Cadmium block of calcium current in frog sympathetic neurons

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ABSTRACT Cd^{2+} blocks whole-cell calcium currents in frog sympathetic neurons by 50% at ~300 nM. Strong depolarizations rapidly reverse that blockade (τ = 1.3 ms at +120 mV). Reblocking follows bimolecular kinetics (rate = 1.2×10^8 M^{-1} s^{-1}) at voltages where channels are mostly open (0 to +30 mV). The unblocking rate is ~50 s^{-1}, so the dissociation constant calculated from the rate constants is ~400 nM. Steady-state block is strong at −80 mV, so closed channels can also be blocked. However, reblocking is extremely slow (τ = 1–2 s) at voltages where the channels are mostly closed. The rates for Cd^{2+} entry and exit are >100-fold lower for closed channels than for open channels, and closed channels appear to be closed at both ends.

INTRODUCTION

The kinetics of blockade of ion channels by drugs and ions have provided much information on the mechanisms of ion permeation (Hille, 1992).

Previous studies have shown that blockade of open calcium channels by Cd^{2+} is voltage dependent, with relief of block at negative voltages (Brown et al., 1983). That is expressed as a decrease in mean closed times in single channel records (Lansman et al., 1986), and as a "hook" in tail currents (Swandulla and Armstrong, 1989; Chow, 1991). Despite this, it is clear that blockade of closed channels can be quite strong at negative voltages (Swandulla and Armstrong, 1989). Many of the effects of Cd^{2+} can be explained well by binding to a site(s) in the ion permeation pathway of the calcium channel (Lansman et al., 1986), but additional actions at sites outside the channel remain possible (Almers and McCleskey, 1984; Taylor, 1988).

Jones and Marks (1989) found that Cd^{2+} block is also relieved at extreme positive voltages. We have investigated that effect further, and have used it as a tool to explore the kinetics of Cd^{2+} block. A preliminary report of some of these results has appeared (Thévenod and Jones, 1990).

METHODS

Neurons were isolated from caudal paravertebral sympathetic ganglia (Kuffler and Sejnowski, 1983) and were voltage clamped in the whole-cell configuration (Hamill et al., 1981) as described (Jones and Marks, 1989). The standard intracellular solution was 86.5 mM *N*-methyl-D-glucamine (NMG) chloride, 2.5 mM NMG·Hepes, 10 mM NMG_2EGTA, 5 mM $Tris_2ATP$, and 4 mM $MgCl_2$, titrated to pH 7.2 with NMG base. Some experiments used 76.5 mM NMG·Cl, 2.5 mM NMG·Hepes, 10 mM $Tris_4BAPTA$, 5 mM $Tris_2ATP$, 0.3 mM Li_2GTP, and 6 mM $MgCl_2$ (pH 7.2). The extracellular solution was 117.5 mM NMG·Cl, 2.5 mM NMG·Hepes, and 2 mM $BaCl_2$ (pH 7.2). Series resistance compensation (nominally 80%) was used. The clamp settling time, estimated from the 10–90% rise time of tail currents, was typically ~0.1 ms. pClamp software v. 5.5 (Axon Instruments, Foster City, CA) was used for data acquisition and analysis; their Clampfit program was used for single and double exponential fits. Linear leakage and capacitative currents were subtracted unless noted otherwise. After subtraction, calcium current is well isolated, except for a small, rapid outward current (possibly a gating current) at the beginning of depolarizing steps (Figs. 3, 6, 10, 11). Filtering is indicated in figure legends, including an analogue anti-alias filter and subsequent digital Gaussian filtering. Values given are mean ± SEM unless otherwise noted.

Under these whole-cell recording conditions, there appears to be one predominant calcium current in frog sympathetic neurons. Since at least 80% of the current is blocked by ω-conotoxin (Jones and Marks, 1989), and <10% is blocked by 1 μM nifedipine (Jones and Jacobs, 1990), the major current closely resembles the N-type current of mammalian sympathetic neurons (Hirning et al., 1988; Plummer et al., 1989).

In most experiments, $CdCl_2$ (ACS grade; Alfa Products, Danvers, MA) was applied by bath superfusion, remotely controlled by solenoid valves. Where noted, a more rapid flow tube system was used (Yellen, 1982; Jones, 1991).

