

Ca²⁺ Currents in Cerebral Artery Smooth Muscle Cells of Rat at Physiological Ca²⁺ Concentrations

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ABSTRACT Single Ca²⁺ channel and whole cell currents were measured in smooth muscle cells dissociated from resistance-sized (100- μ m diameter) rat cerebral arteries. We sought to quantify the magnitude of Ca²⁺ channel currents and activity under the putative physiological conditions of these cells: 2 mM [Ca²⁺]_o, steady depolarizations to potentials between -50 and -20 mV, and (where possible) without extrinsic channel agonists. Single Ca²⁺ channel conductance was measured over a broad range of Ca²⁺ concentrations (0.5–80 mM). The saturating conductance ranged from 1.5 pS at 0.5 mM to 7.8 pS at 80 mM, with a value of 3.5 pS at 2 mM Ca (unitary currents of 0.18 pA at -40 mV). Both single channel and whole cell Ca²⁺ currents were measured during pulses and at steady holding potentials. Ca²⁺ channel open probability and the lower limit for the total number of channels per cell were estimated by dividing the whole-cell Ca²⁺ currents by the single channel current. We estimate that an average cell has at least 5,000 functional channels with open probabilities of 3.4×10^{-4} and 2×10^{-3} at -40 and -20 mV, respectively. An average of 1–10 (-40 mV and -20 mV, respectively) Ca²⁺ channels are thus open at physiological potentials, carrying ~ 0.5 pA steady Ca²⁺ current at -30 mV. We also observed a very slow reduction in open probability during steady test potentials when compared with peak pulse responses. This 4–10-fold reduction in activity could not be accounted for by the channel's normal inactivation at our recording potentials between -50 and -20 mV, implying that an additional slow inactivation process may be important in regulating Ca²⁺ channel activity during steady depolarization.

INTRODUCTION

The flux of Ca²⁺ through voltage-dependent channels in the sarcolemma of arterial smooth muscle cells has been hypothesized to be a critical link in the maintenance of myogenic vascular tone (Nelson et al., 1990), and thus the autoregulation of blood flow. The development of tone in response to transmural pressure *in vitro* is associated with a steady depolarization of the smooth muscle cell membrane potential, e.g., from -60 to -40 mV (Harder, 1984; Brayden and Wellmann, 1989; Brayden and Nelson, 1992; Knot and Nelson, 1995). Conversely, membrane hyperpolarization of these arteries at constant pressure causes vasodilation. The myogenic tone of cerebral arteries is dependent on external calcium, and can be blocked by calcium channel inhibitors such as nimodipine and dilt-

iazem (Brayden and Nelson, 1992; Knot and Nelson, 1995). These observations suggest that cerebral artery tone depends on steady Ca²⁺ entry through voltage-dependent calcium channels in smooth muscle cells at membrane potentials around -40 mV.

Despite the importance of such steady Ca²⁺ entry into arterial smooth muscle cells, little is known about the properties of the underlying Ca²⁺ channels at steady potentials with physiological calcium as the charge carrier. The majority of calcium channel studies in arterial smooth muscle as well as in other preparations have examined the channel properties during relatively brief voltage pulses (usually less than 0.5 seconds) using high concentrations of barium as the charge carrier. Studies of whole cell Ca²⁺ currents can be made in 2 mM extracellular Ca²⁺ but these studies also generally examine responses to brief depolarizations. They also suffer from uncertainty regarding separation of the Ca²⁺ current from other ionic currents, from lack of direct knowledge of the number and unitary amplitude of the Ca²⁺ channels, and from a disturbed intracellular environment. Furthermore, most

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of these studies have been made on cells derived from large, nonmyogenic arteries.

Although single channel measurements overcome some of these difficulties, they introduce different sources of uncertainty. Divalent cation concentration is usually elevated and Ba^{2+} substituted for Ca^{2+} to increase unitary currents. The agonist Bay K 8644 (or its active enantiomer Bay R 5417) has also been extensively used to prolong channel openings. Finally, channels were usually studied during brief pulses. No single experimental method alone, therefore, can provide an accurate picture of Ca^{2+} channel activity in the physiological state of these cells.

Nevertheless, recent measurements have provided tantalizing glimpses of these properties. Gollasch et al. (1992) reported the unitary amplitude of Ca^{2+} channels at 2 mM external Ca^{2+} . Langton and Standen (1993) measured steady whole cell currents in 10 mM Ba^{2+} and used the data from Gollasch et al. and other single channel studies (Worley et al., 1991; Quayle et al., 1993) to calculate steady channel activity. Quayle et al. (1993) measured steady open probability (P_o) in single channels (10 mM Ba^{2+}) both with and without agonist. Ca^{2+} currents have also been detected after 30-s depolarizing pulses in mesenteric artery (Smirnov and Aaronson, 1992).

We sought to provide a more comprehensive picture of Ca^{2+} channel activity under physiological conditions by combining single channel and whole cell techniques using cells from resistance-sized, myogenic (100–150 μm cerebral) arteries. The single channel recording technique was used to measure unitary currents over a broad concentration range and to measure peak and steady state P_o values for the currents. The whole cell recording technique was used to measure peak and steady state whole cell Ca^{2+} currents at 2 mM Ca^{2+} . Such measurements gave a lower bound for the number of calcium channels in each cell by dividing the maximum peak current by the unitary channel current at 2 mM Ca^{2+} . We were therefore able to estimate steady single Ca^{2+} channel open probability at physiological potentials with two independent approaches. First, by dividing the steady whole cell currents by the unitary amplitude and total number of channels; and second, by correcting the steady state single channel P_o measurements for the action of Bay R 5417, which was required to observe sufficient single channel signal at 2 mM Ca^{2+} . The degree of enhancement by Bay R 5417 was estimated either via the whole cell current enhancement reported here, or by the previously reported effect of Bay R 5417 in the presence of 10 mM Ba^{2+} (Quayle et al., 1993). Each method gave comparable estimates for physiological open probability. Furthermore, since each method has different sources of uncertainty, their combination permits a higher degree of confidence in the results.

We provide the following significant new information: (1) The first full single channel conductance- Ca^{2+} relationship below 10 mM Ca^{2+} ; (2) the first measurements of channel open state probability with 2 mM Ca^{2+} as the charge carrier; (3) the first whole cell Ca^{2+} current measurements at steady physiological membrane potentials; and (4) the first evidence for slow inactivation process at the single channel level in smooth muscle.

This study, combined with quantitative information from previous Ca^{2+} channel studies (Nelson et al., 1988; Quayle et al., 1993; Langton and Standen, 1993; Fleischmann et al., 1994) provide strong support for the idea that a small number of open Ca^{2+} channels supply the steady Ca^{2+} influx (~ 0.5 pA) needed to support a maintained constricted state (tone) in small arteries.

METHODS

Cell Isolation

Female Sprague-Dawley rats (12–13 wk old) were anesthetized and decapitated. Posterior cerebral arteries (100–150 μm diameter) were dissected from the brain tissue and placed in low-Ca solution containing (in mM) 55 NaCl, 80 Na-glutamate, 6 KCl, 0.1 CaCl_2 , 10 HEPES, 11 glucose, and 1 mg/ml fat-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO), pH adjusted to 7.3 with NaOH. Arteries were cleaned of adherent meninges and transferred to a vial containing ~ 1 ml of the same solution with papain (Worthington BioChemical, Freehold, NJ; 1.5 mg/ml) and dithioerythritol (Sigma; 1 mg/ml) for 20 min at 35°C. The tissue was then incubated in ~ 1 ml of fresh low-Ca solution containing collagenase (Sigma, type F; 1.5 mg/ml) and hyaluronidase (Sigma, type II-S; 1 mg/ml) for an additional 10 min. The enzyme-treated arteries were placed in fresh, enzyme-free solution of the same composition and triturated through a wide-bore Pasteur pipette until single cells were obtained. Cells were stored in the same solution at $\sim 4^\circ\text{C}$ until use.

