J. Membrane Biol. 178, 49–54 (2000)
DOI: 10.1007/s002320010014

The Journal of
Membrane
Biology

Spontaneous Gating of Olfactory Cyclic-Nucleotide-Gated Channels

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Received: 30 May 2000/Revised: 8 August 2000

Abstract. In vertebrates, cilia on the olfactory receptor neurons have a high density of cyclic-nucleotide-gated (CNG) channels. During transduction of odorous stimuli, cyclic AMP is formed. cAMP gates the CNG channels and this initiates the neuronal depolarization. Here it is shown that the ciliary CNG channels also open spontaneously. In the absence of odorants and second messengers, olfactory cilia have a small basal conductance to cations. Part of this conductance is similar to the cAMP-activated conductance in its sensitivity to channel inhibitors and divalent cations. The basal conductance may help to stabilize the neuronal membrane potential while limiting the sensitivity of odorant detection.

Key words: Olfaction — Receptor neuron — Cilia — Cyclic-nucleotide-gated channel — Spontaneous gating — Electrophysiology

Introduction

In vertebrates, detection of odorous stimuli occurs on the cilia of olfactory receptor neurons (reviewed in Schild & Restrepo, 1998). In the absence of odorants and second messengers, the ciliary membrane has a small basal conductance to cations (Kleene, 1992). This conductance may help to stabilize the ciliary membrane potential at some cost to odorant sensitivity.

Vertebrate olfactory cilia have a high density of cyclic-nucleotide-gated (CNG) channels (Nakamura & Gold, 1988; Kurahashi & Kaneko, 1993; Larsson, Kleene & Lecar, 1997). Evidence suggests that the ciliary basal conductance arises in part from spontaneous (ligand-independent) gating of these channels. Both the basal conductance and the CNG channels permit Na^+, K^+, and Ca^{2+} ions, but not Cl^-, to carry current (Kleene, 1992; Kleene, 1993*b*). Both have outwardly rectifying current-voltage relations in the presence of external divalent cations.

Other channels have been shown to undergo both ligand-activated and spontaneous gating. These include channels activated directly or indirectly by acetylcholine (Jackson, 1984, 1986; Rojas & Zuazaga, 1991; Okabe, Yatani & Brown, 1991; Franco-Obregón & Lansman, 1995), as well as the NMDA receptor (Tureček et al., 1997). In invertebrate (Johnson & Bacigalupo, 1992) and vertebrate (Picones & Korenbrot, 1995) photoreceptors, the cGMP-gated channels undergo spontaneous gating. In the olfactory system, it is known that exogenous expression of the α subunit of the catfish olfactory CNG channel yields homomeric channels that open in the absence of ligand (Tibbs, Goulding & Siegelbaum, 1997). However, native CNG channels include more than one subunit type (e.g., Bönigk et al., 1999) and differ in several ways from expressed homomultimers. Evidence is presented here that the native CNG channels in frog olfactory cilia also gate spontaneously, producing a detectable macroscopic current.

Materials and Methods

Ciliary Patch Procedure

Northern grass frogs (*Rana pipiens*) were decapitated and pithed. Single receptor neurons were isolated from the olfactory epithelium as described elsewhere (Kleene & Gesteland, 1991*a*). Cell suspensions were prepared in a standard extracellular solution (*see* Table 1). A single receptor neuron was placed in an extracellular bath containing this solution. One cilium of a neuron was sucked into a patch pipette until a high-resistance seal formed near the base of the cilium. The pipette contained the K^+- and divalent-free extracellular solution shown in Table 1. Small amounts of bath solution entered the pipette tip during the patch procedure. However, within 1 min the current-voltage relation reached a stable state that depended on the bulk

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Table 1. Compositions of solutions (mM)

	NaCl	KCl	NMDG-Cl	$CaCl_2$	$MgCl_2$	EDTA	NaOH	TRIS base
Extracellular								
Standard	115	3	—	1	2	—	1	—
K^+- and divalent-free	118	—	—	—	—	1	4	—
High-Ca^{2+}	118	—	—	3	—	—	1	—
Na^+- and K^+-free	—	—	118	1	2	—	—	1

