Calcium Channels in Muscle Cells Isolated from Rat Mesenteric Arteries: Modulation by Dihydropyridine Drugs

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The patch clamp technique was used to make whole-cell recordings of calcium channel currents from single muscle cells freshly isolated from rat mesenteric arteries. The cells were found to contain two types of calcium channels; one type is activated by small depolarizations and inactivates quickly, whereas the other requires stronger depolarizations for activation and inactivates more slowly. Nitrendipine blocked the second type of channels with a potency that depended on membrane potential. Interpreted by a modulated receptor hypothesis, these results suggest that nitrendipine binds to the inactivated state of the channel with a $K_d$ of about 0.5 nM, similar to concentrations effective in relaxing blood vessels. The magnitude of the calcium channel current was small compared with other excitable cells, but the "calcium agonist" drug BAY K8644 produced a 10-fold augmentation of calcium channel current, suggesting the existence of a large "reserve" of calcium channel current.

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SMOOTH muscle cells in the walls of blood vessels are widely believed to contain voltage-dependent calcium channels, but little is known about the properties of such channels. Direct voltage clamp studies have been precluded by the small size of the cells, typically embedded in tough connective tissue. The lack of information about vascular muscle calcium channels is especially unfortunate, since they probably play a major role in regulating cell contraction, blood vessel diameter, and blood pressure. Nifedipine, nitrendipine, and other dihydropyridine drugs, increasingly used clinically to treat hypertension and angina, have often been proposed to relax vascular muscle by blocking calcium channels, but direct voltage clamp evidence is lacking and other mechanisms have been proposed. Most voltage and patch clamp studies of the drugs have been done with cardiac muscle cells, even though the clinical efficacy of the drugs depends on the fact that they inhibit vascular muscle contraction much more potently than cardiac contraction. It is not known whether this difference reflects fundamentally different types of calcium channels in the two types of muscle. Using patch clamp techniques to record whole-cell currents from isolated mesenteric artery cells, we have found that there are actually two different types of calcium channels in vascular muscle cells. Both are kinetically and pharmacologically similar (although perhaps not identical) to types of channels recently identified in cardiac and other excitable cells. Our results suggest that the profound difference in dihydropyridine sensitivity between vascular muscle and cardiac muscle arises from a voltage dependence of drug block — and the difference in resting potential between the two tissue types — and not from greatly different channel pharmacology.

Materials and Methods

Pieces of primary mesenteric arteries (~1 cm) were dissected from two adult rats in each experiment. Fatty tissue was removed, and the arterial tissue was soaked for 10 minutes in a zero calcium, high potassium, low chloride solution (KG solution) consisting of (in mM) 135 potassium glutamate, 16 NaHCO$_3$, 0.5 NaH$_2$PO$_4$, 20 HEPES, 16.5 dextrose, 0.014 phenol red, pH 7.40 (37°C). The tissue was minced with scissors into 0.5-mm pieces and incubated at 37°C in 5 ml of KG solution with 3 mg/ml collagenase (Worthington class II), 0.5 mg/ml trypsin (Kansas City Biochemicals), and 5 U/ml elastase (Sigma type III) with gentle (90 rpm) stirring. Every 15 minutes for 1/2 hours, the supernatant fluid was removed, combined with 5 ml horse serum (Kansas City Biochemicals), and stored on ice. The supernatant solutions were pooled, spun at 1200 rpm for 10 minutes, and the pellet was resuspended in a solution (CV3M solution) consisting of 85% Eagle's minimum essential medium and 15% horse serum, with 4 mM glutamate, 20 mM HEPES, and 16 mM NaHCO$_3$, pH 7.4 (37°C). After filtering through a 125-μm glass screen to remove pieces of connective tissue, the cell-containing solution was centrifuged at 1200 rpm for 5 minutes, and the cells were resuspended in 2–3 ml of CV3M solution, where they were stored until use. Cells were used within 12 hours.

