# Interactions between Divalent Cations and the Gating Machinery of Cyclic GMP–activated Channels in Salamander Retinal Rods

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ABSTRACT The effects of divalent cations on the gating of the cGMP-activated channel, and the effects of gating on the movement of divalent cations in and out of the channel's pore were studied by recording macroscopic currents in excised membrane patches from salamander retinal rods. The fractional block of cGMP-activated $Na^+$ currents by internal and external $Mg^{2+}$ as well as internal $Ca^{2+}$ was nearly independent of cGMP concentration. This indicates that $Mg^{2+}$ and $Ca^{2+}$ bind with similar affinity to open and closed states of the channel. In contrast, the efficiency of block by internal $Cd^{2+}$ or $Zn^{2+}$ increased in proportion to the fraction of open channels, indicating that these ions preferentially occupy open channels. The kinetics of block by internal $Ni^{2+}$, which competes with $Mg^{2+}$ but blocks more slowly, were found to be unaffected by the fraction of channels open. External $Ni^{2+}$, however, blocked and unblocked much more rapidly when channels were mostly open. This suggests that within the pore a gate is located between the binding site(s) for ions and the extracellular mouth of the channel. Micromolar concentrations of the transition metal divalent cations $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ applied to the cytoplasmic surface of a patch potentiated the response to subsaturating concentrations of cGMP without affecting the maximum current induced by saturating cGMP. The concentration of cGMP that opened half the channels was often lowered by a factor of three or more. Potentiation persisted after the experimental chamber was washed with divalent-free solution and fresh cGMP was applied, indicating that it does not result from an interaction between divalent cations and cGMP in solution; 1 mM EDTA or isotonic $MgCl_2$ reversed potentiation. Voltage-jump experiments suggest that potentiation results from an increase in the rate of cGMP binding. Lowering the ionic strength of the bathing solution enhanced potentiation, suggesting that it involves electrostatic interactions. The strong electrostatic effect on cGMP binding and absence of effect on ion permeation through open channels implies that the cGMP binding sites on the channel are well separated from the permeation pathway.

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## INTRODUCTION

Ion channels gated by cyclic GMP (cGMP) generate the electrical response to light in retinal rods. cGMP holds channels open in darkness; light closes channels and hyperpolarizes the cell by activating an enzyme cascade that lowers the concentration of cGMP (reviewed in Yau and Baylor, 1989; McNaughton, 1990; Kaupp, 1991; Stryer, 1991).

The cGMP-activated channel has a very low effective unit conductance under physiological conditions (Bodoia and Detwiler, 1985; Gray and Attwell, 1985) due to block of $Na^+$ entry by external $Ca^{2+}$ and $Mg^{2+}$ ions (Haynes, Kay, and Yau, 1986; Zimmerman and Baylor, 1986), which themselves carry a small fraction of the inward current (Nakatani and Yau, 1988*a*). $Ca^{2+}$ entry in the dark provides negative feedback control of cGMP synthesis (Lolley and Racz, 1982; Pepe, Panfoli, and Cugnoli, 1986; Hodgkin and Nunn, 1988; Koch and Stryer, 1988; Dizhoor, Ray, Kumar, Niemi, Spencer, Brolley, Walsh, Philipov, Hurley, and Stryer, 1991). A light-induced drop in internal $Ca^{2+}$ concentration (Yau and Nakatani, 1985; McNaughton, Cervetto, and Nunn, 1986) is important for the recovery of the light response and for light adaptation (Matthews, Murphy, Fain, and Lamb, 1988; Nakatani and Yau, 1988*b*).

The dependence of current through the channel on ion concentrations and voltage has been described by a model which assumes that ions must bind to a single site within the channel in order to permeate (Zimmerman and Baylor, 1988, 1992; Menini, 1990). It is assumed that only a single cation occupies the site at a time and that divalents bind to the site more strongly than monovalents. Against this simple picture there is evidence that under some conditions a channel may bind more than one monovalent cation at a time (Furman and Tanaka, 1990; Menini, 1990).

There is a cubic relationship between current and cGMP concentration in the physiological range (Haynes et al., 1986; Zimmerman and Baylor, 1986), and the channel responds to changes in cGMP within milliseconds (Karpen, Zimmerman, Stryer, and Baylor, 1988*a*, *b*).

One aim of this study was to determine whether binding of $Ca^{2+}$ or $Mg^{2+}$ in the channel's pore alters gating. The strategy was to determine how effectively these ions block the channel at different concentrations of cGMP. A second aim was to locate the channel's gate with respect to its binding site for ions. Here the approach was to examine the kinetics of block by $Ni^{2+}$, which competes with $Mg^{2+}$ but blocks more slowly. An unexpected finding was that $Ni^{2+}$, as well as $Zn^{2+}$, $Cd^{2+}$, and $Mn^{2+}$, at micromolar concentrations that do not block conduction, strongly potentiate the effect of cGMP on the channel.

Preliminary accounts of some of the work have appeared previously (Karpen et al., 1988*b*; Karpen, Stryer, and Baylor, 1989).

## METHODS

### *Preparation and Perfusion*

Retinal rod outer segments were obtained from light-adapted larval tiger salamanders, *Ambystoma tigrinum*. After rapid decapitation the brain and spinal cord were pithed and the retinas were isolated into a Ringer solution. A small piece of retina was placed in the

experimental chamber and teased with fine bent needles to release isolated rod outer segments. The floor of the chamber was made sticky by filling the chamber with 1 mg/ml poly-L-lysine hydrobromide (30,000–70,000 mol wt; Sigma Chemical Co., St. Louis, MO), and then thoroughly rinsing it after 2 min; this treatment prevented outer segments from rolling away when the patch electrode approached. The solution in the experimental chamber was changed by a gravity-driven perfusion system within ~20 s. In some experiments the solution bathing the patch was changed within 5 ms by rapidly switching the patch between two streams emerging from a divided tube (see Karpen et al., 1988*b*). Experiments were performed in visible light at 22–25°C.

### *Patch Excision and Recording*

Patch electrodes with orifices ~1 μm in diameter and resistances near 3 MΩ were pulled from borosilicate glass and lightly fire-polished before use. A miniature $O_2/H_2$ torch was used to produce a right angle bend ~1 mm from the tip. This allowed the tip to be located precisely in the horizontal plane as an outer segment was approached in the direction perpendicular to the chamber floor. Seal resistances ranged from 0.2 to 10 GΩ. Currents from excised, inside-out patches were recorded with an Axopatch 1A or 1D amplifier and low-pass filtered (eight-pole Bessel). Some records were sampled, stored, and analyzed with a PDP 11/73 or Gateway 386 computer. cGMP-induced currents were obtained from the difference in currents with and without the nucleotide. About 40% of the excised patches did not respond to cGMP. When these patches were touched to a bead of Sylgard resin, ~40% of them began to respond, apparently because a vesicle was ruptured. The remaining 60% of unresponsive patches usually broke on contact with Sylgard. It is not known whether these patches were vesicles or simply lacked channels.

### *Solutions*

Isolation of rod outer segments, seal formation, and patch excision were carried out in a Ringer solution containing (mM): 111 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 6.0 $MgCl_2$, 10 D-glucose, 0.02 EDTA, and 3 HEPES (pH 7.6). Unless otherwise noted in the figure legends, cGMP-induced currents were measured with the following solution in the patch pipette and experimental chamber (mM): 130 NaCl, 2 Tris (pH 7.6) with divalent cation salts ($MgCl_2$, $CaCl_2$, $ZnCl_2$, $CdCl_2$, $MnCl_2$, $NiSO_4$, or $NiCl_2$) or EDTA added as indicated.

## THEORY

This section presents equations that describe the block of $Na^+$ currents by divalent cations. We consider three simple limiting mechanisms in which divalent cations bind only to open channels, only to closed channels, or to all states of the channel.

First consider the behavior of the channel in the absence of divalent cation block. The following simple scheme (Karpen et al., 1988*a*, *b*) has been used to account for steady-state cGMP dose–response relations and channel gating kinetics in the absence of divalent cations:

### *No Block (Zero Divalent Cations)*

$$C \xleftrightarrow{K_1} GC \xleftrightarrow{K_2} G_2C \xleftrightarrow{K_3} G_3C \xleftrightarrow{K_C} OPEN$$

In the model C represents the closed channel and G denotes cGMP. Channels open after binding three molecules of cGMP. $K_1$, $K_2$, and $K_3$ are the microscopic or intrinsic

ligand dissociation constants, which describe binding to a single site for the first, second, and third cGMP binding events, respectively. $K_1 = 3[C][G]/[GC]$, $K_2 = [GC][G]/[G_2C]$, and $K_3 = [G_2C][G]/(3[G_3C])$. $K_C$, the equilibrium constant for channel closing, is given by $[G_3C]/[OPEN]$. The macroscopic current through open channels, $I$, is

$$I = I_{OPEN} \frac{[G]^3}{[G]^3(1 + K_C) + 3K_3K_C[G]^2 + 3K_2K_3K_C[G] + K_1K_2K_3K_C} = I_{OPEN}F \quad (1)$$

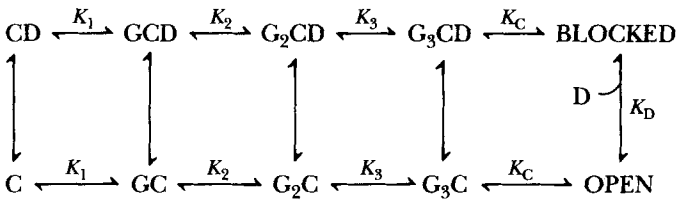
where $I_{OPEN}$ is the current if all channels were open and $F$ is the fraction of open channels. The apparent rate constant, $k_{resp}$, for channel closing after an instantaneous drop in cGMP concentration to zero is

$$k_{resp} = 3k_{off} \frac{[G_3C]}{[G_3C] + [OPEN]} = 3k_{off} \frac{K_C}{1 + K_C} \quad (2)$$

where $k_{off}$ is the microscopic rate constant for the $G_3C$ to $G_2C$ transition.

