescent cells ($K_d = 48 \pm 14$ pM, $B_{max} = 42 \pm 8$ fmoles/10⁶ cells; mean $\pm$ SEM, n = 3).

Displacement of (+)³H-PN-200-110 by nifedipine was also voltage sensitive, with nifedipine exhibiting a lower K_i in a high K⁺

buffer (Fig. III3). When cells were incubated with 50 pM (+)³H-PN-200-110, nifedipine displaced (+)³H-PN-200-110 in a dose dependent manner. In both the high K⁺ and low K⁺-low Ca²⁺ buffers, (+)³H-PN-200-110 displacement by nifedipine was fit by eye according to an equation derived by the law of mass action assuming a single affinity site. Values for K_i were calculated from the fitted curves (Cheng and Prusoff 1973) and were 260 and 1600 pM in the high K⁺ and low K⁺, low Ca²⁺ buffers respectively.

Fig III.1. Time course of (+)³H-PN-200-110 binding to intact A7r5 cells. Specific binding of 50 pM (+)³H-PN-200-110 takes many minutes to reach equilibrium. Nonspecific binding was complete by the earliest measurable time point. No error bars are shown, as the S.E.M. was smaller than the size of the symbol (n = 3)

Figure III.1.

Fig. III.2. Saturable binding of (+)³H-PN-200-110 to intact A7r5 cells. *Open squares* are from experiments with high K⁺ (50 mM) buffer, and *solid squares* are with low K⁺-low Ca²⁺ buffer. Each point represents the mean $\pm$ S.E.M. of three experiments each done in triplicate. Where no error bars are shown the size of the symbol exceeds the S.E.M. Curves are drawn according to the law of mass action assuming a single site in each condition.

Figure III.2.

Fig. III.3. Displacement of (+)³H-PN-200-110 by nifedipine. Cells were incubated with 50 pM (+)³H-PN-200-110 and indicated concentrations of nifedipine. *Open squares*, high K⁺ buffer; *closed squares*, low K⁺-low Ca²⁺ buffer. This is the result of a single experiment which was repeated three times with the same result. Curves are drawn according to the law of mass action assuming a single site and fit by eye.

Figure III.3

DISCUSSION

The affinity of (+)- ^3H -PN-200-110 for binding sites in A7r5 cells under polarizing and depolarizing conditions are in excellent agreement with those reported in intact PC12 cells (Kunze et al. 1987; Greenberg et al. 1986), cardiac cells (Kokubun et al. 1986), myometrial cells (Honore et al. 1989) as well as vascular smooth muscle tissue (Morel and Godfraind 1987), which range from 40 - 65 pM in high K^+ solutions.

Binding studies with intact cells allow for the direct demonstration of the voltage-dependent binding of DHPs as has been suggested by electrophysiological experiments (Bean 1984; Sanguinetti and Kass 1984). Since my data were well fit assuming a single binding site, most if not all binding sites are voltage-sensitive, and thus reside in the plasma membrane.

McCarthy and Cohen (1989) reported a somewhat higher affinity (16 pM) for binding of another DHP (nimodipine) to the open state of the calcium channel in A7r5 cells. This conclusion was drawn from electrophysiological experiments and is strongly model dependent. McCarthy and Cohen speculated that this high affinity binding could be used to distinguish between calcium channels in A7r5 cells and other cell types. The radioligand binding studies reported here do not support dissimilarity between calcium channels in different cell types.

Estimates of channel numbers from binding studies can be compared to those from electrophysiology. At 0 mV in 90 mM Ba^{2+} , the single channel amplitude was 11 pA, and channels were open 70.4% of the time. Under those conditions, the whole-cell current was -20 pA, which corresponds to ~4500 channels per cell. That is ~15% of the number of DHP binding sites. This calculation is subject to error from cell-to-cell variability, especially since 0 mV is near the threshold for activation in 90 mM Ba^{2+} . In skeletal muscle, the discrepancy between DHP binding sites and functional calcium channels is more extreme (Schwartz et al. 1985). It is possible that some DHP binding sites in smooth muscle are not calcium channels, that each channel contains more than one binding site, or that some channels do not gate normally under whole-cell conditions.

Chapter IV.

INTRACELLULAR $[Ca^{2+}]$

It is clear from the data presented in Chapters II and III that DHP sensitive calcium channels are present in A7r5 cells and that they make a significant contribution to the voltage-dependent currents. I was also interested in examining how currents through the DHP sensitive calcium channels could impact cellular Ca^{2+} homeostasis in these cells. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured under a variety of conditions using the fluorescent indicator fura-2 and the effects of changing membrane potential and DHP agonists and antagonists were assessed.

METHODS

Confluent cells were treated with trypsin and suspended in BSS with 0.5% BSA. After washing, cells were incubated with 0.5 μ M fura-2-AM for 30 min at 37° C, rinsed and placed into a glass cuvette which was thermostated at 37 °C and stirred continuously. Monolayers of cells for fura-2 fluorescence experiments were grown on plastic coverslips in 12-well plates as previously described (Mene et al. 1987). Monolayers were incubated with 2 μ M fura-2-AM in DMEM with 0.5% CS for 30 min at 37° C. Excitation and emission wavelengths were 339 and 500 nm respectively. Calibration was

accomplished by adding 20 $\mu\text{g/ml}$ digitonin followed by 12 mM EGTA (suspensions) or 10 μM ionomycin followed by 1 mM MnCl_2 (monolayers). Calculations were made assuming a fura-2- Ca^{2+} K_d of 220 nM.

RESULTS

Suspended cells had resting $[\text{Ca}^{2+}]_i$ of ~ 150 nM. Cells that had been confluent and serum deprived for less than 1 week before experiments showed little or no change in $[\text{Ca}^{2+}]_i$ when Bay K 8644, nifedipine, or K^+ were added. Cells were more responsive with increasing time in culture following confluence (Fig. IV.1).

The resting $[\text{Ca}^{2+}]_i$ in confluent monolayers of fura-2 loaded A7r5 cells ranged from 200-250 nM. However, most monolayers exhibited episodes of spontaneous, rapid changes in $[\text{Ca}^{2+}]_i$. On chelation of extracellular Ca^{2+} ions with excess EGTA or upon addition of nifedipine, these spontaneous transients ceased. Addition of the Ca^{2+} channel agonist Bay K 8644 caused a dramatic increase in the frequency of these Ca^{2+} transients (Fig. IV.2). The Ca^{2+} channel antagonist nifedipine led to an abrupt cessation of the $[\text{Ca}^{2+}]_i$ elevations and a prompt return of $[\text{Ca}^{2+}]_i$ to the resting level.

Fig. IV.1. Cells acquire sensitivity to DHPs and increased K^+ after prolonged time in culture. Fura-2 loaded cells were suspended in the proliferative state and at varying times after reaching confluence.

Figure IV.1.

Fig. IV.2. Effects of dihydropyridines on $[Ca^{2+}]_i$ transients in a monolayer of A7r5 cells. Spontaneous increases in $[Ca^{2+}]_i$ were potentiated by Bay K 8644 and were terminated by nifedipine.

Figure IV.2.

Cytosolic Ca^{2+} , nM

DISCUSSION

Cells that had been confluent for several weeks showed increases in $[Ca^{2+}]_i$ in response to high $[K^+]_o$ or dihydropyridine agonists, whereas cells at earlier stages were consistently unresponsive. This might suggest an increase in the number of functional Ca^{2+} channels, but the number of DHP binding sites per cell was unchanged with the proliferative state of the cells (see Chapter III). The increase in channel activity might result from a more negative resting potential as the cells are in culture longer (Blennerhassett et al. 1989), leading to removal of voltage-dependent inactivation of Ca^{2+} channels. Alternatively, phenotypic modulation during maturation might directly enhance Ca^{2+} channel function.

In most cases, confluent substrate-attached monolayers of A7r5 cells showed spontaneous oscillations in $[Ca^{2+}]_i$. These events depend on Ca^{2+} influx through L-type Ca^{2+} channels, as the transient increases in $[Ca^{2+}]_i$ were prevented by DHP antagonists, and the frequency and amplitude of the transients were increased by DHP agonists. This suggests that the oscillations are due to synchronous action potentials among electrically coupled cells (van Renterghem et al. 1988) throughout the large (0.64 cm^2) optical field of the photomultiplier. Action potential-driven oscillations in $[Ca^{2+}]_i$, reported previously for pituitary cells (Schlegel et al. 1987), contrast with superficially similar oscillations resulting from Ca^{2+} release from intracellular stores (Woods et al. 1986).

