

# Cyclic Nucleotide-gated Channels of Rat Olfactory Receptor Cells: Divalent Cations Control the Sensitivity to cAMP

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**ABSTRACT** cAMP-gated channels were studied in inside-out membrane patches excised from the apical cellular pole of isolated olfactory receptor cells of the rat. In the absence of divalent cations the dose-response curve of activation of patch current by cAMP had a  $K_M$  of 4.0  $\mu\text{M}$  at  $-50$  mV and of 2.5  $\mu\text{M}$  at  $+50$  mV. However, addition of 0.2 or 0.5 mM  $\text{Ca}^{2+}$  shifted the  $K_M$  of cAMP reversibly to the higher cAMP concentrations of 33 or 90  $\mu\text{M}$ , respectively, at  $-50$  mV. Among divalent cations, the relative potency for inducing cAMP affinity shifts was:  $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Mn}^{2+} > \text{Ba}^{2+} > \text{Mg}^{2+}$ , of which  $\text{Mg}^{2+}$  (up to 3 mM) did not shift the  $K_M$  at all. This potency sequence corresponds closely to that required for the activation of calmodulin. However, the  $\text{Ca}^{2+}$ -sensitivity is lower than expected for a calmodulin-mediated action. Brief (60 s) transient exposure to 3 mM  $\text{Mg}^{2+}$ , in the absence of other divalent cations, had a protective effect in that following washout of  $\text{Mg}^{2+}$ , subsequent exposure to 0.2 mM  $\text{Ca}^{2+}$  no longer caused affinity shifts. This protection effect did not occur in intact cells and was probably a consequence of patch excision, possibly representing ablation of a regulatory protein from the channel cyclic nucleotide binding site. Thus, the binding of divalent cations, probably via a regulatory protein, controls the sensitivity of the cAMP-gated channels to cAMP. The influx of  $\text{Ca}^{2+}$  through these channels during the odorant response may rise to a sufficiently high concentration at the intracellular membrane surface to contribute to the desensitization of the odorant-induced response. The results also indicate that divalent cation effects on cyclic nucleotide-gated channels may depend on the sequence of pre-exposure to other divalent cations.

## INTRODUCTION

The stimulation of olfactory receptor neurons by odorants results in the formation of cAMP, which directly gates cation-conducting membrane ion channels, leading to membrane depolarization and spike initiation (for a review, see Reed, 1992). During

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continuous odorant exposure, odorant-induced responses usually display a progressive attenuation, or desensitization. The mechanisms involved in this process have not yet been clearly elucidated, yet are crucial to the understanding of olfactory transduction because desensitization, by modulating the time-dependence of neuronal firing patterns, contributes to the coding of odor information (Kauer, 1987).

The cAMP-gated channels which support the odor-induced current are similar in their permeation properties to the vertebrate photoreceptor cGMP-gated channels in conducting mainly monovalent cations (Nakamura and Gold, 1987; Yau and Baylor, 1989; Frings, Lynch and Lindemann, 1992a), but with a significant permeability to  $\text{Ca}^{2+}$  (Nakatani and Yau, 1988; Kurahashi and Shibuya, 1990). Because the removal of extracellular  $\text{Ca}^{2+}$  dramatically prolongs the duration of these currents (Kurahashi, 1990; Kurahashi and Shibuya, 1990), it is possible that  $\text{Ca}^{2+}$  influx through cyclic nucleotide-gated (CNG) channels may contribute to signal termination. Indeed, two separate mechanisms compatible with this idea have recently been proposed. A  $\text{Ca}^{2+}$ -activated, calmodulin-dependent phosphodiesterase, which is concentrated in olfactory cilia, reduces cAMP concentration leading to channel closure (Borisy, Ronnett, Cunningham, Juilfs, Beavo and Snyder, 1992). However, phosphodiesterase inhibitors only partially remove desensitization (Firestein, Darrow, and Shepherd, 1991; Boekhoff and Breer, 1992), suggesting involvement of at least one additional mechanism.

Evidence has also been presented for a distinct inhibitory mechanism whereby  $\text{Ca}^{2+}$  influx may reduce the sensitivity of the CNG channels to cAMP in catfish olfactory neurons (Kramer and Siegelbaum, 1992). The effect was attributed to an as yet unidentified  $\text{Ca}^{2+}$ -sensitive regulatory protein. The affinity shift was not sensitive to a calmodulin inhibitor, and the time-dependent washout after patch excision was not affected by exogenous calmodulin application (Kramer and Siegelbaum, 1992). In olfactory neurons of the salamander, Zufall, Shepherd, and Firestein (1991) had previously found a strong inhibition at low  $\text{Ca}^{2+}$  concentrations. However, this effect was observed at saturating cAMP concentrations, suggesting either a far larger  $\text{Ca}^{2+}$ -dependent shift than observed by Kramer and Siegelbaum (1992), or perhaps a different inhibitory mechanism.

$\text{Ca}^{2+}$  has also recently been reported to inhibit the cGMP affinity of the photoreceptor CNG channel. In this case, the regulatory protein was identified as calmodulin (Hsu and Molday, 1993). On the other hand, Gordon, Brautigan, and Zimmerman (1992) found that  $\text{Ca}^{2+}$  induced an increase in the cGMP affinity of these channels. This affinity shift was inhibited by a protein phosphatase inhibitor, suggesting modulation of channel function by phosphorylation.

It is not yet possible to reconcile all of these results into a simple scheme. It appears that  $\text{Ca}^{2+}$  may exert multiple actions, with different effects predominating either in different species or under different experimental conditions. In this report, we describe the inhibitory effects of divalent cations on cAMP-gated channels from olfactory cells of the rat. In accordance with Kramer and Siegelbaum (1992), we found that  $\text{Ca}^{2+}$  ions shifted the cAMP-dependence of channel activation to much higher concentrations. However, the effect was significantly different in that higher  $\text{Ca}^{2+}$  concentrations were required and it did not wash out with time in excised patches. We also found that the ability of  $\text{Ca}^{2+}$  to induce the inhibition was strongly

dependent upon the history of patch exposure to divalent cations. In particular, the inhibition was irreversibly abolished by brief pre-exposure to 3 mM  $Mg^{2+}$ , but only in the absence of  $Ca^{2+}$ . We also investigated the relative potency of different divalent cations in causing the inhibitory effect. The potency sequence was found to be similar to that required for the activation of calmodulin. Short summaries of our findings have appeared in abstract form (Lindemann, Frings, and Lynch, 1992; Lynch and Lindemann, 1992).

#### MATERIALS AND METHODS

Cell dissociation and electrophysiological procedures have previously been described in detail (Lynch and Barry, 1991; Frings et al., 1992a). Briefly, the olfactory neuroepithelium from adult rats was enzymatically dissociated using 0.022% trypsin (066-07072E, Gibco Laboratories, Grand Island, NY) in a medium without added divalent cations (Table I) and incubated for 35 min at 37°C. Epithelial pieces were then gently triturated and suspended cells were allowed to settle onto the base of a glass-bottomed recording chamber. Once settled, cells were continuously superfused by a modified Tyrode solution (Table I). Although several cell types were isolated, olfactory neurons were readily identified by their distinct bipolar shape.

TABLE I  
Solutions

	NaCl	KCl	CaCl <sub>2</sub>	MgCl <sub>2</sub>	EGTA	EDTA	NaOH	Glucose
	<i>Concentrations in mM</i>							
Dissociation solution	145	5	—	—	—	—	5	10
Tyrode solution	140	5	2	1	—	—	5	10
Pipette solution	140	—	—	—	10	—	25	—
Low Ca solution	160	—	—	—	—	—	5	—
0 Ca solution	155	—	—	—	2	—	10	—
0 Ca/Mg solution	150	—	—	—	—	5	15	—

The low Ca solution was prepared without added  $Ca^{2+}$  salts. Its free  $Ca^{2+}$  concentration was near 3  $\mu$ M as measured with a  $Ca^{2+}$  sensitive electrode (ETH 1001).

The 0 Ca solution had a free  $Ca^{2+}$  concentration of <1 nM, as calculated with a  $K_d$  for EGTA- $Ca^{2+}$  of 315 nM  $Ca^{2+}$  (Bers, 1982) for a total  $Ca^{2+}$  concentration of 3  $\mu$ M.

All solutions contained 10 mM HEPES and were adjusted to pH 7.4 with NaOH.

Membrane currents were recorded using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). Patch pipettes, pulled in two stages from borosilicate tubing (H15/10, Jenkoms Scientific, Leighton Buzzard, Bedfordshire, UK), typically had tip resistances of 10 M $\Omega$  when filled with the standard divalent cation-free pipette solution (Table I). Cell-attached membrane patches were formed amongst the cilia on the apical knobs of visually identified olfactory neurons and detached into the inside-out configuration by brief air exposure. Patches from the apical knobs typically contained up to several hundred cAMP-activated channels with unitary conductances of 14 pS, whereas those pulled from the soma contained few, if any, cAMP-activated channels.

