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Cyclic Nucleotide Signaling Mediates an Odorant-suppressible Chloride Conductance in Lobster Olfactory Receptor Neurons

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Abstract

We have previously shown that lobster olfactory receptor neurons (ORNs) express an odorant-suppressible Cl^- conductance that modulates the output of the cells. Here, we develop a more complete pharmacological profile of this conductance, showing it is blockable by the Cl^- channel blockers DIDS, 9-AC and flufenamic acid, but not by niflumic acid. We then show that a conductance with this pharmacological profile is mediated by cyclic nucleotide signaling. These findings further our understanding of the cellular mechanisms through which odorants can modulate the output of lobster ORNs.

Key words: Cl^- conductance, cyclic nucleotide signalling, lobster, olfaction

Introduction

Two modes of olfactory signaling, excitation and inhibition, are used by olfactory receptor neurons (ORNs) in diverse animal species to code olfactory information, including lobster (McClintock and Ache, 1989), *Drosophila* (Dubin and Harris, 1997; deBruyne *et al.*, 2001) and other insects (Akers, 1992; Shields and Hildebrand, 2001), squid (Lucero *et al.*, 1992), mudpuppy (Dionne, 1992) and other amphibians (Vogler and Schild, 1999; Sanhueza *et al.*, 2000), catfish (Kang and Caprio, 1995) and rat (Duchamp-Viret *et al.*, 1999). A number of mechanisms have been suggested through which odorants could inhibit the output of ORNs, including biasing the receptor in an inactive state (Hallem *et al.*, 2004), modulating one or more steps of the excitatory signaling cascade (Spehr *et al.*, 2002) and activating or suppressing conductances that would oppose excitation at the cellular level. The latter mechanisms include triggering a concomitant increase in K^+ conductance (lobster: Michel *et al.*, 1991; squid: Lucero and Chen, 1997; toad and rat: Sanhueza *et al.*, 2000) and suppressing a steady-state Cl^- conductance (mudpuppy: Dubin and Dionne, 1993; *Drosophila*: Dubin and Harris, 1997; and lobster: Doolin *et al.*, 2001). The emerging idea is that different mechanisms are used by different animals to achieve a common organizational principle, coding through opponent input.

Efforts to identify intracellular signaling pathways that may mediate inhibition have been limited, but suggest that these, too, differ across animal species. Activation of a K^+ conductance by odorants has been coupled to elevation of intracellular Ca^{2+} in squid (Lucero and Chen, 1997) and toad (Morales *et al.*, 1997) ORNs. Cyclic nucleotide signaling has been associated with odor-dependent changes in a Cl^- conductance in *Necturus* ORNs (Delay *et al.*, 1997), while phosphoinositol signaling was postulated to drive odor-dependent changes in a Cl^- conductance in squid (Danaceau and Lucero, 1995). In lobster ORNs, cyclic nucleotide signaling is thought to mediate inhibition (Michel and Ache, 1992; Boekhoff *et al.*, 1994). Since odorants can activate a K^+ conductance (Michel *et al.*, 1991) as well as suppress a steady-state Cl^- conductance (Doolin *et al.*, 2001) in these cells, it is possible that cyclic nucleotide signaling targets more than one inhibitory mechanism. cAMP directly activates an ion channel in the outer dendritic membranes of lobster ORNs, suggesting that one target of cyclic nucleotide signaling is a cAMP-gated K^+ channel (Hatt and Ache, 1994). It remains to be determined whether cyclic nucleotide signaling additionally or alternately targets the Cl^- conductance in these cells. Here, we first develop a more complete pharmacological profile of the odorant-suppressed Cl^- conductance, showing it is blocked by the Cl^- channel blockers DIDS, 9-AC and flufenamic acid, but not by niflumic acid. We then show that a conductance with this pharmacological profile is mediated by cyclic nucleotide signaling.

Materials and methods

Animals and preparation

Adult specimens of the Caribbean spiny lobster, *Panulirus argus*, were collected in the Florida Keys. They were maintained in the laboratory in running seawater on a mixed diet of frozen fish, squid and shrimp. ORNs were recorded *in situ* as detailed elsewhere (Doolin and Ache, 2002). Briefly, the olfactory organ (lateral filament of the antennule) was cut into sections of four annuli. Each section was split longitudinally in a dorso-ventral plain and individual hemisections containing olfactory sensilla (aesthetascs) were placed in a recording chamber molded from silicone elastomer. The sheath covering the clusters of ORN somata was digested away to allow patch clamping by replacing the *Panulirus* saline (PS) in the recording bath with L-cysteine-activated papain (Sigma Type IV, 0.17 mg/ml in PS) for 1 min. The cells were then rinsed with PS and the bath was replaced with trypsin (1 mg/ml in PS) for 1 min. The cells were then rinsed a final time with PS before recording. The recording chamber was mounted on the stage of an upright microscope (Axioskop, Carl Zeiss, Inc.), and the somata viewed with conventional bright-field optics using a ×40 long working-distance water-immersion objective (Zeiss 440091). Solutions were delivered selectively to the aesthetasc sensilla using a venturi-based 'spritzer'-type olfactometer (Doolin and Ache, 2002).

