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# NIMODIPINE BLOCK OF CALCIUM CHANNELS IN RAT ANTERIOR PITUITARY CELLS

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SUMMARY

1. Ca channels were studied in the $GH_4C_1$ clonal cell line derived from rat anterior pituitary cells. The whole-cell variation of the patch-electrode voltage-clamp technique was used.

2. Two types of Ca channels were found. One type ('slowly inactivating' channels) is insensitive to changes in holding potential, does not inactivate during test pulses lasting several seconds, and deactivates very quickly upon repolarization. For holding potentials $< -40$ mV, a second type of Ca channel is available for opening. This population ('transient' channels) differs from the first type in that it activates at more negative potentials, inactivates rapidly with either Ca or Ba as the charge carrier, deactivates about 10 times more slowly upon repolarization, and is less selective for Ba over Cs.

3. Nimodipine preferentially blocks the slowly inactivating channels. Block of these channels is time- and voltage-dependent, such that block is maximized by long depolarizations.

4. A comparison of the voltage dependence of steady-state nimodipine block with the voltage dependence of channel activation indicates that channel block is directly proportional to the number of open channels. The results are accounted for by a model that postulates 1:1 high-affinity drug binding to open Ca channels. The apparent dissociation constant for binding to open channels is 517 pM. Similar binding constants were previously reported for the inhibition of high-K-induced hormone secretion and high-affinity ligand binding of [$^3$H]nimodipine to isolated plasma membranes.

5. The rate of onset of nimodipine block increases with the test potential, in quantitative agreement with the model of open-channel block. The apparent association rate is about $9{\cdot}6 \times 10^7$ M$^{-1}$ s$^{-1}$; the dissociation rate is about 0·050 s$^{-1}$. At therapeutic concentrations ($< 10$ nM) nimodipine block takes many seconds to reach equilibrium.

6. Nimodipine should have little effect on stimulus–secretion coupling in healthy pituitary cells *in vivo* because: (a) the drug binds very weakly to the transient channels that are open at normal resting potentials, and (b) negligible high-affinity binding occurs during spontaneous activity because the onset of block is very slow.

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## INTRODUCTION

Stimulus–secretion coupling in neurones and endocrine cells can be mediated through an increase in cell Ca (Rubin, 1982; Reichardt & Kelly, 1983). The Ca necessary for this process can be supplied by influx through voltage-gated membrane channels, but the relative importance of this pathway is incompletely understood. A promising approach to this problem is the use of Ca-channel blockers, which in ideal cases selectively eliminate Ca influx.

The most potent and specific Ca-channel blockers in muscle are the 1,4-dihydropyridine derivatives (typified by nifedipine), so these drugs have often been used to analyse stimulus–secretion coupling. Some dihydropyridines potently block secretion and the associated Ca influx induced by membrane depolarization in neurones or endocrine cells (Malaisse & Sener, 1981; Miller & Freedman, 1984; Tan & Tashjian, 1984*a*, *b*; Kojima, Kojima & Rasmussen, 1984; Enyeart, Aizawa & Hinkle, 1985). These studies suggest that Ca channels in neuronal and endocrine cells are very similar to those in smooth muscle and myocardial cells with respect to the binding of dihydropyridines. This idea is supported by ligand binding studies with tritiated dihydropyridines, since similar high-affinity binding sites for dihydropyridines have been found in all of these tissues (see Triggle & Janis (1984) for a review).

These recent studies contrast with earlier *in vitro* and *in vivo* experiments, which found little effect of dihydropyridines on stimulus–secretion coupling in endocrine cells or neurones (see Triggle & Swamy (1983) for a review). *In vivo* experiments, in particular, supported the idea that the clinically useful Ca antagonists modify excitation–contraction coupling with little effect on stimulus–secretion coupling (Millar & Struthers, 1984). Consequently, it is widely thought that subsets of Ca channels differ dramatically in their affinity for dihydropyridines.

Voltage-clamp measurements of Ca currents in secretory cells can provide the most direct measure of Ca-channel block by dihydropyridines, but no reports on the effect of therapeutic doses of dihydropyridines on Ca currents in secretory cells are available. To understand the effects of dihydropyridines on secretory cells better, we have studied Ca channels in $GH_4C_1$ rat anterior pituitary cells, using the whole-cell variation of the patch-electrode voltage-clamp technique. This technique has been used previously to characterize extensively Ca-channel gating in $GH_3$ and $GH_4C_1$ cells (Hagiwara & Ohmori, 1982, 1983; Dubinsky & Oxford, 1984; Matteson & Armstrong, 1984, 1986; Armstrong & Matteson, 1985; Barros, Katz, Kaczorowski, Vandlen & Reuben, 1985).

A GH cell line was chosen for this study because the role of Ca in stimulus–secretion coupling is very well characterized in these cells and because dihydropyridines are potent and selective blockers of this process (Tan & Tashjian, 1984*a*, *b*; Enyeart *et al.* 1985). Both high K and thyrotropin releasing hormone (TRH) stimulate Ca entry and the secretion of prolactin and growth hormone, but the mechanism of action of these two agents differs considerably. High K causes a maintained depolarization, while TRH triggers rapid trains of action potentials by altering a K conductance (Dufy, Vincent, Fleury, Du Pasquier, Gourdji & Tixier-Vidal, 1979; Taraskevich & Douglas, 1980; Barker, Dufy, Owen & Segal, 1984; Dubinsky & Oxford, 1985).

Although Ca entry apparently occurs through voltage-dependent channels in response to both agents, some dihydropyridines block the high-K response about 1000 times more potently than the TRH response (Enyeart *et al.* 1985). Hence, the dihydropyridines are very selective in their effect on Ca channels.

We have focused our study on the mechanism of Ca-channel block by nimodipine. Nimodipine preferentially enhances cerebral blood flow and may be efficacious in the treatment of subarachnoid haemorrhage, migraine, stroke and senile dementia (Scriabine, Battye, Hoffmeister, Kazda, Towart, Garthoff, Schlüter, Ramsch & Scherling, 1985). The therapeutic effects of nimodipine are usually attributed to relaxation of cerebral blood vessels via a direct effect on vascular smooth muscle, but there is some evidence indicating drug effects on neuronal and/or neuroendocrine cells. We have designed our experiments to address four basic questions: (1) do therapeutic doses of nimodipine cause significant Ca-channel block in endocrine cells? (2) is the block of Ca channels in endocrine cells similar to that for myocardial and vascular smooth muscle cells? (3) do studies of Ca fluxes in excitable cell lines accurately reflect *in vivo* drug effects on Ca channels? (4) why are some dihydropyridines more potent at blocking the effects of high K than at blocking the TRH response?

Most earlier reports on GH cells interpreted Ca-channel activity as due to a single population of channels. Armstrong & Matteson (1985) recently reported tail-current measurements that indicate two populations of Ca channels in $GH_3$ cells. The first part of the Results section will expand upon this report. A more detailed analysis of Ca-channel gating indicates that the Ca channels in $GH_4C_1$ cells are very similar to those in sensory and hippocampal neurones and neuroblastoma cells. The two populations of Ca channels can be distinguished not only by their time- and voltage-dependence of activation and inactivation, but also by their ionic selectivity and susceptibility to pharmacological block. The second part of the Results section will characterize the block of these channels by nimodipine. We find that nimodipine is a very potent blocker of one type of Ca channel, but only weakly blocks the other. Nimodipine block of the sensitive channels is time and voltage dependent, such that block is maximized by long depolarizations. The kinetics of block can be explained best by preferential binding of nimodipine to open Ca channels. Preliminary reports of this work have been published previously (Cohen & McCarthy, 1985, 1986).

## METHODS

### *Cell culture*

Subclones of the $GH_4C_1$ rat anterior pituitary cell line were obtained from Dr Priscilla Dannies, Department of Pharmacology, Yale University. Cells were cultured in a 1:1 mixture of Dulbecco–modified Eagle medium and Ham F-10 medium, supplemented with 15% horse serum. Cells were plated at a density of $1–2 \times 10^5$ cells per Petri dish (30 mm) and used 1–4 days after plating. Best results were obtained when the cells were used on the second day after plating.

### *Solutions and drugs*

The cells were transferred to protein-free physiological saline for electrophysiological experiments. Initially the cells were bathed with a modified Tyrode solution containing (mM): NaCl, 150; KCl, 4·0; $CaCl_2$, 9·0; $MgCl_2$, 0·5; dextrose, 5; HEPES, 10; pH 7·5. After formation of a high-resistance seal between the pipette and cell membrane, the bathing solution was changed to one optimized for recording Ca-channel currents. Currents through Na channels were eliminated by

replacing all Na with tetraethylammonium (TEA) and adding 200 nM-tetrodotoxin (TTX; Dubinsky & Oxford, 1984). 20 mM-Ca or Ba were added as current carriers and sucrose was substituted for part of the NaCl, so that the ionic strength and tonicity of the solution was the same as the modified Tyrode solution. The solution contained (mM): TEA Cl, 117; $CaCl_2$, 20 or $BaCl_2$, 20; $MgCl_2$, 0·5; dextrose, 5; sucrose, 32; HEPES, 10; TTX 0·2 $\mu$M; pH 7·5 (adjusted with CsOH). Bath solutions were gassed with 100% oxygen and maintained at room temperature (the temperature did not change by more than 0·5 °C in any one experiment, but there was a 4 °C range of temperature (19–23 °C) between experiments that accounts for some variability in rates of Ca-channel gating). Patch electrodes were filled with a solution designed to eliminate all K-channel currents and to slow run-down of the Ca currents. The composition was usually (mM): CsCl, 130; tetrabutyl ammonium chloride (TBA Cl), 10; EGTA, 11; $CaCl_2$, 0·9; $MgCl_2$, 2·0; ATP, 1·0; HEPES, 10; pH 7·2 (adjusted with CsOH). In later experiments the pipette solution was (mM): CsCl, 108; TBA Cl, 10; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA), 11; $CaCl_2$, 0·9; $MgCl_2$, 6; ATP, 5; HEPES, 20; pH 7·2. This solution is identified in the text as high ATP–BAPTA solution. Nimodipine (BAY E 9736) was synthesized by Bayer AG and was diluted from a 1 mM stock solution in polyethylene glycol 400 (Baker). Sodium vapour lights were used during experiments with nimodipine to prevent photodestruction of the drug. 1% polyethylene glycol had no significant effect on the amplitude or kinetics of the Ca-channel currents.

*Patch voltage-clamp experiments*

Patch electrodes were fabricated from Corning no. 7052 glass pipettes (Rae & Levis, 1984). The electrodes were fabricated according to the method of Hamill, Marty, Neher, Sakmann & Sigworth (1981) and usually had resistances of 2–4 MΩ. The patch pipettes were insulated with Sylgard 184 (Dow Corning). A Mark V patch voltage-clamp amplifier was used to record whole-cell currents (Pan Electronics, New Haven, CT). 50–80% of the series resistance was compensated for by positive feed-back of the current signal. The uncompensated portion of the series resistance was typically 1–2 MΩ and the Ca currents were < 500 pA, so the voltage error due to series resistance was < 1 mV.

Voltage pulse generation, data acquisition and analysis were performed with an LSI 11/23 computer (Digital Equipment Corp.) in conjunction with a programmable stimulator (Cheshire Data; presently sold by INDEC Systems, Sunnyvale, CA, U.S.A.) Whole-cell patch-electrode voltage-clamp measurements were performed as described by Hamill *et al.* (1981). Membrane current was sampled at 5 or 10 kHz and filtered with an 8-pole low-pass Bessel filter (Frequency Devices) with a cut-off frequency (−3 dB) of 2·5 kHz. Linear leak and capacity currents were subtracted digitally by appropriately scaling a test pulse from −90 to −110 mV.