Suppose that a divalent cation, D, can bind to all states of the channel (closed and open) with equal affinity, and that only the open state without a divalent is conducting:

*Mechanism I: Divalent Cation Bound in Closed and Open Channels with Equal Affinity*




For this mechanism, the current through open channels, $I$, is

$$I = I_{OPEN} \frac{F}{1 + [D]/K_D} \quad (3)$$

In this expression, $F$ is the fraction of open channels in the absence of divalent cations (Eq. 1) and [D] is the concentration of divalent cations on one side of the membrane, the concentration on the other side being zero. $K_D$ is the dissociation constant for divalent cation binding. It is not a true equilibrium constant because it is equal to the sum of dissociation rate constants for exiting the pore to both sides divided by the association rate constant for entering the pore from the side on which D is present. $K_D$ reflects the effectiveness of block under steady-state current conditions. The current in the absence of divalents (Eq. 1) divided by the current in the presence of divalents (Eq. 3) is given by

$$I_{-D}/I_{+D} = 1 + [D]/K_D \quad (4)$$

Eq. 4 is a measure of the degree of block by the divalent cation. The dependence of $I_{-D}/I_{+D}$ on [D] gives the apparent dissociation constant for divalent block, $K'_D$, which for this mechanism is equal to $K_D$:

$$K'_D = K_D \tag{5}$$

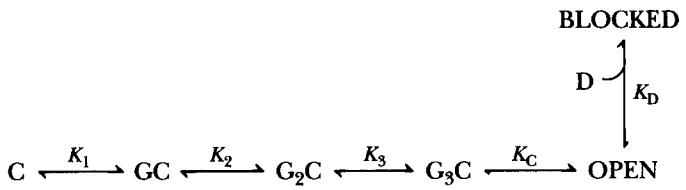
The apparent rate constant, $k_{resp}$, for channel closing after an instantaneous drop in cGMP concentration to zero is

$$k_{resp} = 3k_{off}\frac{[G_3C]}{[G_3C] + [OPEN]} = 3k_{off}\frac{K_C}{1 + K_C} \tag{6}$$

For this mechanism, the apparent dissociation constant for divalent block is independent of the fraction of channels opened by cGMP, and divalent block has no effect on the rate at which channels close after a sudden drop in cGMP concentration.

Suppose that a divalent cation can bind only to an open channel, and that the blocked channel is unable to close:

*Mechanism II: Divalent Cation Bound Only in Open Channels*




The current through open channels, $I$, is

$$I = I_{OPEN}\frac{F}{1 + F[D]/K_D} \tag{7}$$

The current in the absence of divalents (Eq. 1) divided by the current in the presence of divalents (Eq. 7) is now given by

$$I_{-D}/I_{+D} = 1 + F[D]/K_D \tag{8}$$

The apparent dissociation constant for divalent block, $K'_D$, is

$$K'_D = K_D/F \tag{9}$$

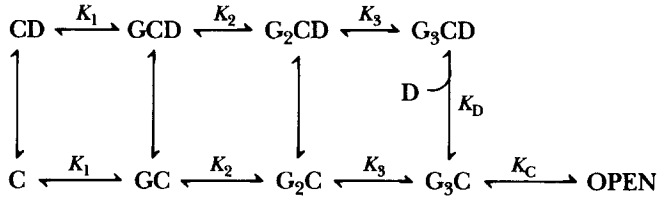
The apparent rate constant, $k_{resp}$, for channel closing following an instantaneous drop in cGMP concentration to zero is

$$k_{resp} = 3k_{off}\frac{[G_3C]}{[G_3C] + [OPEN] + [BLOCKED]} = 3k_{off}\frac{K_C}{1 + K_C + [D]/K_D} \tag{10}$$

For this mechanism, the apparent dissociation constant for divalent block decreases as the fraction of open channels increases, and divalent block slows the rate of channel closing after a sudden drop in cGMP concentration.

Suppose that a divalent cation can bind only to a closed channel, and that the blocked channel is unable to open:

*Mechanism III: Divalent Cation Bound Only in Closed Channels*




The current through open channels, $I$, is

$$I = I_{OPEN} \frac{F}{1 + (1 - F)[D]/K_D} \tag{11}$$

The current in the absence of divalents (Eq. 1) divided by the current in the presence of divalents (Eq. 11) is given by

$$I_{-D}/I_{+D} = 1 + (1 - F)[D]/K_D \tag{12}$$

The apparent dissociation constant for divalent block, $K'_D$, is

$$K'_D = K_D/(1 - F) \tag{13}$$

The apparent rate constant, $k_{resp}$, for channel closing after an instantaneous drop in cGMP concentration to zero is

$$k_{resp} = 3k_{off} \frac{[G_3C] + [G_3CD]}{[G_3C] + [G_3CD] + [OPEN]} = 3k_{off} \frac{K_C(1 + [D]/K_D)}{1 + K_C(1 + [D]/K_D)} \tag{14}$$

For this mechanism, the apparent dissociation constant for divalent block increases as the fraction of open channels increases, and divalent block speeds the rate of channel closing after a sudden drop in cGMP concentration.

The treatment above makes the following simplifying assumptions: (*1*) [G] and [D] are much larger than the channel concentration and are assumed not to change as the result of binding. (*2*) In deriving the expressions for $k_{resp}$, the opening and closing of the fully liganded channel and the binding and unbinding of divalent cation are assumed to be equilibria that are established much faster than the unbinding of cGMP (see text and Karpen et al., 1988*a*, *b*). (*3*) Divalent cations are permeant blockers that bind to a single site in the pore as they pass through the channel (Zimmerman and Baylor, 1988, 1992; Colamartino, Menini, and Torre, 1991). (*4*) Under the conditions studied the current carried by divalent cations is negligible compared with that carried by $Na^+$ (Zimmerman and Baylor, 1988, 1992; Colamartino et al., 1991). (*5*) Occupancy of the channel by $Na^+$ ions and its effect on divalent cation binding have been ignored. With divalent cations absent the channel is occupied by $Na^+$ less than half the time at a concentration of 130 mM (Zimmerman and Baylor, 1992). The concentration of $Na^+$ is constant in each experiment, and

calculations show that including $Na^+$ occupancy would increase the apparent dissociation constant for divalent block by a factor of only 1.6.

*Comparison of the Three Mechanisms*

The three limiting mechanisms make different predictions that were tested experimentally. One difference is in the dependence of the divalent cation blocking efficacy on the fraction of channels open. In mechanism I, the effectiveness of D is independent of $F$ (Eqs. 4 and 5). In mechanism II, D becomes a more effective blocker as $F$ increases because D only binds to the open state (Eqs. 8 and 9). In contrast, for mechanism III, D becomes less effective with increasing $F$ because D only binds to closed states (Eqs. 12 and 13).

A second difference is in the effects of divalent block on the kinetics of channel closing after a sudden drop in cGMP concentration. In mechanism I, divalent block has no effect on channel closing kinetics because open-blocked channels are assumed to close with the same kinetics as open channels (Eqs. 2 and 6). In mechanism II, divalent block slows the closing of channels after a drop in cGMP because blocked channels have to unblock before they can close (compare Eqs. 2 and 10). In mechanism III, divalent block speeds closing because divalent binding shifts the gating equilibrium to closed states (compare Eqs. 2 and 14). In the Discussion we reexamine previous measurements of the rate of channel closing in terms of these three mechanisms.

## RESULTS

*Evidence That the Channel Can Close with $Mg^{2+}$ or $Ca^{2+}$ Bound in the Pore*

The fractional blockage of cGMP-activated $Na^+$ currents by internal $Mg^{2+}$ was nearly independent of cGMP concentration (Karpen et al., 1988*b* and Table I). The efficiency of steady-state current block was slightly higher at low than at high cGMP. At both cGMP concentrations, the $Mg^{2+}$ titrations had the form expected for block at a single binding site (Karpen et al., 1988*b*). At +30 mV the apparent dissociation constant for internal $Mg^{2+}$ block was 70 μM at 5 μM cGMP and 120 μM at 100 μM cGMP. This behavior suggests that $Mg^{2+}$ can be bound in both closed and open channels (compare mechanisms I and II). If only open channels could contain a $Mg^{2+}$ ion (mechanism II), the effectiveness of block would increase at higher cGMP concentrations by the same factor that the fraction of open channels increased. For this mechanism, the apparent dissociation constant for block ($K_D/F$; Eq. 9) would be eight times lower at 100 μM cGMP than at 5 μM cGMP, where one-eighth as many channels were open. If $Mg^{2+}$ were bound to closed and open channels with equal affinity (mechanism I), the apparent $K_D$ for block would be independent of the fraction of open channels (Eq. 5). The slightly higher apparent dissociation constant observed at high cGMP suggests that $Mg^{2+}$ bound with slightly higher affinity to closed states.