	NaCl	NMDG-Cl	$CaCl_2$	$MgCl_2$	BAPTA	NaOH	TRIS base
Pseudointracellular (all low in divalent cations)							
K^+-free	115	—	1	—	2	9	—
High-Mg^{2+}	115	—	1	2	2	9	—
Na^+- and K^+-free	—	115	1	—	2	—	9

Abbreviations used: NMDG, *N*-methyl-D-glucamine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. All solutions contained 5 mM HEPES and were adjusted to pH 7.2. The NaOH and TRIS base listed include amounts necessary to titrate EDTA, BAPTA, and HEPES, which are listed as free acids. $[Ca^{2+}]_{free}$ in the pseudointracellular solutions was 0.15 μM.

pipette solution (Kleene, 1993*a*). The pipette was raised briefly into the air, causing excision of the cilium from the cell. The cilium remained sealed inside the recording micropipette with the cytoplasmic face of the membrane exposed to the bath. The pipette containing the cilium could be quickly transferred through the air to various pseudointracellular baths without rupturing the seal. In most experiments, the pipette solution was also replaced by intrapipette perfusion (Tang et al. 1990; Kleene 1992). Additional details of the ciliary patch procedure have been presented elsewhere (Kleene & Gesteland, 1991*a;* Kleene, 1995*b*).

Solutions (Table 1) were designed to select for Na^+ current through open CNG channels. Since divalent cations can reduce this current by open-channel block, Ca^{2+} and Mg^{2+} were chelated to very low levels except as noted. To eliminate any K^+-selective ciliary conductances, K^+ was eliminated from all recording solutions.

Electrical Recording

Both the recording pipette and chamber were coupled to an Axopatch 200B patch-clamp amplifier by Ag/AgCl electrodes. All recordings were done under voltage clamp at room temperature (25°C). Current was adjusted to zero with the open pipette in the well in which the patching procedure was done. Corrections were made for liquid junction potentials at the pipette tip; these were at most 3 mV.

Voltage ramps (−100 to +100 mV, 1 sec) were generated by pCLAMP software (Axon Instruments, Foster City, CA). Current was sampled at 500 Hz. Potentials are reported as bath (cytoplasmic) potential relative to pipette potential. Results of repeated experiments are reported as mean ± SEM. Student's *t*-test for repeated measures was used for statistical comparisons. Errors were propagated through subsequent calculations as described by Taylor (1982).

Measurement of Basal Ciliary Conductance

The measured input conductance is due to two parallel current pathways: the ciliary membrane itself and a shunt at the seal between the membrane and the recording pipette. The two currents can be resolved on the assumption that large cations can carry current through the shunt but not through the membrane. After measuring the input conductance, solutions on both sides of the membrane were replaced with solutions in which all Na^+ and K^+ were replaced with *N*-methyl-D-glucamine (Table 1). Current remaining after this was attributed to the shunt. This measured shunt was corrected in two ways as described previously (Kleene, 1992). First, a correction was made for the liquid junction potential between the pipette and bath solutions. Second, the slope was increased to compensate for the decrease in conductance caused by replacing Na^+ and K^+ with the larger, less mobile *N*-methyl-D-glucamine.In the present study, the mean corrected conductance of the shunt be-tween −80 and +80 mV was 99 ± 14 pS, which was consistent with a previous measurement in similar solutions (92 ± 6 pS; Kleene, 1992).

With the exception of the phosphodiesterase study, the shunt conductance has been subtracted from all values reported. In Fig. 2*B*, the shunt conductance was not directly measured. Measuring the effects of external Ca^{2+} required changing the pipette solution once by perfusion. Measuring the shunt conductance would have required a second perfusion with the Na^+- and K^+-free extracellular solution, and this was found to be impractical. For these data only, the average current-voltage relation for all measured shunts was subtracted.