In some experiments, such as that shown in Fig. 4, the Ca-channel tail currents were biexponential. The time constants of the two components of tail current differed approximately 10-fold, so the amplitude of each component could be determined accurately by curve peeling: the slow component of tail current was fitted by an exponential (as shown in Fig. 4) and was then subtracted digitally from the total tail current. All other curve fitting was performed by non-linear least-squares analysis, using the Patternsearch algorithm (Colquhoun, 1971).

Quantitative analysis of Ca-channel block was complicated by run-down of the Ca-channel currents in the absence of drug. This problem was particularly severe in experiments that required long-lasting voltage pulses to potentials near the peak of the *I–V* curve (Fenwick, Marty & Neher, 1982). The run-down effect placed several limitations on the experimental protocol, as follows. (1) Control measurements before the addition of drug were only taken after an initial rapid phase of run-down was complete. This period typically lasted about 5 min, but it could also be quickly completed by applying a test pulse to the peak of the *I–V* relationship for > 5 s. After this initial phase, run-down was quite slow and current measurements could typically be made for 30–45 min. This procedure dramatically reduced the magnitude of the Ca current, so that leak currents were often of comparable magnitude during a test depolarization. However, tail currents could still be reliably quantified with minimal correction for leak current. (2) Block by subnanomolar concentrations of nimodipine was not quantified because extremely long time intervals are required for attaining binding equilibrium (Bean, 1984). Instead, we studied nimodipine concentrations ⩾ 10 nM and extrapolated our results to lower concentrations by formulating a model that can predict low-dose effects.

During run-down, inactivation of the slowly inactivating component of Ca-channel current often decreased with the current magnitude, presumably because the amount of inactivation caused by

Ca or Ba influx was reduced. Run-down did not alter the relative magnitude of the two components of Ca-channel current, even when the currents were reduced about 90% (C. J. Cohen & R. T. McCarthy, unpublished results). Armstrong & Matteson (1985) and Matteson & Armstrong (1986) found that the slowly inactivating component of Ca current runs down much faster than the transient component in GH cells, so that preferential reduction of the slowly inactivating current component during drug application could occur as an artifact. A likely explanation for the discrepancy between our finding and theirs is the inclusion of 1 or 5 mM-Mg ATP and the omission of fluoride in the pipette solution in our experiments. Cyclic AMP or catecholamines preferentially enhance the slowly inactivating component of Ca current in other cells with two similar populations of channels (Fedulova, Kostyuk & Veselovsky, 1985; Bean, 1985) and intracellular ATP can slow run-down (Fedulova *et al.* 1985; Forscher & Oxford, 1985). Intracellular fluoride salts enhance run-down (Carbone & Lux, 1985).

## RESULTS

### *$GH_4C_1$ cells have two types of Ca channels*

Fig. 1 shows two Ca current records measured during test pulses to −10 mV. When the test pulse was preceded by a 5 s pre-pulse to −35 mV the current showed little or no decay. An additional transient component of current was elicited if the 5 s pre-pulse was to −90 mV. Note that both current amplitudes are equal by the end of the test pulse.

Changes in current time course with holding potential are not consistent with a Hodgkin–Huxley type inactivation mechanism. Such changes can occur when the rate of inactivation increases with the current magnitude, as found with Ca-induced inactivation (Eckert & Chad, 1984). Alternatively, two distinct populations of Ca channels may be present with differing inactivation kinetics. The results in Figs. 2 and 3 favour the latter alternative. Fig. 2 shows that changes in holding potential alter the $I$–$V$ relationship. Peak currents are plotted *vs.* test potential for the same experiment as shown in Fig. 1. Changing to a more negative holding potential causes a large increase in current for weak depolarizations, but little additional current for very positive test pulses. Thus, the extra component of current seen with more negative holding potentials activates at more negative voltages than the maintained component of current.

Fig. 3 shows that the extra component of current reverses at more negative potentials than the maintained component. For this experiment, Ba was used as the charge carrier. Each panel shows a pair of current records measured during voltage pulses from −35 or −90 mV to the indicated test potential (records with holding potential ($V_h$) = −90 mV are indicated by an asterisk). At all four test potentials, a non-decaying inward current was measured when $V_h$ = −35 mV. For $V_h$ = −90 mV, an extra increment of inward current is seen for test potentials ($V_t$) of −30, 0 and +30 mV. However, at +80 mV the current for $V_h$ = −90 mV is initially less inward than for $V_h$ = −35 mV. Hence, the difference current for these two holding potentials is a decaying inward current at −30, 0 and +30 mV, but a decaying outward current at +80 mV. Similar results were obtained in two other experiments.

The simplest explanation for the results of Figs. 1–3 is that two populations of Ca channels are present. Current through one population of channels is insensitive to changes in holding potential and does not decay during a test pulse of several seconds (the behaviour during very long test pulses will be shown below). In other words, these channels do not inactivate. We refer to these channels as 'slowly inactivating'

because $^{45}Ca$ fluxes through these channels inactivate over several minutes (Tan & Tashjian, 1984*a*). The other population of channels is only available for opening when the holding potential is more negative than −35 mV, and current through these channels inactivates rapidly during a test pulse. We refer to these channels as 'transient'. Fig. 2 shows that only the transient channels activate significantly for


Fig. 1. Ca-channel currents have transient and slowly inactivating components. Two current records measured during test pulses to −10 mV are superimposed. A 5 s pre-pulse to −35 or −90 mV preceded each test pulse. The holding current at −90 mV was defined as zero Ca current. The holding current at −35 mV is inward, indicating that some Ca channels remain open at this potential in the steady state. The bath solution contained 20 mM-Ca as the charge carrier. The first 1·2 ms after the voltage step has been blanked from each current record.

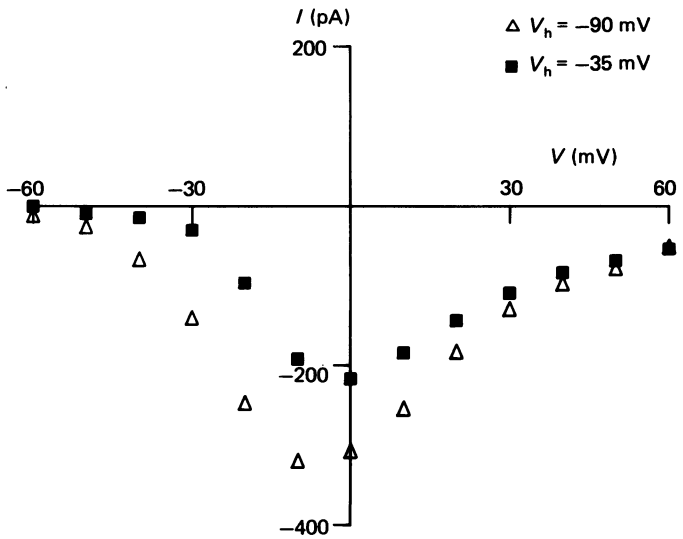


Fig. 2. The *I–V* relationship for Ca-channel current varies with the holding potential. Peak current is plotted *vs.* test potential. The membrane potential was held at −35 mV (■) or −90 mV (△) for 5 s before each test pulse. Same experiment as shown in Fig. 1.

$V < -30$ mV. Fig. 3 indicates that the transient channels are less selective for Ba over Cs than are the slowly inactivating channels.

Similar populations of Ca channels have previously been reported in sensory and hippocampal neurones, neuroblastoma cells, myocardial atrial and ventricular cells and $GH_3$ anterior pituitary cells (Brown & Griffith, 1983; Tsunoo, Yoshii & Narahashi, 1984; Carbone & Lux, 1984*a*, *b*; Fedulova *et al.* 1985; Bossu, Feltz & Thomann, 1985; Nowycky, Fox & Tsien, 1985; Bean, 1985; Nilius, Hess, Lansman & Tsien, 1985; Armstrong & Matteson, 1985; Matteson & Armstrong, 1986). The studies with $GH_3$ cells indicated that the channels could be distinguished by their

rate of deactivation: the slowly inactivating channels deactivate much faster than the transient channels. Hence, tail currents measured after repolarization consist of two exponential components that can be associated with the two populations of channels. We have found that tail-current analysis is a very effective means of separating and quantifying the amount of current through each channel type in $GH_4C_1$ cells. An example of this analysis is shown in Fig. 4.

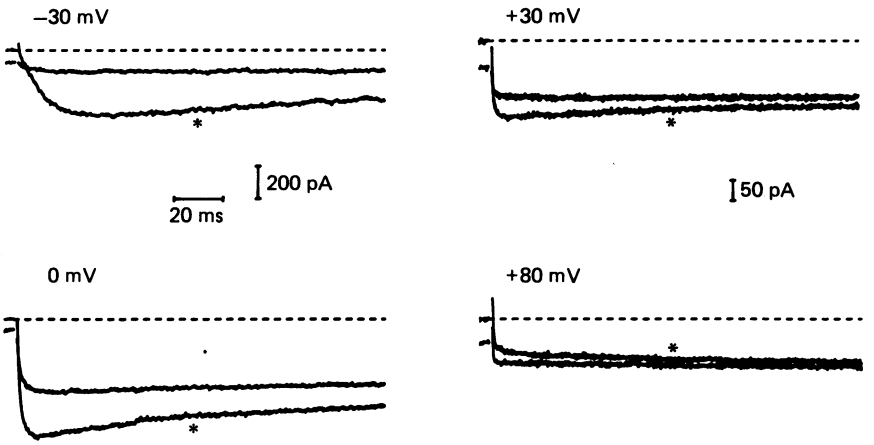


Fig. 3. The transient and slowly inactivating components of Ca-channel current have different reversal potentials. Each panel shows a pair of superimposed current records measured during voltage pulses to the indicated test potential after 5 s pre-pulses to either −35 or −90 mV. Records with pre-pulses to −90 mV are indicated by an asterisk. The dashed line in each panel indicates the holding current at −90 mV (which was defined as zero Ca-channel current). The bath solution contained 20 mM-Ba as the charge carrier. The first 1·2 ms after the voltage step has been blanked from each current record. Note that the two panels on the right are reproduced at higher gain than those on the left.

This Figure shows the use of tail-current measurements to determine the voltage dependence of transient channel activation and inactivation. Fig. 4*A* shows an example of the tail-current measurements. Records for pre-pulses to −50 and −110 mV are superimposed. For the pre-pulse to −110 mV the tail current is biexponential. A single exponential has been fitted to the slower component with a time constant of 10·6 ms. When the pre-pulse was to −50 mV, the slow component of tail current was absent, but the fast component was virtually unchanged. Fig. 4*B* shows a plot of the slow-tail-current amplitude *vs.* pre-pulse potential ($V_p$). Results for three cells are averaged. The continuous curve is defined by Hodgkin–Huxley inactivation kinetics with $V_{\frac{1}{2}} = -75{\cdot}1$ mV and slope factor ($k$) = 5·35. Tail-current analysis was also used to measure channel activation. The protocol is shown in the inset to this Figure and the results are plotted as open triangles. The continuous curve through these points is an $m_\infty$ curve with $V_{\frac{1}{2}} = -31{\cdot}9$ mV and $k = 7{\cdot}95$. Steady-state Ca influx through the transient channels occurs over the voltage range where the activation and inactivation curves overlap. The maximal overlap is at about −60 mV, near the resting potential for these cells (see Discussion). Hence, Ca influx through the transient channels is likely to be important in determining basal levels of hormone secretion.