After formation of an inside-out patch, pipettes were moved along a narrow channel of solution to a separate compartment, into which up to 12 different solutions flowed through adjacently-placed tubes. Pipette tips were placed near the outflow of each tube by moving the microscope stage. When necessary to ascertain the sequence of exposure, the flow from certain tubes was stopped. Current-voltage ( $I[V]$ ) relationships were measured by applying digitally-

generated voltage ramps of 1-s duration in both directions and averaging the responses to (usually) eight ramps. Divalent cations normally reduced the background conductance of the patch in a reversible, concentration-dependent manner. Therefore, for each concentration of each divalent cation (see Table I), the cAMP-gated current was measured as the difference in current responses to voltage ramps applied in the presence and the absence of cAMP.

All patches were excised into Tyrode solution, then transiently exposed to a divalent cation-free solution (0 Ca solution [see Table I]), containing 100  $\mu\text{M}$  cAMP to test for the presence of CNG channels, before commencement of the experiment. Patches exposed only to these solutions are described as naive. Divalent cation sequence protocols were then applied as described in the Results.

The solutions are summarized in Table I. In general, responses were measured under conditions of symmetrical [NaCl]. The patch pipette solution was used in all experiments. The 0 Ca solution (2 mM EGTA and no added  $\text{Ca}^{2+}$ ) was used to measure responses in the absence of  $\text{Ca}^{2+}$ . Responses at different  $\text{Ca}^{2+}$  concentrations were measured using the low Ca solution (3  $\mu\text{M}$   $\text{Ca}^{2+}$  when no  $\text{Ca}^{2+}$  was added) or a Ca solution, where  $\text{CaCl}_2$  was added to the low Ca solution to the concentration indicated. Responses to all other divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Sr}^{2+}$ ) were measured in 0 Ca solution, with the chloride salt of the relevant divalent cation added to give the concentration indicated. The  $\text{Na}^+$  salt of cAMP (Sigma Chemical Co., St Louis, MO) was used in all experiments.

All voltages are expressed as potential of the internal with respect to the external membrane surface. Experiments were performed at room temperature (20–22°C).

## RESULTS

### *Micromolar Concentrations of $\text{Ca}^{2+}$ Do Not Decrease the cAMP-dependent Current in the Rat*

Recent results obtained with membrane patches excised from catfish olfactory cells indicate that  $\text{Ca}^{2+}$  on the cytosolic side of the membrane inhibits the cAMP-induced current with a half-maximal effect near 3  $\mu\text{M}$  (Kramer and Siegelbaum, 1992). We attempted to reproduce this effect, using inside-out membrane patches excised from apical knobs of rat olfactory receptor neurons. In Fig. 1A, the cAMP dose-response measured in the presence of 3  $\mu\text{M}$  [ $\text{Ca}^{2+}$ ] (*circles*) is compared with results from an earlier study performed under identical conditions (Frings et al., 1992a), but where [ $\text{Ca}^{2+}$ ] was < 10 nM (*curves*). There was no significant difference in the sensitivity to cAMP.

Another possibility, inferred from the data of Zufall et al. (1991), is that 3  $\mu\text{M}$  [ $\text{Ca}^{2+}$ ] may inhibit the current by a mechanism unrelated to a shift in the cAMP sensitivity. To test this possibility,  $I(V)$ s were measured in different [ $\text{Ca}^{2+}$ ] in the presence of a saturating concentration (100  $\mu\text{M}$ ) of cAMP. As displayed in Fig. 1B, decreasing the free  $\text{Ca}^{2+}$  concentration from 3  $\mu\text{M}$  to < 3 nM by the progressive addition of EGTA, had no noticeable effect. A similar result was obtained with each of three patches.

Thus, we were unable to reproduce in rat olfactory cells the inhibitory effect of  $\text{Ca}^{2+}$  at the same concentration as has been reported in the salamander or catfish. Although the dose-response experiments (Fig. 1A) were completed within 5 min after patch excision, it is possible that the lack of effect may have been attributable to the rapid washout of a cytosolic factor.

*Inhibition of cAMP-dependent Current by Divalent Cations at Higher Concentrations*

When cAMP concentrations of 1, 3, 10, and 100  $\mu\text{M}$  were sequentially applied to the cytosolic side of a membrane patch in the absence of  $\text{Ca}^{2+}$ , the  $I(V)$  relationships in Fig. 2 A (upper left) were generated. The apparent dissociation constant ( $K_M$ ) for cAMP was near 3  $\mu\text{M}$ . When repeated in the presence of 0.5 mM  $\text{Ca}^{2+}$  (Fig. 2 A, lower left), the current magnitudes were decreased in a manner which depended on the concentration of cAMP. Similar behavior was observed in each of five patches.

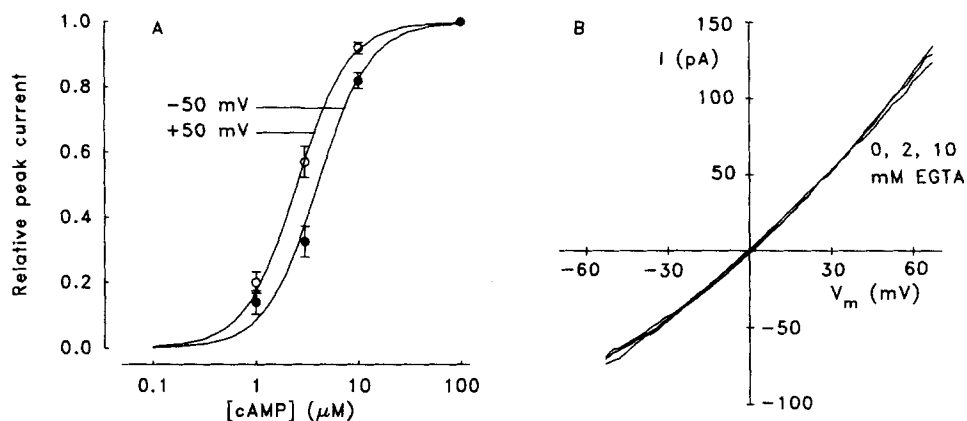


FIGURE 1. Low concentrations of  $\text{Ca}^{2+}$  (near 3  $\mu\text{M}$ ) are without effect on the activation of channels by cAMP. In this and all subsequent figures, the  $I(V)$  relationships show difference currents obtained in the presence and absence of cAMP, for each divalent cation condition. (A) Dose-responses for cAMP at +50 (hollow circles) and -50 mV (filled circles) averaged from seven inside-out patches exposed to 0 Ca solution (see Table I), having a free  $[\text{Ca}^{2+}]$  of <1 nM. Error bars represent SEM. The sigmoid curves represent the corresponding best fits to dose-responses averaged from seven patches measured in low Ca solution (free  $[\text{Ca}^{2+}] \sim 3 \mu\text{M}$ ) reproduced from Frings et al. (1992). The fitted curves had Hill coefficients of 1.8 and  $K_M$  values of 2.5  $\mu\text{M}$  (+50 mV) and 4.0  $\mu\text{M}$  (-50 mV). (B)  $I(V)$  curves obtained from one inside-out patch in the presence of 100  $\mu\text{M}$  cAMP, showing that the addition of 2 or 10 mM EGTA to the low Ca solution had no effect. The three superimposed curves were obtained upon exposure to low Ca solution (no EGTA), 0 Ca solution (2 mM EGTA) and 10 mM EGTA solution. The latter contained (in mM): NaCl 160, EGTA 10, NaOH 25 (pH 7.4). Equivalent results were obtained with two other patches.

The inhibitory effect of  $\text{Ca}^{2+}$  is highlighted by plotting the ratios of currents recorded for [cAMP]s of 3, 10, and 100  $\mu\text{M}$  in the presence of 0.5 mM  $\text{Ca}^{2+}$  with respect to those measured in the absence of  $\text{Ca}^{2+}$ . Fig. 2 A (right) shows such ratio plots averaged from five patches. Although some  $\text{Ca}^{2+}$ -induced suppression occurred at all [cAMP]s, it was progressively overcome at higher cAMP concentrations. Corresponding results were reported for the cGMP-gated channel of the tiger salamander photoreceptor (Colamartino, Menini, and Torre, 1991).