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RESULTS

Unblocking of calcium channels at extreme positive voltages

In Cd^{2+}, depolarization to +80 mV increases the calcium current during a subsequent step to −10 mV (Fig. 1). This could be observed for a step as brief as 0.5 ms, and was effectively complete by 8 ms. The unblocking effect was voltage dependent (Fig. 2). Steps to +40 mV had little effect, but the extent and rate of unblocking increased at more positive voltages. Measured time constants for unblocking were 3.6 ± 0.6 ms at +60 mV, 2.7 ± 0.4 ms at +80 mV, 1.6 ± 0.2 ms at +100 mV, and 1.3 ± 0.3 ms at +120 mV (n = 6–7, at 0.3–3 μM Cd^{2+}).

Reblocking of open channels

Thus far, we have assumed that the increase in current observed at early times in the postpulse (with the protocol of Fig. 1) results from unblocking of calcium channels during the previous step to +80 mV. If so, the subsequent decrease in current during the postpulse would reflect rebinding of Cd^{2+} and should be concentration

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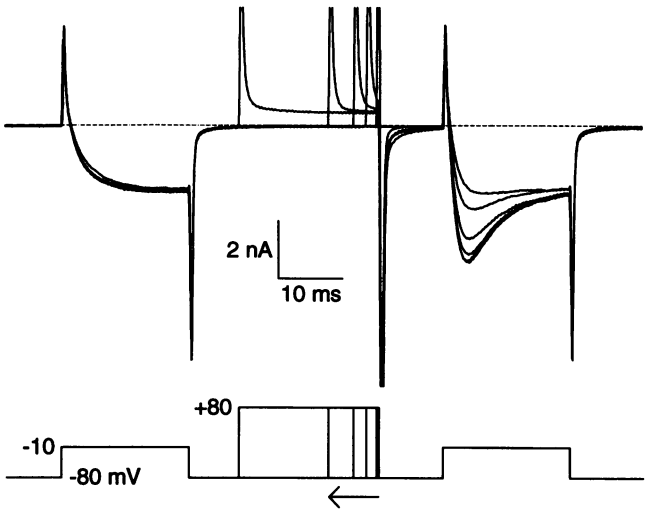

FIGURE 1 Relief of Cd^{2+} block at +80 mV. Six records in 1 μM Cd^{2+} are superimposed, with pre- and postpulses to −10 mV, and middle pulses to +80 mV. The middle pulses increased in duration toward the left (*arrow*), and lasted 0, 0.5, 2, 4, 8, and 22 ms. Cell 89815, filtered at 1.2 kHz, not leak subtracted. The full amplitudes of capacity transients and tail currents are not shown for steps to and from +80 mV.

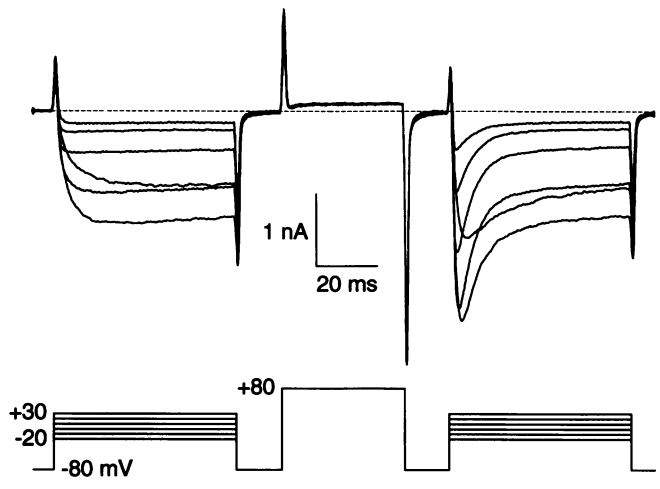

FIGURE 3 Reblocking of open calcium channels at different voltages. Calcium currents (in 1 μM Cd^{2+}) are illustrated for depolarizations to voltages between −20 and +30 mV (prepulse and postpulse), with a 40-ms middle pulse to +80 mV. Cell b9905, filtered at 300 Hz.

dependent. Fig. 3 illustrates the protocol used to test that prediction. Following steps to +80 mV, the current during a postpulse was initially increased, followed by a relaxation toward the steady-state current level. The relaxation rate was slower at more negative voltages. This protocol was performed at three concentrations (0.3, 1.0, and 3.0 μM Cd^{2+}) in four cells.