One might expect neurotransmitters and second messengers which act on calcium currents could regulate calcium spiking in confluent cells. Stimulation of the monolayers with isoproterenol and forskolin resulted in inconsistent results. In some cases these agents lead to increased frequency and amplitude of the spiking activity in a manner similar to that observed for DHP agonists. In other cases, baseline cytosolic calcium concentrations were decreased or no effect was observed. This type of inconsistency may indicate that isoproterenol and forskolin have actions at loci distinct from the calcium channel, including the regulation of K^+ currents or cell-to-cell communication. As the generation of action potentials is undoubtedly a complicated phenomenon involving K^+ currents and gap junctions, adrenergic modulation of these contributing factors may override potentiating effects on the calcium channel.

Although vascular smooth muscle cells do not fire action potentials under normal circumstances (Burnstock et al. 1963), such activity in A7r5 cells may provide important insights into the factors which regulate excitability in pathological conditions such as coronary vascular spasm.

Chapter V.

KINETICS OF CALCIUM CHANNEL ACTIVATION WITH AND WITHOUT DIHYDROPYRIDINE AGONISTS.

INTRODUCTION

Calcium channel gating has generally been described with models containing a series of closed states and an open state. Multiple closed states are required to explain delays in activation kinetics, and nonexponential distributions of channel closed times (Fenwick et al, 1982). Technical difficulties, particularly the rapid gating kinetics and poor signal to noise ratio at the single channel level, have limited the amount of kinetic information available about calcium channels. DHP agonists, which improve resolution of channel activity by greatly prolonging channel open times (Hess et al, 1984), may provide insights into the normal gating kinetics of calcium channels. However, qualitatively different models of DHP agonist action have been proposed (Hess et al, 1984; Sanguinetti et al, 1986; Hering et al, 1989; Lacerda and Brown, 1989). These models have not yet been related to the underlying channel structure.

At one level, a kinetic model for gating of a voltage-dependent ion channel is simply an operational description of channel behavior, little more than a way of summarizing data on the response of the channel to changes in membrane potential. However, such models

can also be viewed as hypotheses about the physical mechanisms that couple changes in voltage to conformational changes in the channel protein. Such hypotheses become increasingly testable as information about channel structure accumulates. Generally, models are considered to be successful if they describe channel kinetics with a minimum of states and free parameters. However, models that can be related to existing structural information about ion channels also gain in plausibility.

Molecular cloning has revealed a large family of proteins that can form sodium, calcium, or potassium channels (Catterall, 1988). Sodium and calcium channel proteins include four repeated homologous domains, each including several putative transmembrane regions (Noda et al., 1986; Tanabe et al., 1987; Mikami et al., 1989). The S4 region, present in each of the four domains, may be the voltage sensor (Stuhmer et al., 1989, Guy and Seetharamulu, 1986). Homologous potassium channel proteins contain only one such domain, and are thus thought to function as oligomers, presumably with four subunits (Tempel et al., 1987).

Recently, Zagotta and Aldrich (1990) and Koren et al. (1990) proposed models for potassium channel gating that involve independent movement of 4 voltage sensors, as in the original Hodgkin and Huxley (1952) model for potassium channel gating. However, there was evidence for an additional voltage-independent transition subsequent to movement of the voltage sensors, prior to channel opening. This scheme is illustrated in Scheme 1 (Fig. V1A).

The postulation of 4 distinct voltage sensors fit well with the structural information, but the physical basis of the voltage-independent step remained unclear.

We report here data on calcium channel kinetics in the A7r5 smooth muscle-derived cell line. Both single-channel and whole-cell kinetics have been studied, in the absence and presence of DHP agonists. Under control conditions, the data are well described by Scheme 1. Much of the data with DHP agonists is consistent with an effect on the closed-open transition in Scheme 1, and no effect on the voltage-dependent steps. However, we find that DHP agonists greatly reduce the latency to first opening of calcium channels (see also Lacerda and Brown, 1989; Hess et al, 1984), which cannot be reproduced by Scheme 1 since most of the latency results from the closed-closed transitions. We also observe both fast and slow components to the open time distribution with DHP agonists, suggestive of multiple open states.

These results can be explained by a scheme (Fig. V1B) based on the MWC model for cooperativity in allosteric enzymes such as hemoglobin (Monod et al., 1965). Here, movement of a voltage sensor corresponds to binding of a ligand, and the closed and open states correspond to the inactive (T) and active (R) states of the enzyme. The physical interpretation is that depolarization induces small, local conformational changes in the protein, presumably movement of the S4 helix. The local changes then facilitate channel opening, which could be a global conformational change involving all domains of the

protein. Hille (1984) has also suggested a possible analogy between allosteric enzymes and ion channel gating.

The MWC model (Fig. V.1B) can reproduce calcium channel gating both in control and with DHP agonists, with a minimum of free parameters (only one more than Scheme 1). We propose that DHP agonists act as allosteric effectors to stabilize the open state, much as 2,3 diphosphoglycerate acts to stabilize the T state of hemoglobin. Under control conditions, our data is best fit with strong cooperativity, where the MWC model reduces to Scheme 1. With DHP agonists, the shift in the closed-open equilibria weakens the cooperativity sufficiently to allow openings to the O_3 state.

METHODS

Current recording

Cells were grown and currents were recorded in a manner similar to that described in Chapter II except as noted below.

Single channels. All experiments were conducted at room temperature. Fifty percent threshold crossing techniques from Fetchan (V. 5.5) were used for event detection.

Whole cell currents. The signal was analog filtered at 25 kHz before digitization at either 20 or 100 kHz. Cell capacitance (15 - 35 pF) and series resistance (25 - 50 M Ω) were determined by optimal transient cancellation. Series resistance was compensated 60 - 70%. This results in a theoretical time constant for voltage clamp < 0.1 ms, and a steady state voltage error of - 2 mV with 1 nA of current. Often, an additional transient component which was difficult to cancel was observed, possibly representing capacitance of internal membranes. Otherwise, space clamp presents little problem in these cells as they are spherical with diameter < 40 μ m. Leak current was corrected for by averaging, scaling, and adding four steps of amplitude -0.25 of the test step. The pipette solution contained (mM) CsCl 120, MgCl₂ 4, Tris₂-ATP 5, Cs₂-EGTA 1, and Cs-HEPES 25, pH 7.2. To maintain consistency with single channel experiments, the bath solution contained BaCl₂ 90 and NMG-HEPES 10, pH 7.4.

Amplitude histograms

Under control conditions, gating kinetics within a burst were too rapid to be resolved directly. A modification of the method of Yellen (1984) was used to estimate rate constants for channel opening and closing from point-by-point amplitude histograms. Ideally, the amplitude histogram for a channel showing simple 2 state (closed-open) kinetics, when filtered heavily, is described by a beta distribution:

$$f(x) = x^{(a-1)}(1-x)^{(b-1)} \quad (1)$$

where $a = k_L \tau$, $b = k_{-L} \tau$, x is the current amplitude (on the scale 0 = closed, 1 = open), k_L and k_{-L} are the opening and closing rates, and τ is the cutoff frequency of the filter. The effect of recording noise can be incorporated by convolving the beta distribution with a Gaussian, fit to the baseline noise (0.07 - 0.1 pA following 300 Hz Gaussian filtering in our experiments).

We were not able to reliably detect the beginnings and ends of bursts using threshold-crossing analysis, so our amplitude histograms are contaminated by baseline noise and data points on the rising and falling edges of transitions to and from bursts. Baseline noise was subtracted by fitting the amplitude distribution on the side of the baseline opposite the open channel current to a Gaussian distribution with mean of 0, and then subtracting that Gaussian from the total amplitude distribution. As the baseline opposite the open channel

current may also contain some open channel signal due to noise, this subtraction will not be strictly accurate, especially at small amplitudes. The effect of transitions to and from bursts is described below.

Strategy for modelling

Two models will be considered in detail, a linear six state scheme (Scheme 1) and the MWC model (Fig. V1). We will argue below that simpler models (such as C-C-O, or Hodgkin-Huxley-type models) cannot fit important features of our data. Data in control conditions and with DHP agonists will be discussed in parallel, since we conclude that the effects of DHP agonists can be explained by a relatively simple modification of normal gating kinetics. Inactivation will not be considered.

