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Cyclic nucleotide- and inositol phosphate-gated ion channels in lobster olfactory receptor neurons

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ABSTRACT **The idea of having two second messenger pathways in olfaction, one mediated by cAMP and the other by inositol 1,4,5-trisphosphate, is supported by evidence that both second messengers directly activate distinct ion channels in the outer dendrite of lobster olfactory receptor neurons. Evidence that both types of second messenger-gated channels can occur in the same patch of membrane suggests that channels of both types can be expressed in one neuron. Evidence of more than one type of inositol phosphate-gated channel in this highly specialized region of the neuron furthers the idea that the output of individual olfactory receptor cells is regulated through multiple effectors and allows that effector diversity may contribute to functional diversity among olfactory receptor cells.**

Odors are generally thought to activate olfactory receptor neurons (ORNs) through guanine nucleotide binding protein-coupled receptors that elicit rapid and transient pulses of intracellular second messengers (for reviews, see refs. 1–3). Attention has focused on two second messenger pathways, one mediated by cAMP and the other by inositol 1,4,5-trisphosphate ($InsP_3$). cAMP and $InsP_3$ olfactory transduction cascades have been identified in four groups of animals (mammals, refs. 4–6; amphibians, refs. 7 and 8; fish, refs. 9 and 10; crustaceans, refs. 11 and 12). The presence of two second messenger pathways in such phylogenetically diverse animals argues that having two transduction pathways may play a fundamental role in olfaction.

Evidence that the two transduction pathways are not equally sensitive to the same odors (mammals, refs. 4–6; fish, ref. 13; crustaceans, ref. 14) implies that the two pathways work in parallel, not sequentially, and that the parallel inputs function in coding *per se* and not in adaptation or other cellular events that presumably should be independent of odor composition. Odors can inhibit and excite the same ORN (amphibians, refs. 15 and 16; fish, ref. 17; molluscs, ref. 18; crustaceans, ref. 19). In lobster ORNs, excitation and inhibition of the cells can be linked to the $InsP_3$ and cAMP transduction cascades, respectively (11, 12). One role of having two parallel transduction pathways in olfaction, therefore, may be to allow ORNs to integrate bipolar input (20).

Two lines of evidence link excitation and inhibition of lobster ORNs to $InsP_3$ and cAMP transduction cascades. (*i*) Plasma membrane $InsP_3$-gated ion channels mediate odor-evoked inward currents in the soma of cultured lobster ORNs (12). Lobster ORNs in culture appear to express elements of the transduction pathways in the soma (21). (*ii*) Outward K^+ currents evoked by odors in lobster ORNs *in situ* (22) are altered selectively by membrane-permeant probes for cyclic nucleotides (11). Preliminary evidence reveals the presence of cAMP-gated ion channels in the plasma membrane of cultured lobster ORNs (23). More direct evidence that excitation and inhibition of lobster ORNs are mediated by $InsP_3$ and cAMP transduction cascades would be to demonstrate the presence of $InsP_3$- and cAMP-activated ion channels at the presumed site of transduction, the outer dendritic membrane, in lobster ORNs *in situ*.

Here, we report that cAMP and $InsP_3$ applied to the inner face of cell-free patches of the outer dendritic membrane of lobster ORNs *in situ* directly activate distinct ion channels, that both types of second messenger-gated channels can occur in the same patch, and that the $InsP_3$-gated channels, and possibly the cAMP-gated channels, can be one of two types. A preliminary description of these results has appeared (24).

MATERIALS AND METHODS

The outer dendrites were isolated from the olfactory receptor cells of the Caribbean spiny lobster, *Panulirus argus*, by excising the tips of ≈100 olfactory sensilla. The sensilla contain only the thin (≈0.1 μm diameter) branches of the outer dendrites of the olfactory receptor cells for 85–90% of their length (25), so the excised tips should contain only outer dendritic membrane and microtubules. Care was taken to include <50% of the length of the sensillum in the excised tips. Outer dendrites were placed in poly(D-lysine)-coated plastic dishes containing 5 ml of isolation buffer (460 mM NaCl/13 mM KCl/10 mM $MgCl_2$/1.7 mM glucose/3 mM Hepes, pH 7.4/930 milliosmolar—estimated free $[Ca^{2+}]$, 1 μM) diluted 80:20 with glass-distilled H_2O. The outer dendrites immediately extruded from the cuticular tip and in many instances began to form membrane vesicles up to ≈3 μm in diameter that could be patch-clamped without enzymatic treatment (Fig. 1).

Seals of >10 GΩ were obtained with fire-polished patch electrodes pulled from borosilicate filament glass (Clark GC150TF-10, Clark Instruments, Pangbourne, U.K.). The electrodes were filled with a pseudo-intracellular solution (180 mM potassium acetate/30 mM NaCl/1 mM $CaCl_2$/10 mM EGTA/10 mM Hepes/696 mM glucose, pH 7.4, 910 milliosmolar—estimated free $[Ca^{2+}]$, 15 nM). Membrane patches were excised from the vesicles, transferred to a microchamber equipped with a liquid filament switch for fast solution exchange (26), and brought through the air–water interface to attain an inside-out configuration. The microchamber was continuously perfused with EGTA-buffered saline (460 mM NaCl/13 mM KCl/10 mM $MgCl_2$/0.1 mM $CaCl_2$/10 mM EGTA/3 mM Hepes/1.7 mM glucose, pH 7.4/930 milliosmolar—estimated free $[Ca^{2+}]$, 1.9 nM) to minimize vesiculation of the patch. The liquid filament,

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; ORN, olfactory receptor neuron.

