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Single nisoldipine-sensitive calcium channels in smooth muscle cells isolated from rabbit mesenteric artery

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ABSTRACT **Single smooth muscle cells were enzymatically isolated from the rabbit mesenteric artery. At physiological levels of external Ca, these cells were relaxed and contracted on exposure to norepinephrine, caffeine, or high levels of potassium. The patch-clamp technique was used to measure unitary currents through single channels in the isolated cells. Single channels were selective for divalent cations and exhibited two conductance levels, 8 pS and 15 pS. Both types of channels were voltage-dependent, and channel activity occurred at potentials positive to −40 mV. The activity of both channel types was almost completely inhibited by 50 nM nisoldipine. These channels appear to be the pathways for voltage-dependent Ca influx in vascular smooth muscle and may be the targets of the clinically used dihydropyridines.**

Calcium ions that enter cells through voltage-dependent pathways, Ca channels, regulate a wide variety of physiological functions, including contraction of vascular smooth muscle (VSM) (1–3); the degree of contraction of VSM cells determines the arterial resistance to blood flow. VSM appears to be the site of action of the dihydropyridines, an important class of therapeutic drugs (4, 5). Because these Ca antagonists are potent vasodilators, the dihydropyridines are prescribed for certain types of cardiovascular disorders, such as angina and hypertension. The selectivity of these drugs in relaxing VSM seems to be related to the greater affinity of these agents for Ca-influx pathways in VSM than similar pathways in such other tissue types as heart and nerve (5, 6).

Voltage-dependent Ca-influx pathways in VSM have been studied primarily by the technique of depolarizing the cell with high external potassium concentration to measure Ca isotope influx and muscle contraction (7–9). However, difficulties in isolating viable single cells from VSM have impeded investigation of the electrophysiological properties of these important Ca-influx pathways.

We report a procedure for isolating physiologically responsive single smooth muscle cells from the rabbit mesenteric artery. Single Ca channels from these cells were identified by measuring unitary current fluctuations in excised membrane patches using the patch-clamp technique. Single Ca channels exhibited two different conductance levels, ≈8 pS and 15 pS (in 80 mM barium). The activity of both channel types was reduced by the dihydropyridine nisoldipine (37).

METHODS AND MATERIALS

Cell Isolation. Single smooth muscle cells were enzymatically isolated from the rabbit (New Zealand White) mesenteric artery (200- to 300-μm diameter). Following dissection, the artery was placed in Hanks' solution (137 mM NaCl/5.4 mM KCl/0.44 mM KH_2PO_4/0.42 mM NaH_2PO_4/4.17 mM $NaCO_3$/5.55 mM glucose/0.02 mM EGTA/2 mM $MgCl_2$/0.2 mM $CaCl_2$/10 mM Hepes/NaOH, pH 7.4), and cleared of connective and fat tissue. A 5-mm segment of the artery was next pulled over a glass cannula to allow a perfusion of Hanks' solution containing collagenase at 1.0 mg/ml (CLS-II, Cooper Biomedicals, Malvern, PA), elastase at 0.5 mg/ml (type II, Sigma), hyaluronidase at 0.3 mg/ml (Sigma), trypsin inhibitor at 0.5–1.0 mg/ml (type II-S, Sigma), bovine serum albumin at 2.0 mg/ml (Sigma), DNase I and II at 0.1 mg/ml (Sigma), at 30°C through the vessel's lumen at a rate of ≈5 ml/min for 1 hr; the Hanks'-enzyme solution had been filtered through a 0.22-μm filter before use. After 1 hr, gentle agitation of the vessel released single smooth muscle cells and a group of these cells in a dish was placed onto a Nikon inverted microscope equipped with Hoffman Optics. Single VSM cells were relaxed in physiological levels of external Ca (mean length = 140 μm; mean diameter at nucleus = 5 μm, tapering to <1 μm). The addition of 30 μM norepinephrine by a pipette caused a typical VSM cell (Fig. 1*A*) to contract (Fig. 1*B*). The addition of either high levels of potassium or caffeine (1 mM) could also induce contraction. The norepinephrine-induced contraction was prevented by phentolamine in an equal concentration. These criteria indicate the Ca regulatory processes of these cells were unaffected by isolation procedures.