A droplet of cell-containing solution was added to the glass-bottomed chamber containing Tyrode's solution consisting of (in mM) 5 CaCl$_2$, 150 NaCl, 4 KCl, 2

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MgCl₂, 10 glucose, 10 HEPES (pH adjusted to 7.40, 25°C). After 10 minutes, many cells adhered to the bottom, and the chamber was rinsed with 5 mM calcium-Tyrode’s solution to remove debris. After isolation, muscle cells were almost always contracted and were oval (typical dimensions 20 x 10 μm) or spherical (mean diameter 13 μm). Patch pipettes (1–4 MΩ) containing potassium glutamate or cesium glutamate solution (see legend to Figure 1) were used to voltage clamp the cells by the method of Hamill et al. Membrane potentials were corrected for a measured junction potential of -10 mV between pipette and bath solutions. Membrane currents were digitized and analyzed on a laboratory computer. Leak and capacity currents were subtracted using currents elicited by a small hyperpolarizing or depolarizing pulse (usually, many such sweeps were averaged to remove noise or a smooth version of the leak current was prepared by exponential fitting of the experimental record). Cell capacity was 17.8 ± 1.5 pF (mean ± SEM, 35 cells with transients recorded on a fast-time base). Nitrendipine and BAY K8644, kind gifts of Dr. Alexander Scriabine, Miles Laboratories, were prepared as 1 mM stock solutions in polyethylene glycol and diluted at the time of use. Applications of polyethylene glycol alone had no effect on calcium channel currents (even at depolarized potentials). All experiments were done at 25°C.

**Results**

Figure 1 shows ionic currents from mesenteric artery cells that were internally dialyzed and voltage clamped with patch pipettes. When the pipette solution contained potassium as the principal cation, the dominant currents were large, depolarization activated outward currents (Figure 1a) that were identified as potassium currents by their sensitivity to block by external tetraethylammonium ions. To our surprise, net inward calcium currents were not evident with internal potassium and 5 mM calcium saline externally. To test the possibility that calcium currents were actually present in the cells but were masked by much larger potassium currents, we replaced internal potassium by cesium and attempted to maximize calcium channel currents by using barium as the external charge carrier, taking advantage of the fact that barium is even more permeant than calcium in many types of calcium channels, as well as being a blocker of some types of potassium channels. Under these ionic conditions, moderate depolarizations activated inward rather than outward currents in most cells (Figure 1, b and c). Membrane conductance was extremely low at the holding potential of −70 mV (cell resistance was typical > 5 GΩ) but depolarizing the cells to ~+20 mV activated inward currents that reached a peak of 15–20 pA in ~10 msec and then decayed with a half-time of 70–200 msec. These were identified as calcium channel currents by their sensitivity to block by both inorganic and organic calcium channel blockers; lanthanum (Figure 1b) or nitrendipine (Figure 1c) completely eliminated the inward current. Under the optimal conditions of internal cesium and external barium ions, clear inward currents like those in Figure 1, b and c, were seen in 44 of 62 successfully voltage-clamped cells. In cells with clear calcium channel current, peak calcium currents were 15–20 pA at 2.8 ± 5.9 sec after the stimulus.

**Figure 1.** Potassium and calcium channel currents in mesenteric artery cells. a) Currents elicited by depolarization in a cell dialyzed with a pipette solution of (in mM) 140 potassium glutamate, 10 K₂ EGTA, 5 MgCl₂, 10 HEPES (pH adjusted to 7.40 with KOH), and bathed in 5 mM calcium Tyrode’s solution (see “Materials and Methods”). Cell B25A, cell capacity 18 pF. b) Current elicited in another cell dialyzed with a pipette solution of (in mM) 140 cesium glutamate, 10 Cs₂ EGTA, 5 MgCl₂, 10 HEPES (pH adjusted to 7.40 with CsOH) and bathed in a solution of (in mM) 110 BaCl₂, 10 HEPES [pH adjusted to 7.45 with Ba(OH)₂]. After recording control current (trace shown is signal averaged from six sweeps and is leak and capacity corrected), 30 μM LaCl₃ was added to the superfusing solution. The trace labelled La³⁺ was signal averaged from six sweeps taken about 5 minutes after lanthanum superfusion; lanthanum acted promptly, within seconds, but traces during the solution exchange were noisy. 100 μM LaCl₃ had no further effect, confirming complete calcium channel block by 30 μM. Cell 53A, cell capacity 11 pF. c) Nitrendipine block of inward current. Internal and external solutions as in b. Control trace is signal averaged from 10 individual sweeps and is leak and capacity corrected; trace labelled “3 μM nitrendipine” is the signal-averaged mean of four sweeps taken about 30 seconds after nitrendipine reached the cell. Cell B37I, cell capacity 10 pF.
current ranged from 4 to 60 pA; values near 20 pA were most typical.

