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Calcium permeability of the *N*-methyl-D-aspartate receptor channel in hippocampal neurons in culture

(glutamate receptors/synapse/single channel/ion permeation)

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ABSTRACT **We have developed a quantitative description for calcium permeability of the *N*-methyl-D-aspartate receptor channel to permit predictions of the reversal potential and calcium influx at different voltages for different extracellular calcium concentrations. Increasing the external calcium concentration markedly shifted the reversal potential to positive values and simultaneously decreased the single-channel conductance at potentials negative to the reversal potential. Very simple quantitative descriptions of calcium permeation and channel block by calcium ions accurately characterize our data and permit the prediction of reversal potentials and magnitudes of calcium influx for a wide range of conditions.**

Calcium ions that flow through *N*-methyl-D-aspartate (NMDA) receptor channels are thought to play a central role in the triggering of long-term potentiation (1, 2), in excitotoxicity (3, 4), and perhaps in other processes such as the activity-dependent reorganization of synaptic connections (5). Because the calcium ion flux through these channels has important consequences, we have carried out experiments to characterize the phenomenon quantitatively. We find, in confirmation of earlier reports (6–10), that NMDA receptor channels are highly permeable to calcium ions, as judged by the effect of calcium ion concentration changes on the NMDA receptor channel reversal potential. But calcium ions also block the channels while they permeate, as seen by the decreased single-channel conductance in the presence of elevated calcium. Such phenomena have previously been described for hydrogen ions in sodium channels (11–13) and calcium ions in acetylcholine receptor channels (14–17). In this paper we present a descriptive model of ion permeation through the NMDA channel that can be used to predict the effect of different external calcium concentrations on the single-channel conductance within the physiological range of membrane potentials. In addition, this model, in conjunction with our earlier model of the blockade of NMDA channels by magnesium (18, 19), can be used to determine the percentage of current carried by calcium ions in different concentrations of external calcium and magnesium.

METHODS

Hippocampi from 1- to 3-day-old rats were grown in long-term cell culture as described (18). Currents were recorded from outside-out patches (20) taken from neurons grown from 1–2 weeks in culture. Solutions containing NMDA (10 μM), glycine (1 μM), and various calcium concentrations were applied by placing the patch inside constantly flowing perfusion tubes to ensure complete changes in the concentration of calcium. The external solution to which NMDA, glycine, and calcium were added contained either 165 mM CsCl and 5 mM Hepes (pH adjusted to 7.4 with CsOH), for steady-state measurements of single-channel conductance, or 172.5 mM NaCl, 2.5 mM NaOH, and 5 mM Hepes (pH 7.4), in experiments using the voltage ramp (1 mM EGTA was added to the zero-calcium solution). The internal solution contained 150 mM CsCl, 5 mM Hepes, and 10 mM EGTA (pH adjusted to 7.4 with CsOH) or, in ramp experiments, 130 mM sodium gluconate, 20 mM NaCl, 25 mM NaOH, 10 mM Hepes, and 10 mM EGTA (pH 7.4). In the ramp experiments, internal and external sodium concentrations were the same (175 mM). Membrane current was filtered at 1–2 kHz (−3 decibels; 8-pole Bessel) and digitally sampled at 5–10 kHz. Reversal potentials were corrected for junction potentials.

RESULTS

Quantitative Relationships. We use three equations, the motivation for which is presented in the *Appendix*. Eq. **1** specifies the reversal potential for various monovalent and divalent extracellular ion concentrations:

$$V_0 = \frac{RT}{F} \ln\left(\frac{m_0 + n_0 w e^{-sFV_0/RT}}{m_1 + n_1 w e^{(1-s)FV_0/RT}}\right), \quad [1]$$

where V_0 is the reversal potential, R is the gas constant, T is the temperature (K), F is the Faraday, m_j is the activity for monovalent permanent ions, n_j is the calcium ion activity, w and s are constants, and the subscript j indicates intracellular ($j = 1$) and extracellular ($j = 0$) activities.

The single-channel conductance also will be seen to depend on the extracellular calcium ion concentration according to

$$\gamma_i = \frac{G_0 + n_0 h}{1 + (n_0 h / g_\infty)}, \quad [2]$$

where γ_i is the single-channel conductance for inward currents, n_0 is the extracellular calcium ion activity, and G_0, g_∞, and h are constants; G_0 gives the limiting conductance (for 175 mM extracellular sodium) in the absence of calcium ions, g_∞ determines the limiting conductance at high calcium ion activities, and h sets the rapidity with which this limiting conductance is reached as calcium ion activity is increased.

