

## Calcium channel block by cadmium in chicken sensory neurons

(tail currents/patch clamp/supercharging/channel gating)

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**ABSTRACT** Cadmium block of calcium channels was studied in chicken dorsal root ganglion cells by a whole-cell patch clamp that provides high time resolution. Barium ion was the current carrier, and the channel type studied had a high threshold of activation and fast deactivation (type FD). Block of these channels by 20  $\mu\text{M}$  external  $\text{Cd}^{2+}$  is voltage dependent.  $\text{Cd}^{2+}$  ions can be cleared from blocked channels by stepping the membrane voltage ( $V_m$ ) to a negative value. Clearing the channels is progressively faster and more complete as  $V_m$  is made more negative. Once cleared of  $\text{Cd}^{2+}$ , the channels conduct transiently on reopening but reequilibrate with  $\text{Cd}^{2+}$  and become blocked within a few milliseconds.  $\text{Cd}^{2+}$  equilibrates much more slowly with closed channels, but at a holding potential of  $-80$  mV virtually all channels are blocked at equilibrium.  $\text{Cd}^{2+}$  does not slow closing of the channels, as would be expected if it were necessary for  $\text{Cd}^{2+}$  to leave the channels before closing occurred. Instead, the data show unambiguously that the channel gate can close when the channel is  $\text{Cd}^{2+}$  occupied.

Calcium channels are found in the membranes of many excitable cells, including nerve, muscle, and endocrine cells, and are essential in the control of cellular processes such as secretion. Calcium channels are voltage gated—i.e., they open and close in response to changes of the membrane voltage ( $V_m$ ), but relatively little is known about their gating mechanism. Analysis of channel block by organic and inorganic cations has yielded much information about the size, shape, and gating mechanism of other ionic channel types, and here we have used cadmium ion as a blocker and a probe for calcium channels and their gates.

It has been noted that cadmium ion block of calcium channels is more intense when the  $V_m$  is positive (1, 2). At the single channel level,  $\text{Cd}^{2+}$  block has been described (3, 4) and the entry and exit of  $\text{Cd}^{2+}$  into single channels can be resolved (3).

We have examined macroscopic calcium currents with a fast patch clamp that employs supercharging (5). This method combined with low-resistance pipettes gives time resolution approaching that of a good squid giant axon clamp, making it possible for us to resolve the time course of  $\text{Cd}^{2+}$  exit from blocked channels. A significant result from our analysis is the discovery that calcium channels can close when occupied by divalent cations. This suggests that occupancy of the channel by a divalent cation is an essential part of the gating mechanism, as is the case for potassium channels. Part of this work has been reported in short form (6). A detailed analysis of  $\text{Cd}^{2+}$  action on squid neurons is in preparation by R. H. Chow, and a short report of his work has appeared (7).

### METHODS

**Cell Culture.** Experiments were performed on primary cultures of chicken dorsal root ganglion cells. Details of the

dissociation and cultivation process were as described elsewhere (8). The cells were used 6–12 hr after plating and were free of any processes. This and their near-spherical shape ensured good control of the membrane potential.

**Solutions.** The composition of the solutions is given in Table 1. Solutions are specified as external/internal in the figure legends. External solutions were sodium and potassium free, and barium ion was the charge carrier in all experiments.

**Recording Conditions.** Coverslips were transferred from 35-mm culture dishes to the recording chamber, which contained  $\approx 0.3$  ml of external recording solution. A flow/suction arrangement continually exchanged the bathing solution. Pipettes were made of Kimax-51 borosilicate capillary tubes (Kimble, Toledo, OH) and had resistances between 0.6 and 1.5 M $\Omega$ .

**Electronics and Data Acquisition.** Details of the patch clamp circuit and its performance are given in refs. 5 and 8. Large voltage spikes of 15  $\mu\text{s}$  duration were added to the leading edge of the command step to charge the membrane potential rapidly. Useful current measurements started 30–40  $\mu\text{s}$  after initiation of the voltage step. An LSI-11/73 computer (DEC, Maynard, MA) was used for data acquisition, storage, and analysis. The sample rate was either 10 or 20  $\mu\text{s}$  per sample point. Currents were corrected by subtracting linear capacitive and current components. For this purpose, 10 current responses to 50-mV hyperpolarizing steps were averaged to give a control trace, which was scaled and subtracted from the test trace. The control trace was updated frequently during the experiment.