When a similar experiment was performed using 0.5 mM  $\text{Mg}^{2+}$  instead of  $\text{Ca}^{2+}$ , the results were different. In the example shown in Fig. 2 B, 0.5 mM  $\text{Mg}^{2+}$  induced a strongly voltage-dependent block, but the ratio plots of current recorded in the

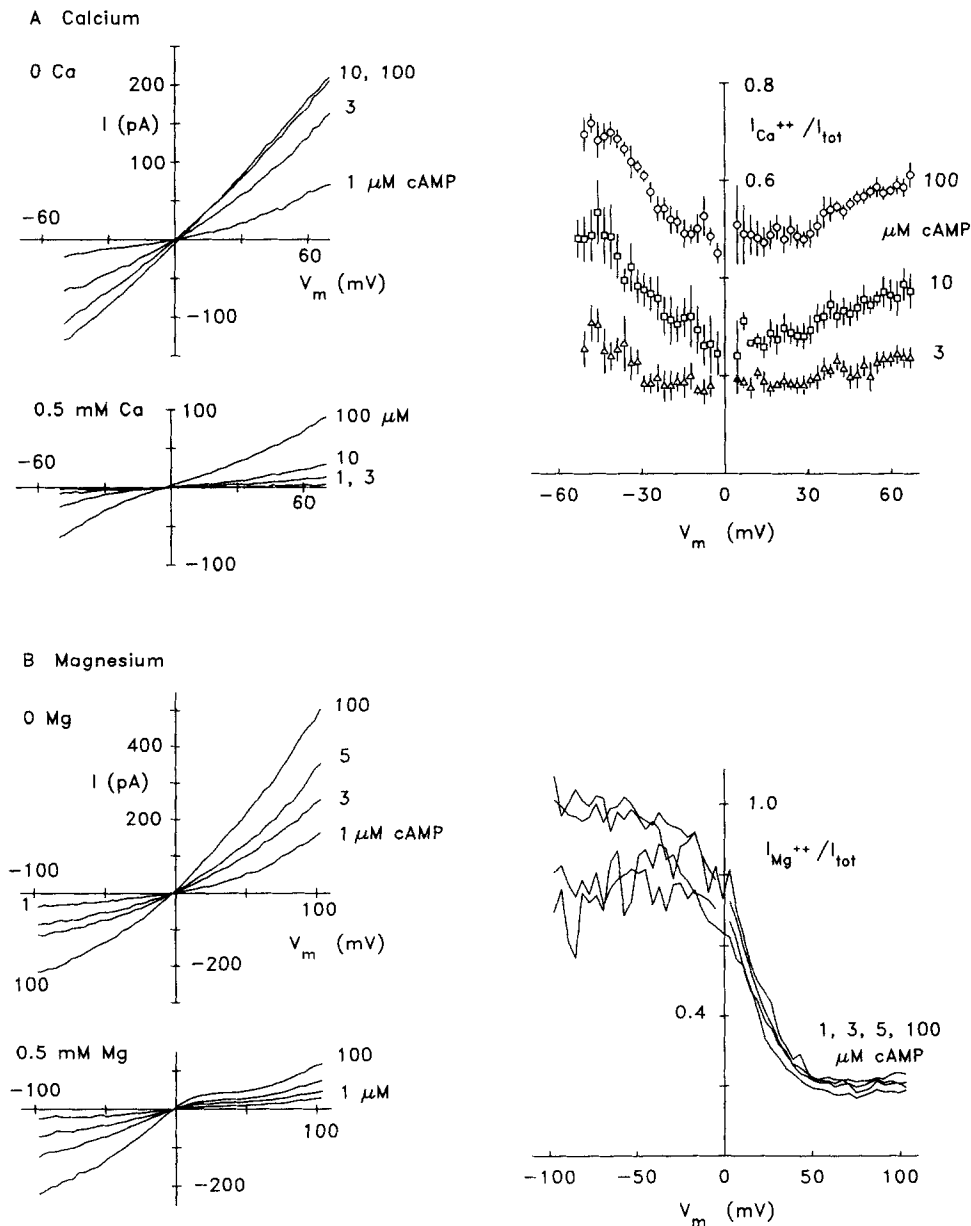


FIGURE 2. The relative current-inhibition caused by  $Ca^{2+}$  appears to depend on [cAMP], while that caused by  $Mg^{2+}$  does not. (A) The upper left panel displays a series of  $I(V)$  relationships for cAMP of 1, 3, 10, and 100  $\mu M$  in the absence of  $Ca^{2+}$ . In the lower left panel, the experiment was repeated after  $[Ca^{2+}]$  had been increased to 0.5 mM (same patch). The right panel shows current inhibition ratios, i.e. current values recorded in the presence of 0.5 mM  $[Ca^{2+}]$  divided by those obtained at 0 mM  $[Ca^{2+}]$ . Inhibition ratios for cAMP concentrations of 3, 10, and 100  $\mu M$  are shown as triangles, squares and circles (averaged from five patches). (B) Similar experiment to A, but using 0.5 mM  $[Mg^{2+}]$  instead of 0.5 mM  $[Ca^{2+}]$ . All data from the same patch. Left panels: examples of  $I(V)$  relationships generated in response to cAMP concentrations of 1, 3, 5 and 100  $\mu M$  in the absence (upper) and presence (lower) of 0.5 mM  $[Mg^{2+}]$ . (Right) Current inhibition ratios of the data displayed in the left panel, indicating a lack of dependence on [cAMP].

presence with respect to the absence of 0.5 mM  $Mg^{2+}$  were not consistently affected by [cAMP]. This was confirmed in each of four patches. For the photoreceptor CNG channel, Colamartino et al. (1991) found a different behavior, i.e., that the  $Mg^{2+}$ -induced suppression had a similar [cGMP]-dependence to that seen for  $Ca^{2+}$ .

To explain this interaction between  $Ca^{2+}$  and cyclic nucleotide effects, it was suggested that a higher cyclic nucleotide concentration opens the channel to a larger conductance state, thereby facilitating  $Ca^{2+}$  permeation and reducing the effectiveness of a supposed pore blocking effect of  $Ca^{2+}$  (Colamartino et al., 1991; Kaupp and Koch, 1992). However, as described in the next section, this effect can be explained by  $Ca^{2+}$ -dependent inhibition of the activation of the channels by cAMP.

#### *Ca<sup>2+</sup> Decreases Sensitivity to cAMP*

The possibility that  $Ca^{2+}$  may modulate the sensitivity of the channels to activation by cAMP was tested by measuring the cAMP dose-responses in the presence of 0.5 mM  $Ca^{2+}$ . One example of such an experiment is shown in Fig. 3A (left). Compared to Fig. 2A, where 3  $\mu$ M cAMP caused about half-maximal activation, a more than 10-fold higher [cAMP] was now needed. The right panel shows cAMP dose-responses averaged from currents recorded in five patches in 0.5 mM  $Ca^{2+}$  (squares), at both +50 mV (hollow squares) and -50 mV (filled squares). Both dose-responses were fitted by curves with a Hill coefficient of 1.7 and  $K_M$  values of 55  $\mu$ M at +50 mV and 90  $\mu$ M at -50 mV. The cAMP dose-response obtained in the absence of divalent cations is also included for comparison (Fig. 3A, circles), using data replotted from Frings et al. (1992a). These data were fitted with a Hill coefficient of 1.8 and  $K_M$  values of 2.5  $\mu$ M (+50 mV, hollow circles) and 4.0  $\mu$ M (-50 mV, filled circles), respectively.  $K_M$  values are indicated on the abscissa of each dose-response plot.

These results show a marked increase in the  $K_M$  for cAMP caused by the presence of 0.5 mM  $Ca^{2+}$ . A lower [ $Ca^{2+}$ ] of 0.2 mM caused a less pronounced inhibition, as will be discussed below. During continuous exposure to a solution of constant [ $Ca^{2+}$ ], time-dependent shifts in the  $K_M$  for cAMP were never observed. Furthermore, the  $Ca^{2+}$ -dependent inhibition was completely and rapidly reversed as  $Ca^{2+}$  was added or removed. The effect indicates that  $Ca^{2+}$  present at the internal surface of the membrane causes desensitization of the channels with respect to their agonist. The shift of the  $K_M$  must have contributed strongly to the apparent interaction between  $Ca^{2+}$  and cAMP as represented in Fig. 2A and suggests that a similar effect may also exist for photoreceptor CNG channels (Colamartino et al., 1991).

The relative abilities of  $Mg^{2+}$ ,  $Ba^{2+}$ ,  $Sr^{2+}$ , and  $Mn^{2+}$  to inhibit cAMP-induced current activation were also examined. Unlike  $Ca^{2+}$ , none of these cations had any effect at concentrations of 0.5 mM at the cytosolic membrane surface. Therefore, their relative effectiveness was compared at concentrations of 3 mM. The effect of 3 mM  $Mg^{2+}$  is demonstrated in Fig. 3B. The  $I(V)$  curves show a strong voltage-dependent block, reminiscent of the  $Mg^{2+}$  effect at inward rectifier  $K^+$  channels (reviewed in Jan and Jan, 1992). However, 3 mM  $Mg^{2+}$  had no clear effect on the  $K_M$  for cAMP. The cAMP dose-response was fitted by a Hill coefficient of 1.6 and with a  $K_M$  of 2.0  $\mu$ M at +50 mV (filled circles) and 5.8  $\mu$ M at -50 mV (hollow circles). These values were not significantly different from those determined in the absence of divalent cations.

