brief communication

External cadmium and internal calcium block of single calcium channels in smooth muscle cells from rabbit mesenteric artery

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ABSTRACT The patch clamp technique was used to record unitary currents through single calcium channels from smooth muscle cells of rabbit mesenteric arteries. The effects of external cadmium and cobalt and internal calcium, barium, cadmium, and magnesium on single channel currents were investigated with 80 mM barium as the charge carrier and Bay K 8644 to prolong openings. External cadmium shortened the mean open time of single Ca channels. Cadmium blocking and unblocking rate constants of 16.5 mM^{-1} ms^{-1} and 0.6 ms^{-1}, respectively, were determined, corresponding to dissociation constant K_d of 36 μM at −20 mV. These results are very similar to those reported for cardiac muscle Ca channels (Lansman, J. B., P. Hess, and R. W. Tsien. 1986. *J. Gen. Physiol.* 88:321–347). In contrast, Cd^{2+} (0.1–10 mM), when applied to the internal surface of Ca channels in inside-out patches, did not affect the mean open time, mean unitary current, or the variance of the open channel current. Internal calcium induced a flickery block, with a K_d of 5.8 mM. Mean blocking and unblocking rate constants for calcium of 0.56 mM^{-1} ms^{-1} and 3.22 ms^{-1}, respectively, were determined. Internal barium (8 mM) reduced the mean unitary current by 36%. We conclude that under our experimental conditions, the Ca channel is not symmetrical with respect to inorganic ion block and that intracellular calcium can modulate Ca channel currents via a low-affinity binding site.

INTRODUCTION

A number of divalent cations are well known to block calcium channels when added to the external solution. Cadmium is usually the most potent, with a dissociation constant, K_d, in the range 10–200 μM (Lansman et al., 1986; Rosenberg et al., 1988). The kinetics of external block have been studied at the single channel level in cardiac muscle (Lansman et al., 1986). Cd^{2+}, in common with Co^{2+} and La^{3+}, chops the long Ca channel openings seen in the presence of the agonist Bay K 8644 into bursts of much briefer events as it blocks and unblocks the channel. Further, internal calcium has been shown to reduce barium currents through single cardiac muscle calcium channels with K_d = 4 mM (Rosenberg et al., 1988). The effects of these blocking ions have not been investigated in smooth muscle.

We examined the effects of external cadmium and cobalt, and internal calcium, cadmium, magnesium, and barium on unitary barium currents through single calcium channels in membrane patches on-cell and excised from smooth muscle cells of rabbit mesenteric artery. We show that the affinity and kinetics of external cadmium and cobalt and internal calcium block of arterial smooth muscle calcium channels are very similar to those measured in cardiac muscle. In addition, we show that cadmium block is very asymmetric, with internal cadmium at concentrations as high as 10 mM having no effect on single channel currents. Internal calcium, however, produced a flickery block.

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METHODS

Single smooth muscle cells were enzymatically isolated from rabbit mesenteric artery as described previously (Worley et al., 1986). The control pipette (extracellular) solution contained 80 mM $BaCl_2$, 10 mM Hepes/$Ba(OH)_2$, pH 7.4. In some experiments external $CdCl_2$ or $CoCl_2$ were added to this solution (see legend to Fig. 1). The bath solution contained 120 mM NaCl, 20 mM CsCl and 10 mM Hepes/NaOH, pH 7.4. The Ca channel agonist Bay K 8644 (1 μM) or its active isomer Bay R 5417 (0.5 μM) was added to the bath solution to increase the frequency of long-duration openings (cf. Lansman et al., 1986). Current and voltage were recorded on a Dagan or Axopatch 1C patch clamp amplifier and stored on video tape. Filtered data (eight-pole Bessel filter, Frequency Devices, Haverhill, MA) were later replayed through a CED 502 or Labmaster A-D converter (Axon Instruments, Burlingame, CA) and analyzed with a PDP (11/23 or 11/73) or Compaq (286 or 386s) computer (Houston, TX). Capacity and leakage currents associated with voltage steps were removed partly by analog means and finally by digital subtraction of averaged records free of channel openings. Open and closed times were measured using a cursor set halfway between open and closed current levels. Power spectral densities

