Contribution of Cyclic-Nucleotide-Gated Channels to the Resting Conductance of Olfactory Receptor Neurons

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ABSTRACT The basal conductance of unstimulated frog olfactory receptor neurons was investigated using whole-cell and perforated-patch recording. The input conductance, measured between −80 mV and −60 mV, averaged 0.25 nS in physiological saline. Studies were conducted to determine whether part of the input conductance is due to gating of neuronal cyclic-nucleotide-gated (CNG) channels. In support of this idea, the neuronal resting conductance was reduced by each of five treatments that reduce current through CNG channels: external application of divalent cations or amiloride; treatment with either of two adenylate cyclase inhibitors; and application of AMP-PNP, a competitive substrate for adenylate cyclase. The current blocked by divalent cations or by a cyclase inhibitor reversed near 0 mV, as expected for a CNG current. Under physiological conditions, gating of CNG channels contributes ~0.06 nS to the resting neuronal conductance. This implies a resting cAMP concentration of 0.1–0.3 μM. A theoretical model suggests that a neuron containing 0.1–0.3 μM cAMP is poised to give the largest possible depolarization in response to a very small olfactory stimulus. Although having CNG channels open at rest decreases the voltage change resulting from a given receptor current, it more substantially increases the receptor current resulting from a given increase in [cAMP].

INTRODUCTION

Transduction of an odorous stimulus into a response is dependent on successful activation of the appropriate signaling pathway (reviewed by Schild and Restrepo, 1998). In one such pathway, binding of odorous molecules to their receptors activates G_{olf}, a GTP-binding protein. This in turn leads to activation of adenylate cyclase and the generation of cyclic AMP. Sufficient cAMP can cause opening of cyclic-nucleotide-gated (CNG) channels, allowing a depolarizing influx of Na^+ and Ca^{2+} (Nakamura and Gold, 1987; Kurahashi, 1990; Firestein et al., 1991b). In isolated cilia (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990) and in intact olfactory receptor neurons (ORNs; see also Frings and Lindemann, 1991; Firestein et al., 1991a; Ronnett et al., 1991), studies have shown basal (unstimulated) activities of both adenylate cyclase and cAMP phosphodiesterase. It is therefore expected that the resting neuron has some steady-state level of cytoplasmic cAMP. However, it is not known whether this level is sufficient to have any physiological effects. In isolated frog olfactory cilia, spontaneous gating of the CNG channels contributes to the resting conductance (Kleene, 2000). Here we show that ~20–40% of the resting conductance in intact frog ORNs is due to open CNG channels. The resting cAMP concentration is estimated to be 0.1–0.3 μM.

Having transduction channels open at rest will decrease the depolarization caused by a given receptor current. One might expect that this would reduce the sensitivity of the neuron to weak stimuli. However, a theoretical model is presented that indicates that an ORN containing 0.1–0.3 μM cAMP at rest is *more* sensitive. Although having the channels open at rest decreases the voltage change resulting from a given receptor current, it more substantially increases the receptor current resulting from a given increase in [cAMP]. Such a mechanism could increase the sensitivity of any neuron that responds with an increase in the concentration of a second messenger.

MATERIALS AND METHODS

Whole-cell voltage-clamp recordings were made in single, isolated ORNs from the grass frog (*Rana pipiens*). Olfactory mucosae were removed from the nasal cavities of the frog after decapitation and pithing. Cells were isolated by gentle compression of the mucosae between two glass surfaces and suspended in either a low-divalent HEPES-buffered amphibian saline (0.1 mM Ca^{2+}, 0 mM Mg^{2+}) or HEPES-buffered amphibian saline with 2 mM Ca^{2+} and 1 mM Mg^{2+} (2Ca:1Mg amphibian saline). The ionic composition of the amphibian saline was (in mM): NaCl, 120; KCl, 3; HEPES, 5. The pH of the solution was adjusted to 7.2–7.3. The osmolarity of the saline was usually between 240 and 250 mOsm. The conductance of the CNG channels increases in solutions low in divalent cations (Zufall and Firestein, 1993; Kleene, 1995; Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002). ORNs were identified by the presence of slowly moving cilia (Pun et al., 1994; Kleene and Pun, 1996). All electrophysiological recordings were done at room temperature (22–25°C) with an Axopatch 1A amplifier (Axon Instruments, Union City, CA).

Two series of experiments were performed. In the first series, the perforated-patch recording method was used. With this method, the cells are kept intact and there is no loss of cellular constituents. The composition of the pipette solution was (in mM): KCl, 64; K_2SO_4, 28; NaCl, 12; EGTA, 0.5; HEPES, 20; $MgCl_2$, 1; glucose, 5. The pH was adjusted to 7.2 with KOH and the osmolarity to between 240 and 250 mOsm with sucrose if necessary. This solution suppresses the development of a Donnan potential that would shift the potential during the course of the recording (Horn and Marty, 1988). In a few experiments, KCl and K_2SO_4 were replaced with equimolar

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amounts of CsCl and Cs_2SO_4 to block outward currents. The antibiotic nystatin (Sigma) was used for perforation (Pun et al., 1994). A stock solution of 50 mg/ml nystatin in DMSO was made freshly for each experiment. The stock was diluted in K^+- or Cs^+-containing pipette solution to a final concentration of 200–400 μg/ml. The current was adjusted to zero with the open pipette in the bath before seal formation. After the formation of a tight seal between the pipette and the membrane, the pipette potential was set to −60 mV and a depolarizing pulse of 5 mV was passed through the pipette. While the evoked current pulse was monitored, the access resistance and membrane capacitance were estimated by adjusting the amplifier to compensate the transients at the beginning and end of the current pulse until the current trace was a flat line. Studies only began when the access resistance dropped below 40 MΩ, which usually took 5–10 min. Then a series of depolarizing steps of 30 mV or more were given to determine whether fast inward currents could be evoked from the cell. The access resistance and membrane capacitance were monitored throughout each study to ensure that resealing of the membrane had not occurred. High access resistance, which would indicate inadequate permeabilization of the membrane, would result in poor voltage control of the fast inward currents or an absence of evoked currents. Breakage of the perforated patch would have been apparent as a dramatic decrease in access resistance. (The access resistance of whole-cell recording is usually 2–3 times lower than that of perforated-patch recording.) We did see a gradual decrease in access resistance with time, which suggests that perforation of the membrane continued as long as the membrane was sealed against the pipette.

In the perforated-patch experiments, the effects of the 2Ca:1Mg amphibian saline, amiloride, or the adenylate cyclase inhibitors MDL12330A (also known as RMI12330A, Calbiochem) and 9-(2-tetrahydrofuryl)adenine (THFA, also known as SQ22536, Sigma) on the slope conductance were evaluated. The slope conductance protocol is described below. Both the 2Ca:1Mg saline and the above-mentioned reagents were applied extracellularly by ejecting them with low positive pressure from a pipette of tip diameter 10–15 μm placed nearby. Since the effects of 2Ca:1Mg saline and of amiloride occurred within seconds of application, the time of application was limited to 2 min. The adenylate cyclase inhibitors were each applied for as long as 10 min.