Recording and data analysis

Current-clamp recordings were made from the soma of the cells using conventional whole-cell patch-clamp recording. Patch pipettes were fabricated from borosilicate filament glass (1.50 mm o.d., 0.86 mm i.d.; Sutter Instrument Co.) and fire-polished to a tip diameter of 1 μm. Pipettes with resistances of 5 to 9 MΩ were filled with patch-pipette solution (see solutions) and formed 4 to 10 GΩ seals. Membrane potentials were adjusted to −80 mV by injection of current (53 ± 6.1 pA, $n = 25$). It is unlikely, however, that this membrane potential was reflected in the transduction zone (outer dendritic filaments) of these long, thin bipolar neurons. Signals were recorded with a commercial amplifier (Dagan 3900), low pass filtered at 1 kHz (−3 dB; 4-pole Bessel filter), directly digitized at 2–5 kHz and analyzed using pClamp 8 software (Axon Instruments, Inc.). A 3 M KCl/agar bridge was used to connect a reference electrode to the bath solution. All potentials were corrected for junction potentials between pipette tip and indifferent electrode as described by Neher (1992). The series resistance was <10 MΩ in all instances. Experiments were carried out at room temperature (20–22°C). Results are expressed as the mean ± SEM.

Solutions

PS consisted of (mM) 458 NaCl, 13.4 KCl, 13.4 Na_2SO_4, 13.6 $CaCl_2$, 9.8 $MgCl_2$, 2 glucose, 3 HEPES, pH 7.4, adjusted with NaOH. Patch pipette solution consisted of (mM) 180 KCl, 30 NaCl, 11 EGTA, 1 $CaCl_2$, 10 HEPES, 522 glucose, pH 7.2, adjusted with Tris base. The channel blockers anthracene-9-carboxylic acid (9-AC, 50 mM) and niflumic acid (50 mM) were dissolved in DMSO and diluted in PS to the concentrations indicated in the text. 4,4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS, 100 μM), flufenamic (100 μM) acid and 4-aminopyridine (4-AP, 4 mM) were added directly to PS. Forskolin, an adenylyl cyclase agonist, was prepared as a 50 mM stock concentration in DMSO and diluted in PS before use. BODIPY-forskolin, a fluorescent version of forskolin, was dissolved in DMSO (3 mM) and diluted to 6 μM as needed. The stock solutions of the two adenylyl cyclase antagonists, MDL12330A (10 mM) and SQ22536 (60 mM), were prepared as stock solutions in DMSO and diluted in PS to the final concentrations indicated in the text. IBMX (2 mM), a phosphodiesterase inhibitor, was dissolved in PS daily. The cAMP analog, 8-Br-cAMP, was prepared as a stock solution (2 mM) in PS and diluted as needed.

The odorant in all instances was proline, prepared by dissolving it in PS to provide a stock concentration of 2 mM and adjusted to a pH of 7.4 (with NaOH). The odorant was then diluted to the reported concentration, in PS. This concentration was diluted an estimated additional two times by the olfactometer (Doolin and Ache, 2002). The concentration of proline applied was purposely high in order to maximize the magnitude of inhibitory receptor potentials and increase the accuracy of measuring imposed changes in receptor potential amplitude. The strategy of using relatively high concentrations of odorants to study single ORNs is often necessary due to the fact that olfactory sensitivity is in part the result of a high degree of central neural integration (convergence) and receptors that exchange affinity for broader sensitivity (Firestein, 2001). Proline can be shown to inhibit these cells down to at least micromolar concentrations by mixing it with an excitatory odorant and measuring subsequent graded reduction in the magnitude of the depolarizing receptor potential (Michel *et al.*, 1991).

All inorganic salts were purchased from Fisher Scientific, Inc. All organic chemicals were obtained from Sigma Chemical Co., except for HEPES (Research Organics, Inc.) and BODIPY-forskolin (Molecular Probe, Eugene, OR).

Imaging

Hemisected pieces of antennule were incubated in BODIPY-forskolin (3 μM, dissolved in PS) for 30 min and imaged for the presence of adenylyl cyclase. As a control, other sections of antennule were preincubated in non-conjugated forskolin (100 μM, dissolved in PS) for 30 min to saturate binding sites prior to incubation in forskolin + BODIPY-forskolin (dissolved in PS) for 30 min. Labeling was visualized using the technique of Liu *et al.* (1998) with confocal microscopy (Leica TCS-SPII). Fluorescent images were acquired at a depth of 1 μm and stored as TIFF files. All steps were performed at room temperature (20–22°C).

Results

Cl^- channel blockers hyperpolarize lobster ORNs and block some hyperpolarizing receptor potentials

When tested on a population of 21 lobster ORNs that were hyperpolarized by proline from a membrane potential set to −80 mV, 9-AC (500 μM) hyperpolarized the cells in 14/14 attempts by −7.3 ± 1.2 mV, DIDS (50 μM) hyperpolarized the cells in 13/13 attempts by −6.6 ± 2.5 mV and flufenamic acid (FFA, 50 μM) hyperpolarized the cells in 10/10 attempts by −2.1 ± 0.8 mV (Figure 1a). Niflumic acid (NA, 1 mM), in contrast, had no significant effect on the cells. Normalizing the relative magnitude of the potential evoked by each drug to that evoked by proline (−8.9 ± 3.4 mV, −80 mV membrane potential), gave a relative potency of 9AC = DIDS >FFA>>>NA (Figure 1b).

The same three active Cl channel blockers also blocked the hyperpolarizing receptor potential in some, but not all, cells, and did so with the same relative effectiveness (Figure 2a). Limited recording time permitted testing all four drugs on only four ORNs, but all cells were screened with at least two drugs. ORNs were rinsed for an average of 3 min after testing each blocker and the order of blockers was randomized to minimize carry-over effects. Pretreatment (1 min) with 9-AC (500 μM) reduced the peak of the hyperpolarizing receptor potential in 8 of 13 cells to 18.0 ± 6.2% of its pretreatment magnitude. Pretreatment with DIDS (50 μM) reduced the hyperpolarizing receptor potential in 7 of 14 cells to 20.5 ± 9.9% of its pretreatment magnitude. Pretreatment with flufenamic acid (50 μM) reduced the hyperpolarizing receptor potential in 5 of 12 cells to 44.6 ± 8.9% of its pretreatment magnitude. Pretreatment with niflumic acid (1 mM) was without significant effect, reducing the hyperpolarizing receptor potential in 13 of 13 cells to 97.3 ± 5.6% of its initial magnitude. Normalizing the relative magnitude of each drug to the magnitude of the hyperpolarizing receptor potential evoked by proline again gave a relative potency of 9AC = DIDS >FFA>>>NA (Figure 2b).