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were estimated by averaging fast Fourier transforms (FFT) from digitized data (see Patlak and Oritz, 1989). Power spectra were obtained from segments of record containing bursts of channel openings, but excluding long closures. Background (closed level) spectra were subtracted to obtain the illustrated difference spectra. Lorentzians were fitted to power spectra using a least squares routine to minimize χ^2 calculated as the sum of the squares of the difference between the observed and expected power at each frequency. The average difference spectra (burst-background) were fitted with one or more Lorentzian components. Filter frequencies and sample rates are given in the figure legends. Mean unitary currents were determined from Gaussian curves fit to the amplitude histograms. Where appropriate, results are given as mean ± SE of the mean.

Patches were excised in the inside-out configuration and were exposed to different internal solutions by moving the patch pipette between a series of flow pipes (Spruce et al., 1985). Single Ca channel currents can be recorded in inside-out (Nelson and Worley, 1989) and outside-out excised patches (Benham et al., 1987) from arterial smooth muscle.

RESULTS

Fig. 1 shows the action of external cadmium and cobalt on single calcium channels in patches from smooth muscle cells isolated from rabbit mesenteric artery at −20 mV. These blocking ions shorten the mean open time, τ_o, because blocking gives an additional route by which the channel can leave the open state, and both induce many short closings. Fig. 1 *C* (*inset*) shows that $1/\tau_o$ is a linear function of cadmium concentration, as expected for the simple blocking scheme described in the figure legend. The rate constant for blocking, k_b, is 16.5 mM^{-1} ms^{-1}. The closed times within the bursts of openings seen with [Cd] of 10 μM or above are dominanted by blocking events (cf. Lansman et al., 1986), so the reciprocal of their mean gives an estimate of the unblocking rate constant, k_{-b}, as ~0.6 ms^{-1}. As expected, this was independent of [Cd] and gives a value for the dissociation constant, $K_d(=k_{-b}/k_b)$, of 36 μM at −20 mV. At 0 mV K_d was slightly higher, around 45 μM (three patches). Both the rate constants and K_d are quite close to those reported for cardiac muscle (k_b = 40 mM^{-1} ms^{-1}, k_{-b} = 0.5–1.0 ms^{-1}, K_d = 20 μM, Lansman et al., 1986). Cadmium also blocks maintained contractions of rabbit mesenteric arterial rings induced by high potassium or by norepinephrine with a similar K_d, ~50 μM; such contractions are caused by calcium entry through voltage-dependent Ca channels (Nelson et al., 1988). External cobalt also blocks (Fig. 1) but with lower affinity; in one patch at 0 mV a similar analysis to that described above gave a K_d of 325 μM.

In contrast to the effects of micromolar concentrations of external cadmium, we found no effect of Cd at concentrations ~1,000-fold higher when applied to the cytoplasmic surface of excised patches. Fig. 2 shows examples of single calcium channel currents recorded in an inside-out patch in response to a voltage step from the holding potential of −75 to 0 mV. There was no obvious change in the appearance of the currents when the patch was moved from a flow pipe with control solution into a pipe containing solution with 10 mM Cd. Depending on the kinetics of the blocking reaction, block by internal Cd might manifest itself in any of a number of ways. First, block with kinetics similar to those for the action of external Cd should be resolvable in single channel records as a reduction in mean open time like that described