A minimal model for currents during the postpulse is C-O-OB, where normal activation kinetics are approximated by a two-state model (Jones and Marks, 1989), and Cd^{2+} binding to the open channel produces a blocked state (Fig. 4, *inset*). On that model, currents would be fit by the sum of two exponentials, with each time constant depending on all four rate constants. However, if channel opening is nearly complete and faster than blocking, the two time constants are well approxi-

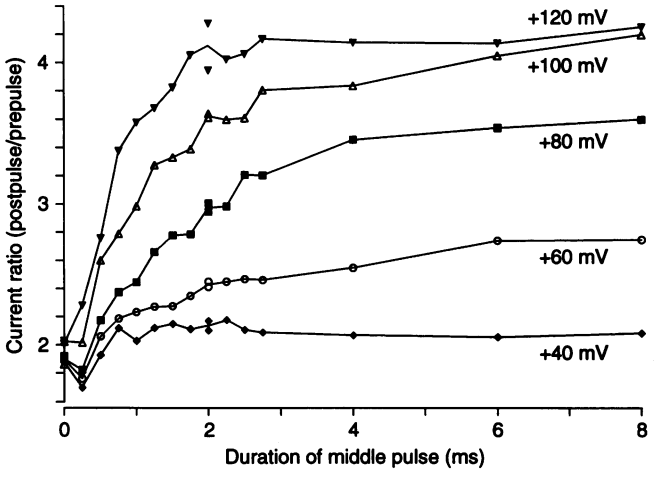

FIGURE 2 Voltage and time dependence of unblocking. Data are from the protocol of Fig. 1, in 1 μM Cd^{2+}. Currents were measured between 2.5 and 3.0 ms after the start of each step to −10 mV, to approximate the initial rate of channel opening (i.e., before reblocking is significant during the postpulse) and to avoid contamination by residual capacity transients. The postpulse/prepulse current ratio is plotted as a function of the duration of the depolarizing middle pulse. The voltages of the middle pulses are shown. The postpulse/prepulse ratio was ~2 with no middle pulse, presumably due to unblocking during the tail current following the prepulse (Swandulla and Armstrong, 1989). Cell 89915.

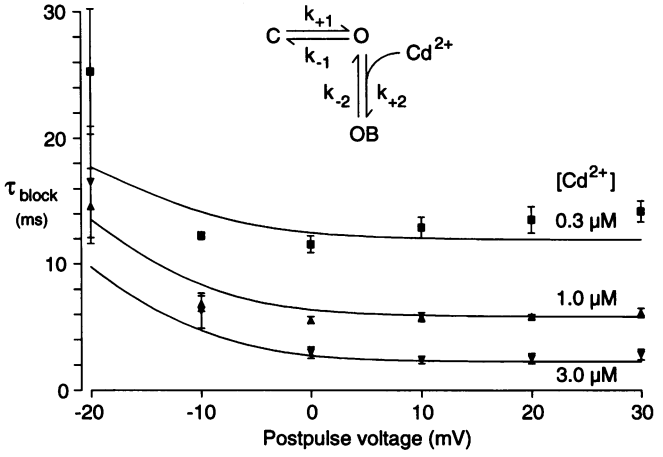

FIGURE 4 Dependence of blocking kinetics on voltage and Cd^{2+}. Values are the slower time constant of two-exponential fits to the time course of postpulse currents from the protocol of Fig. 3 (n = 4). The smooth curves are the slower of the two time constants calculated from the analytic solution (Chiu, 1977; Gutnick et al., 1989) to the three-state model shown, with $k_{+1} = 200\ e^{0.06(V+15)}\ s^{-1}$, $k_{-1} = 200\ e^{-0.06(V+15)}\ s^{-1}$ (Jones and Marks, 1989), $k_2 = 1.2 \times 10^8\ M^{-1}\ s^{-1}$, and $k_2 = 47\ s^{-1}$.

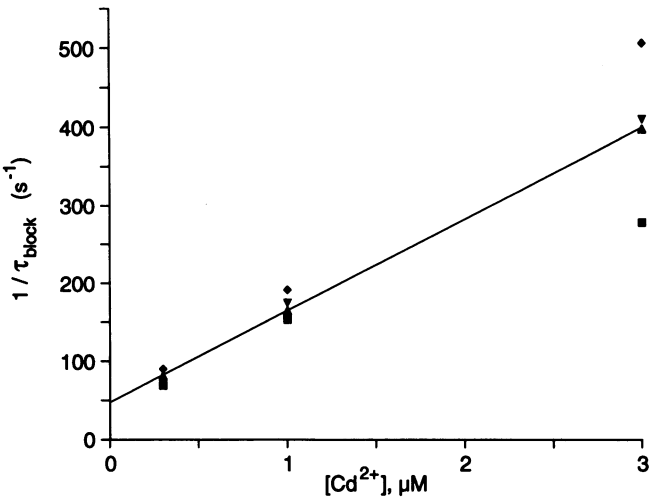

FIGURE 5 Kinetics of open channel block by Cd^{2+}. Reciprocal time constants were averaged over the voltage range 0 to +30 mV and plotted as a function of $[Cd^{2+}]_0$. Each symbol is a different experiment. The straight line was calculated by linear regression.

mated by $1/(k_{+1} + k_{-1})$ and $1/(k_{+2}[Cd^{2+}]_0 + k_{-2})$, so the slower time constant would accurately reflect blocking kinetics.