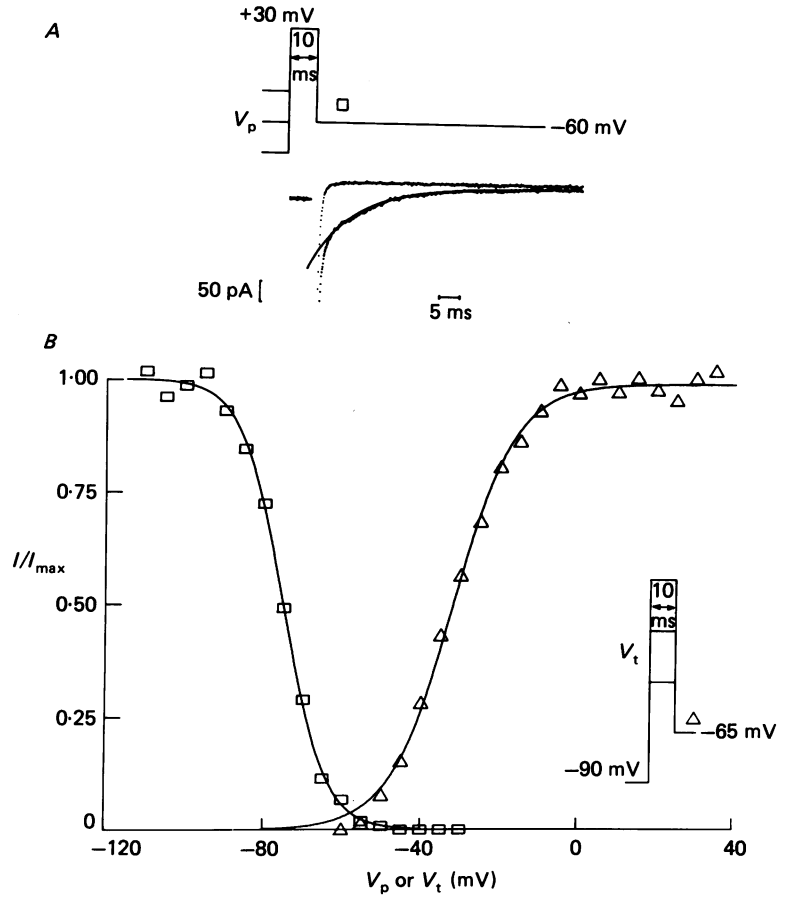


Fig. 4. Voltage dependence of the activation and inactivation of the transient Ca channels. *A*, the transient Ca channels deactivate slowly. The steady-state availability of the transient Ca channels was varied by applying 5 s pre-pulses to $V_p$. Below the pulse protocol are superimposed current records for $V_p = -110$ mV and $-50$ mV, measured when the membrane was repolarized to $-60$ mV after a 10 ms test pulse to $+30$ mV. The record for $V_p = -110$ mV shows a slowly deactivating component that was fitted by an exponential with a time constant of 10·6 ms. The amplitude of this exponential provided a measure of the instantaneous transient Ca-channel current. The bath solution contained 20 mM-Ba. The first 600 $\mu$s after repolarization has been blanked from each current record. *B*, steady-state activation and inactivation of the transient Ca channels. Using the pulse protocol shown in *A*, the steady-state availability of the transient Ca channels was determined. The average result for three cells was normalized and plotted as a function of $V_p$ (□). The data have been fitted by the eqn $I/I_{max} = [1+\exp((V-V_{\frac{1}{2}})/k)]^{-1}$ with $V_{\frac{1}{2}} = -75{\cdot}1$ mV and $k = 5{\cdot}35$. Activation of the transient Ca channels was determined using the protocol shown in the inset to *B*. The magnitude of the slow component of the tail current at $-65$ mV was measured for each test potential ($V_t$). The average result for three cells was normalized and plotted *vs.* $V_t$ (△). The data have been fitted by the eqn $I/I_{max} = [1+\exp((V_{\frac{1}{2}}-V)/k)]^{-1}$ with $V_{\frac{1}{2}} = -31{\cdot}9$ mV and $k = 7{\cdot}95$. For $V_t < -40$ mV, the time to peak current was > 10 ms, so that activation of the transient Ca channels was underestimated. The patch pipette was filled with high ATP–BAPTA solution in two of the experiments.

*Selectivity of Ca-channel block*

The relative importance of each type of Ca channel in mediating stimulus–secretion coupling depends on the pattern of electrical activity. During maintained depolarizations the slowly inactivating channels are most important, but at normal resting potentials only the transient channels conduct significantly. Hence, agents that modify only one population of Ca channels can very selectively affect stimulus–secretion coupling.

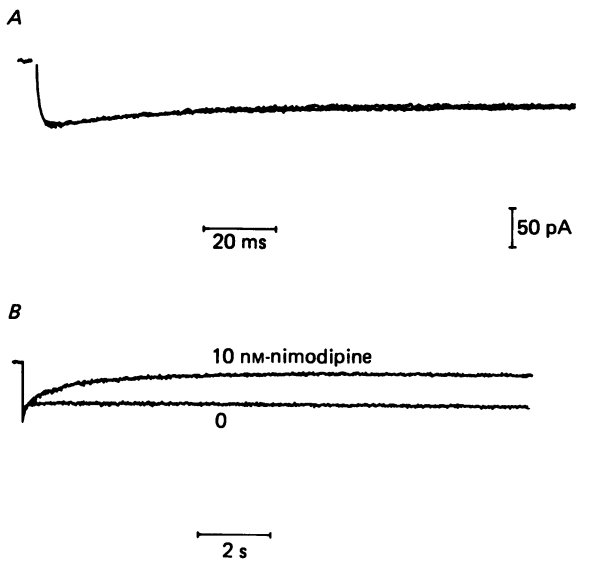


Fig. 5. Time dependence of nimodipine block of the slowly inactivating Ca channels. The effect of 10 nM-nimodipine on current measured during a test pulse from −90 to −20 mV is shown on two time scales. *A*, superimposed current records with and without drug on a faster time scale. Each current record is the average of four successive test pulses. The current was sampled at 5 kHz and the first 1·2 ms after the voltage step has been blanked from each current record. The bath solution contained 20 mM-Ba as the charge carrier. *B*, superimposed current records with and without drug on a 100 times slower time scale. Same experiment as in *A*. Note the absence of inactivation in the control measurement. The current was sampled at 66·7 Hz. Each test pulse was preceded by a 5 s rest at −90 mV for control measurements and by a 15 s pre-pulse in the presence of the drug.

Cd blocks both types of Ca channels with similar potency. The percentage block of each type of channel was equated with the percentage block of each component of tail current. When 20 mM-Ba was in the bath solution as current carrier, 6 $\mu$M-Cd reduced the slow component of tail current at −50 mV by 48·0±0·1 % (±S.E. of mean, $n = 2$) and the fast component by 78·9±0·9 %. 100 $\mu$M-Cd eliminated all time-dependent current, consistent with the idea that it was entirely through Ca channels.

Fig. 5 shows that nimodipine is a very selective Ca-channel blocker. The effect of 10 nM-nimodipine is shown on two different time scales. Both panels show current measurements with and without drug during a test pulse to −20 mV after a long rest at −90 mV. Fig. 5*A* shows that during the first 150 ms there is no drug effect;

Fig. 5*B* shows the effect on a time scale that is 100 times slower. Although there is no drug effect at the beginning of the pulse, about 90 % of the channels are blocked after approximately 10 s. Hence, nimodipine block is time- and voltage-dependent.

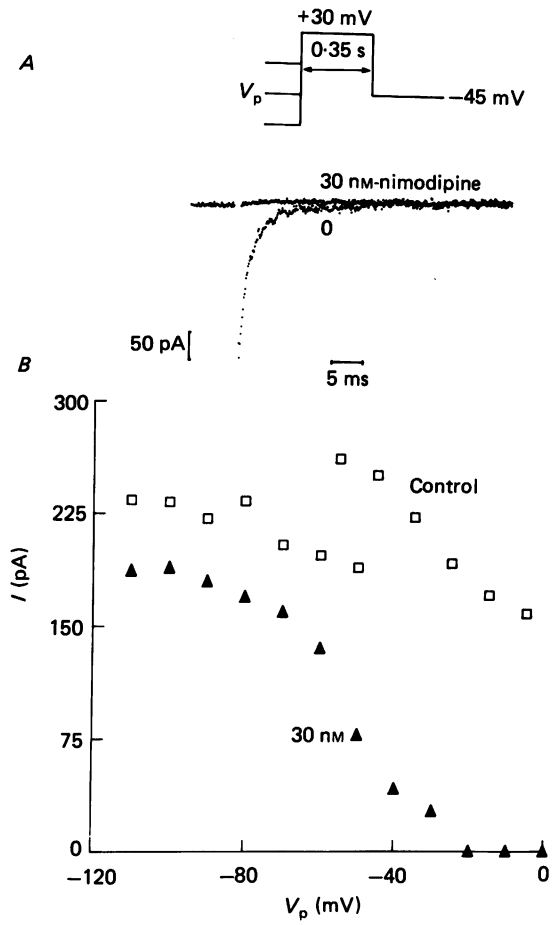


Fig. 6. Steady-state availability of the slowly inactivating Ca channels with and without 30 nM-nimodipine. *A*, nimodipine block of the rapidly deactivating component of Ca-channel current. At top is the pulse protocol used to measure steady-state availability. The pre-pulses to $V_p$ were 5 s without drug and 20 s in 30 nM-nimodipine. Below the pulse protocol are superimposed current records for $V_p = -20$ mV measured when the membrane was repolarized to −45 mV. The tail current without drug was fitted by a single exponential with a time constant of 1·43 ms. This tail current was completely blocked by 30 nM-nimodipine. The bath solution contained 20 mM-Ba. Current was sampled at 10 kHz. The first 900 $\mu$s after repolarization have been blanked from each current record. *B*, isochronal measurements of the tail current amplitude *vs.* $V_p$ for the experiment shown in *A*. □, control data; ▲, in 30 nM-nimodipine. See text for further details.

The absence of block when the depolarization is first applied indicates that nimodipine binds with low affinity to both types of Ca channels at −90 mV. At −20 mV, the amount of block increases exponentially with a time constant of 2·0 s. This represents block of the slowly inactivating Ca channels because the transient channels inactivate rapidly at −20 mV. Nimodipine is a weak blocker of the transient Ca channels at all potentials (as shown below).