Current Recordings. Single channel recordings were accomplished using the patch-clamp technique as described by Hamill *et al.* (10); the patch pipettes (Boralex, Rochester Scientific, Rochester, NY) with resistances of 10–20 MΩ were made with a vertical puller (David Kopf, Tujunga, CA; model 700C). After positioning the patch pipette against the cell, a high resistance seal (1–20 GΩ) formed spontaneously. In the on-cell patch configuration, outward single channel current fluctuations were observed in almost every patch. The detection of small currents through a single Ca channel was difficult in the presence of these outward currents that were not completely blocked by external cesium (40 mM), barium (80 mM), or tetraethylammonium (40 mM). Excision of the membrane patch by detaching the pipette from the cell and removal of potassium from the bathing solution were the only strategies effective in eliminating these outward current fluctuations. The excised patch configuration had the advantage of enabling control over both transmembrane voltage and the composition of the solution at the two membrane surfaces. A Dagan patch-clamp amplifier (model 8900) was used for single channel current recordings; membrane current and voltage were recorded on VCR tape, and the data subsequently were played into an IBM AT computer for off-line analysis.

The intracellular cell surfaces were bathed in solution containing 120 mM sodium-aspartate or 50 mM *N*-methyl-D-glucamine/20 mM CsCl/5 mM EGTA/10–80 mM Hepes, pH 7.4, while the patch pipette contained 80 mM $BaCl_2$ and 10

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Abbreviation: VSM, vascular smooth muscle.

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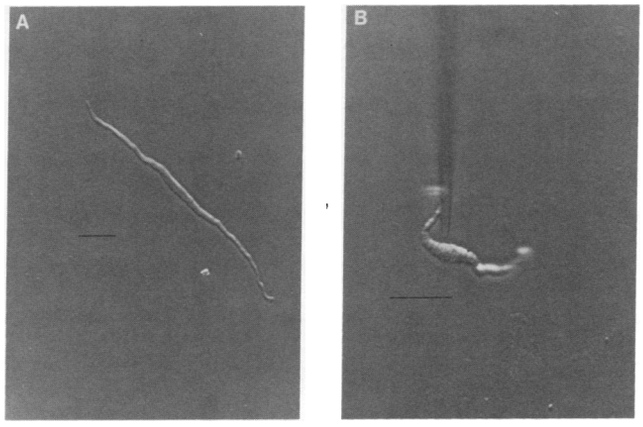

FIG. 1. Single VSM cell isolated from rabbit mesenteric artery. (*A*) Example of a single cell obtained following the cell isolation procedure described in *Methods and Materials*. This cell was relaxed in a physiological saline solution containing 2 mM Ca. (Bar = 20 μm.) (*B*) The same cell as in *A* in a contracted state. The contraction was induced by applying positive pressure to a patch pipette containing 30 μM norepinephrine in physiological saline solution. Similar results have been obtained by applying 140 mM potassium or 1 mM caffeine solutions. (Bar = 20 μm.)

mM Hepes (pH 7.4). To facilitate the detection of single Ca channels in excised membrane patches, the Ca channel agonist Bay K 8644 (1 μM; the kind gift from A. Scriabine, Miles, New Haven) was usually included in the solutions (11). In heart muscle, Bay K 8644 significantly increases the frequency of long-lasting Ca channel openings without affecting the single channel conductance (12, 13). We also found the single channel conductances to be unaffected by the presence of Bay K 8644. Nisoldipine (A. Scriabine, Miles, New Haven) was added from a concentrated stock solution (10 μM) in the dark. Gigaohm seals were obtained in ≈70% of the attempts, and ≈50% of these excised patches contained single Ca channels. In those patches with Ca-channel activity, Ca channels numbered from 1–3, although occasionally patches contained as many as 20 Ca channels. Ca-channel activity has been recorded from >50 patches and did not decline during these experiments; some patch preparations retained activity for as long as 2 hr.

RESULTS

Recordings of single channel current fluctuations in an excised patch from the sarcolemma of a single VSM cell isolated from the rabbit mesenteric artery are displayed in Fig. 2*A*. At each voltage there were two single channel current amplitudes. As seen in the current–voltage relationships depicted in Fig. 2*B*, these single channel events corresponded to single channel conductances of 8 pS and 15 pS. The extrapolated reversal potentials for the 8-pS channel and the 15-pS channel were +63 mV and +60 mV, respectively. In this experiment, the equilibrium potentials for barium, *N*-methyl-D-glucamine, cesium, and chloride were positive infinity, negative infinity, negative infinity, and −30 mV, respectively. The extrapolated reversal potentials for both single channel current–voltage relationships were unaffected by replacing *N*-methyl-D-glucamine with sodium or potassium. These results indicate that these channels are highly selective for divalent cations over sodium, potassium, chloride, *N*-methyl-D-glucamine, and cesium ions.