The postpulse currents were fit to the sum of two exponentials, and the time constants of the slower component are shown in Fig. 4. For postpulses between 0 and +30 mV, the time constants were not detectably voltage dependent, but clearly depended on $[Cd^{2+}]_0$. At −20 mV, the time constants were slower. Qualitatively, this is as predicted by the three-state model (Fig. 4).

For a reversible bimolecular reaction, a plot of $1/\tau_{block}$ vs. $[Cd^{2+}]_0$ should be linear with slope k_{+2} and y-intercept k_{-2}. Using the average of time constants between 0 and +30 mV to approximate τ_{block}, $k_{+2} = (1.2 \pm 0.2) \times 10^8$ M^{-1} s^{-1} and $k_{-2} = 47 \pm 5$ s^{-1} (Fig. 5). This corresponds to a K_D (dissociation constant = k_{-2}/k_{+2}) of 450 ± 120 nM.

Reblocking of closed channels?

At voltages where channels are mostly closed, reblocking was slow (Fig. 6). Following a brief step to +80 mV, the postpulse current remained increased for several seconds (Fig. 7). For 0.3 to 3 μM Cd^{2+}, from −60 to −80 mV, time constants ranged from 0.4 to 5 s in 12 cells tested at one or more Cd^{2+} concentration. The time course of reblocking was not always well fitted by a single exponential. Several minutes were required to measure the time course of reblocking, which could contribute to the variability observed.

Can the slow reblocking be accounted for by direct binding of Cd^{2+} to the closed channel? When reciprocal time constants were plotted, as in Fig. 5, there was a clear concentration dependence at −60 mV, but not at −100 mV (Fig. 8). Dissociation constants, calculated on the assumption of closed channel block, were >1 μM. These values are inconsistent with the strong steady-state block observed in this voltage range.

An alternative possibility is that reblocking is due to binding of Cd^{2+} to the small fraction of channels that might remain open at these negative voltages. The reblocking rate of 1.0 ± 0.3 s^{-1} at −60 mV in 3 μM Cd^{2+} could be fully explained by open channel block (at 1.2×10^8 M^{-1} s^{-1}) if 0.3% of the channels are open at that voltage in the steady-state. The channel open probability cannot be directly measured at such voltages, but the two-state activation model of Jones and Marks (1989) predicts 0.13% of the channels would be open at −60 mV. Thus, open channel block may contribute to the observed reblocking rates, at least at −60 mV.

Rapid application of Cd^{2+}

The slow rate of channel block at negative voltages suggested that the rates of Cd^{2+} binding and unbinding could be observed more directly. With a flow pipe system (Jones, 1991), the 10–90% solution exchange time was typically ~100 ms, measured by the response to a change in Ba^{2+} concentration during a long depolarizing step (Fig. 9 *A*). Cd^{2+} acted equally rapidly when applied during a depolarization, suggesting that the time course was limited by the solution exchange time. However, recovery from Cd^{2+} block was considerably slower (Fig. 9 *B*).

When Cd^{2+} was applied or removed while the cell was held at −80 mV, time-dependent relaxations corresponding to binding or unbinding could be observed during the next depolarization (Fig. 10). Approximately 1 s after application of Cd^{2+}, the current was initially reduced partially, and then decreased further during the first depolarizing step (trace 2 in Fig. 10 *A*). Approxi-

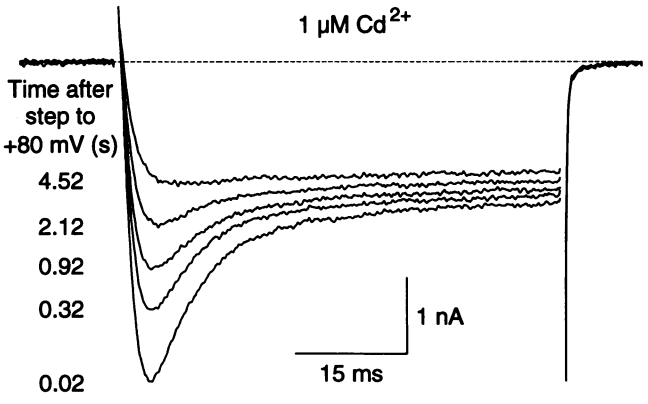

FIGURE 6 Reblocking of calcium channels at −80 mV. Unblocking was induced by a 60 ms depolarizing pulse to +80 mV. Currents shown were evoked by 60-ms pulses to −10 mV, separated from the pulse to +80 mV by hyperpolarizing episodes at −80 mV of the durations indicated. Cell 89n16, filtered at 1 kHz, 0.6 ms blanked.