Data Recording

The whole cell and cell-attached configuration of the patch clamp technique were used to record Ca^{2+} currents (Hamill et al., 1981). Pipettes were fabricated from borosilicate glass and coated with wax to minimize capacitive transients. For the whole cell experiments the external (bathing) solution contained either 137 mM NaCl, 5.6 mM KCl, or 142 mM TEA-Cl with 10 mM HEPES, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose, pH adjusted to 7.4. For the whole cell recording, the pipette (internal) solution contained (in mM) 130 CsCl, 5 EGTA, 1 MgCl_2 , 10 HEPES, 2 Na_2ATP , 0.5 GTP, 10 glucose, pH adjusted to 7.2 with CsOH. Where indicated in the text, the dihydropyridine calcium channel agonist, Bay K 8644, or its active isomer, Bay R 5417, was included at a concentration of 500 nM to promote long-lasting openings (Quayle et al., 1993).

During the cell-attached experiments, the external (bathing) solution contained (in mM) 135 KCl, 10 NaCl, 1 EGTA, 10 HEPES, 1 MgCl_2 , 0.569 CaCl_2 , pH adjusted to 7.4 with NaOH. The estimated free calcium activity was 100 nM. Patch pipette solutions containing 5 mM CaCl_2 or less were prepared by addition

of the appropriate amount of CaCl_2 to Ca^{2+} -free solution containing 120 mM NaCl, 10 mM HEPES, pH adjusted to 7.4 with NaOH. When 10, 20, 50, and 80 mM CaCl_2 were used in the patch pipette solution, the concentration of NaCl was lowered to 105, 80, 20, and 0 mM.

Experiments were performed at room temperature (18–22°C). Single channel and whole cell currents were amplified by an Axopatch 200 A amplifier (Axon Instruments, Foster City, CA). Whole-cell recordings were made from $n = 34$ cells, with a series resistance of $11.1 \pm 1.5 \text{ M}\Omega$ and a cell capacitance of $13.7 \pm 0.4 \text{ pF}$. Whole cell current records during voltage steps were low-pass filtered at 1 kHz (–3 dB, 8-pole Bessel filter), and digitized at 5 kHz. During steady holding potentials, whole-cell currents were filtered at 2 Hz and sampled at 10 Hz. Single-channel currents were filtered at 500 Hz and sampled at 6–7 times this filter setting. Digitized current signals were stored on microcomputer for later analysis.

Data acquisition and command potentials were controlled with commercial software programs (pCLAMP and Axotape, Axon Instruments, Foster City, CA). Analysis of whole cell currents was performed using pCLAMP. Single-channel data were analyzed using custom software. Numerical values are given, where appropriate, as averages \pm SEM.

Because macroscopic inward currents through Ca^{2+} channels in smooth muscle cells from posterior cerebral arteries are relatively small, they may be obscured by leak and other outward currents. To minimize K^+ currents, cells were dialyzed with 130 mM CsCl and bathed in a solution containing 142 mM TEA-Cl. In addition, activities of K_{Ca} and K_{ATP} channels were reduced by buffering intracellular free Ca^{2+} concentration with 5 mM EGTA and by adding 2 mM ATP to the pipette solution, respectively. Leak current was estimated by fitting a line by least squares to the current–voltage relationship in the range –100 to –60 mV. Ca^{2+} currents were leak-corrected by linear extrapolation of this line. In some cases, we also measured the inward current through Ca^{2+} channels by subtracting the residual current in the presence of 2 mM Co^{2+} or diltiazem. The Co^{2+} -sensitive component of the inward current was assumed to be the inward current through Ca^{2+} channels. As previously shown by others (Aaronson et al., 1987;

Langton and Standen, 1993), both methods yielded very similar current–voltage relations for the peak inward current over the voltage range –60 to +20 mV, both in control solution and in the presence of 500 nM Bay K 8644.

Analysis of Single Channel Data

The primary method of single channel analysis used here was the mean–variance (MV) technique, described in detail elsewhere (Patlak, 1993). Briefly, the MV analysis consists of sliding a window, W sample points wide, one point at a time over the original digitized record, examples of which are shown in Fig. 1. The mean and variance of the points in the window were determined for each sample in the record. Each of the mean–variance combinations computed in this way were entered into a two-dimensional histogram displaying mean current, (x -axis) versus variance (y -axis), with the amplitude of each bin encoded by the z -axis for the bin (see Fig. 2 A). For each record, eight MV histograms were constructed simultaneously at window widths between 3 and 100 points. In each MV histogram, the presence of steady current levels within the data yielded low-variance regions (represented as peaks in the three-dimensional representation in Fig. 2 A) that characterize the currents at that level. Average amplitudes from those low variance bins (variances as low or lower than the baseline current variance) were taken to be the unitary current amplitude. The value for the single channel amplitude for each individual patch was finally obtained by averaging the amplitude of low variance points from MV histograms constructed at window widths >5 . The volume of each low variance region, N , (i.e., the total number of instances where all W data points are within a steady current level) was derived from the curves best fitting the mean and variance histograms.

Open state probability of data recorded at steady membrane potentials were determined using the relationship

$$N_k(W) = k \cdot \tau \cdot \exp\left[\frac{-(W-1)}{\tau}\right] \quad (1)$$

where N_k is the total number of instances in the data during which the entire window width is fully within a steady current level, τ is the time constant of that level, W is the window width,

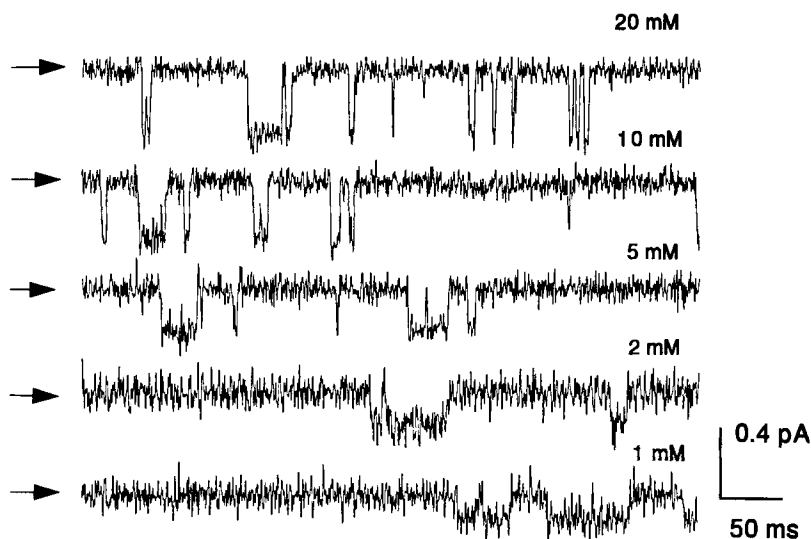


FIGURE 1. The relationship between external calcium concentration and single channel current amplitude. Single calcium channel records with external calcium concentrations of 1–20 mM. The pipette solution also contained 120 mM NaCl (105 and 80 mM NaCl at 10 and 20 mM Ca^{2+}) and 10 mM HEPES at pH 7.4. Current responses to 0.5-s pulses to –40 mV from a holding potential of –70 mV (500 nM Bay R 5417 present). Arrows indicate the closed state. Filter frequency was 500 Hz. Capacitive transients and leak currents were subtracted digitally using average current traces that contained no channel openings.

and k is the number of open events at a window width W (see Patlak, 1993). The volume representing the open level was plotted as a function of time corresponding to the $(W - 1)$ sample points (Fig. 2 B) and subsequently fitted with one or more exponentials to determine the time constants at the open level. When the sum of exponentials best fitting the $N_k(W)$ relationship is extrapolated to $(W - 1) = 0$, N_k equals the total time spent at this level (Fig. 2 B). The single-channel open-state probability at steady membrane potentials, P_o , was then calculated according to the relationship

$$P_o = \frac{\sum_{i=1}^N t_i * i}{TN} \quad (2)$$

where t_i is the time spent at conductance level $i = 1, 2, \dots, N$, N is the maximum number of channels seen in the patch, and T is the duration of the measurement (usually >5 min at each voltage). The lower bound for the number of channels was estimated by examining >50 test pulses (in the presence of Bay R 5417, 500 nM) to voltages (e.g., $-20, -10$ mV) where P_o was relatively high.

