J. Membrane Biol. 147, 1–6 (1995)

The Journal of
Membrane
Biology

Sodium Current Inhibition by Internal Calcium: A Combination of Open-channel Block and Surface Charge Screening?

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Received: 23 January 1995/Revised: 6 April 1995

Abstract. Internal application of millimolar concentrations of calcium to batrachotoxin (BTX)-activated rat skeletal muscle sodium channels, bathed symmetrically in 200 mM NaCl, causes a reduction in apparent single-channel amplitude without visibly increasing noise at a bandwidth of 50 Hz. A greater calcium-induced reduction occurred upon removal of external sodium ions. Internal calcium acted similarly in high ionic strength solutions (3M NaCl), where surface charges are effectively screened, suggesting that calcium acts, in part, by binding within the pore and occluding the conducting pathway. In low ionic strength solutions (20 mM NaCl), internal addition of N-Methyl-Glucamine (NMG) ions decreased the single channel amplitude consistent with screening of negative surface charges. An accurate description of the dose dependence of calcium inhibition, using either a simple blocking model, or rate theory calculations of ion permeation and block, also required surface charge screening. Hence, our data support the view that sodium current inhibition by internal calcium arises from a combination of both open-channel block and surface charge effects.

Key words: Na channel — Batrachotoxin — Bilayers — Surface potential — Energy profile — Divalent cation block

Introduction

Internally applied divalent cations such as calcium or cobalt cause a reduction in apparent single-channel amplitude of BTX-activated rat skeletal muscle sodium channels (Moczydlowski et al., 1986). At the bandwidth used, no increase in noise was observed in single-channel records, and it is not clear to what extent these ions act via rapid open-channel block, or via screening of surface charges (Green & Andersen, 1991). Based on the relatively weak voltage dependence of this effect, and on deviations from Michaelis-Menten kinetics, Moczydlowski et al. (1986) suggested a surface charge effect, or block involving multiple sites, as two possible mechanisms. In this vein, inhibition of high conductance Ca-activated K channels by cytoplasmically applied monovalent and divalent cations appears to result from a combination of surface charge screening and 1:1 open-channel block (MacKinnon, Latorre & Miller, 1989). Green and coworkers have suggested that block of dog brain sodium channels by internal divalents might result entirely from a surface charge mechanism (Green, Weiss, & Andersen, 1987; reviewed by Green & Andersen, 1991). In contrast, two recent papers described internal magnesium as acting on sodium channels exclusively by open-channel block (Pusch, Conti & Stühmer, 1989; Albitz, Magyar & Nilius, 1990). Here, we give new evidence for a distinct pore-blocking action of internal calcium. Fitting of the dose-response relation suggested that surface charge effects also contribute to sodium channel inhibition by internal calcium.

Materials and Methods

Membrane Preparations, Bilayer Recordings, and Solutions

Plasma membrane vesicles from rat skeletal muscle were prepared and incubated with batrachotoxin (BTX), were incorporated into neutral

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lipid bilayers (40 mg/ml PE, 10 mg/ml PC in decane, formed across a 150 μm hole in a planar plastic partition), and were voltage-clamped as described previously (Zamponi, Doyle & French, 1993). Recordings were performed using symmetric solutions at room temperature (≈22°C) and at a pH of 7.0, with either 200 mM NaCl/20 mM 3-N-Morpholinopranesulfonic acid (MOPS), or 3 M NaCl/50 mM MOPS, or 20 mM NaCl/5 mM MOPS. Generally, only single-channel incorporations were used for analysis. Calcium was added to the bath facing the cytoplasmic side of the channel from concentrated stocks chosen to keep the sodium concentration constant (either 600 mM $CaCl_2$/200 mM NaCl/20 mM MOPS or 1 M $CaCl_2$/3 M NaCl/50 mM MOPS at pH 7.0). In some experiments, Na ions were replaced by perfusion of the external bath with 200 mM N-Methyl-Glucamine (NMG)/20 mM MOPS, pH7.0 (with NaOH). In low ionic strength experiments, NMG was added to the cytoplasmic side from a stock of 200 mM NMG/20 mM NaCl/5 mM MOPS, pH 7.0.