### RESULTS

External  $\text{Cd}^{2+}$  in micromolar concentrations is an effective blocker of calcium channels only at positive  $V_m$  values, as illustrated in Fig. 1. The left trace is the control. It shows the sigmoid activation time course of current through calcium channels during a depolarization from  $-80$  to  $+10$  mV (left arrow), and the deactivation of the channels when  $V_m$  is returned to  $-80$  mV (right arrow). The step back to  $-80$  mV causes a large jump in the amplitude of the current because of the increase in driving force, and current magnitude then decreases rapidly as the channels deactivate.

Addition of 20  $\mu\text{M}$   $\text{Cd}^{2+}$  to the external solution almost completely blocks current during the pulse to  $+20$  mV, but there is a very clear tail current after stepping back to  $-80$  mV. Thus, during the pulse it appears that  $\text{Cd}^{2+}$  blocks almost all of the channels, but only  $\approx 60\%$  of them at  $-80$  mV.

A reasonable guess is that  $\text{Cd}^{2+}$  ions rapidly come out of many of the channels on stepping from  $+20$  to  $-80$  mV. By the use of supercharging, we have been able to resolve the time course of  $\text{Cd}^{2+}$  exit at some voltages, as illustrated in

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Abbreviations:  $V_m$ , membrane voltage; NMG, *N*-methyl-D-glucamine. \*Present address: Department of Neurophysiology, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18A, D-8033 Martinsried, Federal Republic of Germany.

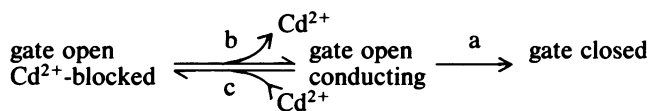
Table 1. Solutions

Solution	Choline chloride, mM	BaCl <sub>2</sub> , mM	MgCl <sub>2</sub> , mM	CsCl, mM	NMG Cl, mM	TEA Cl, mM	MgCl <sub>2</sub> , mM	EGTA, mM
<b>External</b>								
2 mM Ba <sup>2+</sup>	155	2						
5 mM Ba <sup>2+</sup> /10 mM Mg <sup>2+</sup>	135	5	10					
10 mM Ba <sup>2+</sup> /2 mM Mg <sup>2+</sup>	140	10	2					
<b>Internal</b>								
70 mM Cs <sup>+</sup> /50 mM NMG <sup>+</sup> /2 mM Mg <sup>2+</sup>	70	50	20	2	10			
73 mM Cs <sup>+</sup> /50 mM NMG <sup>+</sup>	73	50	20					

The osmolarity of all solutions was adjusted to ≈300 mosM. NMG (Sigma) was titrated with HCl to pH 7.0. EGTA (Sigma) was used as the cesium salt. All solutions were buffered to pH 7.3 with 10 mM Hepes + CsOH. TEA, tetraethylammonium.

Fig. 2. The left traces were taken in the absence of Cd<sup>2+</sup>, with steps back to the voltage indicated in the figure. After ≈30 μs, the current in the control traces declines monotonically. With Cd<sup>2+</sup> in the external medium, the trace at -80 mV is similar to the control, but the other traces show a prominent hook: current magnitude increases, and then decreases with time.

The time course of the tails can be partially explained by the following reaction diagram:



At the end of the pulse to +20 mV, almost all channels are in the gate open Cd<sup>2+</sup>-blocked state, and current is almost zero. When V<sub>m</sub> is stepped to, e.g., -40 mV, current initially increases as Cd<sup>2+</sup> comes out of the blocked channels and then declines as the channel gates close. The traces give a clear impression of the rate of Cd<sup>2+</sup> exit from the channels. At -80 mV, the exodus is too rapid to resolve, while at -20 mV the current continues to increase in magnitude for >0.5 ms.

