Cadmium Block of Squid Calcium Currents

Macroscopic Data and a Kinetic Model

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ABSTRACT The mechanism of Cd$^{2+}$ block of Ca$^{2+}$ currents ($I_{ca}$) was explored in squid neurons using whole-cell patch clamp. Control currents activated sigmoidally, more rapidly at more positive potentials, and did not inactivate significantly. External Cd$^{2+}$ up to 250 μM reduced $I_{ca}$ reversibly. For small depolarizations, the current for a step of 10 ms increased to a maintained value, resembling the control; but for $V_m > 0$ mV, the increase was followed by a decrease, as Cd$^{2+}$ block became greater. Final block was greater for larger depolarizations. At 0 mV the half-blocking concentration was 125 μM. Tail currents, measured as channels close, had an initial "hook" when recorded in Cd$^{2+}$: currents increased transiently, then decreased. This suggests that Cd$^{2+}$ escapes from some channels, which then conduct briefly before closing. Analysis of tail currents shows that Cd$^{2+}$ does not slow channel closing. The data can be explained if Cd$^{2+}$ is a permeant blocker of Ca$^{2+}$ channels and if channels can close when occupied by Cd$^{2+}$. Cd$^{2+}$ permeates the channels, but binds transiently to a site in the pore, obstructing the passage of other ions (e.g., Ca$^{2+}$). Dwell time depends on the transmembrane potential, becoming shorter for more negative internal potentials. A five-state model was used to simulate the steady-state and kinetic features. It combines a Hodgkin-Huxley type m$^2$ gating scheme and a one-site Woodhull ionic blockage model for a permeant blocker and includes a closed blocked state. To fit the data, the binding site for Cd$^{2+}$ had to be near the outer end of the pore, with a well depth of $-12.2 \ RT$, and with a barrier at each end of the pore. The model predicts that the Cd$^{2+}$ entry rate is nearly voltage independent, but the exit rate is steeply voltage dependent (e-fold/17 mV). Analysis further suggests that the channel closes at a normal rate with Cd$^{2+}$ in the pore.

INTRODUCTION

Cadmium and many other inorganic cations traditionally considered to be blockers of calcium channels may actually be permeant, albeit sparingly (macroscopic current data: Fakuda and Kawa, 1977; Kawa, 1979; Anderson, 1979, 1983; Brown et al.,

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Recent macroscopic current analysis has yielded another interesting finding: calcium tail currents recorded with Cd$^{2+}$ present have “hooks” which are absent in the controls, and the tail currents with Cd$^{2+}$ are not significantly prolonged (Chow, 1988; Swandulla and Armstrong, 1989). As will be discussed, these findings suggest that not only does cadmium traverse the channel, but the channel can close when occupied by cadmium.

This paper presents macroscopic current data and a kinetic model of the mechanism of cadmium block of squid calcium channels. The principle hypotheses are that cadmium is a permeant blocker and that the channel can close when occupied by a cadmium ion. There is a site in the permeation pathway to which cadmium binds tightly (more tightly than does calcium), resulting in obstruction to the flux of other ions through the channel. Although it binds tightly, it can be expelled from the channel by a sufficiently large electrical driving force.

Parts of this work have been presented previously in abstract and dissertation form (Chow, 1988; Chow and Armstrong, 1988).

MATERIALS AND METHODS

Culture

Two types of squid were used for the studies reported here. In Woods Hole, MA, *Loligo pealei* were supplied by the Marine Biological Laboratory. In Philadelphia, *Loliguncula brevis* were shipped by air freight from the Marine Biomedical Institute in Galveston, TX. The method of preparation of dispersed giant fiber lobe (GFL) neurons was identical for the two species, and has been described previously (Llano and Bookrnan, 1986; Parsons and Chow, 1989). The only difference in handling the cultured cells from these two species was in the temperature of incubation: 15°C for *Loligo* and 21°C (room temperature) for *Loliguncula*.

Electronics

The patch clamp amplifier for whole cell recording incorporated most of the standard features (Hamill et al., 1981), including series resistance compensation. Using a low feedback resistor (10 MΩ) for the headstage current-to-voltage converter permitted faster time resolution at the expense of a noncritical increase in noise. To ensure rapid and accurate clamp of membrane potential, the “supercharger” modification was used (Armstrong and Chow, 1987). This method involves the addition of a 15-μs spike on the leading edge of the command potential; the spike speeds the injection of current through the series resistance to charge the membrane capacitance. The combination of these methods and the use of low resistance electrodes (see below) typically permitted measurements of current to be made starting 30–50 μs after a voltage clamp step.