Data Analysis

Data were acquired for up to 1 min under each condition, filtered at 100 Hz, and sampled at 250 Hz during transcription into a Compaq personal computer, and digitally filtered at 20 or 50 Hz during analysis with pCLAMP v 5.5 (Axon Instruments, Foster City, California). Apparent single-channel amplitudes were determined by cursor using pCLAMP. Preparation of figures was carried out in Sigmaplot (Jandel Scientific, Corte Madera, California).

The dose dependence of calcium action was described by calculating net ionic currents from barrier profiles, with either a single occupancy 4B3S model, or a double occupancy 3B2S model with ion-ion repulsion, using the program AJUSTE which has been previously described in detail (Alvarez, Villaroel & Eisenman, 1992; as adapted by French et al., 1994). These rate theory calculations include the effect of surface potential on both the electrical potential gradient within the pore and the concentration of blocking ion at the channel-solution interface. Independently, surface potential calculations were also performed using the Gouy-Chapman theory in the form of Eqs. 1 and 2. Equation 1 combines a 1:1 blocking isotherm with the Boltzman equation, which, in this form, predicts the effective calcium concentration at the channel-solution interface as a function of the surface potential (*see* Latorre et al., 1992).

$$I([Ca])/I([Ca = 0]) = 1/(1 + ([Ca]\exp(-\Psi/12.8))/K_d) \quad (1)$$

Here, $I([Ca])$ and $I([Ca = 0])$ are respectively, the apparent single-channel amplitudes in the presence and absence of calcium, K_d is the apparent equilibrium dissociation constant for calcium block, [Ca] is the calcium activity in the bulk solution, and the surface potential, ψ in mV, is determined from the Grahame equation. However, Eq. 1, unlike AJUSTE, does not provide a mathematical treatment for the dependence of the intrapore voltage gradient on the surface potential. MacKinnon et al. (1989) have made an attempt to physically account for this effect with an expanded version of Eq. 1 in order to describe divalent cation block of K(Ca) channels (*see* Eq. 2).

$$I([Ca])/I([Ca = 0]) = \{1 + (\psi([Ca = 0]) - \psi([Ca]))/(V_m - E_{rev})\}/\{1 + ([Ca]/K_d)(\exp(-\psi([Ca])/12.8))\}. \quad (2)$$

Here, V_m is the command potential (+40 mV in our experiments), E_{rev} is the reversal potential (0 mV), and $\psi([Ca = 0])$ and $\psi([Ca])$ are respectively, the surface potentials determined from the Grahame equation in the absence and presence of calcium. Equation 2 is written differently from the original equation proposed by MacKinnon et al. in that the conductance, γ, is replaced by $I([Ca = 0])/(V_m - E_{rev})$. In Eq. 2, the change in surface potential is assumed to add to the voltage gradient and driving force, and thus, ion permeation, across the pore. This contribution of surface potental changes to the overall driving force is reflected in the term $(\Psi([Ca = 0]) - \Psi([Ca]))/(V_m - E_{rev})$. Hence, Eq. 2 is an attempt to provide a mathematical description for the reduction in apparent single-channel amplitude without the requirement of physical occlusion of the pore by a blocking ion (i.e., at very large K_d values). We note that Eq. 2 has serious limitations (for discussion *see* MacKinnon et al., 1989) and is only defined at potentials sufficiently far from the reversal potential. However, it provides an intuitive basis for contributions of block and changes in sodium conductance in the unblocked pore due to surface charge screening.

Statistics

Numerical values are provided as mean ± SD, with n being the number of experiments. The statistical significance of changes was evaluated with a two-tailed paired Student's *t*-test, with probability, *P*.