As calcium channel currents were recorded from more cells, it became evident that there were two distinct patterns of current in different cells. Figure 2, a and b, shows a pattern that was typical of many cells. Depolarization to potentials of -10 mV or below elicited little or no current; current began to be evident at about 0 mV, and peak inward current occurred at about +30 mV. In cells exhibiting this voltage dependence, currents at 0 or +10 mV decayed very slowly (half-time > 300 msec) or not at all. Other cells showed a very different pattern of currents (Figure 2, c and d): substantial currents were elicited by pulses to -30 or -20 mV, and the largest inward current occurred at about +10 mV. In cells exhibiting this voltage dependence of activation, decay of the currents was invariably much more rapid, with half-times of 20—60 msec near 0 mV.

Recent studies have indicated that a variety of excitable cells, including neuronal, pituitary, and cardiac cells, contain two distinct kinds of calcium channels: typically, one type is activated by small depolarizations and inactivates relatively quickly ("fast" channels), and another requires larger depolarizations for activation and inactivates relatively slowly ("slow" channels). In a given cell type, the two types of calcium channels have different selectivity, single channel conductance, and pharmacology.5-11 It already seems clear that the two types of channels are not invariant across all cell types; for example, the "slow" channels of cardiac cells (which probably contribute most of the classical ICa of multicellular preparations) actually inactivate quite rapidly in physiological calcium concentrations, especially at positive potentials,10 in contrast to the "slow" or "long-lasting" components of neuronal calcium current.5-8 However, common to all of these cell types is the finding that the components of current arising from the two types of channels can be conveniently separated by holding potential: "fast" channels are inactivated by steady holding potentials positive to about -30 mV, while

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**Figure 2.** Two patterns of calcium channel currents. a) Currents elicited by 350-msec depolarizations from a holding potential of -70 mV. Each current shown is a signal-averaged record of 4—6 sweeps except for that at -10 mV, which is a single sweep. Cell B49B, 115 BaCl2 glutamate. Cell capacity 35 pF. b) Peak inward current vs. test potential for the cell in panel a. c) Currents from another cell with different time and voltage dependence. Currents elicited by 350-msec depolarizations from a holding potential of -60 mV. Each current shown is signal averaged from 20—45 sweeps. Cell B36B, 115 BaCl2 glutamate. Cell capacity 34 pF. d) Peak inward current vs. test potential for the cell in panel c. e) Steady state inactivation curves for two different cells. Holding potential was varied and peak inward current elicited by a test pulse (to near the peak of the current voltage curve) was measured; holding potentials were established for at least 2 seconds in order to reach a quasi steady state. •: steady state inactivation in a cell with a current pattern like that in panel a. Cell B54H. Test pulse, +30 mV; maximal peak current, 25 pA. □: steady state inactivation in a cell with a current pattern like that in panel b. Cell B37C. Test pulse, +10 mV; maximal peak current, 11 pA. Data have been fit by a smooth curve drawn according to $I = \frac{1}{1 + \exp(V - V_h)/k}$, with $V_h = -41$ mV and $k = 5.1$ f) Fast-inactivating and slow inactivating current components in the same cell. A 350-msec depolarization was given to +10 mV from a holding potential of either -70 mV or -30 mV. Raw current (no leak or capacity correction) is shown for each case; capacity transients have been truncated to save space. Cell B52C, 115 BaCl2 glutamate, cell capacity 20 pF.
“slow” channels need much more positive holding potentials for steady state inactivation. These observations prompted us to see if the two patterns of current in vascular smooth muscle cells could be distinguished by steady state inactivation properties. Figure 2e shows that this was so. In a cell with currents like those in Figure 2a (midpoint of I-V curve +10, slow decay at +10), fairly depolarized holding potentials were needed to inactivate the channels (midpoint -6 mV), whereas, in a cell with currents like those in Figure 2c (midpoint of I-V curve -10 mV, fast decay at +10 mV), channels were inactivated at much more negative holding potentials (midpoint -41 mV).