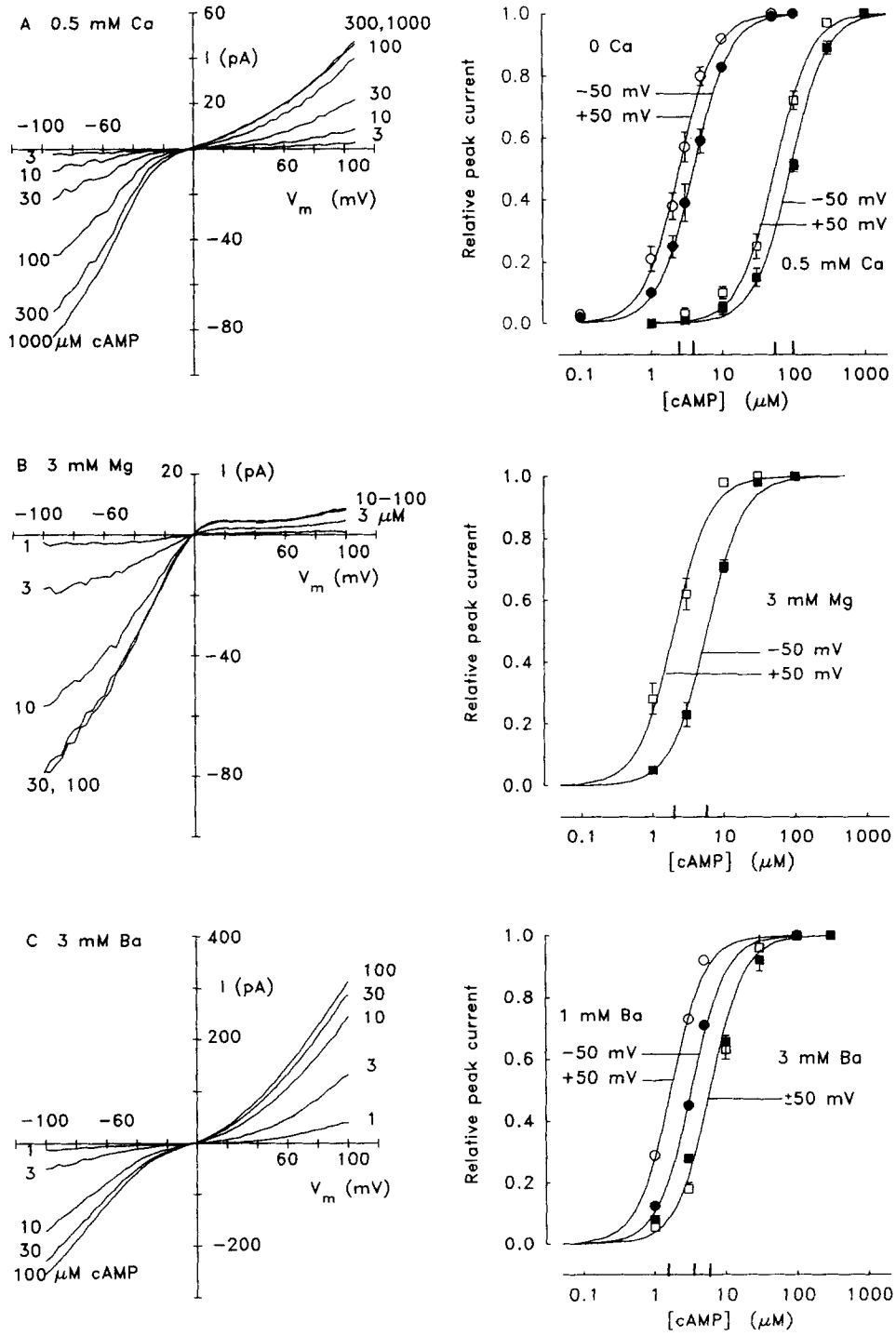


FIGURE 3.



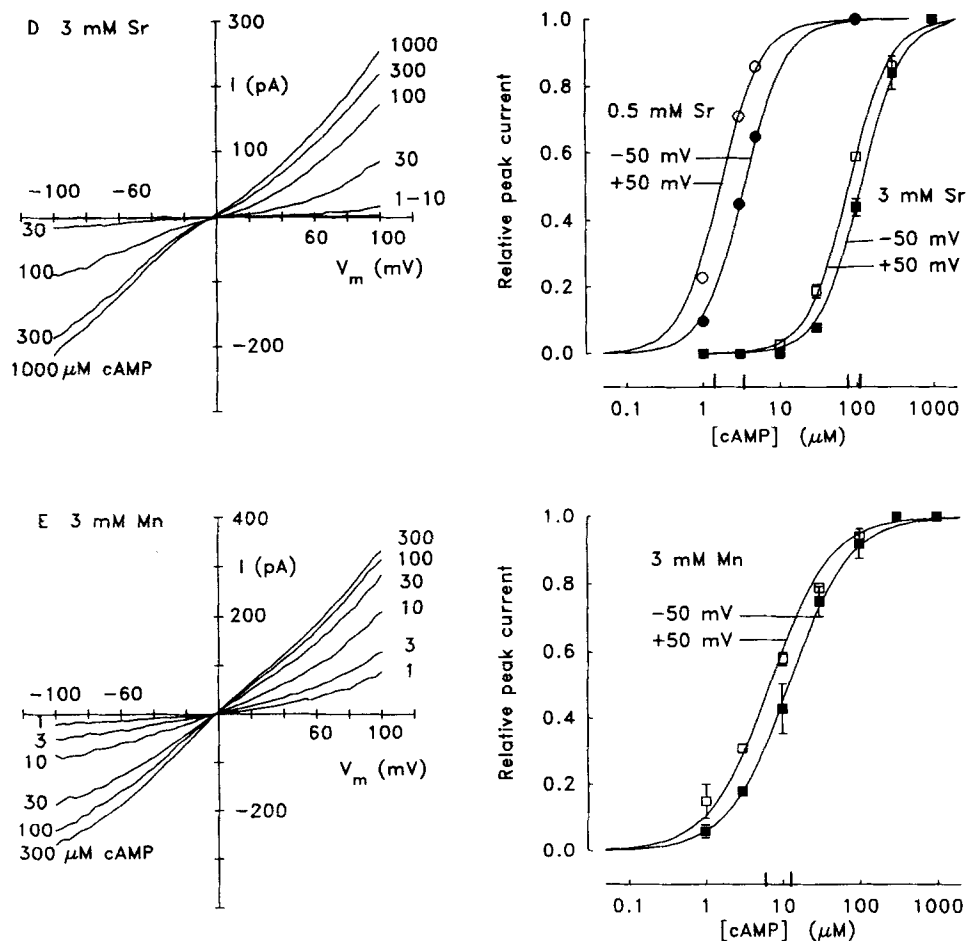
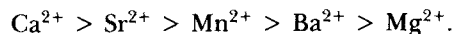


FIGURE 3. The effects of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ , and  $\text{Mn}^{2+}$  on the cAMP dose-response. For each divalent cation, cAMP dose-responses were obtained from  $I(V)$  curves, using currents at +50 and -50 mV. Because divalent cations also caused a direct channel block, the 'relative peak currents' are the currents normalized with respect to the maximum observed in each divalent cation condition. Unless otherwise indicated, each point represents data averaged from multiple patches and error bars ( $\pm$ SEM) are shown when larger than symbol size.  $K_M$  values are indicated as short lines on the [cAMP] scales. (A) The left panel shows a typical sequence of  $I(V)$  relationships recorded in one patch at the indicated [cAMP] in the presence of 0.5 mM  $\text{Ca}^{2+}$ . The right panel shows averaged cAMP dose-responses in both the absence of  $\text{Ca}^{2+}$  (circles) and in 0.5 mM  $\text{Ca}^{2+}$  (squares). The data for 0  $\text{Ca}^{2+}$  were replotted from Fig. 2 F of Frings et al. (1992a), with the fitted curves having a Hill coefficient of 1.8 and  $K_M$  values of 2.5  $\mu\text{M}$  (+50 mV) and 4.0  $\mu\text{M}$  (-50 mV). Data were averaged from eight patches. The data for 0.5 mM  $\text{Ca}^{2+}$  were averaged from five patches, with the fitted curves having a Hill coefficient of 1.7 and a  $K_M$  of 55  $\mu\text{M}$  (+50 mV) and 90  $\mu\text{M}$  (-50 mV). (B) The left panel shows an example of  $I(V)$  relationships activated by the indicated [cAMP] in the presence of 3 mM  $\text{Mg}^{2+}$ . The right panel shows the dose-response averaged from two patches, with fitted curves having a Hill coefficient of 1.6 and a  $K_M$  of 2.0  $\mu\text{M}$  (+50 mV) and 5.8  $\mu\text{M}$  (-50 mV). (C) The left panel shows an example of  $I(V)$  relationships activated by the indicated cAMP in 3 mM  $\text{Ba}^{2+}$ . The right panel

In the presence of 3 mM Ba<sup>2+</sup>, a slight increase in the K<sub>M</sub> was observed (Fig. 3 C, *squares*) although there was no effect at a concentration of 1 mM (*circles*). The voltage-dependence of cAMP activation was diminished in 3 mM Ba<sup>2+</sup>, both +50 and -50 mV having a K<sub>M</sub> of 6.0 μM. However, the Hill coefficient remained unchanged. In 3 mM Sr<sup>2+</sup>, a dramatic increase in K<sub>M</sub> was observed (Fig. 3 D, *squares*), while 0.5 mM Sr<sup>2+</sup> did not elicit this effect (*circles*). The cAMP dose-response in 3 mM Sr<sup>2+</sup> was fitted with a K<sub>M</sub> of 80 μM at +50 mV (*filled squares*) and 115 μM at -50 mV (*hollow squares*), and a Hill coefficient of 1.6. In 3 mM Mn<sup>2+</sup>, the cAMP dose-response was shifted to a value of 6 μM at +50 mV (Fig. 3 E, *filled squares*) and 12 μM at -50 mV (*hollow squares*). It also reduced the apparent Hill coefficient from 1.8 to 1.1.