Data Acquisition

Data were sampled at 10, 20, or 50 μs per point and stored in digital form on computer floppy disks by a PDP-11/73 computer (Digital Equipment Corp., Marlboro, MA). Correction for linear capacity transient and leak currents was accomplished by subtracting from experimental traces a scaled representation of 10 averaged hyperpolarizing current sweeps.
Pipettes and Solutions

Pipettes were made of borosilicate glass (Kimax-51; Kimble Glass Inc., Vineland, NJ) and had resistances of 0.30–0.80 MΩ (~3 μm tip diameter).

The compositions of internal and external solutions are given in Table I. The internal solutions contained 2 mM MgATP (A-0770; Sigma Chemical Co., St. Louis, MO) to slow the rate of calcium current rundown (Kostyuk et al., 1981, 1986; Byerly and Yazejian, 1986; Cota, 1986).

Recording Conditions

All experiments were performed within 1–5 d of culture preparation, using cells without processes, 25–35 μm in diameter. Cells attached to glass coverslip fragments were transferred to a 0.3-ml chamber mounted on a Nikon Diaphot microscope stage. The chamber was perfused continuously, with the inflowing solution selected by a manual valve and the outflow controlled by a suction pipette. This system permitted complete bath exchange within 30 s. A feedback-regulated Peltier device was used to cool the chamber and precool all inflowing solutions to 15.0 ± 0.1°C.

The initial seal was formed in standard artificial seawater (ASW), after which the external solution was changed to one containing 50 mM CaCl₂. Using this procedure, it was possible to routinely obtain seals of 1–5 GΩ. No records were taken for at least 15 min after breaking into the cell.

The first set of records was always taken in control solution. After this, test cadmium solutions were alternated with control solution (bracketing) to permit assessment of the reversibility of cadmium effects. The effects were reliably reversible for short exposures (<10 min), except with concentrations >250 μM. Records were taken starting ~90 s after switching to a new solution. When analysis revealed that a pair of bracketing controls were significantly different

| TABLE 1 |
| Recording Solutions |

| External* | NaCl | Choline-Cl | KCl | CaCl₂ | MgCl₂ |
|-----------|------|------------|-----|-------|-------|
| STD nASW  | 440  | 0          | 5   | 50    | 10    |
| 50 Ca²⁺ choline | 0 | 500 | 0 | 50 | 0 |
| 50 Ca²⁺ 440 Na⁺ | 440 | 0 | 0 | 50 | 0 |

*Concentrations in millimolar. All external solutions contained 200–500 nM TTX and 10 mM HEPES, titrated to a final pH of 7.3 with NaOH or CsOH. Cadmium chloride was added directly to external solution from a 100 mM stock solution. The final osmolarity of external solutions was 1,010–1,030 mosM.

| Internal† | CsCl | NMG-glutamate | Cs·F | EGTA | HEPES |
|-----------|------|---------------|------|------|-------|
| NMG-Cs⁺4 | 125  | 350           | 25   | 10   | 10    |
| NMG-Cs⁺5 | 125  | 350           | 25   | 2    | 10    |

†Internal solutions contained 2 mM MgATP unless specified otherwise. The pH of all internal solutions was 7.3, titrated with CsOH. The internal osmolarity was 20–50 mosM less than the external.
(> 10% difference for any given voltage), the data were discarded except in special instances. The exceptions to this rule were the few experiments in which it was necessary to use very high (irreversible) cadmium concentrations.

**Computer Modeling**

The kinetic model used to fit the data is given in Fig. 10A, and is considered at length in the Discussion. The scheme can be described by five simultaneous first order linear differential equations of the general form

\[
\frac{dx}{dt} = ax + b
\]

Rates of channel opening and closing for the calcium currents in the absence of cadmium were obtained empirically by standard methods (Hodgkin and Huxley, 1952).

The rates of cadmium entry and exit into the channel are voltage dependent, and were assumed to be exponentially related to energy barrier heights, as is usual in Eyring models of ion movements in channels (Woodhull, 1973; Hille, 1984). Fig. 10B shows the single site, two barrier model that was used. An example of a rate constant determined from the model is

\[
f_1 = f_1(0) \exp \left( - \frac{zF}{RT} \delta_D \frac{D_1 V_m}{a_1} \right)
\]

where \(f_1(0)\) is the zero-voltage rate constant, the rate measured when the transmembrane potential is 0.