In the second series of experiments, tight-seal whole-cell recording was used. This approach allowed the direct introduction of ATP and its analogs into the cell. After the formation of a high-resistance seal against the pipette, a potential of −60 mV was imposed. Gentle suction was applied to rupture the membrane and gain entry into the cell. Intracellular recording was evidenced by the appearance of capacitive transients. The composition of the pipette solution was (in mM): KCl, 115; NaCl, 5; EGTA, 1.1; HEPES, 5; $MgCl_2$, 1; glucose, 5. The pH was adjusted to 7.2 and the osmolarity to between 240 and 250 mOsm. The effects of the following compounds on the slope conductance were individually examined: adenosine 5′-triphosphate (ATP); adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S); and 5′-adenylylimidodiphosphate (AMP-PNP). These reagents were added via the pipette solution.

In the standard whole-cell pipette solution, $[Ca^{2+}]$free was <10 nM. For one set of experiments, a solution containing 200 nM free Ca^{2+} was prepared by adding 0.65 mM $CaCl_2$ to the standard pipette solution. We calculated $[Ca^{2+}]$free using an apparent association constant for EGTA and Ca^{2+} of 7,120,000 M^{-1}, estimated as described by Marks and Maxfield (1991).

Voltage protocols were executed by pCLAMP software (version 5.5; Axon Instruments) interfaced with a TL-1 analog-to-digital converter. The effects of the various manipulations on the neuronal slope conductance were studied as follows. After the establishment of whole-cell recording, the membrane potential was held at −60 mV and a voltage ramp (from −100 to 0 mV or +50 mV) of duration 1 s was given. Evoked current ramps were low-pass filtered at 0.5 or 2 kHz, sampled at 1 kHz, stored on the hard disk of an IBM-compatible computer, and later analyzed. Each record was made by averaging four ramps collected at 0.5 Hz. The slope conductance was measured before the application of drugs and at various times after the application. Where reagents were applied from the intracellular recording pipette, the control conductance was measured immediately upon breaking into the cell.

The linear portion of the slope conductance between ~−80 mV and −60 mV was analyzed. This eliminated from the analysis an inward rectifier seen in some cells at potentials of −90 mV and more negative, as well as fast inward currents generated at potentials of ~−55 mV, especially in 2Ca:1Mg saline. The slope conductance was obtained by linear regression fit of the data points collected for each file (between 150 and 200 points). The measured input conductance depends in part on the seal resistance between the pipette and the membrane. A frog ORN has a resting membrane potential of ~−50 mV and an input resistance of ~1 GΩ (Pun and Gesteland, 1991). If the seal resistance is sufficiently high that all of the injected current is needed to hold the membrane potential, then a current of ~−10 pA should suffice to clamp the cell at −60 mV. Thus we set a limit of −10 pA for the holding current in our studies. Cells in which the holding current was more negative than −10 pA or increased to beyond −10 pA during drug application were deemed to have poor seal resistance and were not included in the final analysis. Results of the slope conductance are expressed as mean ± SD unless otherwise stated. Statistical comparisons were made by analysis of variance. Statistical significance is taken if the P value is less than 0.05.

Estimates of resting [cAMP] and the theoretical model in the Appendix rely on Hill-type equations with parameters empirically determined in frog olfactory cilia. The form of the Hill-type equation and its parameters are defined in the Appendix (Eq. 1). For studies in which external divalent cations were present, parameters measured in the presence of 1 mM external Ca at −50 mV were used (unpublished values from Kleene, 1999; $n = 7$): k, 10.3 ± 2.2 μM; n, 1.28 ± 0.08; and I_{max}, 283 ± 68 pA. I_{max}, the macroscopic current through CNG channels, was estimated as the product of the mean ciliary current times 6, a typical number of cilia for the receptor neurons. When a low-divalent external solution was used, these parameters applied (unpublished values from Kleene, 1999; $n = 7$): k, 1.70 ± 0.19 μM; n, 1.70 ± 0.07; and I_{max}, 1838 ± 537 pA. Note that k is decreased and I_{max} increased on reduction of external divalent cations. Variability in the Hill parameters was the greatest source of variability in the estimated [cAMP]. In the original dose-response study (Kleene, 1999), the three Hill parameters were estimated in each of seven experiments. If each measured parameter value is considered equally probable, the number of possible Hill-type equations is 7^3 (=343). For each such equation, the [cAMP] corresponding to a measured conductance was calculated. The 343 [cAMP] estimates were evaluated as a mean ± SD, as reported in Results.

Current variance was predicted to be $i^2Np\,(1-p)$ (Kleene, 1997), where i is the unitary (single-channel) current; N is the total number of CNG channels; and p is the open probability of the CNG channels at a given [cAMP]. The value p was calculated by assuming that macroscopic current values (I) of the Hill-type relation are equal to iNP_m, where P_m is the maximum open probability of the CNG channels (i.e., the open probability at a saturating [cAMP]). Values inferred from noise analysis in physiological solutions at −50 mV (Kleene, 1997; $n = 5$–7) are: i, 0.028 ± 0.001 pA; N, 14,820 channels/neuron (2470 ± 820 channels/cilium × 6 cilia/neuron); and P_m, 0.68 ± 0.03.

RESULTS

In an external medium containing 0.1 mM Ca^{2+}, the input conductance of the unstimulated olfactory receptor neuron (ORN) between −80 mV and −60 mV averaged 0.39 ± 0.15 nS (n = 91, range 0.13–0.83 nS). Sources of variability in this value were not determined. Sources likely include the resistance of the membrane-pipette seal, although we rejected cells with holding currents more negative than −10 pA. Comparisons of the control conductance were made among all of the experimental groups described below, and there were no significant differences. Except as noted, all measurements were made with perforated-patch recording.

External application of 2Ca:1Mg amphibian saline and amiloride

Under physiological conditions, CNG channels are partially blocked by external divalent cations (Zufall and Firestein, 1993; Kleene, 1995; Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002). If these channels contribute to the resting neuronal conductance, then the resting conductance should also be reduced by external divalent cations. In external medium containing 0.1 mM Ca^{2+}, the slope conductance was 0.39 ± 0.08 nS ($n = 12$). Application of saline with 2 mM Ca^{2+} and 1 mM Mg^{2+} significantly reduced the slope conductance to 0.24 ± 0.04 nS ($n = 12$; Fig. 1 *A*; Table 1). Recovery was almost immediate upon termination of application (0.39 ± 0.10 nS). Amiloride, which also blocks the CNG channels (Frings and Lindemann, 1998; Frings et al., 1992; Kleene, 1994), had a similar effect. At 1 mM, amiloride significantly decreased the slope conductance from 0.34 ± 0.07 nS to 0.20 ± 0.05 nS ($n = 10$; Fig. 1 *B*; Table 1). At a lower concentration (500 μM), amiloride decreased the slope conductance less, from 0.47 ± 0.15 nS to 0.39 ± 0.13 nS ($n = 5$).