Because Ca<sup>2+</sup> was the only divalent cation to be effective at 0.5 mM, the relative potencies for inhibiting the cAMP-induced activation may be assigned as follows:



When plotted as a function of ionic radius, this sequence forms a pyramid-shaped distribution, with Ca<sup>2+</sup> at the apex. Thus, the closer the ionic radius to Ca<sup>2+</sup>, the better the divalent cation acts as a substitute for Ca<sup>2+</sup>. Clearly, the abilities to block the channel and to shift cAMP sensitivity are distributed differently. For example, Mg<sup>2+</sup> is an efficient channel blocker but the poorest shifter.

It was recently reported that low (μM) concentrations of Ca<sup>2+</sup> dramatically sensitized the photoreceptor cGMP-gated channel to activation by cGMP (Ildefonse and Bennett, 1991). In the present study we were unable to demonstrate any effect of Ca<sup>2+</sup> at concentrations of 10 μM (*n* = 2), 100 μM (*n* = 3) or 1 mM (*n* = 3).

*cAMP Affinity Shifts Were Abolished by Pre-exposure to Mg<sup>2+</sup>*

Exposure of the cytosolic surface of an inside-out patch to Mg<sup>2+</sup> prevented a subsequent K<sub>M</sub> shift induced by the first divalent cation. An example of this is given in Fig. 4, where the sequence of divalent cation exposure was:



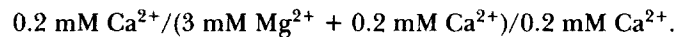
The first exposure of the newly-excised (or naive) patch to 3 mM Sr<sup>2+</sup> caused the K<sub>M</sub> to be shifted to near 100 μM (Fig. 4 A), where it remained stable while the exposure

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shows averaged cAMP dose-responses measured in 1 mM Ba<sup>2+</sup> (*circles*) and 3 mM Ba<sup>2+</sup> (*squares*). The data for 1 mM Ba<sup>2+</sup> were averaged from four patches and fitted curves have a Hill coefficient of 1.6 with a K<sub>M</sub> of 1.5 μM (+50 mV) and 3.3 μM (-50 mV). The data for 3 mM Ba<sup>2+</sup> were averaged from two patches with the fitted curve having a Hill coefficient of 1.8 and a K<sub>M</sub> of 6.0 μM. (D) The left panel shows an example of *I(V)* relationships activated by the indicated [cAMP] in 3 mM Sr<sup>2+</sup>. The right panel shows cAMP dose-responses recorded in 0.5 mM Sr<sup>2+</sup> (*circles*) and 3 mM Sr<sup>2+</sup> (*squares*). The data for 0.5 mM Sr<sup>2+</sup> were from one patch and fitted with a Hill coefficient of 1.8 and a K<sub>M</sub> of 1.5 μM (+50 mV) and 3.5 μM (-50 mV). The data for 3 mM Sr<sup>2+</sup> were averaged from two patches and fitted curves had a Hill coefficient of 1.6 and a K<sub>M</sub> of 80 μM (+50 mV) and 115 μM (-50 mV). (E) The left panel shows an example of *I(V)* relationships activated by the indicated cAMP in 3 mM Mn<sup>2+</sup>. The right panel shows the cAMP dose-response averaged from two patches, with the fitted curve having a low Hill coefficient (1.15) and a K<sub>M</sub> of 6 μM (+50 mV) and 12 μM (-50 mV).

to 3 mM  $\text{Sr}^{2+}$  was continued for 20 min (Fig. 4 *B*). After brief exposure (2 min) to a solution containing 3 mM  $\text{Mg}^{2+}$  as the only divalent cation (Fig. 4 *C*), the  $K_M$  subsequently measured in 3 mM  $\text{Sr}^{2+}$  (after the removal of  $\text{Mg}^{2+}$ ) was near the value typically seen in the absence of divalent cations (Fig. 4 *D*). This  $K_M$  remained constant, during the continued exposure to 3 mM  $\text{Sr}^{2+}$ , for the following 90 min (Fig. 4 *E*). The time courses of three relevant parameters are given in *F–H*. After the transient exposure to  $\text{Mg}^{2+}$ , there was a  $\sim 20\%$  stepwise reduction in the maximum current (*F*). However, this decrease was not observed in all patches and was not studied in detail. Immediately after  $\text{Mg}^{2+}$  exposure, the  $K_M$  decreased to  $< 1/10$ th of its previous value (*G*). The Hill coefficient remained unchanged (*H*). As shown below,  $\text{Mg}^{2+}$  pre-exposure also abolished the  $\text{Ca}^{2+}$ -induced cAMP affinity shifts.

The inhibitory effect of  $\text{Mg}^{2+}$  was further investigated using 0.2 mM  $\text{Ca}^{2+}$ , which also induced a large  $K_M$  shift. An example of  $I(V)$  relationships recorded from one patch at increasing [cAMP] are given in Fig. 5 *A*, and dose-responses averaged from four patches are shown in Fig. 5 *C*. At  $-50$  mV, the addition of 0.2 mM  $\text{Ca}^{2+}$  increased the cAMP affinity from 4 to 33  $\mu\text{M}$ . In Fig. 5 *B*, it is apparent that exposure for 60 s to a solution containing both  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  had no effect on the cAMP sensitivity. This experiment represents the following sequence:



This negative result was confirmed in three other patches. Yet, as demonstrated below, exposure for 60 s to a solution containing 3 mM  $\text{Mg}^{2+}$  and no  $\text{Ca}^{2+}$  completely abolished the desensitising  $K_M$  shift induced by 0.2 mM  $\text{Ca}^{2+}$ . Thus, the continuous presence of 0.2 mM  $\text{Ca}^{2+}$  effectively eliminates the ability of  $\text{Mg}^{2+}$  to prevent  $\text{Ca}^{2+}$ -induced  $K_M$  increases. This suggests why patch excision into bath Tyrode (which contained 2 mM  $\text{Ca}^{2+}$  and 1 mM  $\text{Mg}^{2+}$ ) did not prevent subsequent shifts of  $K_M$ .

From Fig. 4, it cannot be concluded whether  $\text{Mg}^{2+}$  induced a general increase in the sensitivity to cAMP, or whether it abolished the  $K_M$  shift induced by  $\text{Sr}^{2+}$ . Using 0.2 mM  $\text{Ca}^{2+}$  instead of 3 mM  $\text{Sr}^{2+}$  to induce the shift, the following exposure sequence was used to distinguish between these two possibilities:



An example of such an experiment is given in Fig. 6. In Fig. 6, *A* and *B*, it is clear that a 60-s pre-exposure to 3 mM  $\text{Mg}^{2+}$  had no effect on the cAMP dose-response measured in the absence of divalent cations, although in the same patch (Fig. 6, *C–E*) it completely abolished the  $K_M$  shift induced by 0.2 mM  $\text{Ca}^{2+}$ . Similar results were obtained in two other patches. To average results from different patches, the ratio of currents obtained with 10 with respect to 100  $\mu\text{M}$  cAMP was chosen as an assay of a desensitising  $K_M$  shift because 10  $\mu\text{M}$  cAMP is almost saturating in a zero divalent solution, but near half maximal in 0.2 mM  $\text{Ca}^{2+}$ . This ratio, which was measured at  $-50$  mV, is termed  $I_{10/100}$ . The  $I_{10/100}$  in zero divalent solution, averaged from three patches, was  $0.87 \pm 0.03$  ( $\pm\text{SEM}$ ,  $n = 3$ ). Addition of 0.2 mM  $\text{Ca}^{2+}$  reduced  $I_{10/100}$  to  $0.46 \pm 0.09$ . Subsequent exposure to 3 mM  $\text{Mg}^{2+}$  for 60 s increased this ratio to  $0.85 \pm 0.06$ , which was not significantly different from control. No significant shift in the zero divalent cation cAMP dose-response was measured in any of the three