Finding the rates for cadmium entry and exit was done in two steps. For steady-state fitting, the equation used was

\[
\frac{I_{CAD}}{I_{CONTROL}} = \frac{K_{CAD}}{K_{CAD} + [Cd^{2+}]_o K_{CHANNEL}}
\]

This equation was obtained by equating the five derivatives to zero and solving simultaneously for current magnitude in the presence (\(I_{CAD}\)) and absence (\(I_{CONTROL}\)) of \(Cd^{2+}\). \([Cd^{2+}]_o\) is the external cadmium concentration. \(K_{CAD}\) is a function of the rates of cadmium entry and exit from the channel:

\[
K_{CAD} = \frac{r_1}{f_1} + \frac{f_2}{f_1}
\]

\(K_{CHANNEL}\) is a term composed of equilibrium constants among the channel states:

\[
K_{CHANNEL} = \frac{K_{OB} + 1}{(1 + KC1 + KC1*KC2)*K_{OB}}
\]

where

\[
KC1 = b1/a1, \quad KC2 = b2/a2, \quad \text{and} \quad KOB = rb1/fb1 = a1/b1.
\]

The parameters of the fit were \(\delta\), the location of the blocking site; \(D2\), the location of the second barrier; and \(r1(0)/f1(0)\) and \(f2(0)/f1(0)\), the ratios of the zero-voltage rate constants. \(D1\) was fixed at 0.5.

For kinetic fits of calcium currents with cadmium present, the five simultaneous differential equations were solved numerically, with the locations of the cadmium binding site and the energy barriers given by the preceding steady-state fits. The resulting simulated current trace was superimposed on an experimental record. The zero-voltage rate constants were adjusted to
RESULTS

Calcium Currents in GFL Neurons

Squid GFL calcium currents activate sigmoidally and rise to near steady state within 5 ms at -20 mV and within 1 ms at +40 mV, as can be seen in the control traces in Fig. 1A. The currents become detectable near -35 mV (in 50 mM Ca\(^{2+}\) and 0 mM Mg\(^{2+}\), and the I-V relation peaks near 0 mV (see the control I-V curve in Fig. 2A). The currents inactivate minimally or not at all even for large depolarizations that are several hundreds of milliseconds long (e.g., less than or equal to a 10% decrement for a 300-ms step to 0 mV in five cells). As illustrated in Fig. 3A, deactivation shows evidence of two components, fast and slow. The latter component is very small and will be ignored in the analysis.

Cd\(^{2+}\) Block of Calcium Pulse Currents

External cadmium alters the kinetics of the "pulse currents," i.e., the current during an activating pulse (Fig. 1A). In Cd\(^{2+}\), for small depolarizations the records have nearly the same time course as the controls, while at large positive potentials the currents increase and then decay (see, for example, the traces for depolarizations to +20 or +40 mV in Fig. 1A).

The calcium currents with cadmium are reduced more severely the larger the...
depolarization (up to +60 mV). For example, at -20 mV the fraction of current blocked at 5 ms is ~20%, whereas at +40 mV the fraction is ~88% (Fig. 1 A). These findings are surprising in that one might expect an externally applied divalent cation blocker to be driven out of the channels at more positive potentials, resulting in less block.

A plot of the I-V curves in the presence and absence of cadmium is illustrated in Fig. 2 A. The I-V curves superimpose in the voltage range of activation of the channels; however, as the membrane potential is made more positive, cadmium block becomes more severe.

Fig. 2 B shows the ratio of steady-state current with cadmium present, I_{CAD}, divided by the steady-state control current, I_{CONTROL}, plotted as a function of the clamp potential. As expected from the initial survey of the data, this ratio decreases as voltage is made more positive and more channels become blocked.
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Cd²⁺ Block as Channels Are Deactivating

Fig. 3 A shows superimposed tail currents in the presence and absence of Cd²⁺. Tail currents are recorded when the membrane potential is repolarized after a depolarizing voltage clamp step which opens the channels. At the instant of repolarization, the driving force for calcium current is suddenly increased, leading to a jump in the current magnitude, and the current then diminishes as the channels close.

Tail records begin 50 μs after the voltage transition, the earliest time (for this cell) at which the capacity transient has settled and the data are considered valid. In the presence of Cd²⁺, the tails have an initial hook which is absent in the controls; i.e., they increase transiently in amplitude before decreasing. Hooked tail currents have been described with pore-blocking ions and drugs (see, for example, quaternary ammonium derivative block of K channels in Armstrong [1971], pancuronium block of Na channels in Yeh and Narahashi [1977], N-methyl-strychnine block of Na channels in Cahalan and Almers [1979], and cadmium and calcium channels in Swandulla and Armstrong [1989]), and are believed to be due to the clearing out of blockers from the channels that briefly conduct before they close. The hook is present at all repolarizing voltages illustrated in Fig. 3 A.