It remained possible that divalent cations and amiloride might be blocking different sets of channels. We examined the effects of amiloride on the input conductance measured in physiological 2Ca:1Mg saline. In this saline, the slope conductance was 0.25 ± 0.05 nS ($n = 7$), which is consistent with the value reported above after addition of divalent cations. In 2Ca:1Mg saline, application of amiloride (1 mM) further reduced the slope conductance, to 0.19 ± 0.06 nS ($n = 7$; Fig. 1 *C*). Note that application of 1 mM amiloride reduced the conductance to the same value (0.19–0.20 nS) in both 2Ca:1Mg and low-divalent saline. Thus it can be concluded that divalent cations did not block some population of channels differently from those blocked by amiloride. The conductances in the presence of 1 mM amiloride were the lowest recorded in any of our tests.

To determine whether a Cs^+-sensitive inward rectifier contributes to the resting conductance, K^+ in the cytoplasmic (pipette) solution was replaced with Cs^+. Under this condition, the slope conductance near the resting potential was 0.37 ± 0.04 nS ($n = 7$). Application of saline with 2 mM Ca^{2+} and 1 mM Mg^{2+} significantly reduced the slope conductance to 0.21 ± 0.03 nS ($n = 7$). Neither of these values is significantly different from those measured in the absence of cytoplasmic Cs^+. It is therefore unlikely that any Cs^+-sensitive conductance contributes significantly near the resting potential.

If there is a resting current due to CNG channels, it should have a reversal potential near 0 mV under our conditions. The experiments shown in Fig. 1 could not test this hypothesis. At positive potentials, an outward current was apparent that was sometimes reduced by 2Ca:1Mg saline or by amiloride (Fig. 1). Any shift in reversal potential may have been due to effects on both this conductance and any CNG channels present. Therefore the outward current was eliminated (see Fig. 2 legend). After this, the current blocked by application of 2Ca:1Mg saline reversed at +1.0 ± 1.6 mV ($n = 9$; Fig. 2).

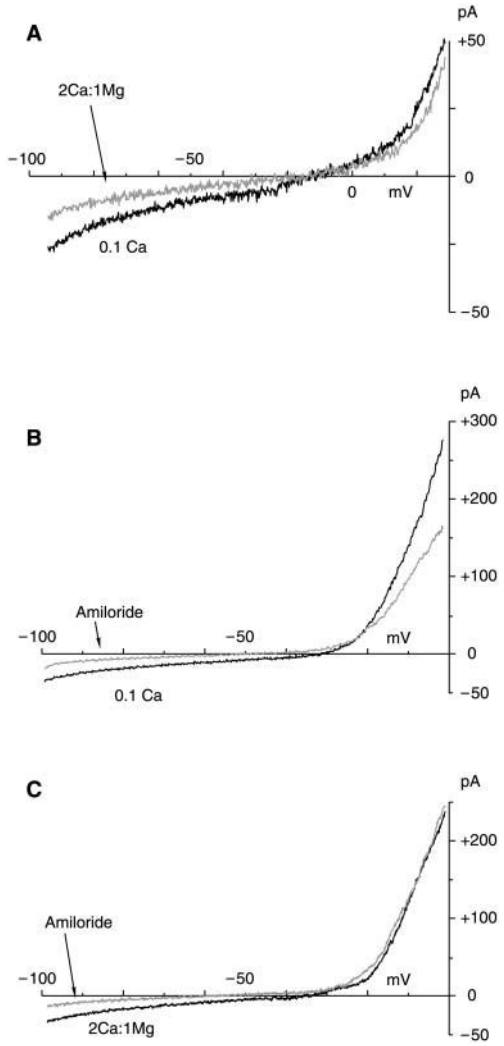

FIGURE 1 Effects of 2Ca:1Mg amphibian saline solution (*A*) and amiloride (*B* and *C*) on current in three different olfactory receptor neurons. (*Black curves*) The ramp currents before application of 2Ca:1Mg saline (*A*) or amiloride (1 mM, *B* and *C*). (*Gray curves*) The currents during the first 1–2 min of application of 2Ca:1Mg saline or amiloride. The experiments shown in *A* and *B* were performed in 0.1 Ca saline, whereas *C* was performed in 2Ca:1Mg saline. The slope conductance, which was obtained from a linear fit of the data between −80 and −55 mV, was reduced during the application of the 2Ca:1Mg solution (*A*) or amiloride (*B* and *C*). In *A*, 2Ca:1Mg solution reduced the slope conductance from 0.35 nS to 0.21 nS. Amiloride (1 mM) reduced the slope conductance from 0.57 nS to 0.34 nS in the cell shown in *B*, and from 0.45 nS to 0.25 nS in the cell shown in *C*. Measurements were made with perforated-patch recording.

Most experiments were done with nystatin perforated-patch recording, which preserves the normal level of cytoplasmic free Ca^{2+} (Kleinberg and Finkelstein, 1984). To test the effect of increasing cytoplasmic Ca^{2+} on the resting conductance, standard whole-cell recordings were done with 200 nM free Ca^{2+} in the pipette. Initially, low-divalent extracellular solution was present. The slope conductance was 0.36

TABLE 1 Effects of externally applied 2Ca:1Mg saline, amiloride, MDL12330A, and THFA on slope conductance of frog olfactory receptor neurons

	Slope conductance (nS)			
	control	treatment	reduction	recovery
2Ca:1Mg saline $n = 12$	0.39 ± 0.08	0.24 ± 0.04*	0.15 ± 0.06	0.39 ± 0.10
amiloride (1 mM)				
Low divalents ($n = 10$)	0.34 ± 0.07	0.20 ± 0.05*	0.14 ± 0.07	0.29 ± 0.10
2Ca:1Mg ($n = 7$)	0.25 ± 0.05	0.19 ± 0.06*	0.06 ± 0.03	0.24 ± 0.04
MDL12330A (1 μM)				
$n = 7$	0.54 ± 0.22	0.41 ± 0.18*	0.12 ± 0.07	0.48 ± 0.20
THFA (100 μM)				
$n = 6$	0.47 ± 0.12	0.37 ± 0.10*	0.10 ± 0.06	0.29 ± 0.06

Electrophysiological recordings were performed with perforated-patch recording. Results shown are the mean ± SD. The column labeled *reduction* shows the difference between the *control* value and the *treatment* value.
*Statistically significant ($P < 0.05$) when compared to the controls.

± 0.10 nS ($n = 10$). On application of 2Ca:1Mg external saline, the conductance was reduced to 0.21 ± 0.09 nS ($n = 10$). Neither conductance was significantly different from those measured with the perforated-patch method.