In many mesenteric artery cells, both types of current appeared to be present simultaneously. In a cell similar to that shown in Figure 2f, the current at +10 mV consists of two components, a fast inactivating component that is eliminated by holding at -30 mV and a slowly inactivating component that is not inactivated by holding at -30 mV. By analogy to the closely similar behavior in neurons, heart cells, and pituitary cells, these results suggest the existence of two distinct types of calcium channels in vascular smooth muscle cells, although other explanations (for example, localized differences in surface charge within a cell) are possible, and it remains to be determined whether the channels underlying the two types of currents have different single channel current and selectivity.

As already shown (Figure 1c), we found that vascular muscle calcium channels are sensitive to dihydropyridine block. To study the mechanism of block in more detail, we used a lower concentration of nitrendipine and investigated whether drug block might be modulated by holding potential, as has recently been found in cardiac cells. Since preliminary experiments suggested that the fast type of channel is nearly insensitive to dihydropyridines (similar to results in cardiac and pituitary cells9,10,12), our experiments with nitrendipine were done with cells displaying predominantly the slow type of calcium channel. We found that nitrendipine blocked the slow type of calcium channel with a pronounced voltage dependence. In the experiment shown in Figure 3a, 100 nM nitrendipine blocked calcium channel current elicited from a negative holding potential (-70 mV) by about 35%, while completely eliminating current elicited from a depolarized holding potential (-10 mV) where, in the absence of drug, calcium channel current was about half inactivated. In this cell, comparison of current kinetics at various holding potentials suggested that about 80% of the control current elicited from -70 mV was slow channel current, with about a 20% contribution from fast channels, whereas all of the current from -10 mV would be from slow channels. Thus, even taking into account the presence of some fast current, it is clear that nitrendipine blocked the slow current much more effectively at the more positive holding potential. Quantitatively similar results were obtained in repetitions of the experiment with two other cells. This sort of voltage-dependent block can be understood by hypothesizing that drug molecules bind much more tightly.
When complete inactivation curves were determined in the same cell in the absence and presence of the drug, nitrendipine was found to shift the midpoint of the inactivation curve in the hyperpolarizing direction without changing its slope factor (Figure 3b), exactly the changes that are predicted by the modulated receptor model. The changes in the inactivation curve can be used to estimate the strength of nitrendipine binding to the resting state ($K_{d} = 222$ nM) and inactivated state ($K_{d} = 0.46$ nM) of the channel (see legend to Figure 3).

The 1,4-dihydropyridine derivative, BAY K8644, though structurally similar to blocking compounds, produces opposite effects on the circulation, causing vasoconstriction and hypertension. Figure 4 shows that BAY K8644 has potent effects on vascular muscle cells. The increase in current is larger for small depolarizations than for larger depolarizations, and the rate of inactivation is faster in the presence of drug.

**Discussion**

Our results suggest that the voltage-dependent calcium channels present in vascular muscle cells are much like those found in cardiac cells. There are two distinct patterns of voltage- and time-dependent current in the mesenteric artery cells, and they are very similar in voltage dependence and kinetics to the two components of calcium currently recently identified in whole-cell recordings from cardiac cells, where the two components clearly arise from two distinct types of channels. It should be emphasized that the voltage-dependence and kinetics described in the present work were recorded in high barium concentrations and are undoubtedly quite different from those in physiological calcium concentrations. The goal of elucidating the role of the channels under physiological conditions — for example, determining whether there may be a steady "window" current at normal resting potentials — will require vascular muscle cells with larger calcium currents. Klockner and Isenberg recently reported the existence of relatively large calcium currents in smooth muscle cells from bovine coronary arteries. Like the currents we recorded, the currents in coronary artery cells had a voltage-dependence similar to that seen in cardiac cells, although the distinction between two types of channels was not reported.