An important feature is that the tails in the presence of Cd²⁺ do not cross over the control tails. The late time courses in both are nearly identical. This observation indicates that cadmium does not significantly slow channel closing. As discussed below, this is evidence that the channels close at normal rates with cadmium inside the pore.

![Figure 3](image-url)
Cadmium Effect: Not Merely a Scaling Down of Calcium Current Amplitude

To further illustrate the properties of the block, Fig. 4 shows the currents in response to a multistep voltage clamp protocol. The potential is jumped from −80 to 0 mV for 10 ms, to +30 mV for 10 ms, and then back to 0 mV. The top trace is the control...
As cadmium concentration was increased, the initial and peak tail currents were reduced. Tail currents were scaled to compensate for rundown. Scaling factors were the same as in Fig. 5. Tail currents displayed start 50 ms after the step transition. 15°C. Solutions: 50 Ca$^{2+}$ 440 Na$^+$ (200 nM TTX) // NMG-Cs-4 with 2 mM MgATP. Cell 7SE14A.N1. (B) Model of simulation of tail currents at -40 mV after a 10-ms step to +20 mV, in 0, 31, 62, and 125 μM Cd$^{2+}$. Initial tail current point in the simulation is the point 20 μs after the simulated voltage transition. Modeling parameters are in Table II.

record. At 0 mV the current activates sigmoidally. On stepping to +30 mV, it drops instantaneously due to the decreased driving force, then it increases slightly as additional channels open. The third step to 0 mV leads to a sudden increase in amplitude, and then a quick relaxation to a smaller value as some of the channels activated at +30 mV close.

The behavior of the current in cadmium is quite different (Fig. 4 B). The current approaches a final level more quickly than in the control, but this final level is
smaller. At +30 mV, the current increases slightly, as in the control, but instead of reaching a steady-state level, it decays to a smaller level over 5 ms. After the third step (to 0) the time course of the current is quite unlike that in the control. The initial jump is smaller, showing that fewer pores are conducting. Then, instead of decreasing in amplitude, the current increases as Cd\(^{2+}\) comes out of some of the channels.

**Figure 8.** Relation of pulse current block to tail current block. The two experiments in this figure were performed on *Loliguncula brevis*. No attempt at quantitation was made on the calcium currents of this species; however, at least qualitatively the currents are very similar to those of *Loligo pealei*. (A) Tail currents show progressively more block when recorded at different times as the pulse current activates and then decays. At the top of the figure the pulse currents in the presence and absence of 62.5 \(\mu\)M Cd\(^{2+}\) for a step to +30 mV are plotted together. Below, tail current records are illustrated with the initial tail current plotted directly beneath the appropriate time point in the pulse current. The time relative to the initiation of the pulse is indicated in milliseconds. HP = -80 mV. 15°C. 50 Ca\(^{2+}\) 440 Na\(^{+}\) (200 nM TTX) // NMG-Cs-4, 2 mM MgATP. Cell: 7N017C.N1. Calibration for pulse current: 5.5 nA, 2.6 ms; for tail currents, 16 nA, 5.2 ms. (B) Increased block of pulse currents at large depolarizations is correlated with more block of tail currents. The membrane potential was stepped to the voltage indicated below the traces for 5 ms, during which the pulse currents activated and (if Cd\(^{2+}\) was present) became blocked. Only the tail currents are illustrated here, for repolarizations to -40 mV. For each pulse potential, there are two tail records superimposed, one in the presence and one in the absence of Cd\(^{2+}\) (62.5 \(\mu\)M). The control records are normalized to the same initial amplitude to emphasize the effect of the pulse potential on block of tails in Cd\(^{2+}\) relative to the tails in the absence of Cd\(^{2+}\). HP = -80 mV. 15°C. 50 Ca\(^{2+}\) 440 Na\(^{+}\) (200 nM TTX) // NMG-Cs-4, 2 mM MgATP. Cell: 7N016A.N1.

The traces in the presence and absence of cadmium have been superimposed in Fig. 4 C, with the pulse current amplitudes normalized for 0 mV. If the effect of cadmium were merely to scale down the current amplitude, then one would expect the records to superimpose even when the voltage is changed. This is obviously not the case.
**Effect of Changing Cadmium Concentration on Blocking Kinetics and Steady-State Current Reduction**

Fig. 5 A shows the effect of changing cadmium concentrations on calcium pulse currents. Note that as the cadmium concentration is increased, the block is faster, and the steady-state current is smaller.