Finally, the inward current and outward current limbs of the current–voltage relationship conform approximately to Ohm's Law, with a transition between the two limbs according to

$$i = (V - V_0)\{\gamma_i K(V) + \gamma_0[1 - K(V)]\}, \quad [3]$$

with

$$K(V) = \frac{1}{1 + e^{p(V-V_0)}}, \quad [4]$$

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Abbreviation: NMDA, *N*-methyl-D-aspartate.

where γ_i is the single-channel conductance for the inward current limb (given by Eq. 2), γ_0 is the single-channel conductance for the outward current limb, V is the membrane potential, V_0 is the reversal potential (given by Eq. 1), and $K(V)$ is the function that determines the way in which the transition occurs between the two limbs and makes use of only a single constant, p, that characterizes the sharpness of the transition.

Current–Voltage Relationships. We have measured current–voltage relationships for single NMDA receptor channels in two ways. First, we recorded single channel activity at a variety of voltages between −80 and +80 mV and determined the single-channel currents directly by averaging measured values for a number of separate openings. A current–voltage relation established in this way is presented in Fig. 1*A*. Second, we applied voltage ramps during the time that the channels were open and averaged the currents that flowed for a number of openings; an example of a current–voltage relationship determined in this way is presented in Fig. 1*B* for extracellular calcium concentrations of 0, 5, and 100 mM. The theoretical curve given by Eq. 3 is superimposed on the data points in Fig. 1 *A* and *B*. Note that the inward and outward current limbs are nearly, but not exactly, linear; a more accurate treatment would have to use an additional term in the expansion that led to Eq. 3.

Because estimates for average single-channel conductance and reversal potential are more accurate for the voltage ramp data, we have restricted the remainder of our analysis to current–voltage relationships determined in this way. Current–voltage relationships like those illustrated in the figures have been measured for 14 patches with extracellular calcium concentrations ranging from 0 to 100 mM, in the presence of 165 or 175 mM sodium, and for extracellular calcium concentrations of 114, 130, and 160 mM without extracellular sodium. In each case, the reversal potential and slope of the inward and outward current limbs have been measured by fitting Eq. 3 to the current–voltage relationships with a nonlinear least-squares procedure. These data are presented in succeeding sections. The value for p in Eq. 4 was taken to be 20 mV^{-1} throughout.

In Fig. 1*C*, the current–voltage relationships in Fig. 1*B* have been shifted along the voltage axis to a reversal of 0 mV and normalized to their current magnitudes at −100 mV. This illustrates that the (slight) voltage dependence of the inward limbs is not altered by the external calcium concentration.

Reversal Potential Is Strongly Dependent on the Extracellular Calcium Concentration. Fig. 2 presents the reversal potential for single NMDA receptor channels as a function of extracellular calcium concentration. Most striking is the fact that a change of calcium concentration from 20 mM to 100 mM (in the presence of 175 mM sodium) produces a shift in reversal potential of 14 mV. When Eq. 1 is fitted to these data as illustrated in the figure, the value w found is 13 as compared to 1 for sodium. That is, each 1 mM calcium is as effective as 13 mM sodium in shifting the reversal potential. An indistinguishable fit of the data is obtained with the Goldman–Hodgkin–Katz equation (17) using a P_{Ca}/P_{Na} of 5.

Calcium Ions Block as They Permeate. Even though the permeability of calcium is 13 times greater than that of sodium as measured by reversal potential, increasing concentrations of this ion dramatically decrease the single-channel conductance. As the calcium concentration is increased from 0 to 100 mM (always in the presence of 175 mM sodium), the single-channel conductance decreases from 67 to 15 pS. This dependence is presented graphically in Fig. 3, with a theoretical curve from Eq. 2 superimposed. According to the fit of Eq. 2, the single-channel conductance should approach 65.6 pS as calcium is eliminated from the extracellular medium and should approach 15.2 pS as a large con-