Forskolin/IBMX hyperpolarizes lobster ORNs in a Cl^--dependent manner

Consistent with previous evidence that hyperpolarizing receptor potentials are mediated by cyclic nucleotide signaling in lobster ORNs (Michel and Ache, 1992), forskolin (50 μM), an activator of adenylyl cyclase, together with the phosphodiesterase inhibitor IBMX (1 mM), hyperpolarized 12 of 29 cells from a membrane potential of −80 mV. Forskolin/IBMX had no effect on the remaining cells. The same three active Cl^- channel blockers reduced the magnitude of the forskolin/IBMX-evoked hyperpolarizing potentials with the same relative effectiveness (Figure 3). Once again, limited recording time prevented the testing of all four blockers on every cell, although a minimum of two blockers was tested on any one cell. The results from three cells on which all four blockers could be tested mirrored the population response. Of the 12 cells hyperpolarized by forskolin/IBMX

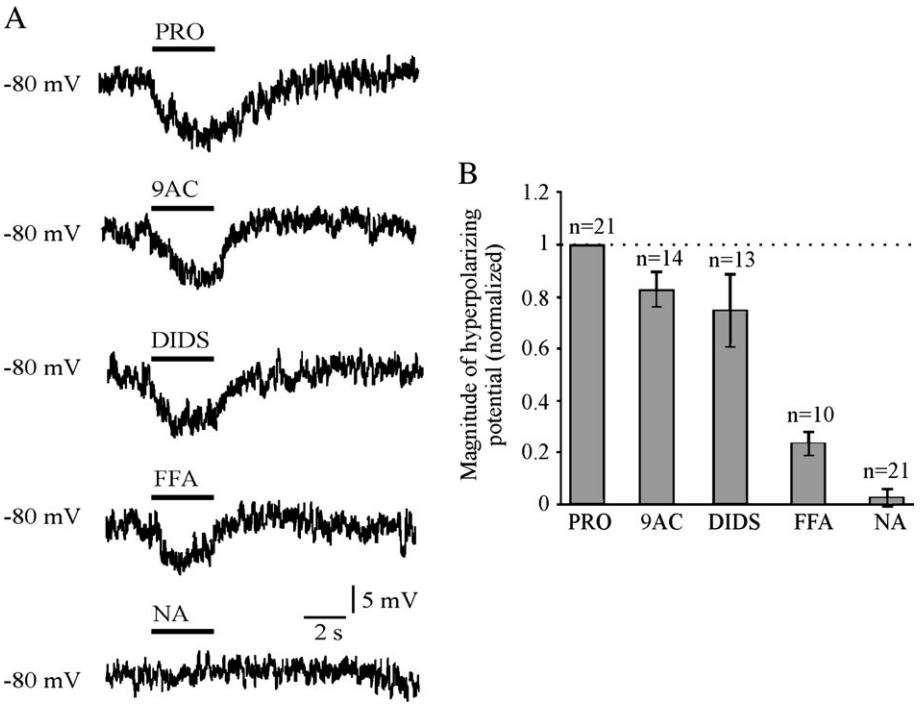

Figure 1 Cl^--channel blockers hyperpolarize some, but not all, lobsters ORNs that are hyperpolarized by odorants. **(A)** Five traces from one cell showing the response of the cell to an odorant (proline 1 mM) and four Cl^--channel blockers (9-AC, 500 μM; DIDS, 50 μM; flufenamic acid, 50 μM; niflumic acid, 1 mM) presented in the same manner as the odorant. Bar: 3 s exposure. E_m = −80 mV. **(B)** Bar chart of 21 such ORNs.