Tail currents also change as cadmium concentration increases, as illustrated in Fig. 6 A: a hook appears and then becomes more pronounced. The initial tail current and the peak of the tail current are progressively reduced.

Fig. 7 is a Cd²⁺ dose–response curve measured at 0 mV. The ordinate is the ratio, at steady state, of current in the presence of Cd²⁺ divided by the control current. The steady-state current diminishes, asymptotically approaching zero at higher Cd²⁺ concentration. The half-block concentration at 0 mV is ~125 μM in 50 mM Ca²⁺. The continuous line through the points was obtained from the model (Eq. 1, Materials and Methods).

**Relation of Pulse Current Block to Tail Current Block**

Fig. 8 shows that more severe block of pulse currents is correlated with a more pronounced hook in the tail currents. This is seen whether the block is deepened by progressively longer pulses (Fig. 8 A) or by progressively larger depolarizations (Fig. 8 B).

**Instantaneous I-V Curve**

Fig. 9 shows the open channel I-V curve for the calcium current in 0–250 μM external cadmium. The voltage was stepped from −80 to +20 mV for 10 ms to open the channels. Then the voltage was changed to a test potential and the current was measured as soon as possible. In this cell, the 50-μs point is plotted versus the test potential. The instantaneous I-V relations are curvilinear and concave downward in all cases; i.e., current flows more easily at negative potentials. For the control, the relation is nearly linear below 0 mV, whereas with Cd²⁺ the curves continue to bend.
downward at more negative potentials, gradually approaching the control. While there is pronounced block at the positive potentials, there is proportionately less block at negative potentials.

**DISCUSSION**

**Kinetic Model**

The kinetic model for Cd²⁺ block developed here is based on the idea that Cd²⁺ is a "permeant blocker." The assumption that Cd²⁺ is permeant was not tested directly, but is in accord with data and models presented by numerous others (see references in the Introduction); and, as will be seen below, when the model incorporates this feature, the data are fit reasonably well. Channel kinetics were simulated using a Hodgkin-Huxley type m² gating scheme (Hodgkin and Huxley, 1952), while block of open channels was simulated using a Woodhull-type ionic blockage model (Woodhull, 1973).

The state diagram is given in Fig. 10 A. C2 and C1 represent closed states of a channel with two or one of its hypothetical Hodgkin-Huxley gating particles in the closed position (Fitzhugh, 1965). O represents the conducting channel that has an open activation gate. A cadmium ion from the outside (Cdₒ) enters the conducting channel (rate constant f₁) and binds to a site in the pore to give an open blocked channel (OB). It then exits either back the way it came (rate constant r₁) or forward through the channel (rate constant f₂), to yield an open channel (O) and an internal cadmium ion (Cdᵢ). Open blocked channels can close with cadmium still in the pore, yielding a closed blocked state (CB; further discussion below). Because there was

**Figure 10.** (A) Kinetic scheme. The kinetic scheme combines features of Hodgkin-Huxley type m² gating and Woodhull-type ionic blockage schemes. Details of the model are described in the text. (B) Energy profile for ion permeation of a hypothetical one-site channel. Uᵢ's are the energy barrier heights, and fᵢ's and rᵢ's are forward and reverse rate constants. δ is the electrical distance to the binding site defined from outside to inside, and D₁ and D₂ give the location of the energy barrier peak as a fraction of the electrical distance between two energy wells, from outside to inside. For the present model, D₁ was set equal to 0.5 (symmetrical barrier assumption). D₂ was a free parameter.
internal EGTA, the concentration of internal free cadmium was assumed to be negligible (EGTA affinity for Cd$^{2+}$ is nearly six orders of magnitude greater than for Ca$^{2+}$), and rate $r_2$ was set to zero. The energy barrier diagram illustrating the components used to define the Cd$^{2+}$ entry and exit rates is shown in Fig. 10 B. An example of derivation of one of the rates was given in Material and Methods.

The steps used to obtain the parameters of the model were outlined in Materials and Methods. Simulations of the time course of control currents provided estimates of the Hodgkin-Huxley rates $\alpha$ and $\beta$, some of which are summarized in Table II. Examples of such simulations can be seen in Fig. 1 B (pulse currents) and 3 B (tail currents).