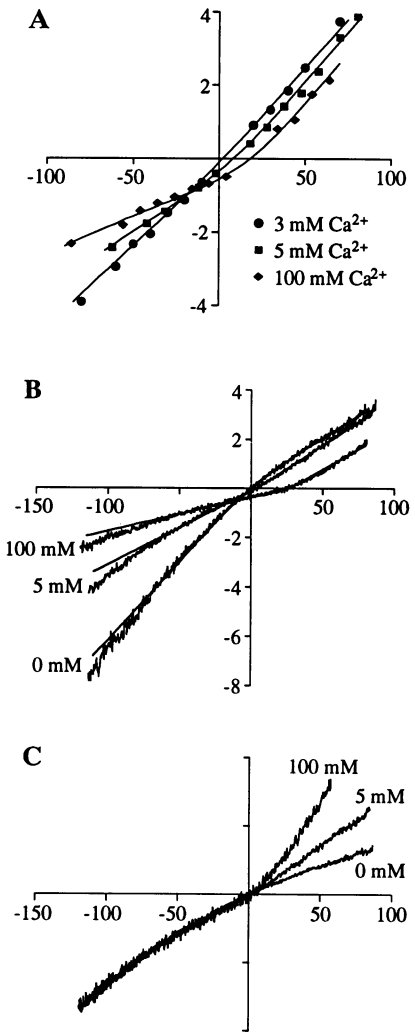

FIG. 1. Current–voltage relations of single-channel NMDA receptors depend on extracellular calcium concentration. (*A*) Current–voltage relations from steady-state single-channel recordings in 3, 5, and 100 mM extracellular calcium. Ordinate, picosiemens; abscissa, millivolts. (*B*) Current–voltage relations derived from openings during voltage ramps in 0, 5, and 100 mM calcium. The smooth curves are the theoretical fits using Eq. 3. (*C*) Current–voltage relations from *B*, normalized as described in the text.

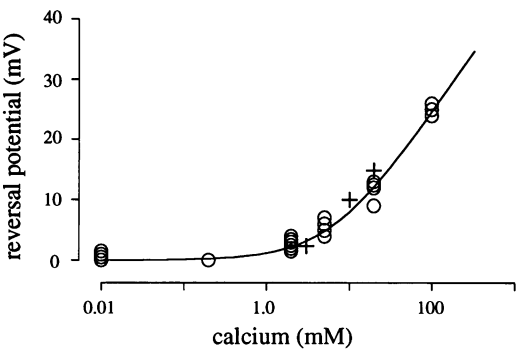

FIG. 2. Reversal potentials of NMDA receptor single-channel currents depend on extracellular calcium concentration. Open circles represent reversal potentials obtained from voltage ramp experiments as in Fig. 1*B*. The points plotted at 0.01 mM were actually recorded in nominally zero calcium (1 mM EGTA). Crosses represent the mean measured reversal potentials of the NMDA component of synaptic currents elicited from pairs of hippocampal neurons grown in cell culture (J. Bekkers and C.F.S., unpublished data). The smooth curve is the theoretical fit from Eq. 1 using values in Table 1.

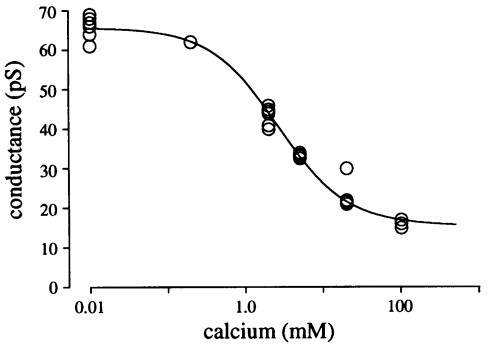

FIG. 3. Dependence of single-channel conductance on extracellular calcium. The smooth curve is the theoretical fit using Eq. 2 with values of constants given in Table 1.

centration is added to the normal 175 mM sodium; the constant h, which specifies how rapidly the transition is made between these limiting conductances, is 11.3 pS/mM. When calcium is set at 130 mM and sodium at 0, Eq. 2 predicts a single-channel conductance of 14.8 pS; the observed value is 16 ± 0.6 pS (mean ± SE, n = 3), a value within our experimental error.