P_o in response to voltage pulses was estimated from the $N_k(W)$ relationships using the procedure as outlined for continuously recorded data. For test potentials to -30 mV and more positive (where inactivation was seen during test pulses), P_o was determined from a segment ± 50 ms the time point at which peak inward current occurred in whole cell Ca^{2+} current recordings in the presence of Bay K 8644 (500 nM). Little inactivation was seen for test pulses negative to -30 mV, and P_o was determined from the whole current trace during the voltage pulse. Test pulses were delivered at a rate of 0.2 Hz.

When the number of entries into the open-state component was very low, it was not possible to obtain adequate fits to the mean-variance histograms using the methods described in Patlak (1993). In these cases, the volume of the low variance region was estimated by integrating all the entries in the component, assuming that the variance of the fully open state was close to that of the baseline. Volumes determined in this way were quantitatively identical to those from standard fits but did not provide independent simultaneous estimates of open channel variance.

Materials

Papain was obtained from Worthington Biochemical (Freehold, NJ). Collagenase type F, hyaluronidase, dithioerythritol, ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), tetraethylammonium ions (TEA⁺), were all obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

The Relationship between External Calcium Concentration and Single Channel Current

Currents through single calcium channels were measured in membrane patches on myocytes freshly isolated from rat posterior cerebral artery, with different levels of external calcium as charge carrier. We measured unitary current amplitude over a wide range of calcium concentrations. The calcium channel agonist Bay K 8644 or its active isomere, Bay R 5417, was used

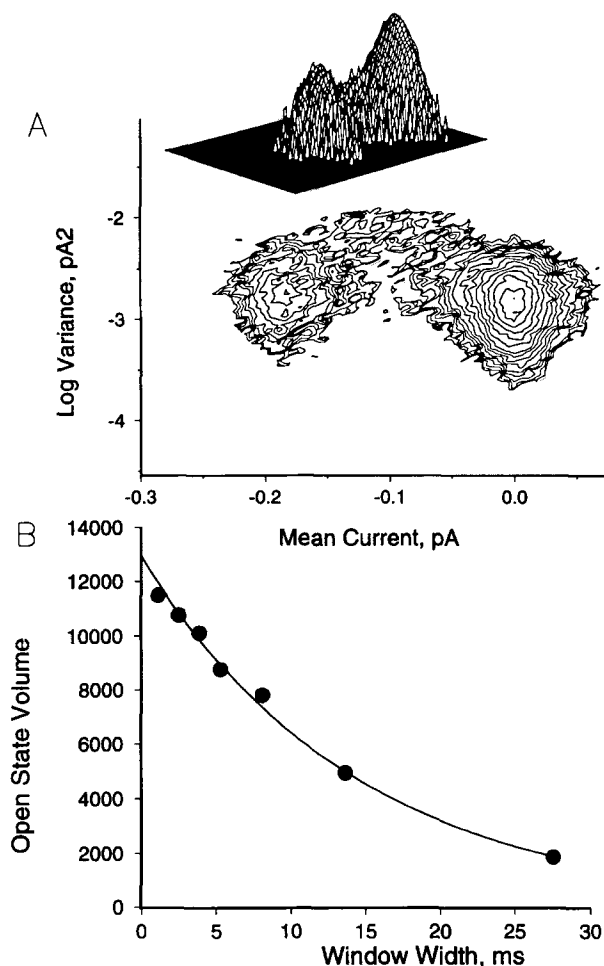


FIGURE 2. Mean-variance analysis of amplitude and open probability. (A) Mean-variance relationships of currents recorded during test pulses to -50 mV with 2 mM Ca^{2+} as charge carrier. For each sample point in the raw data, mean and variance of that point and 19 consecutive points were calculated. The primary plot illustrates the number of observations for each mean-variance combination as a contour plot of the three-dimensional mean-variance histogram (see *inset*). The coding for height has been log scaled. Baseline noise results in the large peak on the right. Data entries from periods where the channel was open are shown as the peak on the left. (B) Open component volume of the mean-variance histogram as a function of window width. Data were from a record at a steady membrane potential $V = -50$ mV. Volumes at each window width were determined using the fitting procedure outlined in the text. The volume was then plotted as a function of the time corresponding to the $(W - 1)$ sample points. The continuous line shows best-fit single exponential. The time constant for the open state was 14.3 ms.

in all the single channel experiments to prolong channel openings. This agonist, in combination with the mean-variance analysis, permitted quantitative analysis of the unitary currents and open probability of single channels at 2 mM external Ca^{2+} and below. Bay K 8644 does not appear to alter the single channel conduc-

tance of Ca^{2+} channels (Hess et al., 1984, 1986; Worley et al., 1991), and its effects on P_o have been well defined in this (Quayle et al., 1993) and other preparations (see McDonald et al. [1994] for review); it increases P_o 30- to 45-fold in the hyperpolarized voltage range, in part by shifting the activation voltage sensitivity of the channels by 10–20 mV in the hyperpolarized direction.

Fig. 1 illustrates currents through single calcium channels at five different calcium concentrations during 500-ms depolarizing voltage steps to -40 mV from a holding potential of -70 mV in the presence of Bay R 5417. We were able to resolve single channel openings at levels of Ca^{2+} as low as 0.5 mM, where the unitary current amplitude was 0.09 pA at -40 mV.

The single channel current–voltage relationships at each external calcium concentration are summarized in Fig. 3 A. The slope conductances (negative to -10 mV) are indicated on the figure, and ranged from 1.5 pS at 0.5 mM ($n = 5$ cells) to 7.8 pS at 50 mM and 80 mM ($n = 6$ cells). The unitary current amplitude with 2 mM calcium as charge carrier was -0.18 pA at -40 mV ($n = 19$ cells).

The single channel slope conductances (γ) are plotted as a function of calcium concentration in Fig. 3 B. The relationship can be fitted empirically with the Hill equation:

$$\gamma = \frac{\gamma_{\max}}{1 + (K_d/[Ca]_o)^n} \quad (3)$$

with an apparent dissociation constant (K_d) equal to 7.4 mM, a Hill coefficient (n) equal to 0.55, and a maximum conductance (γ_{\max}) equal to 10.1 pS.

Membrane Depolarization Increases Peak and Steady State Open State Probability of Calcium Channels with 2 mM Calcium as the Charge Carrier

To test the prediction that Ca^{2+} channels carry steady currents in the range of potentials experienced during myogenic tone by the smooth muscle cells in myogenic cerebral arteries, we measured the properties of calcium channels at constant membrane potentials with 2 mM calcium as the charge carrier. We also compared the steady state activity to the activity evoked by brief voltage pulses.

Fig. 4 A shows sample traces selected to illustrate the difference between Ca^{2+} channel activity at a constant membrane potential of -50 mV and that during 500-ms depolarizing steps (from a holding potential of -70 mV) to the same potential. Fig. 4 B summarizes the dependence of steady state and peak open state probability (P_o) on membrane potential (see also Table I). In the range of membrane potentials tested (-50 to -20 mV), peak P_o in response to pulses increased ~ 10 -fold with depolarization. Steady-state P_o at -50 mV (mea-