The traces just described suggest that clearing of the channels is more effective at negative voltage, and this is confirmed by plotting the maximum value reached by the tail current as a function of the potential during the second step (Fig. 3). At the instant the current measurement is made, most of the channels have open gates, and current flow mainly reflects driving force and the degree of Cd<sup>2+</sup> block. In the absence of Cd<sup>2+</sup>, the current plot is approximately linear from -100 to -10 mV, and the data points then begin to approach the zero current axis asymptotically. In the presence of Cd<sup>2+</sup>, there is no tail current until the voltage is

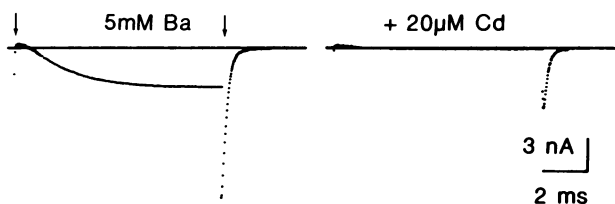


FIG. 1. Cadmium does not effectively block tail current through calcium channels. Currents were activated by pulses to +10 mV (left arrow) with repolarization to -80 mV (right arrow). Left trace, control; right trace, effect of Cd<sup>2+</sup> on pulse and tail current. While pulse currents were almost completely blocked by adding 20 μM Cd<sup>2+</sup> to the external medium, tail current (recorded on repolarization to -80 mV) is still clearly visible. Currents in this and the following experiments were evoked ≈60 s after changing external solutions. Holding potential, -80 mV. External solution, 5 mM Ba<sup>2+</sup>/10 mM Mg<sup>2+</sup>; internal solution, 50 mM Cs<sup>+</sup>/50 mM N-methyl-D-glucamine (NMG<sup>+</sup>)/2 mM Mg<sup>2+</sup>; 20°C.

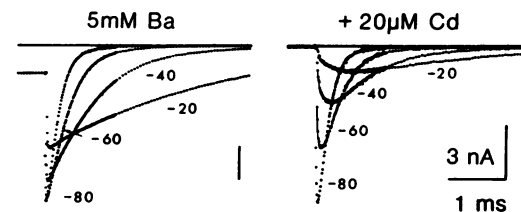
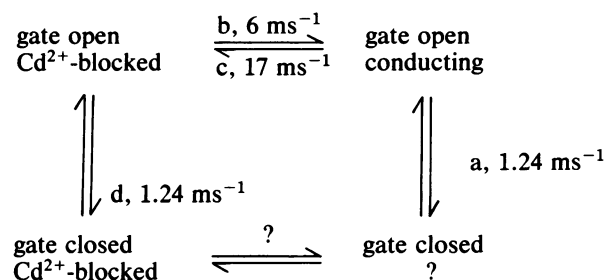


FIG. 2. Cadmium leaves calcium channels at a detectable rate. Tail currents were recorded on repolarization to the potentials indicated following 10-ms activating pulses to +20 mV. Left traces, average of two tail currents, one taken before external Cd<sup>2+</sup> application, and the other after washing it away. Tail currents in Cd<sup>2+</sup> (right traces) show hooks, which were particularly prominent at potentials positive to -80 mV. Note the difference in current scales. Holding potential, -80 mV. External solution, 5 mM Ba<sup>2+</sup>/10 mM Mg<sup>2+</sup>; internal solution, 50 mM Cs<sup>+</sup>/50 mM NMG<sup>+</sup>/2 mM Mg<sup>2+</sup>; 20°C.

negative to -20 mV, and the curve then steepens progressively, approaching the control curve. Thus, it appears that at -20 mV, with 2 mM Ba<sup>2+</sup> as current carrier, almost all of the channels remain blocked, while at -100 mV, 62% of the channels are cleared of Cd<sup>2+</sup> ions before they close.