Both models assume that the channel contains four identical voltage-sensors, which move independently (except for the cooperativity introduced by closed-open transitions). Scheme 1 thus is determined by four parameters (k_C , k_{-C} , k_L , k_{-L}) at a given voltage. We will assume that the rate constants for movement of each voltage sensor depend exponentially on voltage (Stevens, 1978), with equal voltage dependence for movement of the sensor in each direction:

$$k_C = A e^{(V/V_s)}, k_{-C} = B e^{(-V/V_s)} \quad (2)$$

where A and B are the rate constants at 0 mV, and V_s is inversely proportional to the amount of charge moved across the membrane field. These assumptions mean that the microscopic voltage dependence of the model is determined by a single parameter, the amount of charge moved by one voltage sensor.

To reduce the number of free parameters on the MWC model, we assume symmetrical changes both in the voltage-dependent transitions between closed and open states, and between the closed-open rate constants for transitions with different numbers of voltage sensors moved. Those assumptions mean that the MWC model is determined by one free parameter, f , in addition to those in Scheme 1. The parameter c in the original Monod et al. (1965) model is equal to f^2 . We also assume that the action of DHP agonists can be explained by symmetrically increasing k_L and decreasing k_{-L} by the scaling factor, Δ_{DHP} .

The order in which the data are presented below corresponds roughly to the sequence used for determination of the model parameters. The rate constants for channel opening and closing in control conditions (k_L , k_{-L}) are determined primarily by channel gating during bursts. Information about closed-closed transitions (k_C , k_{-C}) depends in part on the steady-state activation curve (which determines the *ratio* k_C/k_{-C} and the voltage dependence). The shift in the activation curve with DHP agonists sets the factor by which k_L and k_{-L} are changed. The kinetics of whole-cell currents, and the distribution of latency to first opening of single channels, determine

the absolute magnitudes of k_c and k_{-c} . The parameter f , which strongly influences the cooperativity, is not directly determined by the data, but constraints on f are discussed below.

One difficulty that we have encountered is the lack of a general method for fitting a model to data when qualitatively different sorts of data are involved (e. g., single channel and whole cell experiments). We have resorted to an iterative process based heavily on simulations, where we guess a set of rate constants, simulate whole cell currents with realistic series resistance, and single channels with realistic noise. We use modified versions of AXOVACS programs for whole cell currents, and CSIM (Axon Instruments) for single channels. We then analyze the simulated data as we would real data, including filtering. Next, the rate constants are changed based on any discrepancies between model and experimental data, and the entire cycle is repeated.

RESULTS

Single channel currents

Activation of calcium channels is clearly voltage-dependent (Fig. V2). Under control conditions, openings are rare and brief at negative voltages. At positive voltages, openings occur mostly in bursts. Channel opening events with DHP agonists are more frequent and longer, with "tail" events indicating a slow channel closing rate even at -80 mV. Gating within a burst is not obviously voltage dependent, but bursts are longer and more frequent at more positive potentials.

In order to determine whether the open channel conductance was the same for control and DHP stimulated channels, we examined the relationship between event duration and amplitude at 0 mV, where the signal to noise ratio is most favorable. It is clear from Fig. V2 that the open channel current is easily measurable with DHP agonists, but most openings in control are attenuated. Fig. V3A shows that the longest events in control have amplitudes comparable to those with DHP agonists, suggesting that the true open channel current is the same under both conditions.

With DHP agonists, the conductance is nearly linear with voltage in the measurable region (Fig. V3B). The conductance was 20 pS with an extrapolated reversal potential of +54 mV.

Amplitude histograms

Direct measurements of channel open and closed times using threshold-crossing analysis yielded a single mean open time - 0.3 ms and multiple closed times, with most closed times falling in the most rapid distribution of τ - 0.2 ms. We consider those values to be completely unreliable, as the heavy filtering needed for a favorable signal to noise level (- 1 kHz) distorts the signal to the extent that most events would be missed. Although corrections for missed events can be made in some cases (Roux and Sauve, 1985; Blatz and Magleby, 1986), we are not aware of any way to correct these values when open and closed times are both so fast in relation to the system dead time.

We next considered point-by-point amplitude histograms (Fig. V.4), which contain information on channel gating (Yellen, 1984). In control conditions, two peaks were observed, one at zero current, and the other between zero and the open channel amplitude with DHP agonists. Subtraction of baseline noise produces a distribution (Fig. V.4C) that is too broad to be fit by a beta distribution convolved with baseline noise (Fig. V.5). This might result from contamination of the distribution by points from transitions into and out of bursts, which would broaden the curve on the closed channel side.

To explore this further, we compared amplitude distributions from experimental data, simulated data, and beta distributions (Fig. V.5). The position of the experimental peak along the amplitude axis, and the shape of the curve on the open channel side of the distribution, are well fit with $k_L = 12 \text{ ms}^{-1}$ and $k_{-L} = 6 \text{ ms}^{-1}$ (Fig. 4A).

Changes in the equilibrium constant shift the position of the peak. The simulated data, using the rate constants described below for closed-closed transitions to produce realistic bursting, was also well fit by the beta distribution, except for the shoulder on the closed-channel side (Fig. 4B). This suggests that our assumptions about closed-closed kinetics have little effect on the open channel side of the amplitude histogram. The histograms depend critically on the equilibrium constant, but are less sensitive to the absolute rate constants (Fig. 4C,D). A 3-fold slowing of kinetics from values $k_L = 12 \text{ ms}^{-1}$ and $k_{-L} = 6 \text{ ms}^{-1}$ is clearly distinguishable, but 3-fold faster gating produces a distribution that is only slightly narrower. This loss of resolution when the rate constants become very fast is due to the relatively large amount of noise that must be convolved with the beta distribution. Varying the filter frequency should change the noise characteristics; and one might suppose that heavier filtering might improve resolution with faster rate constants. However, if the signal is *very* heavily filtered (Fig. V.6), then the unconvolved beta distribution becomes very narrow at moderately fast rate constants and the final resolution is no better than with less filtering. Additionally, very heavy filtering (i.e. 100 Hz) increases the distortion due to crossings in and out of bursts.

For $k_L = 12 \text{ ms}^{-1}$ and $k_{-L} = 6 \text{ ms}^{-1}$, simulated histograms and beta distributions agree well with the experimental data over a 40 mV range (Fig. V.7). This suggests that the microscopic rate constants for channel opening and closing are not voltage-dependent.

Additionally, the shoulders on the closed channel side of the histograms are well approximated by the simulated distributions. For both experimental and simulated data, the shoulders are more prominent at negative voltages, where burst length is shorter, and crossings into and out of bursts make a larger relative contribution to the histogram. This qualitative agreement suggests that the model predicts bursting behavior similar to that of the real channel.

Gating with DHP agonists

In the presence of DHP agonists, most openings are long enough to be well resolved at a 1 kHz bandwidth. However, very fast events, both openings and closings, were observed (see Fig. V.2). In order to test the voltage-dependence of gating we examined the kinetics directly. Good fits to open time distributions in the presence of 1 μ M (+) 202-791 require at least two exponentials (Fig. V.8). Although the fast component (~ 0.3 ms) is poorly quantified given the limited bandwidth, its presence is clear. This fast component is indicative of a unique open state and not expected to be due to a "phantom state", as the long open times are much longer than the short closed times (Roux and Sauve, 1985). The time constants for both the fast and slow open events appear to be voltage-independent between -10 and +10 mV (Fig. V.9). The fast closings were too rapid to analyze directly. The occasional observation of very rapid closings in single channel currents after repolarization to negative potentials (Fig. V.10) also suggests that the primary closed-open reaction is

voltage independent, as a voltage-dependent opening would almost never occur at -80 mV. Additionally, the long open times during single channel tail currents qualitatively resemble those observed at more depolarized potentials.

A biexponential distribution of open times is not expected with the linear six state model (Scheme 1), and simulations using such a scheme yield only a single open time distribution, regardless of missed closing events. Thus, there must be at least two open states in the presence of DHP agonists. The MWC model can explain a population of short openings as transitions from C_3 to O_3 . Furthermore, inspection of records in the presence of DHP agonists at weak depolarizations reveals that the short openings often appear in close proximity to long openings (Fig. V.11A). As the channel is very likely to be in state C_3 immediately before and after a burst, the MWC model predicts that the shorter openings should occur preferentially, although not exclusively, in close temporal relation to the bursts (Fig 11B).