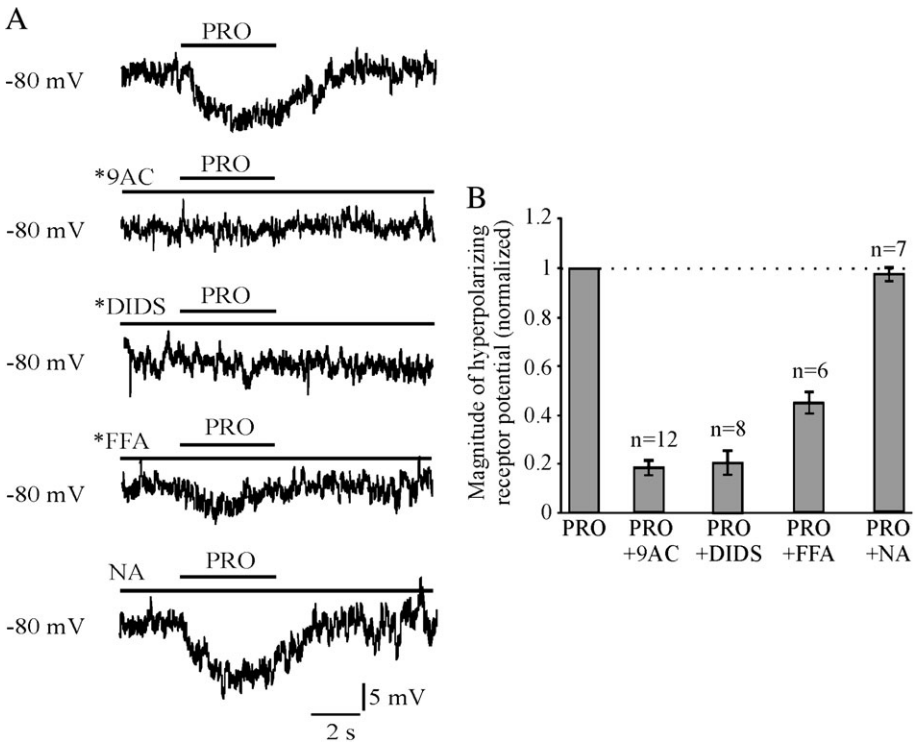

Figure 2 Cl^--channel blockers reduce odorant-evoked hyperpolarizations in some ORNs. **(A)** Five traces from one cell illustrating the effect four Cl^--channel blockers (9-AC, 500 μM; DIDS, 50 μM; flufenamic acid, 50 μM; niflumic acid, 1 mM) on an odorant (proline, 1 mM)-evoked hyperpolarization. E_m = −80 mV. Short bar: 2 s odorant exposure. Long bar: exposure to the drug indicated. In this, and all subsequent figures involving pretreatments with Cl^--channel blockers, current was injected to adjust the E_m to −80 mV during pretreatments but before secondary odorant/drug exposure. **(B)** Bar chart of 21 such ORNs on which 1–4 blockers were tested (numbers as indicated).

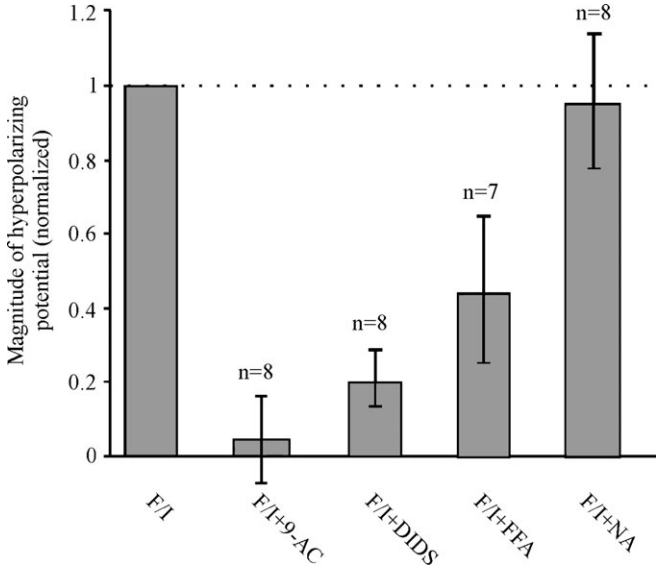

Figure 3 Cl^- blockers inhibit forskolin-induced hyperpolarizations. Bar chart of lobster ORNs (numbers as indicated) that are hyperpolarized by forskolin/IBMX (F/I; 50 μM and 1 mM, respectively) and then subsequently pretreated (1 min) with 1–4 of the Cl^--channel blockers (9-AC, 500 μM; DIDS, 50 μM; flufenamic acid, 50 μM; niflumic acid, 1 mM) indicated before being re-exposed to forskolin/IBMX.

(50 μM/1 mM), −5.9 ± 1.7 mV, pretreatment (1 min) with 9-AC (500 μM) reduced the magnitude of forskolin/IBMX-evoked hyperpolarization in 8 of 8 cells to 3.7 ± 10.6% of its initial magnitude. Pretreatment with DIDS (50 μM) reduced the magnitude of the forskolin/IBMX-induced hyperpolarization in 8 of 8 to 19.9 ± 7.7% of its pretreatment magnitude. Pretreatment with flufenamic acid (50 μM) reduced the magnitude of the forskolin/IBMX-induced hyperpolarization in 7 of 7 cells to 44.2 ± 20.0% of the pretreatment magnitude. Pretreatment with niflumic acid (1 mM) was without significant effect, reducing the magnitude of the forskolin/IBMX-evoked hyperpolarization in 7 of 7 cells to 95.5 ± 18.1% of its initial magnitude. These results suggest that cyclic nucleotide signaling mediates the hyperpolarizing Cl^- conductance.

cAMP also hyperpolarizes lobster ORNs in a Cl^--dependent manner

As would be predicted from the previous finding, a membrane-permeant form of cAMP, 8-Br-cAMP (1 mM), also hyperpolarized 13 of 24 cells from a membrane potential of −80 mV, in this instance by an average of −3.9 ± 1.7 mV (Figure 4a). 8-Br-cAMP had no effect on the remaining cells. Again, the same three active Cl^- channel blockers reduced the magnitude of the 8-Br-cAMP-evoked hyperpolarizing potentials with the same relative effectiveness (Figure 4b).

