

# Nimodipine Block of Calcium Channels in Rat Vascular Smooth Muscle Cell Lines

## *Exceptionally High-Affinity Binding in A7r5 and A10 Cells*

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**ABSTRACT** Calcium channel currents were studied in the A10 and A7r5 cell lines derived from rat thoracic aorta muscle cells. The whole-cell variation of the patch voltage clamp technique was used. Results with each cell line were nearly identical. Two types of Ca channels were found in each cell line that are similar to the L-type and T-type Ca channels found in excitable cells. Nimodipine block of the L-type Ca channels in both cell lines is more potent than in previously studied tissues. The kinetics of nimodipine block are accounted for by a model that postulates 1:1 drug binding to open Ca channels with an apparent dissociation constant ( $K_O$ ) of 16–45 pM. In A7r5 cells, 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 ( $f$ ) is  $1.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ; the dissociation rate ( $b$ ) is about  $0.024 \text{ s}^{-1}$ . In anterior pituitary cells (GH<sub>4</sub>C<sub>1</sub> cells),  $K_O$  is 30 times larger;  $b$  is only twice as fast, but  $f$  is 15 times slower. The comparative kinetic analysis indicates that the high-affinity binding site for nimodipine is similar in both GH<sub>4</sub>C<sub>1</sub> and A7r5 cells, but nimodipine diffuses much faster or has a larger partition coefficient into the plasmalemma of A7r5 cells than for GH<sub>4</sub>C<sub>1</sub> cells. Unusually high-affinity binding was not observed in earlier <sup>45</sup>Ca flux studies with A10 and A7r5 cells. The model of open channel block accounts for the discrepancy; only a small fraction of the Ca channels are in the high affinity open state under the conditions used in <sup>45</sup>Ca flux studies, so an effective binding constant is measured that is much greater than the dissociation constant for high-affinity binding.

### INTRODUCTION

Voltage clamp measurements of Ca currents in vascular smooth muscle cells can provide the most direct measure of Ca channel gating and block. The patch electrode voltage clamp technique overcomes the difficulties associated with earlier voltage clamp studies with vascular smooth muscle, but requires the use of dissociated

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cells (Bolton et al., 1981; Marty and Neher, 1983). Unfortunately, freshly dispersed vascular smooth muscle cells are relatively difficult to prepare and are not available in large enough quantities for routine biochemical studies. In the hope of obtaining a vascular smooth muscle preparation that could be routinely used for electrophysiological and biochemical studies of Ca channels, we have studied Ca channels in the A10 and A7r5 cell lines derived from rat thoracic aorta muscle cells. These cell lines were chosen for this study because each has retained a number of properties characteristic of aortic muscle cells. The cell morphology is typical of vascular smooth muscle and the cells contain  $V_1$  vasopressin, atriopeptin II,  $\beta_2$ -adrenergic, and serotonin 2 receptors and the MM isoenzyme of creatinine phosphokinase (Kimes and Brandt, 1976; Baker et al., 1985; Doyle and Ruegg, 1985; Heckman et al., 1985; Aiyar et al., 1986; Doyle et al., 1986; Nambi et al., 1986; Napier et al., 1986; Neuser and Bellemann, 1986). As for intact aortic muscle, high K increases the influx of  $^{45}\text{Ca}$  in A10 and A7r5 cells and some 1,4-dihydropyridines are potent inhibitors of this increased influx (Kongsamut et al., 1985; Ruegg et al., 1985). A10 and A7r5 cells have two types of Ca channels similar to those subsequently found in cells from mesenteric and ear arteries and azygous, portal and saphenous veins (Bean et al., 1986; Friedman, et al., 1986; Loirand et al., 1986; Sturek and Hermsmeyer, 1986; Benham et al., 1987; Yatani et al., 1987; Fish et al., 1988). In addition, BAY k 8644 modifies Ca channel gating in A10 cells and ear artery muscle cells in a similar manner (Fox et al., 1986; Benham et al., 1987; Hering et al., 1988).

A major goal of these experiments was to determine whether block of Ca channels by 1,4-dihydropyridine derivatives in vascular muscle cell lines differs from the block in other types of excitable cells. Nimodipine and its congeners are thought to preferentially affect vascular smooth muscle *in vivo* by blocking Ca channels. However, there is little evidence that the tissue specificity of drug action occurs because Ca channels in vascular smooth muscle bind these drugs with especially high affinity. High-affinity block of Ca channels by nimodipine, nitrendipine, nisoldipine or nifedipine is similar in vascular muscle, myocardial and anterior pituitary cells (Bean, 1984; Sanguinetti and Kass, 1984; Uehara and Hume, 1985; Gurney et al., 1985; Brown et al., 1986; Bean et al., 1986; Cohen and McCarthy, 1987; Simasko et al., 1988). Ligand binding studies also indicate similar high affinity binding sites for 1,4-dihydropyridine derivatives in many different tissues (Triggle and Janis, 1984; Godfraind et al., 1986). To our surprise, we found that the high-affinity binding of nimodipine to L-type Ca channels in A10 and A7r5 cells is about 20 times more potent than in previously studied tissues. This result raised a number of questions that we address in this article: (a) Is the model used to calculate the binding affinity of nimodipine for Ca channels correct? (b) Are the L-type Ca channels in A10 and A7r5 cells structurally different with respect to nimodipine binding from similar channels in other cell types? and (c) Why do voltage clamp studies of Ca channel block differ from studies of  $\text{K}^+$ -induced  $^{45}\text{Ca}$  influx?

The first part of the Results will show that tail current analysis can be used to separate and quantitate current through two types of Ca channels in A10 and A7r5 cells. The second part of the Results will characterize the block of the Ca channels by nimodipine. A preliminary report of this work has been published (McCarthy and Cohen, 1987).

## METHODS

*Cell Culture*

The A10 and A7r5 cell lines were obtained from the American Type Culture Collection, Rockville, MD (cell repository lines 1476 and 1444, respectively). Both cell lines were derived from rat thoracic aorta (Kimes and Brandt, 1976). Cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. Cells were plated on glass coverslips and used 1–4 d after plating. Best results were obtained when the cells were used on the third day after plating. A10 cells divided much more slowly than A7r5 cells and had to be plated at a higher density.

*Solutions and Drugs*

The bath solution used for recording Ca channel currents contained (in millimolar): tetraethylammonium Cl 117; BaCl<sub>2</sub> 20 or CaCl<sub>2</sub> 20; MgCl<sub>2</sub> 0.5; dextrose 5; sucrose 32; HEPES 10; tetrodotoxin  $2 \times 10^{-4}$ ; pH 7.5 (adjusted with CsOH). Ba was used as the charge carrier for all of the experiments described in the figures. Bath solutions were gassed with 100% oxygen and maintained at room temperature (20–23°C). The patch pipettes were filled with a solution designed to eliminate K channel currents and to slow rundown of Ca currents. The composition usually was (in millimolar): CsCl 108; tetrabutylammonium Cl (TBA-Cl), 10; 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) 11; CaCl<sub>2</sub> 0.9; MgCl<sub>2</sub> 6; Na<sub>2</sub>-ATP 5; HEPES 20; pH 7.2 (adjusted with CsOH). This solution is identified in the text as high ATP-BAPTA solution. In some early experiments, the pipette solution was (in millimolar): CsCl 130; TBA-Cl 10; EGTA 11; CaCl<sub>2</sub> 0.9; MgCl<sub>2</sub> 2; Na<sub>2</sub>-ATP 1; HEPES 10; pH 7.2. Nimodipine (BAY E 9736) was synthesized by Bayer AG and stored as 1 mM stock solutions in polyethylene glycol 400. Sodium vapor lights were used during experiments 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*

The methods for patch voltage clamp experiments used in our laboratory have been described previously (Cohen and McCarthy, 1987). Patch electrodes were fabricated from No.7052 glass (Corning Glass Works; Corning, NY). A List EPC/7 patch clamp amplifier was used to record whole-cell currents (Medical Systems Corp., Greenvale, NY). Membrane current was sampled at 10 or 20 kHz and filtered with an eight-pole low-pass Bessel filter with a cutoff frequency (–3 dB) of 2.5 or 5 kHz, respectively. Linear leak and capacity currents were subtracted digitally by appropriately scaling a test pulse from –90 to –110 mV. L-type Ca channels deactivate very rapidly, but tail current measurements could be reliably made in A10 and A7r5 cells because the cells are relatively small ( $\approx 15 \mu\text{m}$  in diameter), nearly spherical and the currents measured were small (typically  $< 300 \text{ pA}$ ). The rate of deactivation was slowed by using fairly positive repolarization potentials (usually –45 or –55 mV) and by carefully compensating for series resistance. Nonlinear least squares curve fitting was performed using the Patternsearch algorithm (Colquhoun, 1971).