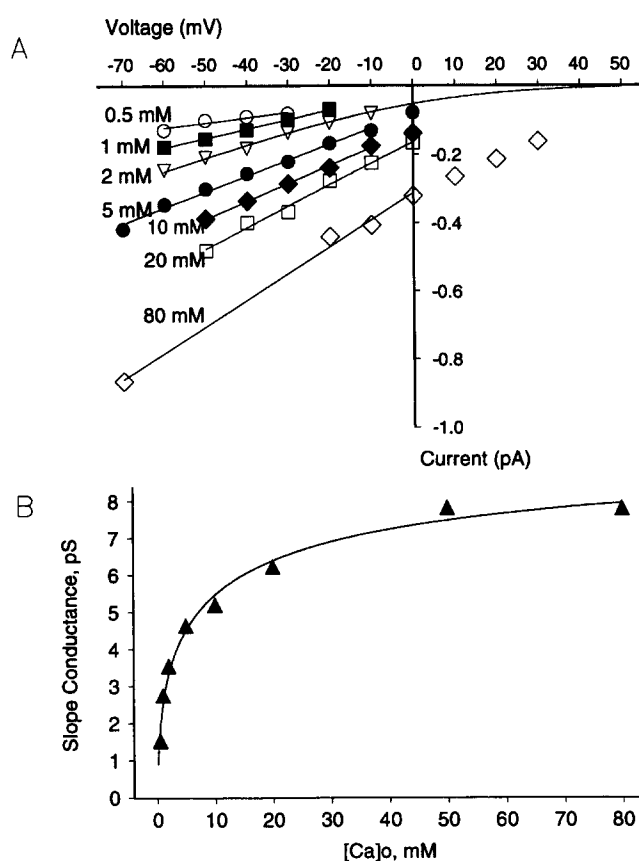


FIGURE 3. Concentration dependence of Ca^{2+} channel conductance. (A). Single-channel current–voltage relationships at 0.5 mM through 80 mM Ca^{2+} . Unitary currents at each voltage were determined from means of those values obtained by mean–variance analysis. Solid lines were fitted to points between -20 and -70 mV using linear regressions. The fit of the Goldman–Hodgkin–Katz equation to the points at 2 mM is also shown. Slopes were 1.5 pS in 0.5 mM Ca, 2.8 pS in 1 mM Ca, 3.5 pS in 2 mM Ca, 4.6 pS in 5 mM Ca, 6.2 pS in 20 mM Ca, and 7.8 pS in 80 mM Ca. (B) Dependence of single-channel conductance on concentration of calcium ions in the patch pipette. The continuous line is a least-squares fit to the Hill equation as given in the text.

sured at >5 min, but excluding the first 30 s) was 0.0017 ± 0.00042 ($n = 9$), increased steeply (~ 12 -fold) during steady depolarization to -30 mV (0.02 ± 0.012 ; $n = 11$), and decreased again slightly at -20 mV (0.012 ± 0.007 ; $n = 7$). Values for steady-state P_o were between $\sim 12\%$ (at -50 mV) and $\sim 28\%$ (at -30 mV) of their respective values for peak P_o elicited during brief pulses, suggesting that a slow inactivation process reduces P_o by as much as \sim ninefold in the range of membrane potentials occurring in the intact pressurized artery.

These data indicate that single Ca^{2+} channels could indeed carry steady currents under conditions seen during myogenic tone. To verify that our microscopic results were representative of the entire cell's response

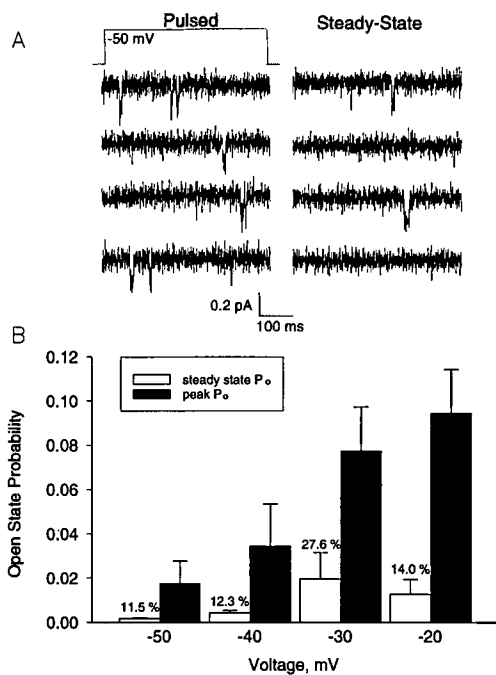


FIGURE 4. Voltage dependence of peak and steady state open state probability of calcium channels with 2 mM calcium as the charge carrier. (A) Comparison of pulsed and steady state data records. Representative pulsed and steady state single channel data at -50 mV recorded from same patch in the presence of 500 nM Bay R 5417. Filter frequency was 500 Hz. (B) Voltage dependence of peak (during pulsed stimuli) and steady state–open state probability (P_o), as measured with the mean–variance technique. The numbers above each bar give the relative magnitude of steady state P_o compared with peak P_o .

and to measure these responses in the absence of Bay R 5417, we also recorded Ca^{2+} currents in the whole cell configuration.

Voltage Dependence of Pulsed Whole Cell Calcium Currents with 2 mM Ca^{2+} as the Charge Carrier

The properties of whole cell Ca^{2+} currents were studied in isolated myocytes in the absence and presence of the

calcium channel activator, Bay K 8644. Fig. 5 A illustrates typical records of currents measured in response to 200-ms depolarizing voltage steps from a holding potential of -70 mV, whereas Fig. 5 B shows the subsequent responses of the same cell in the presence of Bay K 8644. The Ca^{2+} channel agonist increased maximum whole cell currents up to 10-fold (at -30 mV, decreasing to about twofold at more depolarized potentials), and shifted the activation voltages to more negative voltages.

Fig. 6 A summarizes the effects of Bay K 8644 on the current–voltage relationship for peak inward currents in response to 200-ms test pulses for five paired measurements. Macroscopic calcium currents could be resolved at potentials positive to -40 mV, and the currents were largest at $+10$ mV, with a mean current of -53 ± 5 pA. (Current–voltage relations recorded in a total of 21 cells under control conditions showed a similar value for maximal peak current [-40 ± 3 pA], with an identical current–voltage sensitivity.) Bay K 8644 (500 nM) increased the maximal peak calcium current to -112 ± 21 pA, which occurred at 0 mV.

Voltage Dependence of Whole Cell Calcium Current Activation

Instead of attempting to estimate Ca^{2+} conductance from the current–voltage curves at strong depolarization (where nonlinearities and other uncertainties are significant), activation curves were directly obtained by dividing the peak whole-cell current, I , by the unitary current, i (measured above), at each voltage. This ratio should be equal to the product of the number of channels per cell (N) and the open-state probability (P_o) or NP_o . Since N is unlikely to change with voltage, the relationship between voltage and NP_o should reflect the voltage-dependence of P_o , and thus be a scaled representation of the channel's activation curve. At voltages > -10 mV the unitary currents were too small to be accurately measured. At these potentials, we obtained estimates of the unitary currents by extrapolating fits of the Goldman–Hodgkin–Katz constant field equation

TABLE I
Comparison between Open Probability Measurements Using Different Methods

Membrane Potential	Steady Ca^{2+} -sensitive Current (Whole Cell)	P_o , Single Channel (with Bay R 5417)	P_o , Whole Cell Calculated from $P_o = I/iN$	P_o , Single Channel (Bay R-corrected from Whole Cell)	P_o , Single Channel (Bay R-corrected from 10 mM Ba)
-40 mV	0.23 ± 0.04 pA	$4.2 \cdot 10^{-3}$	$0.34 \cdot 10^{-3}$	$(0.4 \cdot 10^{-3})^*$	$0.06 \cdot 10^{-3}$
-30 mV	0.42 ± 0.04 pA	$2.0 \cdot 10^{-2}$	$0.84 \cdot 10^{-3}$	$2.0 \cdot 10^{-3}$	$0.50 \cdot 10^{-3}$
-20 mV	0.79 ± 0.1 pA	$1.2 \cdot 10^{-2}$	$2.0 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$	$0.75 \cdot 10^{-3}$

The first two columns show our two sets of direct measurements: (1) The steady Ca^{2+} current in the whole cell at three different potentials, and (2) the open probabilities of single channels when Bay R 5417 was present. The third column gives the open probability values derived from the whole cell currents using our estimates for the single channel current and the maximum number of channels in the cell (see text). The final two columns show the corrected single channel P_o values using the measured Bay R 5417 increment from paired whole cell peak currents and from direct measurements previously reported by Quayle et al. (1993) in 10 mM Ba. *The value given in parentheses used the correction factor from -30 mV, since peak currents were too small at this potential for reliable measurements.