Predictions of steady-state block vs. voltage (Eq. 1, Materials and Methods) and the effect of varying the model parameters on the predictions are illustrated in Fig. 11, A–C. In each, the ordinate is $I_{CAD}/I_{CONTROL}$ determined as described in Fig. 2 B, and the abscissa is membrane potential. The experimental finding that block is 50% at 0 mV for 125 $\mu$M Cd$^{2+}$ was used as a constraint in the modeling. As seen in Fig. 11 A where $\delta$ is varied, the direction of the voltage dependence can reverse depending on the location of the binding site for cadmium. Changing the location of the inner barrier (parameter D2) affects the maximal degree of block and the steepness of the voltage dependence of block (Fig. 11 B). R1 and R2 represent defined ratios of the rate constants at 0 mV and are related to the depth of the energy well, as well as the relative height of the barriers. As demonstrated in Fig. 11 C, the particular pair of values for R1 and R2 chosen also affects the steepness of the voltage dependence and the maximal degree of block.

Simultaneous fits for five cadmium concentrations in the same cell are illustrated in Fig. 12. To obtain the correct direction and steepness for the voltage dependence of block, the blocking site had to be located near the outer end of the pore ($\delta = 0.1$) and the inner barrier near the inner end (D2 = 0.85). In addition, the blocking site had an energy well depth of about $-29$ kJ/mol or $-12.2$ RT.

The next step involved adjusting the values of the rate constants at 0 mV to give the best fits of kinetic simulations, using the information gained from the steady-state

| $V_m$ | $a1^*$ | $b_1$ | $b_2$ | $f_1^*$ | $r_1$ | $f_2$ |
|-------|--------|-------|-------|--------|-------|-------|
| mV    | ms$^{-1}$ | ms$^{-1}$ | ms$^{-1}$ | M$^{-1}$·ms$^{-1}$ | ms$^{-1}$ | ms$^{-1}$ |
| -80   | 0.000  | 15.037 | 0.000  | 7.512  | 12650.0 | 0.033  | 135.501 |
| -60   | 0.000  | 9.852  | 0.000  | 4.926  | 11670.0 | 0.036  | 40.673  |
| -40   | 0.000  | 5.540  | 0.000  | 2.770  | 10770.0 | 0.039  | 12.209  |
| -20   | 0.316  | 1.519  | 0.632  | 0.660  | 9936.0  | 0.042  | 3.665   |
| 0     | 0.950  | 0.459  | 1.860  | 0.220  | 9167.0  | 0.046  | 1.100   |
| +20   | 2.010  | 0.199  | 4.020  | 0.100  | 8458.0  | 0.050  | 0.530   |
| +30   | 2.659  | 0.162  | 5.318  | 0.081  | 8124.0  | 0.052  | 0.181   |
| +40   | 3.451  | 0.090  | 6.903  | 0.045  | 7803.0  | 0.054  | 0.099   |

*To simulate $m^1$ kinetics, $a_2 = 2 \cdot a_1$ and $b_1 = 2 \cdot b_2$.

For all cadmium rate constants, the model parameters were $\delta = 0.10$, $D2 = 0.83$, $f_1(0) = 9,167$ M$^{-1}$·ms$^{-1}$, $r_1(0) = 0.0458$ ms$^{-1}$, $f_2(0) = 1.10$ ms$^{-1}$, where $f_1(0)$, $r_1(0)$, and $f_2(0)$ are the zero-voltage rate constants.
fitting. The final model predicts with fair accuracy the major features of the data for a wide range of voltages and Cd\(^{2+}\) concentrations. Figs. 1B and 3B show examples of simulations of the Cd\(^{2+}\) block. For calcium pulse currents, the deepening of block at more positive potentials is well reproduced, as is the appearance of a decaying time course at positive potentials. Increasing the Cd\(^{2+}\) concentration in the simulations speeds the decay of pulse currents and leads to more severe reduction (Fig. 5B). The dose–response curve is predicted accurately, as seen in Fig. 7. Simulated tail currents in Cd\(^{2+}\) have a hook, and they do not cross over the control currents (Fig. 3B). Increasing cadmium concentration decreases the initial and peak tail currents (Fig. 6).

**Figure 11.** Effect of choices of model parameters on predictions of how \(I_{\text{CAD}}/I_{\text{CONTROL}}\) depends on the voltage. (A) Effect of varying \(\delta\). (B) Effect of varying \(D_2\). (C) Effect of varying \(r_1(0)/f_1(0)\) and \(f_2(0)/f_1(0)\) (called R1 and R2, respectively, in the figure). R1 and R2 are denoted in the figure as multiples of \(10^{-6}\). The sum of R1 and R2 was held constant \((125 \times 10^{-6})\) to give a predicted half block concentration of \(\approx 125 \mu\text{M}\). In each panel, the selected parameter was varied while all others were held constant. The constant values were \(\delta = 0.1\), \(D_2 = 0.83\), \(r_1(0)/f_1(0) = 5 \times 10^{-6}\), \(f_2(0)/f_1(0) = 120 \times 10^{-6}\). These were the best-fit values for the squid neuron calcium channel. The values for channel gating appear in Table II.
To reproduce the near-identical late time course of tail currents with and without cadmium and the lack of crossover of the tail currents, it was essential to include a CB state in the model; that is, channels can close when occupied by Cd$^{2+}$. A significant point is that the rates of channel opening and closing with Cd$^{2+}$ were found empirically to be the same as those of the control currents. Examples of simulations with and without the CB state are illustrated in Fig. 13. Without the CB state, the tail current with Cd$^{2+}$ crosses over the control trace.