Time- and voltage-dependent Ca-channel block by dihydropyridines has previously been reported in myocardial cells and neurones. The voltage dependence of block is thought to occur because drug binding is modified by channel gating. Drug binding is promoted either by channel activation (Lee & Tsien, 1983; Sanguinetti & Kass,

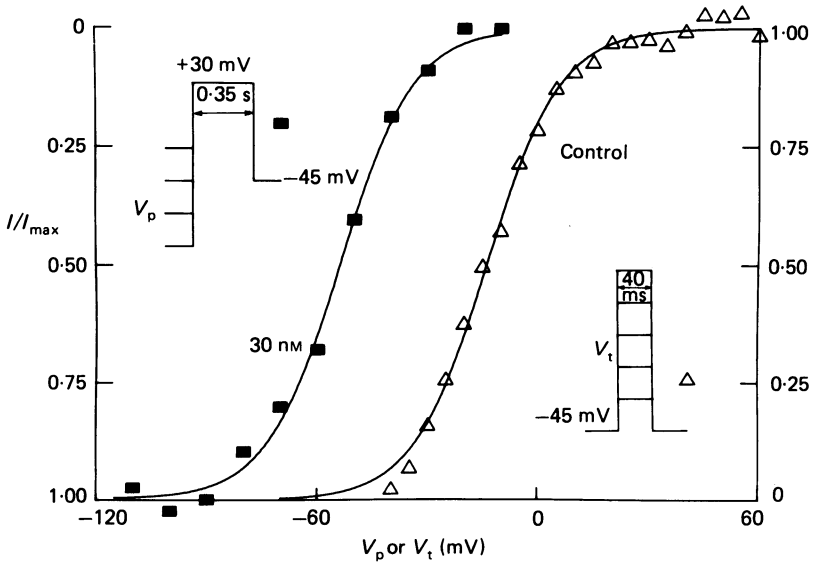


Fig. 7. Steady-state voltage dependence of nimodipine block of the slowly inactivating Ca channels *vs.* the voltage dependence of Ca-channel activation. The steady-state availability of the slowly inactivating Ca channels in 30 nM-nimodipine was determined in three experiments using the same experimental protocol as in Fig. 6 (the pulse protocol is given in the left inset). The normalized tail-current measurements for each value of $V_p$ were averaged and plotted *vs.* $V_p$ (■). The ordinate for this plot is inverted and at the left of the Figure. The continuous curve through these data points is a non-linear least-squares fit to the eqn $I/I_{max} = [1+\exp((V-V_{\frac{1}{2}})/k')]^{-1}$ with $V_{\frac{1}{2}} = -53{\cdot}9$ mV and $k' = 9{\cdot}85$. The voltage dependence of activation of the slowly inactivating Ca channels was determined using the pulse protocol shown in the right inset. Tail currents at −45 mV following a 40 ms test pulse were fitted by a single rapidly decaying exponential. The average normalized tail current amplitude for six experiments is plotted *vs.* the test potential (△). The ordinate for this plot is on the right. The continuous curve through these data points is a non-linear least-squares fit to the eqn $I/I_{max} = [1+\exp((V_m-V)/k)]^{-1}$ with $V_m = -13{\cdot}8$ mV and $k = 9{\cdot}90$. For $V_t \leqslant -40$ mV, the time to peak current was > 40 ms, so that steady-state activation of the slowly inactivating Ca channels was underestimated. The bath solution for all experiments contained 20 mM-Ba.

1984; Gurney & Nerbonne, 1984; Gurney, Nerbonne & Lester, 1985) or inactivation (Bean, 1984; Sanguinetti & Kass, 1984; Uehara & Hume, 1985; Kunze, Hawkes, Hamilton & Brown, 1985; Gurney *et al.* 1985). Very-high-affinity binding, comparable to that found in ligand binding experiments, has been attributed to preferential binding to inactivated channels. The results in Fig. 5 suggest that inactivation is not required for high-affinity binding in $GH_4C_1$ cells. In this experiment, Ca-induced inactivation was minimized by using Ba as the charge carrier and by buffering cell Ca to about 10 nM. During the 14 s control measurement shown in Fig. 5*B* there is no evidence of inactivation. The rapid decay seen at the beginning of the pulse is due to inactivation of the second population of channels (the transient channels).

In myocardial cells, the steady-state voltage dependence of Ca-channel block by dihydropyridines parallels the voltage dependence of Ca-channel inactivation. Fig. 6 shows that this is not the case in $GH_4C_1$ cells. The number of channels available for opening was assayed by tail-current measurements, as shown in Fig. 6*A*. A 5 s pre-pulse was followed by a 350 ms test pulse to +30 mV. Almost all of the transient channels inactivated during the long test pulse, so the tail currents that accompany repolarization are described by a single exponential associated with the slowly inactivating channels (for the most negative pre-pulse potentials a second slow exponential component of the tail current was present that was subtracted out by curve peeling). Fig. 6*A* also shows that 30 nM-nimodipine blocked all of the tail current when $V_p = -20$ mV.

Fig. 6*B* shows a plot of tail-current amplitudes *vs.* $V_p$. In the absence of drug, there was little inactivation over the 100 mV voltage range studied. There may be a small amount of inactivation at the most positive potentials, but most of the decline in current size was probably due to run-down because large inward currents were elicited during these long pre-pulses (Fenwick *et al.* 1982). Although there is little evidence of Ca-channel inactivation, nimodipine block has a sigmoid voltage dependence very similar to that reported for myocardial cells. Hence, the voltage dependence of nimodipine block is not easily attributed to channel inactivation.

In some other experiments, inactivation of the slowly inactivating Ca currents was much more pronounced, and occurred over a voltage range similar to that for channel activation. The presence of inactivation had little or no effect on the steady-state voltage dependence of nimodipine block. The steady-state voltage dependence of nimodipine block does not preclude high-affinity binding to inactivated channels. Nimodipine may bind to inactivated channels with high affinity, but channels need not inactivate before high-affinity binding occurs.

*Nimodipine preferentially binds to open Ca channels*

Fig. 7 shows a striking parallel between the voltage dependence of nimodipine block and the voltage dependence of Ca-channel activation. The filled squares show the effect of 30 nM-nimodipine on the availability of the slowly inactivating Ca channels. The experimental protocol is the same as in Fig. 6, but the results shown here are the average of three cells and the ordinate (shown at left) has been inverted to emphasize the similarity between nimodipine block and channel activation.

The open triangles indicate the voltage dependence for activation of the slowly inactivating Ca channels. The pulse protocol is shown in the inset, and the results are the average for six cells. The number of open channels was assayed by measuring tail currents, and current through the transient channels was eliminated by using a depolarized holding potential. The voltage dependence of channel activation is well fitted by a Hodgkin–Huxley type $m_\infty$ curve. The continuous curve through the data points is a non-linear least-squares fit to the equation:

$$m_\infty = I/I_{max} = [1 + \exp((V_m - V)/k)]^{-1}, \quad (1)$$

with the potential for half-maximal activation ($V_m$) = −13·8 mV and the slope factor ($k$) = 9·90.

The effect of nimodipine on channel availability is described by a similar equation: $I/I_{max} = [1 + \exp((V - V_{\frac{1}{2}})/k')]^{-1}$. A non-linear least-squares fit to this equation

(shown as a continuous curve) indicated $V_{\frac{1}{2}} = -53{\cdot}9$ mV and $k' = 9{\cdot}85$. Since there is negligible inactivation over the voltage range for which block develops, this curve also describes the percentage block by 30 nM-nimodipine in the steady state. The parallel between the two curves in Fig. 7 indicates that the fraction of channels blocked by nimodipine at the pre-pulse potential is directly proportional to the fraction of open channels at that potential. This result strongly suggests that nimodipine preferentially binds to open (activated) Ca channels.

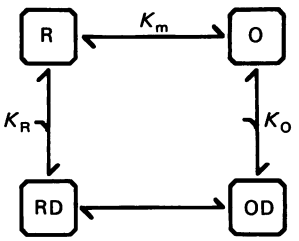


Fig. 8. A four-state model to account for the time and voltage dependence of nimodipine block of the slowly inactivating Ca channels. See text for a definition of terms and an explanation of the model.

Fig. 8 shows a simple four-state model that can account quantitatively for the steady-state voltage dependence of nimodipine block. The model is adapted from the modulated receptor theory, which describes the block of Na channels by local anaesthetics (Hille, 1977; Hondeghem & Katzung, 1977). In the absence of drug, channels are either in the resting (R) state or are open (O). The corresponding drug-bound states are RD and OD, respectively. Although a complete description of activation of Ca channels in GH cells requires at least two closed states (Hagiwara & Ohmori, 1982, 1983), the effects of nimodipine can be accounted for with only one closed state. An inactivated state is not included. It was reasonable to ignore the occupancy of this state because there often was little or no inactivation. However, the model is also applicable to instances where inactivation subsequent to activation is reversible and does not alter drug binding. The equilibrium between R and O is defined by the $m_\infty$ curve of Fig. 7:

$$K_m = [\mathrm{O}]/[\mathrm{R}] = m_\infty/(1-m_\infty) = \exp((V-V_\mathrm{m})/k),$$

where symbols in brackets indicate the fractional occupancy of a state. It is assumed that $m_\infty \cong 1$ at very positive potentials in $GH_4C_1$ cells, as in $GH_3$ cells (Hagiwara & Ohmori, 1982). At more negative holding potentials, more of the channels are in the resting state and nimodipine block is reduced. Hence, the dissociation constant for drug binding to channels in the resting state ($K_\mathrm{R}$) must be greater than the dissociation constant for binding to open channels ($K_\mathrm{O}$). The data shown in Fig. 7 can be used to obtain values for $K_\mathrm{R}$ and $K_\mathrm{O}$.

Drug binding is allowed to equilibrate at the pre-pulse potential during a long pre-pulse. All drug-free channels are opened by a test pulse to +30 mV ($m_\infty \cong 1$). If we assume that no significant change in drug binding occurs during the test pulse, then the tail current is proportional to the steady-state value of ([R]+[O])/([R]+[O]+[RD]+[OD]) at $V_\mathrm{p}$. The normalized fraction of drug-free

channels at $V_p$ is this quantity divided by [R]/([R]+[RD]) and is defined as $m'_\infty$. If we also assume that $K_R$ and $K_O$ are not voltage dependent, then

$$\begin{aligned} m'_\infty &= (1+K_m)(1+[D]/K_R)/(1+K_m+[D]/K_R+K_m[D]/K_O) \\ &= [1+K_m([D]/K_O-[D]/K_R)/(1+K_m)(1+[D]/K_R)]^{-1}, \end{aligned} \tag{2}$$

where [D] is the drug concentration. Empirically, we found that

$$\begin{aligned} m'_\infty &= [1+\exp((V-V_{\frac{1}{2}})/k')]^{-1} \\ &= [1+\exp((V-V_m-\Delta V)/k)]^{-1} \\ &= [1+K_m\exp(-\Delta V/k)]^{-1}. \end{aligned} \tag{3}$$

Combining eqns. (2) and (3) gives

$$\Delta V = -k\ln[([D]/K_O-[D]/K_R)/(1+[D]/K_R)(1+K_m)]. \tag{4}$$

For $V<-40$ mV, $1+K_m \cong 1$ (i.e. [O] $\ll$ [R] before the test pulse to +30 mV). For the experiments in Fig. 7, [D] = 30 nM, $\Delta V = V_{\frac{1}{2}}-V_m = -40{\cdot}1$ mV, and $k = 9{\cdot}88$ (the average slope factor for the two curves). $K_R$ is determined by measuring the amount of block following a strong hyperpolarization ($V_p = -120$ mV), so that binding occurs when all channels are in states R or RD. As shown in Fig. 11, $K_R \cong 7\ \mu$M. Solving eqn. (4) for $K_O$ yields $K_O = 0{\cdot}517$ nM.