Channel gating kinetics with DHP agonists help define the cooperativity factor f . Extremely strong cooperativity ($f \ll 1$) reduces the model to Scheme 1, and weak cooperativity ($f \sim 1$) produces a voltage-independent channel. With $f = 1/15$, channel gating with DHP agonists was well reproduced. A 2-fold increase in f produces too many fast openings and closings, and a 2-fold decrease in f practically eliminates openings to the O_3 state.

Concentration dependence of DHP agonist action

An alternate interpretation of the two open states with DHP agonists is that one state is the normal open state, and the other is the DHP-modified channel. If so, the intermingling of short and long openings requires that binding and unbinding of DHP agonists be extremely rapid, and the channel open time(s) might be concentration-dependent (Lacerda and Brown, 1989). If DHP binding is slow, concentration dependence would be reflected in sweep-by-sweep changes in channel activity rather than in gating kinetics on the millisecond time scale (Hess et al., 1984).

As the concentration of DHP agonist was increased from 10 nM to 100 nM to 1 μ M, an increasing number of sweeps with long openings was observed (Fig. V.12). No long openings were observed in the absence of drug in this patch. A complex distribution of open times is evident from Fig. V.12. Biexponential fits to open time histograms for this patch and two others reveal that the time constants are not detectably concentration-dependent, but the relative number of short and long openings changes in a concentration-dependent manner.

We examined the probability of being open [$p(\text{open})$] in each sweep that had at least one long opening, as a function of DHP agonist concentration. If binding and unbinding are rapid with respect to the test pulse length (135 ms) then increasing the concentration should increase $p(\text{open})$ within a sweep, as DHP rebinding is more likely to occur at high concentrations. Conversely,

if binding and unbinding are slow with respect to the sweep length, sweeps showing long openings should have the same $p(\text{open})$ regardless of concentration, as it is unlikely that association or dissociation will occur over the time of the test pulse. There was a wide range of $p(\text{open})$ among sweeps with long openings, at all concentrations (Fig. V.13A). Only a few sweeps contained openings longer than 3 ms in control, and their mean $p(\text{open})$ was relatively low. The mean $p(\text{open})$ was virtually the same at all DHP agonist concentrations (Fig. V.13A), despite a 5-fold increase in the number of sweeps with long openings (Fig. V.13B).

If binding and unbinding were *very* slow (τ on the order of seconds) then we expect sweeps with long openings to be consecutive at low DHP agonist concentration. We do not observe this consistently, which argues against very slow kinetics. Clearly, most if not all of the concentration dependence of the effect of DHP agonists appears as changes in gating from sweep to sweep, consistent with relatively slow binding and unbinding, on the order of hundreds of milliseconds. Thus, DHP-modified channels must have access to at least two open states.

Whole cell currents

Properties of whole cell currents in these cells with lower concentrations of Ba^{2+} and Ca^{2+} have been reported (Galizzi et al., 1987; Van Renterghem et al., 1987; Fish et al., 1988; McCarthy and Cohen, 1989, Marks et al., 1990)(see also chapter II). High external Ba^{2+}

concentrations shifted the current-voltage curves in the positive direction, probably due to a surface charge effect.

Examples of currents evoked by steps to +20 mV in the absence and presence of (+)202-791 are shown in Fig. V14A, with current voltage relationships for the same cell in Fig. V14B. In order to estimate the steepness of the voltage dependence of opening and the voltage of half maximal activation, we fitted the currents empirically to a Boltzmann function:

$$I(V) = N i [1 + \exp\{(V_{0.5} - V)/K\}]^{-1} \quad (3)$$

where N is the number of channels open at extreme positive potentials, i is the single channel current at the test potential V , $V_{0.5}$ is the voltage of half maximal activation, and K is inversely related to the steepness of the voltage dependence. At potentials $> +30$ mV we noticed variability both among cells and in the same cell over the course of the experiment, possibly due to small errors in leakage subtraction. We therefore confined the analysis to potentials between -40 and $+30$ mV, a range where the single channel conductance is linearly dependent on voltage (Fig. V.3). Addition of (+) 202-791 shifted $V_{0.5}$ to more negative voltages, decreased K , and increased N (Table V.1).

Naively, the increase in N would indicate that DHP agonists increase the number of available calcium channels. Instead, we interpret the effect as primarily an increase in the saturating level of

$p(\text{open})$ reached at extreme positive potentials. The MWC model, with our parameters, predicts that $p(\text{open})$ at positive voltages will approach the voltage-independent $p(\text{open})$ during a burst, which is 0.67 in control and 1 with DHP agonists. It is possible that DHP agonists also reduce the amount of inactivation, which would also appear as an increase in N .

In cases where the voltage clamp was of good enough quality to accurately measure tail currents, the shape of the activation curve determined from tail currents agreed well with the relative conductance measurements described above (Fig. V14C). Relative conductance values for five cells in the absence and presence of (+) 202-791 are plotted in Fig. V15 along with a fit using the MWC model. To compare results in control and with DHP agonists, the maximal tail current amplitudes were normalized to 1 in the presence of (+) 202-791 and to 0.667 for controls.

The steady-state activation curve provides information on the voltage-dependent steps in the MWC model. The value $V_{0.5}$ is strongly affected by the ratio of the rate constants describing the movement of a single voltage sensor within a closed channel, and the steepness is a function of the charge moved in association with each voltage sensor (which depend on A/B and V_s respectively, Eq. 1). The activation curve was well fit with $A/B = 0.62$ and $V_s = 26$ mV, or -1 elementary charge per voltage sensor (Fig. V15). The changes in $V_{0.5}$ and K with (+) 202-791 were modeled as a 12.25-fold change in the equilibrium constant for each of the voltage independent C - O steps

(i.e. $\Delta_{\text{DHP}} = 3.5$). Note that the changes in macroscopic voltage dependence with DHP agonists, including a change in the steepness of the activation curve, can be explained fully with no effect of DHP agonists on the voltage sensors themselves.

Absolute values for A and B can be obtained from kinetic data. In control conditions, current activation kinetics can be well fit using a H-H model with $n = 4$ (Fig. V.16). This is to be expected from the MWC model as long as most of the openings are to O_4 (i.e. strong cooperativity), and the $C_4 - O_4$ transition rates are much faster than the voltage dependent transition rates between closed states. We determined the coefficients for closed channel voltage sensor movement by fitting the model by eye to control whole cell activation kinetics, constraining the ratio A/B to be 0.62: $A = 0.26 \text{ ms}^{-1}$ and $B = 0.38 \text{ ms}^{-1}$ (Fig. V.17). The change in the voltage-insensitive equilibrium constants caused by (+) 202-791 was assumed to involve an increase of the opening rates and decrease of the closing rates, each by a factor (Δ_{DHP}) of 3.5 (i.e., $k_L = 42$ and $k_{-L} = 1.71 \text{ ms}^{-1}$).

At negative voltages, tail currents in control conditions were generally too fast to be accurately clamped. However, DHP-modified tail currents can be measured in well clamped cells. Tail currents in the presence of $1 \mu\text{M}$ (+) 202-791 show a clear dependence on voltage, and are usually well fit with a single exponential decay. The MWC model, with parameters described above, accurately simulates tail currents with DHP agonists (Fig. V.18). Simulations of control

conditions produce tail currents with fast time constants (e. g., $\tau \sim 200 \mu\text{s}$ at -40 mV).

The data from whole cell experiments define the voltage-dependent rate constants for closed-closed transitions. As the value of f was fixed above, defining the microscopic rate constants for the movement of a voltage sensor in a closed channel also serves to determine the rate constants for the sensor moving in an open channel. All independent variables needed to fully define Scheme 2 in the absence and presence of DHP agonists are presented in Table V.2.

Distribution of latencies to first opening

The first latency distribution provides an independent picture of the voltage dependence of channel gating. Since latencies reflect the rate of movement of the channel through closed states, they are expected to be strongly voltage dependent. As event detection is more reliable when the channels are DHP-modified, we were able to measure first latencies over a wide voltage range in the presence of (+) 202-791. The cumulative latency plots rise in a voltage-dependent manner (Fig. V.19A) following a brief but clear delay (Fig. V.19B). Simulations with the MWC model using the rate constants derived above show similar kinetics, including the delay (Fig. V.19C,D).