Both channel types were voltage-dependent; membrane depolarization increased the probability of the open state. Voltage steps from a holding potential of −75 mV to −50 mV did not open either type of channel. However, for example, in the experiment shown in Fig. 2, changing the pulse

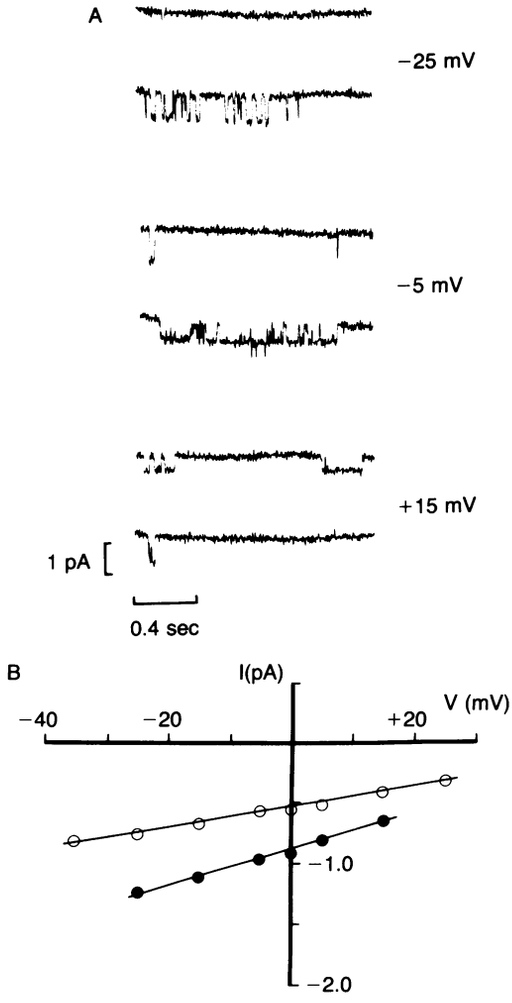

FIG. 2. Effect of voltage on currents through single Ca channels in an excised patch of VSM membrane. (*A*) Original records at −25 mV, −5 mV, and +15 mV. Single Ca-channel openings are downward and activity was observed only at pulse potentials positive to −40 mV. In these experiments the holding potential was set at −75 mV, and the depolarizing pulse potential was held for 3.1 sec at the indicated voltage. At −25 mV and −5 mV the top record shows a single opening of the 15-pS channel and the bottom record shows openings of the 8-pS channel. At +15 mV, the top and bottom records show the 8-pS and 15-pS channel, respectively. To illustrate both types of single Ca channels, two records are shown for each potential. Single channel records were filtered at 200 Hz on playback. The solution in the pipette was 80 mM $BaCl_2$/10 mM Hepes, and the bath contained 50 mM *N*-methyl-D-glucamine/20 mM CsCl/5 mM EGTA/80 mM Hepes, pH 7.4. Both the pipette and bath solutions contained 1 μM Bay K 8644. (*B*) Single channel current–voltage relationships of the two channel types. The slope conductance for these two channel types is 8 pS (open circles) and 15 pS (solid circles). Under the experimental conditions, the equilibrium potential for barium is positive infinity, for cesium and *N*-methyl-D-glucamine is negative infinity, and for chloride is −30 mV. The extrapolated reversal potentials for the 8-pS and 15-pS channels are +63 mV and +60 mV, respectively, indicating that both types of channels have similar divalent cation selectivities. Each current level depicted is the mean of at least 50 determinations; the standard deviation < the width of a circle.

potential from −50 mV to −35 mV increased the percentage of pulses that activated channels from <2% to ≈20%. Depolarizing voltage steps to −25 mV further increased the percentage of pulses that activated channels to ≈50%. Thus, the voltage sensitive range of these channels is within the physiological range of those membrane potentials that initiate Ca influx and cellular contraction (14, 15).