The studies were initiated with A10 cells because earlier reports indicated that these cells contain dihydropyridine-sensitive Ca channels (Fox et al., 1986; Friedman et al., 1986). We subsequently found that A7r5 cells are easier to grow and better suited to whole-cell patch electrode voltage clamp measurements of Ca channel currents. The gating of Ca channels and block by nimodipine is virtually identical in these two cell lines.

## RESULTS

*A10 and A7r5 Cells Have Two Types of Calcium Channels*

The results shown in Figs. 1 and 2 indicate that A10 and A7r5 cells have two populations of Ca channels that can be distinguished by their rate of deactivation. As in some other types of excitable cells, the T-type (or low threshold) Ca channels deactivate slowly when the membrane is repolarized and the L-type (or high threshold)

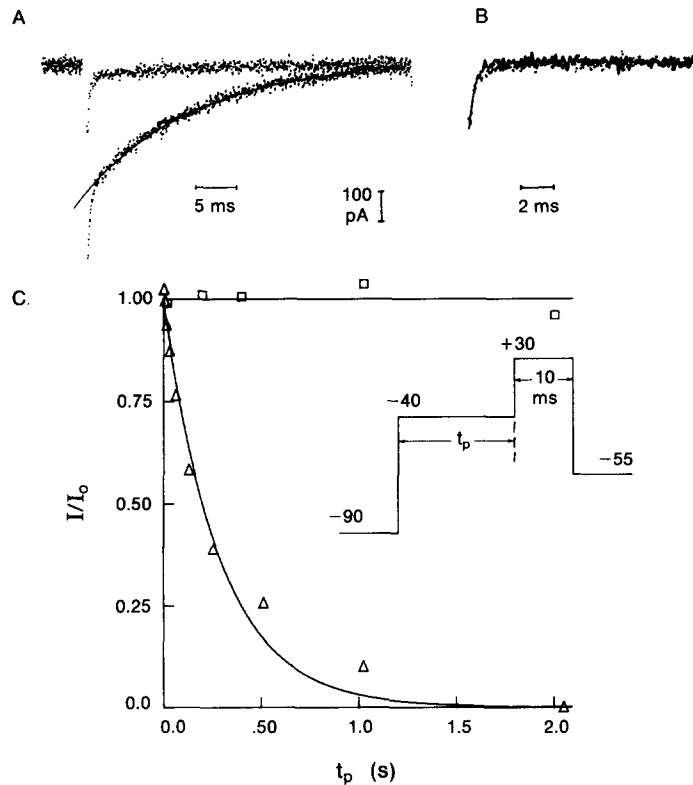


FIGURE 1. A7r5 cells have two components of tail current through Ca channels that differ in the voltage dependence of inactivation. The rate of inactivation of Ca channels was measured using the pulse protocol shown in the inset to panel C. (A) Superimposed tail current records made when the membrane potential was repolarized to  $-55$  mV for prepulse durations ( $t_p$ ) of 20 ms and 2.0 s. The tail current was biexponential when  $t_p = 20$  ms, but had only one rapidly deactivating exponential component when  $t_p = 2.0$  s. (B) The same current records as shown in A after the slowly decaying exponential component was digitally subtracted from each current record. (C) A plot of the relative amplitude of each component of tail current vs. the prepulse duration. The triangles indicate the amplitude of the slowly decaying exponential component and the squares indicate the amplitude of the rapidly decaying exponential component. Current was sampled at 20 kHz and filtered with  $f_c = 2.5$  kHz. The first 450  $\mu$ s after repolarization has been blanked from each current record. See text for further details.

Ca channels deactivate rapidly (Cota, 1986; Matteson and Armstrong, 1986; Carbone and Lux, 1987; Cohen et al., 1988; Hiriart and Matteson, 1988).

Fig. 1 shows that A7r5 cells have two components of tail current that differ in the voltage dependence of inactivation. The pulse protocol used to measure the rate of inactivation is shown in the inset to C. Fig. 1 A shows an example of the tail current measurements made when the membrane was repolarized to  $-55$  mV. Records for prepulse durations of 20 ms and 2.0 s are superimposed. For the 20-ms prepulse, the tail current was biexponential. An exponential with a time constant of 13.29 ms was fit to the slow component. When the prepulse lasted 2.0 s, the tail current could be fit by a single rapidly decaying exponential. Fig. 1 B shows that the amplitude of the fast component of tail current was unchanged when the prepulse duration was increased from 20 ms to 2 s. The slow exponential component of tail current (the solid curve shown in A) was digitally subtracted from the total tail current measured after a 20-ms prepulse and the remaining fast component is shown superimposed with the tail current measured after a 2-s prepulse. Fig 1 C shows a plot of the amplitude of the two components of tail current as a function of prepulse duration ( $t_p$ ). The amplitude of the slow component is indicated by triangles and the solid curve through this data is a nonlinear least squares fit to a single exponential with a time constant of 288 ms. For each  $t_p$ , the amplitude of the fast exponential component of tail current was determined by peeling the slow exponential component from the tail current measurement and by fitting an exponential with a time constant of 0.54 ms to the remainder. These amplitudes are indicated by squares in C. There was little or no inactivation of the fast component of tail current. Hence, only the slow component of tail current inactivates rapidly at  $-40$  mV, consistent with the idea that the slow component of tail current indicates the instantaneous conductance of the T-type Ca channels. The fast component of tail current likewise indicates the instantaneous conductance of the L-type Ca channels.

Fig. 2 shows the use of tail current analysis to determine the voltage dependence of activation of each component of Ca channel current in A10 cells. The top left panel shows the pulse protocol used to measure the voltage dependence of activation of the L-type Ca channels and the corresponding tail current measurements. Current through the T-type Ca channels was reduced by using a holding potential of  $-55$  mV (in addition, this cell had very few T-type Ca channels). Each of the tail currents was well fit by an exponential with a time constant of 0.38 ms. Stronger depolarizations elicited tail currents of greater amplitude. The top right panel shows tail current measurements made from a different cell using a more negative holding potential so that the T-type Ca channels were available to open during the test pulses. A slow component of the tail current is now evident which was fit by an exponential with a time constant of 15.1 ms. The bottom panel shows that the components of tail current differ in the voltage dependence of activation. The amplitude of each component of tail current is plotted vs. test potential ( $V_t$ ). The open triangles indicate the normalized amplitude of the slow component of tail current and each point represents the mean value for three experiments. The solid curve through this data is a nonlinear least squares fit to a two-state Boltzmann distribution with midpoint ( $V_m$ ) at  $-17.0$  mV and slope factor ( $k$ ) of 11.76. The filled squares indicate the mean relative amplitude of the fast component of tail current

for three experiments. The activation curve describing this component of Ca channel current is described by  $V_m = +1.6$  mV and  $k = 13.93$ . Similar results were obtained with A7r5 cells (see Fig. 8).