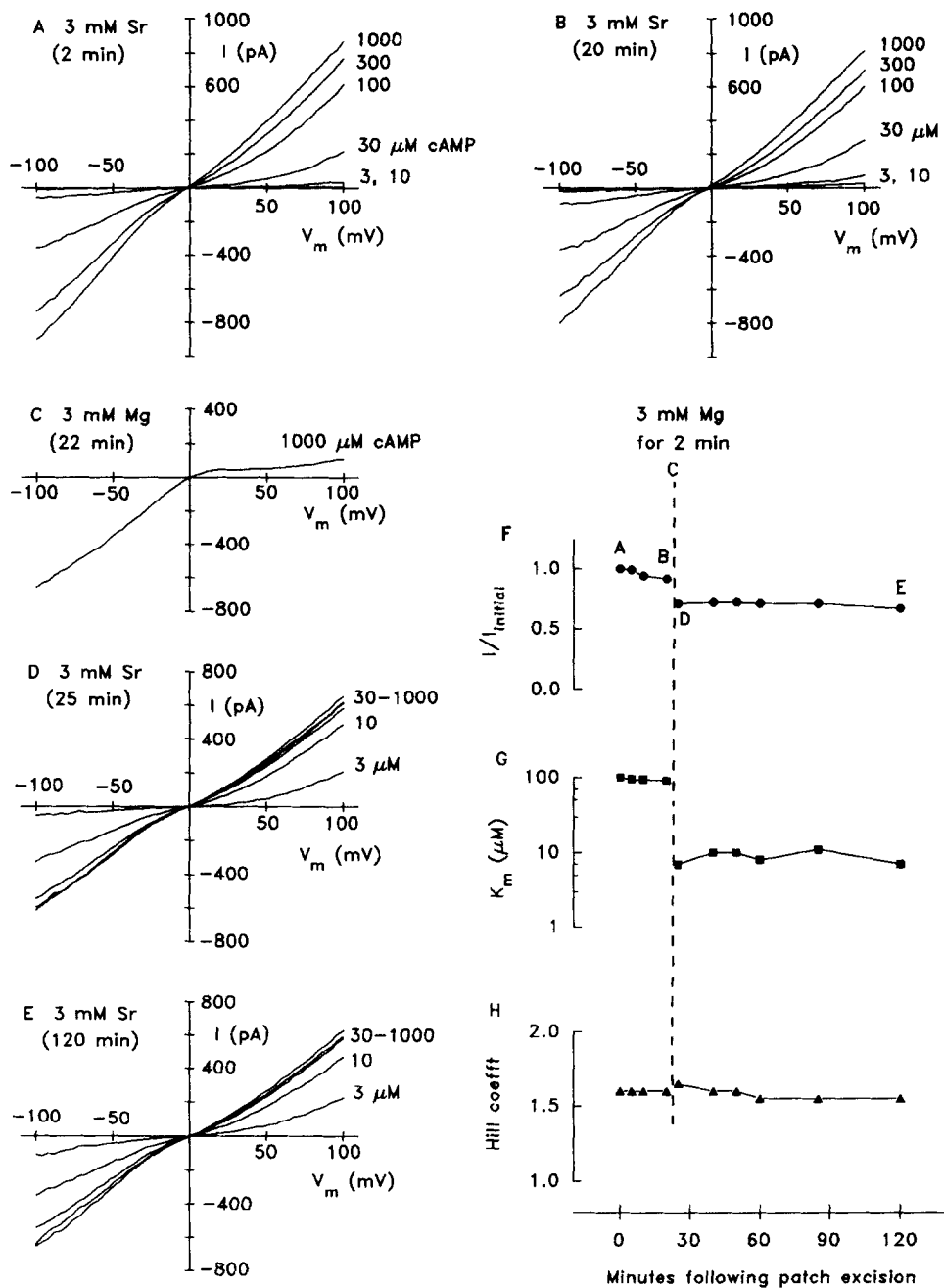


FIGURE 4. The decrease in the cAMP sensitivity, induced in this case by 3 mM Sr<sup>2+</sup>, is abolished following a 2-min exposure to 3 mM Mg<sup>2+</sup>. Data from 1 patch. *I/V* relationships activated by the indicated [cAMP] in 3 mM Sr<sup>2+</sup> are displayed for the following times after patch excision: 2 min (A), 20 min (B), 25 min (D) and 120 min (E). At 22 min (C), the patch was exposed for 2 min to a solution containing 3 mM Mg<sup>2+</sup> and 1 mM cAMP. Eight 1-s voltage ramps between -100 and +100 mV were also applied during this period. Thereafter, the Mg<sup>2+</sup>

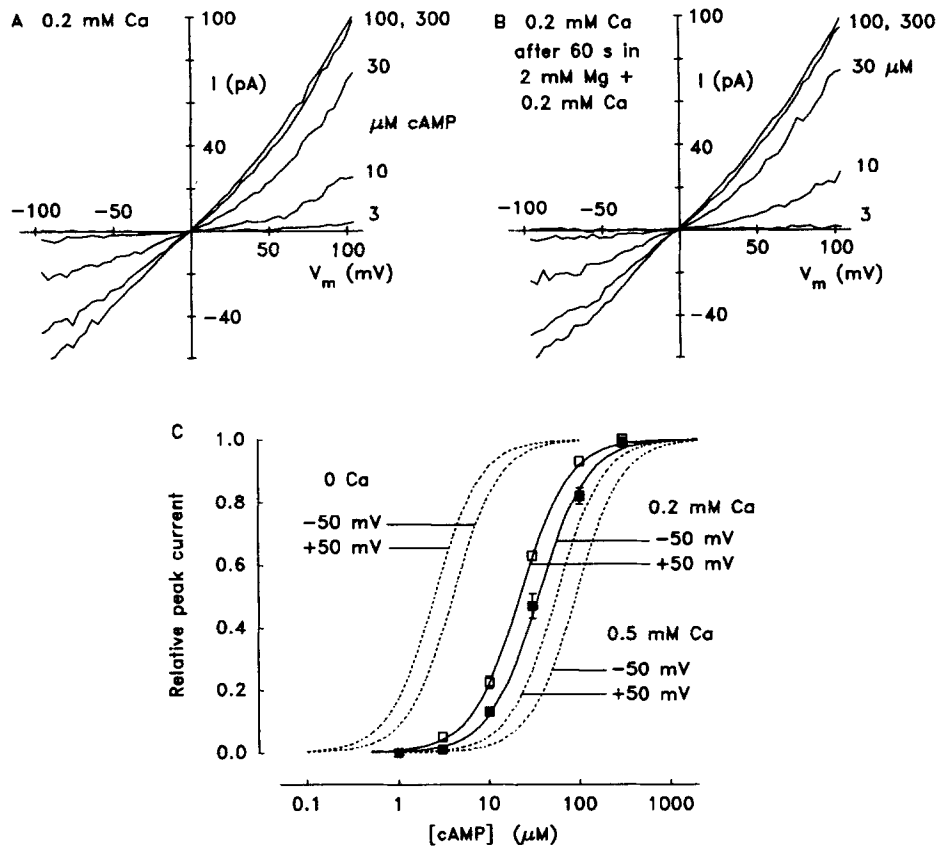


FIGURE 5. The cAMP dose-response shift induced by the lower  $[Ca^{2+}]$  of 0.2 mM. An example of a cAMP dose-response in 0.2 mM  $Ca^{2+}$  before (A) and after (B) exposure of the patch to a solution (low Ca solution in Table I) containing 2 mM  $Mg^{2+}$  + 0.2 mM  $Ca^{2+}$  for 60 s. (C) The dose-responses of cAMP at +50 mV (hollow squares) and -50 mV (filled squares) were averaged from four patches. Error bars ( $\pm$ SEM) are displayed when larger than symbol size. The fitted curves had a Hill coefficient of 1.6 and a  $K_M$  of 22  $\mu$ M (+50 mV) or 33  $\mu$ M (-50 mV). Corresponding cAMP dose-responses for 0 and 0.5 mM  $Ca^{2+}$  from Fig. 2 A (dashed lines) are included for comparison.

patches. Thus, the effect of  $Mg^{2+}$  was simply to abolish the affinity shift induced by 0.2 mM  $Ca^{2+}$ .

Because the protective effect of  $Mg^{2+}$  required the absence of  $Ca^{2+}$ , it is possible that either, (a) both  $Mg^{2+}$  and  $Ca^{2+}$  compete for a site (or sites) at which only  $Mg^{2+}$  is

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was washed away. Data in A-E represent sample recordings. A summary of all cAMP dose-responses measured in this patch, plotted as a function of time following patch excision, is presented in F, G, and H. (F) Ratio of current activated by saturating cAMP at indicated time following patch excision, measured with respect to the current activated by saturating cAMP at time  $t = 120$  s. (G) The  $K_M$  of the cAMP dose-response recorded at -50 mV. (H) Hill coefficient of the cAMP dose-response.