Fig. 14 shows the estimates of the rate constants for block and unblock as a function of voltage. The rate of block does not change very much with voltage. The rate of unblock, however, increases $e$-fold per $-16.6$ mV.

![Figure 12](image-url)

**Figure 12.** Steady-state voltage dependence of cadmium block at five cadmium concentrations. Currents were recorded in the presence and absence of cadmium at five concentrations. Test cadmium solutions were bracketed by control solution in all cases. Calculation of the fraction of current not blocked was performed as described in Fig. 2 B, and the values were plotted as a function of voltage. Open circles, 15 µM Cd$^{2+}$; closed circles, 31 µM; open triangles, 62 µM; closed triangles, 125 µM; open squares, 250 µM. HP = -80 mV. 15°C. 50 Ca$^{2+}$ 440 Na$^+$ (200 mM TTX) // NMG-Cs-4, 2 mM MgATP. Cell: 7SE08A.N1. Predictions of the model are superimposed (continuous line) on the data points. The Cd$^{2+}$-related model values were $\delta = 0.1$, $D_2 = 0.83$, $r_1(0)/f_1(0) = 5 \times 10^{-5}$, $f_2(0)/f_1(0) = 120 \times 10^{-5}$. The channel rates appear in Table II.

How does the model qualitatively explain the features of cadmium block? Cd$^{2+}$ permeates Ca$^{2+}$ channels, but binds transiently to a site in the pore, obstructing the passage of Ca$^{2+}$ ions. At negative internal potentials, cadmium resides only very briefly at the binding site, exiting so rapidly into the cell that there is very little net block. At positive internal potentials, on the other hand, exit from the site into the cell is electrostatically inhibited, resulting in the accumulation of blocked channels. The accumulation of blocked channels is seen as a decay in the calcium current.

The hooked tail currents with cadmium are due to the escape of cadmium from some of the channels that conduct briefly before closing. Tail currents with cadmium are not slowed compared with the controls and, in fact, have near-identical late time
FIGURE 13. Simulations of tail currents with and without the closed blocked (CB) state. (A) Tail current simulations without the CB state. The superimposed records (+125 μM Cd 2+) show the last 1 ms of the pulse currents elicited by 10-ms pulses to +20 mV, followed by tail currents at -40 mV. Note that the tails cross one another due to the slowing of channel closing in the presence of Cd 2+. The simulation was performed with the rates fbl and rbl set to 0. Model parameters are listed in Table II. (B) Tail current simulations with the CB state. The rates rbl and fbl are set equal to a1 and b1, respectively (refer to the scheme in Fig. 10 A). The voltage protocol is the same as that for (A). The tail currents ± 125 μM Cd 2+ do not cross. Model parameters are listed in Table II. courses. This is due to the ability of the channels to close at normal rates with cadmium in the pores.

Comparison with Previous Work

The results in this paper are in accord with observations by others that the degree of Cd 2+ block of macroscopic Ca 2+ currents changes with voltage, becoming greater at positive potentials within the range of potentials from -30 to +60 mV (Brown et al., 1983; Byerly et al., 1984). Of interest is the Cd 2+-induced decay of calcium pulse currents, which appeared at positive potentials. This finding may have been obscured in other studies by the normal current decline of inactivating calcium channels or by the use of concentrations of Cd 2+ that completely block pulse currents. Swandulla and Armstrong (1989) reported a similar finding in chick dorsal root ganglion (DRG) neurons, but only with a two-pulse protocol in which the calcium channels were activated by a prepulse and then cleared of cadmium (as evidenced by the tail current kinetics) by a step to a highly negative potential. During the succeeding pulse, the pulse currents displayed activation followed by decay.