The slow component of tail current measured after a strong test depolarization is unaffected by substituting Ca for Ba, but the amplitude of the fast component of

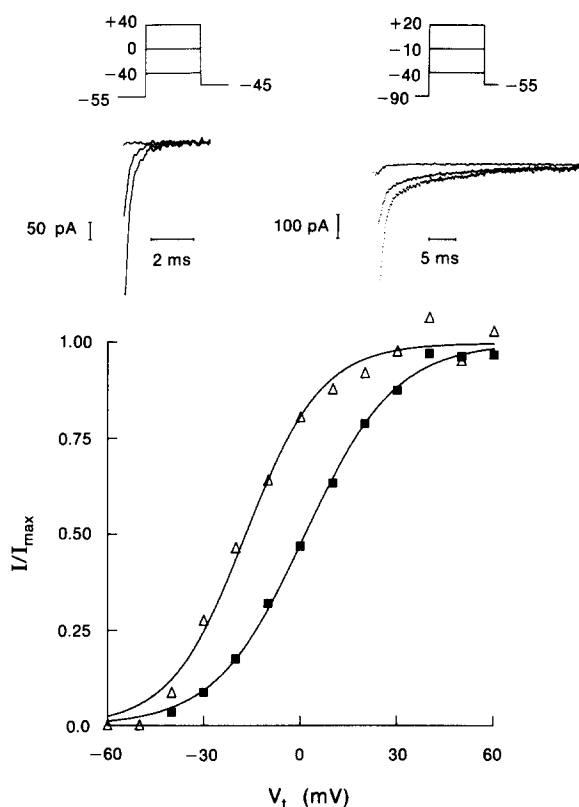


FIGURE 2. A10 cells have two components of tail current through Ca channels that differ in the voltage dependence of activation. *Upper left panel:* Superimposed tail current measurements following 20 ms test pulses to  $-40$ ,  $0$  and  $+40$  mV. Current was sampled at 20 kHz and filtered with  $f_c = 5$  kHz. The first  $400 \mu\text{s}$  after repolarization has been blanked from each current record. *Upper right panel:* Superimposed tail current measurements following 10 ms test pulses to  $-40$ ,  $-10$ , and  $+20$  mV. Each current record is the average of two measurements. Current was sampled at 10 kHz and filtered with  $f_c = 2.5$  kHz. The first  $200 \mu\text{s}$  after repolarization has been blanked from each current record. *Lower panel:* The normalized amplitude of each exponential component of tail current is plotted vs. test po-

tential. Each data point represents the mean of three experiments. ( $\Delta$ ) Amplitude of the slowly decaying component. The solid curve through this data is a nonlinear least squares fit to the equation  $I/I_{\max} = \{1 + \exp [(V_m - V_t)/k]\}^{-1}$  with  $V_m = -16.96$  mV and  $k = 11.76$ . ( $\blacksquare$ ) Relative amplitude of the rapidly decaying component of tail current. The solid curve through this data is defined by the same equation with  $V_m = +1.56$  and  $k = 13.93$ . For  $V_t < -30$  mV, the time to peak current was  $>10$  ms, so that activation of the T-type Ca channels was underestimated in this voltage range.

tail current is decreased (data not shown). This result is most easily explained by invoking two populations of Ca channels with differing ionic selectivities (Matteson and Armstrong, 1986; Hiriart and Matteson, 1988).

Many laboratories have inferred the properties of Ca channels in vascular smooth muscle cells from studies of high K induced vasoconstriction and  $^{45}\text{Ca}$  influx. Ca

channels are assumed to be involved because high K depolarizes the cells and increases  $^{45}\text{Ca}$  uptake and vasoconstriction is eliminated by removing external Ca (Johansson and Somlyo, 1980). In these studies we infer a maintained influx of Ca during depolarization: (a) vasoconstriction can be initiated by adding Ca to a preparation that has been depolarized by high K for many minutes (Hinke et al., 1964); (b)  $^{45}\text{Ca}$  influx is stimulated by high K for many minutes (Van Breeman et al., 1972); and (c) nimodipine and other Ca channel blockers can relax an artery that has been contracted by high K for hours (Towart, 1981). If A10 and A7r5 cells have Ca channels that are characteristic of aortic smooth muscle, then there should be a noninactivating component of Ca channel current that is blocked by 1,4-dihydropyridines with high affinity. The results of Fig. 1 indicate that the L-type Ca channels may provide this current pathway in A7r5 cells. Fig. 3 shows a more stringent test for a noninactivating component of Ca channel current in an A10 cell. In this experiment, there was little or no inactivation of the L-type Ca channels during a 14-s test pulse to 0 mV. The partial decay of the current during the first 500 ms of depolar-

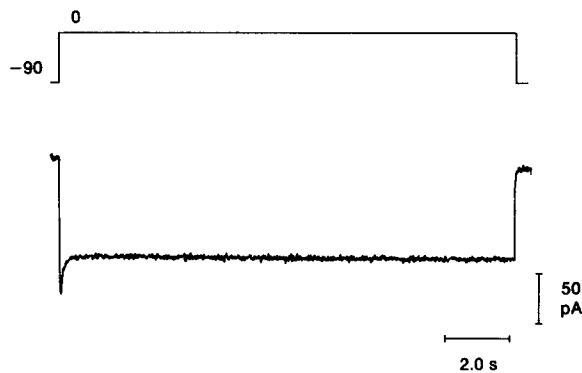


FIGURE 3. Steady-state current through the L-type Ca channels in an A10 cell. The current measured during a 14-s test pulse from  $-90$  to  $0$  mV is shown. The current decay during the first second is primarily due to inactivation of the T-type Ca channels. Current was sampled at 66.7 Hz. This cell was atypical in that currents with  $20$  mM Ba as the charge carrier were usually much smaller than  $100$  pA.

ization was probably due to inactivation of the T-type Ca channels. Note that in this experiment, Ca induced inactivation was minimized by using Ba as the charge carrier and by buffering intracellular Ca to  $\sim 10$  nM.

#### *Nimodipine Binds to Open L-Type Ca Channels with High Affinity*

In this section, we will characterize the time and voltage dependence of nimodipine block of L-type Ca channels in A10 and A7r5 cells. Our earlier study of nimodipine block of Ca channels in  $\text{GH}_4\text{C}_1$  cells served as a paradigm for this part of the study (Cohen and McCarthy, 1987). The studies with  $\text{GH}_4\text{C}_1$  cells lead to the following predictions: (a) nimodipine preferentially blocks the L-type Ca channels; (b) steady-state block of the L-type Ca channels is time and voltage dependent, so that maximal block occurs during maintained depolarizations; and (c) the time and voltage dependence of nimodipine block of the L-type Ca channels can be accounted for by a sequential model that postulates that channels must open (activate) before high-affinity binding can occur. A test of these predictions is shown in Figs. 4–7.

Fig. 4 shows a striking parallel between the voltage dependence of nimodipine block and the voltage dependence of activation of the L-type Ca channels in an A7r5 cell. The filled triangles show the effect of 30 nM nimodipine on the steady state availability of the L-type Ca channels. The pulse protocol is indicated at the top left of the figure. Drug binding was allowed to equilibrate at the prepulse potential ( $V_p$ ) for 30 s. The fast component of the tail current indicated the instantaneous

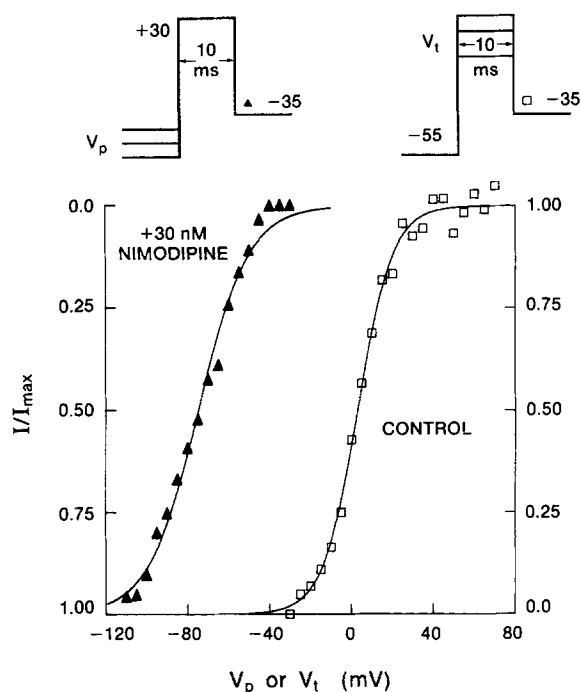


FIGURE 4. Steady-state voltage dependence of nimodipine block of the L-type Ca channels in an A7r5 cell vs. the voltage dependence of Ca channel activation. The steady-state availability of the L-type Ca channels in 30 nM nimodipine was determined using the experimental protocol shown in the upper left panel. The prepulses to  $V_p$  were 30 s in duration. The normalized tail current amplitudes are plotted vs.  $V_p$  ( $\blacktriangle$ ). The ordinate for this plot is inverted and at the left of the figure. The solid curve through these data points is a nonlinear least squares fit to the equation  $I/I_{\max} = \{1 + \exp [(V_p - V_{1/2})/k']\}^{-1}$ , with  $V_{1/2} = -74.6$  mV and  $k' = 12.29$ . The voltage dependence of activation of the L-type Ca channels was determined using the same pulse protocol as in Fig. 2. Tail currents at  $-35$  mV after a 10-ms test pulse were fit by a single rapidly decaying exponential. The normalized tail current

amplitude is plotted vs. the test potential ( $\square$ ). The ordinate for this plot is on the right. The solid curve through these data points is a nonlinear least squares fit to the equation  $I/I_{\max} = \{1 + \exp [(V_m - V_t)/k]\}^{-1}$ , with  $V_m = +3.2$  mV and  $k = 8.57$ . For  $V_t < -30$  mV, the time to peak current was  $>10$  ms, so that steady-state activation of the L-type Ca channels was underestimated. See text for further details.

conductance of the L-type Ca channels at the end of the test pulse to  $+30$  mV. The relative amplitude of the fast component of the tail current is plotted vs.  $V_p$ . The ordinate for this plot is shown at left; note that the range has been inverted from normal. In the absence of drug, there is little or no inactivation for  $V_p < -30$  mV (as shown in Figs. 1 and 3). Hence, this data indicates the voltage dependence of Ca channel block by 30 nM nimodipine.