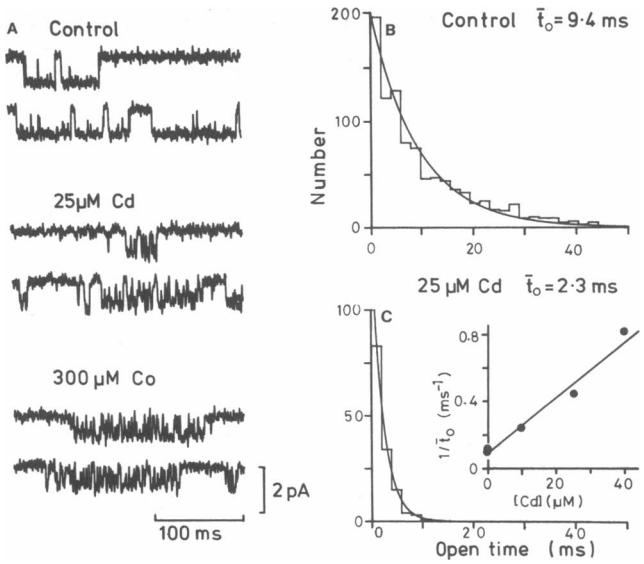

FIGURE 1 Ca channel block by external divalent cations. (*A*) Records of unitary Ca channel currents at −20 mV in control pipette solution (80 mM $BaCl_2$, 10 mM Hepes/$Ba(OH)_2$, pH 7.4, upper records). Middle records are from a different patch at the same potential but recorded with a pipette containing solution to which 25 μM $CdCl_2$ was added. Lower records are from a third patch at 0 mV with 300 μM $CoCl_2$ added to the pipette. Records were from inside-out patches and filtered at 1 kHz (eight-pole Bessel, −3 dB) and sampled at 4 kHz. Holding potential was −75 mV. (*B*) Open time histogram in the absence of divalent cation blockers formed from the patch shown in *A*. Line shows a single exponential fit with a Marquardt algorithm (Marquardt, 1963) giving a mean open time, τ_o, of 9.4 ms. (*C*) Open time histogram in 25 μM cadmium. The fitted exponential has τ_o = 2.3 ms. The inset shows $1/\tau_o$ plotted against the external $[Cd^{2+}]$. Blocking scheme:

$$\text{Closed} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Open} \underset{k_{-b}}{\overset{k_b[\text{Cd}]}{\rightleftharpoons}} \text{Blocked}$$

The mean dwell time in the open state will be given by $\tau_o = 1/(k_{-1} + k_b[\text{Cd}])$, so that $1/\tau_o$ should be linear function of $[Cd^{2+}]$ with a slope equal to the blocking rate constant, k_b, and an intercept at zero cadmium of k_{-1}, the rate constant for closing. The straight line gives k_b = 16.5 mM^{-1} ms^{-1}. The results shown are from patches excised in the inside-out configuration, except that at 10 μM cadmium and one control which remained cell attached.