External application of adenylate cyclase inhibitors

Reduction of the slope conductance by 2Ca:1Mg saline and by amiloride suggests the presence of a CNG conductance even in the absence of odorous stimulation (i.e., in the basal or resting state). It should be possible to reduce this conductance by inhibiting the production of cAMP, which gates the CNG channels. Each of two membrane-permeant adenylate cyclase inhibitors (MDL12330A and THFA) reduced the slope conductance. At 1 μM, MDL12330A significantly reduced the conductance from 0.54 ± 0.22 nS to 0.41 ± 0.18 nS ($n = 7$; Table 1). An example is shown in Fig. 3 *A*. After the removal of the compound, the slope conductance was still significantly reduced (0.48 ± 0.20 nS). We examined the time course of the effect of 10 μM MDL12330A applied for 6 min in another population of cells. As shown in Fig. 3 *B*, the slope conductance was reduced within 2 min upon the application of the drug. No recovery from inhibition was seen even 10 min after the application was terminated. Inhibition by MDL12330 has been reported to be irreversible (Guellaen et al., 1977). The reversal potential of the current blocked by MDL12330A, determined after first blocking the Cs^+-sensitive outward current, averaged −10.5 ± 2.6 mV ($n = 4$; Fig. 3 *C*).

The cyclase inhibitor THFA (100 μM) had comparable effects (Table 1; Fig. 4 *A*), reducing the slope conductance from 0.47 ± 0.12 nS to 0.37 ± 0.10 nS ($n = 6$). The effect of

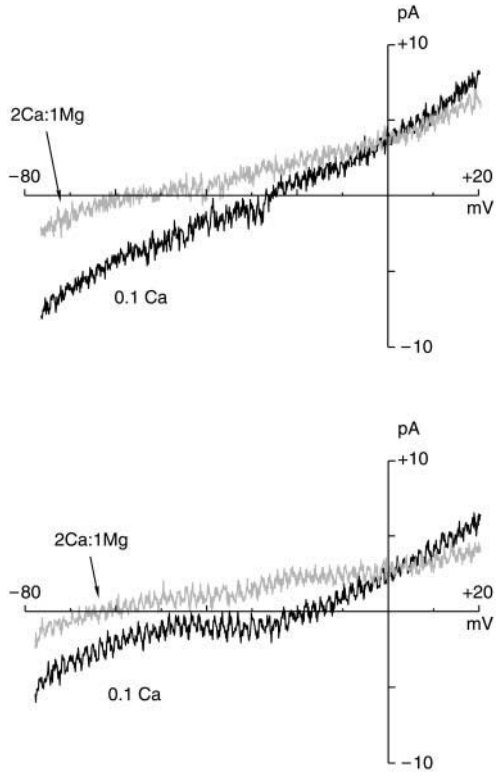

FIGURE 2 Reversal potential of the current blocked by external application of 2Ca:1Mg saline to two olfactory receptor neurons. The outwardly rectifying current visible in Fig. 1 was eliminated by replacing K^+ in the cytoplasmic (pipette) solution with Cs^+ and by adding 2 mM $CoCl_2$ and 5 mM tetraethylammonium chloride to the extracellular bath. Measurements were made with perforated-patch recording. Equilibrium potentials in the solutions used were +59 mV for Na^+ and −9 mV for Cl^-. Since K^+ was not included in the cytoplasmic solution, the equilibrium potential for K^+ was very positive.

THFA also lasted several min after the application was terminated (0.29 ± 0.06 nS). We studied the time course of the effect of THFA (10 μM) in another population of seven cells (Fig. 4 *B*). The slope conductance was reduced by 11%, which is less than the reduction caused by 100 μM THFA (20%). The time course was similar to that of MDL12330A. There was no further reduction in slope conductance through 8 min of application, and the reduction was maintained for up to 10 min after the application. (The reduction from 6 to 10 min after application was not statistically significant, perhaps because fewer cells were available for those points.)

Intracellular application of ATP analogs

We determined the effects of several ATP analogs on the input conductance. AMP-PNP, a competitive substrate for adenylate cyclase (Maguire and Gilman, 1974), significantly reduced the slope conductance of ORNs within 30 s after establishment of whole-cell recording (Fig. 5 *A*). The reduction was gradual. At 2 min, the reduction was similar to those produced by 2Ca:1Mg saline and by amiloride

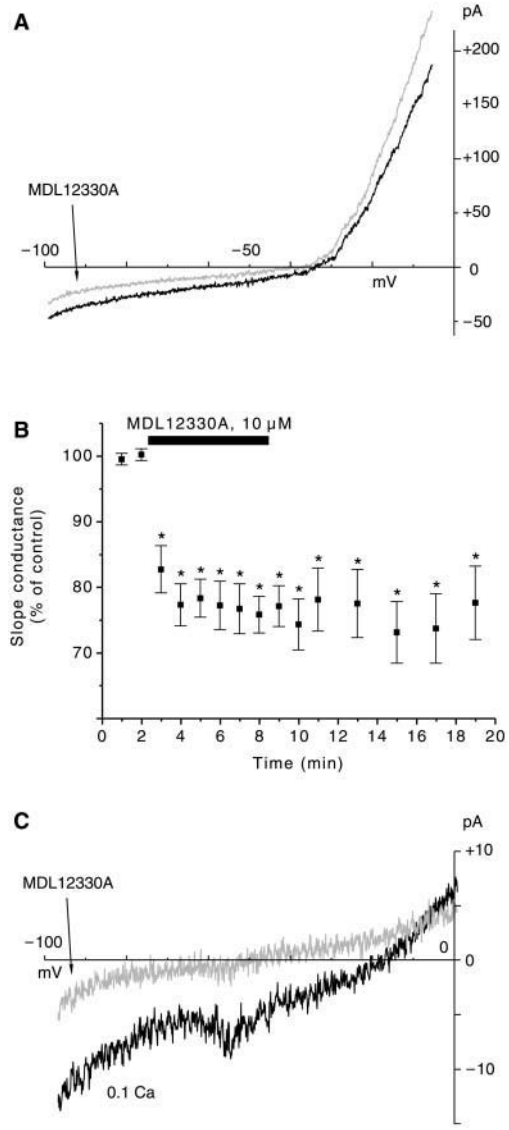

FIGURE 3 Effects of the adenylate cyclase inhibitor MDL12330A on current in olfactory receptor neurons. (*A*) The ramp current before (*black*) and 10 min after (*gray*) the onset of application of 10 μM MDL12330A. In this cell, MDL12330A decreased the slope conductance from 0.52 nS to 0.32 nS. (*B*) Time course of the action of 10 μM MDL12330A on three cells. Data shown are the mean ± SE. A reduction in the slope conductance is evident within the first 2 min of application and persisted throughout the 6 min of application. The slope conductance remained suppressed even 10 min after the application of MDL12330A had ceased. (*C*) Reversal potential of the current blocked by 10 μM MDL12330A. An outwardly rectifying current was blocked by replacing K^+ in the cytoplasmic (pipette) solution with Cs^+. Measurements were made with perforated-patch recording.