The open squares indicate the voltage dependence of activation. The pulse protocol is indicated at the top right of the figure and channel activation was assayed by tail current analysis, as in Fig. 2. The relative amplitude of the fast component of tail current is plotted vs. test potential ( $V_t$ ) and the ordinate for this plot is at the right of the figure. The solid curve through the data points is a nonlinear least squares fit to the equation:

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

with  $V_m = +3.2$  mV and  $k = 8.57$ .

The effect of nimodipine on channel availability is described by a similar equation:  $I/I_{\max} = \{1 + \exp[(V_p - V_{1/2})/k']\}^{-1}$ . A nonlinear least squares fit to this equation (shown as a solid curve) indicated  $V_{1/2} = -74.6$  mV and  $k' = 12.3$ . The parallel between the two curves in Fig. 4 indicates that the fraction of channels blocked by nimodipine is directly proportional to the fraction of open channels. This result suggests that nimodipine preferentially binds to open (activated) Ca channels.

As in previous studies with  $\text{GH}_4\text{C}_1$  cells, a simple four-state model can quantitatively account for the steady-state voltage dependence of nimodipine block (Cohen and McCarthy, 1987). In the absence of drug, channels are either in the rested (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 A10 and A7r5 cells may require more than one rested state, the effects of nimodipine can be accounted for with only one. An inactivated state is not explicitly included in this model. The amount of inactivation was quite variable, but generally was negligible for  $V < -40$  mV and appreciable for  $V > 0$  mV. As will be shown below, it is reasonable to ignore drug binding to inactivated channels because the rate of inactivation is much slower than the rate of channel block for the drug concentrations that were studied.

The equilibrium between R and O is defined by the  $m_\infty$  curve of Fig. 4:  $K_m = [\text{O}]/[\text{R}] = m_\infty/(1 - m_\infty) = \exp((V - V_m)/k)$ , where symbols in brackets indicate the fractional occupancy of a state. At more negative holding potentials, more of the channels are in the rested state and nimodipine block is reduced. Hence, the dissociation constant for drug binding to channels in the rested state ( $K_R$ ) must be greater than the dissociation constant for binding to open channels ( $K_O$ ). The data shown in Fig. 4 can be used to obtain values for  $K_R$  and  $K_O$ . As shown previously,

$$\Delta V = -k \ln [(D/K_O - D/K_R)/(1 - D/K_R)(1 + K_m)], \quad (2)$$

where  $\Delta V$  is the difference between the midpoints of the two curves of fig. 4. For  $V < -40$  mV,  $1 + K_m \approx 1$  (i.e.,  $[\text{O}] \ll [\text{R}]$  before the test pulse to +30 mV). For the experiment in Fig. 4,  $D = 30$  nM,  $\Delta V = V_{1/2} - V_m = -77.8$  mV, and  $k = 10.4$  (the average slope factor for the two curves).

$K_R$  is determined by measuring the amount of block after a strong hyperpolarization ( $V_p = -110$  mV), so that binding occurs when all channels are in R or RD. The number of drug-free channels was assayed by a 10-ms test pulse that maximally activated the channels, followed by a tail current measurement. Unfortunately, drug binding increases very rapidly during the test pulse (see Fig. 5), so that the amount of block before the pulse is overestimated. It is not feasible to vary the test pulse duration and extrapolate the amount of block back to the beginning of the test

pulse (as in Fig. 11 of Cohen and McCarthy, 1987) because the rate of nimodipine block will be comparable to the rate of channel activation when drug concentrations comparable to  $K_R$  are used. A lower limit for  $K_R$  can be obtained by ignoring drug block during the test pulse, so that  $K_R > 300$  nm. Fortunately, the value of  $K_O$  varies very little for  $K_R > 300$  nm, so we chose  $K_R = 1$   $\mu$ M. Solving Eq. (2) for  $K_O$ , we find  $K_O = 16.4$  pM. Thus, substantial block occurred at potentials for which  $m_\infty < 0.01$  because the concentration of nimodipine used was  $\approx 1,000$  times greater than  $K_O$ .

The experiment shown in Fig. 4 was difficult to perform because it required a rapidly settling voltage clamp, fairly large L-type Ca channel currents, and a long-

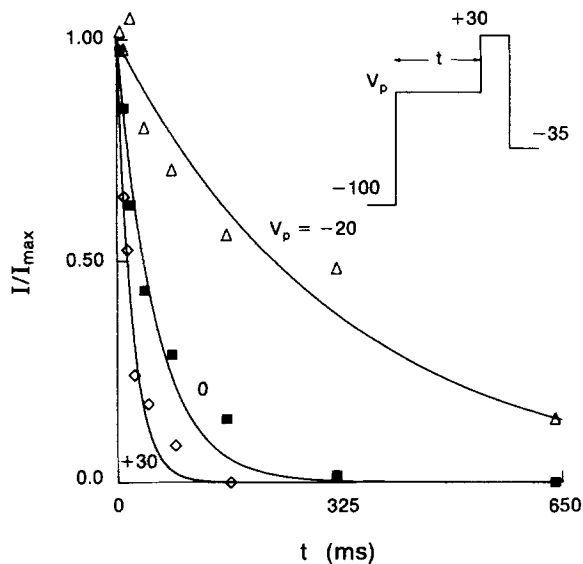


FIGURE 5. The rate of onset of nimodipine block is voltage dependent. The time course of block of the L-type Ca channels by 30 nM nimodipine was determined at four potentials:  $-40$  mV (data not shown),  $-20$  mV ( $\Delta$ ),  $0$  mV ( $\blacksquare$ ), and  $+30$  mV ( $\circ$ ). For each data point, the following pulse protocol was used (see *inset*): the membrane potential was held at  $-100$  mV for 20 s; the potential was then stepped to the prepulse potential ( $V_p$ ) for a time  $t$  (the abscissa); a 10-ms voltage pulse to  $+30$  mV produced maximal activation of the L-type Ca channels, and tail currents were measured

upon repolarization to  $-35$  mV. The tail currents contained a rapidly decaying exponential component ( $\tau = 0.92$  ms) and the amplitude of this component defines the ordinate. For each  $V_p$ , the results were fit to the equation  $I = I_0 \cdot \exp(-t/\tau)$ . For  $V_p = -40$  mV,  $I_0 = 58.14$  pA,  $\tau = 3.32$  s; for  $V_p = -20$  mV,  $I_0 = 53.77$  pA,  $\tau = 330.6$  ms; for  $V_p = 0$  mV,  $I_0 = 60.8$  pA,  $\tau = 56.1$  ms; and for  $V_p = +30$  mV,  $I_0 = 104.2$  pA,  $\tau = 22.0$  ms. Ideally,  $I_0$  should be the same for each set of data. However, the assumptions of the modeling were not fulfilled because drug binding was changed by a 10-ms pulse to  $+30$  mV. For  $V_p = +30$  mV, there is no error introduced, but for all other  $V_p$ ,  $I_0$  indicates the number of drug-free channels after a 10-ms pulse to  $+30$  mV.

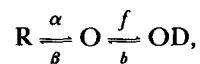
lasting experiment with little run down of Ca currents. These criteria were fulfilled one other time with an A7r5 cell, and we obtained a similar result with  $K_O = 40.7$  pM. We also obtained a nearly identical result in one successful experiment with an A10 cell, with  $K_O = 45.7$  pM.