Results

Evidence that Calcium Ions Block the Pore

Figure 1*A* depicts single-channel records from BTX-activated rat skeletal muscle sodium channels. In the absence of blockers, these channels are open most of the time at +40 mV, showing only sporadic gating closures. Internal application of 36 mM $CaCl_2$ resulted in a decrease in apparent single-channel amplitude without either a visible increase in noise at a bandwidth of 50 Hz, or a resolvable change in the reversal potential, consistent with data previously reported by Moczydlowski et al. (1986). When external sodium ions were replaced by the impermeant NMG, the effect of calcium was enhanced ((I(36 mM Ca)/I(0 mM Ca): ext. Na: 0.70 ± 0.02, ext. NMG: 0.57 ± 0.04, $P < 0.0004$, n = 5). These data suggest that calcium ions block the sodium channel in the narrow region of the pore, where their action can be inhibited by sodium ions entering from the outside.

Pore block is also suggested by the records in Fig. 1*B*. Here, we recorded from a single sodium channel bathed in symmetric 3M NaCl/50 mM MOPS. In such high ionic strength solutions, surface charges are essentially completely screened by sodium ions (Cukierman, 1993) thereby helping to separate block from putative surface charge effects. In addition, at this high concentration sodium would compete more effectively for intrapore sites. As can be seen from the records in Fig. 1*B*, calcium, although at higher concentrations, (250 mM), still reduced the apparent single-channel amplitude (I(250 mM Ca)/I(0 Ca): 0.80 ± 0.04, $P < 0.00004$, n = 6). This strongly implies a surface charge-independent com-

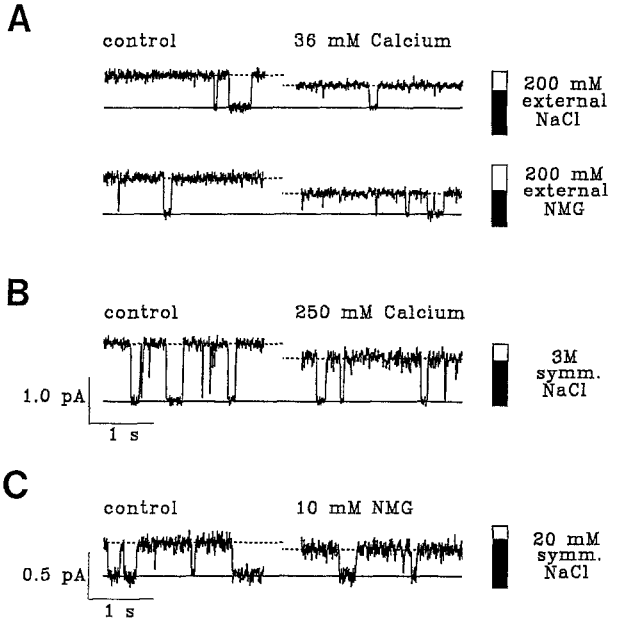

Fig. 1. Single-channel current traces from BTX-activated rat skeletal muscle sodium channels (V = +40 mV). The records were filtered at 50 Hz, the solid lines indicate the closed level, the broken lines indicate the apparent open level. The solid bars, reflect on a scale from 0 to 1, the normalized apparent single channel current amplitude. (A) Dependence of calcium block on extracellular sodium ions. In the presence of 200 mM external sodium (upper traces), 36 mM calcium decreased the apparent single-channel amplitude to 70% of the control level. When external sodium was replaced by 200 mM NMG, the amplitude was further decreased to 58% of the control level. (B) Calcium block in high ionic strength solutions. In symmetric 3 M NaCl, application of 250 mM $CaCl_2$ reduces the apparent single channel amplitude to 80% of the control level. (C) Reduction of the single-channel current amplitude due to surface charge screening by NMG. In symmetric 20 mM NaCl, internal addition of 10 mM NMG reduced the apparent single channel amplitude by 20%.

ponent of calcium action. However, the experiments shown in Fig. 1 *A* and *B* do not rule out an additional contribution from surface charge screening to the overall action of calcium.

Surface Charge Screening Contributes to Sodium Current Inhibition

To test for a possible involvement of surface charge screening, we investigated the effect of internally applied NMG, a commonly used "inert" ion substitute, in low ionic strength solutions (20 mM NaCl). As can be seen from Fig. 1*C*, application of 10 mM NMG causes a 20% reduction in apparent single channel amplitude (I(10 mM NMG)/I(0 NMG): 0.80 ± 0.06, $P < 0.04$), n = 3), suggesting that surface charge screening is a sufficient mechanism to reduce sodium flux through the channel. This implies that surface charge screening would contribute to the overall action of calcium.