A high sensitivity of VSM Ca channels to the dihydropyridinetype Ca channel antagonists underlies the therapeutic specificity of these agents (see ref. 16). Therefore, we tested the effects of nisoldipine on the activity of single channels (Fig. 3),

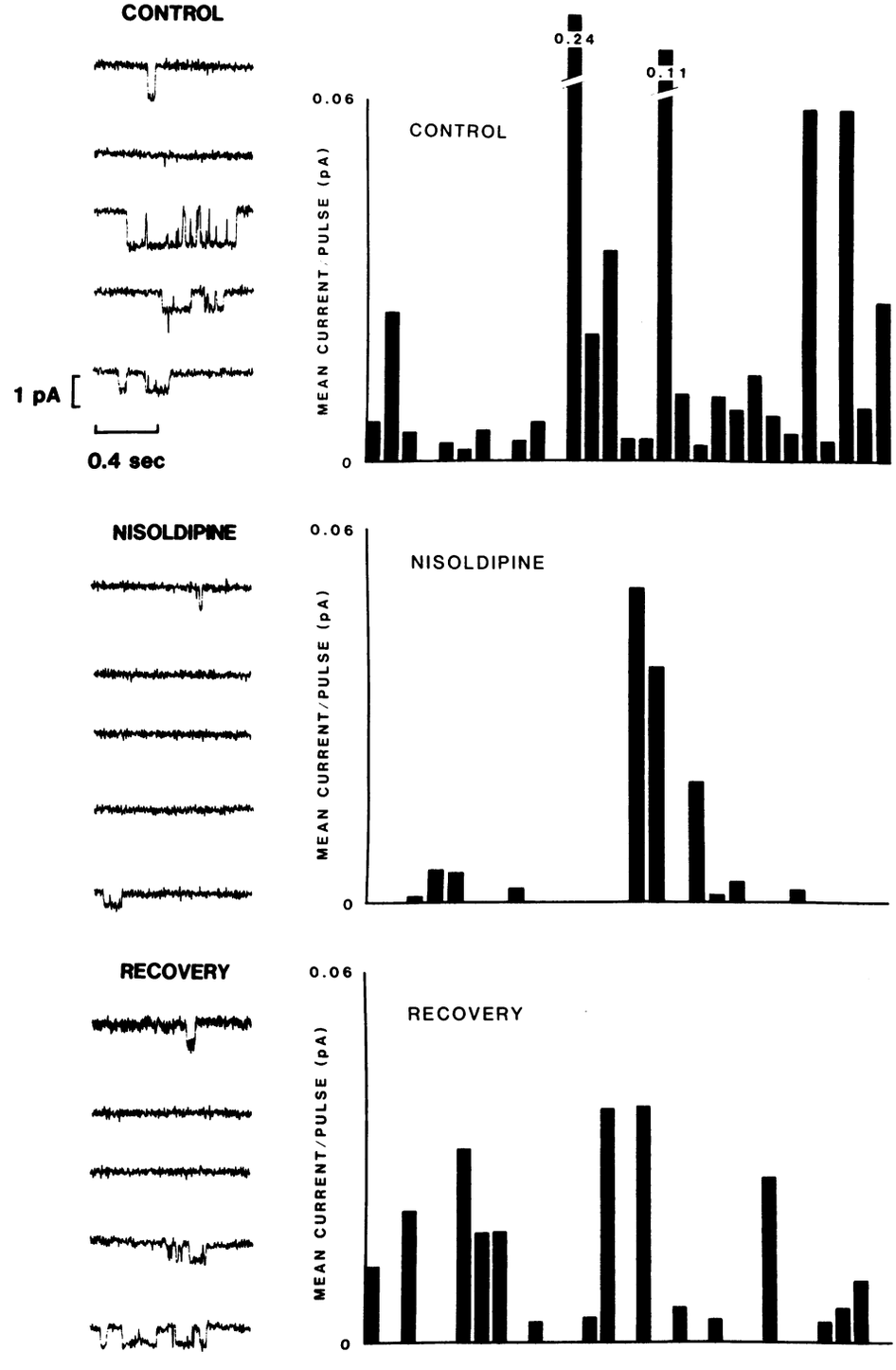

FIG. 3. Effect of nisoldipine on single Ca channel current fluctuations. (*Left*) Original records. Control: Single Ca channel current fluctuations before the addition of nisoldipine to the bath. All single channel current recordings were obtained by pulsing from a holding potential of −75 mV to 0 mV for 3.1 sec, and channel openings are downward current deflections as in Fig. 2*A*. Before the addition of nisoldipine, single channel current fluctuations were recorded for 25 min without a decline in single Ca channel activity. Nisoldipine: The same membrane patch 15 min after the addition of 30 nM nisoldipine. This phase of the experiment was conducted under dark conditions. Recovery: The same membrane patch after exposing the patch to bright light (Dolan Jenner, series 170-D) for 6 min. At the peak wavelength known to inactivate nisoldipine, ≈400 nm (17), the light source delivers ≈10 μW at 0.25 m. The additional noise in the first current trace in the Recovery is due to the light source. The current traces in Control, Nisoldipine, and Recovery were taken from five consecutive voltage pulses. Ionic conditions in this experiment were the same as indicated for Fig. 2; 1 μM Bay K 8644 is present. All current records were filtered at 500 Hz for data analysis. (*Right*) Mean current per pulse through the 8-pS channel. The mean current for each pulse is defined as the total charge movement through a single 8-pS channel divided by the pulse duration (3.1 sec), and each panel shows mean currents in consecutive pulses. In 29 consecutive control pulses, 26 pulses activated the 8-pS channel; the average current for all pulses was 23.6 fA (Control). In the presence of 30 nM nisoldipine, 10 of 27 pulses instigated single channel activity, and the average current for all the pulses was 4.8 fA (Nisoldipine). After a 5-min patch exposure to light, 50% of the pulses (n = 30) had single channel activity; the average current was 8.8 fA (Recovery). The average current for the 15-pS channel is not shown.

first measuring activity in the absence of nisoldipine for 25 min (Fig. 3). To verify that single channel activity was constant during the experiment, this activity was monitored at the beginning and end of the control run using 30 pulses of 3.1-sec duration to 0 mV; no differences were found between the averages of these two activities. After the addition of 30 nM nisoldipine to the bath, single channel activity was substantially reduced (Fig. 3). In this experiment the membrane potential was held at −75 mV, and the pulse potential was 0 mV. Note that the average current through the 8-pS channel was reduced to ≈20% of control levels. Average current