#### *Kinetics of Nimodipine Binding to Open Ca Channels*

Our calculation indicates that channel opening increases the affinity for nimodipine binding by over four orders of magnitude. Preferential block of open Ca channels by nimodipine was previously found in  $\text{GH}_4\text{C}_1$  cells, but with  $K_O = 517$  pM rather

than 16.4–45.7 pM, as in three experiments with vascular smooth muscle cell lines. The value for  $K_O$  for GH<sub>4</sub>C<sub>1</sub> cells seemed reasonable, based on earlier studies of the effect of nimodipine on high K-induced Ca influx and on ligand binding studies with [<sup>3</sup>H]nimodipine. However, the very low values for  $K_O$  obtained with A10 and A7r5 cells were totally unexpected and raised suspicions that either the model used to calculate  $K_O$  was incorrect or that these two cell lines are anomalous. One of the strong points of the model is that it predicts the kinetics of nimodipine binding to open channels, and this prediction provides a stringent test for the validity of the model. Measurements of the kinetics of drug binding are also of interest in understanding the tissue specificity of nimodipine action: is  $K_O$  smaller in A10 and A7r5 cells than in GH<sub>4</sub>C<sub>1</sub> cells because the association rate for binding to open channels is faster or is the dissociation rate slower?

The rate constants for nimodipine binding were measured using the same analytical techniques previously developed in studies with GH<sub>4</sub>C<sub>1</sub> cells (Cohen and McCarthy, 1987). For sufficiently low concentrations of nimodipine, the rate of block of open channels is much slower than the rate of channel activation and block of rested state channels is negligible. Hence, one can ignore the detailed kinetics of channel activation and the model reduces to a three-state sequential scheme:



where  $\alpha$  and  $\beta$  are the rate constants describing channel activation and  $f$  and  $b$  are the association and dissociation rates for nimodipine binding to open channels. In this case, the model predicts that open channel block will increase with an exponential time course following a voltage step and the time constant ( $\tau$ ) will be:

$$\tau = (m_{\infty}fD + b)^{-1} \quad (3)$$

In other words, the rate of block will increase in proportion to the fraction of channels that are activated ( $m_{\infty} = \alpha/(\alpha + \beta)$ ). If  $f$  and  $b$  are voltage independent (which is likely because nimodipine is electroneutral), then the voltage dependence of  $\tau$  is derived entirely from the voltage dependence of steady state Ca channel activation, given by Eq. (1).

The pulse protocol used to determine  $\tau$  is shown in the inset of Fig. 5. The rate constants for drug binding were determined after 30 nM nimodipine had equilibrated in the bath. The membrane potential was held at  $-100$  mV for  $>20$  s to drive all channels into the rested state and to reverse drug binding. Binding was initiated by depolarizing the membrane to  $V_p$  and it was allowed to continue for a time  $t$ . The fraction of drug-free channels was then assayed by measuring the amplitude of the fast component of tail current that followed a 10-ms voltage pulse to  $+30$  mV. The relative amplitude of the tail current is plotted vs. the duration of the prepulse ( $t$ ). Fig. 5 shows the rate of onset of block for  $V_p = -20, 0,$  and  $+30$  mV for the same experiment shown in Fig. 4. From the activation curve of Fig. 4, we expect a sixfold change in  $m_{\infty}$  when  $V_p$  is increased from  $-20$  to  $0$  mV, but only a twofold change for  $V_p = 0$  mV vs.  $V_p = +30$  mV. If  $m_{\infty}fD \gg b$ , then comparable changes in  $\tau$  should occur. The solid curves through the three sets of data points are nonlinear least squares fits to an exponential with  $\tau = 330.6, 56.1,$  and  $22.0$  ms at

$V_p = -20, 0, \text{ and } +30 \text{ mV}$ , respectively.  $\tau$  was also measured at  $-40 \text{ mV}$  (data not shown because the time course of block was much slower).

Fig. 6 shows a semilogarithmic plot of  $\tau$  vs.  $V_p$  for this experiment. The solid curve is a least squares fit to Eq. (3) with the constraint that  $b/f = K_O = 16.4 \text{ pM}$  (hence, there is one free parameter). The optimal fit is for  $f = 1.44 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  and  $b = 0.0236 \text{ s}^{-1}$ . As the prepulse potential was increased from  $-40$  to  $+30 \text{ mV}$ ,  $\tau$  decreased by more than two orders of magnitude, and all of this change is accounted for by the model for open channel block. The excellent agreement between experiment and theory strongly supports the validity of the model and severely limits the number of possible alternative explanations. The occupancy of the high-affinity state for nimodipine binding must increase with voltage with the same slope factor as the activated state in order to account for the results of Fig. 4

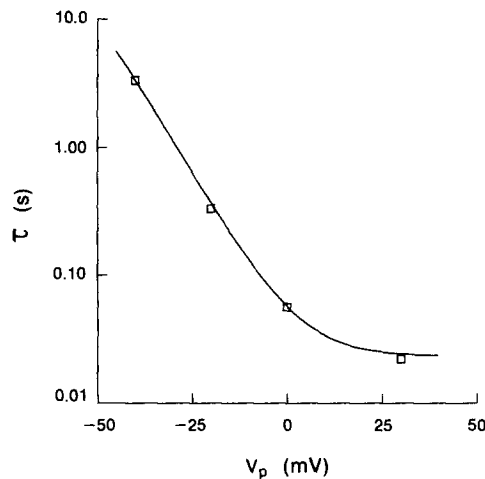


FIGURE 6. The voltage dependence of Ca channel activation can account for the voltage dependence of the rate of onset of nimodipine block. The rate of onset of block of the L-type Ca channels by  $30 \text{ nM}$  nimodipine was measured by the exponential fitting procedure shown in Fig. 5. The time course of block at each potential was fit by a single exponential and the logarithm of the time constant ( $\tau$ ) is plotted vs. prepulse potential. The solid curve is a nonlinear least squares fit to the equation  $\tau = (fDm_{\infty} + b)^{-1} = \{1 + \exp [(V_m - V)/k]\} / \{(fD + b) + (b) \exp [(V_m - V)/k]\}$  with  $V_m = +3.2 \text{ mV}$ ,  $k = 8.57$ ,  $f = b/K_O = b/0.0164 \text{ nM}$ , and  $D = 30 \text{ nM}$ . There is one free parameter in the fit. See text for a definition of terms and further details.

and it must have half-maximal occupancy at the same potential as  $V_m$  in order to account for the results of Fig. 5.

#### *Nimodipine Block of T-Type Ca Channels*

The extraordinarily potent block of the L-type Ca channels in A10 and A7r5 cells by nimodipine suggested that block of the T-type Ca channels might be more potent than in other tissues with T-type Ca channels (see the Discussion). Fig. 7 shows the effect of nimodipine on the T-type Ca channels. The tail current records at the top of Fig. 7 show that  $100 \text{ nM}$  nimodipine causes significant block of the T-type Ca channels in A7r5 cells only when drug binding is allowed to equilibrate at depolarized potentials. Each panel shows superimposed tail current measurements and the record in  $100 \text{ nM}$  nimodipine is indicated by an asterisk. When drug binding equilibrated at  $-110 \text{ mV}$ , there was little or no change in the amplitude of the slow

component of tail current, but the rate of deactivation was modestly accelerated by drug (this was a consistent finding). However, when drug binding equilibrated at  $-50$  mV, about half of the channels were blocked by drug. The effect of nimodipine on the steady state availability of the T-type Ca channels is shown by plotting the relative amplitude of the slow component of tail current as a function of  $V_p$ . The open squares show the control measurements and the solid curve through these