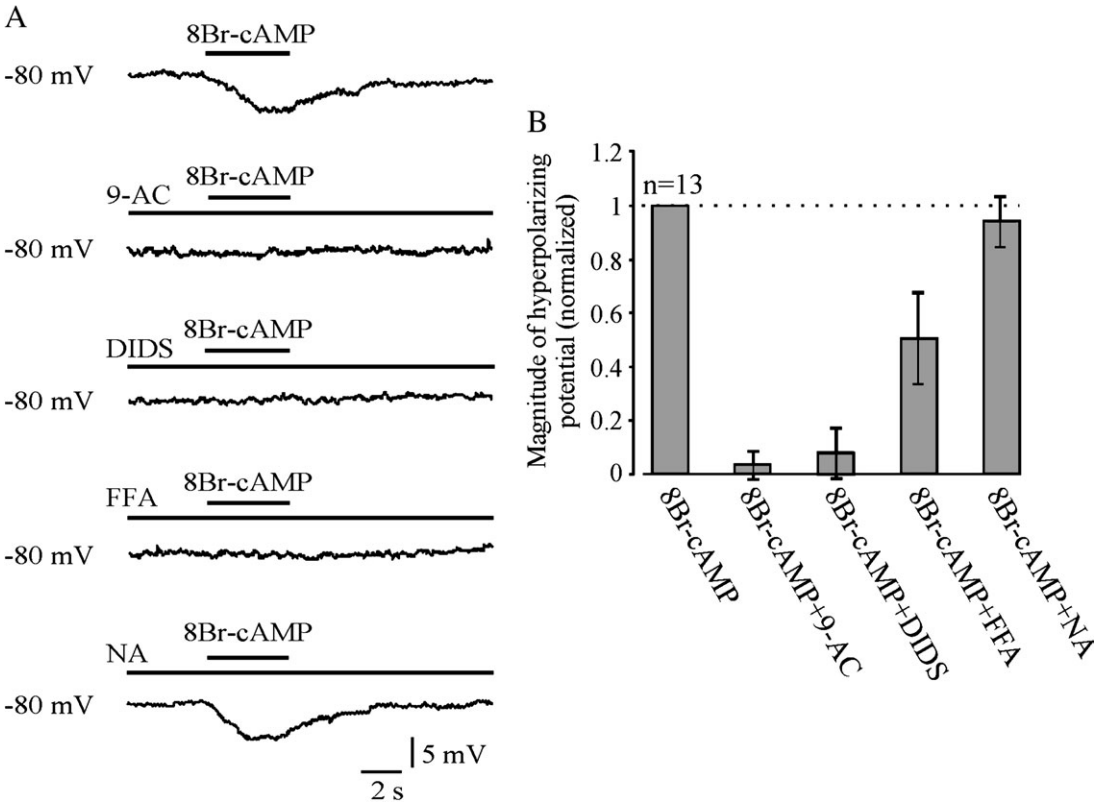

Figure 4 Cl^- blockers inhibit cAMP-induced hyperpolarizations. **(A)** Five traces from one cell showing the effect of four Cl^--channel blockers (1 min pretreatments; 9-AC, 500 μM; DIDS, 50 μM; flufenamic acid, 50 μM; niflumic acid, 1 mM) on hyperpolarizations evoked by 8Br-cAMP (1 mM). E_m = −80 mV. Short bar: 4 s exposure to 8Br-cAMP. Long bar: exposure to drug indicated. **(B)** Bar graph of 13 such ORNs pretreated with 1–4 of the Cl^--channel blockers indicated.

Limited recording time prevented testing all four blockers on every cell hyperpolarized by 8-Br-cAMP, but three cells were exposed to all four blockers and the effect on these four cells mirrored the population response. Pretreatment (1 min) with 9-AC (500 μM) reduced the magnitude of the cAMP-induced hyperpolarization in 8 of 8 cells to 3.1 ± 5.3% of its initial magnitude. Pretreatment with DIDS (50 μM) reduced the magnitude of the cAMP-induced hyperpolarization in 6 of 6 cells to 7.7 ± 9.5% of its initial magnitude. Pretreatment with flufenamic acid (50 μM) reduced the magnitude of the cAMP-evoked hyperpolarization in 7 of 7 cells to 5.0 ± 17.2% of its initial magnitude. Pretreatment with niflumic acid (1 mM) had no significant effect on the magnitude of the cAMP-induced hyperpolarization, reducing it in 8 of 8 cells to 94.3 ± 9.5% of its initial magnitude. These results further the contention that cyclic nucleotide signaling mediates the hyperpolarizing Cl^- conductance.

Odorant- and forskolin-evoked hyperpolarizing potentials in lobster ORNs are blocked by inhibitors of adenylyl cyclase

If, as suggested by the previous two findings, cyclic nucleotide signaling mediates inhibition of lobster ORNs, antagonists of adenylyl cyclase should block hyperpolarizing receptor potentials. Consistent with this prediction, two antagonists of adenylyl cyclase, MDL12330A and SQ22536, either blocked or reduced the hyperpolarizing receptor potential in lobster ORNs (Figure 5a). Pretreatment (1 min) with MDL12330A (100 μM) reduced the magnitude of the hyperpolarizing receptor potential in 8 of 11 cells to 20.7 ± 8.8% of its pretreatment magnitude. Pretreatment with SQ22536 (300 μM), tested on the same eight cells, also reduced the magnitude of the hyperpolarizing receptor potential in 7 of 8 of the cells to 43.2 ± 10.2% of its pretreatment magnitude (Figure 5b). One cell was unaffected by SQ22536. The effect of both drugs was reversible. To test the assumption that MDL12330A and SQ22536 were indeed targeting adenylyl cyclase in these cells, we tested their effect on forskolin/IBMX-evoked hyperpolarizing potentials (Figure 6a). Forskolin/IBMX (50 μM/1 mM) hyperpolarized 9 of 21 cells −5.3 ± 1.9 mV from a membrane potential of −80 mV. Pretreatment (1 min) with MDL12330A (100 μM) reduced the magnitude of the forskolin/IBMX-induced hyperpolarization in 9 of 9 cells to 2.2 ± 7.4% of its pretreatment magnitude. Pretreatment with SQ22536 (300 μM) also reduced the magnitude of the forskolin/IBMX-induced hyperpolarization in 8 of 9 cells to 10.7 ± 12.7% of its pretreatment magnitude (Figure 6b). One cell was unaffected by SQ22536. The effect of both drugs was again reversible. These results are