In the absence of DHP agonists, the first latencies clearly appeared to be longer. It is, however, difficult to obtain control data over a wide potential range. At positive test potentials ($\geq +10 \text{ mV}$)

threshold crossing schemes were not reliable, due to the low signal to noise ratio. At negative potentials (≤ -10 mV) the latencies were so long that it was not possible to accurately estimate the saturating probability of channel opening. It was possible to measure first latencies in the presence and absence of DHPs at 0 mV in two patches (Fig. V.20A,B). In each case the first latencies were considerably shortened in the presence of (+)202-79L.

The MWC model predicts such a DHP induced reduction in the first latencies, as the channel would be more likely to open with fewer than all four voltage sensors in the + position in the presence of DHP agonists (Fig. V.20C). Scheme 1 predicts a much smaller effect, as nearly all of the latency is in the voltage-dependent closed-closed steps. In fact, the slight shortening of the first latencies with Scheme 1 (Fig. V.20D) is primarily artifactual, as very brief openings which would go unresolved in control conditions will be lengthened and thus detected in the presence of DHPs. According to our simulations, the maximum change predicted by Scheme 1 is too small to explain the observed effect of DHP agonists on the first latency distributions.

Figure V.1. Two structurally based schemes for calcium channel gating. **A:** Scheme 1, a linear six state model. *Vertical* transitions reflect movement of the voltage sensor within one of the four repeated domains of the channel. *Horizontal* transitions reflect channel opening, which is assumed to be a voltage-independent change in the quaternary structure of the protein. Since the voltage sensors are assumed to be identical, the rate constants within each vertical column are identical except for statistical factors. **B:** Scheme 2, an adaptation of the MWC model for allosteric proteins to calcium channels. Voltage-independent transitions between closed and open states are theoretically possible with any number of voltage sensors moved, but are much more likely with an increasing number of sensors in the + position. The factor f relates rate constants for the movement of a voltage sensor in the closed conformation to those in the open state, as well as the voltage-independent rate constants with different numbers of voltage sensors moved. The voltage sensors move from the - to + position more readily when the channel is in the open state.

Figure V.2. Gating of single calcium channels. Gating of single calcium channels in A7r5 cells. Depolarizing steps (135 ms) were given at 2 second intervals. (+) 202-791 was added to the bath. These traces were digitally filtered at 1500 Hz on final analysis. This patch contained only one channel.

Figure V.2.

Figure V.3. Conduction through single calcium channels. **A:** Measured single-channel current amplitudes as a function of event duration. Traces at 0 mV were filtered at 1000 Hz and events were detected using a threshold crossing routine. **B:** Single-channel currents between -30 and +40 mV in the presence of (+) 202-79L. Peaks of the point-by-point amplitude histograms were fit by eye in order to determine the open channel current.

Figure V.3.

Figure V.4. Construction of point-by-point amplitude histograms. **A:** Currents from a patch containing a single channel in the absence (*upper*) and presence (*lower*) of 1 μ M (+) 202-791. Traces were digitally filtered at 300 Hz. **B:** All digitized points from sweeps with openings at +20 mV were binned according to amplitude. The *solid* line represents control conditions, and the *dashed* line is from traces after (+) 202-791 was added to the bath. Baseline noise distributes around the 0 current level. **C:** Histograms from the same data, after subtraction of baseline noise (see Methods).

Figure V.4.

Figure V.5. Comparison of beta distributions and single channel gating simulations to experimental amplitude histograms. **A:** An experimental amplitude histogram at +20 mV (*squares*) is compared to beta distributions convolved with Gaussian noise, with k_L and k_{-L} equal to 10 and 6.67 (*dashed line*), 12 and 6 (*solid line*) and 15 and 5 ms^{-1} (*interrupted line*). **B:** The same beta distributions are compared to amplitude histograms from simulated data (*symbols*) using the same rate constants. **C:** The experimental histogram shown in A is compared to beta distributions with k_L and k_{-L} equal to 36 and 18 (*interrupted line*), 12 and 6 (*solid line*), and 4 and 2 ms^{-1} (*dashed line*). **(D)** The beta distributions from C are shown together with amplitude histograms from simulated data using the same rate constants.

Figure V.5.

Figure V.6. Filtering at different cutoff frequencies results in amplitude distributions which are predicted by beta distributions programmed with rate constants $k_L = 12 \text{ ms}^{-1}$ and $k_{-L} = 6 \text{ ms}^{-1}$. Openings at +20 mV were filtered at 100 (A), 300 (B), and 1000 Hz (C) and fit with beta distributions convolved with baseline noise measured at each frequency.

Figure V.6.

A.

B.

C.

Figure V.7. Analysis of amplitude histograms over a 40 mV voltage range, with fixed rate constants. Experimental amplitude histograms (*squares*), beta distributions (*solid lines*), and amplitude histograms from simulated data (*dashed lines*) are compared at 0 (A), +10 (B), +20 (C) and +30 mV(D). All beta distributions and simulations were programmed with rate constants $k_L = 12 \text{ ms}^{-1}$ and $k_{-L} = 6 \text{ ms}^{-1}$

Figure V.7.

Figure V.8. Kinetics of DHP-modified calcium channel gating. **A:** Distribution of open channel dwell times at -10 mV in the presence of 1 μ M BAY K 8644. Times are binned every 0.5 ms. 3343 events were measured. **B:** All events ≤ 20 ms (3177 events) were examined at a higher time resolution. The dwell time distribution was poorly fit with a single exponential function ($\tau = 15$ ms). **C:** The same distribution was well fit with two exponentials ($\tau_f = 0.24$ and $\tau_s = 3.85$ ms).

$r_1 = 1.50$ $r_1 = 0.236$ $r_2 = 3.65$

Figure V.9. Voltage-dependence of gating kinetics of DHP modified channels. Distributions of open channel dwell times in the presence of 1 μM (+) 202-791. A biexponential distribution can be seen at all potentials. The slower time constants were 92 ms (-10 mV), 86 ms (0 mV) and 6.0 ms (+10 mV).

Figure V.10. Single-channel tail currents on repolarization. Records were selected for those showing long openings at -80 mV. When open single channels are repolarized they show fast closing events, similar to those observed at more depolarized potentials.

Figure V.10.

Figure V.11. Short and long openings are temporally related. **A:** Five consecutive sweeps with openings at -10 mV are shown. Regions under the *solid lines* are shown on an expanded time scale (*right*). **B:** The MWC model predicts that fast openings will be frequently found in association with bursts. Although we assume that the states O_3 and O_4 have the same conductance, they are shown here at different amplitudes to allow for visual discrimination.

Figure V.11.

A.

B.

Figure V.12. Concentration dependence of DHP-modified gating. All measured open times are plotted on a sweep-by-sweep basis, in control and in different (+) 202-791 concentrations. Each column of *squares* represents all measured openings in each 135 ms sweep. Null sweeps are denoted by a *diamond*.

Figure V.12.

Figure V.13. P(open) for sweeps with at least one long opening is DHP agonist concentration-independent. **A:** The probability of being open was measured during each sweep with at least one opening longer than 3 ms. *Squares* represent the p(open) in individual sweeps and *diamonds* represent the mean p(open). **B:** The proportion of active sweeps with at least one long (> 3 ms) opening at different (+) 202-791 concentrations. The *solid line* is drawn according to the law of mass action for a single DHP binding site with $K_D = 150$ nM.

Figure V.13.

Figure V.14. Whole-cell currents in the absence and presence of (+) 202-791. **A:** Inward currents before (*upper trace*) and after (*lower trace*) addition of 1 μ M (+) 202-791 to the bath. Currents to the left of the vertical dashed line were digitized at 20 kHz and filtered at 1 kHz, and those to the right were digitized at 100 kHz and filtered at 5 kHz. **B:** Current-voltage relationship. *Squares* are in control conditions and *triangles* are after addition of 1 μ M (+) 202-791 to the bath. Steady-state currents were measured at the end of 50 ms pulses. The *solid lines* are fits between -40 and +30 mV using a Boltzmann function (Eq. 3), and assuming a linear single-channel current-voltage relationship (Fig. 2C). **C:** Activation curves measured from the current-voltage relationship and from tail currents. The *solid lines* are drawn according to the same Boltzmann functions as in B, which were derived from the steady state inward currents. Tail currents measured 400-550 μ s after repolarization to 0 mV were scaled to a maximum of 1 in the presence of (+) 202-791 and 0.4 for controls.

Figure V.14.