Materials

DCB (3′,4′-dichlorobenzamil, an amiloride derivative also known as DCPA) was generously provided by Dr. Gregory Kaczorowski (Merck, Rahway, New Jersey). *l-cis*-Diltiazem hydrochloride was a gift of Dr. Pal Vaghy, who obtained it from Tanabe Seiyaku, Osaka. All other reagents were from Sigma. DCB was diluted from a 25 mM stock solution in dimethyl sulfoxide, and W-7 from a 10 mM stock solution in ethanol. Solutions diluted from nonaqueous solvents were sonicated to promote solution of the inhibitor.

Results

Electrical recordings were made from single frog olfactory cilia. Each cilium was sealed inside a patch pipette

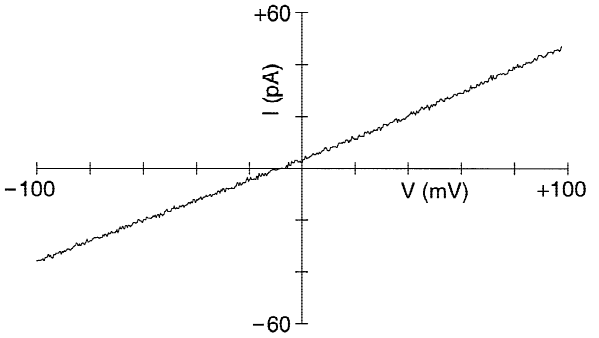

Fig. 1. Basal conductance of frog olfactory cilia. The current-voltage (*I-V*) relation of a frog olfactory cilium was measured in K^+-free, low-divalent solutions. Current attributed to a nonspecific shunt at the seal between the membrane and the pipette has been subtracted as described in Materials and Methods. The *I-V* relation shown is the average of all 31 cilia studied.

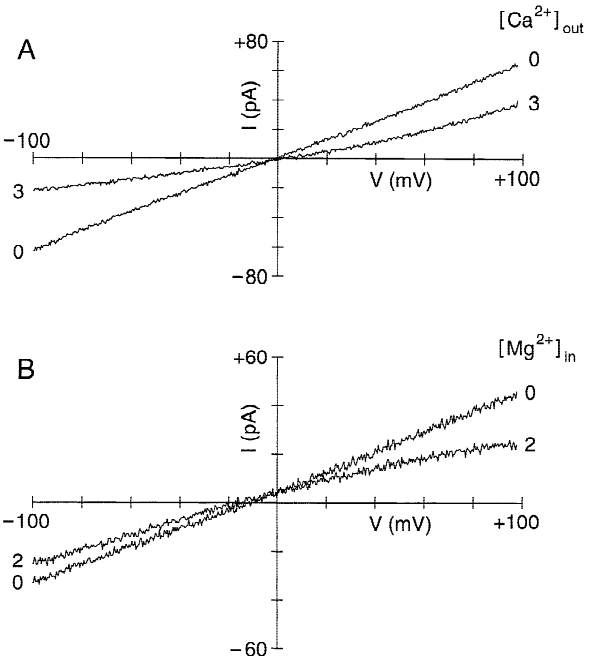

Fig. 2. Rectification of the ciliary basal conductance by divalent cations. (*A*) Outward rectification by 3 mM external Ca^{2+}. For each cilium, the current-voltage (*I-V*) relation was first measured in low-divalent solutions (curve labeled '0'). The external (pipette) solution was replaced with the high-Ca^{2+} solution (Table 1) by intrapipette perfusion. After 2 min, the *I-V* relation was stable again (curve labeled '3'). Each curve shown is the average of the 6 cilia studied. (*B*) Inward rectification by 2 mM cytoplasmic Mg^{2+}. For each cilium, the current-voltage (*I-V*) relation was first measured in low-divalent solutions (curve labeled '0'). The cytoplasmic (bath) solution was replaced with the high-Mg^{2+} solution (Table 1), and the *I-V* relation was stable again (curve labeled '2'). Each curve shown is the average of the 7 cilia studied. Current attributed to a nonspecific shunt at the seal between the membrane and the pipette has been subtracted as described in Materials and Methods.