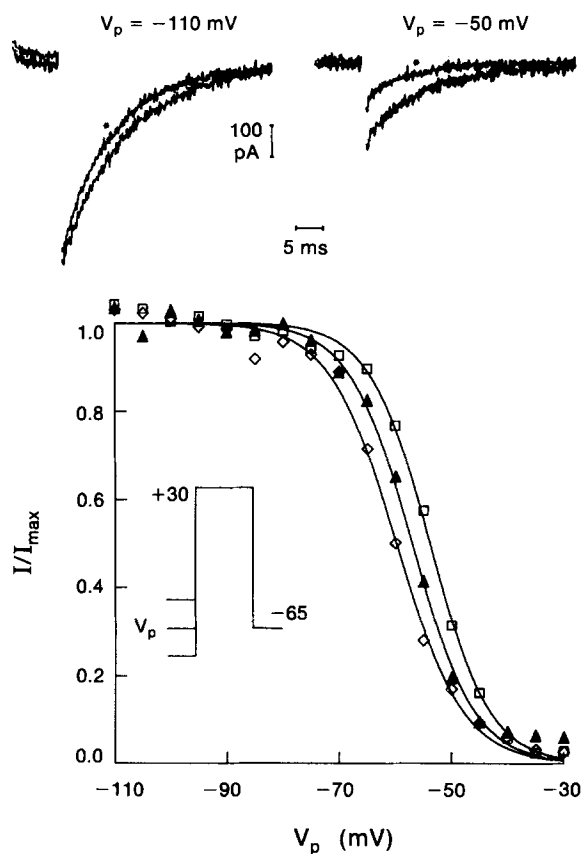


FIGURE 7. The steady-state voltage dependence of nimodipine block of the T-type Ca channels in an A7r5 cell. The pulse protocol used is shown in the inset of the lower panel. The prepulse duration was 5 s for control measurements and 10 s in drug. The panels at top show superimposed tail current measurements with and without 100 nM nimodipine for the indicated prepulse potentials. Current records in the presence of drug are the average of two measurements and are indicated by an asterisk. Current was sampled at 10 kHz and filtered with  $f_c = 2.5$  kHz. The first 900  $\mu$ s after repolarization has been omitted from each current record. The tail currents were fit by an exponential with a time constant of decay of 10.9–11.9 ms. The lower panel shows a plot of the relative amplitude of the exponential fit of the tail currents vs.  $V_p$ . The steady-state availability of the T-type

Ca channels was determined in the absence of drug ( $\square$ ) and in 100 nM and 500 nM nimodipine ( $\blacktriangle$  and  $\diamond$ , respectively). The solid curve through each set of data points indicates the least squares best fit to the equation  $I/I_{\max} = \{1 + \exp [(V_p - V_{1/2})/k]\}^{-1}$ . In the absence of drug,  $V_{1/2} = -53.8$  mV and  $k = 5.36$ ; for 100 nM nimodipine,  $V_{1/2} = -57.0$  mV and  $k = 5.58$ ; and for 500 nM nimodipine,  $V_{1/2} = -59.8$  mV and  $k = 5.95$ .

data is defined by a two-state Boltzmann distribution with  $V_{1/2} = -53.8$  mV and  $k = 5.36$ . This result was typical of three other experiments with A7r5 cells. The filled triangles show the effect of 100 nM nimodipine. The curve through this data was fit with  $V_{1/2} = -57.0$  mV and  $k = 5.58$ . The availability curve is shifted to still more negative potentials by 500 nM nimodipine (open diamonds):  $V_{1/2} = -59.8$  mV and  $k = 5.95$ .

The voltage-dependent block of T-type Ca channels by nimodipine can be accounted for by the modulated receptor theory. The model attributes the voltage dependence of block to the voltage dependence of inactivation; i.e., it is assumed that nimodipine preferentially binds to inactivated T-type Ca channels. The data shown in Fig. 7 can be used to calculate the dissociation constants for binding to the rested and inactivated states (Bean et al., 1983):

$$\Delta V = -k \ln [(1 + D/K_I)/(1 + D/K_R)], \quad (4)$$

where  $K_I$  is the dissociation constant for binding to inactivated channels and  $\Delta V$  is the shift between the availability curves in Fig. 7.  $K_R$  is calculated from the amount of block when binding equilibrates at  $-110$  mV, and is  $1.33 \mu\text{M}$ . This value could be an underestimate because of possible run down in currents during application of drug. The results with  $100$  nM nimodipine indicate that  $K_I = 108$  nM. The results with  $500$  nM nimodipine indicate that  $K_I = 168$  nM, so the model is fairly successful in accounting for the voltage and concentration dependence of block. Voltage-dependent block of T-type Ca channels by nimodipine was found in three other experiments with concentrations of drug ranging from  $30$  to  $500$  nM. The average value for  $K_R$  was  $6.74 \mu\text{M}$ . A nonlinear least squares fit to Eq. (4) indicated  $K_I = 266$  nM. Hence, nimodipine block of T-type Ca channels in A10 and A7r5 cells is much weaker than block of the L-type Ca channels, but more potent than most reports of block of T-type Ca channels in other preparations.

#### DISCUSSION

Our studies indicate that A10 and A7r5 cells contain two types of Ca channels similar to the L- and T-type Ca channels found in vascular smooth muscle. Nimodipine block of the L-type Ca channels in A10 and A7r5 cells is much more potent than block of similar Ca channels in  $\text{GH}_4\text{C}_1$  cells. This finding differs from earlier studies of Ca channel block by 1,4-dihydropyridines in vascular smooth muscle cell lines using  $^{45}\text{Ca}$  flux and ligand binding techniques. These earlier studies suggested that high affinity block of Ca channels by 1,4-dihydropyridines is quite similar in many different cell types and that block is not unusually potent in vascular smooth muscle. Our studies support the idea that the K-induced Ca influx and  $^3\text{H}$ nitrendipine binding techniques measure block of L-type Ca channels, but our studies also reveal limitations of these techniques for quantitative analysis of drug binding.

#### *The Ca Channels in A10 and A7r5 Cells Are Similar to Those in Intact Aortic Muscle*

A10 and A7r5 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 nimodipine. The results can best be explained by invoking two distinct populations of Ca channels similar to the L- and T-type Ca channels found in neurons, muscle cells, fibroblasts, and endocrine cells (Bean, 1989). The existence of two populations of Ca channels in A10 cells was first reported by Friedman et al. (1986) and recently two types of Ca channels have been described in A7r5 cells (Fish et al., 1988).

Several voltage clamp studies of Ca channels in vascular smooth muscle cells are available for comparison with our results, but the results with freshly dispersed cells are far from uniform. The Ca channels in A10 and A7r5 cells are similar to those in most of the reports, but they are not identical to those of any of the cell types that have been studied. For example, two populations of Ca channels similar to those in A10 and A7r5 cells have been identified in cells from rat cerebral arterioles (Hirst et al., 1986), rat mesenteric arteries (Bean et al., 1986), rat azygous vein (Sturek and Hermsmeyer, 1986), rat portal vein (Loirand et al., 1986), rabbit ear arteries (Benham et al., 1987), and canine saphenous vein (Yatani et al., 1987), but only one type of Ca channel was found in guinea pig aorta cells (Caffrey et al., 1986) and two populations of Ca channels that are highly sensitive to block by dihydropyridines were found in rabbit mesenteric artery (Worley et al., 1986). Most studies with freshly dispersed cells found that the T-type Ca channels activate at more negative potentials than the L-type Ca channels (in agreement with our findings), but the L-type Ca channels activate at more negative potentials in rat cerebral arterioles (Hirst et al., 1986). The time and voltage dependence of Ca channel gating in A10 and A7r5 cells is fairly similar to that in vascular smooth muscle in primary culture, with the notable exception that much slower inactivation of the L-type Ca channels is seen in our studies than previously reported. Studies with intact tissue indicate that there is a noninactivating component of Ca channel current, but most studies with primary cultures indicate that only a small fraction of the L-type Ca current is noninactivating, even when Ca induced inactivation is minimized. High-affinity block of L-type Ca channels in vascular smooth muscle cells by dihydropyridines has not been reported to be unusually potent, but the binding affinity for the open state was not evaluated (Bean et al., 1986; Yatani et al., 1987). Apparent rested state block appears to be unusually potent in vascular smooth muscle (Bean et al., 1986; Yatani et al., 1987; Aaronson et al., 1988), which may indicate unusually rapid block during the test depolarization (see the discussion of Fig. 4).

#### *Tissue Specificity of High-Affinity Nimodipine Binding*

The time and voltage dependence of nimodipine block indicate that nimodipine binds with high affinity to channels in the open state. The steady-state voltage dependence of nimodipine block is well fit by a two-state Boltzmann distribution (see Fig. 4). This suggests that the voltage dependence of drug binding is derived from the voltage dependence of channel gating. Channel activation has the appropriate slope factor ( $k$ ), as shown in Fig. 4, but the model for open channel block lead to a surprisingly low value of  $K_O$ . However, the voltage dependence of the rate of onset of nimodipine block provided a second measure of the occupancy of the high-affinity state for nimodipine binding. The 100-fold increase in the rate of nimodipine block as the voltage is increased from  $-40$  to  $+30$  mV indicates a proportionate increase in the occupancy of the high-affinity state for nimodipine binding over the same voltage range. The results of Fig. 6 thus serve to precisely define the voltage for half-maximal occupancy of the high-affinity state for nimodipine binding. Together, the results of Fig. 4 and 6 completely specify the voltage dependence of occupancy of the high-affinity state; that is, both the midpoint and the slope factor for the Boltzmann distribution are defined. The open (activated) state has the same

voltage dependence. Nimodipine may also bind to channels in the inactivated state with high affinity, but the time dependence of nimodipine block shown in Fig. 5 is not consistent with binding to the inactivated state because inactivation develops very slowly in A10 and A7r5 cells (see Figs. 1 and 3).