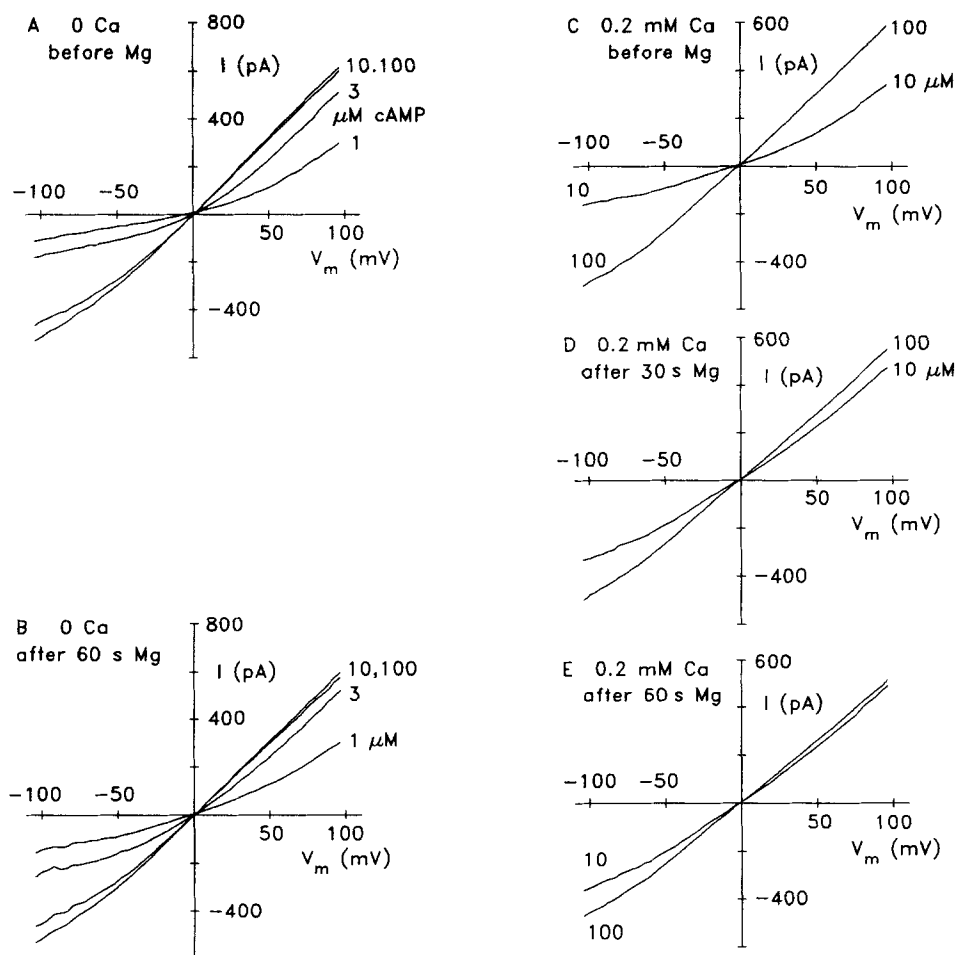


FIGURE 6. Protection from channel desensitization: effect of 3 mM  $Mg^{2+}$  on the cAMP dose-responses in 0 and 0.2 mM  $Ca^{2+}$ . (A and B) Dose-response in 0  $Ca^{2+}$  before (A) and after (B) a 60-s application of 3 mM  $Mg^{2+}$ . (C-E)  $I/V$  relationships recorded in 10 and 100  $\mu M$  cAMP in the presence of 0.2 mM  $Ca^{2+}$  before (C), after 30 s (D), and after 60 s (E) exposure to 3 mM  $Mg^{2+}$ . All data are from the same patch. In these experiments, neither voltage ramps nor the addition of cAMP significantly increased the rate of induction of the  $Mg^{2+}$  effect ( $n = 2$  each). Furthermore, the  $Mg^{2+}$  effect was not reversed by exposure to a strongly  $Mg^{2+}$  chelating solution, containing 2 mM EDTA ( $n = 2$ ).

effective but for which it has a lower affinity than  $Ca^{2+}$ , or (b), before  $Mg^{2+}$  can be effective, the desensitising shift must be reversed by removal of  $Ca^{2+}$ . These can be tested by observing whether a higher  $[Ca^{2+}]$  can protect against a shift caused by a lower  $[Ca^{2+}]$ . The following exposure sequence was used:

$$0.2 \text{ mM } Ca^{2+} / 3 \text{ mM } Ca^{2+} / 0.2 \text{ mM } Ca^{2+}.$$

Before exposure to 0.2 mM  $Ca^{2+}$ , the  $I_{10/100}$  was  $0.81 \pm 0.02$  ( $\pm SEM$ ,  $n = 2$ ), which reduced to  $0.37 \pm 0.12$  upon exposure to 0.2 mM  $Ca^{2+}$ . After exposure to 3 mM

Ca<sup>2+</sup> for 2 min, the  $I_{10/100}$  increased to  $0.81 \pm 0.05$ . The  $K_M$  in the absence of divalent cations did not change. Thus, 3 mM Ca<sup>2+</sup> protected against the desensitising shift induced by 0.2 mM Ca<sup>2+</sup>, suggesting that shifting sites are functionally distinct from the protection sites, but that higher divalent concentrations are necessary for activation of protection sites. The longer time required to establish the protective effect of Ca<sup>2+</sup> (120 s, c.f., 60 s for Mg<sup>2+</sup>) may indicate a relatively reduced affinity for the protection site.

Because the  $K_M$  shift induced by 0.2 mM Ca<sup>2+</sup> was abolished by a subsequent exposure to 3 mM Mg<sup>2+</sup>, it may be anticipated that reversing the exposure sequence would have the same effect. That is, exposure of a naive patch to 3 mM Mg<sup>2+</sup> should prevent a  $K_M$  shift induced by a subsequent exposure to 0.2 mM Ca<sup>2+</sup>. This was tested using the following exposure sequence:

3 mM Mg<sup>2+</sup>/0 divalent/0.2 mM Ca<sup>2+</sup>/3 mM Mg<sup>2+</sup>/0.2 mM Ca<sup>2+</sup>.

Immediately after excision into normal Tyrode solution, patches were exposed to 3 mM Mg<sup>2+</sup> solution for 120 s. In each of three patches tested, the cAMP dose-response measured in the absence of divalent cations was normal and did not change throughout the experiment. But surprisingly, after a 120-s pre-exposure to 3 mM Mg<sup>2+</sup>, the usual desensitising  $K_M$  shift was observed upon exposure to 0.2 mM Ca<sup>2+</sup>. The  $I_{10/100}$  was originally  $0.82 \pm 0.04$  ( $n = 3$ ) and reduced to  $0.42 \pm 0.10$  in 0.2 mM Ca<sup>2+</sup>. Then, after re-exposure to 3 mM Mg<sup>2+</sup>, the removal of the Ca<sup>2+</sup>-induced  $K_M$  shift occurred as before. Thus, pre-exposure of naive patches to Mg<sup>2+</sup> was ineffective in preventing the Ca<sup>2+</sup>-induced  $K_M$  shift.

## DISCUSSION

### *Summary of Results*

Ca<sup>2+</sup>, acting at the cytosolic membrane surface, decreased the sensitivity of the CNG channels to activation by cAMP. The sensitivity decrease could also be achieved with other divalent cations, with a relative potency dependent on the closeness of the ionic radius to Ca<sup>2+</sup>. Mg<sup>2+</sup> was the only divalent cation tested which did not cause a shift. Once a shift had been induced by simultaneous exposure to cAMP and a shifting divalent cation, brief transient exposure to a relatively high (3 mM) concentration of Ca<sup>2+</sup>, Mg<sup>2+</sup>, or Ba<sup>2+</sup> (not shown) protected against subsequent shifts. Surprisingly, mixtures of divalent cations were not effective in achieving this protection. Our results can be summarized by the reaction scheme in Fig. 7. To simplify discussion of this scheme, only Ca<sup>2+</sup> is considered as a shifting divalent cation and Mg<sup>2+</sup> is considered as a protecting divalent. In this model, the term protection is used descriptively and is not intended to suggest a physical action of Mg<sup>2+</sup>.

The conversion from the high affinity ( $R'$ ) to the low affinity ( $R^S$ ) state was rapidly achieved by the addition of 0.2 mM Ca<sup>2+</sup>. It was readily reversible upon removal of Ca<sup>2+</sup>, did not wash out with time in excised patches (e.g., Fig. 4, *A* and *B*) and was independent of the presence of 3 mM Mg<sup>2+</sup> (Fig. 5, *A* and *B*). However, once Ca<sup>2+</sup> was removed from the solution, 3 mM Mg<sup>2+</sup> induced the apparently irreversible reaction to state ( $R''$ ) where desensitising shifts could no longer be induced. This step did not require cAMP. Surprisingly, this reaction ( $R' \rightarrow R''$ ) could not be induced in

naive patches which had been pre-exposed only to bath Tyrode solution (containing 2 mM  $\text{Ca}^{2+}$  and 1 mM  $\text{Mg}^{2+}$ ) and to a zero divalent solution containing 100  $\mu\text{M}$  cAMP. Apparently, the  $R^S$  state had first to be achieved before  $\text{Mg}^{2+}$  exposure was effective. Thus, it was necessary to define an additional state ( $R$ ), corresponding to that of naive patches. The step  $R \rightarrow R^S$  required both  $\text{Ca}^{2+}$  and cAMP, while  $\text{Mg}^{2+}$  was optional. It was not established whether excision into bath Tyrode induced any changes in the channels from their state in the intact cell.