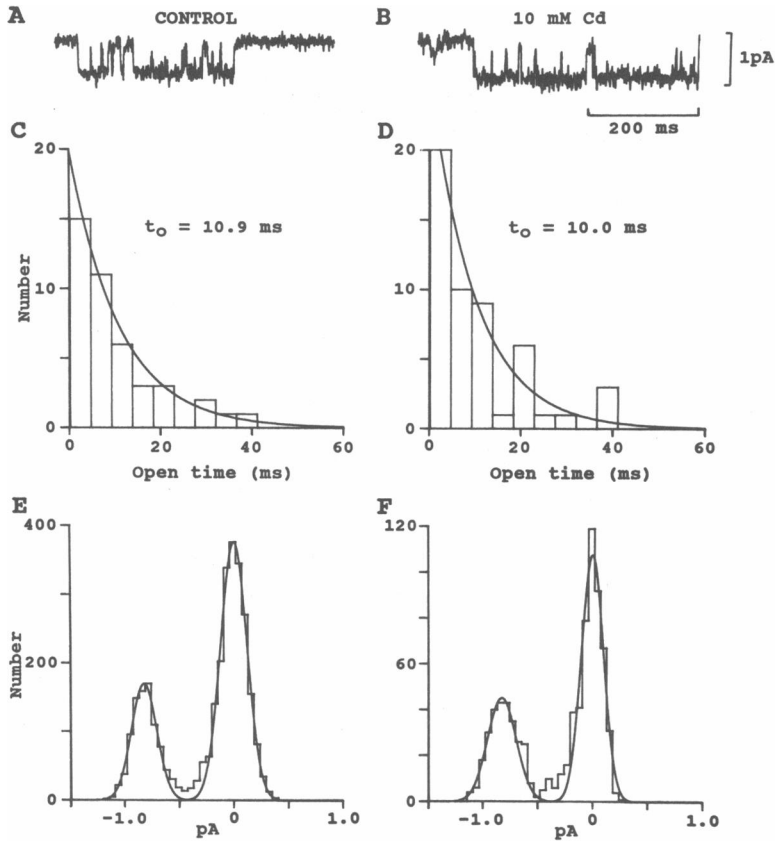

FIGURE 2 Lack of block by internal cadmium. Control pipette solution throughout. (*A*) Current records for a step to 0 mV in an excised inside-out patch. The cytoplasmic surface of the patch was exposed to solution containing 120 mM NaCl, 20 mM CsCl, 10 mM EGTA, 1 μM Bay K 8644, and 10 mM Hepes/NaOH, pH 7.4. Holding potential was −75 mV. (*B*) Records from the same patch after it has been moved into a flow pipe containing the same solution as in *A*, but without EGTA and with 10 mM $CdCl_2$. Holding potential was −75 mV. (*C* and *D*) Open time histograms from the patch in *A* and *B*. The mean open time was 10.9 ms in control and 10.0 ms in 10 mM cadmium. (*E* and *F*) Amplitude histograms from the patch in *A* and *B*. The single channel current, i, measured from the fitted Gaussian distributions, was 0.83 pA both in control solution and in 10 mM cadmium.

above. Fig. 2, *C* and *D*, show examples of open time histograms obtained from the same patch, first under control conditions and then after the patch had been moved into a solution containing 10 mM Cd. Open times were effectively unchanged. In similar experiments on four patches τ_o was 9.2 ± 2.3 ms in control solution and 12.0 ± 3.9 ms in 10 mM Cd, values quite similar to the τ_o of 9.1 ± 2.1 ms of cardiac Ca channels at the same potential (Lansman et al., 1986).

A second type of block might be seen if the blocking kinetics were rather faster, so blocking events were only partly resolved. This would give a flickery block, characterized by reduction in unitary current and an increase in open channel noise. Thirdly, if the blocking kinetics were so fast that blocked and unblocked periods were averaged by the recording system, unitary current would be reduced, but without any measurable increase in noise. This type of block has been reported for the effect of Cd on single Ca channels of rat brain recorded in lipid bilayers (Nelson, 1986). We measured unitary current size from amplitude histograms like those shown in Figs. 2, *E* and *F*, and also the variance of the open channel current. Neither current amplitude nor variance was changed by Cd^{2+}. At 0 mV, the current amplitude was 0.77 ± 0.06 pA in control and 0.76 ± 0.06 pA in 10 mM Cd (five patches). The open level variance was 1.5 ± 0.5 × 10^{-26} A^2 in control and 1.3 ± 0.3 × 10^{-26} A^2 in 10 mM Cd (four patches). These results suggest that Cd does not block smooth muscle Ca channels when applied to their cytoplasmic side. In similar experiments on four patches we could not find any evidence for block by 10 mM magnesium at the internal face of the membrane.

We have considered the possibility that the blocking ions were somehow unable to reach the inner surface of the membrane, perhaps because of inadequate perfusion of the cytoplasmic membrane face by the flow system.