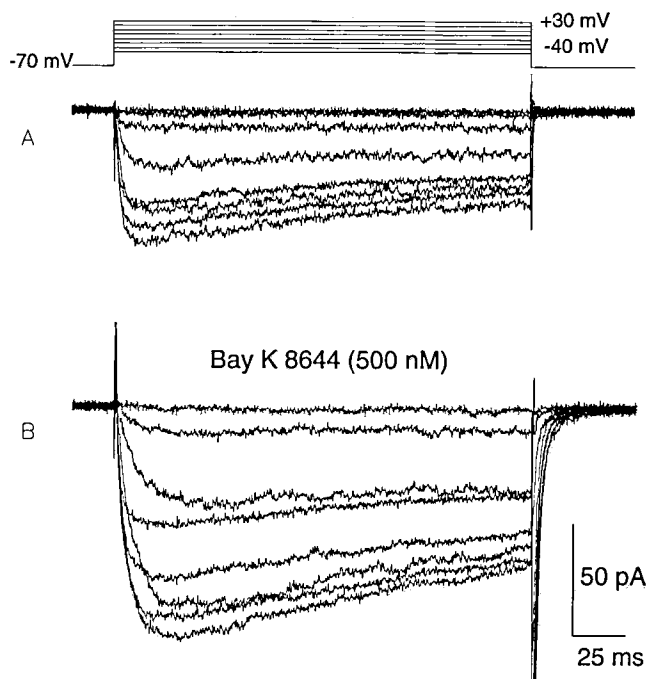


FIGURE 5. Voltage dependence of whole cell calcium currents with 2 mM calcium as the charge carrier with and without Bay K 8644. (A) Original records of whole cell calcium currents elicited without Bay K 8644 in response to 200-ms step depolarization from a holding potential of -70 mV to various test potentials as indicated. Interval between test pulses was 5 s. Extracellular solution contained 2 mM Ca^{2+} and 142 mM TEA-Cl, intracellular solution contained 120 mM CsCl. Current traces have been corrected for leak currents by subtracting currents in the presence of Co^{2+} from those in its absence. (B) Records are shown for the same cell and pulse potentials in the presence of 500 nM Bay K 8644.

(as shown fitted to the 2-mM points in Fig. 3 A) to the measurable parts of the single channel current-voltage relationship.

Fig. 6 B summarizes the relationship between NP_o and voltage for these five cells before and after Bay K 8644 (500 nM). Peak inward currents during the voltage steps were used for our calculations. NP_o at each voltage is shown fitted with a Boltzmann equation of the form

$$\frac{NP_o}{NP_{o \max}} = \frac{1}{1 + \exp\left[\frac{V_h - V_m}{k}\right]} \quad (4)$$

where V_h is the voltage at which NP_o is half-maximal, k is the steepness factor, and $NP_{o \max}$ is the maximal value for NP_o . (Single channel measurements in our study have been made in the absence of extracellular Mg^{2+} , whereas macroscopic currents were recorded using extracellular solution containing 1 mM Mg^{2+} . Since Mg^{2+} at physiological concentrations [1 mM] is known to reduce macroscopic Ca^{2+} currents in arterial smooth muscle cells [rat basilar artery] by 17% [Langton and

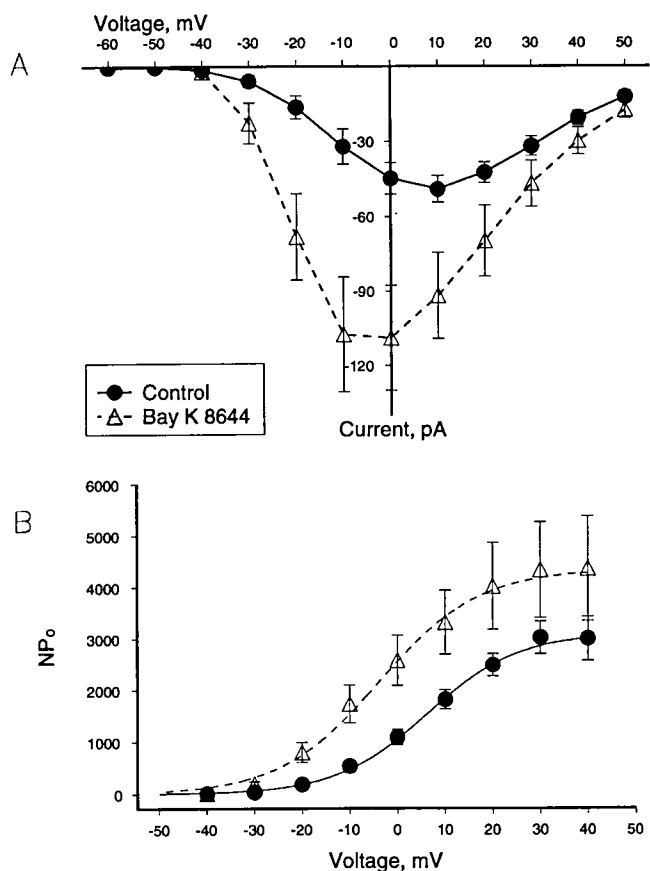


FIGURE 6. Activation of Ca^{2+} currents as a function of potential. (A) Effect of Bay K 8644 (500 nM) on the current-voltage relation for peak inward currents elicited by 200-ms depolarizing voltage steps from a holding potential of -70 mV. Symbols show means \pm SEMs. The values plotted were the Co^{2+} -sensitive peak inward currents. (B) Voltage dependence of whole cell calcium current activation. Voltage-dependence of NP_o calculated as I/i for five cells before (filled circles) and in the presence of Bay K 8644 (500 nM, open circles). Bars show \pm SEM. Because whole-cell currents were recorded with 1 mM MgCl_2 in the external solution, values for i have been reduced by 17% to account for the effect of external Mg ions on the single channel conductance with 2 mM Ca^{2+} . Lines are best fits to Eq. 1, as described in the text.

Standen, 1993], the activation curves were scaled by a factor of 1.20.) Bay K 8644 shifted the activation curves to more negative potentials, with V_h values before and after Bay K 8644 of 6.2 ± 2.8 mV and -3.8 ± 2.1 mV, respectively ($P < 0.05$; Student's t test). The steepness factors did not differ significantly between control and Bay K-treated cells, being 9.5 ± 0.5 mV and 10.4 ± 0.8 mV, respectively ($P > 0.05$; Student's t test). Bay K 8644 significantly increased maximal NP_o to 4377 from a control value of 3008. If P_o approached 1 at positive potentials, these numbers would give estimates of the number of channels that could open in a cell. However, because P_o is likely to be less than unity even in

the presence of Bay K 8644 (e.g., 0.44 in Quayle et al., 1993), NP_o at strong depolarization must be interpreted as a lower bound for the actual number of functional channels present in the cell.

Voltage Dependence of Macroscopic Ca^{2+} Current Inactivation with 2 mM Ca^{2+}

Currents during 200-ms depolarizing test pulses to moderate potentials (e.g., -30 mV) decline little over the course of the pulse. On the other hand, steady-state P_o measured over minutes of single channel recording was substantially lower than peak P_o elicited during test pulses to these same potentials. This observation suggests that steady state inactivation can reduce P_o (and so whole cell current) by as much as ninefold, even at -50 mV. To gain further insight into the time and voltage dependence of inactivation of our macroscopic Ca^{2+} currents, we investigated inactivation using a two-pulse protocol. A 2-s conditioning prepulse to a broad range of potentials is followed by a 200-ms test pulse to +10 mV. A fixed 10-ms recovery interval at the holding potential was used between conditioning and test pulses to deactivate any non-inactivated channels (Gillespie and Meves, 1980). For each cell, steady state inactivation was measured as the ratio of I/I_{max} , where I_{max} is the maximum current amplitude elicited during

the test pulse to +10 mV after the most hyperpolarizing prepulse (-90 mV).

$$\frac{I}{I_{max}} = \frac{(1 - C)}{\left\{ 1 + \exp \left[\frac{V_h - V_m}{k} \right] \right\}} + C \quad (5)$$

where V_h and k are as described above, and C is a non-inactivating component. Fig. 7 summarizes the results from 10 cells. Inactivation was half-maximal at -24.2 mV and increased e -fold per 9.6 mV. The non-inactivating component, C , was 0.27.

Taking these inactivation parameters, conditioning prepulses of 2-s duration in the range -50 to -20 mV should reduce whole cell Ca^{2+} currents by only ~10% to ~45%, whereas our steady state measurements of single channel activity showed much greater reduction of activity (i.e., 75% to 89% reduction at the same potentials). These results suggest that a slow inactivation process (i.e., much longer than 2 s) is responsible for the reduction in P_o . We confirmed our single channel observations at the whole cell level, as described below.