The reaction diagram shown above assumes that a channel cannot begin to close until the Cd<sup>2+</sup> ion blocking it has come out. If this were the case, closing of the average channel would be delayed until Cd<sup>2+</sup> leaves, and the time course of the tail current would be prolonged. Whether this is indeed the case can be tested by comparing the time course of the tail current with and without Cd<sup>2+</sup> ion, as in Fig. 4A. Traces i and ii in Fig. 4B show the prediction of the reaction scheme without (trace i) and with (trace ii) Cd<sup>2+</sup>. According to this model, the delay in closing prolongs the Cd<sup>2+</sup> tail and causes the Cd<sup>2+</sup> trace to cross above the control. The experimental traces in Fig. 4A never cross, and thus prediction and experiment do not agree.

The findings just described can be accommodated by adding a "gate closed Cd<sup>2+</sup>-blocked" state to the reaction diagram:



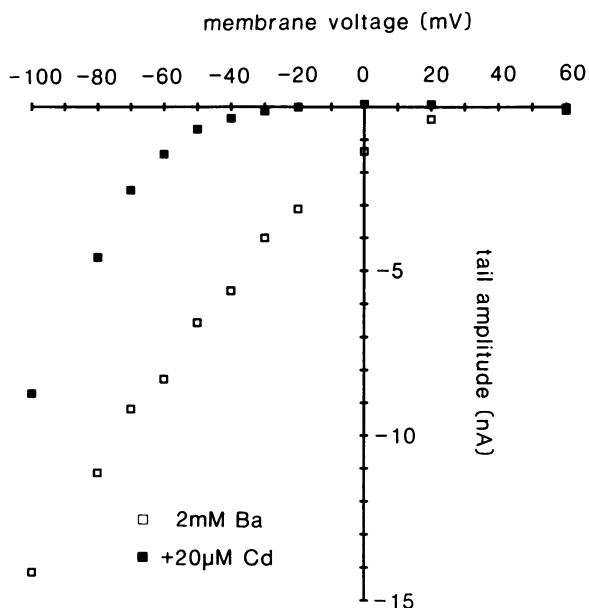


FIG. 3. Tail current as a function of voltage with (■) and without (□)  $\text{Cd}^{2+}$ . The maximum value of tail current was measured at a variety of repolarization potentials following activation of calcium channels by a 10-ms step to +20 mV. Tail amplitudes are plotted as a function of repolarization potential. Holding potential, -80 mV. External solution, 2 mM  $\text{Ba}^{2+}$ ; internal solution, 80 mM  $\text{Cs}^+$ /50 mM  $\text{NMG}^+$ ; 15°C.

The rate constants shown in the diagram were obtained by empirical fitting of the -60-mV traces in Fig. 4A. The predictions of this scheme (trace iii in Fig. 4B) do not cross over the control trace and are in good quantitative agreement with the data.

The findings so far show that  $\text{Cd}^{2+}$  ions come out of many of the channels at negative voltage. It was of interest to see how rapidly they reentered and blocked the channels after being forced out. To examine this point we applied three voltage steps in quick succession. The first step to +20 mV activated the channels. A second step to -130 mV provided a strong field for driving  $\text{Cd}^{2+}$  ions from the channels. The third step to +20 mV provided a measure of the fraction of channels that were not blocked.

Results of the procedure are shown in Fig. 5. Traces i and ii are for single steps to +20 mV without (trace i) and with (trace ii)  $\text{Cd}^{2+}$ . Comparing traces i and ii at the time indicated by the arrow, only 10% of the channels were conducting in 20  $\mu\text{M}$   $\text{Cd}^{2+}$  (trace ii). The block deepens slightly with time at