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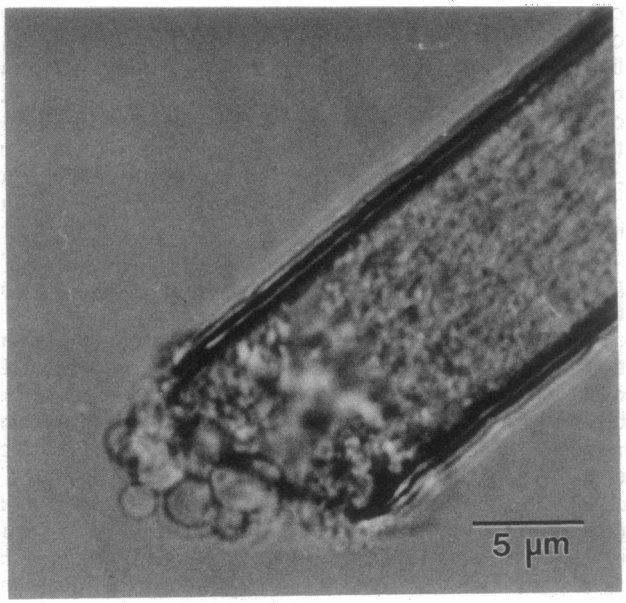

FIG. 1. Laser scanning micrograph of the cut end of the distal tip of one of some 2400 hair-like olfactory sensilla (aesthetascs) on the lobster olfactory organ (antennule). Each hair contains some 8000 outer dendritic branches of the ≈320 primary ORNs associated with each sensillum. Note the numerous outer dendritic membrane vesicles that form after excision.

which transiently bathed the inner face of the patch during stimulation, contained higher Ca^{2+} (≈1 μM) to provide any Ca^{2+} necessary for channel activation. Single-channel currents were recorded with a List EPC7 patch-clamp amplifier and stored on video tape for subsequent analysis. Records were digitized at 20 kHz with a cut-off (3 db) frequency of 3 kHz and stored on a Hewlett–Packard HP 9800 microcomputer. Multiple measurements are given as the mean ± SD.

All chemicals were obtained from Sigma (product numbers are indicated). cAMP (A-6885) was prepared as a 10 mM stock in isolation solution and frozen in 100-μl aliquots, which were thawed and diluted in the same saline to the concentrations indicated. InsP_3 (I-4009) was prepared as a 0.2 mM stock solution and otherwise treated as described for cAMP. This is a racemic mixture of 80–90% of the 1,4,5-isomer, with the balance being primarily the 2,4,5-isomer. Inositol 1,3,4,5-tetrakisphosphate (InsP_4, I-8636) was prepared as a 64 μM stock solution and otherwise treated as described for cAMP. Heparin (H-3125) was prepared as needed at 25 μg/ml.

RESULTS

Channel activity was observed in only a few of the >100 patches in the absence of second messenger application or with voltage stimulation, suggesting that the outer dendritic membrane is electrotonically tight in the absence of stimulation. Applying InsP_3 (InsP_4 in a few instances) and/or cAMP to the inner face evoked channel activity in most patches tested, suggesting that the density of InsP_3- and cAMP-activated channels is relatively high in the outer dendrite, although the actual density was not determined. Most patches revesiculated during the experimental protocol and were not analyzed; 24 patches yielded usable data.

InsP_3 (2 μM, unless specified) activated channels of two different conductances. In at least one instance, both types of channels clearly occurred in the same patch of membrane; otherwise they occurred in separate patches. The smaller conductance channel showed longer openings with characteristic flickering behavior (Fig. 2*A*). The mean unitary current was calculated from a Gaussian distribution fit to current–amplitude histograms (Fig. 2*B*). The channel had a linear current–voltage relationship between +70 and −100 mV and conducted an outward current at positive potentials that reversed polarity near −10 mV in our ionic conditions (Fig. 2*C*). The channel had an average slope conductance of 27 ± 3 pS (n = 5). The open probability was strongly voltage-dependent, with the channels having the highest probability of being open (0.78 ± 0.12, n = 4) at +70 mV, decaying to almost 0 at −70 mV (Fig. 2*D*). Dwell-time distributions were determined in this and subsequent exper-