Measurement of Macroscopic Calcium Currents during Steady Potentials with 2 mM Ca^{2+}

Although the measurement and interpretation of transient Ca^{2+} current responses to brief depolarizing

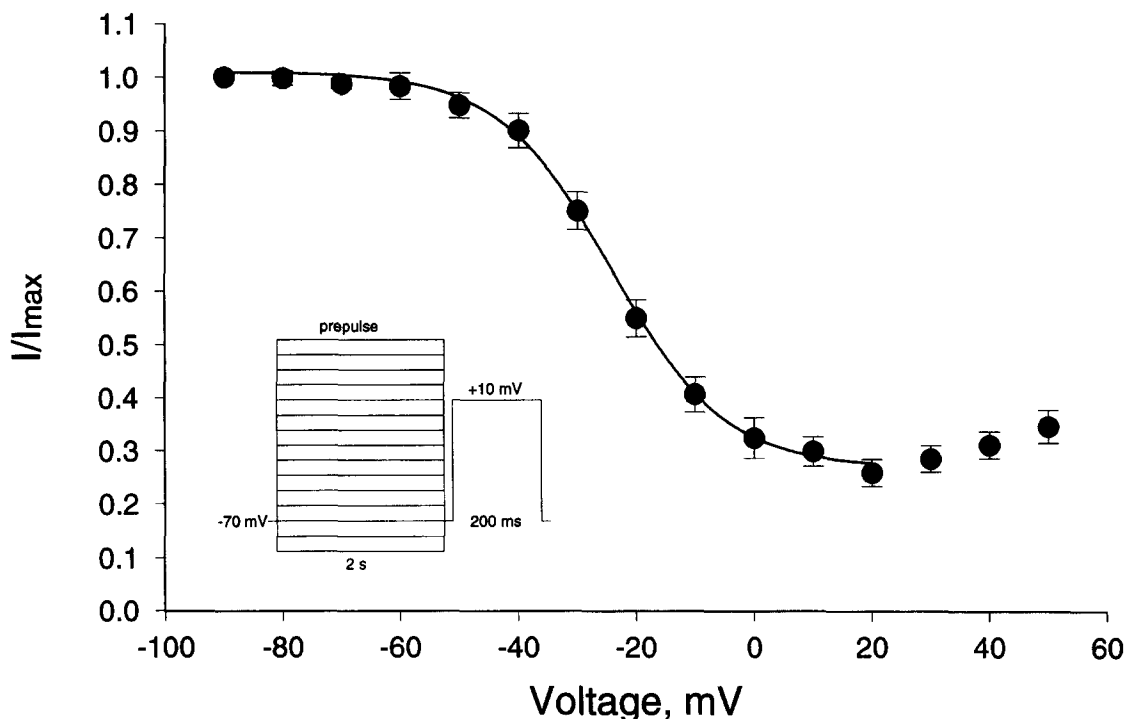


FIGURE 7. Steady-state inactivation of the whole cell Ca^{2+} currents studied with the two-pulse protocol in the absence of Bay K 8644. A 2-s conditioning prepulse to various potentials was followed after a fixed 10 ms interpulse interval to the holding potential by a 200-ms test pulse to +10 mV (see inset). Pulses were applied every 10 s. Peak inward current during test pulses was normalized by its value in the absence of a conditioning prepulse (I/I_{max}) and plotted as a function of prepulse potential. Continuous line is best fit to Eq. 5, as described in the text.

pulses are straightforward, such responses present a skewed view of channel function in a cell that normally has steady or very slowly changing membrane potentials. The steady state P_o 's from single channel measurements reported above make a major step toward observing steady function, but they still relied on Bay R 5417, and are thus skewed 10–20 mV negative of the true voltage sensitivity of the channel. Such measurements thus give a partial view of the channel's physiological function, but they can only be correlated directly with cell or tissue function by taking into account the effect of Bay R 5417 or Bay K 8644. We have extended our study to provide the direct measurements of steady whole-cell Ca^{2+} currents at 2 mM Ca^{2+} in the absence of Bay K 8644.

Fig. 8 A shows an example of a steady state current recorded at different holding potentials using an extracellular solution containing 142 mM TEA-Cl and 2 mM CaCl_2 . Ca^{2+} currents were identified by their sensitivity to block by cobalt (2 mM; Huang et al., 1989; Langton and Standen, 1993) and diltiazem (10 μM). As the holding potential was shifted from -70 to -40 mV, a small, cobalt-sensitive inward current of ~ -0.2 pA was observed. At holding potentials of -30 mV and -20 mV, these inward currents showed a slow decline with time from an initial peak value before they reached a steady state level. Similar results were observed with diltiazem blockade (not shown).

The non-inactivating, Co-sensitive component of the inward current increased with depolarization to ~ -0.6 pA at -30 mV and ~ -0.9 pA at -20 mV. After wash-out of Co^{2+} , steady state currents returned to their respective non-inactivating levels, suggesting that these steady state inward currents were indeed caused by Ca^{2+} channel activity. Fig. 8 B shows mean current–voltage relationships for 19 cells in which both the current at steady holding potentials in the range of -40 – -20 mV and the peak inward current in response to 200-ms depolarizing voltage steps to the same potentials were measured. Cells that showed significant run-down of the currents, as determined with brief pulses before and after the measurements, were discarded from the analysis. Mean steady state, cobalt-sensitive Ca^{2+} current was 0.23 ± 0.04 pA at -40 mV and increased with depolarization to 0.42 ± 0.04 pA at -30 mV and 0.79 ± 0.1 pA at -20 mV. Steady state currents at -40 , -30 , and -20 mV were 29%, 25%, and 18% of their respective peak currents elicited during voltage steps to the same potential. Dividing these whole cell currents by the unitary current at each voltage gives an estimate for the average number of open Ca^{2+} channels carrying this current. The numbers of channels open on average at -40 mV, -30 mV, and -20 mV were 1.5, 3.7, and 8.9 channels out of at least $\sim 4,300$ per cell, corresponding to steady state open probabilities of $0.34 \times$

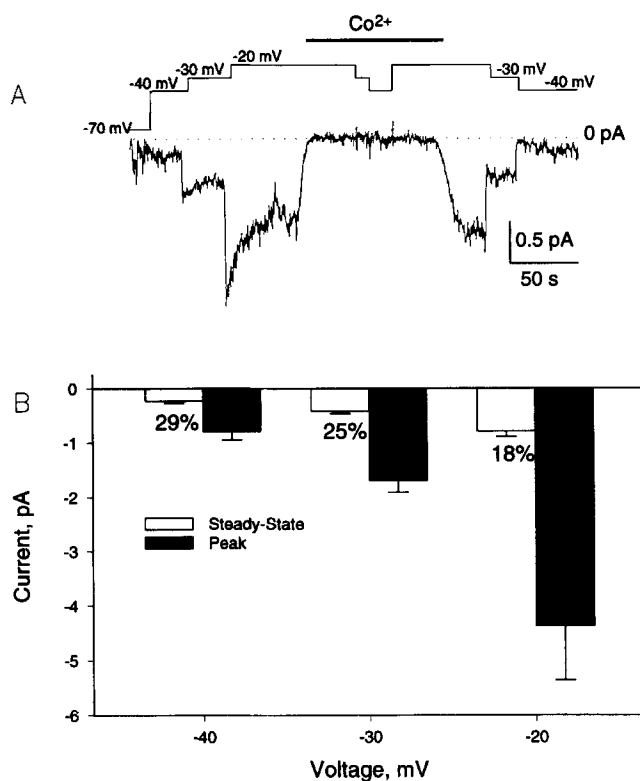


FIGURE 8. Whole cell steady-state calcium currents in the absence of Bay K 8644. (A) Original record of Co-sensitive inward currents at steady holding voltages as indicated. The bathing solution contained 2 mM Ca^{2+} and 142 mM TEA-Cl, and the whole cell electrode had 130 mM CsCl (see Methods for full solution compositions). 2 mM CoCl_2 was applied during the indicated period to block currents through calcium channels. The data shown are the difference between the responses with and without Co^{2+} . (B) Peak versus steady state whole cell currents at -40 mV, -30 mV, and -20 mV. The numbers show the relative magnitude of the steady state currents compared with the peak currents that were measured during the responses to pulses to the same potentials.

10^{-3} , 0.84×10^{-3} , and 2×10^{-3} (summarized in Table I), in reasonable accord with the direct P_o measurements from the single channels after accounting for the effects of Bay R 5417 (see discussion below).