*The kinetics of nimodipine binding to open Ca channels*

Our calculation indicates that channel opening increases the affinity for nimodipine binding by over 4 orders of magnitude. Preferential block of open Ca channels by organic molecules has not previously been analysed quantitatively, but quantitative models of open-channel block have been formulated to describe the block of K channels (Armstrong, 1969), pronase-treated Na channels (Yeh & Narahashi, 1977; Cahalan, 1978) and motor end-plate channels (see Adams (1981) for a review). The models for block of K and Na channels considered cases when the rate of drug binding was comparable to the rate of channel gating. Hence, it was necessary to consider the kinetics of channel gating in detail, and the rate constants for drug binding were obtained by numerical simulation. Fortunately, the block of Ca channels by therapeutic doses of nimodipine is about 1000 times slower than the rate of Ca-channel activation (see Fig. 5), so we can simplify the model for drug block to a three-state model analogous to the one that describes the block of end-plate channels:

$$\mathrm{R} \underset{\beta_m}{\overset{\alpha_m}{\rightleftharpoons}} \mathrm{O} \underset{b}{\overset{f}{\rightleftharpoons}} \mathrm{OD}.$$

Here, $\alpha_m$ and $\beta_m$ are the rate constants describing channel activation and $f$ and $b$ are the forward and backward rate constants for drug binding. The linear differential equations that describe transitions between states R, O, and OD in response to a voltage step can be integrated and solved analytically. The solution allows us to predict the rate of nimodipine block as a function of voltage. In order to test the validity of our model of nimodipine action and to derive 'on' and 'off' rate constants for binding to open channels, we measured the rate of nimodipine block at various voltages and compared the results with theory.

The equations that describe the binding of nimodipine to open Ca channels are

identical to those for a two-step enzymatic reaction (Hammes & Schimmel, 1970). Following a voltage step, there is a relaxation to a new equilibrium with the following time course:

$$[\mathrm{R}] = \overline{[\mathrm{R}]} + A_{11}\exp(-t/\tau_f) + A_{12}\exp(-t/\tau_s),$$

$$[\mathrm{O}] = \overline{[\mathrm{O}]} + A_{21}\exp(-t/\tau_f) + A_{22}\exp(-t/\tau_s),$$

$$[\mathrm{OD}] = \overline{[\mathrm{OD}]} + A_{31}\exp(-t/\tau_f) + A_{32}\exp(-t/\tau_s),$$

where the overbar denotes an equilibrium value and $\tau_f$, $\tau_s$ (the fast and slow time constants) and the $A_{ij}$ values are defined by these equations. The time constants of relaxation are:

$$\tau^{-1} = [(\alpha_m+\beta_m) \pm (\alpha_m+\beta_m)[1-4(\alpha_m f[\mathrm{D}]+\alpha_m b+\beta_m b)/(\alpha_m+\beta_m)^2)]^{\frac{1}{2}}]/2.$$

The last term on the right-hand side is $\ll 1$ for therapeutic doses of drug, so we can use the approximation $(1-x)^{\frac{1}{2}} \cong 1-x/2$ for $x \ll 1$. Hence,

$$\tau_s^{-1} = \alpha_m f[\mathrm{D}]/(\alpha_m+\beta_m)+b = m_\infty f[\mathrm{D}]+b, \tag{5}$$

$$\tau_f^{-1} = \alpha_m+\beta_m-m_\infty f[\mathrm{D}]-b = \tau_m^{-1}-\tau_s^{-1}, \tag{6}$$

where $m_\infty = \alpha_m/(\alpha_m+\beta_m)$ and $\tau_m = (\alpha_m+\beta_m)^{-1}$. For [D] = 30 nM, $\tau_m \cong \tau_s/1000$, so $\tau_f \cong \tau_m$. After a long rest at −90 mV, nearly all channels are in state R. When a constant depolarization is applied, the channels quickly redistribute between states R and O, so that $[\mathrm{R}] \cong \beta_m/(\alpha_m+\beta_m)$, $[\mathrm{O}] \cong \alpha_m/(\alpha_m+\beta_m)$ and [OD] ≅ 0. Since $\alpha_m$ and $\beta_m$ are much greater than $f[\mathrm{D}]$ and $b$, $[\mathrm{R}]/[\mathrm{O}] \cong \overline{[\mathrm{R}]}/\overline{[\mathrm{O}]}$ at all times. The occupancy of state OD then increases exponentially with time constant $\tau_s \cong (m_\infty f[\mathrm{D}]+b)^{-1}$, while the occupancy of both states R and O decay exponentially at $\tau_s$. If $f$ and $b$ are voltage independent (which is likely because nimodipine is electroneutral), then the voltage dependence of $\tau_s$ is derived entirely from the voltage dependence of steady-state Ca channel activation, given by eqn. (1).

Two methods for experimentally determining $\tau_s$ were used. The first is shown in Fig. 5*B*. The membrane voltage was held at −90 mV long enough to allow nearly all channels to enter state R. The occupancy of the open state was then monitored continuously during a long test pulse. Since there is negligible decay of the current in the absence of drug, the time course of block (O + D → OD) can be obtained by fitting an exponential to the current measurement in the presence of drug. This method takes good advantage of the absence of inactivation and obtains the time constant for the onset of block ($\tau_{onset}$) in one 15 s pulse, but it is limited to test voltages near the peak of the *I–V* relationship. The most critical test of the theory involves voltages near the foot of the activation curve, where $\tau_{onset}$ should be steeply voltage dependent. In order to study voltages over a broader range, we used the pulse protocol shown in the inset of Fig. 9.

Drug binding is initiated by stepping from −90 mV to various pre-pulse potentials. The fast component of the tail current that follows a 10 ms pulse to +30 mV is proportional to the fraction of drug-free channels at time $t$. The rate of onset of block is shown for $V_p = -40$, −20 and 0 mV. From the activation curve of Fig. 8, we expect a 5-fold change in $m_\infty$ when $V_p$ is increased from −40 to −20 mV, but only a 2-fold change for $V_p = -20$ mV *vs.* $V_p = 0$ mV. If $m_\infty f[\mathrm{D}] \gg b$, then $\tau_{onset}$ should change in inverse proportion to $m_\infty$. The continuous curves through the three sets of data

points are non-linear least-squares fits to a single exponential; $\tau_{onset}$ = 3·98, 1·15 and 0·60 s at $V_p$ = −40, −20 and 0 mV, respectively.

Fig. 10 summarizes the results of several experiments of this type. The open squares indicate measurements of $\tau_{onset}$ using a single long test pulse as in Fig. 5*B*, while the filled squares are for values obtained using the protocol shown in Fig. 9. The former procedure measures d[O]/d*t*, while the latter measures d([O]+[R])/d*t*. The similar results obtained with both techniques indicate that the occupancy of state R and

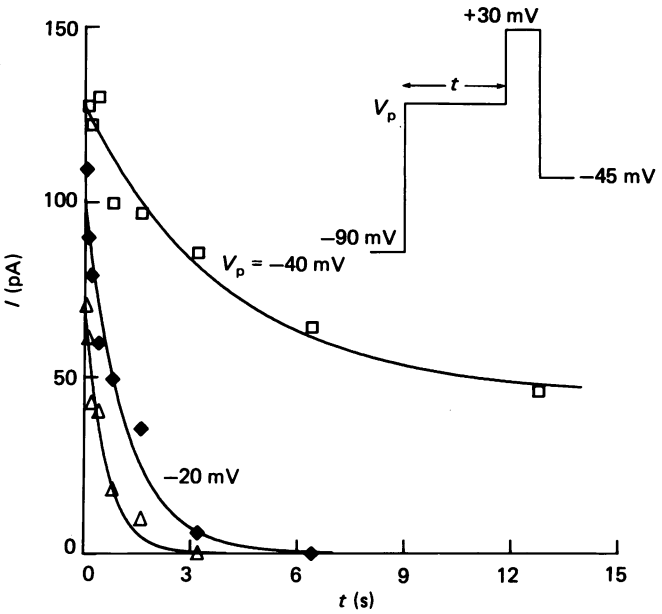


Fig. 9. The rate of onset of nimodipine block is voltage dependent. The time course of block of the slowly inactivating Ca channels by 30 nM-nimodipine was determined at three potentials: −40 (□), −20 (◆) and 0 mV (△). For each data point the following pulse protocol was used (see inset): the membrane potential was held at −90 mV for > 50 s; the potential was then stepped to the pre-pulse potential ($V_p$) for a time *t* (the abscissa); a 10 ms voltage pulse to +30 mV produced maximal activation of the slowly inactivating Ca channels, and tail currents were measured upon repolarization to −45 mV. The tail currents were analysed as the sum of two exponentials, and the amplitude of the fast component (measured isochronally 800 μs after repolarization) defines the ordinate. For each value of $V_p$, the results were fitted to the equation:

$$I = (I_0 - I_\infty)\exp(-t/\tau_{onset}) + I_\infty,$$

for $V_p$ = −40 mV, $I_0$ = 127·3 pA, $I_\infty$ = 44·8 pA, $\tau_{onset}$ = 3·98 s; for $V_p$ = −20 mV, $I_0$ = 100·6 pA, $I_\infty$ = 0, $\tau_{onset}$ = 1·15 s; and for $V_p$ = 0 mV, $I_0$ = 71·5 pA, $I_\infty$ = 0, $\tau_{onset}$ = 0·602 s. The progressive decrease in $I_0$ during the experiment was presumably due to slow run-down of the Ca current, since the set of measurements at each value of $V_p$ required > 6 min. The bath solution contained 20 mM-Ba.

O decay at the same rate, as predicted by the model. The continuous curve is a least-squares fit to eqn. (5) with the constraint that $b/f = K_O$ = 0·517 nM (hence, there is one free parameter). The optimal fit is for $f = 9{\cdot}59 \times 10^7$ M$^{-1}$ s$^{-1}$ and $b$ = 0·0496 s$^{-1}$. The goodness-of-fit indicates that the model can account satisfactorily for the time

and voltage dependence of nimodipine block and severely limits the number of alternative explanations. If the time and voltage dependence of nimodipine binding derives from the voltage dependence of Ca-channel gating, then the high-affinity state must have the same Boltzmann distribution for occupancy as the open state: both the slope factor of the distribution and the potential for half-maximal occupancy must be the same as for the open state. The model allows one to predict the time

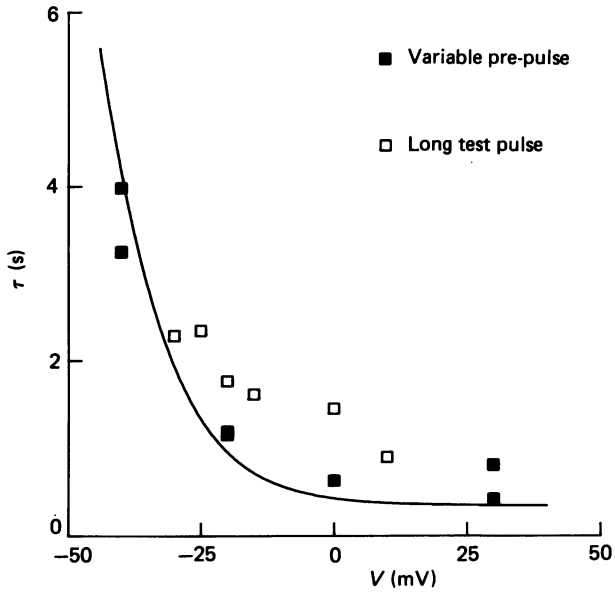


Fig. 10. The voltage dependence of the rate of onset of nimodipine block: a comparison between the model for open-channel block ($\tau\alpha 1/m_\infty$) and experimentally determined time constants. The rate of onset of block of the slowly inactivating Ca channels by 30 nM-nimodipine was measured by two procedures as described in the text. For each experiment, drug binding was initiated by depolarization of the membrane after the drug had equilibrated in the bath. The bath contained 20 mM-Ba. The time course of block was well fitted by a single exponential in each experiment, and the time constant is plotted *vs.* test potential. The continuous curve is a non-linear least-squares fit to the equation:

$$\tau_s = (m_\infty f[\mathrm{D}]+b)^{-1} = [1+\exp((V_m-V)/k)]/[(f[\mathrm{D}]+b)+(b)\exp((V_m-V)/k)],$$

with $V_m = -13{\cdot}8$ mV, $k = 9{\cdot}88$, $f = b/K_0 = b/0{\cdot}517$ nM, and $[\mathrm{D}] = 30$ nM. There are thirteen data points and one free parameter in the fit. See text for a definition of terms and further details.

course and steady-state level of nimodipine block over a wide range of drug concentrations and membrane potentials. It is likely that the time and voltage dependence of Ca-channel block is critical in determining the tissue specificity and efficacy of nimodipine *in vivo*, so our model allows us to explain the drug's therapeutic action in molecular terms (see Discussion).