As in cardiac cells, the slowly inactivating type of channel in vascular muscle is sensitive to block by nitrendipine or enhancement by BAY K8644. The potency of nitrendipine block changes dramatically with holding potential, just as has been seen in cardiac tissue and in gut smooth muscle, in a manner consistent with very tight binding to the inactivated state of the calcium channel and much weaker binding to the resting state. Even quantitatively, the $K_{d}$ estimated for binding to inactivated slow channels in mesenteric artery cells (0.46 nM) is similar to that estimated for binding to inactivated cardiac channels (0.2–1 nM). This $K_{d}$ is also very similar to that measured for the high-affinity binding site for nitrendipine in vascular tissue (0.1–1 nM), supporting the idea that this binding site is, in fact, the calcium channel, specifically, the inactivated state of the calcium channel. The $K_{d}$ for inactivated channel binding is also very similar to concentrations that begin to be effective in relaxing blood vessels.

Although nitrendipine binding to inactivated slow channels seems very similar in cardiac and mesenteric artery cells, the potency of nitrendipine is different in the two cell types when assayed from negative holding potentials. Currents elicited from −70 or −80 mV were consistently half-blocked by 100–300 nM nitrendipine in mesenteric artery cells, whereas, under similar or identical ionic conditions, currents in canine atrial or ventricular cells required about 700 nM nitrendipine (Bean and unpublished results). This suggests that the resting state of the channel binds nitrendipine more tightly in vascular muscle than in cardiac muscle. However, the concentrations required for resting channel block are still two orders of magnitude higher than for high-affinity binding to inactivated channels and for relaxation of vessels. More detailed experiments (preferably on a preparation with larger currents) will be needed to explore more thoroughly drug binding to the various states of the channel. Also, it is empha-
sized that the form of the modulated receptor hypothesis used to fit the data in Figure 3 is likely to be oversimplified; for example, it ignores possible multiple inactivated states and possible high-affinity binding to the open state of the channel.

These results suggest that there are pharmacological differences between slow calcium channels in vascular muscle and those in cardiac cells, but that the differences are unlikely to be important at clinical concentrations of nifedipine. If high-affinity binding to inactivated channels is similar in the two tissues, why is the contraction of vascular muscle so much more potently inhibited? A simple explanation could be that in vascular muscle a higher fraction of channels is in the high-affinity inactivated state at rest. This would be consistent with the observation that resting potentials in vascular muscle are typically more positive than in cardiac muscle, where almost all channels would be in the low-affinity resting state. However, rigorous tests of this idea will require more complete information on the voltage dependence of inactivation under physiological ionic conditions and temperature. Some vascular muscle cells can have resting potentials near −70 mV,27,28 where it seems likely that most channels of the slow type are in the low-affinity resting state.

The calcium channel currents in adult mesenteric artery cells are very small, compared with those in adult mammalian cardiac cells. Measured in isotonic BaCl2 currents through the slow type of channels in mesenteric artery cells were typically about 20 pA (cell capacitance −18 pF), whereas, in canine ventricular cells, they were −3000 pA (cell capacitance −100 pF), approximately a 30-fold difference in current density. A similar difference exists in the concentration of high-affinity nifedipine-binding sites in the two tissues,27 further supporting the identification of the calcium channel as the high-affinity binding site. (However, canine ventricle has a somewhat higher density of binding sites than cardiac tissue from other species, and the results of Klockner and Isenberg25 already suggest that other types of vascular muscle cells may have larger calcium currents than the mesenteric artery cells we studied.) Besides the intrinsically low density of calcium channels in vascular muscle cells, the large increase in current produced by BAY K8644, which appears to act by increasing the probability of a channel’s being open,29,31 suggests that the channels may normally have a rather low probability of being open during a depolarizing pulse. A tantalizing possibility is that large increases in calcium current could also be produced by contraction-regulating hormones or neurotransmitters if these modulated calcium channel gating. In preliminary experiments, applications of 10 μM norepinephrine (to two cells) had no effect on calcium channel current; neither did norepinephrine induce current in a cell with no current, but the internal dialysis could disrupt any mechanism dependent on intracellular metabolism. With increasing evidence for a variety of mechanisms by which hormones and neurotransmitters modulate calcium channels in neurons and cardiac cells, it would almost seem surprising if further experiments did not reveal modulation of calcium channels in vascular muscle as well.

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