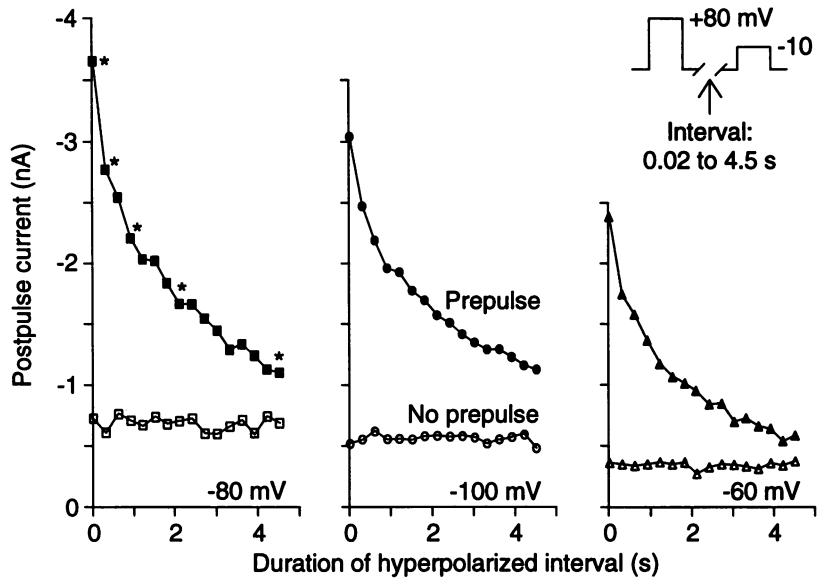

FIGURE 7 Time dependence of reblocking at negative voltages. Postpulse currents were measured at 2.5 to 3.0 ms after the start of each voltage step to −10 mV by the protocol of Fig. 6 (see *inset*), in 1 μM Cd^{2+}. With no prepulse to +80 mV, changes in the holding potential to −60 or −100 mV for up to 4.5 s had little effect (*open symbols*). Currents are plotted as a function of the hyperpolarized interval between the pre- and postpulses. Asterisks indicate the currents illustrated in Fig. 6. The decreasing current magnitude in the three panels resulted from rundown during the timecourse of the protocol.

mately 1 s after removal of Cd^{2+}, the initial rate of channel opening was intermediate between the control and fully blocked levels (trace 6; see also Fig. 10 *B*), and the current returned nearly to control levels by the end of the 50-ms prepulse. These observations also confirm that both blocking and unblocking are slow ($\tau \sim 1$ s) at −80 mV.

The time course of initial Cd^{2+} block (trace 2, prepulse) was similar to that of Cd^{2+} reblocking following the step to +80 mV (traces 2–5, postpulse), confirming the interpretation that the relaxation in the postpulse current is indeed due to block by Cd^{2+}. The time constant of unblocking during the prepulse was 17.9 ± 0.5 ms (n = 6), measured from the slower of two time constants in a fit to the first prepulse current after removal of Cd^{2+} (*e.g.*, trace 6, Fig. 10); that corresponds to an unblocking rate of 56 ± 2 s^{-1}, which agrees reasonably well with the value of 47 ± 5 s^{-1} calculated from the concentration dependence of the time constant for reblocking (Fig. 5).

The initial rate of channel opening during the postpulse is almost as fast in Cd^{2+} as in control (Fig. 10 *C*), which suggests that unblocking can be nearly complete at extreme positive voltages.

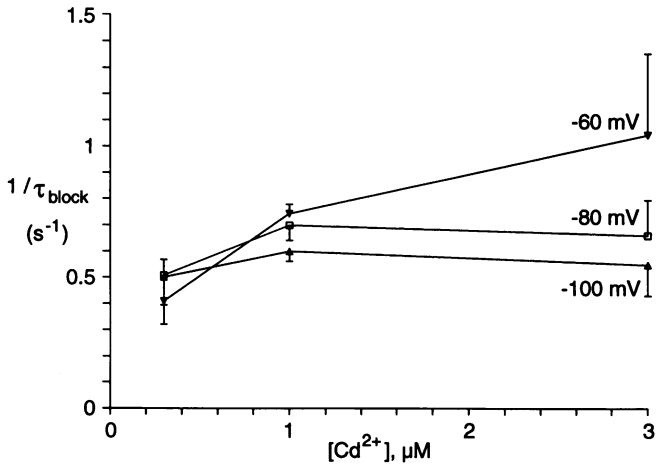

FIGURE 8 Kinetics of block at hyperpolarized voltages. Reciprocal time constants for Cd^{2+} block from the protocol of Fig. 6 (n = 3–6 cells per point) are plotted as a function of $[Cd^{2+}]_0$ at −60, −80, and −100 mV.