and excised from the cell so that the cytoplasmic face of its membrane was exposed to the bath (Kleene & Gesteland, 1991*a*). In the absence of odorants and second messengers, the ciliary membrane shows a basal conductance to cations but not to Cl^- (Kleene, 1992). In solutions lacking divalent cations and K^+, the current-voltage (*I-V*) relation of the conductance showed slight outward rectification (Fig. 1). Rectification was measured as the ratio of the slope conductance at positive potentials (+20 to +80 mV) to the conductance at negative (−80 to −20 mV) potentials. The ratio averaged 1.2 ± 0.04 ($n = 31$) for the basal conductance. The slope conductance between −80 and +80 mV averaged 405 ± 52 pS ($n = 31$, range 113 to 1350 pS). The reversal potential averaged −9 ± 1 mV; a value near 0 mV was expected because the external and cytoplasmic solutions were nearly identical.

Some of the basal ciliary conductance may come from spontaneous gating of the ciliary cyclic-nucleotide-gated (CNG) channels. To test this possibility, compounds known to block the CNG channels were tested for their ability to also block the basal conductance. Of four such compounds tested, all reduced the basal conductance substantially (Table 2). In all cases, the inhibitors blocked the basal conductance somewhat less effectively than they blocked the conductance activated by a saturating dose of cAMP (Table 2).

The olfactory CNG channels also show voltage-dependent block by divalent cations. External Ca^{2+} reduces cAMP-activated current at all potentials. However, inward current is more strongly blocked, resulting in outward rectification of the *I-V* relation (Zufall & Firestein, 1993; Kleene, 1995*a;* Frings et al., 1995). External Ca^{2+} (3 mM) produced a similar effect on the ciliary basal conductance in the absence of cAMP (Fig. 2*A*). In the absence of external Ca^{2+}, the rectification ratio of the basal conductance was 1.1 ± 0.1 ($n = 6$). By intrapipette perfusion, the external solution was replaced with one containing 3 mM Ca^{2+}. This produced outward rectification (Fig. 2*A*, curve labeled "3"), increasing the current ratio by a factor of 1.8 ± 0.2. The increase was significant ($P < 0.01$). In a previous study, the effect of external Ca^{2+} on current activated by a saturating level of cAMP was greater. In that case, increasing external Ca^{2+} from 0.1 μM to 3 mM increased the current ratio by a factor of 6.9 ± 2.4 (*unpublished analysis* from Kleene, 1995*a*).

Increasing cytoplasmic Mg^{2+} greatly reduces cAMP-activated current at positive potentials, causing inward rectification (Yau & Baylor, 1989; Colamartino, Menini & Torre, 1991; Zimmerman & Baylor, 1992; Kleene, 1993*a*). Cytoplasmic Mg^{2+} (2 mM) produced a similar effect on the ciliary basal conductance in the absence of cAMP (Fig. 2*B*). The rectification ratio decreased by a factor of 0.63 ± 0.07 ($n = 7$), which was significant ($P < 0.01$). In a previous study, the effect of cytoplasmic Mg^{2+} on current activated by a saturating level of cAMP

Table 2. Inhibitors of the ciliary basal conductance

	Conc. (μM)	Basal conductance % Inhibition			cAMP-activated conductance % Inhibition		
		Mean ± SEM	Range	*n*	Mean ± SEM	Range	*n*
W-7	100	84 ± 6	64–124	6	98 ± 1	95–100	9
Amiloride	1000	46 ± 2	42–53	5	94 ± 2	84–101	8
DCB	300	93 ± 3	73–109	8	100	102–103	2
l-cis-diltiazem	1000	66 ± 5	48–78	5	89 ± 1	88–91	6

For each cilium in this study, ciliary basal conductance between −80 and +80 mV was measured in cytoplasmic baths with or without the inhibitor (*see* Materials and Methods). Current attributed to a nonspecific shunt at the seal between the membrane and the pipette was subtracted as described in Materials and Methods. When the measured inhibition was >100%, the value was reduced to 100% before figuring the mean. Values for inhibition of the cAMP-activated conductance are taken from Kleene (1993*b*, 1994). In those studies, the conductance activated by 100 μM cAMP in low-divalent solutions was measured between −50 and +50 mV in the presence or absence of the cytoplasmic inhibitor.

was greater. Increasing cytoplasmic Mg^{2+} from 0 to 2 mM decreased the current ratio by a factor of 0.026 ± 0.009 (*unpublished analysis* from Kleene, 1993*a*).