We conclude, therefore, that calcium permeates effectively but that its presence within the channel inhibits the flux of sodium (and other) ions. The relationship

$$\theta = \frac{1}{1 + (G_0/0.5ch)},$$

developed in the *Appendix*, gives the fraction θ of the single-channel current carried by calcium ions; here c is the extracellular calcium concentration (mM), 0.5 is the activity coefficient for calcium ions in solutions at physiological concentrations, and the other symbols have the meaning given earlier (see Eq. 2 and the values in Table 1). According to this relationship, about 5% of the single-channel current is carried by calcium when the extracellular calcium concentration is 1 mM, about 10% when it is 2 mM, and about 20% when it is 4 mM. Note that this estimate does not represent the actual calcium ion flux near the reversal potential. At the reversal potential, no net electrical charge is carried inward by calcium or extracellular sodium ions, but these ions still are flowing in through the channel with their current balanced by the outward sodium or cesium flux. Even when the net current is outward, still some calcium influx will occur, and we estimate that this flux declines exponentially with the driving voltage, as does the function $\exp[-(V - V_0)/20]$. Thus the calcium influx at 20 mV positive to the reversal potential would be 37% of that occurring at the reversal potential.

DISCUSSION

Of special interest is the small fraction of the synaptic current through NMDA receptor channels carried by calcium ions. Although the calcium ion contribution to the total single-channel current at −20 mV (with an extracellular calcium concentration of 2 mM) would be only 0.1 pA, this amount of current would be sufficient to raise the calcium concentration of a 1-μm^3 volume, the approximate size of a spine head, to 0.5 μM in 1 ms (assuming no diffusional losses through the spine neck during this time and no local depletion of extracellular calcium). Since the average burst duration for an NMDA receptor channel is about 10 ms, a single NMDA receptor channel opening once at a membrane potential of −20 mV would be sufficient to have a large effect on the calcium concentration of a spine head, even with diffusional losses and a considerable amount of magnesium block. Clearly, then, even the small fraction of the synaptic current carried by calcium ions could have profound effects on spine calcium levels. Increases in spine calcium of 1 μM attributable to synaptic activation of NMDA receptors have been reported by using microfluorometry (21).

The self-block of calcium flux has the interesting effect of tending to maintain a constant influx of calcium ions at a given voltage even when the extracellular calcium concentration changes. For a calcium concentration c (mM) in the physiological range, the calcium conductance is, according to the equations developed in the appendix,

$$\gamma_{Ca}(c) = \frac{0.5ch}{1 + (0.5ch/g_\infty)},$$

where the activity coefficient has been taken to be 0.5, h = 11.3 pS/mM, and g_∞ = 15.2 pS (Table 1). If calcium ions did not block, the calcium conductance would be given by just the numerator of this expression. If we take the derivative of the preceding expression (and also just of the numerator) with respect to c, we find that the calcium conductance changes 5.7 pS/mM without calcium block and 1.5 pS/mM with the calcium-blocking mechanism operating. Thus, the effect of calcium block is to attenuate about 3.8-fold the decrease in calcium influx that accompanies a decline in extracellular calcium concentration. We propose that the calcium block provides a regulatory mechanism to minimize the reduction of the calcium influx through NMDA receptors at a given voltage in the face of depletion of extracellular calcium in the vicinity of the postsynaptic membrane.

Two other characteristics of the NMDA receptor channel are also addressed by our results. First, because the relationships between both the conductance and the reversal potential as a function of extracellular calcium concentration are monotonic (i.e., there is no anomalous mole-fraction effect), the NMDA receptor channel behaves like a single-occupancy pore that generally contains at most one ion at a time. Second, the equation used to predict the reversal potential does not require a term for surface potential. Rather it uses the ionic concentrations of the bulk solution. Thus, we are not compelled to consider a significant surface potential effect at the extracellular entrance of the NMDA receptor channel (6).

Although the equations presented here provide an accurate summary of our data, we must emphasize some limitations of our analysis. All of the channels we have studied have been extrajunctional, and studies of the acetylcholine receptor channel suggest that one cannot necessarily assume that junctional and extrajunctional channels will have identical properties (22, 23), although the available evidence for calcium permeability of synaptic NMDA receptor channels indicates similar properties (e.g., ref. 24). Bekkers and Stevens (J. Bekkers and C.F.S., unpublished observations) have measured the effect of various calcium concentrations on the reversal potential of the NMDA component of synaptic currents in hippocampal pyramidal cells in culture and found values very similar to the ones reported here (see Fig. 2). Clear differences do, nevertheless, exist between NMDA receptors. Two laboratories have recently reported develop-

Table 1. Values for constants in equations

Eq.	Constant	Value
1	w	13
	s	0.85
2	G_0	65.6 pS
	h	11.3 pS/mM
	g_∞	15.2 pS

mental changes in the kinetics of both synaptic and extrasynaptic NMDA receptor channels in tissue slice preparations (25, 26). Two forms of channel function are apparently also represented in cultured hippocampal neurons (27).