equals the total charge movement through the open 8-pS channel in 27 or more pulses divided by the total time at that pulse potential (0 mV). This reduction in average current can partially be explained by an increase in the percentage of pulses that failed to activate the 8-pS channel—10% before and 63% after the addition of nisoldipine. Because nisoldipine is photolabile (17, 18), these experiments were conducted in the dark. Thus, the specificity of the action of nisoldipine on single Ca channel activity was tested by partially reversing its action with light (17, 18). As shown in Fig. 3 (Recovery), light-exposure of the same membrane patch bathed in 30 nM nisoldipine increased the average current through the 8-pS channel to 37% of the control. Moreover, the percentage of pulses that did not open channels decreased from 63% to 50%.

In the control run of this experiment, activity of the 15-pS channel was observed with 34% of the depolarizing pulses. Following nisoldipine addition, no activity of this channel was seen. When nisoldipine was partially light-inactivated, activity of the 15-pS channel was observed on ≈3% of depolarizing pulses to 0 mV. At longer light exposures, the activity of the 15-pS channel increased at other pulse potentials. In patches containing both types of channels, 50 nM nisoldipine reduced the average current through both channel types by >95%. At these nisoldipine concentrations, the single channel current–voltage relationships of the 8 pS- and 15 pS-Ca channels were not affected.

DISCUSSION

Single VSM cells were enzymatically isolated from the rabbit mesenteric artery, and single channel current fluctuations in excised cellular membrane patches were recorded using the patch-clamp technique. These channels were identified as the pathways that allow Ca entry into VSM based on the criteria of: (*i*) ionic selectivity—these channels select for divalent cations over monovalent ions, (*ii*) single channel conductances—the single channel conductances (8 pS and 15 pS) are similar to those reported for Ca channels from heart muscle (12), (*iii*) voltage-activation—the probability of channel activity increases steeply as membrane depolarization exceeds −40 mV (14, 15), and (*iv*) antagonist-sensitivity—nisoldipine (50 nM) almost completely inhibits both types of single Ca channels. This result agrees with observations on intact VSM in which nisoldipine (>50 nM) inhibited 100% of the potassium-stimulated Ca influx and contraction, 50% inhibition (K_i) occurring at about 1 nM (19, 20). At 0 mV, under our experimental conditions, the apparent K_i for nisoldipine inhibition was ≈8 nM nisoldipine. This high sensitivity of single Ca channels to nisoldipine is particularly striking because of the presence of Bay K 8644 (1 μM), a possible competitor for the nisoldipine binding site in these experiments (21, 22). The apparent K_i may also be a function of the pulse and holding potential (18, 23, 24).