Similar results were obtained with $Ca^{2+}$ (Table I). At +50 mV the apparent dissociation constant for internal $Ca^{2+}$ block was 490 μM at 7.5 μM cGMP and 560 μM at 125 μM cGMP. If the ion were bound exclusively in the open channel, the apparent dissociation constant would be about four times lower at 125 μM cGMP

(Eq. 9). Colamartino et al. (1991) have recently reported that block by internal $Ca^{2+}$ at −60 mV is slightly more effective at low than at high cGMP.

Because $Mg^{2+}$ and $Ca^{2+}$ inhibit $Na^+$ currents with roughly equal efficiency at low and high cGMP, the presence of these ions in the cytoplasmic solution will have little effect on the shape of the steady-state dose–response curve for cGMP.

Fig. 1 shows that external $Mg^{2+}$ also had little effect on the dose–response relation for cGMP. Dose–response relations from two patches determined with 1 mM $Mg^{2+}$ in the patch pipette are compared with those of two patches from the same animal determined with $< 10^{-7}$ M $Mg^{2+}$ in the pipette. Steady-state current amplitudes were normalized by the value at saturating cGMP. The relations are very similar, indicating that external $Mg^{2+}$ did not preferentially occupy the channel when it was open. Had

TABLE I

*Block of cGMP-activated $Na^+$ Currents by Internal Divalent Cations at High and Low cGMP*

| Ion | $V_m$ | $K'_{D(L)}$ | $K'_{D(H)}$ | $K'_{D(H)}/K'_{D(L)}$ | $I_L/I_H$ |
|---|---|---|---|---|---|
| | *mV* | *μM* | *μM* | | |
| $Mg^{2+}$ | +30 | 70 | 120 | 1.7 | 0.12 |
| $Ca^{2+}$ | +50 | 490 | 560 | 1.1 | 0.26 |
| $Ni^{2+}$ | +50 | 13 | 40 | 3.1 | 0.35 |
| $Zn^{2+}$ | +50 | 170 | 60 | 0.35 | 0.32 |
| | +50 | 380 | 50 | 0.13 | 0.11 |
| $Cd^{2+}$ | +50 | 930 | 77 | 0.083 | 0.077 |

$V_m$ is membrane potential. $K'_{D(L)}$ and $K'_{D(H)}$ are apparent dissociation constants for divalent block measured at low and saturating cGMP, respectively. They were calculated from the following relation: $K'_{D(L)}$ or $K'_{D(H)} = DI_{+D}/(I_{-D} - I_{+D})$, where D is divalent cation concentration, $I_{-D}$ is cGMP-induced current in the absence of divalent cations, and $I_{+D}$ is cGMP-induced current in the presence of divalent cations. Currents at a given membrane potential were measured after the steady state was reached. $K'_{D(H)}$ was measured at 100, 125, or 500 μM cGMP; $K'_{D(L)}$ was measured at 5 μM cGMP for $Mg^{2+}$ and $Cd^{2+}$, 7.5 μM cGMP for $Ca^{2+}$ and $Ni^{2+}$, and both 5 and 2 μM cGMP on the same patch for $Zn^{2+}$ (upper and lower entries, respectively). For the patch in which $Zn^{2+}$ was tested, $K'_{D(H)}$ was measured at two different times and the values differed slightly. $I_L/I_H$ is the ratio of currents induced by the low and high cGMP concentrations in the absence of divalent cations.

this been the case, $Mg^{2+}$ would have caused a leftward shift in the dose–response relation, because currents at high cGMP would have been depressed more than currents at low cGMP (Eq. 7). $Mg^{2+}$ would have caused the relation to saturate at a lower cGMP concentration by shifting the equilibrium toward the open state. The magnitude of the shift to be expected in the dose–response relation depends on the fractional occupancy of the open channel at 1 mM external $Mg^{2+}$ concentration. This has been estimated (Zimmerman and Baylor, 1992) by measuring how much 1 mM external $Mg^{2+}$ shifts the titration curve for internal $Mg^{2+}$ block to higher concentrations, when most of the channels were opened by saturating cGMP. The apparent dissociation constant for block by external $Mg^{2+}$ was estimated to be 150 μM at −30 mV. In the absence of divalent cations, the fraction of maximum current produced by

any cGMP concentration is

$$I/I_{max} = F/F_{SAT} \tag{15}$$

where $F$ is given by Eq. 1 and $F_{SAT}$ is the fraction of open channels at saturating cGMP concentration. If divalent cations are bound to all states with equal affinity (mechanism I), the fraction of maximum current is also given by Eq. 15, since currents will be depressed to the same extent at every cGMP concentration (Eq. 3). If divalent cations are only bound when the channel is open (mechanism II), it follows from Eq. 7 that the fraction of maximum current is given by

$$I/I_{max} = (F/F_{SAT})\frac{1 + F_{SAT}[D]/K_D}{1 + F[D]/K_D} \tag{16}$$

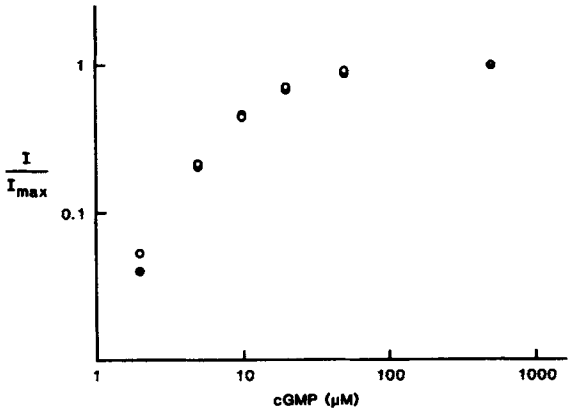



FIGURE 1. Double logarithmic plot of dose–response relations for cGMP in the absence and presence of external $Mg^{2+}$. Steady-state cGMP-activated currents measured at −30 mV were normalized by the saturating value ($I_{max}$). Results are from four patches obtained from a single animal. ○, Data averaged from two patches with nominally 0 external $Mg^{2+}$ (inside the patch pipette); ●, data averaged from two patches with a free external $Mg^{2+}$ concentration of 1 mM. The solution in the patch pipette and experimental chamber contained (mM): 130 NaCl, 5 sodium phosphate (pH 7.6), and 0.5 EDTA. 1.5 mM $MgCl_2$ was added to the solution in the patch pipette to obtain a free external $Mg^{2+}$ concentration of 1 mM. $I_{max}$ values were −236 and −315 pA in the absence of external $Mg^{2+}$, and −55 and −98 pA in the presence of external $Mg^{2+}$.

In the experiment of Fig. 1, $[D]/K_D$ was 6.7. Mechanism II predicts that divalent cation binding should have caused a large shift in the dose–response relation. For $I/I_{max} = F/F_{SAT} = 0.1$ in the absence of divalents, and $F_{SAT}$ taken to be 0.77 at −30 mV (Karpen et al., 1988*a*), 1 mM external $Mg^{2+}$ should have increased $I/I_{max}$ by a factor of ~4.1 (Eq. 16). No such shift was observed in Fig. 1. Although the concentration of cGMP that opens half the channels varies among different patches (Zimmerman and Baylor, 1986), these variations are unlikely to obscure such a shift.

*Evidence That the Channel Can Close with $Ni^{2+}$, But Not $Zn^{2+}$ or $Cd^{2+}$, Bound in the Pore*

Steady-state block of cGMP-activated $Na^+$ currents by internal $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ was measured as described above for $Mg^{2+}$ and $Ca^{2+}$. Internal $Ni^{2+}$ blocked

steady-state currents more strongly at low than at high cGMP (Table I). This suggests that $Ni^{2+}$ blocks closed channels with higher affinity than open channels (see mechanism III and Eq. 13). Estimating the degree to which $Ni^{2+}$ prefers closed over open states from the ratio of apparent dissociation constants measured at high and low cGMP (Table I) requires knowing the absolute values of $F$, the fraction of channels open in the absence of divalents (see Eq. 13). Here we have measured the ratio of $F$ at low and high cGMP ($I_L/I_H$ in Table I); absolute values of $F$, determined at +50 mV by single channel recording, are not yet available.

In contrast to $Ni^{2+}$, the effectiveness of block by $Zn^{2+}$ and $Cd^{2+}$ increased at higher cGMP concentration by the same factor that the fraction of open channels increased (Table I). This behavior is consistent with mechanism II (Eq. 9), in which only open channels can contain these ions.