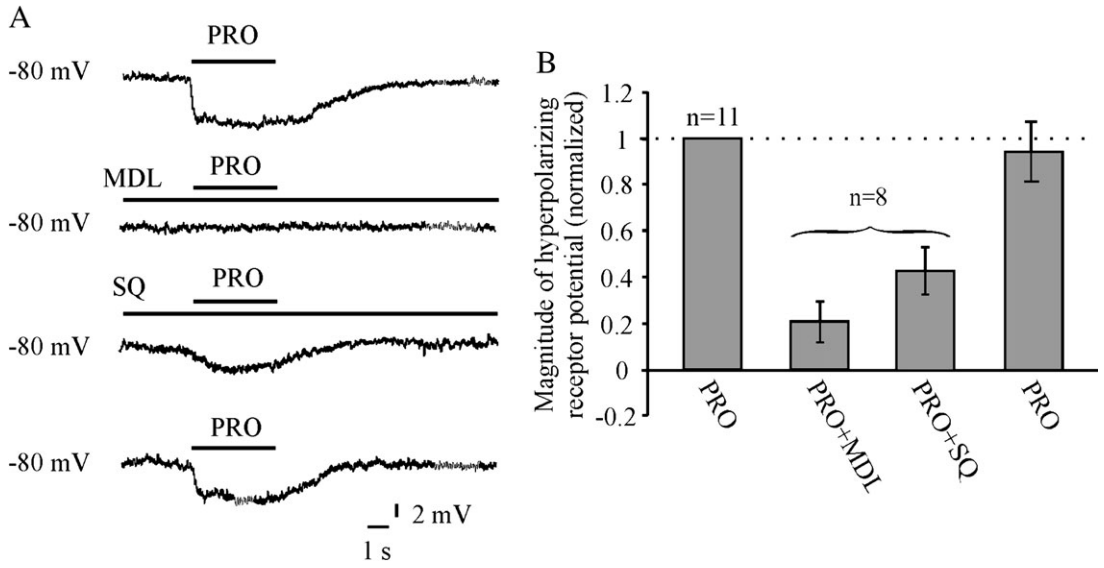

Figure 5 Blockers of adenylyl cyclase inhibit odorant-evoked hyperpolarizations in some lobster ORNs. **(A)** Four traces from one cell showing the effect of two adenylyl antagonists, MDL12330A (MDL, 100 μM) and SQ22536 (SQ, 300 μM) (1 min pretreatment) on the odorant (proline, 1 mM)-evoked hyperpolarization. E_m = −80 mV. Short bar: 4 s odorant exposure. Long bar: exposure to the drug indicated. **(B)** Bar chart of 11 such cells treated with both antagonists, eight of which were blocked by the drugs (one of these cells failed to respond to SQ).

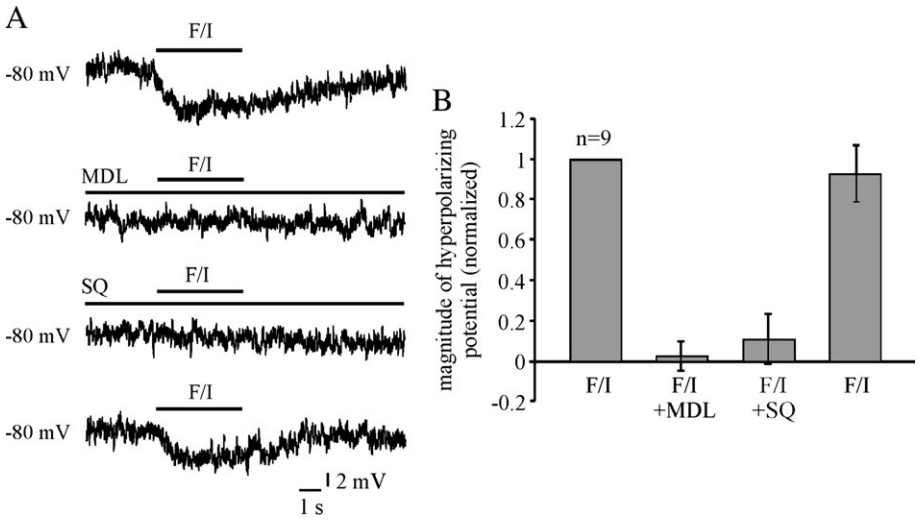

Figure 6 Blockers of adenylyl cyclase inhibit all forskolin-evoked hyperpolarizations. **(A)** Four traces from one cell illustrating the effects of the same two adenylyl antagonists tested in Figure 5 (1 min pretreatments; MDL, 100 μM and SQ, 300 μM) on forskolin/IBMX (50 μM/1 mM)-evoked hyperpolarizations. E_m = −80 mV. Short bar: 4 s exposure to forskolin/IBMX. Long bar: exposure to the drug indicated. **(B)** Bar chart of nine such cells, all of which were blocked by the drugs (one of these cells failed to respond to SQ).

consistent with the hypothesis that cyclic nucleotide signaling mediates the hyperpolarizing Cl^- conductance.

Adenylyl cyclase occurs in the outer dendrites of lobster ORNs

To verify the presumed presence of adenylyl cyclase in the transduction compartment of the cells, we incubated olfactory sensilla (aesthetascs) in BODIPY-forskolin (3 μM, 30 min) to label adenylyl cyclase in the outer dendrites, which fill the lumen of the hairs. Incubating the olfactory sensilla in BODIPY-forskolin (3 μM, 30 min) labeled the outer dendrites (Figure 7). Preincubating the sensilla in non-conjugated forskolin (100 μM, 30 min) prior to BODIPY-forskolin (3 μM, 30 min) eliminated this labeling, leaving only the autoflorescence of the tissue. The latter result suggests that the labeling was specific for adenylyl cyclase, and is consistent with the functional evidence implying that adenylyl cyclase occurs in the transduction zone of the cells.