The rate of onset of nimodipine block should increase with drug concentration, as expected from the principle of mass action (see eqn. (5)). Since the forward rate constant for drug binding is very fast, 1 μM-nimodipine should cause appreciable open-state block during brief test pulses. Fig. 11 shows that this is indeed the case. As in Fig. 9, drug binding to open channels was initiated by applying a test pulse

after a long rest at −90 mV. The fast component of the tail current measured upon repolarization indicated the number of drug-free slowly inactivating Ca channels. In the absence of drug, there is no inactivation of these channels. In 1 μM-nimodipine, block is an exponentially increasing function of test-pulse duration, with $\tau_{onset}$ = 53·9 ms at +30 mV. This onset rate is approximately 10 times faster than

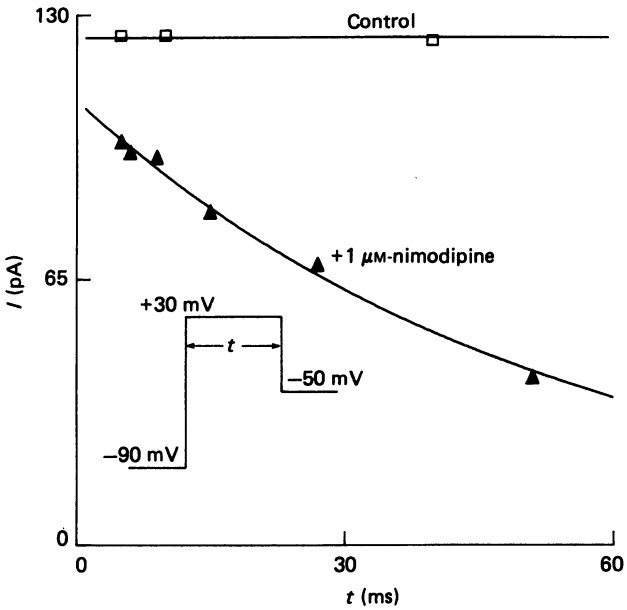


Fig. 11. Time-dependent block of the slowly inactivating Ca channels by 1 μM-nimodipine. The amplitude of the fast component of the tail current is plotted *vs.* test-pulse duration. The pulse protocol is shown in the inset. The membrane potential was held at −90 mV for 3 s in control measurements and 21 s in drug. All control tail-current measurements were fitted by an exponential with a time constant of 0·45 ms; tail currents in presence of the drug had a time constant of 0·44 ms. Current measurements were sampled at 10 kHz. Each control value (□) and the 5 ms time point in the drug is the average of two measurements. The continuous line through the control data is $I_0$ = 124·5 pA. The continuous curve through the data in presence of the drug (▲) is a non-linear least-squares fit to $I = I_{max} \exp(-t/\tau)$, with $I_{max}$ = 108·9 pA and $\tau$ = 53·9 ms. The fractional block at time zero was assumed to be resting-state block, so that $K_R$ was calculated from the eqn: $(1+[D]/K_R)^{-1} = I_{max}/I_0$, with [D] = 1 μM. The patch pipette was filled with the high ATP–BAPTA solution.

found for 30 nM-nimodipine, but the model predicted that $\tau_{onset}$ should be about 40 times faster. Extrapolating the amount of block back to the beginning of the test pulse indicates the amount of resting-state block. For this experiment we found that $K_R$ = 6·98 μM. In other experiments we found that block was not reduced by holding at −120 mV rather than −90 mV. More apparent resting-state block ($K_R \cong 1$ μM) was found in some experiments, but we suspect that part of the decrease in current after application of drug was due to run-down. Fortunately, the calculated value of $K_O$ is changed by only 2 % if $K_R$ is 1 μM rather than 7 μM.

Experiments with high concentrations of nimodipine also revealed block of the transient Ca channels. Fig. 12 shows that 2 μM-nimodipine speeds the rate of decay

of current through the transient Ca channels. In the absence of drug, the amplitude of the slow component of the tail current is an exponentially decreasing function of the test-pulse duration. Note that rapid inactivation is incomplete when Ba is the charge carrier (as also seen in Fig. 3), although currents inactivate completely when Ca is the charge carrier (see Fig. 1). 2 μM-nimodipine increases the rate and extent of transient channel inactivation. These effects are consistent with preferential binding to open transient channels by nimodipine, but this scheme was not tested in detail.

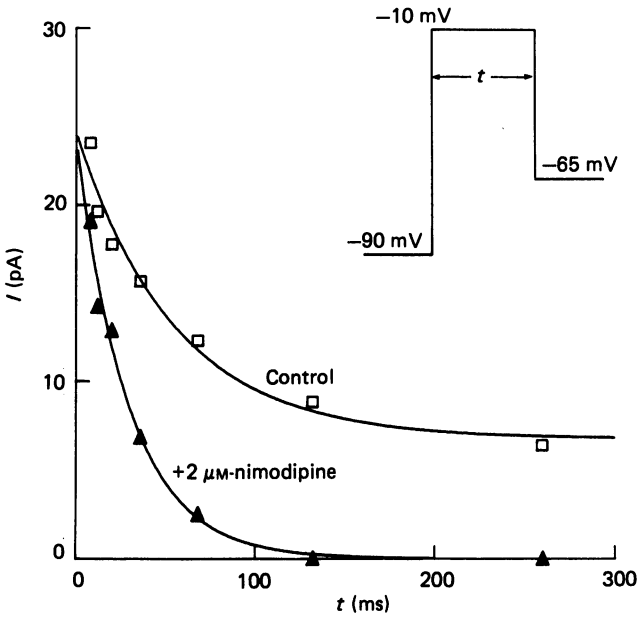


Fig. 12. Block of the transient Ca channels by 2 μM-nimodipine. The rate of inactivation of the transient Ca channels was measured at −10 mV using the pulse protocol shown in the inset. The membrane potential was held at −90 mV for 5 s without drug and for 15 s in 2 μM-nimodipine. The tail currents measured at −65 mV after repolarization were biexponential. The slow component of the tail current had a time constant of 6·5 ms. The amplitude of this component 3·0 ms after repolarization is plotted *vs.* test-pulse duration ($t$). The bath solution contained 20 mM-Ba. The continuous curves are a non-linear least-squares fit to the eqn: $I = (I_0 - I_\infty)\exp(-t/\tau) + I_\infty$. For the control measurement (□), $\tau = 54{\cdot}6$ ms, $I_0 = 24{\cdot}2$ pA, $I_\infty = 6{\cdot}7$ pA. In 2 μM-nimodipine (▲), $\tau = 29{\cdot}1$ ms, $I_0 = 23{\cdot}9$ pA, $I_\infty = 0$. In a second experiment of this type, $\tau = 65{\cdot}6$ ms without drug and 28·4 ms with 2 μM-nimodipine.

Fig. 12 indicates that nimodipine block of the transient Ca channels is time- and voltage-dependent and raises the possibility that long-lasting depolarization might greatly enhance the amount of channel block. However, as shown in Fig. 13, 100 nM-nimodipine only weakly affects the steady-state availability of the transient Ca channels. Both sets of data were determined using the same pulse protocol as in Fig. 4, except that in the presence of drug the pre-pulses lasted 30 s. 100 nM-nimodipine shifted the mid-point of the availability curve by only −1·5 mV. In a second similar experiment 300 nM-nimodipine shifted the mid-point by −3·5 mV. From these results we conclude that therapeutic concentrations of nimodipine do not cause significant block of the transient Ca channels.

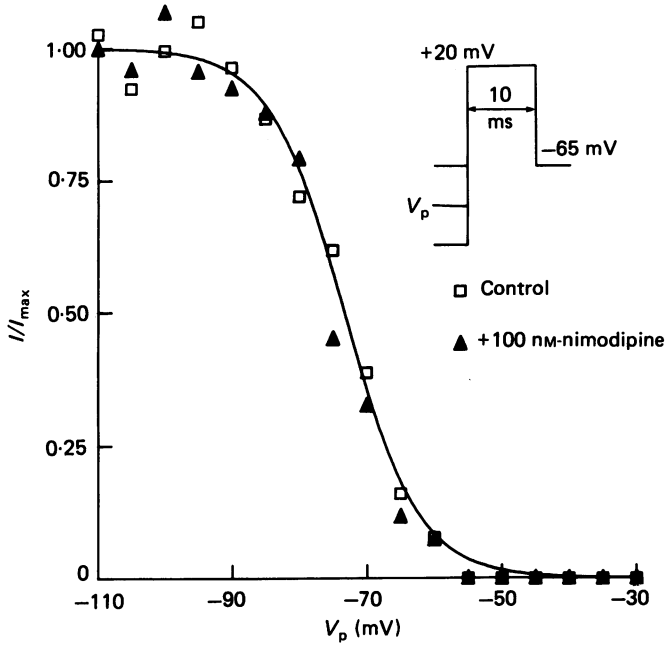


Fig. 13. The steady-state availability of the transient Ca channels with and without 100 nM-nimodipine. The pulse protocol used is shown in the inset. The pre-pulses to $V_p$ were 5 s without drug and 30 s in 100 nM-nimodipine. The tail currents measured at −65 mV had a slow exponential component that decayed with a time constant of 8·1 ms. Isochronal measurements of the amplitude of the slow component are plotted *vs.* $V_p$. The continuous curve is a non-linear least-squares fit of the control measurements to the equation:

$$I/I_{max} = [1+\exp((V-V_h)/k)]^{-1},$$

with $V_h = -73{\cdot}2$ mV, $k = 5{\cdot}5$, $I_{max} = 84{\cdot}0$ pA. The measurements in 100 nM-nimodipine (▲) were fitted to the same equation with $V_h = -74{\cdot}7$ mV, $k = 5{\cdot}1$, $I_{max} = 73{\cdot}8$ pA. The bath solution contained 20 mM-Ba and the patch pipette contained high ATP–BAPTA solution.

## DISCUSSION

Our studies clarify three important aspects of the mechanism of action of nimodipine. We found that dihydropyridines can block Ca-channel currents in endocrine cells with very high affinity, but only during maintained depolarizations. Moreover, not all Ca channels are susceptible to high-affinity nimodipine block. Finally, a model was formulated and tested that predicts the amount of nimodipine binding as a function of time, voltage and drug concentration. These findings modify our understanding of the role of Ca channels in stimulus–secretion coupling. They also suggest that some of the therapeutic effects of nimodipine are due to a direct action on endocrine and/or neural tissue.