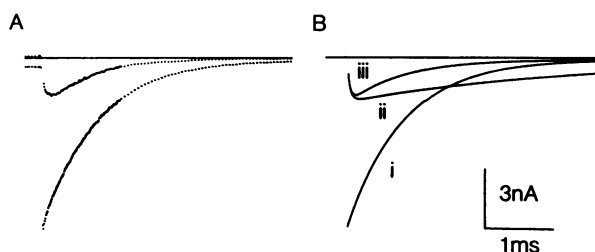


FIG. 4. Cadmium block does not slow calcium-channel closing. (A) Tail currents with (upper trace) and without (lower trace) 20  $\mu\text{M}$   $\text{Cd}^{2+}$  were recorded on repolarization to -60 mV following 10-ms activating pulses to +20 mV. (B) The time course of the tail currents was modelled for  $\text{Cd}^{2+}$ -free (trace i), and  $\text{Cd}^{2+}$ -blocked channels, assuming that  $\text{Cd}^{2+}$  has to leave channels before they can close (trace ii), or that the channels are able to close with  $\text{Cd}^{2+}$  ion inside (trace iii; for details see text). Holding potential, -80 mV. External solution, 2 mM  $\text{Ba}^{2+}$ ; internal solution, 80 mM  $\text{Cs}^+$ /50 mM  $\text{NMG}^+$ ; 15°C.

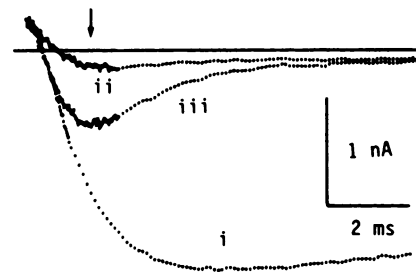


FIG. 5. Kinetics of calcium-channel block by cadmium.  $\text{Ba}^{2+}$  currents through calcium channels were activated by pulses to +20 mV (control, trace i). In 20  $\mu\text{M}$   $\text{Cd}^{2+}$  (trace ii), pulse currents were almost completely blocked, and only 10% of the channels were not blocked at the time indicated by the arrow. Hyperpolarization after a first activating pulse forced  $\text{Cd}^{2+}$  ions out of the channels, temporarily restoring the ability of the channels to carry current. Trace iii shows the current during the second of a pair of activating pulses, which were separated by a 2-ms pulse to -130 mV. In this case, a maximum of 50% of the channels were conducting during the second activating pulse. As  $\text{Cd}^{2+}$  ions reentered and blocked the open channels the current "inactivated" with a time course that was well approximated by a single exponential (fit not shown) with a time constant of 1.6 ms. Holding potential, -80 mV. External solution, 10 mM  $\text{Ba}^{2+}$ /2 mM  $\text{Mg}^{2+}$ ; internal solution, 70 mM  $\text{Cs}^+$ /50 mM  $\text{NMG}^+$ ; 20°C.

+20 mV, and only  $\approx 5\%$  of the channels are conducting at the trace's end. The prepulse to -130 mV cleared enough channels (trace iii) that the current at the arrow is half of the control value (trace i) at the same point in time. The current in trace iii then declines with a time constant of 1.6 ms as  $\text{Cd}^{2+}$  ions reenter and block the conducting channels. At the end of the trace, only  $\approx 6\%$  of the channels are conducting.

These results strongly suggest that, in 20  $\mu\text{M}$   $\text{Cd}^{2+}$ ,  $\approx 90\%$  of the closed channels at -80 mV are  $\text{Cd}^{2+}$  blocked. An obvious next question is whether  $\text{Cd}^{2+}$ -occupied channels gate normally—i.e., do they open at a normal rate, and is the voltage dependence of activation changed by  $\text{Cd}^{2+}$  block? To address these questions, we studied activation of the channels, using tail current amplitude as a measure of the number of channels open at the end of the activating pulse. Fig. 6A shows a plot of maximum tail amplitudes after activating pulses (to 0 mV) for the time given on the abscissa, with and without  $\text{Cd}^{2+}$ . Although the maximum current amplitude is smaller in  $\text{Cd}^{2+}$ , the time course of activation is just the same as in the control case.

To ascertain the effect of  $\text{Cd}^{2+}$  on the voltage dependence of activation, we measured the amplitude of tails after 10-ms activating pulses to a range of voltages (Fig. 6B). Although there is some scatter, the points with and without  $\text{Cd}^{2+}$  are very close to each other, leading to the conclusion that the presence of  $\text{Cd}^{2+}$  in a closed channel does not alter the tendency of a channel to open on depolarization.