In seven cilia, the basal conductance was compared to the conductance activated by 100 μM cAMP, which saturates the CNG channels. In these cilia, the mean basal conductance was 387 ± 61 pS (range 270 to 644 pS), while the cAMP-activated conductance was 9.6 ± 1.5 nS (range 4.0 to 15.9 nS). The ratio of basal to cAMP-activated conductance averaged 0.04 ± 0.01, but there was no significant correlation between the two conductances.

If an excised cilium retains a small amount of cAMP or cGMP on excision from the cell, this could gate the ciliary CNG channels and account for part of the measured conductance. However, the cilium has an endogenous cAMP phosphodiesterase activity sufficient to eliminate low levels of cAMP. In the absence of added cAMP, the input conductance in five cilia averaged 761 ± 215 pS ($n = 5$) in low-divalent solutions. Addition of 0.3 μM cAMP increased this to 791 ± 195 pS; the increase was not significant ($P > 0.3$). However, simultaneous addition of 0.3 μM cAMP and 100 μM IBMX (3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor) increased the ciliary conductance to 1036 ± 226 pS. This increase was significant ($P < 0.01$). In a separate population of cilia, it was shown that IBMX had no effect on the ciliary basal conductance in the absence of added cAMP. Adding 100 μM IBMX increased the conductance from 507 ± 44 pS to 510 ± 29 pS, which was not a significant change ($n = 4$, $P > 0.90$).

Discussion

In the absence of odorants and second messengers, frog olfactory cilia have a basal conductance. Each cilium has a high density of cyclic-nucleotide-gated (CNG) channels (Nakamura & Gold, 1988; Kurahashi & Kaneko, 1993; Larsson et al., 1997), and prior evidence has suggested that ligand-independent gating of these channels might account for part of the basal conductance. Like the CNG channels, the basal conductance is permeable to Na^+, K^+, and Ca^{2+} but not Cl^- (Kleene, 1992; Kleene 1993*b*). Like the cAMP-activated conductance (Zufall & Firestein, 1993; Kleene, 1995*a;* Frings et al., 1995), the ciliary conductance shows outward rectification when external divalent cations are present (Kleene, 1992).

The present study supports the hypothesis that ciliary CNG channels gate spontaneously, producing a detectable macroscopic conductance. In the absence of divalent cations, the ciliary basal current-voltage (*I-V*) relation shows slight outward rectification (Figs. 1 and 2). Under similar ionic conditions, the *I-V* relation of the cAMP-activated ciliary conductance also shows slight outward rectification (Colamartino et al., 1991; Frings, Lynch & Lindemann, 1992; Kleene, 1993*a;* Kurahashi & Kaneko, 1993; Lynch & Lindemann, 1994; Kleene, 1995*a;* Frings et al., 1995; Balasubramanian, Lynch & Barry, 1995). Divalent cations, through open-channel block, cause distinct rectification of the cAMP-activated conductance. External Ca^{2+}, for example, reduces current at negative potentials (Zufall & Firestein, 1993; Kleene, 1995*a;* Frings et al., 1995). Conversely, cytoplasmic Mg^{2+} greatly reduces current at positive potentials (Yau & Baylor, 1989; Colamartino et al., 1991; Zimmerman & Baylor, 1992; Kleene, 1993*a*). In the absence of cAMP, external Ca^{2+} and cytoplasmic Mg^{2+} produced qualitatively the same effects on the *I-V* relation of the basal ciliary conductance (Fig. 2). Although qualitatively the same, the rectification of the basal conductance was less than that seen with the cAMP-activated conductance.