#### Comparison with Other Studies

This report confirms a recent study where  $\text{Ca}^{2+}$  was shown to reduce the sensitivity of catfish olfactory CNG channels to activation by cAMP (Kramer and Siegelbaum,

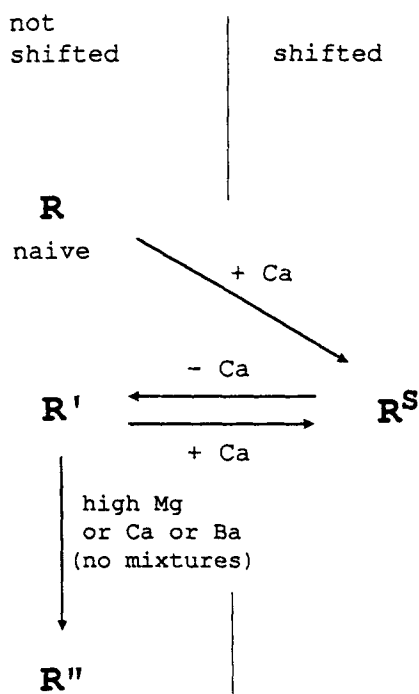


FIGURE 7. Scheme of possible transitions to explain divalent cation-cAMP interactions. In state  $R^S$  the cAMP-dose response was shifted to higher  $K_M$  values (channel desensitization). The transition  $R \rightarrow R^S$  was achieved by exposure to cAMP +  $\text{Ca}^{2+}$  (with and without  $\text{Mg}^{2+}$ ), whereas cAMP +  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  +  $\text{Mg}^{2+}$ , cAMP alone, or  $\text{Mg}^{2+}$  alone were ineffective. In excised patches, the transitions  $R \rightarrow R^S$  and  $R' \rightarrow R''$  appeared to be irreversible. The attainment of state  $R''$  protected from further desensitization.

1992). However, the two effects differ dramatically in that the catfish channel had a much higher sensitivity to  $\text{Ca}^{2+}$  and the effect was labile. These differences are considered further below.  $\text{Ca}^{2+}$ -dependent inhibition of photoreceptor CNG channel activation has also been reported. The cooperative binding of a fluorescent analog of cGMP was decreased by  $\text{Ca}^{2+}$  present in concentrations of 0.1–1  $\mu\text{M}$  (Caretta, Cavaggioni, Grimaldi, and Sorbi, 1988). Recently, the  $\text{Ca}^{2+}$ -binding protein calmodulin was shown to decrease the affinity of cGMP for these channels (Hsu and Molday, 1993). It had previously been established that calmodulin is closely associated with the channel (Molday, Cook, Kaupp, and Molday, 1990). An effect like that described in Fig. 2A was previously observed in the salamander photoreceptor CNG channel (Colamartino et al., 1991). Studying the same channels, Karpen, Loney, and



Baylor (1992) reported smaller CNG currents in patches excised into  $\text{Ca}^{2+}$ -containing, compared to  $\text{Ca}^{2+}$ -free solutions, which is also consistent with a  $\text{Ca}^{2+}$ -induced affinity reduction. Thus,  $\text{Ca}^{2+}$ -mediated shifts from high to low cyclic nucleotide affinity may be a common form of CNG channel regulation.

A recent study demonstrated that  $\text{Ca}^{2+}$ -dependent increases in cGMP affinity can also occur. Gordon et al., (1992) found that cGMP-gated channels in photoreceptor cells displayed a spontaneous sensitization with time that appeared to be due to a  $\text{Ca}^{2+}$ -activated phosphatase present at the internal membrane surface. Patches exposed to low  $\text{Ca}^{2+}$  for minutes lost the ability of spontaneous sensitization. Because spontaneous or divalent cation-induced sensitization were never observed in our experiments, such a phosphatase effect was unlikely to have contributed to the effects reported here. Ildefonse and collaborators (Ildefonse and Bennett, 1991; Ildefonse, Crouzy, and Bennett, 1992) reported that micromolar concentrations of certain divalent cations ( $\text{Fe}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ) induced a strong sensitization of photoreceptor CNG channels to activation by cGMP. However,  $\text{Co}^{2+}$ , which exerted a particularly potent effect at micromolar concentrations (Ildefonse and Bennett, 1991), had no effect in the concentration range 10  $\mu\text{M}$ –1 mM used in the present study.

As illustrated in Fig. 7, the effect of  $\text{Mg}^{2+}$  is dependent on the sequence of channel preexposure to other divalent cations. This result suggests that other divalent cation effects on CNG channels may also be sequence-dependent. In addition, the use of  $\text{Mg}^{2+}$  to eliminate the cAMP affinity shift offers an opportunity to separate these effects from other types of divalent cation effects on CNG channels.

#### *Possible Mechanism of Action*

From the experiments described in this report, direct inhibitory effects of divalent cations on the CNG channel cannot be ruled out. However, recent studies (Kramer and Siegelbaum, 1992; Hsu and Molday, 1993) provide strong evidence for the existence of a regulatory protein in mediating the  $\text{Ca}^{2+}$ -induced inhibition. A regulatory protein, possibly calmodulin, may also have mediated the  $\text{Ca}^{2+}$ -induced effects in our system either directly (Hsu and Molday, 1993), or via a  $\text{Ca}^{2+}$ -activated calmodulin-dependent phosphodiesterase (Borisy et al., 1992). The sequence of divalent cation potency in reducing cAMP affinity ( $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Mn}^{2+} > \text{Ba}^{2+} > \text{Mg}^{2+}$ ) corresponds closely to that required for the activation of calmodulin (Chao, Suzuki, Zysk, and Cheung, 1984). Another similarity is that  $\text{Mg}^{2+}$  also inhibited the  $\text{Ca}^{2+}$ -dependent affinity shift mediated by this enzyme (Hsu and Molday, 1993). Calmodulin also contains two classes of divalent cation binding sites, which corresponds to the minimum number required to explain the scheme in Fig. 7. However, it is not yet possible to reconcile all aspects of this model with the known properties of calmodulin. A potential problem is that the observed  $\text{Ca}^{2+}$ -sensitivity was about two orders of magnitude lower than required for calmodulin activation. On the other hand, the experiments described here were not a direct assay of calmodulin activation, and it is possible that if a limiting concentration of calmodulin was present in the excised patches, the apparent  $\text{Ca}^{2+}$ -sensitivity of the effect at the channel may have been skewed to much higher concentrations.

Kramer and Siegelbaum (1992) found no effect of either the calmodulin antagonist, calmidazolium, or of exogenous calmodulin application. However, because

calmodulin exerts multiple effects in olfactory neurons, these observations do not necessarily refute the hypothesis that calmodulin was involved. As well as a possible direct inhibitory effect on the olfactory CNG channel, calmodulin also activates olfactory adenylate cyclase (Anholt and Rivers, 1990; Frings, 1993) leading to an increase in cAMP levels, or a phosphodiesterase (Borisy et al., 1992), leading to a decrease. A particularly significant difference was that Kramer and Siegelbaum (1992) reported a strong time-dependent response rundown, whereas none was observed in the present study. It is difficult to attribute this to differences in experimental technique, because we used micro-patches from the apical knob, whereas they used mainly macro-patches, which should have reduced rundown rate, and indeed found an even greater rundown rate in micro-patches. The other significant difference was the higher  $\text{Ca}^{2+}$  concentration required in the present study. Taken together, these differences possibly suggest that the two effects are discrete, with the one reported here becoming evident at higher  $\text{Ca}^{2+}$  concentrations after washout of the effect reported by Kramer and Siegelbaum (1992).

It remains to be investigated why  $\text{Mg}^{2+}$ -induced protection did not occur either in intact cells or in naive patches. In excised patches,  $\text{Mg}^{2+}$ -induced protection was effective only after simultaneous  $\text{Ca}^{2+}$  and cAMP exposure (Fig. 7), such as the channels would normally experience during odorant stimulation in vivo. Thus, odorant exposure may change the regulatory protein-CNG channel complex in a way that would make itself susceptible to  $\text{Mg}^{2+}$  action in an excised patch. This implies the intervention of a cytosolic factor that is washed out after simulated odorant stimulation of excised patches.

#### *Physiological Role*

In isolated olfactory cells, both the odorant-induced and the cAMP-induced current display a desensitization which can be abolished by removing extracellular  $\text{Ca}^{2+}$  (Kurahashi, 1990; Kurahashi and Shibuya, 1990). This points to  $\text{Ca}^{2+}$  inflow as an important element in desensitization. Because in other systems, high ( $>100 \mu\text{M}$ ) local  $\text{Ca}^{2+}$  concentrations can be generated at the internal surface of  $\text{Ca}^{2+}$ -permeant channels (Augustine and Neher, 1992; Llinas, Sugimori, and Silver, 1992), it is possible that the effect reported here may contribute significantly to the termination of olfactory signaling.

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