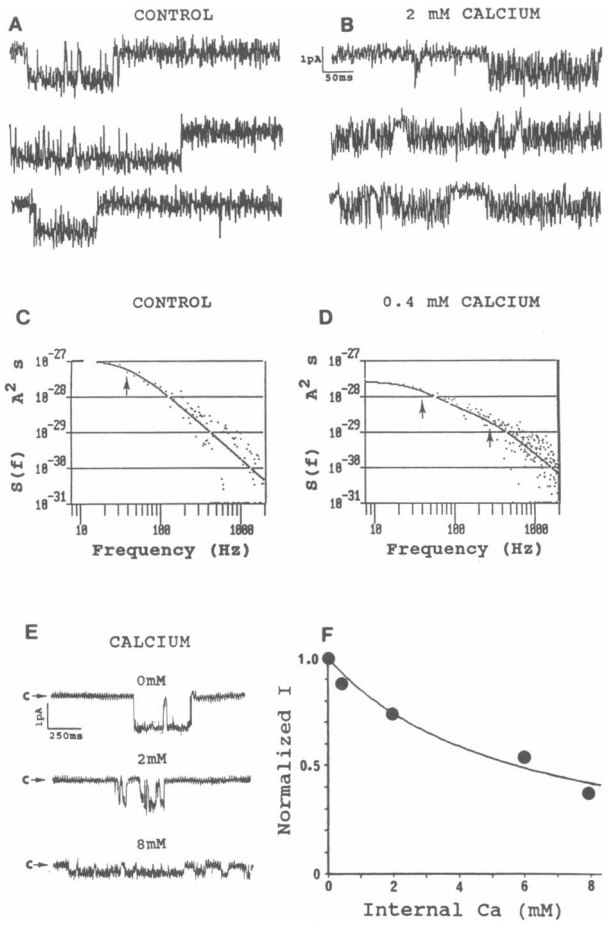

FIGURE 3 Internal calcium block of single Ca channels. Control pipette solution throughout. (*A*) Current records from an on-cell patch at −10 mV bathed in a solution with nominally zero calcium, 5 mM EGTA, 0.5 μM Bay R 5417, 120 mM NaCl, 20 mM CsCl, and 10 mM Hepes/NaOH, pH 7.4. Filtered at 2 kHz. The power spectrum of the channel currents was fitted by a single Lorentzian with f_c = 68.2 Hz (not shown). Holding potential was −75 mV. (*B*) Current records from an excised, inside-out patch at −10 mV with 2 mM internal calcium. The cytoplasmic solution contained 0.5 μM Bay R 5417, 120 mM NaCl, 20 mM CsCl, 2 mM $CaCl_2$, and 10 mM Hepes/NaOH, pH 7.4. Filtered at 2 kHz. The power spectrum of the channel currents was fitted with two Lorentzians with corner frequencies of 32.7 Hz and 1,139 Hz (filtered at 5 kHz, sampled at 20 kHz) (not shown). Holding potential was −80 mV. (*C*) Difference power spectrum of single calcium channel currents from an excised, inside-out patch bathed in nominally calcium-free solution with 1 mM EGTA and 0.5 μM Bay R 5417, 120 mM NaCl, 20 mM CsCl, and 10 mM Hepes/NaOH, pH 7.4. The membrane potential was −20 mV, filtered at 1.5 kHz, and sampled at 6 kHz. The difference spectrum in the absence of internal calcium was fitted with a single Lorentzian (f_c = 39.5 Hz, arrowed). Holding potential was −75 mV. (*D*) Difference power spectrum of single calcium channel currents from the same patch as in *C* after increasing internal calcium to 0.4 mM. Total calcium in the bath was elevated by adding 1.4 mM $CaCl_2$ from a 100 mM stock to the bath solution containing 1 mM EGTA. The difference power spectrum was fitted with two Lorentzians in the presence of calcium (f_{c1} = 39.1 Hz and f_{c2} = 282.0 Hz, arrowed). (*E*) Relationship between internal calcium concentration and mean unitary current of heavily filtered data (200 Hz). Unitary currents filtered at 200 Hz relative to the control value should represent the average time a channel spends unblocked. This filter frequency should permit resolution of most of the intrinsic gating events since they occur with f_c of ~40 Hz. Membrane potential was −20 mV. Holding potential was −80 mV. Current records in 0 and 2 mM internal calcium are from the same patch. (*F*) Plot of mean unitary current against internal calcium concentration at −20 mV. The point at 8 mM is the mean of two measurements. The line was drawn assuming 1:1 block by internal Ca with a K_d of 5.8 mM, and is the best fit by eye to the points.