Forskolin-evoked hyperpolarizing potentials are selectively inhibited by Cl^- channel blockers

The question remains whether cyclic nucleotide signaling exclusively targets the hyperpolarizing Cl^- conductance. To address this we tested whether any of the hyperpolarizing potentials induced by forskolin/IBMX were sensitive to the K^+ channel blocker, 4-aminopyridine (4-AP), or only the Cl^- channel blocker 9-AC (Figure 8a). Forskolin/IBMX (50 μM/1 mM) hyperpolarized 13 of 30 cells from a membrane potential of −80 mV by an average of −5.7 ± 2.7 mV. Pretreatment (1 min) with 9-AC (500 μM) reduced the magnitude of the forskolin/IBMX-induced hyperpolarization in 13/13 cells to 4.1 ± 12.3% of its pretreatment magnitude (Figure 8b). In contrast, pretreatment (1 min) with 4-AP (2 mM) did not significantly affect the magnitude of the response of the same group of 13 cells, reducing it to 92.0 ± 30.0% of its pretreatment magnitude (Figure 8b). The effect of 9-AC was reversible. These results suggest that the hyperpolarizing potentials induced by forskolin/IBMX either are not K^+-dependent, or have a minimum K^+-dependency.

Discussion

The present study confirms our earlier finding that lobster ORNs express an odorant-suppressible Cl^- conductance (Doolin *et al.*, 2001) and expands the pharmacological profile of this conductance. In agreement with the previous study, 9-AC but not niflumic acid blocked the odorant-suppressed Cl^- conductance. We further show that DIDS and flufenamic acid target the same conductance. DIDS and flufenamic acid are known to block Cl^- channels in both olfactory (DIDS, Dubin and Dionne, 1994; Sato and Suzuki, 2000; flufenamic acid, Sato and Suzuki, 2000) and non-olfactory (DIDS, salivary glands, reviewed by Melvin, 1999; rat sympathetic neurons, Sacchi *et al.*, 1999; vascular smooth muscles, Davis *et al.*, 2000; flufenamic acid, Ca^{2+}-activated Cl^- channel family, reviewed by Pauli *et al.*, 2000; *Xenopus* oocytes, Weber *et al.*, 1995; mouse spinal cord, Yamazaki *et al.*, 2000; toad skin, Nagel *et al.*, 2001) cells. Although a growing number of Cl^- channels now have been pharmacologically characterized (e.g. CLCA channels, Pauli *et al.*, 2000; CLC channels, Pusch *et al.*, 2000), the pharmacological profile of the odorant-suppressable Cl^- conductance in lobster ORNs does not match that of any previously characterized Cl^- conductance, suggesting that lobster ORNs may express a novel transduction related Cl^- channel.

Several lines of evidence support the conclusion that cyclic nucleotide signaling targets the transduction related Cl^- channel. The same profile of Cl^- channel blockers that blocked the steady-state Cl^- conductance also blocked hyperpolarizing potentials evoked by forskolin and IBMX, both drugs known to increase intracellular levels of cAMP. The same profile of Cl^- channel blockers also blocked hyperpolarizing potentials evoked by 8-Br-cAMP, a membrane permeant analog of cAMP. Lastly, MDL12330A and SQ22536, drugs known to

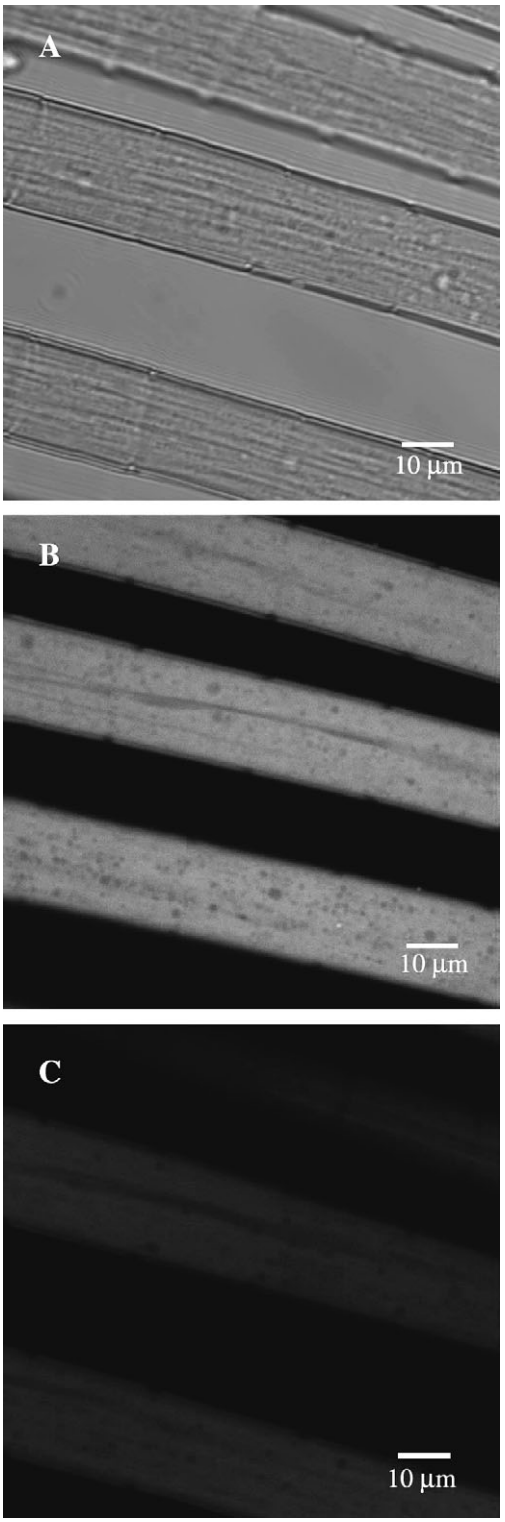

Figure 7 Adenylyl cyclase occurs in the transduction compartment of the lobster ORNs. **(A)** Light micrograph of outer dendrites (aesthetascs). **(B)** Confocal image of lobster olfactory sensilla incubated in BODIPY-forskolin (3 μM) for 30 min and viewed at 488 nm. **(C)** Confocal image of the same structures preincubated in non-conjugated forskolin (100 μM) for 30 min followed by forskolin + BODIPY-forskolin (3 μM) for 30 min.