*Patch-to-Patch Variability in the Efficiency of Block*

The data in Table I were obtained from one patch for each ion. Very similar results on the *relative* efficiencies of block at high and low cGMP were obtained on other patches from different animals (number of patches in parentheses): $Mg^{2+}$(1), $Ca^{2+}$(3), $Ni^{2+}$(1), $Zn^{2+}$(4), and $Cd^{2+}$(3). The *absolute* blocking efficiency, however, varied widely from patch to patch. The range of apparent dissociation constants of internal divalent block measured at saturating cGMP and +50 mV were: $Zn^{2+}$, 14–60 μM; $Cd^{2+}$, 32–77 μM; $Ni^{2+}$, 40–190 μM; $Ca^{2+}$, 560–710 μM. For $Mg^{2+}$ the value of 120 μM at +30 mV in Table I contrasts sharply with 370 μM at +50 mV for the patch in Fig. 2 (see below). The basis for these large variations is unknown. It should be noted that patch-to-patch variations in the sensitivity of channels to cGMP ($I_L/I_H$ in Table I; see also Zimmerman and Baylor, 1986; Zhainazarov and Kolesnikov, 1990) do not affect the conclusions about block in this study. We have measured how block varies as the fraction of open channels in a patch varies; the cGMP concentration required to open a given fraction of channels is not relevant here.

*Where Is the Gate in Relation to the Binding Site(s) for Ions?*

We have investigated this question by measuring how fast internal or external divalent cations can block $Na^+$ current when channels are mostly closed or mostly open. If a gate that controls the flow of ions exists between the bulk solution on one side of the membrane and the pore's ion binding site, the kinetics of block from that side should be much slower when the channels are mostly closed. We have used $Ni^{2+}$ to study the kinetics of divalent block because it moves in and out of the channel more slowly than $Mg^{2+}$ or $Ca^{2+}$. This makes the time course of block easier to measure. More importantly, to determine how gating affects the movement of ions through the channel, the kinetics of block should be comparable to or slower than the kinetics of gating. Gating here refers to the overall process of cGMP binding and consequent channel opening. $Mg^{2+}$ and $Ca^{2+}$ move through the channel quickly on the time scale of channel gating: 10–100 μs for divalent movement vs. several milliseconds for gating at low cGMP concentration (Karpen et al., 1988*a*, *b*; Zimmerman and Baylor, 1992).

There are three indications that $Ni^{2+}$ blocks in a manner similar to $Mg^{2+}$ and $Ca^{2+}$: (*1*) $Ni^{2+}$ blocked $Na^+$ current in a voltage-dependent manner from both sides of the

membrane, similar to $Mg^{2+}$ and $Ca^{2+}$ (see below). (*2*) $Ni^{2+}$, like $Mg^{2+}$ and $Ca^{2+}$, was apparently bound inside a closed channel. (*3*) Internal $Ni^{2+}$ competed with internal $Mg^{2+}$ for block of $Na^+$ current as shown in Fig. 2. The ratio of steady-state currents in the absence and presence of $Ni^{2+}$ is plotted vs. internal $Ni^{2+}$ concentration, where most of the channels were held open by saturating cGMP (500 μM). In the absence of internal $Mg^{2+}$, the results followed the straight line behavior predicted by Eq. 4, indicating block at a single site with a dissociation constant of 190 μM. If $Mg^{2+}$ competed with $Ni^{2+}$ for the same site, the ratio of currents in the absence and presence of $Ni^{2+}$ would be

$$I/I_{Ni} = 1 + [Ni^{2+}]/\{K_{Ni}(1 + [Mg^{2+}]/K_{Mg})\} \quad (17)$$

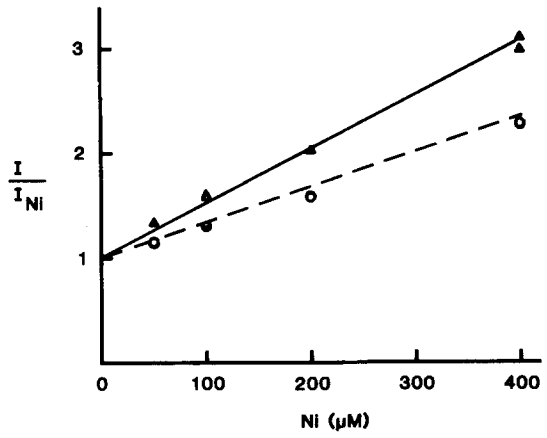



FIGURE 2. Evidence that $Mg^{2+}$ and $Ni^{2+}$ compete for a common binding site within the channel pore. Blockage of cGMP-activated $Na^+$ currents by internal $Ni^{2+}$ in the absence and presence of internal $Mg^{2+}$. The ratio of currents in the absence and presence of $Ni^{2+}$ is plotted vs. $Ni^{2+}$ concentration. Results are from one patch, cGMP concentration 500 μM, steady-state currents measured at +50 mV, maximum current in the absence of divalent cations 795 pA. △ and ▲, Results with 0 added $Mg^{2+}$; ○ and ●, results with 200 μM $Mg^{2+}$ added. Open and filled symbols represent measurements at two different times during the experiment. The solid line was obtained by linear regression on the 0 $Mg^{2+}$ data, with a fixed y-intercept of 1. The dashed line indicates the expected behavior in the presence of 200 μM $Mg^{2+}$ if $Ni^{2+}$ and $Mg^{2+}$ compete for a common blocking site (see text). $MgCl_2$ and $NiSO_4$ were added to the chamber perfusion solution at the concentrations indicated.

where $K_{Ni}$ and $K_{Mg}$ are the dissociation constants for block. Eq. 17 predicts that $Mg^{2+}$ will increase the apparent dissociation constant for $Ni^{2+}$ block by the factor $(1 + [Mg^{2+}]/K_{Mg})$. Fig. 2 shows that this was observed. The dashed line is the behavior predicted by Eq. 17 for $Ni^{2+}$ block in the presence of 200 μM $Mg^{2+}$; $K_{Mg}$ was determined in the absence of $Ni^{2+}$ to be 370 μM for this patch. The data in the presence of $Mg^{2+}$ fell close to the dashed line, indicating competition between $Ni^{2+}$ and $Mg^{2+}$. If the two divalents blocked $Na^+$ currents at distinct, noninteracting sites, the apparent dissociation constant for $Ni^{2+}$ block would be unaffected by $Mg^{2+}$ and the data in the presence of $Mg^{2+}$ would fall on the same line as the data in the absence of $Mg^{2+}$. The result indicates that the binding of one ion prevents block by the other.

The kinetics of $Na^+$ current block by internal and external $Ni^{2+}$ are shown in Fig.

3. With $Ni^{2+}$ on the inside surface (bath applied), blocking kinetics were measured with channels mostly opened by 100 μM cGMP, or with ~25% of channels opened by 7.5 μM cGMP. The fraction of channels opened by cGMP was estimated from currents measured on the same patch in the absence of $Ni^{2+}$ (see Fig. 3 legend). Blocking kinetics were measured after rapidly switching the membrane potential, taking advantage of the voltage dependence of $Ni^{2+}$ block. After a switch from −50 to +50 mV, there was a slow decline in current at both 100 and 7.5 μM cGMP (Fig. 3, *left*). The decline was not observed in the absence of $Ni^{2+}$ (data not shown); instead, a

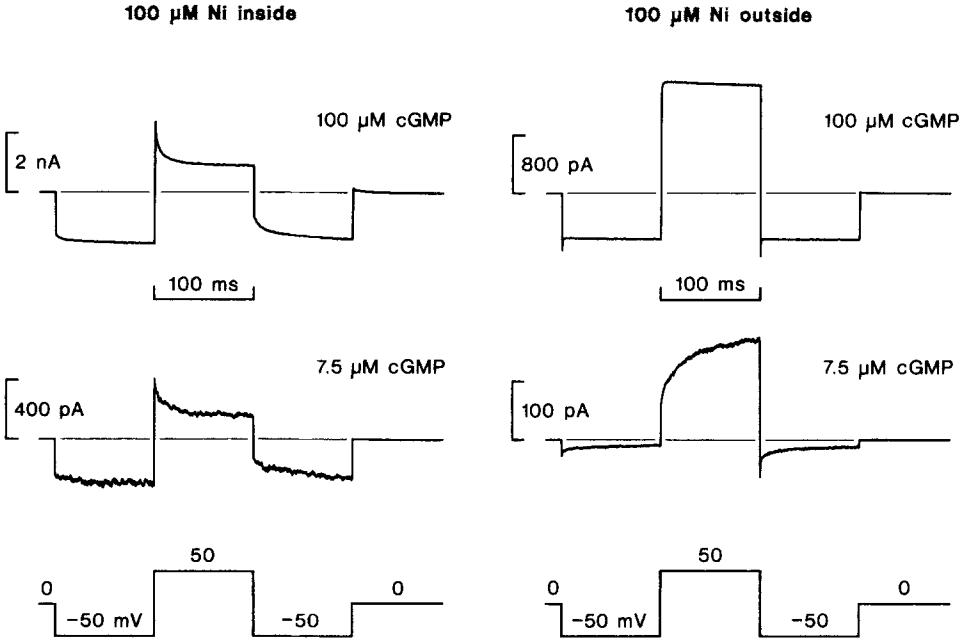



FIGURE 3. Kinetics of block of cGMP-activated $Na^+$ currents by internal and external $Ni^{2+}$. *Left*, 100 μM $NiSO_4$ added to chamber perfusion solution; *right*, 100 μM $NiSO_4$ added to patch pipette solution. Each panel is from a different patch. Voltage was changed as shown at the bottom, and cGMP concentrations were as indicated. Each trace was averaged from five to eight trials. Bandwidth 0–2 kHz, 5 kHz sampling. Steady-state currents in the absence of $Ni^{2+}$ for the patch in the left panel: 100 μM cGMP, 2,229 pA at +50 mV and −1,125 pA at −50 mV; 7.5 μM cGMP, 545 pA at +50 mV and −206 pA at −50 mV.