Figure V.15. Steady-state activation curves fit with the MWC model. Whole-cell currents from five cells in the absence (*triangles*) and presence (*squares*) of 1 μ M (+) 202-791. Relative conductance was derived from by normalizing the Boltzmann fits to currents at the end of 50 ms test pulses as in Figure V.14 B and C. The relative conductance was normalized to 1 for (+) 202-791 and 0.67 for control data. The *solid lines* are fits using the MWC model with $A/B = 0.62$, $\Delta_{\text{DHP}} = 3.5$, and $V_s = 26$ mV.

Figure V.15.

Figure V.16. Activation kinetics can be well fit assuming four independent identical gates. **A:** Steps from -40 to +30 mV are fit with H-H kinetics with $m = 2$ (upper), 3 (middle) and 4 (lower). The *arrow* denotes the beginning of the voltage step. **B:** Currents at different test potentials in the same cell. The smooth curves are best fits with $m = 4$. The signal was digitized at 65 kHz.

Figure V.16

Figure V.17. The time course of whole-cell currents fit with the MWC model. The noisy traces are currents resulting from 10 ms steps from a holding potential of -40 mV to the indicated test potentials in control (*left*) and with 1 μ M (+) 202-791 (*right*). Currents were digitally filtered at 5 kHz. 500 μ s after each voltage step was blanked. Currents calculated from the MWC model ($A = 0.38$, $B = 0.26$) are superimposed.

Figure V.17

Figure V.18. Tail currents with 1 μ M (+) 202-791. The noisy traces are tail currents on repolarization to indicated potentials after steps to +50 mV, with tail currents simulated using the MWC model superimposed. Filtering was at 5 kHz.

Figure V.18

Figure V.19. Latencies to first opening are strongly voltage-dependent. **A:** Cumulative plots of first latencies with 1 μ M (+) 202-791. The holding potential was -80 mV and test potentials were -20, -10, 0, +10 and +20 mV. Events were detected by threshold crossing, with digital filtering at 1000 Hz (-20 to 0 mV) and 300 Hz (+10 and +20 mV). The number of events at each voltage were 31 (-20), 49 (-10), 87 (0), 43 (+10) and 34 (+20). At voltages where saturation was apparent (≥ 0 mV) the data were normalized to a p(open) of 1.0, on the assumption that null sweeps result primarily from sweeps spent in an inactivated state. In this patch, measured probabilities at saturation were 0.64 (0 mV), 0.56 (+10 mV), and 0.453 (+20 mV). At potentials where saturation was not evident, the data were normalized to the average p(open) from the more depolarized traces, 0.55. Data at early times are shown on an expanded scale (**B**). **C-D:** Simulated first latency distributions. 500 sweeps were simulated for each potential. The voltages used for the simulations were shifted 5 mV in the depolarized direction in order to agree better with this patch. Simulated data were analyzed in the same manner as the experimental data, including filtering and addition of simulated noise.

Figure V.19.

Figure V.20. DHP agonists shorten latencies to the first opening. **A-B:** Latencies to first opening at 0 mV from two separate experiments before (*solid line*) and after (*dashed line*) addition of DHP agonist to the bath. **C:** Latencies predicted from the MWC model with and without DHP agonists. **D:** Latencies predicted from Scheme 1 with the same values for parameters A , B , V_S , k_L and k_{-L} .

Figure V.20.

Table V.1. Effects of (+) 202-791 on steady-state whole-cell calcium currents.

	change in N	$V_{0.5}$ (mV)	K
Control	-	17.5 ± 2.2	7.5 ± 0.2
1 μ M (+)202-791	2.2	3.1 ± 2.2	5.7 ± 0.3

1 μ M (+) 202-791 increases the relative conductance (N), shifts the voltage of half-maximum activation ($V_{0.5}$) to more negative potentials, and increases the slope (1/K) of the activation curve. Values are the mean $\pm$ SEM (n = 5).

Table V.2. A summary of independent variables needed to describe scheme 2.

A	0.26 ms ⁻¹
B	0.38 ms ⁻¹
V _s	26 mV
k _L	12 ms ⁻¹
k _{-L}	6 ms ⁻¹
f	1/15
Δ _{DHP}	3.5

The values A, B, and V_s define k_c and k_{-c} as described in equation 2, and are derived primarily from whole-cell kinetics and steady-state activation. k_L and k_{-L} describe the gating within a burst and were obtained by the amplitude histogram analysis. Assignment of a value for the scaling factor f was discussed in the text. Δ_{DHP} defines the change in the voltage-independent rate constants on addition of DHP agonists, and is obtained from steady-state activation curves with and without DHP agonist.

DISCUSSION

Model-independent results

Since we have chosen to present our results in the context of a novel model, we should indicate the features of the experimental data that do not depend on the model. Many but not all of these features are familiar from previous studies of calcium channel kinetics in other cell types.

Calcium channel gating is extremely rapid (Fig. V.2), so it is impossible to reliably obtain rate constants for channel opening and closing directly from measurement of channel open and closed times. Analysis of point-by-point amplitude histograms is also complicated by baseline noise and finite burst length, but it seems clear that the fraction of time the channel spends open during a burst (~ 0.67) is not strongly dependent on voltage in the measurable region. The absolute opening and closing rates are less well determined, but as it seems unlikely that both would change in the same direction with voltage, our data imply a voltage-independent transition between the open state and a closed state. That closed state need not be on the normal pathway for channel opening, but that is the simplest assumption, and the marked decrease in channel closing rate with DHP agonists makes explanations such as channel block unlikely. Voltage-independent intra-burst kinetics were also noted by Hagiwara and Ohmori (1983) in pituitary cells.

The data under control conditions are consistent with a single open state, but the bursting kinetics clearly indicate at least two closed states, as previously reported (Fenwick et al, 1982; Cavalie et al, 1983; Brown et al, 1984). However, our values for k_L and k_{-L} are considerably faster than previous estimates of the rate constants to and from the open state. This is likely to be due the effect of missed events in direct measurements of open and closed times, which are severe when both open and closed times are fast. Actually, k_L and k_{-L} could be considerably faster than we estimate, but values more than 2 times slower would not fit our data well (Fig. V5,C-D).

Macroscopic currents show a clear delay (Figs. V16 and V17), as do distributions of latencies to first opening (Fig. V19), which also indicate the existence of multiple closed states. It is noteworthy that macroscopic activation kinetics were slightly slower with DHP agonists than in control, although the first latencies were considerably faster with DHPs.

Data with DHP agonists require the existence of at least two open states (Figs. V8 and V9). As fast and slow openings can occur close together (Fig. V11), and DHP agonists appear to bind and unbind relatively slowly (Figs. V12 and V13), we conclude that two kinetically distinct open states are available to the DHP-modified channel.

Schemes 1 and 2

A model based on Scheme 1 (Fig. V1) can explain all of our data in control conditions, and most of the data in DHP modified conditions if we assume that DHP agonists simply act to stabilize the open state. According to this scheme DHP agonists would lengthen channel open times, slow deactivation kinetics, and shift the steady-state activation curve in the negative direction while increasing its slope, as we have observed. However, the reduced latencies to first opening and multiple open states observed in the presence of DHP agonists can be explained by Scheme 2 but not Scheme 1.

Our preferred (MWC) model may seem complex, with 10 states and 26 rate constants (Fig. V1B). However, the rate constants are determined by relatively few free parameters, given the cyclic nature of the model and our assumptions of symmetry: the kinetics are fully defined at a particular voltage by 5 parameters (k_C , k_{-C} , k_O , k_{-O} , and f), 1 additional parameter (V_s) defines the voltage dependence, and 1 more (Δ_{DHP}) defines the effect of DHP agonist binding. This is only one more parameter than for Scheme 1. More significantly, a state diagram such as Fig. V1B does not do justice to the simplicity of the underlying physical picture, which involves only two states for a voltage sensor (+ and -) and two global conformations of the channel protein (O and C).

It might be argued that the MWC model is *too* simple to be realistic for calcium channels. The four internal repeat domains are homologous, but not identical. In particular, the S4 regions contain

varying amounts of positive charge (Mikami et al., 1989). However, none of our data requires asymmetrical behavior of the subunits.