From average sizes of isolated single VSM cells (Fig. 1*A*), the apparent surface area (exclusive of surface membrane invaginations) is estimated at ≈1600 μm^2. If each excised patch contains ≈5 μm^2 of surface membrane (25) and one Ca channel (see *Methods and Materials*), the total number of functional Ca channels in one VSM cell can be estimated at ≈320. However, if invaginations account for at least 50% of the total membrane surface area (26), the minimal number of Ca channels per cell would be doubled.

Two Types of Single Ca Channels. According to differences in single channel conductances, we have identified two types of single Ca channels. The single channel conductances were ≈8 pS and 15 pS (with 80 mM barium) and are similar to the single channel conductance levels reported for cardiac Ca channels in on-cell patches (12) and in planar lipid bilayers (27). That Ca channels in two different types of muscle have similar conductances under almost identical experimental conditions suggests that the Ca channel pore(s) may be conserved among different tissue types. In support of this hypothesis, a survey of single channel recordings reveals that single Ca channel conductances fall primarily into two groups: (*i*) a 7- to 10-pS group—e.g., brain synaptosomes (28), clonal pituitary cell line (29), snail neurons (30), dorsal root ganglion cells (31), heart muscle (12), and VSM (this report)— and (*ii*) a 15- to 25-pS group—e.g., heart muscle (12), dorsal root ganglion cells (31), and VSM (this report). Our observation of two types of Ca channels in VSM agrees with the recent report that there are two components to the macroscopic Ca current in rat mesenteric artery (32).

Although the rates at which divalent cations permeate single open Ca channels from heart and VSM are similar, there appears to be at least one apparent difference between the Ca channels in these two preparations—their dihydropyridine sensitivity. In heart muscle, micromolar concentrations of nitrendipine are required for a 50% reduction in the average current through the 15- to 20-pS channel in on-cell patches (13) and the 7- to 10-pS Ca channel has been reported as insensitive to the dihydropyridine agonist Bay K 8644 (12). In contrast we find that both types of single Ca channels in VSM are sensitive to nanomolar concentrations of nisoldipine (Fig. 3). In heart muscle, nitrendipine and nifedipine inhibition of Ca currents is most potent when the holding potentials maintain the Ca channels primarily in an inactivated state (18, 23, 24). It has consequently been proposed that the resting membrane potential of smooth muscle may be more positive than heart muscle thus inactivating a large fraction of the smooth muscle Ca channels so that these have a greater affinity for dihydropyridines. But differences in resting membrane potentials between smooth muscle cells and other cell types cannot solely account for the observed variations in Ca channel sensitivity to the dihydropyridines (cf. refs. 33 and 34). In excised patches from VSM we have observed high affinity inhibition of single Ca channels even when the holding potential was −100 mV. A comparison of VSM with heart muscle Ca channels under identical conditions should be conducted.

Another observed difference between heart muscle and VSM is that the 20-pS channel in heart becomes nonfunctional in excised patches (12); under our experimental conditions both Ca channel types in VSM functioned for hours without loss of Ca channel activity (see Fig. 3 and *Methods and Materials*). However, in the presence of Bay K 8644 the 20-pS Ca channel from heart muscle appears to function normally in the planar lipid bilayer system (27, 35). The survival of single Ca channels in isolated patches of VSM cell membranes may be aided by Bay K 8644 (cf. ref. 36) or may reflect differences in the metabolic regulation of Ca channels.

In summary, a procedure for isolating physiologically responsive single cells from the mesenteric artery of the rabbit has been developed. Two types of single voltage-dependent Ca channels in these cells, with unitary conductances of 8 pS and 15 pS, have been identified. Furthermore, both types of Ca channels were inhibited by nanomolar concentrations of the dihydropyridine nisoldipine. The high-affinity inhibition of these single Ca channels by nisoldipine

suggests that these are the sites of action of the clinically used dihydropyridines. Since in the excised patch configuration the intracellular surfaces of these single Ca channels are exposed to the bathing solution, this system should be uniquely suited to investigate channel regulation by Ca, pH, phosphorylation, and a variety of therapeutic agents.

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