smaller time-dependent increase was observed that reflects the slight voltage sensitivity of channel gating by cGMP (see Karpen et al., 1988*a*). We interpret the decline in the presence of internal $Ni^{2+}$ to result from $Ni^{2+}$'s being driven into the pore at +50 mV. The currents declined along a very similar time course at both cGMP concentrations, indicating that the rate of block was nearly independent of the fraction of open channels. Apparently internal $Ni^{2+}$ reached the site at which it blocks $Na^+$ current at approximately the same rate in both closed and open channels. If $Ni^{2+}$ were only able to reach the site when the channel was open, assuming a single blocking site and

channel gating kinetics that are rapid compared with the rate of $Ni^{2+}$ block, the apparent rate constant for block would be given by

$$k_{block} = k_{assoc}[Ni^{2+}]F + k_{dissoc} \quad (18)$$

where $F$ is the fraction of open channels or the probability that a channel is open, $k_{assoc}$ is the bimolecular rate constant for $Ni^{2+}$ association with the pore binding site, and $k_{dissoc}$ is the dissociation rate constant. If the channel's ability to close with $Ni^{2+}$ in the pore is taken into account, $k_{dissoc}$ may also be a function of the proportion of channels that are open if $Ni^{2+}$ dissociates at different rates from closed and open channels. In any case, the apparent rate constant for block in this type of model depends strongly on the probability that a channel is open. The existence of more than one site at which $Ni^{2+}$ can block would change the form of Eq. 18 but not the conclusion. Similarly, a model in which $Ni^{2+}$ only enters closed channels would give different rates of block at low and high cGMP. The data indicate that internal $Ni^{2+}$ has roughly equal access to the blocking site in both closed and open channels. The same conclusion is also reached by examining the kinetics of the relief of block after switching the voltage from +50 to −50 mV. Very similar results were obtained on a second patch.

With $Ni^{2+}$ on the outside surface (inside the patch pipette), blocking and unblocking kinetics were quite different at low and high cGMP (Fig. 3, *right*). When the membrane potential was switched from −50 to +50 mV, block was relieved as $Ni^{2+}$ was driven from the pore to the outside. The kinetics of unblocking were dramatically slower at 7.5 μM cGMP than at 100 μM cGMP, suggesting that $Ni^{2+}$ exits the pore to the outside much more rapidly in open than in closed channels.

The record in the upper right corner of Fig. 3 does not show the progress of unblocking, which occurred with a time constant of ~0.6 ms. For this patch, currents were not measured in the absence of $Ni^{2+}$, which would have required exchanging solutions inside the patch pipette. The degree of outward rectification after a steady current has been reached is evidence that the overall time-dependent increase in current after a switch from −50 to +50 mV is due to both unblocking and to a previously described rapid (60 μs) increase in the number of open channels (see Karpen et al., 1988*a*, *b*). In the absence of divalent cations, the ratio of steady-state currents at +50 and −50 mV is ~1.8 at saturating cGMP (Karpen et al., 1988*a*, *b*). With $Ni^{2+}$ outside in Fig. 3, the ratio of steady-state currents at +50 and −50 mV was 2.4. The increased outward rectification arises from voltage-dependent block by external $Ni^{2+}$. Very similar results were obtained on a second patch. The small, slow droop in the current at +50 mV results from ion depletion due to hindered diffusion at the cytoplasmic surface of the patch (see Zimmerman, Karpen, and Baylor, 1988).

The much slower unblocking time course observed at 7.5 μM cGMP (Fig. 3, *lower right record*) strongly suggests that $Ni^{2+}$ has to wait for channels to open before escaping to the outside. Because the unblocking of open channels described earlier is rapid compared with channel gating kinetics at 7.5 μM cGMP (time constants of 0.6 vs. 2.5 ms; Karpen et al., 1988*a*, *b*), distinguishing between models in which $Ni^{2+}$ escapes to the outside from both closed and open channels versus only open channels would normally be difficult. This is because channel gating kinetics, rather than unblocking, would still be rate limiting in both cases. The observed time course of

unblocking is much slower, however, than either normal channel gating kinetics or unblocking of open channels. This leads to the conclusion that $Ni^{2+}$ bound in the pore is not only waiting for channels to open in order to escape to the outside, but is also slowing the opening process itself. The slow opening of blocked channels is consistent with the earlier observation that $Ni^{2+}$ binds with higher affinity to closed than open states and therefore stabilizes the closed conformation.

Three gating models that incorporate the channel's ability to close with a divalent cation lodged in the pore are shown in Fig. 4. For simplicity, we assume that there is a single ion binding site in the pore (but see Discussion). A gate could flank the extracellular side of the binding site (model A), the intracellular side (model B), or both sides (model C) as suggested by Miller, Latorre, and Reisin (1987) for $Ca^{2+}$-activated $K^+$ channels of rat skeletal muscle. The $Ni^{2+}$ blocking kinetics seem most consistent with model A, in which internal ions have access to the ion binding

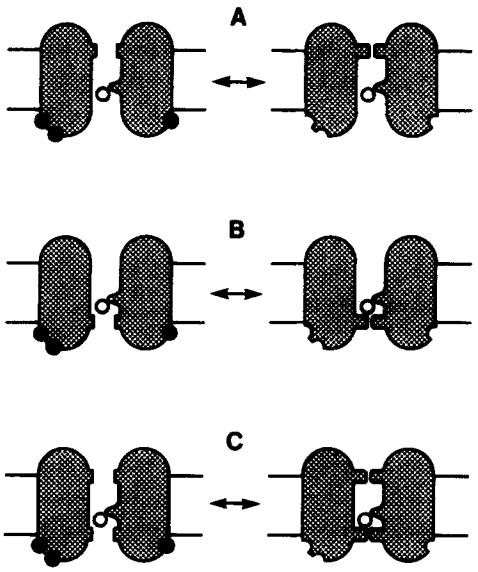



FIGURE 4. Three models of how a channel might close with a divalent cation bound in pore. In each model, the external surface is above and the internal surface below. The channel is open with three cGMPs (●) bound on the inside, and closed with no cGMP bound. A gate is external to the ion binding site in model A and internal to the site in model B. In model C, gates open and close together on both sides of the ion binding site.

site in both open and closed channels, but external ions only have access when the gate is open.

Stimulated by the work of Miller et al. (1987), we attempted to trap $Ni^{2+}$ in the closed channel as a further test of the models in Fig. 4. If gates flanked both the cytoplasmic and external sides of the $Ni^{2+}$ binding site (model C in Fig. 4), a channel that closed on a $Ni^{2+}$ would trap the ion inside it. The ion would not be trapped by a single gate located on only one side of the binding site (models A and B). Trapping was looked for in the following way. The channels in a patch were opened by a saturating concentration of cGMP (100 μM). In the presence of cGMP, 1 mM $Ni^{2+}$ was added, strongly blocking the current. The channels were closed in the presence of $Ni^{2+}$ by removing cGMP. Having closed the channels, any untrapped $Ni^{2+}$ was removed by washing the patch for 2 min with a solution containing neither $Ni^{2+}$ nor cGMP. The channels were then opened suddenly by applying to the patch a high

concentration (5 mM) of cGMP from a divided tube that was moved pneumatically in the chamber (Karpen et al., 1988*b*). Trapping would delay the response to cGMP, as current would not flow until trapped $Ni^{2+}$ was released by channel opening. The channels opened in as little as 2 ms, but there was no evidence that the attempt to load the channel produced any delay attributable to trapping (results not shown); a lag longer than ~1.5 ms would have been resolvable.