FIGURE 14. Rates of block and unblock vs. V_m. Using the parameters obtained from fits for cell 7SE08A.N1 (Table II), the rates of unblock (r1 and r2) and block (f1[Cd 2+]0) in 125 μM Cd 2+ are plotted as functions of voltage. Whereas the rate of block is nearly independent of voltage, the rates of unblock, in particular r2, are steeply dependent on voltage, changing e-fold per -16.6 mV.
The estimates of cadmium entry and exit rates are strikingly similar to estimates obtained from the single-channel data of Lansman et al. (see Fig. 13 of Lansman et al., 1986, and Fig. 14 of this paper). The rates of unblock in both cases are steeply dependent on voltage, while the rates of block are nearly voltage independent.

The concentration for half block in squid neurons (125 \( \mu \text{M} \) at 0 mV) is the same as for the calcium currents of the squid presynaptic terminal (Augustine et al., 1989), but it is different from that reported for the calcium currents in a number of other preparations (for example, molluscan neurons \textit{Lymnaea stagnalis}, \textit{Helix pomatia}, or \textit{Planorbis corneus}: 72 \( \mu \text{M} \) in 10 mM Ca\(^{2+}\) and 4 mM Mg\(^{2+}\) (Kostyuk and Krishtal, 1977); \textit{Lymneae} neurons: < 100 \( \mu \text{M} \) in 4 mM Ca\(^{2+}\) and 4 mM Mg\(^{2+}\) (Byerly et al., 1984); guinea pig ventricle: 20 \( \mu \text{M} \) in 110 mM barium (Lansman et al., 1986); \textit{Helix aspersa} neurons: 2.2 mM in 10 mM Ca\(^{2+}\) and 15 mM Mg\(^{2+}\) (Akaikle et al., 1978). The measured \( K_d \) is almost certainly affected by the membrane potential, by the current carrier and its concentration (see below), and by the channel type.

The Calcium Channel Closes with Cadmium in It

The data on squid calcium currents strongly suggest that the channel closes readily with a cadmium ion inside it. Given the similarity in the ionic radii and charge of cadmium and calcium, one is led to wonder whether the channel may also close when occupied by calcium.

Other investigators have found evidence that calcium channels can close with divalents within the pore. Swandulla and Armstrong (1989) observed a hook in the tail currents of barium currents recorded from Cd\(^{2+}\)-blocked calcium channels in chick DRG cells. As for the squid calcium channels, the tail currents were not slowed by cadmium, suggesting that the channels close when occupied by Cd\(^{2+}\).

Lansman et al. (1986) noted that as divalent blocker concentration is increased, there is no clear increase in calcium single-channel current burst length, and they point out that this can be explained if the channel closes with a divalent inside (cf. Neher and Steinbach, 1978). Chesnoy-Marchais (1985) found that the mean open and closed times of single calcium channels in excised patches from \textit{Aplysia} neurons depended on the permeant divalent cation present. She suggested that an ion may be bound to the closed state of the channel. On the other hand, Nelson (1986) found that changing the species of permeant divalent influenced only the mean open time.

The recognition that the channel may close normally with a divalent cation occupying it leads one to consider a more complex kinetic diagram, as illustrated in Fig. 15. The states formerly referred to as C2, C1, O, OB, and CB in Fig. 10A have been replaced by states C2(Ca), C1(Ca), O(Ca), O(Cd), and C1(Cd), respectively;
C2(Cd) is a new state. The new diagram, while incorporating all the previously suggested state transitions, raises questions about further possible transitions. In particular, is there more than one closed state occupied by cadmium? Are there possible transitions between closed states occupied by cadmium and those occupied by calcium? The possibility of these transitions is suggested by the question marks.

Is there functional significance to the observation that the calcium channel may close when occupied by a divalent cation? Recent investigations in certain other channels suggest that normal gating (e.g., Armstrong and Lopez-Barneo, 1987; Swandulla and Armstrong, 1989) and inactivation (Grissmer and Cahalan, 1989) may require that the channels close with calcium in them. This may apply to squid calcium channel gating.

**Multiion and Multisite Models: How Many Ions and How Many Sites?**

Recently several laboratories have published data and models supporting the idea that the calcium channel has at least two divalent cation binding sites (Kostyuk et al., 1982; Almers and McCleskey, 1984; Hess and Tsien, 1984). The data presented in this paper were very well fit using only one site. Undoubtedly models with multiple sites would also fit the same data, but these were felt to be unnecessarily complicated for the data obtained thus far.

Given the findings in other calcium channels and in most other channels, it seems likely that the squid calcium channel will turn out to be a multiion pore. Then the rates obtained in this paper are apparent rates, which include the implicit effects of ion interactions in the channel. Experiments designed to evaluate ion-ion interaction within the channel—specifically to examine the effect of calcium on cadmium entry and exit rates—are still needed in the squid preparation. Multiion features should be readily accommodated into the model presented here.

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