### *The role of Ca channels in stimulus–secretion coupling*

$GH_4C_1$ cells have two components of Ca-channel current. These components have different activation, inactivation and deactivation kinetics, and different ionic

selectivities and susceptibilities to block by Cd or nimodipine. The results can be explained best by invoking two distinct populations of channels. The slowly inactivating channels correspond to the Ca channels first characterized in $GH_3$ cells by patch voltage-clamp experiments (Hagiwara & Ohmori, 1982, 1983; Dubinsky & Oxford, 1984; Matteson & Armstrong, 1984). The transient channels have some similarity to the Na channels found in $GH_3$ cells, but a number of observations rule out the possibility that the two are the same: (1) only Ba, Ca or TEA were present in the bath solution as potential current carriers, and we found high selectivity for the external cations over internal Cs; (2) the transient component of current is completely blocked by 100 $\mu$M-Cd when Ba is the charge carrier (data not shown), but is resistant to block by 200 nM-TTX; (3) unlike $GH_3$ cells, $GH_4C_1$ cells have very few Na channels (Dubinsky & Oxford, 1984; C. J. Cohen & R. J. McCarthy, unpublished experiments); (4) the rates of transient channel inactivation and deactivation are much slower than for Na channels (see Fig. 5 of Dubinsky & Oxford (1984) and Figs. 4 and 5 of Matteson & Armstrong (1984)) and the rate of inactivation is slowed by substituting Ba for Ca as the charge carrier. The existence of two populations of Ca channels in GH cells was first suggested by Taraskevich & Douglas (1980). Our evidence in support of this idea confirms and extends the voltage-clamp studies of Armstrong & Matteson (1985) and Matteson & Armstrong (1986) in $GH_3$ cells.

The two types of Ca channels found in GH cells are not a peculiarity of transformed cell lines. Two similar types of Ca channels have recently been identified in freshly dispersed cells or primary cultures of sensory and hippocampal neurones and myocardial atrial and ventricular cells (Brown & Griffith, 1983; Carbone & Lux, 1984*a*, *b*; Fedulova *et al.* 1985; Bossu *et al.* 1985; Nowycky *et al.* 1985; Bean, 1985; Nilius *et al.* 1985). More importantly, evidence for both types of channels can be found in studies with primary cultures of rat anterior pituitary cells as well as posterior pituitary cells. Some anterior pituitary cells contain Ca channels that can sustain spiking activity in Na-free solution (Ozawa & Sand, 1978). Regenerative electrical activity is observed at potentials more negative than −40 mV, suggesting that the transient Ca channels are involved. Ca-dependent spiking activity has also been found in pars intermedia cells of the rat posterior pituitary (Douglas & Taraskevich, 1982). This spiking is inhibited by Co, but not by 100 $\mu$M-nifedipine, again indicating that the transient Ca channels are involved.

High K concentrations cause a maintained increase in hormone secretion in rat anterior pituitary cells that is dependent on bath Ca and is blocked by nimodipine at concentrations comparable to those that block Ca channels in our experiments (Enyeart *et al.* 1985). Hence, the slowly inactivating Ca channels are also present in normal tissue and $GH_4C_1$ cells are likely to indicate faithfully Ca-channel function in normal tissue.

The effects of high K on GH cell lines are similar to those on primary cultures of anterior pituitary cells. High K stimulates Ca influx, increases cell Ca ion concentration, and stimulates hormone secretion in GH cells (Albert & Tashjian, 1984; Tan & Tashjian, 1984*a*; Snowdowne & Borle, 1984). Our studies verify two predictions of these $^{45}Ca$ influx studies. First, GH cells contain Ca channels that are open after many seconds of depolarization. It is worth noting that less inactivation probably

occurs in our experiments than in intact cells because we have minimized Ca-induced inactivation. In intact cells, high K produces a maximal $^{45}Ca$ influx equivalent to about 40 pA/cell in 20 mM-Ca at 37 °C (calculated from the data of Tan & Tashjian (1984*a*)), which is much smaller than the slowly inactivating currents that we measure at about 20 °C (see Fig. 2). A likely explanation is that many Ca channels inactivate within seconds in intact cells. Voltage-clamp studies with GH cells using micro-electrodes also provide evidence for Ca-induced inactivation (Dufy, Dupuy, Georgescauld & Barker, 1984).

Secondly, experiments with high K predicted that GH cells contain Ca channels that bind dihydropyridines with high affinity. Our experiments provide the first electrophysiological evidence for high-affinity Ca-channel block in endocrine cells. The dissociation constant for nimodipine binding to open slowly inactivating Ca channels is very similar to the concentration producing 50% inhibition ($IC_{50}$) of high-K-induced hormone secretion or $^{45}Ca$ influx (Enyeart *et al.* 1985; Shangold, Kongsamut & Miller, 1985). The potency of Ca-channel block also suggests that the high-affinity ligand binding sites for dihydropyridines in pituitary cells are the slowly inactivating Ca channels (Trumble & Kaczorowski, 1983; Titeler, De Souza & Kuhar, 1985).

Although nimodipine can block very potently the effects of high K on GH cells, the drug weakly inhibits basal levels of hormone secretion or the effects of stimulatory peptides, like TRH (Conn, Rogers & Seay, 1983; Enyeart *et al.* 1985). Likewise, therapeutic doses of dihydropyridines have little effect on the secretion of prolactin or growth hormone *in vivo* (Struthers, Millar, Beastall, McIntosh & Reid, 1983; Abadie, Gauville & Brisson, 1984; Isles, Baty & Dow, 1985). Numerous studies have substantiated the importance of Ca channels in mediating both basal and stimulated hormone secretion in GH cells (see Taraskevich & Douglas (1984), Tan & Tashjian (1984*b*) and Enyeart *et al.* (1985) for reviews of the literature), so the selectivity of nimodipine action has not been accounted for. Our results provide a rationale for the selectivity of nimodipine action and indicate that nimodipine can be used to define the role of each type of Ca channel in mediating stimulus–secretion coupling.

The transient Ca channels seem to have a particularly important function in some cells of the anterior pituitary. Anterior pituitary cells are spontaneously active, and this electrical activity promotes Ca entry and hormone secretion. TRH, through an effect on K conductance, promotes Ca entry by increasing the frequency of electrical activity and by prolonging the action-potential duration (Kaczorowski, Vandlen, Katz & Reuben, 1983; Barker *et al.* 1984; Dubinsky & Oxford, 1985). In most striated muscle cells and neurones, Na-channel currents generate the upstroke of the action potential, but some cells of the anterior pituitary have very few Na channels (Taraskevich & Douglas, 1977). The transient channels activate significantly at normal resting potentials (about −50 mV), but the slowly inactivating Ca channels do not. Hence, current through the transient channels initiates the action potential. Nimodipine binds very weakly to the transient Ca channels, so that action potential firing should not be suppressed by drug.

Ca current through the transient channels may also be important for determining basal levels of hormone secretion. As seen in Fig. 4, the curves describing steady-state activation and inactivation overlap. In the voltage range spanned by the overlap,

the transient channels will remain open in the steady state. This voltage range encompasses the normal resting potential, so that transient channels probably conduct at rest. Consequently, much of the basal Ca influx is probably through Ca channels that are insensitive to nimodipine block.

Although our results can account for the lack of effect of nimodipine on basal levels of secretion in GH cells, extension of the explanation to account for *in vivo* drug effects will require further experimentation. The voltage range of Ca-channel activation and inactivation found in our experiments may differ from that of non-transformed cells *in vivo*. Our experiments are conducted at room temperature, in unphysiological bathing solutions, with dialysed cells. These factors can alter the voltage dependence of channel gating from that found in intact cells (Hagiwara & Ohmori, 1982; Fernandez, Fox & Krasne, 1984). Also, if the resting membrane potential of anterior pituitary cells *in vivo* is significantly less than that of GH cells, then steady-state currents through the slowly inactivating Ca channels could be significant, and would then be sensitive to nimodipine block. Indeed, nimodipine partially inhibits basal levels of hormone secretion in primary cultures of rat anterior pituitary cells (Enyeart *et al.* 1985).

If stimulus–secretion coupling in anterior pituitary cells is inhibited by nimodipine with high potency, then the Ca which triggers the process enters the cell through the slowly inactivating Ca channels. However, the converse is not true: Ca influx through the slowly inactivating Ca channels is not always highly sensitive to nimodipine block. High-affinity block occurs only during long-lasting depolarizations. The rate of onset of block depends both on the drug concentration and on the degree of channel activation. Therapeutic concentrations of nimodipine are about 1 nM. (Plasma levels in clinical trials are 10–60 ng $ml^{-1}$ and approximately 2 % of this amount is free (Krol, Noe, Yeh & Raemsch, 1984; Scriabine *et al.* 1985). Since the molecular weight of nimodipine is 418·5, the free concentration is 0·5–3 nM. The concentration in cerebrospinal fluid may be significantly different.) At these concentrations, maximal block will not occur unless the membrane is depolarized for many seconds. The action potential of anterior pituitary cells (as well as that of most neurosecretory cells) lasts only about 10 ms, so the membrane is not depolarized long enough for significant block to develop (see Fig. 5). Even though some Ca influx is through the slowly inactivating Ca channels during spontaneous and neuropeptide-stimulated electrical activity in GH cells, nimodipine is a very weak inhibitor of this Ca movement.

In summary, nimodipine will have little effect on stimulus–secretion coupling in healthy anterior pituitary cells for three reasons: (1) nimodipine is a very weak inhibitor of Ca entry through the transient Ca channels; (2) high-affinity block of the slowly inactivating Ca channels does not occur to a significant extent under physiological conditions; (3) nimodipine does not inhibit the intracellular release of Ca (as evidenced by the lack of effect on TRH-induced secretion, which involves release of cell Ca in response to an increase of inositol triphosphate (Ronning, Heatley & Martin, 1982; Gershengorn, Geras, Spina, Purello & Rebecchi, 1984)).

The effects of nimodipine on Ca channels in GH cells are likely to be representative of the effects of nimodipine and similar dihydropyridines on a wide variety of excitable cells. Both myocardial cells and dorsal root ganglion neurones contain a population of Ca channels that have gating kinetics similar to the transient channels

of GH cells, and that weakly bind 1,4-dihydropyridine derivatives (Bean, 1985; Nilius *et al.* 1985; Nowycky *et al.* 1985; Boll & Lux, 1985). Similar dihydropyridine-insensitive Ca channels are likely to be present in a variety of neurones (Miller, 1985). In addition, high-affinity time- and voltage-dependent block of Ca channels by dihydropyridines has been reported in studies with myocardial cells (Bean, 1984; Sanguinetti & Kass, 1984; Uehara & Hume, 1985) and neuronal cell lines (Kunze *et al.* 1985). High-affinity binding sites for [$^3$H]nimodipine and [$^3$H]nitrendipine similar to those in GH cells are present in neurones, striated and smooth muscle, and other endocrine cells (Triggle & Janis, 1984). Hence, the tissue specificity of nimodipine action derives only in part from the limited distribution of an appropriate receptor.

*Preferential block of open Ca channels*

Studies of nitrendipine block of Ca channels in cardiac ventricular cells led to the conclusion that the kinetics of dihydropyridine binding are critically important in determining the tissue specificity of drug action (Bean, 1984). Since we have reached a similar conclusion working with a very dissimilar tissue, it is likely that this feature of drug action is generally important.

Our interpretation of the mechanism of action of dihydropyridines differs from most previous reports by attributing the high-affinity drug binding to channels in the open state, rather than in the inactivated state. The steep voltage dependence of steady-state block suggested that drug binding is strongly modulated by channel gating. The simplest formulations of the modulated receptor theory predict that the occupancy of the high-affinity state should have the same steepness with respect to voltage (the slope factor $k$ in eqn. (1)) as does channel block. The voltage dependence of activation satisfies this requirement, but other gating states might do so as well. However, the occupancy of the high-affinity state must also increase in parallel with the rate of channel block, so the voltage at which occupancy of the high-affinity state is half-maximal is also defined. The voltage dependence of the rate of block, in conjunction with the voltage dependence of steady-state block, completely specifies the voltage dependence of occupancy of the high-affinity state. Channel activation has the same voltage dependence.