*Potentiation of the Response to Subsaturating cGMP by Low Concentrations of $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Mn^{2+}$*

In the course of studying block by different divalent cations, we unexpectedly found that certain transition metal divalents ($Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Mn^{2+}$), at low concentrations (micromolar or less) that do not block conduction, potentiated the response to subsaturating cGMP. The effect was discovered when patches were perfused with subsaturating cGMP and blocking concentrations of the divalents, immediately after perfusing the patch with the same cGMP alone. The current first rose, often to a level close to that caused by saturating cGMP alone, and then fell to a level below that induced by the low cGMP alone (data not shown). The same divalent concentrations caused only block of the current at saturating cGMP. These results suggested high affinity sites at which the divalents enhance the binding of cGMP, in addition to the lower affinity blocking site in the pore. As the chamber filled with divalents, the higher affinity sites would be occupied first. This led to the prediction that a concentration of divalents too low to block might enhance the response to low cGMP. Such was found to be the case, as shown in Fig. 5. Patch currents were measured in response to brief +50-mV pulses, first in the presence and then in the absence of saturating (500 μM) cGMP. 7.5 μM cGMP was then applied to the patch, followed by the same cGMP plus 10 μM $Ni^{2+}$. The current rose within 15 s to a level close to that caused by saturating cGMP. The response to 7.5 μM cGMP remained potentiated even 10 min after $Ni^{2+}$ was removed. During this period, the response to 500 μM cGMP was the same as that observed before the application of $Ni^{2+}$. Because potentiation persisted after the chamber was perfused with divalent-free solution and fresh cGMP, it can not have resulted from an interaction between the divalents and cGMP in solution. This observation, as well as the absence of an effect on the current activated by saturating cGMP, argues that $Ni^{2+}$ bound to sites on or near the channel and enhanced the binding of cGMP. Potentiation is unlikely to result from inhibition of phosphodiesterase activity associated with excised patches (Ertel, 1990) for three reasons. First, the experiments were performed in the absence of added $Mg^{2+}$. Millimolar concentrations of $Mg^{2+}$ have been shown to be required for maximum phosphodiesterase activity (Chader, Johnson, Fletcher, and Bensinger, 1974; Yee and Liebman, 1978). Using deionized and distilled water from our laboratory, no cGMP hydrolysis was detected with trypsin-activated bovine PDE in the absence of added $Mg^{2+}$. Second, $Mn^{2+}$, which can substitute for $Mg^{2+}$ in *activating* PDE (Chader et al., 1974; Yee and Liebman, 1978), was as effective as $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ in potentiating the channel response. Third, potentiation results in higher channel sensitivities to cGMP than have been observed previously, even in the presence of EDTA.

The potentiation in the experiment of Fig. 5 was reversed by applying 1 mM EDTA

for 12 min. In some patches, potentiation was reversed within 2–3 min by 1 mM EDTA or a solution in which isotonic $MgCl_2$ substituted for NaCl. In Fig. 5, after potentiation was reversed, the currents at both 7.5 and 500 μM cGMP were slightly lower than those observed at the beginning of the experiment; this did not occur on other patches. It appears that the number of active channels in the patch decreased slightly, but 7.5 μM cGMP opened the same percentage of channels as early in the patch before $Ni^{2+}$ was applied.

Fig. 6 shows dose–response relations for cGMP measured before and after potentiation by $Ni^{2+}$. $Ni^{2+}$ shifted the relation toward lower cGMP concentrations, as

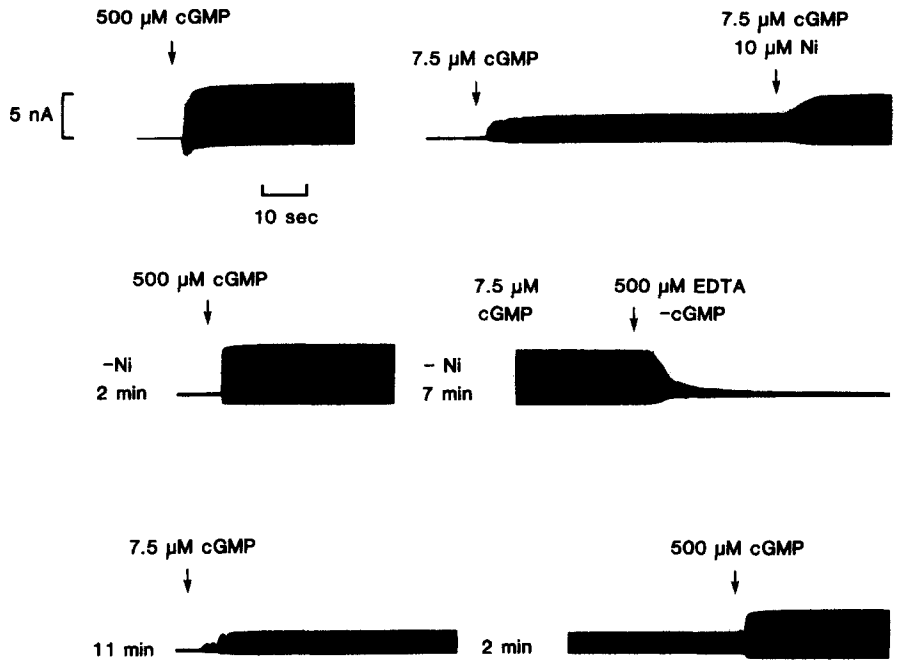



FIGURE 5. Potentiation of the response to subsaturating cGMP by a low concentration of $Ni^{2+}$. Patch current as a function of time. The patch was depolarized by +50-mV pulses lasting 10 ms, applied every 100 ms. Solutions were applied to the cytoplasmic surface of the patch by chamber perfusion at the times indicated by the arrows. The lag in the response is the time required to change the chamber solution. Bandwidth 0–1 kHz.

expected from the results in Fig. 5. The dose–response behavior was first measured in the absence of $Ni^{2+}$, and the patch was then exposed to 10 μM $Ni^{2+}$ for several minutes. The potentiated dose–response relation was measured by alternately perfusing the patch with a cGMP solution (without $Ni^{2+}$) and with 10 μM $Ni^{2+}$ in control solution lacking cGMP to measure the leak and keep the patch potentiated. Nearly identical dose–response relations were obtained on another patch that was initially potentiated by 10 μM $Ni^{2+}$, but in which the subsequent dose–response relation was measured with 1 μM $Ni^{2+}$ in both the cGMP and control solutions.

Exposure to $Ni^{2+}$ caused two notable changes in the dose–response relation. First, the concentration of cGMP that gave a half-maximal current ($K_{1/2}$) shifted from 8.3 to 3.4 μM. Second, the dose–response relation became shallower. The smooth curves are fits to the Hill equation:

$$I/I_{max} = [G]^n/(K^n_{1/2} + [G]^n) \quad (19)$$

where $n$ is an index of cooperativity. Before $Ni^{2+}$ the relation was fitted with $n = 1.8$, while after $Ni^{2+}$ the relation was fitted with $n = 1.1$. The value of 1.8 before $Ni^{2+}$ is lower than the value of 3 required to fit very small currents (Haynes et al., 1986; Zimmerman and Baylor, 1986). The value of 1.1 after $Ni^{2+}$ suggests that over the concentration range tested channel opening was effectively controlled by the binding of one molecule of cGMP. Since the binding of at least three molecules is thought to be required for significant opening, it is possible that potentiation caused the first two or more molecules of cGMP to bind with extremely high affinity at concentrations

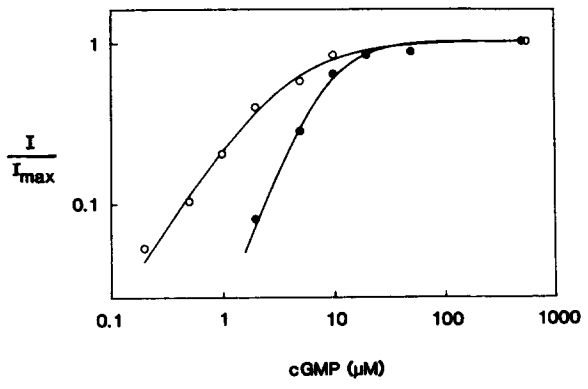



FIGURE 6. Double logarithmic plot of dose–response relations for cGMP before (●) and after (○) potentiation by $Ni^{2+}$. Results from one patch. Steady-state cGMP-activated currents measured at +50 mV were normalized by the saturating value ($I_{max}$). 10 μM $NiCl_2$ was applied to the patch as described in the text. Smooth curves are fits to the Hill equation (see text): pre-$Ni^{2+}$ – $n = 1.8$, $K_{1/2} = 8.3$ μM; post-$Ni^{2+}$ – $n = 1.1$, $K_{1/2} = 3.4$ μM. $I_{max}$ was 1,330 pA before $Ni^{2+}$ and 1,590 pA after $Ni^{2+}$ potentiation.

below those tested in Fig. 6. Exposure of the patch to 1 mM EDTA for 40 min shifted the dose–response relation back to the right, near the original relation (data not shown). An 11-min exposure to EDTA caused only a partial shift. Interestingly, the relation after EDTA exposure appeared less cooperative than the original, as if this effect of potentiation did not completely reverse.

The ions $Cd^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ also potentiated the response to cGMP. $Ca^{2+}$, $Mg^{2+}$, or the polycation spermine, from nonblocking to blocking concentrations of all three (1–200 μM), did not potentiate. Ildefonse and Bennett (1991) have recently reported that $Co^{2+}$, $Ni^{2+}$, and $Fe^{2+}$ enhance the bovine rod channel response to low cGMP in planar lipid bilayers.