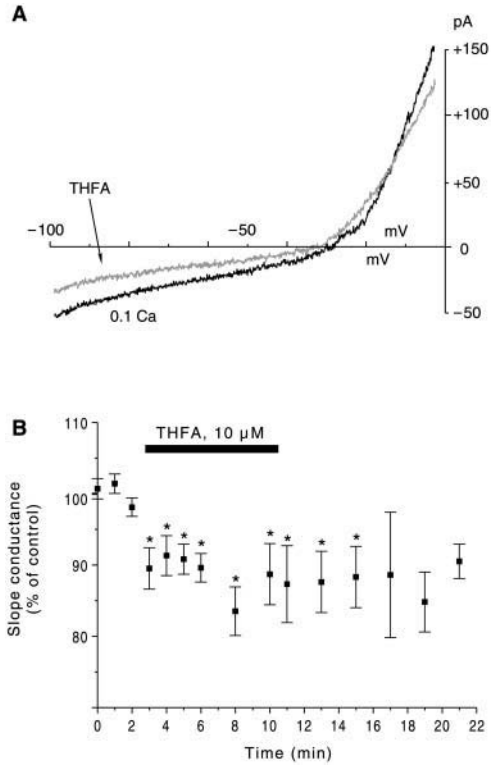

FIGURE 4 Effects of the adenylate cyclase inhibitor THFA on current in olfactory receptor neurons. (*A*) The ramp current before (*black*) and during (*gray*) bath application of 100 μM THFA (recorded with the first 1 min of application). In this cell, THFA reduced the slope conductance from 0.55 nS to 0.39 nS. (*B*) Time course of the action of 10 μM THFA in seven cells. Data shown are the mean ± SE. A reduction in slope conductance was observed within the first 2 min of application. This reduction was sustained throughout the 8 min of application of THFA and persisted for up to 10 min after the application had ceased. Note that in this experiment the reduction was smaller, presumably due to the lower concentration of THFA used. The time course of action of MDL12330A (not shown) was similar to the time course for THFA. Measurements were made with perforated-patch recording.

(Table 2). The reduction persisted for up to 5 min but was most pronounced within the first 3 min (Fig. 5 *B*). The recording solution alone did not have any significant effect on the input conductance (Table 2).

ATP (200 μM) and ATP-γ-S (100 and 200 μM) had no significant effect on the slope conductance within the first 2 min after establishment of whole-cell recording (Table 2). At 3–5 min of recording, both ATP and ATP-γ-S increased the slope conductance. A higher concentration of ATP (2 mM) caused a large increase in slope conductance within the first 2 min of whole-cell recording (data not shown). This increase did not appear to be related to a breakdown of the seal between the pipette and the membrane, because associated with this increase was a concurrent shift of the holding current at −60 mV to very positive values. A breakdown of the seal would have induced a negative shift in the holding current. The increase in outward current caused by 2 mM ATP likely reflects activation of a K^+ conductance.

Estimate of resting cAMP concentration

The relation between [cAMP] and CNG conductance in olfactory cilia is well described by a Hill-type dose-response equation. Since the parameters for this equation have been measured in olfactory cilia, knowing the current or conduc-

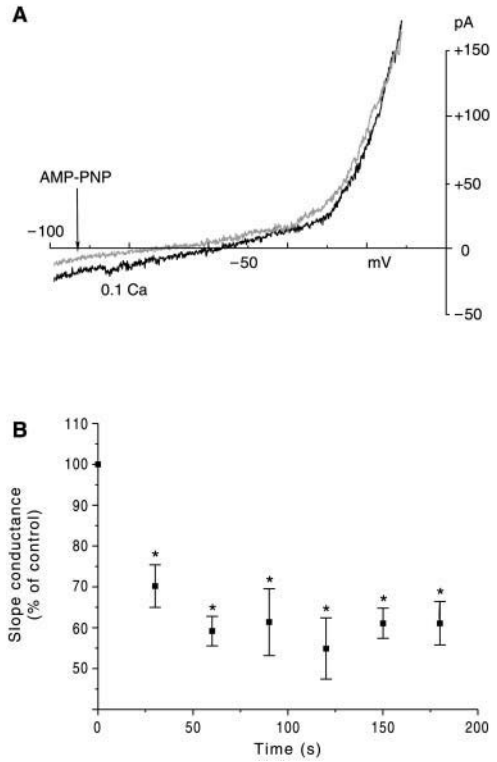

FIGURE 5 Effects of intracellular application of AMP-PNP on current in olfactory receptor neurons. (*A*) The ramp current immediately upon establishing whole-cell recording (*black*) and after recording for 1.5 min (*gray*). For this cell, the slope conductance was reduced from 0.57 nS to 0.33 nS. (*B*) The time course of action for the first 3 min of recording in six cells. Data points shown are the mean ± SE. A reduction in slope conductance was observed within 30 s of establishing whole-cell recording. This reduction persisted throughout the 3 min of recording.

tance due to CNG channels allows an estimate of the resting concentration of cAMP. (See the Appendix for the Hill-type equation, Eq. 1, and definitions of its parameters.) In a physiological saline (2Ca:1Mg saline), addition of amiloride reduced the conductance on average by 0.06 ± 0.03 nS (from 0.25 nS to 0.19 nS; $n = 7$). This difference (0.06 nS) is an estimate of the CNG conductance in a resting neuron in physiological saline; it should produce a current of −3 pA at −50 mV. According to the known dose-response relation and equation parameters previously measured in these cells, this current would result from a cAMP concentration of 0.3 ± 0.1 μM. Parameters for the dose-response relation in physiological solutions are k, 10.3 ± 2.2 μM; n, 1.28 ± 0.08; and I_{max}, 283 ± 68 pA (unpublished values from Kleene, 1999). The variability in the estimated cAMP concentration was primarily due to variability in the measured Hill parameters, as described in Methods.

In a nonphysiological saline low in divalent cations, addition of amiloride reduced the resting conductance on average by 0.14 ± 0.07 nS (from 0.34 nS to 0.20 nS; $n = 10$). The difference (0.14 nS) is an estimate of the resting CNG conductance in low-divalent solutions; it should produce a current of −7 pA at −50 mV. In these solutions, a resting cAMP level of

TABLE 2 Effects of intracellularly applied AMP-PNP, ATP, ATP-γ-S, and recording solution on the slope conductance of frog olfactory receptor neurons

	Slope conductance (nS)			
	control	30 s	1 min	2 min
AMP-PNP, 200 μM $n = 7$	0.52 ± 0.26	0.36 ± 0.16*	0.31 ± 0.14*	0.29 ± 0.15*
ATP, 200 μM $n = 8$	0.35 ± 0.16	0.32 ± 0.17	0.38 ± 0.15	0.40 ± 0.16
ATP-γ-S, 100 and 200 μM $n = 7$	0.35 ± 0.15	0.32 ± 0.24	0.37 ± 0.27	0.45 ± 0.21
Recording solution $n = 12$	0.36 ± 0.16	NM	0.37 ± 0.16	0.40 ± 0.14

Electrophysiological recordings were made with whole-cell tight-seal recording. Only results from the first 2 min of recording are shown. With ATP-γ-S in the pipette, four cells showed a transient decrease at 30 s and 1 min that was not statistically significant. These cells were grouped together with the remaining cells. Results shown are the mean ± SD.
*Symbol denotes statistical significance ($P < 0.05$) compared to controls.
NM, not measured.