Steady-state block

The large difference in blocking and unblocking rates for open and closed channels could produce different levels of steady-state block. This effect can be observed, especially for Cd^{2+} concentrations near the K_D, but it is subtle. At −10 mV, currents in 0.3 μM Cd^{2+} seem to inactivate more slowly than in control, due to a component of the current that appears to activate slowly (Fig. 11). The fraction of current blocked by Cd^{2+} decreases with time, with a time constant (12 ± 2 ms, n = 4) similar to the time constant for Cd^{2+} reblocking (16 ± 4 ms, measured from the protocol of Fig. 3 for the same four cells). This agreement suggests that both processes involve equilibration of Cd^{2+} with the channel. The decrease in block during the step to −10 mV indicates that steady-state block is slightly stronger at −80 mV than at −10 mV.

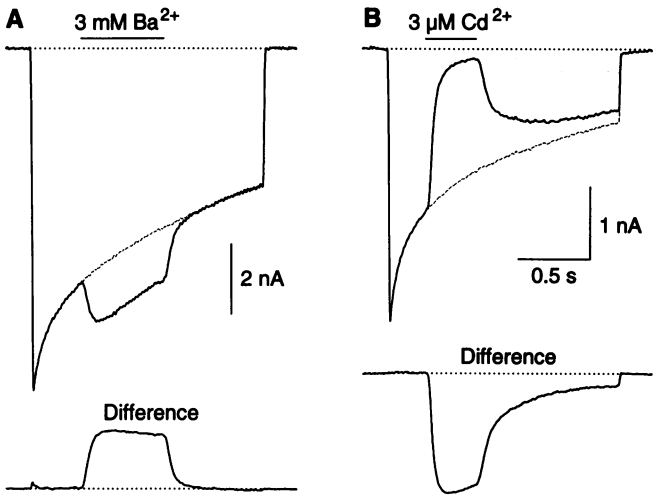

FIGURE 9 Effects of changes in Ba^{2+} and Cd^{2+} on calcium currents, using the flow tube system. Records are for steps to −10 mV, with extracellular solution changes to and from 3 mM Ba^{2+} (*A*) or 3 μM Cd^{2+} (*B*) during the step (*solid lines*). The solid lines above the records indicate the approximate times of the solution changes, which were done manually and thus were not precisely timed. Dashed lines are weighted averages of currents during steps of equal duration recorded before and after, scaled to match the portion of the record before the solution change. This was necessary to compensate for use-dependent inactivation/rundown produced by long depolarizing steps. The difference currents (*below*) illustrate the time course of the effects. The changes in control current amplitude and timecourse between *A* and *B* resulted from rundown over 50 min of recording. Cell e0728, filtered 100 Hz.

DISCUSSION

Open channel block by Cd^{2+}

It is curious that we find strong unblocking at extreme positive potentials, while others found unblocking at extreme negative potentials (Brown et al., 1983; Byerly et al., 1984; Swandulla and Armstrong, 1989; Chow, 1991). However, such behavior is expected from symmetrical barrier models for ion permeation through calcium channels. For example, the model of Almers and McCleskey (1984) predicts that the exit rate for divalent cations from the channel should be the lowest near 0 mV, and should increase with either de- or hyperpolarization.

However, the strong voltage dependence of unblocking above +40 mV (Fig. 2) contrasts with the weak voltage dependence of block at intermediate voltages. The time constant for reblocking was not detectably different between 0 and +30 mV (Fig. 4). Also, the fraction of current blocked by Cd^{2+} was nearly constant for voltage steps between −30 and +30 mV (Jones and Marks, 1989), although close examination of current–voltage curves suggests slightly weaker block at more negative voltages (not shown). The explanation of this discrepancy is not clear. One possibility is that the unblocking is current and not voltage dependent, so that Cd^{2+} ions are "pushed" out of the channel at extreme positive voltages. These experiments were done in the nominal absence of permeant ions in the cell, but small outward currents are usually observed above +50 mV.