This seems unlikely as we were able to record internal Ca-activated K^+ channels and internal ATP-inhibited K^+ channels in similar inside-out patches and to show that they responded to changes in $[Ca^{2+}]_i$ or $[ATP]_i$ as expected (Davies et al., 1989). As a more direct test we investigated the effects of adding the permeant ions calcium and barium to the flow solution. Internal calcium has been shown to reduce the mean unitary current of single calcium channels from cardiac muscle incorporated into planar lipid bilayers that were heavily filtered (200 Hz) (Rosenberg et al., 1988). Fig. 3 shows current records in control and in 2 mM $[Ca]_i$ at higher resolution (filtered at 2 kHz). Internal calcium at millimolar concentrations induced a flickery block. The power spectra from the channel currents in control could be fitted by a single Lorentzian with a corner frequency between 30 and 80 Hz, a value consistent with gating. In $[Ca]_i$, a second higher frequency component appeared which we ascribe to blocking events. This is supported by the observation that the corner frequency of this shifted with calcium concentration from 1,139 Hz in 2 mM calcium (from the experiment shown in Fig. 3 *B*) to 282 Hz in 0.4 mM calcium (Fig. 3 *D*). The low frequency Lorentzian components fitted to the spectra in Fig. 3, *C* and *D*, were 39.5 Hz in control and 39.1 Hz in 0.4 mM calcium, thus calcium ions do not appear to alter intrinsic channel gating. The apparent dissociation constant for calcium block was estimated from the percent reduction in mean unitary current of heavily filtered data by internal calcium and was 5.8 mM, assuming one-to-one binding (Fig. 3 *E*), which compares with K_d of 4 mM found for cardiac calcium channels. Assuming the simple blocking scheme shown in the legend to Fig. 1, the corner frequency (f_c) of the component associated with calcium block allows estimation of the underlying blocking (k_b) and unblocking (k_{-b}) rate constants from (cf. Horie et al., 1987):

$$K_d = \frac{k_{-b}}{k_b} \quad (1)$$

$$2\pi f_c = k_b([Ca]_i + K_d). \quad (2)$$

Eq. 2 becomes a good approximation if open-closed transitions contribute little to the higher corner frequency

(Colquhoun and Hawkes, 1983). Even at the lowest $[Ca]_i$ used, 0.4 mM, where corner frequencies were separated by a factor of 7, we calculate that the measured higher corner frequency will be within 1% of that predicted from Eq. 2. The blocking rate constants calculated this way from three experiments were 0.29, 0.59, 0.79 mM^{-1} ms^{-1} and the unblocking rate constants were 1.66, 3.40, 4.59 ms^{-1}.

We also investigated the effect of internal barium at concentration one-tenth of that in the external solution. Fig. 4 shows current records and amplitude histograms recorded from a patch first in $[Ba]_o$ = 0 mM, and then after it was moved into a solution with $[Ba]_o$ = 8 mM. There was a clear reduction in unitary current amplitude; in four patches it fell from a control value at 0 mV of 0.80 ± 0.08 pA to 0.51 pA ± 0.15 pA in 8 mM barium. This reduction to 63.8% of control is greater than that predicted by constant field theory (90% of control) (Goldman, 1943). One possibility for this difference is that it represents fast barium block of the channel. Alternatively, the difference might be due to ion–ion interactions in the pore which render constant field theory inaccurate to describe ion permeation in calcium channels.