0.07 ± 0.03 μM would give rise to a conductance of 0.14 nS. For this estimate, equation parameters measured in low-divalent solutions were used: k, 1.70 ± 0.19 μM; n, 1.70 ± 0.07; and I_{max}, 1838 ± 537 pA (unpublished values from Kleene, 1999). Taken together, the conductances measured in physiological and low-divalent solutions imply that the olfactory cilia in a resting neuron contain 0.1−0.3 μM cAMP.

DISCUSSION

In unstimulated ORNs, both adenylate cyclase and cAMP phosphodiesterase are active (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Frings and Lindemann, 1991; Firestein et al., 1991a; Ronnett et al., 1991). This likely results in a small, steady-state concentration of cytoplasmic cAMP. The effects of this basal level of cAMP have not previously been examined. We evaluated the effects of divalent cations, amiloride, adenylate cyclase inhibitors, and ATP analogs on the resting slope conductance of unstimulated frog ORNs with voltage-clamp recording. We found that, in the absence of any odorous stimulation, there is a CNG conductance active at resting membrane potential in ORNs. We propose that this active CNG conductance, which contributes 20–40% of the resting or basal slope conductance of an ORN, is due to a basal level of cAMP within the cell. We further propose that this resting level of cAMP improves the sensitivity of the ORN to weak odorous stimulation.

The conductances measured with whole-cell tight-seal recording and perforated-patch recording were similar. One might expect that the perforated-patch configuration would preserve the basal cAMP level, while whole-cell recording would allow the cAMP to diffuse into the patch pipette. In

this case the measured conductance would be lower with whole-cell recording. However, it is likely that the resting cAMP is largely generated in the cilia, where the olfactory type III adenylate cyclase (Bakalyar and Reed, 1990) and the CNG channels are concentrated. The concentration of cAMP would be determined by the activities of the cyclase and phosphodiesterase, as well as by the rate of diffusion of cAMP from the cilium into the much greater volume of the dendrite and soma. It is unlikely that further diffusion into the pipette would significantly reduce [cAMP] in the cilia.

In low-divalent extracellular solutions, the neuronal input conductance averaged 0.39 nS. Addition of extracellular divalent cations (2 mM Ca^{2+} plus 1 mM Mg^{2+}), which partially block CNG channels (Zufall and Firestein, 1993; Kleene, 1995; Zagotta and Siegelbaum, 1996; Kaupp and Seifert, 2002), reduced the conductance to 0.25 nS. The latter value represents an estimate (upper limit) of the total resting neuronal conductance from all sources in physiological solutions, which contain divalent cations. Addition of the CNG-channel blocker amiloride (1 mM) reduced the conductance to 0.19 nS, the lowest value measured. Unlike divalent cations, 1 mM amiloride almost completely blocks the CNG conductance (Frings et al., 1992; Kleene, 1994). Thus the amount of reduction by amiloride in physiological solutions (0.06 nS) is our best estimate of the conductance through CNG channels in a resting ORN.

This resting CNG conductance could be due to cyclic-nucleotide-dependent gating of the underlying channels, spontaneous gating, or both. Spontaneous gating of CNG channels has been demonstrated in isolated olfactory cilia (Kleene, 2000) and in other systems (Picones and Korenbrot, 1995; Tibbs et al., 1997; Ruiz and Karpen, 1997). Our results allow some discrimination between the two gating modes. In low-divalent solutions, the two adenylate cyclase inhibitors each decreased the resting conductance by an amount averaging 0.11 $\pm$ 0.06 nS (mean $\pm$ SD, $n = 13$). These reagents should reduce ligand-dependent gating but not spontaneous gating. Divalent cations and amiloride, which should block CNG channels gated by either mechanism, each reduced the conductance by an amount averaging 0.15 $\pm$ 0.06 nS ($n = 22$). The inhibitions produced by the two types of reagents were different, but only at the 90% confidence level. Clearly, though, most of the resting CNG conductance was blocked by the cyclase inhibitors, suggesting that most of the open CNG channels were gated by a resting level of cAMP. At the concentrations used, the cyclase inhibitors block the odorant-induced current but do not directly block the CNG channels (Chen et al., 2000; THFA is the same as SQ22536).

We also studied the effects of several ATP analogs on the slope conductance of unstimulated ORNs with whole-cell voltage clamp. AMP-PNP reduced the slope conductance within 30 s after establishing whole-cell recording. AMP-PNP is a substrate for adenylate cyclase, but it allows a much lower rate of cAMP production than does the usual substrate, ATP (Maguire and Gilman, 1974). This result supports the hypothesis that in unstimulated ORNs, ATP is being converted into cAMP by basal adenylate cyclase activity, which then activates CNG channels. With ATP, ATP-γ-S, or the recording solution in the pipette, there was no change in the slope conductance within the first several min. An increase in slope conductance with ATP and ATP-γ-S was observed after 2 min of recording. This observation is consistent with the expected actions of ATP and ATP-γ-S (i.e., providing more substrate to be converted to cAMP). However, we also saw a small but statistically insignificant increase with just the recording solution in the whole-cell experiments (Table 2). Since this may have simply been due to a gradual decrease in seal resistance, we confined our observations with the whole-cell method to the first 2 min of recording. We did not test for an effect of cytoplasmic GTP. Theoretically, GTP might increase the resting conductance by either of two mechanisms. It could increase the activity of the adenylate cyclase, or it could be hydrolyzed to cGMP, which also gates the CNG channels.

We have identified five reagents that should reduce the CNG conductance, and each of these also reduces the neuronal input conductance. However, it must be acknowledged that each of these reagents could work by more than one mechanism. (No specific blocker of the native CNG channel is known.) The reduction of slope conductance by cyclase inhibitors suggests that the resting neuron produces cAMP, which gates CNG channels. It is also possible, though, that cAMP contributes indirectly to the gating of other neuronal channels that have not yet been identified. Divalent cations and amiloride block CNG channels but might also block other, cAMP-independent conductances in the cilium. However, addition of amiloride produced the same low conductance (0.19–0.20 nS) in both low-divalent and 2Ca:1Mg salines. Thus it can be concluded that divalent cations did not block some population of channels differently from those blocked by amiloride. An outwardly rectifying current seen at positive potentials was reduced by 2Ca:1Mg saline and by amiloride (Fig. 1). This current could be blocked with cytoplasmic Cs^{+}, but such block had no effect on the slope conductance near the resting potential. Thus the outward rectifier does not contribute to the resting conductance. When the outward rectifier was blocked, it was apparent that 2Ca:1Mg saline and the cyclase inhibitor MDL12330A each reduced a current with a reversal potential near 0 mV (Figs. 2 and 3 *C*). Under our experimental conditions, this is the expected reversal potential for CNG channels and is inconsistent with the possibility of Na^{+}- or K^{+}-selective channels. Although more complicated explanations are possible, the most straightforward interpretation of the combined results is that the unstimulated ORN has a significant conductance due to the CNG channels.