A surface charge component of calcium action is also supported by the dose-response curve (Fig. 2) obtained for the calcium-induced reduction in apparent single channel amplitude at +40 mV (n = 12). The solid line is a transform based on a 4B3S model with single-occupancy and an effective surface charge density of 2.45×10^{-2} e/nm^2, and thus incorporates both open channel block and surface charge screening. A fit using a 3B2S double-occupancy model with the same surface charge density yielded essentially identical results (but *see* Discussion). For comparison, the broken line indicates the best fit to the data set based on 1:1 block without surface charge screening. The latter model, however, systematically overestimated the degree of block with high calcium solutions and yielded a worse fit to the data set (sums of squares: solid line, 0.029 pA^2, dashed line, 0.059 pA^2). Overall, our data suggest that besides very rapid open channel block, internal calcium inhibits sodium flux through the channel via screening of negative surface charges.

Discussion

Because internally applied divalent cations act on sodium channels by reducing the apparent single-channel amplitude without measurably increasing noise in the range of bandwidths used in single-channel experiments, it is difficult to determine whether these ions exert their actions via screening of surface charges, via rapid open-channel block, or via both mechanisms (Moczydlowski et al, 1986; Green et al., 1987; Pusch et al., 1989; Albitz et al., 1990; Green & Andersen, 1991). We have obtained two new lines of evidence for open-channel block: First, the action of calcium was sensitive to the removal of external sodium ions, suggesting that calcium acts within the conducting pathway (*cf.* Zamponi et al., 1993; Zamponi & French, 1993). Second, even in high ionic strength solutions, where the surface charges should be almost completely screened (Cukierman, 1993), calcium induced a reduction in apparent single channel amplitude, consistent with a surface charge-independent blocking component. The antagonistic effect of external sodium, together with the previously reported voltage dependence of calcium action ($z\delta \approx 0.15$, Moczydlowski et al., 1986) suggests that calcium blocks the channel within the narrow region of the pore. The internal action of another divalent ion, magnesium, was recently described as exclusively open-channel block (Pusch et al., 1989; Albitz et al., 1990). However, this ion acted at much lower concentrations (K_d (0 mV) = 3 mM, Pusch et al., 1989) at which contributions due to surface charge screening would be small (<5% reduction in current for a surface charge density of 3×10^{-2} e/nm^2).

The role of surface charge screening in the overall action of calcium is less clear cut. In low ionic strength

solutions, application of 10 mM NMG inhibited 20% of the sodium current through the channel. However, NMG, although widely considered an inert ion for sodium channels, might to some degree block the channel, since it is structurally related to a range of pore-blocking amines (Zamponi & French, 1994). The sodium current inhibition in 20 mM NaCl occurred with an IC_{50} of 40 mM. In contrast, in 200 mM NaCl, NMG reduced the apparent single-channel amplitude with an IC_{50} of >200 mM (*not shown*). For comparison, a complete removal of external sodium ions only results in a twofold reduction in the apparent K_d for calcium action (*this paper*), and a 1.5-fold reduction in the apparent K_d values for block by the cytoplasmic monovalent amine pore blockers diethylamine (Zamponi & French, 1993), QX-314 (Zamponi et al., 1993), and cocaine (Wang, 1988), with little or no effect from changes in the internal concentration of sodium ions (Wang, 1988). If NMG were to interact similarly with sodium, and if the NMG-induced reduction in single channel current in 200 mM NaCl were exclusively caused by block, one would expect pore block by NMG to amount to no more than about one quarter of the 20% inhibition observed in Fig. 1*C* using 20 mM NaCl. Hence, even if NMG is not viewed as an ideally inert ion, our data suggest that NMG ions predominantly act to reduce sodium conductance by reducing the contribution of negative surface charges to the local electrostatic potential.