Alternative models

Activation kinetics. The simplest model worthy of serious consideration for calcium channel activation is a linear C_1 - C_2 -O model. However, if the C_2 -O transition rates are fast and voltage-independent, as our data suggest, then the C_1 - C_2 transition would be the only voltage dependent step. Activation would thus be essentially described by a single exponential, with no appreciable lag, contrary to data presented here and elsewhere. A four state C_1 - C_2 - C_3 -O model (Hagiwara and Ohmori, 1983; Brown et al., 1983; Taylor, 1988) can better describe our data, but again, if the last transition is voltage-independent and fast with respect to the others, the two rate-limiting voltage-dependent steps would primarily determine the activation kinetics. If we assume independent identical gates, then good fits to current activation require at least three gates. If we relax the requirement that the gates be identical then this model could explain many of our results. However, such a model has more free parameters (6 at any one voltage; and 4 in order to determine the voltage dependence of the C-C transitions, assuming that C_3 -O is voltage independent) than other models considered and bears no relation to any known structural features of the channel.

Dihydropyridines. Several qualitatively distinct models for the actions of DHP agonists have been proposed. Hess et al. (1984)

proposed that DHP agonists act to enhance "Mode 2" gating of calcium channels, where the channel has an unusually high probability of being open. The channel can be in Mode 2 under normal conditions, but DHP agonists greatly increase the proportion of time in Mode 2. A mode is thought of as a collection of closed and open states, with transitions among states within a mode much faster than transitions between modes.

Our data favoring slow kinetics for DHP action is consistent with a mode-based model. One interpretation is that our model for normal channel gating is Mode 1 of Hess et al. (1984), and gating with k_L and k_{-L} modified by DHP agonists is Mode 2. On theoretical grounds, it can be argued that any state of the protein accessible with DHPs bound can also exist without DHPs, but at a higher energy level, which could explain the occasional observation of Mode 2 gating under control conditions or after β -adrenergic stimulation (Yue et al., 1990). We also see rare sweeps with high open probability and long openings without DHP agonists.

Other proposals use more conventional state models for DHP action. Sanguinetti et al., (1986) proposed that DHP agonists simply slow the rate constant for channel closing. This is similar in spirit to our proposal that only the immediate closed-open transition is affected by DHP agonists. This model cannot explain the DHP agonist induced decrease in latency to first opening.

The model of Lacerda and Brown (1989) requires very rapid binding of DHP agonists to calcium channels. Their scheme also

predicts a concentration dependence of open times, and several distinct single channel conductance levels. We have not observed any concentration dependence of open times. Although we have observed long-lasting openings to intermediate conductance levels in the presence of DHP agonists, this was not a common occurrence, and we have no evidence for the presence of subconductance states in control conditions. However, other features of the data of Lacerda and Brown, including shortened first latencies in the presence of DHP agonists can be naturally explained by Scheme 2.

Hering et al. (1989) have investigated directly the time course of DHP agonist action by a rapid flow technique. They observed a slow action of 1 μ M (+) 202-791 (τ - 1 s) at -20 mV, with a clear delay, which they attributed to binding of DHP agonists to closed as well as open states of the channel. At 0 mV, the action was more rapid (τ - 400 ms) and no delay was observed, suggesting rapid binding to the open state. These slow effects support our conclusion that the binding of DHP agonists is slow with respect to one of our 135 ms depolarizations. It could be argued that if DHP agonists act by first partitioning into the membrane and then binding to the channel, application of (+) 202-791 via the aqueous medium might produce a slow effect. However, if partitioning into the membrane is the rate-limiting step, the timecourse should not be voltage-dependent.

The model of Hering et al. (1989) assumed that DHP agonists bind equally rapidly to all states of the channel, but DHP-modified channels gate abnormally. We prefer the MWC model, which does

imply state dependent binding, as it allows microscopic reversibility. Their model also required that DHP agonists bind in a voltage-dependent manner, which seems unlikely for nonpolar drugs.

Possible implications for other channels

Calcium channels are members of a homologous family of voltage-dependent ion channel proteins. The S4 helix is strongly conserved among these proteins, suggesting that voltage-dependent ion channels share a common gating mechanism. Thus, our model for cooperative gating of the calcium channel may be equally applicable to other voltage-dependent ion channels. It may also provide a general mechanism for the regulation of ion channels by drugs and neurotransmitters.

Chapter VI.**CONCLUSIONS***The A7r5 cell line as a model system.*

The A7r5 cell line offers many advantages for the *in vitro* study of vascular smooth muscle calcium currents. The cell culture system allows one to combine modalities such as ligand binding, electrophysiology and fluorescence in the same preparation, and thus be able to examine an issue from several perspectives at the same time. In the future molecular biological and genetic approaches could also be introduced. Because the preparation is relatively homogeneous, the confounding influence of other vascular components such as neurons, fibroblasts, and endothelial cells can either be excluded, or studied in a systematic manner by co-culturing with different cell types. The rounded morphology after cytochalasin treatment and the relatively small size of the cells make them ideally suited for whole cell voltage clamp experiments.

Phenotypic instability presently represents a drawback of the use of the A7r5 cells as a model system. This instability can be noted in the present work, as well as when reviewing work done by others. In particular, the magnitudes of the current, the relative expression of T-type current, and the responsiveness to neurotransmitters was variable in our hands.

Some clues as to factors which influence the magnitude of the currents might be gained from experiments measuring $[Ca^{2+}]_i$ (Fig IV.1). Withdrawal from the cell cycle may be accompanied by a greater functional expression of calcium channels. It is also possible that other factors such as varying K^+ current density and/or changes in membrane potential may cause increased response of cells after time in a nonproliferative state. I also noted large variability in current density between preparations of cells that were quiescent for several weeks, arguing that withdrawal from the cell cycle alone is insufficient for optimal expression of functional currents. My impression was that the electrophysiological properties of the cells changed relatively slowly over time in culture, although no systematic study of this phenomena was undertaken.

Phenotypic instability was also observed in relation to neurotransmitter regulation of the current. When present, the β -adrenergic potentiation of the current was obvious, however, many cells appeared completely unresponsive. Lack of a response could be due to alterations in the β -adrenergic signalling cascade including the lack of a surface receptor, G protein, adenylate cyclase, or protein kinase. Alternatively, the basal cAMP levels in nonresponsive cells could be higher than needed for maximum stimulation. It is also possible that changes in the calcium channel protein underlie the variable responsiveness.

I have tried to manipulate cell phenotype primarily by maintaining cells in low serum medium (0.5 % CS) for several weeks,

but, as discussed, this does not generally result in a stable phenotype. Other approaches might also prove useful. In BC3H cells β -transforming growth factor (β -TGF) was found to play a central role in the functional expression of Ca^{2+} channels (Caffrey et al., 1989). It might be useful to test the effect of this, as well as other growth factors, on ion channel expression in A7r5 cells. Other methods for manipulating cell phenotype include growing cells on different substrates, use of a more well defined culture media, and co-culturing with different cell types (i.e. endothelial cells).

Unfortunately, cell-to-cell variability appears to be the rule rather than the exception in A7r5 cells; and single cell electrophysiology is a rather laborious assay. It would therefore be helpful to develop other more convenient assays for the expression of marker proteins under different culture conditions. Such a systematic investigation into factors which regulate phenotypic changes in A7r5 cells, while beyond the scope of the present work, might shed light on important regulatory mechanisms which can influence cell excitability *in vivo*.

The 'L'-type calcium channels found in A7r5 cells appear to be similar to those found in other cell types, including cardiac, pancreatic, neural and smooth muscle cells in terms of their kinetics and permeability. The DHP sensitive calcium currents of skeletal muscle can be easily distinguished from these channels by their slow kinetics, and appear to be substantially different than the channels observed here. It has been suggested that calcium currents in vascular

smooth muscle cells are more sensitive to DHP agonists than in cardiac myocytes (Bean et al, 1986). McCarthy and Cohen (1989) came to a similar conclusion regarding the action of DHP antagonists in A7r5 cells. I also observed a potent effect of nifedipine on calcium currents even at very depolarized holding potentials (Figure II.8). Such high affinity block may be due to actual differences between the calcium channels and/or associated proteins. Alternatively, the lipophilic DHP's may partition differently into the membranes of different cell types. I did not study the concentration dependence of DHP antagonists in the A7r5 cell, so I cannot address this point directly. However, as noted in Chapter III, the binding affinity for (+)-³H-PN-200-110 was identical to that in a wide variety of other cell types.

In my hands, calcium channels in A7r5 cells can be regulated in a manner similar to channels in cardiac cells. This argues for similarity between the channel in the two cell types. As the regulation of calcium currents by neurotransmitters involving second messenger systems involves many components in addition to the channels, *differences* in ion channel regulation between cell types are difficult to relate to differences of the channels involved. For example, I saw no effects of protein kinase-C activators on calcium currents in A7r5 cell, contrary to reports of potentiation (Sperti et al. 1987; Fish et al. 1988) and attenuation (Galizzi et al. 1987) of currents by these agents in the same cell line. The reasons for the discrepancy

are unclear, and I am unable to conclude that differences in the channel protein are responsible.