## DISCUSSION

Cadmium is used widely as a blocker of calcium channels, but it is clear that its mechanism of action is not as simple as, e.g., the block of sodium channels by tetrodotoxin, which, in effect, simply reduces the number of channels at all voltages. Our results agree with those of previous authors (1, 2), who found that  $\text{Cd}^{2+}$  does not effectively block the channels when the membrane potential is very negative. Other aspects of  $\text{Cd}^{2+}$  block have been examined by Lansman *et al.* (3), who observed "flickery block" in the presence of 20  $\mu\text{M}$   $\text{Cd}^{2+}$ , due to the rapid blocking and unblocking of the channels. They were able to determine the rate constants for block and unblock over the range from +20 to -20 mV. The blocking rate was constant, but the unblocking rate increased sharply

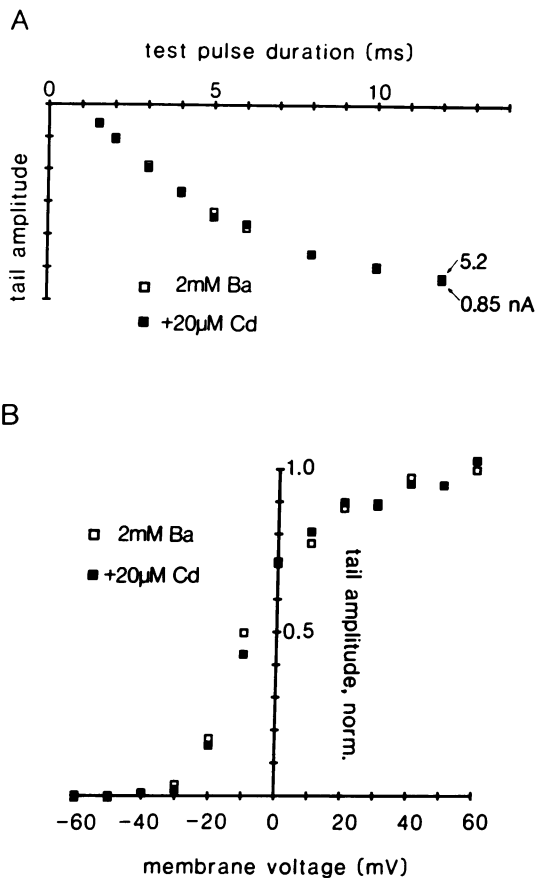


FIG. 6. (A) Cadmium does not alter calcium-channel opening kinetics or voltage dependence. Tail currents with (■) and without (□) 20  $\mu\text{M}$   $\text{Cd}^{2+}$  were recorded at  $-80$  mV following activating pulses to  $+20$  mV of varied duration. Tail current amplitudes, which are a measure of activation during the preceding pulse, were plotted as functions of pulse duration. Amplitudes in  $\text{Cd}^{2+}$  have been scaled up by a factor of 6.12. Maximum tail current amplitudes were as indicated. (B) Voltage dependence of channel activation. Tail currents with (■) and without (□)  $\text{Cd}^{2+}$  were recorded following activation of calcium channels by 10-ms pulses to a variety of potentials. Normalized tail current amplitudes were plotted as a function of the activating potential. Holding potential,  $-80$  mV. External solution, 2 mM  $\text{Ba}^{2+}$ ; internal solution, 50 mM  $\text{Cs}^+$ /50 mM  $\text{NMG}^+$ ;  $15^\circ\text{C}$ .

as  $V_m$  was made negative. Consistent with this, our macroscopic recordings and those of others (1, 2) show that the almost complete block seen at  $+20$  mV (Fig. 1) is progressively less as  $V_m$  is made negative (Fig. 2). In agreement with the single channel data, we find that the main effect of more negative  $V_m$  is to decrease the residence time of a  $\text{Cd}^{2+}$  ion in a channel.