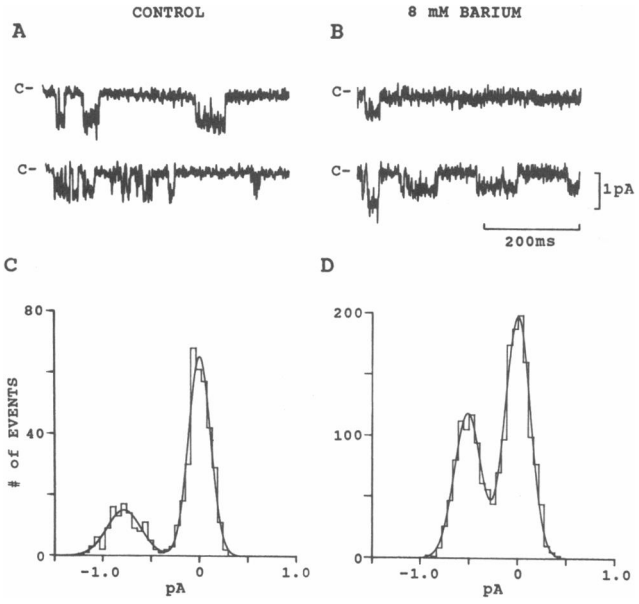

FIGURE 4 Internal barium decreases single channel currents. Control pipette solution throughout. (*A*) Current records for a voltage step to 0 mV in an inside-out patch recorded in control bathing solution as described in Fig. 2 *A*. Holding potential was −75 mV. (*B*) Records from the same patch after it was moved into a flow pipe containing the same solution, but without EGTA and with 8 mM $BaCl_2$. The larger amplitude opening in the bottom trace in internal barium represents two channels open simultaneously. (*C* and *D*) Amplitude histograms in control and 8 mM barium internal solution. The unitary current amplitude was 0.78 pA in control and 0.51 pA in 8 mM barium.

DISCUSSIONS

Comparison to cardiac Ca channels

The dissociation constants and kinetics of block of external cadmium and cobalt in single Ca channels from mesenteric artery are essentially similar to those found for single Ca channels in cardiac muscle (Lansmann et al., 1988). Internal calcium blocked single Ca channels from arterial smooth muscle with K_d of ~5.8 mM, a value similar to one found for cardiac muscle Ca channels. Rosenberg et al. (1988) found that the internal calcium reduced unitary barium currents through cardiac calcium channels without an increase in open channel noise ($[Ca]_i > 1$ mM) when the data were filtered at 200 Hz. This is consistent with our data; i.e., with $[Ca]_i > 1$ mM, most blocking and unblocking events would escape resolution at 200 Hz.

Rosenberg et al. (1988) found that internal calcium had no effect on inactivation time course of average unitary barium currents of cardiac muscle Ca channels. In agreement with these findings, we found that internal calcium did not affect intrinsic gating.

Mechanism of calcium block

The kinetics of calcium block are very slow (mean bound time, ~310 μs) relative to mean transit time for a calcium or barium ion through the calcium channel (~0.5 μs). A simple explanation is that the calcium binds to internal site that is not in the pore and induces a nonconducting conformation of the calcium channel. The low affinity of this site would suggest that calcium block would be of little physiological significance.

Implications for a permeation model

The block of cardiac Ca channels by external cadmium has been explained in terms of current two-site permeation models of the pore (Almers and McCleskey, 1984, Hess and Tsien, 1984; however see Marban and Yue, 1989). For such a model K_{app}, the $[Ba^{2+}]_o$ that gives 50% double occupancy by Ba^{2+}, can be measured from the dependence of unitary Ba currents on external $[Ba^{2+}]$, and is ~27 mM for cardiac Ca channels (Hess et al., 1986). The probability that both sites are unoccupied is close to zero if the $[Ba^{2+}]_o$ is above the micromolar range. Thus the blocking rate constant for external cadmium represents the blocking rate when the inner site is already occupied by Ba^{2+}. External cadmium cannot block when the outer site is occupied by Ba^{2+}, so the blocking rate constant for cadmium will be given by the primary entry

rate coefficient for cadmium multiplied by the probability that the outer site is empty. As expected, therefore, the rate constant for blocking falls as $[Ba^{2+}]_o$ is raised (Lansman et al., 1986). For a symmetrical channel, a Ba^{2+} ion will have equal free energy at the sites when the potential is 0 mV, so P[inner site occupied] = P[outer site occupied]. In other words the occupancy considerations are the same for the inner as for the outer site and the blocking rate constant for Cd^{2+} at a given $[Ba^{2+}]_o$ should be the same. Thus, a symmetrical model predicts that 10 mM cadmium, applied internally, should block the channel by >99%, compared with the complete absence of block we observed experimentally. We conclude that arterial smooth muscle Ca channels are asymmetric, at least with respect to cadmium block.

In cardiac muscle, the blocking rate of external cadmium was not voltage dependent, whereas the unblocking rate constant increased with membrane hyperpolarization (Lansman et al., 1986). This voltage dependence of unblock may represent cadmium ions being driven through the Ca channel. However, contrary to this idea, blocking ions with different charges (lanthanum and cadmium) have the same voltage dependence, suggesting that the voltage dependence of unblock might reside elsewhere (cf. Moczydlowski et al., 1984). It is not clear at present how the voltage dependence of external cadmium block relates to lack of block from the inside.

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