DISCUSSION

The close link between membrane potential and myogenic tone in arterial smooth muscle cells suggests that voltage-dependent Ca^{2+} channels play a critical role in this excitation/contraction coupling. Such a hypothesis makes a number of testable predictions: Ca^{2+} channels must be present, they must have sufficient activity in physiological conditions to support a Ca^{2+} current, the steady Ca^{2+} current must increase with depolarization over the range of potentials seen in myogenic activity, the currents must persist in the steady state, and finally, the magnitude of that current must be large

enough when extracellular Ca^{2+} is ~ 2 mM to account for significant Ca^{2+} entry.

Although some of these predictions are well established (e.g., the presence of substantial numbers of voltage-dependent Ca^{2+} channels in vascular smooth muscle), others have been more difficult to demonstrate quantitatively. Single Ca^{2+} channel currents are small, and the channel openings are quite brief under physiological conditions. Whole cell measurements are a mixture of inward Ca^{2+} currents and several other types of outward currents. In addition, currents are most easily measured when they respond briskly to a rapid depolarization of the membrane from a negative holding potential. All of these reasons have constrained previous experiments to abnormal conditions for their recordings: Ba^{2+} substituted for Ca^{2+} , concentrations of divalent ions >10 mM, pulsed rather than steady state current measurements, and use of the dihydropyridine agonists, Bay K 8644 or Bay R 5417.

We report here several more precise confirmations of this model's predictions. We examined these properties of Ca^{2+} channels in smooth muscle cells from small (100–150 μm diameter) myogenic arteries. Previous studies have used larger arteries or nonmyogenic preparations. We have extended the range of measurement for single Ca^{2+} channel currents down to concentrations as low as 0.5 mM, permitting us to demonstrate clearly the properties of the channel in 2 mM Ca^{2+} . We have also used pulsed, whole cell recordings, with and without agonist and in conjunction with our single channel measurements, to estimate the total number of Ca^{2+} channels present in each cell. Finally, we have recorded whole cell Ca^{2+} currents under conditions of prolonged depolarization, showing that their activity is both finite, has the proper voltage dependence, and should be sufficient to elevate intracellular Ca^{2+} significantly. We discuss below the detailed implications of these observations.

Permeation Properties of Voltage-dependent Ca^{2+} Channels

Our measurements of single Ca^{2+} channel conductance properties were qualitatively and quantitatively similar to those of others in the range of overlapping Ca^{2+} concentrations [see McDonald et al. (1994) for review]. Our value of 3.5 pS is slightly smaller than that reported by Gollasch et al. (1992) and about the same as that given by Klöckner and Isenberg (1991) when this latter value was corrected for temperature difference. Therefore we are confident that our measurements are appropriate as we extended our studies to previously unreported concentrations.

Given early estimations of single Ca^{2+} channel currents of <0.1 pA at 2 mM Ca^{2+} (e.g., Hess et al., 1986), it appears surprising that we were able to measure sin-

gle channel currents at concentrations as low as 0.5 mM. A number of factors permitted such measurements. First, we used more sensitive analysis tools (mean-variance analysis) to minimize the effects of noise on our ability to estimate quantal current amplitudes and open probability. Second, channel voltage sensitivity shifts to more negative values as the divalent ion concentration was reduced. Such shifts might be caused by fixed negative (surface) charges near the channel (e.g., see Hille, 1992). Because Ca^{2+} channel currents were active at more negative potentials, we could measure them with increased driving force and therefore larger absolute current amplitudes at any given slope conductance.

Such negative surface charge near the external mouth of the channel would also cause a local accumulation of a higher Ca^{2+} concentration than the bulk solution. The presence of surface charge effects on kinetics would argue for simultaneous effects on local divalent concentrations. Furthermore, local ion accumulation might explain our finding of power factor (n) of 0.55 for the Hill equation used to fit our conductance/concentration data, since local accumulation would flatten this relationship. In contrast, Yue and Marban (1990) provided experimental evidence that this may not be the case for L-type cardiac channels with low barium as the charge carrier. Also, Kuo and Hess (1992) have shown that the negative surface potentials are small at the L-type Ca^{2+} channel mouth in ambient ionic strength of 110 mM or higher. This issue therefore remains to be resolved for smooth muscle Ca^{2+} channels.

Steady State Voltage Dependence of Ca^{2+} Channel Activity

We measured Ca^{2+} channel open probability during voltage pulses and at steady potentials by two independent methods. Single channel recordings have several well known advantages: (1) Currents through Ca^{2+} channels could be unambiguously separated from unitary currents through other channels; (2) the values and voltage dependence of channel conductance and open probability could be determined directly; and (3) with the on-cell configuration, the cell's cytoplasm remained intact, eliminating possible artifacts from disruption of enzymatic or Ca^{2+} buffering systems. However, the single channel measurements required Bay R 5417 to resolve the small unitary currents at 2 mM Ca^{2+} . We cannot therefore rely solely on these P_o measurements.

Our whole cell recordings of Ca^{2+} channel P_o depended on accurate separation of the Ca^{2+} current, and they were made under conditions where the cell's cytoplasm was dialyzed with an artificial solution. Also, estimating the number of channels in a cell depends

on independent knowledge of the maximum P_o for the channels. Nevertheless, we would argue that they do provide an accurate picture of the cell's Ca^{2+} channel activity.

In particular, other conductances were suppressed by replacing all Na^+ and K^+ with TEA^+ and Cs^+ . Calcium currents were identified by their characteristic voltage dependence, Bay K 8644 activation, and block by cobalt and diltiazem. This approach permitted the measurement of steady Co^{2+} -sensitive currents as small as 0.2 pA. Furthermore, the P_o values that we estimated were consistent with those from other types of recordings.

The voltage dependence displayed by the single channel recordings was the same as that in the whole cell records (Figs. 4, 6, and 8). The difference in P_o values measured at the single channel level and those estimated from whole cell data can be explained by the effect of Bay R 5417. Table I shows our single channel-open probabilities corrected in two different ways for the effects of Bay R 5417. First, the enhancement of whole cell peak currents by Bay R 5417 was estimated from paired current measurements such as that shown in Fig. 5, A and B. Second, previous measurements from this laboratory in the same preparation with 10 mM Ba^{2+} as charge carrier (Quayle et al., 1993) estimated the Bay R 5417 enhancement during steady single channel recordings. In this latter study, Bay R 5417 shifted the steady state voltage dependence by some 20 mV, increasing P_o of the channels by 17–105-fold in the potential range of -20 to -40 mV, respectively. This is also consistent with the 6–24-fold difference reported here for the whole cell P_o without Bay R 5417 and the single channel value with this agonist. Indeed, the steady state P_o values reported here (e.g., 0.00034 at -40 mV) are nearly identical with those seen during the earlier single channel records (0.0003 at -40 mV in 10 mM Ba^{2+} without Bay R 5417) by Quayle et al., and they are approximately twofold lower than the steady state P_o estimates of Langton and Standen (1993; recordings made with 10 mM Ba^{2+} in basilar artery myocytes).

The combination of information from single channel and whole cell recordings also strengthens our conclusions in other ways. The observation by Quayle et al. (1993) of the maximum P_o of 0.44 (during pulses in the presence of Bay R 5417) gives us an indication that our lower bound estimate of about 5,000 channels per cell is accurate to within a factor of approximately two. Also, the observation of a long-term diminution of currents during steady application of depolarizing potentials was observed both at the single channel and whole cell levels (Figs. 4 and 8). This implies that the slow inactivation process is not the result of some kind of buffering or cytoplasmic disturbance.

This combination of several different recording strat-

egies (using values reported here, and from Quayle et al. made in the same laboratory and preparation) provides a set of measurements on which we can cross check results and assumptions. It is because of this cross-correlation that we are confident our observations of unitary current, number of channels per cell, and steady open probability are accurate to well within an order of magnitude, and thus realistically reflect the physiological behavior of the channels.