Our results provide an important clue regarding the gating mechanism of calcium channels. There is evidence that  $\text{Ca}^{2+}$  ion or a suitable divalent substitute is an essential factor in the gating of a potassium channel. The first hint of this was the finding that potassium channels can close when occupied by the blocking ion barium (9, 10). This suggested that the channels normally close when occupied by a divalent cation,  $\text{Ca}^{2+}$  being the most likely candidate in physiological conditions. Subsequently, it was shown that external monovalent cations slow the closing of potassium channels by competing with  $\text{Ca}^{2+}$  for occupancy (11). Finally, squid neuron potassium channels have been shown not to close in the absence of added external  $\text{Ca}^{2+}$  (12). The overall picture is that gating results from two factors: conformational changes produced by movement of intrinsic charges within the channel mole-

cule and the binding or unbinding of a  $\text{Ca}^{2+}$  ion in the channel lumen.

Our work here is a step in constructing a similar chain of evidence for calcium channels, in that it shows that calcium channels close when  $\text{Cd}^{2+}$  occupied. It is easily possible to resolve the relatively slow time course of the exit of  $\text{Cd}^{2+}$  ions from the channels (Figs. 2 and 4). If exit of the  $\text{Cd}^{2+}$  ion were a prerequisite for closing, the time course of closing would be prolonged. This is clearly not the case, as shown in Fig. 4, where the closing rate is the same with or without  $\text{Cd}^{2+}$ . A similar conclusion is suggested from single-channel recordings, in that single-channel lifetime was not prolonged by  $\text{Cd}^{2+}$  (3).

From comparing Figs. 1 and 5, it is further clear that  $\text{Cd}^{2+}$  ions occupy most of the closed channels at rest and are present there when an activating pulse is applied. In Fig. 5, the channels were first cleared of  $\text{Cd}^{2+}$  by a hyperpolarizing pulse, and  $\text{Cd}^{2+}$  then equilibrated with and blocked the channels, with a time constant of 1.6 ms. If there is no hyperpolarizing pulse to clear the channels (Fig. 1 Right), no current transient like the one in Fig. 5 is seen, because the channels are already  $\text{Cd}^{2+}$ -blocked when the pulse is applied.

Finally, the gating of the  $\text{Cd}^{2+}$ -blocked channels seems to be normal, in that the channels open with the same time course as in the absence of  $\text{Cd}^{2+}$  and have the same conductance-voltage curve (Fig. 6).

The fact that calcium channels can close when occupied by  $\text{Cd}^{2+}$  suggests strongly that they are occupied by a divalent cation when normally closed. The arguments are similar to the ones presented previously for potassium channels. The conformational energy of a channel with a divalent cation bound inside would certainly be very different from a channel occupied by a monovalent cation or no cation at all. From Fig. 6, it is apparent that the stability of the  $\text{Cd}^{2+}$ -blocked-closed state is about normal, for the opening rates and conductance-voltage curves are not detectably different from normal. Thus, a divalent-occupied-closed channel seems probable, even in the absence of  $\text{Cd}^{2+}$ . In our experimental conditions, the occupying ion, when there is no  $\text{Cd}^{2+}$ , would be  $\text{Ba}^{2+}$ .

The considerations just described imply that closing of the channels should be slowed as the external  $\text{Ca}^{2+}$  concentration is lowered, as is the case for potassium channels in squid neurons (12). There is no direct evidence on this point for calcium channels, but it is known that the selectivity of calcium channels changes when the calcium concentration is reduced below  $1 \mu\text{M}$ . Under these conditions, the channels become permeant to  $\text{Na}^+$  ions (13–16). As judged from the extreme left shift of the conductance-voltage curve in low calcium, the gating properties are also affected, and the channels clearly have an exaggerated tendency to be open at negative voltages. This has been interpreted as a surface charge or surface potential effect (17). An alternative view is that unusually negative voltages are required to close the calcium channels in the virtual absence of  $\text{Ca}^{2+}$ . In this view, channel opening would be driven by both movement of charged helices in the channel molecule (18–20), and, perhaps as the last of several steps, the liberation of a  $\text{Ca}^{2+}$  ion bound in the channel lumen.

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