It is important to consider whether the resting level of cytoplasmic free Ca^{2+} might affect the basal CNG conductance. Ca^{2+} could reduce [cAMP] by two mechanisms. First,

Ca^{2+}/calmodulin-dependent protein kinase II can phosphorylate the olfactory type III adenylate cyclase and reduce its activity (Wei et al., 1998). However, the kinase is half-maximally activated by 0.7–0.9 μM free Ca^{2+} and is inactive at typical resting Ca^{2+} levels (100 nM; Schulman, 1984, 1988). Ca^{2+} and calmodulin also activate a phosphodiesterase in olfactory receptor neurons (Borisy et al., 1992; Yan et al., 1995); activation begins when $[Ca^{2+}]$free exceeds 100 nM (Borisy et al., 1992). Ca^{2+} and endogenous factors such as calmodulin reduce the sensitivity of the CNG channels to cAMP (Chen and Yau, 1994). Again, though, this effect is negligible at typical resting Ca^{2+} levels (Chen and Yau, 1994). In a resting olfactory receptor neuron, the cilia contain only ~40 nM free Ca^{2+} (Leinders-Zufall et al., 1998). It is thus unlikely that the basal CNG conductance is modulated by Ca^{2+} in the resting state. In most of the present study, we preserved the resting Ca^{2+} level by recording with the nystatin perforated-patch method. Nystatin allows low-resistance access to the cell but prevents diffusion of Ca^{2+} between the cytoplasm and the pipette (Kleinberg and Finkelstein, 1984). We also tested for possible effects of higher (200 nM) cytoplasmic Ca^{2+} using standard whole-cell recording. The increased Ca^{2+} had no significant effect on the neuronal resting conductance.

Our results indicate that the CNG channels cannot account for the total resting neuronal conductance. We have not yet characterized the other channels that open at rest. However, it is unlikely that the ciliary Ca^{2+}-activated Cl^- channels contribute to the resting conductance. These channels are detectable only when cytoplasmic Ca^{2+} exceeds 2 μM (Kleene and Gesteland, 1991), which is ~50 times the resting ciliary Ca^{2+} level (Leinders-Zufall et al., 1998). Consistent with this, replacement of cytoplasmic Cl^- with methanesulfonate$^-$ has no effect on the resting conductance (unpublished observations).

It is generally assumed that for signal transduction and conduction to occur effectively and efficiently in an excitable cell, the cell should have a high input resistance. This assures that even a small current generated as a result of any stimulation will produce a sufficiently large change in membrane voltage. The sensitivity of detection would thus depend on the high input resistance of the cell, ensuring the appropriate transduction and conduction of a signal. Indeed, the opening of a single channel has been reported to be sufficient to generate an action potential (Fenwick et al., 1982; Lynch and Barry, 1989). However, for a small sensory neuron such as the frog ORN, there may be other considerations. First, an ORN needs to discriminate between genuine and false signals. A high input resistance may not always be beneficial. Small increases in conductance from spontaneous opening of channels or random fluctuations in the transduction cascade could lead to generation of a false signal (i.e., one not arising from an odorous stimulus). A second disadvantage occurs because the transduction of a stimulus depends on a cascade system (activation of G_{olf} and cAMP formation). If the initial concentration of the second messenger is very close to zero, it will take a very large amplification before the concentration of the second messenger reaches the threshold sufficient to evoke a signal. An optimal compromise might be for the neuron to have a somewhat lower input resistance while maintaining a slightly subthreshold level of second messenger. We have estimated that the resting level of cAMP in a frog ORN is in the range of 0.1–0.3 μM. A model (see the Appendix) based on the dose-response curve for CNG channels to cAMP suggests that an ORN with this resting level of cAMP is poised to generate the largest possible depolarization in response to a very small increase in cytoplasmic cAMP. This should enhance the sensitivity of the neuron to weak odorous stimuli.

In summary, a cAMP-dependent conductance contributes to the resting slope conductance of unstimulated frog ORNs. This cAMP-dependent conductance is reduced by high divalent cation concentrations, amiloride, adenylate cyclase inhibitors, and the ATP analog AMP-PNP. This conductance is 20–40% of the total resting conductance and plays an important role in determining, and perhaps even setting, the threshold of response to odorous stimulation.

APPENDIX

Our results indicate that ORNs, in the absence of applied olfactory stimuli, maintain a level of cytoplasmic cAMP sufficient to gate the CNG channels. Based on the following theoretical considerations, we propose that this increases the sensitivity of the neurons to very weak stimuli.

Current through the ciliary CNG channels increases with increasing concentration of cytoplasmic cAMP. The relation between this macroscopic current I and [cAMP] is well fit by a Hill-type equation (Nakamura and Gold, 1987; Kleene, 1999):

$$I = I_{max}\frac{c^n}{c^n + k^n}, \quad (1)$$

where I_{max} is the maximum macroscopic current, c is [cAMP], k is the half-maximally effective concentration of cAMP, and n is the Hill coefficient. Fig. 6 *A* shows part of this dose-response relation after substituting parameters measured in frog ORNs in physiological solutions. The parameters used are k, 10.3 μM; n, 1.28; and I_{max}, 283 pA; see Methods. The relation deviates substantially from linearity at low c. Near $c = 0$, the slope of the relation approaches 0. There a slight increase in c produces very little increase in I, the macroscopic CNG current. At somewhat higher concentrations, an incremental increase in [cAMP] produces a greater increase in I. When a weak odorous stimulus causes an incremental increase in [cAMP], a neuron that already has a small concentration of cAMP will generate a greater receptor current than one with no cAMP.

Fig. 6 *B* shows the slope (first derivative) of the Hill-type equation as a function of [cAMP]. The slope reaches a peak (second derivative = 0) when:

$$c = k\left(\frac{n-1}{n+1}\right)^{1/n}. \quad (2)$$

The function shown has a peak at $c = 2.0$ μM, where there is an inflection point in the Hill-type equation. A frog ORN with a resting [cAMP] of 2.0 μM would be poised to give the greatest possible CNG receptor current in response to a small increase in [cAMP].