Magnesium ions block the NMDA receptor channel (28, 29), and we have shown here that calcium ions also act as blockers, although the block is so rapid that individual blocking events cannot be resolved as they can for the magnesium ion effect. Three groups have recently shown that substitution of glutamine or arginine for the putative transmembrane-region residue asparagine-598 alters not only calcium permeability but also the concentration dependence of the magnesium block (30–32), and these papers suggest that calcium and magnesium may bind to the same site in the pore. The magnesium blocking site is, judging from the voltage dependence of its block, about 80% of the way through the membrane field (18). The calcium ion block, however, exhibits very little voltage dependence, because the inward current limb has the same shape over the full voltage range for all of the calcium ion concentrations from 0 to 160 mM (see Fig. 1*C*). Thus, calcium ions interfere with the flux of other ions from a site that either, in terms of membrane field, is near the membrane surface or is located within the membrane field but has a configuration that causes the effects of the membrane field to cancel. Interestingly, magnesium ions seem not to bind as readily to the calcium "site" as does calcium itself, because the single-channel conductance does not decrease as the extracellular magnesium concentration is increased (8, 29).

We hope that the quantitative relationships we have presented will provide a basis for evaluating the role NMDA receptors play in neuronal function and for determining the molecular mechanisms of channel properties.

APPENDIX

Our goal is to develop the simple relationships **1–3** used above. Two general approaches to the problem can be considered. First, we could develop a specific model—a multiple-barrier model of the channel pore, for example—that yields the relations we seek. Alternatively, we could adopt a perturbation approach that is motivated in so far as possible by the physics of the situation we wish to describe. We have adopted the second alternative because it gives simpler equations and depends less on particular mechanistic assumptions.

Let m_j be the monovalent cation (here, sodium) activity, with $j = 0$ for the extracellular medium and $j = 1$ for the intracellular medium; n_j denotes the corresponding activity of another ion (calcium, in the current context) with valence z. We require that the Nernst relation hold whenever only a single ion species is present on both sides of the membrane. Write the reversal potential as

$$e^{\frac{FV_0}{RT}} = \frac{m_0 + n_0 f_0}{m_1 + n_1 f_1}$$

where F, R, and T have their usual meanings, and f_0 and f_1 are two functions that are chosen to give the correct reversal potential for any activities n_j. Because this equation must reduce to the Nernst relation for $n_j = 0$ or for $m_j = 0$, we have

$$\frac{f_0}{f_1} = e^{-\frac{FV_0}{RT}(z-1)}$$

The functions f_j may depend on the reversal potential V_0 and on the species of ion, but not on activities (for then the Nernst relation would not result). Expand $\ln(f_0)$ around zero reversal potential:

$$\ln(f_0) = \ln(w) + \frac{F(z-1)}{RT} sV_0 + t(V_0),$$

with w and s defined by

$$w = f_0(0)$$

and

$$s = \frac{RT}{F(z-1)}\left[\frac{\partial \ln(f_0(V_0))}{\partial V_0}\right]_{V_0=0}.$$

The sum of all remaining terms is represented by $t(V_0)$. To first order,

$$f_0 = we^{-sV_0F(z-1)/RT}$$

and (use the expression for the ratio f_0/f_1)

$$f_1 = we^{(1-s)V_0F(z-1)/RT}.$$

The final result, then, is

$$e^{\frac{FV_0}{RT}} = \frac{m_0 + n_0 we^{-sFV_0(z-1)/RT}}{m_1 + n_1 we^{(1-s)FV_0(z-1)/RT}},$$

with w and s dependent on the ionic species. Eq. **1** in the text arises from this relation by taking natural logarithms and selecting $z = 2$ for calcium ions. This is the same relation that would have resulted from a single-barrier model of the pore, but the equation derived above should be valid for any of a wide range of permeation mechanisms whenever ion concentrations, and thus reversal potentials V_0, are varied only over an appropriately small range. For the more usual treatment, s is the position of the barrier expressed as a fraction of the way through the membrane field and w is the permeability ratio.