We have observed an initial rising phase to tail currents in Cd^{2+} at negative voltages (data not shown), as reported by Swandulla and Armstrong (1989). However, the limited clamp speed in our experiments (~0.1 ms) prevented detailed analysis of that effect. Since tail currents are quite brief, relatively little unblocking would be expected to occur during one tail current, in contrast with the effectively complete unblocking possible at +80 to +120 mV (Figs. 2 and 10 *C*).

The rate for Cd^{2+} exit from open channels found here is considerably lower than previously reported from single channel data (Lansman et al., 1986), as is the equilibrium dissociation constant (Jones and Marks, 1989). It is possible that the N-type calcium channels studied here are more sensitive to Cd^{2+} than are L-type channels, but

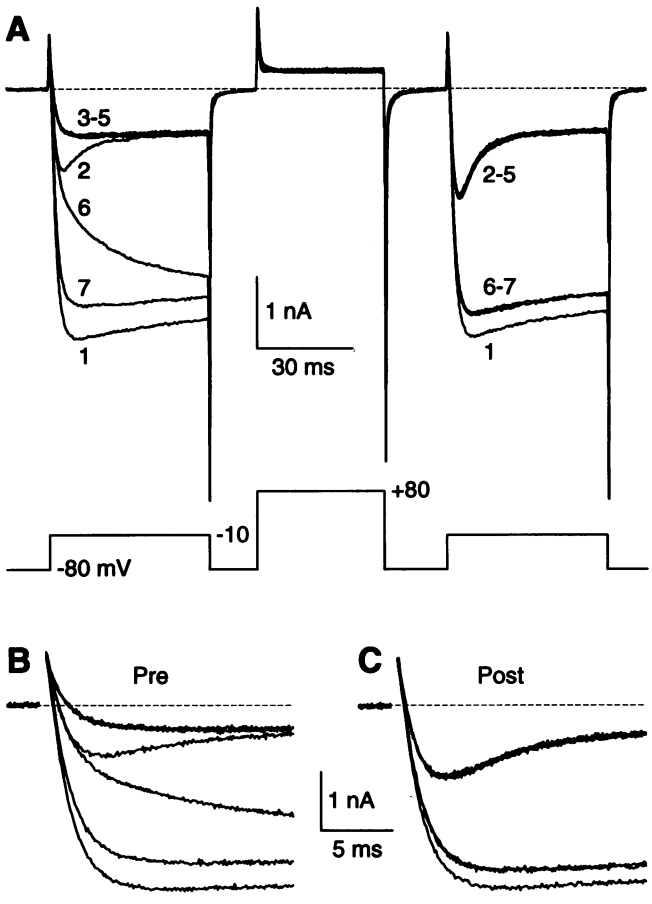

FIGURE 10 Effects of Cd^{2+} applied by the flow tube system at −80 mV. (*A*) The protocol illustrated was given every 2 s, with a change to 3 μM Cd^{2+} after the first record shown, and return to the normal extracellular solution after the fifth record. Expanded views of prepulses (*B*) and postpulses (*C*) are shown below to illustrate the initial rates of channel opening. Cell b0407, filtered 800 Hz (*A*) or 3 kHz with 0.4 ms blanked (*B* and *C*).

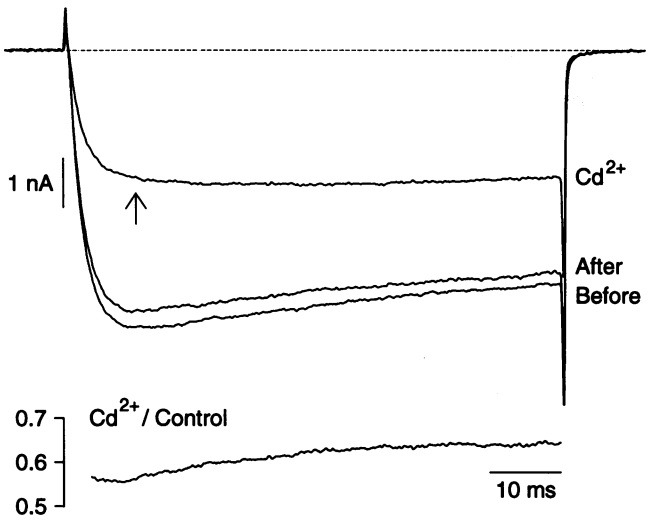

FIGURE 11 Effect of partial blockade by Cd^{2+} on the time course of calcium currents. The main figure illustrates currents upon voltage steps from −80 to −10 mV recorded before, during, and after bath superfusion with 0.3 μM Cd^{2+}. The arrow marks the slow activation kinetics in Cd^{2+}. The record below is the ratio of the current in Cd^{2+} to the control current (average of currents recorded before and after). Cell a0n14, 1 kHz filter.

most if not all of the effect can be attributed to our use of a relatively low concentration (2 mM) of a divalent ion (Ba^{2+}) that binds relatively weakly to sites in the channel (Byerly et al., 1985). The standard models for ion permeation through calcium channels (Hess and Tsien, 1984; Almers and McCleskey, 1984) hold that ion–ion repulsion within the channel speeds the exit rates of bound ions. The relatively high affinity of Cd^{2+} for the channel in our experiments allowed the use of low concentrations, where relaxations due to binding and unbinding are slow enough to be experimentally resolved.