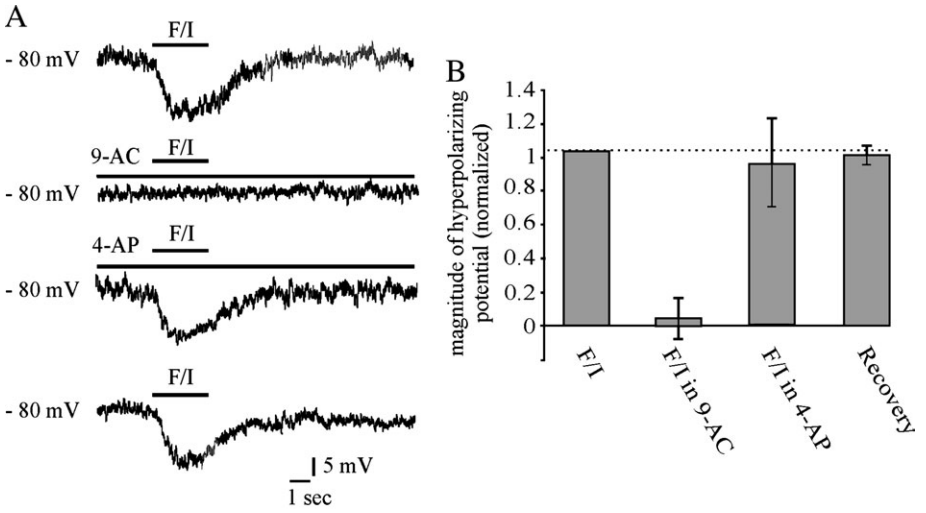

Figure 8 Cl^- blockers selectively inhibit forskolin-induced hyperpolarizations. **(A)** Four traces from the same cell demonstrating that pretreatment (1 min) with 9-AC (500 μM), but not pretreatment (1min) with 4-AP (2 mM), blocks forskolin/IBMX-evoked hyperpolarizations in lobster ORNs set to E_m = −80 mV. Short bar: 3 s exposure to forskolin/IBMX. Long bar: continuous exposure to drug indicated. **(B)** Bar chart of 13 such ORNs.

inhibit the production of cAMP by antagonizing adenylyl cyclase, blocked the hyperpolarizing receptor potentials. Given that the drugs were applied selectively to the transduction compartment (outer dendrites), these findings are consistent with the hypothesis that activation of adenylyl cyclase leads to membrane hyperpolarization due to suppression of a steady-state Cl^- conductance.

We cannot resolve whether cAMP acts on the underlying Cl^- channel directly. Cyclic AMP regulates Cl^- channels in other systems both indirectly (e.g. heart, Hume *et al.*, 2000) and directly (e.g. lung, Welsh and Liedtke, 1986), as may hold for ORNs. The Ca^{2+}-activated Cl^- conductance that carries the majority of the receptor current in vertebrate ORNs is activated indirectly by the cAMP-dependent increase in intracellular Ca^{2+} (for a review, see Frings, 2001). In *Necturus* ORNs, in contrast, the ability of intracellular cAMP to activate a Cl^- conductance in a Ca^{2+}- and PKA- independent manner was interpreted to suggest that cAMP acts on the channel directly (Delay *et al.*, 1997). While ion channels in cell-free patches pulled from the outer dendrites of lobster ORNs can be activated by cAMP (Hatt and Ache, 1994), presumably these are not the channel of interest here since they are activated and not suppressed by cAMP. We tentatively assume, therefore, that cAMP suppresses the Cl^- channel through one or more soluble components that dialyzes in cell-free patches, but further work is required to clarify this assumption.

Our finding that fewer cells were hyperpolarized by the Cl^--channel blockers than were hyperpolarized by odorants suggests that not all hyperpolarizing receptor potentials are dependent on suppression of a steady-state Cl^- conductance, and is consistent with earlier evidence that a K^+ conductance also underlies inhibition in lobster ORNs (Doolin *et al.*, 2001; Michel *et al.*, 1991). Such a dual ionic basis for mediating inhibition would not be unusual in that it was also proposed for squid ORNs (Lucero *et al.*, 1992; Danaceau and Lucero, 2000), and indeed could be a more general principle of peripheral olfactory organization. However, our present findings would argue that cyclic nucleotide signaling does not target the K^+ conductance since essentially all hyperpolarizing potentials induced by manipulating cyclic nucleotide signaling were subject to blockade by the Cl^- channel blockers, and since the K^+ channel blocker 4-AP that was shown previously to block some hyperpolarizing receptor potentials in lobster ORNs (Michel *et al.*, 1991) failed to block hyperpolarizing potentials evoked by forskolin/IBMX in the present study. However, these findings are not necessarily definitive since we were able to sample only a limited subset of cells in the population and since there is always some concern about the effective concentrations of the drugs used. As mentioned earlier, cAMP activates a putative K^+-selective ion channel in cell-free patches pulled from the outer dendrites of lobster ORNs (Hatt and Ache, 1994), suggesting that the steady-state Cl^- channel is not the sole target of cyclic nucleotide signaling in these cells. Again, further work will be required to resolve this issue.

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