The current–voltage relationships are well represented by the simple empirical equation

$$i = \{\gamma_i K(V) + \gamma_0[1 - K(V)]\}(V - V_0),$$

where γ_i and γ_0 are the conductances for the inward and outward limbs, V_0 is the reversal potential defined above, and $K(V)$ is a function that determines the nature of the transition between the two limbs:

$$K(V) = \frac{1}{1 + e^{p(V-V_0)}};$$

here the constant p specifies the sharpness of the transition between the inward and outward limbs.

Now consider only the inward limb of the current–voltage relationship with (say) sodium and calcium ions present with activities m_0 and n_0. Whenever calcium ions are present within the pore we suppose that they preemptively occupy it and thereby briefly exclude sodium and other calcium ions for a fraction ϕ of the time during which the occupancy occurs. For simplicity, we assume that sodium ions dwell such a short time within the pore that they do not influence the sodium or calcium current. From Ohm's law above, the current should be

$$i = (m_0(1 - \phi)g + n_0(1 - \phi)h)(V - V_0),$$

where g and h are the limiting conductances per mole of sodium and calcium ions in the range of activities for our experiments. The occupancy fraction ϕ, to first order, should be directly related to the calcium current i_{Ca} and inversely

related to the driving potential $(V - V_0)$: occupancy increases with current and decreases with greater driving voltages as each ion dwells for a shorter time in the channel. The simplest approximation for the fraction of the time channels are preemptively occupied by calcium ions, then, is

$$\phi = \frac{i_{Ca}}{g_\infty (V - V_0)},$$

where g_∞ is a constant to be determined experimentally. The calcium contribution to the total single-channel current is

$$i_{Ca} = n_0(1 - \phi)h(V - V_0).$$

Thus the fraction of time ϕ that the channel is occupied is (eliminate i_{Ca} between the preceding two equations and solve for ϕ)

$$\phi = \frac{n_0 h}{n_0 h + g_\infty}.$$

The single-channel conductance γ is defined as $i/(V - V_0)$ and can be found by eliminating ϕ with the immediately preceding equation:

$$\gamma_i = (1 - \phi)m_0 g + (1 - \phi)n_0 h = \frac{(m_0 g + n_0 h)}{1 + (n_0 h / g_\infty)}.$$

If we define $G_0 = m_0 g$, then this equation reduces to Eq. 2 in the text:

$$\gamma_i = \frac{G_0 + n_0 h}{1 + (n_0 h / g_\infty)}.$$

To find the fraction θ of the single-channel current carried by calcium ions, divide the calcium contribution i_{Ca} by the total single-channel current i:

$$\theta = \frac{i_{Ca}}{i} = \frac{[n_0(1 - \phi)h](V - V_0)}{[m_0(1 - \phi)g + n_0(1 - \phi)h](V - V_0)}$$

$$= \frac{n_0 h}{m_0 g + n_0 h}$$

$$= \frac{1}{1 + (G_0 / n_0 h)},$$

where we have used $G_0 = m_0 g$. The activity coefficient for calcium ions in physiological solutions is about 0.5 (33), so that $n_0 = 0.5c$, where c is the extracellular calcium concentration (mM). The fraction of single-channel current carried by calcium ions is thus

$$\theta = \frac{1}{1 + (G_0 / 0.5ch)}.$$

Most of the equations above use activities rather than concentrations. If c is an ion's concentration (mM) and n_0 its activity, then $n_0 = rc$, where r is the activity coefficient. We have calculated activity coefficients with equation 9.14 from Robinson and Stokes (33):

$$r(z,c) = 10^{[-0.509|z|\sqrt{I(c)}]/[1 + \sqrt{I(c)}]},$$

where c is the extracellular calcium concentration, z its valence, and $I(c)$ the ionic strength of the extracellular solution. Ionic strength is calculated by (ref. 33, p. 143)

$$I(c) = \frac{0.350 + 0.006c}{2};$$

the extracellular calcium concentration is c (expressed in mM) and the quantity 0.350 represents the contribution to the ionic strength of the extracellular sodium (175 mM), chloride (172.5 mM) and hydroxyl (2.5 mM) ions.

Note that the preceding treatment of pore block by calcium ions is invalid near the reversal potential. The diffusional entry of calcium ions and their block of the channel even at voltages for which the net current is outward accounts for the gradual transition between the single-channel conductance for the inward and outward limbs of the current–voltage relationship; the equations above are really valid only for the regions where the transition is complete.

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