We were not able to fit the dose dependence of calcium action using a model based on pore block with no contribution from surface charge effects (Fig. 2). Ion permeation through BTX-activated rat skeletal muscle (Ravindran et al., 1992) and rat brain sodium channels (French et al., 1994) has been modeled with the use of a 3B2S double occupancy model with no contribution from surface charge. In contrast, for canine brain channels, Green et al. (1987) were able to describe concentration-conductance relations using a single occupancy model based on the Gouy-Chapman theory with a large internal surface charge density, and concluded that block by internal divalents might arise entirely from surface charge screening. Here, we used a 3B2S model based on the previously published energy profile for sodium ions passing through BTX-activated rat skeletal muscle sodium channels (Ravindran et al., 1992), combined with a barrier profile for calcium similar to the one reported by French et al. (1994) for the rat brain sodium channel. This model yielded a dose-response curve for calcium action indistinguishable from a simple 1:1 binding isotherm. When surface charge was introduced, the quality of the fit to the calcium dose dependence improved. However, the model predicted concentration-conductance relationships that deviated from the low sodium concentration-conductance data published by Ravindran et al. (1992).

The 4B3S single occupancy model described in Fig. 2 also required the introduction of surface charge (2.45×10^{-2} e/nm^2) to adequately reflect the dose dependence of calcium action. For comparison, a threefold larger surface charge density resulted in a pronounced overestimate of calcium block at low calcium concentrations. For the experiments carried out in high ionic strength solutions, the simulation predicted 18% block which is very close to the experimentally determined 20%. However, as in the case of the 3B2S model, the introduction of surface charge resulted in deviations from our conductance-concentration data and those of Ravindran et al. (1992), although the effect was not as drastic as with the 3B2S model. In 20 mM Na, the simulation predicted a current which underestimated our experimental data by 20%. Allowing asymmetric surface charge densities on the external and on the cytoplasmic side did not alleviate this problem. Clearly, the models most commonly used to describe permeation through Na channels show certain limitations, and more complex models, or perhaps fundamentally different approaches, may be required (*see also* Moczydlowski, 1993; Naranjo & Latorre, 1993). Nonetheless, regardless of the number of barriers or

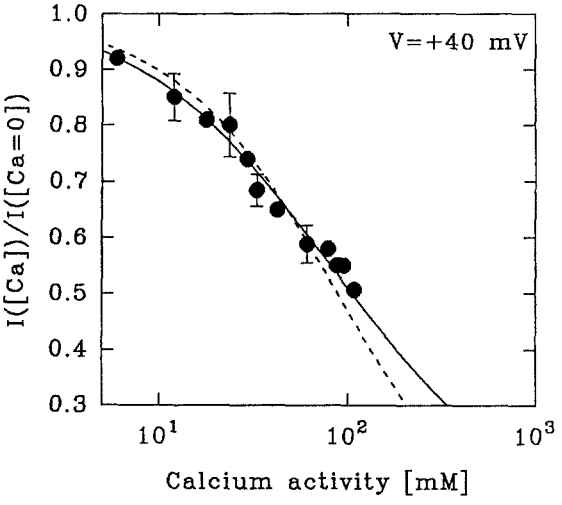

Fig. 2. Dose dependence of calcium action in symmetric 200 mM Na (V = +40 mV, 12 experiments, 1 to 7 determinations per point). The error bars indicate standard deviations, the continuous line is a simulation based on a 4B3S model with internal and external surface charge densities of 2.45×10^{-2} e/nm^2, using the computer program AJUSTE (Alvarez et al. 1992; French et al., 1994). The barrier profile parameters used in the simulation are as follows: Na: G1 = 7.29, G2 = 7.638, G3 = 7.837, G4 = 7.9045, U1 = 3.7005, U2 = 3.8007, U3 = 3.82; Ca: G1 = 11.85, G2 = 8.68, G3 = 10.65, G4 = 10.46, U1 = 6.08, U2 = 6.77, U3 = 6.86; D1 = D2 = 0.0545, D3 = D4 = 0.1744, D5 = D6 = 0.2, D7 = 0.0711, where G is the peak height, U the well depth, and D the fractional distance, numerical indices indicate the position across the transmembrane voltage from the cytoplasmic side. The long-dashed line is a fit to the data based on simple 1:1 block without surface charge screening, using the equation $I([Ca])/I([Ca = 0]) = 1/(1 + [Ca]/K_d)$, where [Ca] is the calcium activity, and K_d is the equilibrium dissociation constant for 1:1 calcium block (88 mM).

channel occupancy, the picture emerged that negative surface charge was necessary to describe our data set.