There is substantial Ca channel block by 30 nM-nimodipine at potentials where few channels are open (see Fig. 7). Hence, our modelling makes the assumption that the Boltzmann distribution that describes channel activation for $V > -40$ mV is also applicable to more negative potentials. The model for drug binding predicts that this assumption could have been avoided if we studied block by much lower concentrations of nimodipine. However, the time required for attaining binding equilibrium would become prohibitively long ($> 2$ min for $V < -40$ mV in 3 nM-nimodipine), so that run-down of Ca channel currents would obscure the drug effects (see Methods). An advantage of our model is that it allowed us to measure $K_O$ without using subnanomolar concentrations of drug. Earlier applications of the modulated receptor theory also made use of this trick (Bean, Cohen & Tsien, 1983; Bean, 1984; Sanguinetti & Kass, 1984.

Subnanomolar concentrations of nimodipine have been used with other techniques for studying Ca channels in anterior pituitary cells, and the binding constants obtained from these studies indicate that our model yields feasible values for $K_O$. The

value for $K_O$ is in good agreement with the value for $IC_{50}$ for block of high-K-stimulated $^{45}Ca$ influx and with the $K_D$ for binding of tritiated dihydropyridines to anterior pituitary cells (Trumble & Kaczorowski, 1983; Enyeart *et al.* 1985; Shangold *et al.* 1985; Titeler *et al.* 1985). The value for the association rate ($f$) is very fast, but it is reasonable for a very lipophilic compound. To our knowledge, the binding rate for nimodipine is the fastest rate reported for an organic molecule. Other studies of open-channel block by neutral molecules have indicated somewhat slower rates of binding. For example, benzocaine binds to open motor end-plate channels at about $2 \times 10^6$ M$^{-1}$ s$^{-1}$ (Ogden, Siegelbaum & Colquhoun, 1981) and to open Na channels at $> 10^7$ M$^{-1}$ s$^{-1}$ (estimated from Fig. 5 of Neumcke, Schwarz & Stampfli (1981)). The binding rate for nimodipine is nearly $10^8$ M$^{-1}$ s$^{-1}$. However, the partition coefficient of nimodipine between sarcolemmal membrane and aqueous solution is 4100 (Rhodes, Sarmiento & Herbette, 1985). Hence, the 'on' and 'off' rates for nimodipine binding to open Ca channels represent the rates of movement within the lipid bilayer. For the diffusion of nimodipine in two dimensions, binding rates of up to $10^{12}$ M$^{-1}$ s$^{-1}$ are feasible (Rhodes *et al.* 1985).

In ligand binding experiments with [$^3$H]nimodipine, the binding rate is due to both the aqueous diffusion of drug until contacting the lipid bilayer, followed by two-dimensional diffusion within the bilayer. The rate of [$^3$H]nimodipine binding to high-affinity sites ($1{\cdot}0 \times 10^7$ M$^{-1}$ s$^{-1}$ at 25 °C in cardiac sarcolemma) is much slower than our value, suggesting that ligand binding is rate-limited by aqueous diffusion (Rhodes *et al.* 1985; see Triggle & Janis (1984) for a review of dihydropyridine ligand binding rates). This result was predicted from theoretical considerations (Rhodes *et al.* 1985). Our studies indicate that high-affinity ligand binding of dihydropyridines was correctly identified as binding to Ca channels, but that the kinetic studies may not accurately measure the 'on' and 'off' rates for the channel. The rates of binding of nitrendipine, nisoldipine or nifedipine to Ca channels in myocardial cells are much slower than the rate of nimodipine binding in $GH_4C_1$ cells (Bean, 1984; Sanguinetti & Kass, 1984; Gurney *et al.* 1985). Hence, the ligand binding rates for these drugs may be more reliable than those for nimodipine.

Our results are not inconsistent with high-affinity binding to the inactivated state. However, it is not necessary for channels to inactivate before high-affinity binding can occur. Rather, our results indicate that binding is enhanced by activation, and whether or not the channels subsequently inactivate has little effect on the affinity for drug. Two observations in GH cells support this idea: (1) in some experiments, we found that the slowly inactivating Ca channels inactivated substantially, yet the steady-state voltage dependence of nimodipine block was virtually unchanged from that shown in Fig. 6; (2) in intact GH cells, there is probably substantial Ca-induced inactivation, yet the inhibition of high-K-stimulated $^{45}Ca$ influx is similar to that in our studies, where inactivation is removed.

It is difficult to account for the time and voltage dependence of nimodipine block with models that postulate high-affinity binding to resting or hypothetical pre-activated states. Our interpretation of the mechanism of action of dihydropyridines thus differs from that of Gurney *et al.* (1985), who concluded that Ca channel block by nifedipine in ventricular cells at $V_h = -50$ mV represents binding to channels in the resting state. Although nearly all channels are in the resting state at this potential

in the absence of drug, our results indicate that nimodipine binds with very high affinity to a small fraction of open channels. If drug binding is to channels in the resting state, then binding at more negative potentials ($V_h = -90$ mV) should be approximately the same as for $V_h = -50$ mV (assuming that $K_R$ is not voltage dependent, which is reasonable for a permanently neutral molecule). We and others studying related dihydropyridines find less block at more negative holding potentials (see Fig. 6). In a single experiment with 30 nM-nifedipine using a $GH_3$ cell, we found no detectable resting-state block and an apparent $K_O = 0{\cdot}44$ nM, so differences in molecular structure are unlikely to account for our differing interpretations. However, differences in preparation may be significant: Gurney *et al.* (1985) specifically tested for open channel block by nifedipine and found the value of $K_O$ was significantly larger than that in our study.

It is also unlikely that our results can be accounted for by a model that postulates preferential binding to a pre-activated state intermediate to the resting and open states. The work of Hagiwara & Ohmori (1982, 1983) indicates that one or more pre-activated states exist in GH cells, although it was not necessary to consider this state explicitly in our modelling. In $GH_3$ cells nearly all slowly inactivating Ca channels are open at very positive potentials (Hagiwara & Ohmori, 1982) and the same is likely to be true for $GH_4C_1$ cells. If nimodipine binds primarily to channels in a pre-activated state, then only a few per cent of the channels are in the high-affinity state at positive potentials. In order to account for the fast rate of block at +30 mV, one would need to postulate binding to a few per cent of the channels in the pre-activated state with a dissociation constant much smaller than 500 pM and/or with a binding rate much faster than $10^8$ $M^{-1}$ $s^{-1}$.

Studies with dihydropyridines in myocardial cells provide evidence for high-affinity open-channel block similar to that found in GH cells. In ventricular cells and Purkinje fibres, high concentrations of nitrendipine or nisoldipine speed the rate of decay of the Ca current during test pulses of several hundred milliseconds (Lee & Tsien, 1983; Sanguinetti & Kass, 1984). Nimodipine has a similar effect in GH cells (see Fig. 11), which is accounted for by high-affinity drug binding to open channels.

The steady-state voltage dependence of Ca-channel block by dihydropyridines in Purkinje fibres and atrial cells is described by a two-state Boltzmann distribution with the same slope factor as channel inactivation (Sanguinetti & Kass, 1984; Uehara & Hume, 1985). Although this result suggests that drug block is proportional to the occupancy of the inactivated state, it is also consistent with binding to the open state because activation has a voltage dependence similar to that of inactivation. In Purkinje fibres, activation is described by an $m_\infty$ curve with a slope factor similar to that for inactivation (compare Fig. 10 of Kass & Sanguinetti (1984) with Fig. 10 of Sanguinetti & Kass (1984)). The mid-points of the activation and inactivation curves are unequal, so the two models predict different binding affinities. The voltage dependence of the rate of onset of block could distinguish between the two alternatives, but this has not been reported.

In atrial cells, as in GH cells, nearly all inactivation of the dihydropyridine-sensitive Ca channels is Ca induced (Mentrard, Vassort & Fischmeister, 1984; Bechem & Pott, 1985), so the voltage dependence of inactivation derives largely from the voltage dependence of activation. Ca channels in atrial cells are still sensitive to block by

dihydropyridines when inactivation is mostly eliminated by using Ba as a charge carrier and by buffering cell Ca to low levels (Bean, 1985).

In the light of our studies in GH cells, we think that the mechanism of Ca-channel block in myocardial cells should be re-evaluated. Earlier studies led to the suggestion that Ca channels must inactivate before high-affinity binding of dihydropyridines can occur (Bean, 1984; Sanguinetti & Kass, 1984; Uehara & Hume, 1985; Kunze *et al.* 1985). It seems likely that the mechanism of drug action in myocardial cells is similar to that in GH cells: binding is enhanced by activation, and whether or not the channels subsequently inactivate has little effect on the affinity for drug. In some cells, like ventricular cells, inactivation develops very rapidly at most potentials, so that drug binding is mostly to inactivated channels during a long-lasting depolarization (but see Cavelie, Ochi, Pelzer & Trautwein (1983) for examples of incomplete inactivation in ventricular cells). In other cells, such as atrial cells, inactivation goes to completion over a very limited voltage range, so that high-affinity binding to open channels can occur during maintained depolarizations.

The mechanism of Ca-channel block by some other organic molecules also needs to be re-evaluated. Since inactivation often does not go to completion during a long-lasting depolarization, and since drug binding to open channels may increase slowly, one cannot conclude that drug block during maintained depolarizations must represent binding to inactivated channels. This assertion has been made for diltiazem (Kanaya, Arlock, Katzung & Hondeghem, 1983; Uehara & Hume, 1985), but is probably not generally true. Inactivation of Ca channels is reduced at very positive potentials in atrial cells because of smaller Ca influx, yet diltiazem block monotonically increases with test potential (Uehara & Hume, 1985). Preferential block of activated channels can account best for this result. Verapamil, gallopamil (D600) and pentobarbitone also preferentially block open Ca channels (Nishi & Oyama, 1983; McDonald, Pelzer & Trautwein, 1984; Sanguinetti & Kass, 1984).

*The therapeutic use of nimodipine*

An interesting parallel exists between the block of Ca channels by nimodipine and the block of Na channels by lidocaine. Lidocaine binds to Na channels in nerve, skeletal muscle and cardiac muscle with about the same affinity, and the binding affinity is modulated by channel gating. Na-channel block is very potent only in depolarized tissue, so that the drug preferentially affects ischaemic tissue (see Bean *et al.* (1983) for a brief review). Likewise, nimodipine should specifically suppress Ca entry in ischaemic tissue, with little effect on healthy, well polarized cells.

Nimodipine can reduce the neuronal damage caused by cerebral ischaemia or subarachnoid haemorrhage (see Scriabine *et al.* (1985) for a review). The therapeutic efficacy has usually been attributed to vasodilatation of cerebral arteries, but in light of our studies direct effects on neuronal and/or neuroendocrine cells seem likely. High-affinity binding sites for nimodipine are present throughout the brain, and they are probably Ca channels that mediate stimulus–secretion coupling, as in $GH_4C_1$ cells (Triggle & Janis, 1984; Turner & Goldin, 1985). During cerebral ischaemia or spreading cortical depression, extracellular K can rapidly increase to 50 mM or more, thereby causing Ca entry during long-lasting depolarizations (Hansen, 1985). Nimodipine is likely to block potently Ca entry under these pathological conditions, but not affect Ca entry under normal physiological conditions.

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