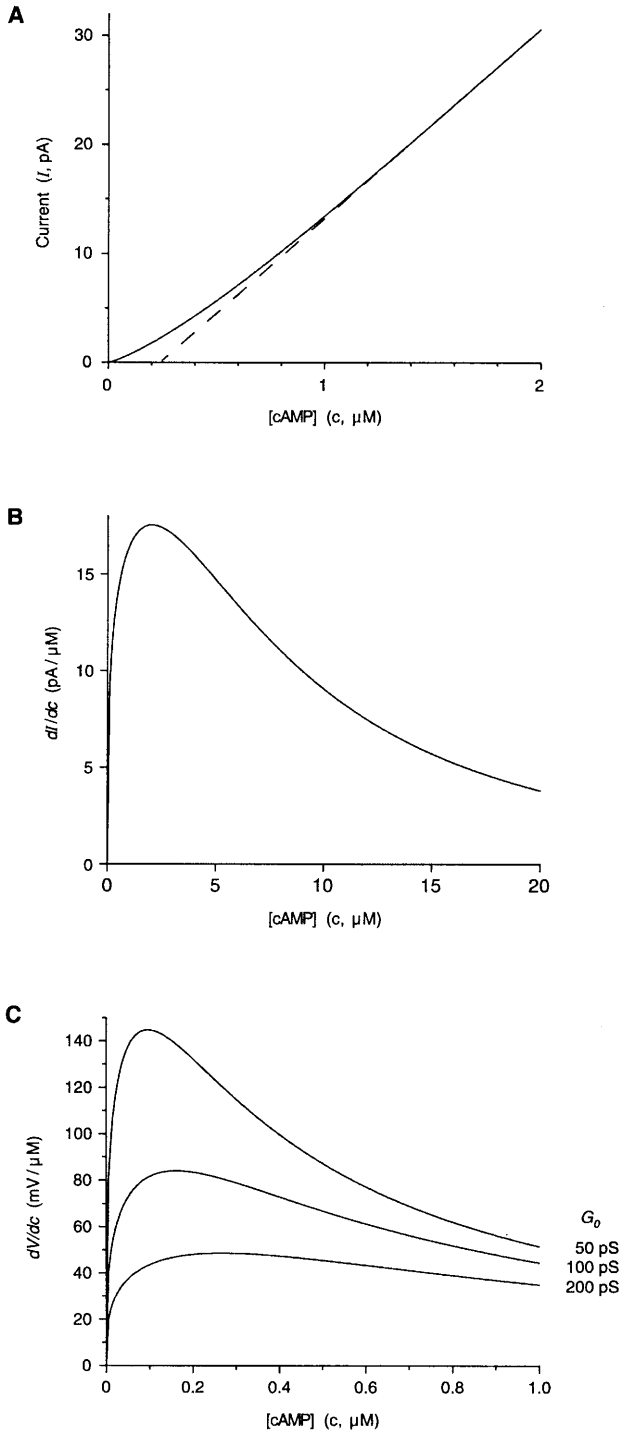

FIGURE 6 Theoretical effects of a small resting level of cAMP. (*a, Solid line*) The macroscopic current through CNG channels as a function of [cAMP] in the cilium. A Hill-type equation was used as described in the Appendix. (*Dashed line*) A linear extension from the point of maximum slope (i.e., the inflection point, where [cAMP] = 2.0 μM). (*B*) Slope (first derivative) of the relation in *A*, shown over a wider range of [cAMP]. (*C*) The change in voltage as a function of [cAMP] (*dV*/*dc*). The change in current as a function of [cAMP] (*dI*/*dc*, shown in *B*), was divided by the resting membrane conductance as defined in the text. *I* was calculated with the Hill-type equation, and the resting membrane potential V_m was assumed to be −50 mV. Three values of G_0 were assumed as indicated. Maxima for the curves occur at $c = 0.096$ μM ($G_0 = 50$ pS), 0.16 μM ($G_0 = 100$ pS), and 0.27 μM ($G_0 = 200$ pS). Although the model represents inward currents at −50 mV, currents are shown as positive for simplicity.

It is necessary to consider other consequences of CNG channels that open at rest. Opening and closing of the channels contributes electrical noise that could make reliable detection of a small receptor event more difficult. In this study, we found that the resting neuron has a cytoplasmic cAMP concentration of 0.1–0.3 μM. This corresponds to a mean channel open probability of 0.003–0.010 (assuming parameters given in Methods) and should produce a current variance of just 0.03–0.12 pA^2. Thus the noise arising from the CNG channels is probably negligible compared to other noise sources.

Having CNG channels open at rest also reduces the neuronal resistance. We found that 20–40% of the input conductance is attributable to open CNG channels. In a simple ohmic model, decreasing the membrane resistance by 30% decreases the voltage response to a given receptor current by 30%. One might guess that this would make it more difficult for a small increase in cAMP to depolarize the neuron. In fact, the voltage response of the resting neuron is optimized by having a nonzero level of cAMP. The resting membrane conductance G_m of the neuron is:

$$G_m = G_0 + \frac{I}{V_m}, \tag{3}$$

where I is again the current through CNG channels at rest, V_m is the resting membrane potential, and G_0 is the conductance at rest from all sources other than the CNG channels. Dividing the change in current with concentration (*dI*/*dc*, Fig. 6 *B*) by the resting membrane conductance G_m yields the change in voltage as a function of [cAMP] (*dV*/*dc*, Fig. 6 *C*). This function takes into account the decrease in membrane resistance caused by CNG channels that are open at rest. For the three values of G_0 modeled, a small increment in [cAMP] produces the greatest voltage change when the starting [cAMP] is in the range of 0.1–0.3 μM. Our measurements (see Results) suggest that the resting cAMP concentration indeed lies within this range.

A sufficient Ca^{2+} current through CNG channels causes gating of Ca^{2+}-activated Cl^- channels, which greatly amplify the receptor current (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993). This gating becomes significant when [Ca^{2+}] in the cilium exceeds 2 μM (Kleene and Gesteland, 1991). It is not yet known what level of cAMP causes a Ca^{2+} influx sufficient to gate the Cl^- channels. At that level, though, the increase in total current resulting from a given increase in [cAMP] should assume values even greater than those shown in Fig. 6 *B*. It is likely that the advantage of having a nonzero resting cAMP level is much greater than is indicated by considering the CNG channels in isolation.

It is known that the dose-response relationship of the CNG channel is well fit by a Hill-type equation over a substantial range of cAMP concentrations (Nakamura and Gold, 1987; Kleene, 1999). However, two caveats must be mentioned. First, the precise shape of the dose-response relation at very low concentrations has not been directly measured. Second, the Hill equation can only approximate the exact dose-response relation (Weiss, 1997). The optimum in *dI*/*dc* (Fig. 6 *B*) depends on the existence of an inflection point in the dose-response relation. An inflection point is assured by having a Hill coefficient $n > 1.0$, and this is established in the case of the olfactory CNG channel (Nakamura and Gold, 1987; Kleene, 1999). This is also expected, since the native olfactory CNG channel is a multimer of subunits, each of which has a cyclic-nucleotide-binding site (Bönigk et al., 1999). Theoretical alternatives to the Hill equation have been proposed (Weiss, 1997), and these also have inflection points if $n > 1.0$.

In summary, having a small number of CNG channels open at rest is predicted to increase the neuronal depolarization in response to a weak odorous stimulus. Although having the channels open at rest decreases the voltage change resulting from a given receptor current, it more substantially increases the receptor current resulting from a given increase in [cAMP].

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