It is also possible to account for the time and voltage dependence of nimodipine block by hypothesizing that the drug dramatically speeds the transition rate from the open state to the inactivated state. However, this hypothesis does not predict voltage-dependent drug binding to Ca channels and ligand binding studies with congeners of nimodipine indicate that drug binding is steeply voltage dependent (Kokubun et al., 1986).

One of the most surprising and important results of this study is provided by a comparison of the kinetics of nimodipine binding to L-type Ca channels in  $\text{GH}_4\text{C}_1$  and A7r5 cells. In  $\text{GH}_4\text{C}_1$  cells,  $K_O = 517 \text{ pM}$ ,  $f = 9.65 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  and  $b = 0.0496 \text{ s}^{-1}$  (Cohen and McCarthy, 1987). In the exemplar experiment with A7r5 cells,  $K_O = 16.4 \text{ pM}$ ,  $f = 1.44 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  and  $b = 0.0236 \text{ s}^{-1}$ . Hence, nimodipine binds with greater affinity to Ca channels in vascular smooth muscle cells, and nearly all of the difference in binding affinity is due to a faster association rate in vascular smooth muscle cells.

The association rate in both cell lines is too fast to represent the three dimensional diffusion of nimodipine from an aqueous phase to a membrane receptor site. However, nimodipine is very lipophilic and the association rates presumably represent the two dimensional diffusion of nimodipine within the plasmalemma. For diffusion in two dimensions, association rates of up to  $10^{12} \text{ M}^{-1}\text{s}^{-1}$  are physically plausible (Rhodes et al., 1985; Herbette et al., 1986; Cohen and McCarthy, 1987).

If the difference in  $K_O$  between the two cell lines were due to structural variations in the Ca channel binding site, then tighter binding of nimodipine to the channel would be reflected in a much slower dissociation rate. The near constancy of  $b$  suggests that the binding site is quite similar in A7r5 and  $\text{GH}_4\text{C}_1$  cells. A simple way to account for the much faster association rate in vascular smooth muscle cells is to postulate that the lipid composition in the plasmalemmas of the cell lines differ, so that nimodipine diffuses much faster or has a larger partition coefficient into the plasmalemma of A7r5 cells than for  $\text{GH}_4\text{C}_1$  cells. Hence, the comparative kinetics of nimodipine binding suggest that the lipid composition of the plasmalemma can have a large effect on the potency of calcium channel block by 1,4-dihydropyridines.

The importance of lipid composition in determining the potency of nimodipine block could best be assessed by measuring the partition coefficient of nimodipine into the plasmalemma of A7r5 and  $\text{GH}_4\text{C}_1$  cells (see Rhodes et al. [1985] for measurements of the partition coefficient in myocardial cells). We were not equipped to make this measurement, so we substituted an electrophysiological test. If the lipid composition of the plasmalemma determines the relative potency of nimodipine block, then any channel common to both A7r5 and  $\text{GH}_4\text{C}_1$  cells should be more potently blocked by nimodipine in A7r5 cells. In particular, nimodipine block of the T-type Ca channels should be ~15 times more potent in A7r5 cells than in  $\text{GH}_4\text{C}_1$  cells. We found that nimodipine blocks the T-type Ca channels in A7r5 cells or A10 cells more potently than in most other cell types that have been studied, but the dissociation constant for high-affinity binding ( $K_1$ ) is not as low as anticipated. In



GH<sub>3</sub> cells,  $K_1 \approx 400$  nM (Cohen and McCarthy, 1987; unpublished results), while in A7r5 and A10 cells,  $K_1$  is about one-half as large (see Fig. 7). The lack of quantitative agreement with our theoretical predictions does not conclusively disprove our hypothesis because: (a) the T-type Ca channels may not be identical in the different cell lines with respect to nimodipine binding (Van Skiver et al., 1989); or (b) the partition coefficient for nimodipine may decrease at high nimodipine concentrations.

*Do <sup>45</sup>Ca Influx Studies Accurately Indicate the Pharmacological Properties of Ca Channels in Vascular Smooth Muscle Cells?*

<sup>45</sup>Ca influx and ligand binding studies both seem to be at odds with our results because neither technique indicated a large variation in high-affinity binding of dihydropyridines between vascular smooth muscle and anterior pituitary cells. The apparent discrepancy is presented in Table I, which shows a comparison of three

TABLE I  
*A Comparison of Three Measures of Nimodipine Binding to Ca Channels in Vascular Smooth Muscle and Anterior Pituitary Cells*

Cell type	$K_O$ (Voltage clamp)	IC <sub>50</sub> ( <sup>45</sup> Ca flux)	$K_d$ ( <sup>3</sup> H-ligand)
	nM	nM	nM
A7r5 or A10	0.02*	2 <sup>‡</sup>	—
GH <sub>4</sub> C <sub>1</sub> or GH <sub>3</sub>	0.5 <sup>‡</sup>	2 <sup>‡</sup>	0.1**
Aortic muscle	—	1.6 <sup>‡</sup>	0.1–0.4 <sup>‡‡</sup>

\*This study.

<sup>‡</sup>Cohen and McCarthy (1987).

<sup>‡</sup>Kongsamut et al. (1985).

<sup>‡</sup>Enyeart et al. (1985).

<sup>‡</sup>Godfraind et al. (1985).

\*\*Trumble and Kaczorowski (1983).

<sup>‡‡</sup>See Triggle and Janis (1984) for references.

techniques that have been used to measure nimodipine binding to L-type Ca channels. The IC<sub>50</sub> for nimodipine block of Ca influx stimulated by high K is about 2 nM in A10 and GH<sub>4</sub>C<sub>1</sub> cells and in intact aorta (Enyeart et al., 1985; Godfraind et al., 1985; Kongsamut et al., 1985). These Ca flux studies indicate that A10 and A7r5 cells are representative of intact vascular smooth muscle with respect to nimodipine binding, so our low values of  $K_O$  cannot be dismissed as an anomaly arising from the use of cell lines. However, the isotopic flux studies give no evidence of intertissue variations in nimodipine binding and the IC<sub>50</sub> for block is much larger than  $K_O$  in our studies. Ligand binding studies with tritiated 1,4-dihydropyridines have also indicated similar dissociation constants for high-affinity binding in vascular smooth muscle and other tissues and much slower association rates for binding than reported here (Triggle and Janis, 1984; Godfraind et al., 1986), but Williams and Tremble (1982) found a lower  $K_d$  in aorta than in ventricle.

The results using the voltage clamp technique are in fact consistent with both Ca influx and ligand binding studies, and the apparent discrepancy can occur because

previously used techniques are limited to voltages  $\leq 0$  mV. Since all channels are in the high-affinity (open) state only for  $V > 0$  mV, neither Ca flux nor ligand binding studies directly measure the dissociation constant of the drug for the open state. Rather, these techniques have been used to determine the effective binding constant ( $K_{\text{eff}}$ ) at  $\leq 0$  mV, which does not show as much variation with cell type as  $K_{\text{O}}$ .