Ca²⁺ Channels and the Voltage Dependence of Arterial Tone

When subjected to increases in transmural pressure, small, resistance-sized cerebral arteries develop (myogenic) tone. The development of myogenic tone in vitro is associated with graded depolarization of the smooth muscle cell membrane (Harder, 1984; Nelson et al., 1990; Brayden and Nelson, 1992; Knot and Nelson, 1995). For example, physiological pressure depolarizes smooth muscle cells of rabbit-middle cerebral arteries from ~ -60 mV at 10 mm Hg to ~ -35 mV at 80 mm Hg (Brayden and Nelson, 1992; Knot and Nelson, 1995). Under these conditions, arterial smooth muscle cells have constant or slowly changing membrane potentials. Conversely, myogenic tone in these arteries is abolished by membrane hyperpolarization, by the removal of external Ca^{2+} , and by calcium channel blockers. These observations suggest that voltage-dependent Ca^{2+} channels play an important role in controlling steady state Ca^{2+} entry, and thus the contractile state of smooth muscle in resistance-sized arteries. We would therefore expect that Ca^{2+} channel activation in the steady state should be sufficient to explain these cell and tissue findings.

In the absence of a calcium channel agonist, membrane depolarization from -40 to -20 mV increased the mean steady state Ca^{2+} current by approximately fourfold, being 0.23 pA at -40 mV and 0.79 pA at -20 mV. Whole cell calcium currents of 0.23 pA correspond to a steady calcium influx rate of 600,000 ions/s or 1.3 attoM per second. Assuming a cell volume of ~ 1 pL (Aaronson et al., 1987), a macroscopic current of this size would deliver Ca^{2+} at a rate sufficient to raise Ca^{2+} $1.3 \mu\text{M/s}$ at 22°C in the absence of buffering and sequestration. With normal buffering, the actual rate of rise in $[\text{Ca}]_i$ would probably be 50-fold or more slower, but this would still be more than enough considering the steady depolarizations in this tissue.

The final change in steady state $[\text{Ca}^{2+}]_i$ resulting from Ca^{2+} entry will depend on the way in which Ca^{2+} is extruded from the cell. For example, measurements of changes in intracellular free calcium in voltage-clamped airway smooth muscle cells (35°C , 1.8 mM extracellular calcium; Fleischmann et al., 1994) have shown that maintaining depolarization between -40 and -30 mV caused voltage-dependent steady state in-

creases in intracellular free calcium. Similarly, Marchetti et al. (1995) found steady state rises in intracellular Ca^{2+} in cultured rat cerebellar granule cells during sustained depolarizations to between -45 and -25 mV that were dependent on external calcium and could be abolished by nimodipine. The mean increase in both studies' $[\text{Ca}^{2+}]_i$ was ~ 20 nM and ~ 100 nM after sustained depolarizations of -40 mV and -30 mV, respectively. Calculations by Fleischman et al. (1994) of the steady state calcium current required to produce a 100-nM rise in $[\text{Ca}^{2+}]_i$ yielded a value of ~ 0.5 pA at -30 mV, assuming saturable fast buffers and a linear Ca^{2+} extrusion mechanism (as quantified previously in other cell types). Steady state rises in $[\text{Ca}^{2+}]_i$ were completely blocked by DHP calcium channel blockers and were augmented and shifted to more negative potentials by Bay K 8644, suggesting that rises in $[\text{Ca}^{2+}]_i$ were mediated, as in our study, by steady state calcium influx through L-type calcium channels.

A calculated steady state calcium current of ~ 0.5 pA (Fleischmann et al., 1994) is in close agreement with direct measurements of the steady state calcium current in our study. We therefore suggest that the magnitude of the total steady state Ca^{2+} current over the physiological range of membrane potential is sufficient to produce steady state rises in intracellular Ca^{2+} that are required to maintain myogenic tone.

Steady State Inactivation of Ca^{2+} Currents

Our observations in this report point to several different inactivation processes for the Ca^{2+} channels in arterial smooth muscle. Whole cell currents decline in Ca^{2+} -containing solutions over the time course of hundreds of milliseconds. The potential that would cause half-maximal inactivation during a 2-s depolarization was -23.8 mV with a slope factor of -9.2 mV. Similar steepness factors but slightly more negative values for $V_{1/2}$ have been reported in barium for other smooth muscle cell preparations (human mesenteric artery: -30.4 mV [Smirnov and Aaronson, 1992]; rabbit ear artery: -47 mV [Aaronson et al., 1987]; rat basilar artery: -37 mV [Langton and Standen, 1993]; rabbit coronary artery: -28 mV [Matsuda et al., 1990]).

Nevertheless, the decrease in P_o generated by this fast inactivation process is not sufficient to explain the differences we saw during pulsed versus steady test potentials. There are discrepancies in both the expected time course of the phenomenon, and in its voltage sensitivity. The decline of pulsed currents occurs over hundreds of milliseconds, and would be expected to be near completion after our 2-s prepulses. Others have similarly measured the time course of inactivation in the same time range. In smooth muscle, monoexponential (Matsuda et al., 1990), double exponential (Katzka and Morad, 1989; Katzka et al., 1992; Kuga et

al., 1990; Nakazawa et al., 1988; Sims, 1992; Vogalis et al., 1992), and triple exponential (Klößner and Isenberg, 1985) decays have been described during test pulses of <5 s. Values for the fast time constant were in the range of 4–200 ms, for the slower time constant in the range of 27–470 ms when physiological calcium concentrations were used as charge carrier. In contrast, Fig. 8 A shows relaxations occurring over a time course of 10–15 s. Thus, even the longest standard inactivation time constant reported for L-type calcium channel in smooth muscle with physiological $[\text{Ca}]_o$ is $<1,000$ ms (e.g., Smirnov and Aaronson, 1992); this would inadequately describe the slow inactivation process seen during steady depolarizations to membrane potentials negative to -10 mV.

The voltage sensitivity of the fast inactivation process is not enough to explain the final steady state values for P_o . For example, at -40 mV our two-pulse inactivation measurements show $\sim 10\%$ inactivation, whereas steady currents or single channel P_o were measured at 29% of peak (71% inactivated). This same discrepancy holds at more positive potentials as well: at -20 mV, inactivation is at 55%, but steady state currents are 82% inactivated.

With strong buffering of intracellular Ca^{2+} , and with the small magnitude of the steady currents, one would not expect changes in Ca^{2+} ion driving force or single-channel conductance to underlie the observed slow changes. Finally, since we observed similar inactivation in single-channel records from cell-attached patches, it is unlikely that cellular metabolic or regulatory phenomena disrupted by our cytoplasmic dialysis are involved.

By elimination, we conclude that an additional, slow inactivation process also controls P_o for the Ca^{2+} channels over the physiologically relevant range of membrane potentials. Note also that Kass and Scheuer (1982) found a very slow inactivation time constant of the cardiac L-type calcium channel in Purkinje fibers with a time constant of 3.4 s, Schouten and Morad (1989) observed slow inactivation in frog ventricular myocytes with a time constant of 37 s at -40 mV, and Boyett et al. (1994) recently observed a voltage-dependent slow inactivation in guinea pig ventricular myocytes with a time constant of 6 s at 0 mV and 37°C .

Conclusions

Our results satisfy the experimental tests that we suggested above were critical to the hypothesis that voltage-dependent Ca^{2+} channels are responsible for delivering cytoplasmic Ca^{2+} during sustained tone in arterial smooth muscle. We estimate that $\sim 5,000$ channels are present in the smooth muscle cell's membrane (a density of 4 per square micrometer), and that membrane

potentials seen during myogenic tone (-50 to -20 mV) are sufficient to cause an average of 1–10 of those channels to be active at any moment, even in the steady state. The whole cell Ca^{2+} currents through these 1–10 channels, 0.2 to 2.0 pA, would carry sufficient Ca^{2+} into the cell to cause an unbuffered rise of $[\text{Ca}^{2+}]_i$, on the order of micromolar per second. Others have provided evidence indicating that such currents are indeed large enough to raise cytoplasmic free Ca^{2+} (Fleischman et al., 1994). Finally, the voltage dependence of these Ca^{2+} currents is consistent with the increasing activa-

tion of tone with increasing depolarization up to membrane potentials of -20 mV.

In addition to supporting this hypothesis, our study provides new quantitative measurement of single and whole cell Ca^{2+} currents in conditions not previously reported. Finally, the new observation of a slow inactivation process for Ca^{2+} channels in arterial smooth muscle cells has significant potential implications for cellular function, since modulation of the degree of this slow inactivation would directly influence the gain of this excitation/contraction coupling in the steady state.

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