### *Effect of Potentiation on the Kinetics of cGMP Binding*

Previous work has shown that channel gating by cGMP is mildly voltage sensitive, and that rapid voltage changes can be used to measure the gating kinetics of the channel

(Karpen et al., 1988*a*, *b*). At subsaturating cGMP concentrations (<50 μM), channel gating kinetics were limited by the binding and unbinding of cGMP, rather than the opening and closing of liganded channels. In the experiment of Fig. 7 the voltage-jump technique was used to examine how $Ni^{2+}$ potentiation affected the kinetics of channel activation by cGMP. Relaxations in cGMP-activated currents after rapid voltage changes were measured before and after potentiation by 1 μM $Ni^{2+}$ (3-min application). After a voltage change from −50 to +50 mV, the current transient in Fig. 7 *A* at 7.5 μM cGMP rose with a time constant of ~5 ms. After potentiation by $Ni^{2+}$, the upward-going current transient in Fig. 7 *B* at 7.5 μM cGMP was too rapid to be seen on the same time scale; this was also the case at 100 μM cGMP. The droop in the larger currents at +50 mV resulted from ion depletion due to hindered

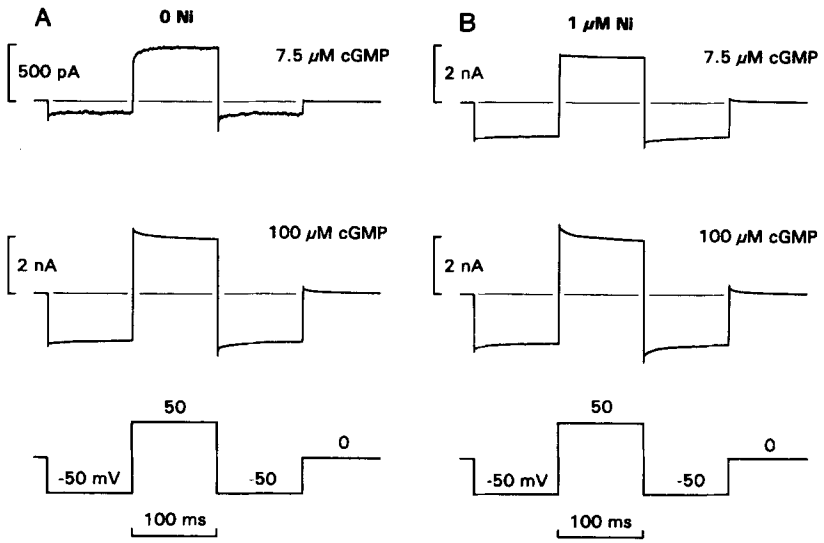



FIGURE 7. Evidence that potentiation by 1 μM $Ni^{2+}$ of the response to cGMP involves an increase in the rate of cGMP binding. Relaxations of cGMP-activated currents in response to voltage changes (shown at the bottom of each panel) were measured in the absence (*A*) or presence of 1 μM $NiSO_4$ (*B*). Data are from one patch, cGMP concentrations were as indicated, and each trace was averaged from five or six trials. Bandwidth 0–2 kHz, 5 kHz sampling.

diffusion at the cytoplasmic patch surface (Zimmerman et al., 1988). Our interpretation is that potentiation was associated with a speeding of cGMP's binding to the channel rather than a slowing of its dissociation from the channel. Similar results were obtained on three other patches.

*Effect of Low Ionic Strength on Potentiation*

Fig. 8 shows that potentiation by $Ni^{2+}$ was stronger at low than at moderate ionic strength. The points plot the patch currents evoked by brief +50-mV pulses. Initially the response to 10 μM cGMP was less than half that to 500 μM. After the patch was exposed to 5 μM $Ni^{2+}$ for 8 min, the response to 10 μM cGMP (without added $Ni^{2+}$)

was 85% of that to 500 μM cGMP and the response to 500 μM was unchanged. The response to 5 μM cGMP was approximately half that to saturating cGMP. When 5 and 500 μM cGMP were applied immediately thereafter in a low ionic strength solution (compensated for osmolarity, see Fig. 8 legend), the current at 5 μM was >80% of the current at saturation. With 5 mM NaCl bathing the cytoplasmic surface of the patch and 130 mM NaCl inside the patch pipette, +50-mV pulses caused a reduction in inward current rather than an increase in outward current because the Na equilibrium potential was near +80 mV. The change in current caused by +50-mV pulses was small because of the low $Na^+$ concentration in the bath

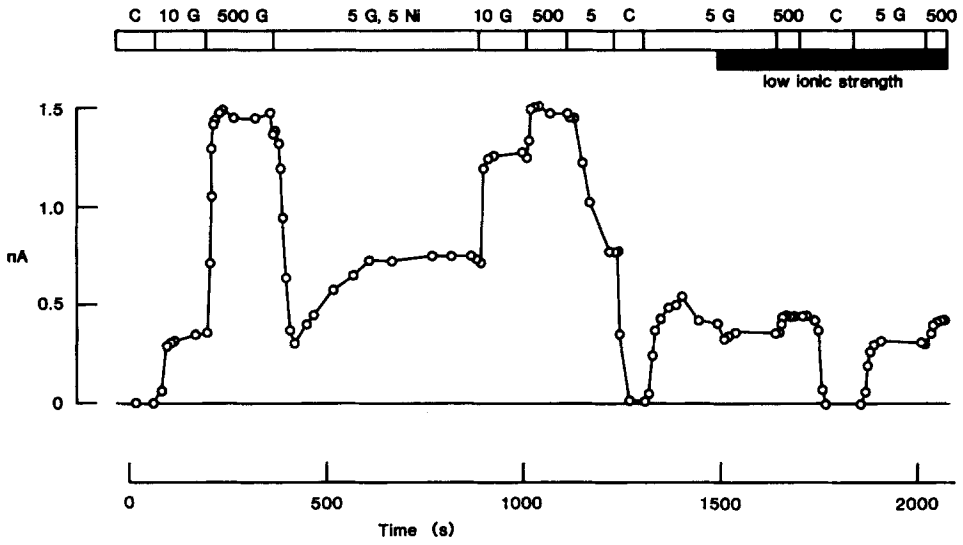



FIGURE 8. Effect of low ionic strength on potentiation of the cGMP response by $Ni^{2+}$. Solutions (indicated by bars above the record) were applied by chamber perfusion as in Fig. 5. *G* represents cGMP; *C* represents control solution lacking nucleotide (see Methods). Patch currents evoked by +50-mV pulses were measured from chart records (*open symbols*). Low ionic strength solution contained (mM): 5 NaCl, 2 Tris (pH 7.6), and 216 sucrose. In this solution the net current was inward and the change in current caused by the +50-mV pulse is plotted. The current at 500 μM cGMP was reduced by the low internal $Na^+$ concentration (see text). Bandwidth 0–1 kHz.

(Zimmerman and Baylor, 1992). Upon returning to normal ionic strength, 5 μM cGMP was again relatively less effective (results not shown). The currents activated by low cGMP remained larger than those early in the experiment. Just before the application of the low ionic strength solution, however, some loss of potentiation in the response to 5 μM cGMP was apparent. Similar effects of low ionic strength on potentiation were observed in two other patches. In the absence of $Ni^{2+}$ or other potentiating ions, low ionic strength had little effect on the dose–response relation for cGMP.

## DISCUSSION

### *The Channel Can Close on $Mg^{2+}$ or $Ca^{2+}$ and Retain Rapid Gating Kinetics*

The results indicate that $Mg^{2+}$ or $Ca^{2+}$ ions bind in closed and open channels with similar affinity. Consequently, block by these ions does not appreciably change the proportion of channels that are open in the steady state. Block also does not appear to change the kinetics of channel gating by cGMP, which is rapid. Cobbs and Pugh (1987) found that after an intense flash of light the photocurrent shut off with a 2–3-ms time constant; the experiments were done with millimolar concentrations of $Ca^{2+}$ and $Mg^{2+}$ outside the cell. In a previous study of channel kinetics in excised patches in the absence of divalent cations (Karpen et al., 1988*a*), we estimated that channels close with a 2-ms time constant after an instantaneous drop in cGMP. The similarity of the rates suggests that a drop in cGMP closes channels as rapidly when they are blocked over 99% of the time by divalent cations as when they are occupied less than half the time by monovalent cations. If channels were unable to close with a divalent cation lodged in the pore, block should slow the closing of channels in response to a drop in cGMP by a factor of $>100$. This is because channels would have to unblock before they could close. For mechanism I, in which channels can close on a bound divalent, the apparent rate constant for closing after an instantaneous drop in cGMP, $k_{resp}$, is independent of the fraction of channels that are blocked (Eq. 6), because blocked channels close as rapidly as open (unblocked) channels. For mechanism II, in which channels cannot close on a divalent, $k_{resp}$ decreases as the fraction of blocked channels increases (Eq. 10). Divalent block slows channel closing in mechanism II even if the blocking and unblocking rates are rapid compared with the rate at which cGMP dissociates from the channel. Therefore, mechanism II is inconsistent with the previous kinetic findings. The conclusion that the channel is able to rapidly close with $Mg^{2+}$ or $Ca^{2+}$ bound in the pore is not unique to the particular gating mechanisms presented here. The mechanisms proposed here do not account for a second open state of smaller conductance observed in single channel recording (Haynes et al., 1986; Zimmerman and Baylor, 1986). However, analysis of more complex gating schemes in which cGMP changes the equilibrium between collections of closed and open states still leads to the conclusion that the channel can close on $Mg^{2+}$ or $Ca^{2+}$.