We independently tested the putative involvement of surface charges using equations 1 and 2 (Materials and Methods). For a surface charge density of 9.8×10^{-2} e/nm^2, Eq. 1 produced a fit to our data set which was virtually indistinguishable from that obtained from the barrier calculations, suggesting that ability of negative surface charges to concentrate calcium ions near the pore mouth is sufficient to account for the apparent deviation of the dose-response curve from a simple 1:1 binding isotherm. The fit yielded an equilibrium dissociation constant for calcium block of 224 mM (which corresponds to a bulk calcium concentration of 106 mM). These data support the view that surface charges are a crucial factor for determining the overall action of calcium. However, Eq. 1 cannot account for our data obtained with NMG, if NMG predominantly acts via a nonblocking mechanism. To include the possibility of inhibiting sodium permeation by altering the cytoplasmic surface potential, we also fitted our data set with Eq. 2 (Materials and Methods), which has been previously used by MacKinnon et al. (1989) to describe block of K(Ca) channels by monovalent and divalent cations as a combination of surface charge screening and open-channel block. As noted in Materials and Methods, this is an approximate expression and must be used with caution. Nonetheless, the barrier modeling (Fig. 2) which is not restricted to the limiting case invoked by Eq. 2 accounts for the effects of surface potential changes on the single-channel conductance (i.e., in the barrier models, the single-channel conductance depends on both internal and external surface charge density even in the absence of blocking ion). Using a surface charge density of 9.8×10^{-2} e/nm^2 we were able to describe both the calcium dose dependence (with fits virtually identical to those obtained with Eq. 1, but a higher effective equilibrium dissociation constant for calcium block; $K_d = 320$ mM) and the effects of NMG in low NaCl solutions (assuming complete lack of pore block, the predicted reduction in apparent single-channel amplitude due to application of 10 mM NMG was 18%). The value for the surface charge density agrees well with that obtained from the rate theory calculations, and is also consistent with results of Naranjo and Latorre (1993). The equilibrium dissociation constants for the pore-blocking part of calcium action obtained from Eqs. 1 and 2 indicate a rather low affinity binding of calcium to the sodium channel pore. The values are consistent with observations in 3M NaCl (estimated IC_{50} for Ca block, 1000 mM) if Na competitively inhibits Ca binding with an inhibition constant of 840 mM. Such a high value might be expected if Na has to enter an occupied pore to inhibit Ca block (*cf.* French et al., 1994).

The previously reported voltage dependence for calcium action (Moczydlowski et al., 1986) is shallower than that of block by local anesthetics such as lidocaine or QX-314 ($z\delta$ between 0.33 and 0.4, Zamponi et al., 1993). Although we did not investigate the voltage dependence of calcium block here, it is possible that the superimposed screening of surface charges might mask a steeper intrinsic voltage dependence of calcium block when studied in a limited range. If so, calcium and local anesthetics might be acting at overlapping receptors. This would be consistent with the steeper intrinsic voltage dependence of block by internal magnesium ($z\delta$ = 0.28; Pusch et al., 1989; but *see* Albitz et al., 1990) and preliminary observations indicating competition between tetrapropylammonium and calcium (Zamponi, *unpublished observations*).

In summary, combining pore block and surface charge screening provides an accurate description of our observations. At present, however, we know of no single model that can encompass all of the pore properties of even this well-studied channel. Qualitative features of our data provide novel evidence for a pore-blocking component of internal calcium action.

This research was supported by grants from the Medical Research Council of Canada, and by a Scholarship to R.J. French from the Alberta Heritage Foundation for Medical Research. We thank Dr. John Daly for providing batrachotoxin, and Drs. Lawrence Haynes, Christopher Miller, David Naranjo, and John Hanrahan for helpful comments on various versions of the manuscript.

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