The ciliary basal and cAMP-activated conductances

are also similar pharmacologically. Several inhibitors of the cAMP-activated conductance have been identified (summarized in Kleene, 1994). All four such inhibitors tested were also effective in reducing the ciliary basal conductance (Table 2). On average, the blockers reduced the cAMP-activated conductance by about 95%. At the same concentrations, they were slightly less effective against the basal conductance, inhibiting it on average by 72%. None of the available inhibitors are completely specific for the CNG channels. However, all four inhibitors tested block the basal conductance, suggesting that much of this conductance arises from spontaneous gating of the CNG channels.

Several lines of evidence indicate that the CNG channels do not account for all of the ciliary basal conductance. In solutions that are primarily NaCl, the *I-V* relation of the basal conductance is nearly linear and reverses near 0 mV (Figs. 1 and 2; *see also* Kleene, 1992). However, in normal solutions (external NaCl and cytoplasmic KCl), the *I-V* relation shows outward rectification and reverses near −50 mV (Kleene, 1992). As judged by the slope conductance, the basal conductance is 2.3 times more conductive to K^+ than to Na^+ (Kleene, 1992). However, the CNG channels are more permeable to Na^+; this would result in a positive reversal potential in normal solutions (Frings, Lynch & Lindemann, 1992; Balasubramanian, Lynch & Barry, 1995). Thus it is reasonable to suppose that the ciliary basal conductance arises in part from other channels that are more permeable to K^+. This would also explain why CNG-channel inhibitors and divalent cations have greater effects on a pure cAMP-activated conductance than on the basal conductance. If the basal conductance were entirely due to the CNG channels, one would expect a strong positive correlation between the basal and ligand-activated conductances across a population of cilia. Such a correlation was observed in the outer segments of cone photoreceptors, in which CNG channels account for the total membrane conductance (Picones & Korenbrot, 1995). In frog olfactory cilia, no such correlation could be demonstrated.

It must be asked whether the cilium retains some cyclic nucleotide or ATP on excision. A sufficient concentration of cAMP or cGMP could gate the CNG channels directly and account for the basal conductance. In addition, adenylate cyclase activity in the ciliary membrane could convert retained ATP to cAMP, which would gate the channels (Kleene, Gesteland & Bryant, 1994). That the excised cilium retains such small molecules is very unlikely. The conductance changes seen on bathing a cilium in cAMP are fully and quickly reversible, suggesting that diffusion of small molecules into and out of the cilium is fast. Furthermore, the cilium retains a cyclic-nucleotide phosphodiesterase sufficient to hydrolyze low levels of cAMP. A low level of added cAMP (0.3 μM) causes a conductance increase of 275 pS, but only if the endogenous phosphodiesterase is inhibited by IBMX. If a cilium were to retain endogenous cAMP, one would expect to see a conductance increase in the presence of IBMX. In fact, there was no effect of IBMX on the cilium in the absence of added cAMP.

On average, the basal conductance was just 0.04 times the conductance measured in the presence of a saturating level of cAMP. With saturating cAMP and no divalent cations, the maximum open probability of the CNG channel has been inferred from noise analysis to be 0.70 (Larsson et al., 1997). Thus the open probability due to spontaneous gating cannot exceed 0.03 (0.04×0.70). The true value must be less than this, assuming that only part of the basal conductance arises from the CNG channels. Exogenous expression of the α subunit of the catfish olfactory CNG channel yields channels with an open probability due to spontaneous gating of 0.002 (Tibbs et al., 1997). Estimates of this probability for the nicotinic acetylcholine receptor range from 3×10^{-7} (Jackson, 1986) to 0.005 (Franco-Obregón & Lansman, 1995).

The consequences of spontaneous gating of the ciliary CNG channels on the functioning of the whole neuron have yet to be determined. The resting membrane conductance of intact olfactory receptor neurons also has some pharmacological similarities to the conductance activated by cAMP (R.Y.K. Pun and S.J. Kleene, *unpublished observations*). However, in the resting neuron, current through the CNG channels could result from either spontaneous gating or from a low level of cAMP or cGMP (Lowe & Gold, 1995).

This work was supported by research grant number 5 R01 DC 00926-07 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health. I am grateful to Tom Nickell for helpful comments on the manuscript.

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