Closed channel block

As noted by Swandulla and Armstrong (1989), Cd^{2+} strongly inhibits the initial rate of channel opening upon depolarization, which is evidence for closed channel block. That conclusion is strengthened by our measurement of channel blocking rates. The current is clearly reduced as early as 2 ms after the start of the depolarization in 0.3 μM Cd^{2+} (Fig. 11), which cannot be explained by open channel block, which has a time constant of ~12 ms in 0.3 μM Cd^{2+} (Fig. 4). It is interesting that closed channel block is strong at −80 mV, where block of the open channel is weak (Swandulla and Armstrong, 1989). In contrast, closed calcium channels of squid neurons are blocked weakly by Cd^{2+} at negative voltages (Chow, 1991).

It appears that Cd^{2+} can enter and exit closed channels, but at rates ~100-fold lower than for open channels (Fig. 7). This conclusion is tentative, as the observed rates could be explained by relatively small numbers of open channels, and there is no direct way to determine the fraction of channels open at voltages negative to −40 mV. However, standard models for voltage dependence of channel gating would predict significantly fewer open channels at −100 mV than at −80, so that block mediated exclusively by binding to open channels should be considerably slower at −100 mV. Furthermore, block of open calcium channels is weak at extreme negative voltages due to rapid unbinding (Swandulla and Armstrong, 1989), so it is unlikely that the strong steady-state block at −80 mV (Fig. 11) results from blocking of open channels. The lack of concentration dependence to the reblocking rates, except possibly at −60 mV, is puzzling, but reblocking rates were difficult to measure accurately at negative voltages. Also, effects of Cd^{2+} on channel gating cannot be ruled out. We emphasize here the qualitative point that reblocking of closed channels is much slower than reblocking of open channels.

The slow blocking rate for closed channels suggests a barrier between the external solution and the Cd^{2+} binding site. The strong block of closed channels at −80 mV, where open channels unblock rapidly (presumably by Cd^{2+} entering the cell), suggests a barrier between the Cd^{2+} binding site and the cytoplasm. That is, calcium channels must be closed at both ends. This implies that the closed channel can contain a Cd^{2+} ion (Swandulla and Armstrong, 1989; Chow, 1991). However, "closed" channels may not be completely impermeable to Cd^{2+}, because slow unblocking can occur at −80 mV (Fig. 10).

If the closed channel is not completely closed to Cd^{2+}, perhaps more permeant cations can also leak through "closed" calcium channels. A small amount of Ca^{2+} leakage through "closed" calcium channels could have physiological consequences for regulation of intracellular Ca^{2+}.

Recovery from Cd^{2+} block is slow during long depolarizing pulses (Fig. 9 *B*). One possible explanation is that Cd^{2+} can also be trapped inside inactivated channels, so that channels must recover from inactivation before Cd^{2+} can exit.

SUMMARY

Cd^{2+} block of calcium channels in frog sympathetic neurons can be rapidly relieved at extreme positive voltages. This observation allowed analysis of the kinetics of Cd^{2+} binding in two voltage regions: 0 to +30 mV, where channels are mostly open; and −60 to −100 mV, where channels are mostly closed. Cd^{2+} binding to the open channel is rapid (10^8 M^{-1} s^{-1}), essentially diffusion limited. The exit rate for Cd^{2+} is ~50 s^{-1}, measured from either the concentration dependence of the time constant of rebinding (Fig. 5), or more directly from unbinding during a depolarization following washout of Cd^{2+} (Fig. 10).

Steady-state block of closed channels is strong, but the rates of Cd^{2+} block and unblock are at least 100-fold

lower for closed channels. This provides additional evidence that the primary site of Cd^{2+} action is within the ion permeation pathway. Conversely, these observations show that closed channels are (mostly) closed to Cd^{2+} ions as well as to more permeant ions such as Ba^{2+}. Since both blocking and unblocking are slow, the closed channel must be closed at both ends.

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