Future directions in the study of gating kinetics.

As the kinetics of calcium channels in A7r5 cells are kinetically indistinguishable from those of 'L' type calcium channels in many other cell types, I feel that the allosteric model for channel activation and modulation by DHP agonists presented here may be generalized to calcium channels found in a variety of cells. Also, given the structural similarity between calcium channels and other voltage-dependent ion channels, the model may be generalizable to other voltage-dependent ion channels. Certain predictions of the model are amenable to testing, although this was deemed beyond the scope of the present work.

When the voltage sensing component of an ion channel moves in the membrane field in response to changes in membrane potential, the movement of the charge of that sensor can be measured. This gating charge movement can offer much information about the events leading to channel opening (Armstrong, 1981). According to the MWC model, the gating current curve should begin to rise at more negative voltages than current activation (Conti and Stuhmer, 1989), as transition through closed states involves charge redistribution. DHP agonists should shift the curve to more negative potentials and steepen it slightly, with no change in the overall amount of charge moved. We have not modeled the kinetics of the

gating charge movement, but expect that they may also be helpful in evaluating the MWC model. Unfortunately, no preparation has been identified as appropriate for calcium channel gating charge measurement. Bean and Rios (1989) have measured gating charge movement in cardiac myocytes, but they found it difficult to isolate the calcium channel charge movement. A7r5 cells probably have too few channels to obtain reliable measurements. It would be useful to over-express the cloned cardiac calcium channel in a cell type with little contaminating current in order to conduct such an experiment.

A question that remains unanswered relates to how many DHP agonist binding sites are to be found on one calcium channel. If each homologous domain contains a binding site, then we expect cooperative binding of the DHP. Binding experiments can not presently address this point, as only the DHP antagonists are available in a radiolabeled form. Even when available, this technique will most likely fall short. DHP agonist binding is probably state-dependent, and control over membrane potential is poor in binding studies, it will be difficult to accurately define conditions for equilibrium binding. Also, the high degree of nonspecific binding will make for an unfavorable signal to noise ratio. Dose-response curves for DHP agonists are difficult to interpret, as drug mediated potentiation is usually obscured by current rundown. One possibility is to assess the dose-response relationship using a fast solution exchange (Hering et al., 1989) in order to avoid the problem of rundown. Dose-response relationships for single channels (i.e. Fig.

V13) are unlikely to be accurate enough to determine Hill coefficients.

Several physio-chemical approaches including changing temperature, pressure, and the replacement of H_2O with D_2O , can be used in an attempt to dissect the movement of charge from channel opening and closing; and may thus be useful in evaluating the MWC model. Ideally, channel activation and gating charge movement should be measured under the same conditions. Currents could be measured at the level of the whole cells and single channels. Interpreting these experiments suggested below in terms of the MWC model for state changes can be difficult. This is largely due to the fact that even though voltage sensing and channel opening occur in distinct regions of the protein, according to the MWC model, the events are coupled. The coupled nature of the system makes it difficult to manipulate each mechanism independently. All results should be analyzed and compared to simulated results using an appropriate model.

As the MWC model predicts that different parts of the molecule are involved the processes of voltage sensing and channel opening, we might expect that the temperature dependence of each mechanism might be different. An interesting observation is that the DHP sensitive cardiac calcium channel seems to show slower kinetics for openings and closings within bursts at higher temperatures (Trautwein and Pelzer, 1988). This may be due to an important entropic component of the system, whereby association of

hydrophobic portions of the channel with the lipid act to stabilize certain conformations. If the movement of the charge sensors is isolated from interactions with the lipids, we might expect that the temperature dependence of these rates (and thus channel activation) may be temperature dependent in both a quantitatively and qualitatively different manner. Temperature may even have opposite effects on the kinetics within a burst than on charge movement and the movement into and out of bursts.

Increase in pressure may also aid in the dissection of charge movement from channel opening. According to our interpretation of the MWC model the movement of the charge sensors should involve relatively small conformational changes, whereas the change associated with the open-closed transition may involve large quaternary changes, possibly involving realignment between subunits. This type of quaternary change is likely to have a large activation volume, and thus the kinetics of this reaction should be strongly pressure dependent. Also differences in the volumes of the open and closed states may make the open-closed equilibrium pressure sensitive. On the other hand, the change in activation volume for the movement of a charge sensor should not be significant, and thus the isolated movement of a sensor is not expected to be very pressure sensitive. Experiments with increased pressure have demonstrated that the opening of sodium channels is accompanied by a step with a large activation volume but no charge movement (Conti et al, 1984),

however, no evidence for a similar step in the activation of calcium channels has been observed (Heinemann et al., 1987).

Replacement of H₂O with D₂O can also help to distinguish mechanisms in the state transitions leading to channel opening. In the case of sodium channels, this approach has been used to demonstrate that charge movement precedes and is independent from channel opening (Schauf and Bullock, 1979; Schauf and Bullock, 1982; Alicata et al., 1990). It would be of interest to determine what the effects of D₂O on single channel and whole cell calcium currents as well as calcium channel gating charge movement.

Several surface-active substances such as *n*-alkanols affect sodium currents in a manner similar to the way that DHP agonists modulate calcium currents, but in the opposite direction (Haydon and Urban, 1982). In particular, these agents reduce the peak current at all voltages, and act to shift the steady state activation curve to more positive potentials and flatten it. As these agents are likely to interact with the membrane and act on the protein in domains where it interfaces with the lipid membrane, it seems possible that they act as allosteric effectors to stabilize the closed state of the channel. To my knowledge, no investigation of this phenomena has been undertaken at the level of the single sodium channel, or for any other channel type.

Site directed mutagenesis is another potentially useful technique for the study of ion channel activation mechanisms. Efforts have been made to mutate the S4 region of potassium and

sodium channels in order to determine if this region acts as a voltage sensor (Stuhmer et al., 1989). However, results are often difficult to interpret. In order to test the MWC model, I would also favor the mutagenesis of regions of the proteins distant from the putative voltage sensors. Catterall (1986) has identified regions of the sodium channel where homologous domains are thought to associate. Such regions might make excellent target regions for mutations which might alter the voltage-independent channel gating processes, as I view voltage-independent conformational changes as changes in alignment between subunits. A general problem with this approach is that mutagenesis is likely to disrupt protein structure and function in unexpected ways making interpretation difficult.

Inactivation has not been addressed by the MWC model in its present form. Inactivation is thought to involve regions of the protein distinct from those involved in activation (Stuhmer et al., 1989; Hescheler and Trautwein, 1988). In the case of sodium channels the microscopic inactivation rate appears to be voltage-independent (Cota and Armstrong, 1989). Zagotta and Aldrich (1990) also demonstrated this for a potassium channel with the additional finding that inactivated states can be directly linked to closed states. We could add a third column to Scheme 2 (Fig. V.1.) so that each vertical level would be either a closed loop or a linear sequence containing a closed, open and inactivated conformation. These modifications would make for much greater complexity in defining

the model. The addition of calcium dependent inactivation to the model might prove more challenging.

It would also be beneficial if the MWC model could be expanded in order to incorporate the actions of DHP antagonists. As it is thought that these agents bind preferentially to the closed and/or inactivated state of the channel, this would most likely necessitate the incorporation of voltage-dependent inactivation into the model as discussed above. These drugs are very similar in structure to the DHP agonists, and one would hope that their mechanisms of action are somehow related. The observed state dependent binding of the antagonists (Bean, 1984; McCarthy and Cohen, 1989) is fundamentally consistent with the MWC model if the antagonists, like the agonists, are allosteric effectors.

Summary

I have investigated the fundamental properties of the DHP sensitive calcium channels in the A7r5 cell line. These channels are very similar to those found in a variety of cell types, including the well studied channels of cardiac myocytes. I have proposed a novel allosteric model in order to explain calcium current activation. This model is formally identical to the Monod-Wyman-Changeux model and is based on structural considerations. The actions of DHP agonists can easily be explained if one makes the simple assumption that these drugs act as allosteric effectors. As voltage gated ion channels share structural features, this model may also be applicable

to other channel types. Several suggestions for the further testing of this model have been offered.

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