Because cGMP dissociation from the channel is slower by about an order of magnitude than the closing of liganded channels in the absence of divalent cations (Karpen et al., 1988*a*, *b*), it is not clear whether blocked channels close at precisely the same rate as open, unblocked channels. Nevertheless, the closing of blocked channels does not seem to limit the channel response to a drop in cGMP. The channel retains rapid gating kinetics in the presence of strong divalent block.

### *The Channel Cannot Close on All Blocking Ions*

Internal $Zn^{2+}$ and $Cd^{2+}$ blocked cGMP-activated $Na^+$ current in a voltage-dependent manner, yet in contrast to $Mg^{2+}$, $Ca^{2+}$, and $Ni^{2+}$, the efficiency of steady-state block was proportional to the fraction of open channels. This behavior suggests that these ions preferentially bind to open channels and prevent or significantly impede closing. It seems unlikely that potentiation of the response to low cGMP, in parallel with

block, made it appear that these ions blocked less effectively at low cGMP. This is because block by $Ni^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ at low cGMP was only measured after potentiation had leveled off. Potentiation had a slow onset and was very slowly reversible (see Results). It is difficult to rule out the possibility that a more rapidly reversible form of potentiation can occur in parallel with block. However, $Ni^{2+}$, which potentiated very similarly to $Zn^{2+}$ and $Cd^{2+}$, blocked currents with higher efficiency at low cGMP (Table I).

The inability of the channel to close on certain blocking ions indicates that the structure of the ion binding site is different in closed and open channels. The ionic radius of $Zn^{2+}$ is smaller than that of $Ca^{2+}$ (0.74 vs. 0.99 Å; Hille, 1984), so that unhydrated ionic size does not appear to be the sole determinant of whether a closed channel can contain an ion.

### *A Gate External to the Ion Binding Site(s)*

Model A (Fig. 4) is consistent with the asymmetry of $Ni^{2+}$ blocking kinetics on the inside and outside (Fig. 3), and the inability to trap $Ni^{2+}$ in a closed channel. It also provides a possible explanation for why internal $Mg^{2+}$, $Ca^{2+}$, and especially $Ni^{2+}$ appear to bind with higher affinity to closed than open channels. In model A, an ion bound in a closed channel can only exit to the inside. In an open channel, a bound ion can exit to the inside or outside. The additional exit path would result in a higher effective "off" rate and a lower apparent affinity. Since the structure of the ion binding site appears to be different in closed and open channels, it is also possible that the $Mg^{2+}$, $Ca^{2+}$, and $Ni^{2+}$ simply have a higher intrinsic affinity for the closed channel site.

The results in Fig. 1 seem difficult to reconcile with model A, however. If a gate is located between the ion binding site and the external solution, then external $Mg^{2+}$ should block current more effectively at high cGMP, where more channels are open. As described earlier, this would shift the dose–response relation for cGMP to lower concentrations. No such behavior was observed in Fig. 1. Instead, external $Mg^{2+}$ appears to occupy open and closed channels about equally in the steady state. This might be consistent with model A if the dwell time of $Mg^{2+}$ in closed channels were long compared with the time the channel is closed. Under these conditions, $Mg^{2+}$ dissociation to the inside from its pore site would be relatively slow, and the pore would remain occupied even during periods in which $Mg^{2+}$ entry from the outside is precluded by a closed gate. It remains to be determined whether $Mg^{2+}$ behaves in this way.

### *Divalent Block at More Than One Site?*

Previous results have been explained by assuming that $Na^+$ and divalent cations bind one at a time to a single site within the pore (Karpen et al., 1988*b*; Zimmerman and Baylor, 1988, 1992; Menini, 1990; see also Fig. 2 above). The results in Fig. 3 are difficult to explain if $Ni^{2+}$ ions from both the inside and outside bind to only a single site in the pore. If this were the case, the kinetics of block by internal $Ni^{2+}$ at +50 mV and saturating cGMP (upper left) should reflect the rapid dissociation rate to the outside observed when block by external $Ni^{2+}$ is relieved at the same voltage (upper right). The block by internal $Ni^{2+}$ should have a much lower affinity and occur much

more rapidly when a higher fraction of channels are open. Instead, the blocking kinetics by internal $Ni^{2+}$ are slow at both low and high cGMP. One interpretation would be that block by internal and external $Ni^{2+}$ occurs at different sites within the pore and that $Ni^{2+}$ moves from an internal to an external site on a time scale slower than the blocking kinetics from the inside. Although internal $Ni^{2+}$ competes with internal $Mg^{2+}$ (Fig. 2), and $Mg^{2+}$ passes through the channel (Nakatani and Yau, 1988*a*; Zimmerman and Baylor, 1988, 1992; Colamartino et al., 1991), we do not know whether $Ni^{2+}$ can pass through the channel. This, as well as the fractional electrical distance of the blocking sites from both sides of the membrane, are subjects for future study.

Furman and Tanaka (1990) have suggested that the channel processes more than one ion at a time, because the permeabilities of $NH_4^+$ and $Li^+$ relative to $Na^+$ depended on absolute ion concentrations (see Hille, 1984). It remains to be determined whether monovalent cations bind to the different sites suggested by the kinetics of internal and external $Ni^{2+}$ block. Menini (1990) has proposed that the channel may contain cation binding sites in an inner vestibule because inward $Na^+$ currents at −100 mV were reduced by internal monovalent cations.

*Comparison with Other Channels*

Delayed rectifier $K^+$ channels from squid axon can close with a divalent cation bound in the pore (Armstrong, Swenson, and Taylor, 1982). Furthermore, it appears that $Ba^{2+}$ can block both closed and open channels from the external medium, but can only enter the channel from the inside when activation gates are open. This behavior is similar to the cGMP-activated channel, but with a gate internal rather than external to the binding site or sites. $Ba^{2+}$ binds much more tightly to closed channels, which is true of $Ni^{2+}$ for the cGMP-activated channel. Despite these similarities, a striking difference is that external monovalent cations impede the closing of the squid axon $K^+$ channel (Matteson and Swenson, 1986), and $Ca^{2+}$ appears to be required for normal channel closing (Armstrong and Matteson, 1986). The cGMP-activated channel closes very rapidly in the absence of divalent cations (Karpen et al., 1988*a*, *b*; Matthews and Watanabe, 1988), and physiological divalents have little or no effect on the ability of the channel to close. Marchais and Marty (1979) have proposed that occupancy by permeant ions impedes the closing of acetylcholine receptors from *Aplysia* neurons, while Swandulla and Armstrong (1989) have shown that $Ca^{2+}$ channels from chicken dorsal root ganglion cells can close when occupied by $Cd^{2+}$. Voltage-dependent $Ca^{2+}$-activated $K^+$ channels from rat skeletal muscle (Miller et al., 1987) and voltage-dependent $K^+$ channels from human T lymphocytes (Grissmer and Cahalan, 1989) not only close on divalent cations, but trap $Ba^{2+}$ in the closed channel. We were unable to trap $Ni^{2+}$ in the cGMP-activated channel, and internal $Ni^{2+}$ appears to have access to a blocking site when channels are closed.

*Potentiation of the Response to Low cGMP by Transition Metal Divalent Cations*

The potentiating cations $Ni^{2+}$, $Cd^{2+}$, $Zn^{2+}$, and $Mn^{2+}$, at nonblocking concentrations, speeded the binding of cGMP. Enhancement of the effect by low ionic strength argues for an electrostatic mechanism. The potentiating cations presumably bind on or near the channel protein and attract cGMP, which carries a negative charge at

physiological pH. The divalents could increase the effective concentration of cGMP near the binding sites, the bimolecular rate constants for cGMP binding, or both. A strong electrostatic effect on cGMP binding with no effect on $Na^+$ permeation argues that the cGMP binding sites are well separated from the permeation pathway.

Although potentiation was not observed with $Ca^{2+}$, $Mg^{2+}$, or spermine, it was reversed by isotonic $MgCl_2$. $Mg^{2+}$ and the other ions may bind to the same sites, but with much lower affinity. The binding sites may resemble those in a chelator such as EDTA, which binds the four transition metal ions 5–10 orders of magnitude more tightly than $Mg^{2+}$.

Might potentiation regulate the sensitivity of channels to cGMP under physiological conditions? Rod outer segments have been reported to contain bound $Zn^{2+}$ at an overall concentration of 300 μM (McCormick, 1985). Furthermore, the effective half-saturating concentration of cGMP has been found to vary significantly from patch to patch (Zimmerman and Baylor, 1986; Zhainazarov and Kolesnikov, 1990). Sensitivity to cGMP may be regulated by potentiation or an analogous mechanism (e.g., phosphorylation) that changes the charge density near cGMP binding sites.

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