The model for open channel block can be used to derive the voltage dependence of  $K_{\text{eff}}$ :  $K_{\text{eff}} = [(1 - m_{\infty})/K_{\text{R}} + m_{\infty}/K_{\text{O}}]^{-1}$ . Since  $K_{\text{R}} > 10^4 K_{\text{O}}$ ,  $K_{\text{eff}} \approx K_{\text{O}}/m_{\infty}$  for  $m_{\infty} > 0.001$ . The upper panel of Fig. 8 shows the activation curves for L-type Ca channels

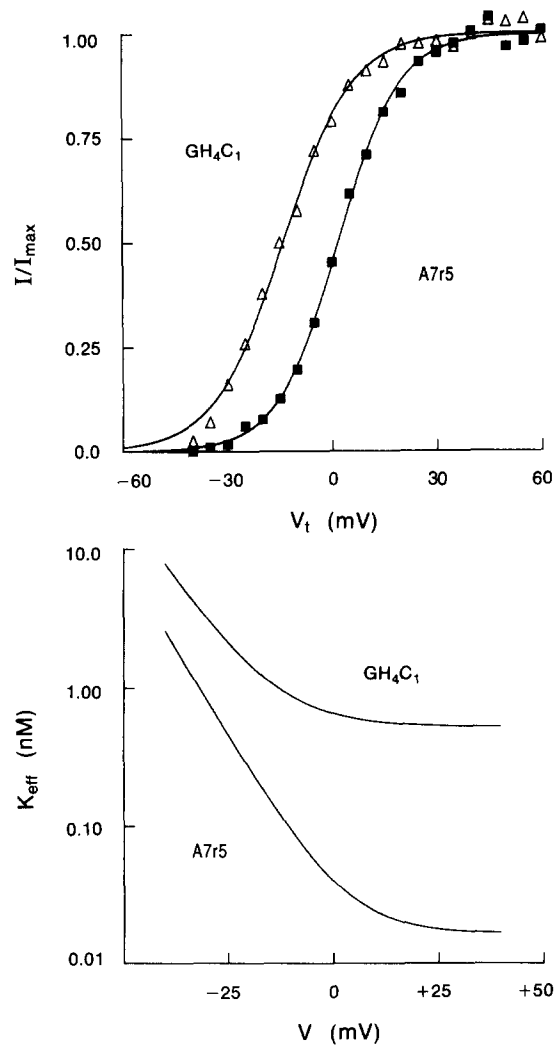


FIGURE 8. A comparison of the effective binding constants for nimodipine binding to L-type Ca channels in GH<sub>4</sub>C<sub>1</sub> and A7r5 cells. *Upper panel:* The voltage dependence of activation of the L-type Ca channels in GH<sub>4</sub>C<sub>1</sub> and A7r5 cells. The activation curve for GH<sub>4</sub>C<sub>1</sub> cells has the midpoint at  $V_m = -13.8$  mV and the slope factor ( $k$ ) equal to 9.90. This curve represents the mean result for six experiments and is the same data used in Fig. 7 of Cohen and McCarthy (1987). The activation curve for A7r5 cells is defined by  $V_m = +1.8$  mV and  $k = 8.86$ . Each point represents the mean of three experiments. *Lower panel:* A semilogarithmic plot of the effective dissociation constant for nimodipine binding ( $K_{\text{eff}}$ ) as a function of voltage. For GH<sub>4</sub>C<sub>1</sub> cells, the solid curve is defined by  $K_{\text{eff}} = (0.517) [1 + \exp\{(-13.8 - V)/9.90\}]$ . The solid curve for A7r5 cells is based on the exemplar experiment shown in Figs. 4 and 5 because that experiment gave the lowest value for  $K_{\text{O}}$  and is therefore the hardest to reconcile with Ca flux studies. The curve is defined by  $K_{\text{eff}} = (0.0164)[1 + \exp(3.2 - V)/8.57]$ . See text for further details.

that we determined in both GH<sub>4</sub>C<sub>1</sub> and A7r5 cells under identical experimental conditions. The lower panel shows a plot of  $K_{\text{eff}}$  vs.  $V$  for GH<sub>4</sub>C<sub>1</sub> and A7r5 cells.  $K_{\text{eff}}$  at each voltage was calculated using the least squares best fit  $m_{\infty}$  curves and experimentally determined values for  $K_{\text{O}}$ . The curves are dissimilar in shape because the activation kinetics differ between the two cell types.

In studies of high K-induced <sup>45</sup>Ca influx, concentrations of external K of 40–100 mM are usually used. Because most cell membranes behave as K electrodes in high K,  $-40 < V < -15$  mV under the conditions used in the isotopic flux studies. Although the ratio of  $K_{\text{O}}$  in GH<sub>4</sub>C<sub>1</sub> cells to  $K_{\text{O}}$  in A7r5 cells is  $\approx 30$ ,  $K_{\text{eff}}$  differs by a factor of only 3–6 for  $-40 < V < -20$  mV. Furthermore,  $K_{\text{eff}}$  at  $-30$  mV is quite close to the  $\text{IC}_{50}$  in the isotopic flux studies.

While similar arguments apply to ligand binding studies with isolated membranes, it is not clear how large an error exists because the effect of disrupting ionic gradients (particularly for Ca) on the activation curve is unknown. To a first approximation, one might expect ligand binding to be a measure of  $K_{\text{eff}}$  at 0 mV. As expected, the apparent  $K_{\text{d}}$  (typically 0.1–0.3 nM) is lower than the  $\text{IC}_{50}$  in Ca influx studies, but larger than  $K_{\text{O}}$  in vascular smooth muscle cell lines. In ligand binding experiments with [<sup>3</sup>H]nimodipine, the ligand 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 [<sup>3</sup>H]nimodipine binding to high-affinity sites ( $1.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  at 25°C) is much slower than our value, suggesting that ligand binding is rate limited by aqueous diffusion (Rhodes et al., 1985); see Triggle and Janis (1984) for a review of dihydropyridine ligand binding rates. This result was predicted from theoretical considerations (Rhodes et al., 1985).

In summary, the quantitative differences between our voltage clamp studies and Ca flux studies are probably not due to inherent difficulties in measuring <sup>45</sup>Ca influx (Borle, 1981; Karaki and Weiss, 1981) nor to our use of cell lines. Our studies strongly support the conclusion that <sup>45</sup>Ca influx and ligand binding studies with 1,4-dihydropyridines indicate high affinity block of Ca channels in vascular smooth muscle cells. However, the voltage clamp studies also indicate that the alternative techniques are severely limited in quantitatively analyzing the binding of drugs to Ca channels when the affinity of drug binding is altered by changes in channel gating.

#### *Therapeutic Use of Nimodipine*

Nimodipine can reduce the neuronal damage caused by some types of cerebral ischemia or subarachnoid hemorrhage (Scriabine et al., 1985). The efficacy of the drug is usually attributed to cerebral vasodilation caused by the block of Ca influx into vascular smooth muscle cells. Most previous discussions of the selectivity of nimodipine action have stressed that nimodipine can be a more potent vasodilator of cerebral arteries than of arteries from other vascular beds. This presumably accounts for the reports that nimodipine can increase cerebral blood flow with little change in blood pressure (Kazda et al., 1982; Haws et al., 1983); but Kanda and Flaim (1986) report conflicting results. The selectivity of Ca channel block has most often been evaluated in vitro by assaying inhibition of K-induced contractions and significant differences in potency of block have been found between smooth muscle preparations, even though the affinity for <sup>3</sup>H-ligand binding is nearly constant (You-

sif and Triggle, 1986). Our studies suggest two possible explanations for the tissue specificity of block: (a) the voltage dependence of activation (or inactivation) can vary significantly between cell types so that the fraction of channels in the high affinity state at a given voltage varies (see Fig. 8); and (b) the partition coefficient of drug into membrane may differ between cell types, presumably because of variations in lipid composition.

When treating focal injuries, it is most desirable to specifically dilate a localized region that is experiencing vasospasm, rather than to generally increase cerebral blood flow. Nonspecific cerebral vasodilation would probably be detrimental in the treatment of subarachnoid hemorrhage or cerebral ischemia because vascular resistance would be decreased in all parts of the cerebral circulation, resulting in the "steal" of blood from vasoconstricted areas. Nimodipine is a particularly exciting drug because it should specifically suppress Ca entry in pathological conditions that result in maintained depolarizations, with little effect on healthy, well-polarized cells. The changes in affinity for nimodipine binding associated with Ca channel activation are orders of magnitude larger than the variations in dissociation constant between different vascular beds, so the time and voltage dependence of Ca channel block are probably more important than intertissue variations in high-affinity nimodipine binding. Studies with cerebral arterioles also indicate voltage-dependent nimodipine block. Our laboratory has previously reported that nimodipine has little effect on Ca dependent electrical activity in well polarized rabbit middle cerebral arterioles, but abnormal automaticity in partially depolarized tissue is potently blocked by drug (Cohen and Chung, 1985). Likewise, nimodipine can have little effect on cerebral blood flow when administered to healthy animals (Kanda and Flaim, 1986), but this drug preferentially increases local cerebral blood flow in areas of ischemic stroke (Gelmers, 1982; Gaab et al., 1985; but see Barnett et al. 1986).

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