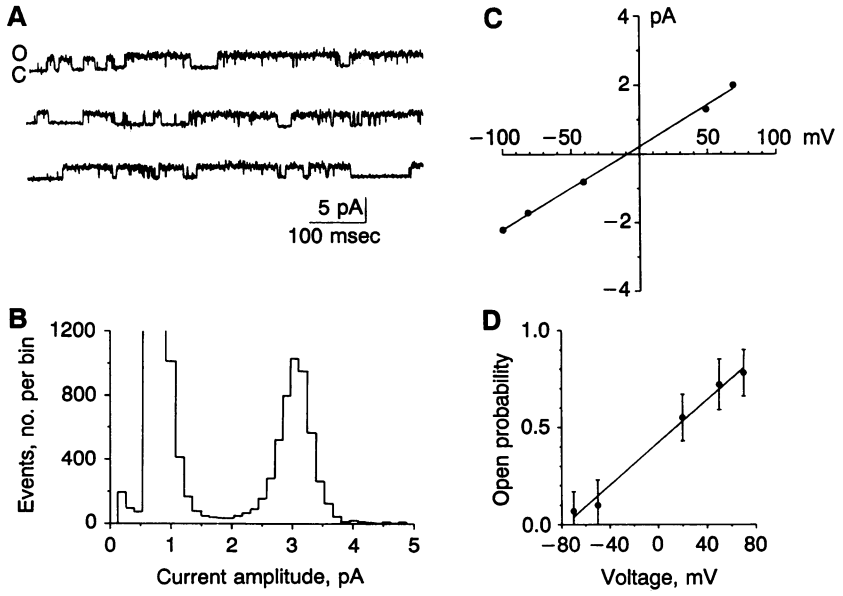

FIG. 2. Unitary currents at 27 pS activated by applying 2 μM InsP_3 to the inner face of a cell-free patch taken from outer dendritic membrane vesicles of lobster ORNs. (*A*) Records after exposure to the ligand. Membrane potential was +70 mV. Records were filtered at 2 kHz. In this and subsequent figures, C denotes the closed state, whereas O or O_X denotes one or more open states. (*B*) Amplitude histogram of 4682 open-time events from the membrane patch shown in *A*. In this and subsequent amplitude histograms, the first peak represents the closed state of the channel. (*C*) Plot of the current (ordinate)–voltage (abscissa) relationship of the channel shown in *A*. The line was fit "by eye." (*D*) Plot of the open probability (X ± SD) of this channel (ordinate) as a function of membrane voltage (abscissa) (n = 4). Line was fit by linear regression. Note the strong voltage dependence of the open probability.

iments from records in which only one channel was activated. The open dwell-time distribution could be best fit by a single exponential, with $\tau_1 = 0.81 \pm 0.08$ msec ($n = 4$). The closed dwell-time distribution could be best fit by a double exponential, with $\tau_1 = 1.68 \pm 0.14$ msec and $\tau_2 = 40.21 \pm 2.14$ msec ($n = 4$).

The larger conductance channel also showed longer openings with characteristic flickering behavior that appeared to be greater than that of the smaller conductance channel (Fig. 3*A*). The mean unitary current was calculated from a Gaussian distribution fit to current–amplitude histograms (Fig. 3*B*). The channel had a linear current–voltage relationship between +70 and −100 mV and conducted an outward current at positive potentials that reversed polarity between −10 and −20 mV in our ionic conditions (Fig. 3*C*). The channel had an average slope conductance of 64 ± 5 pS (n = 7). The open probability of the larger conductance channel was much less voltage-dependent than was that of the smaller conductance channel (Fig. 3*D*) and was lower overall. The highest probability of being open (0.21 ± 0.09, $n = 5$) occurred at +70 mV. The open dwell-time distribution could be best fit by a single exponential, with $\tau_1 = 0.71 \pm 0.1$ msec ($n = 5$). The closed dwell-time distribution could best be fit by a double exponential, with $\tau_1 = 2.06 \pm 0.19$ msec and $\tau_2 = 22.4 \pm 2.08$ msec ($n = 5$). Heparin at 25 μg/ml completely blocked the activation of the larger conductance channel by 0.1 μM InsP_3; 2 μM InsP_3 overcame 80–90% of the heparin-induced blockade ($n = 3$, data not shown). Heparin was not tested on the smaller conductance channel.

InsP_4 (2 μM) activated a third type of channel in one of three attempts to test this ligand (data not shown). This channel differed markedly from those activated by InsP_3 in that it remained in the open state for long periods with no evidence of flickering and had an extremely large chord conductance (200 pS at +90 mV). Long silent periods separated the openings, as indicated by the relatively low open probability (0.15 at +90 mV). The limited duration of the recording prevented further and more detailed characterization of this channel.

cAMP (1 mM) most frequently activated a channel with relatively long openings without the flickering behavior of the channels activated by InsP_3 (Fig. 4*A*). The mean unitary current was calculated from a Gaussian distribution fit to current–amplitude histograms (Fig. 4*B*). The channel had a linear current–voltage relationship at membrane potentials between −40 and −70 mV, with a mean slope conductance of 28 ± 5 pS ($n = 4$) in this voltage range (Fig. 4*C*). In contrast to the InsP_3-activated channels, the cAMP-activated channels strongly rectified at positive potentials, a behavior consistent with the channel being permeant to K^+ under our ionic conditions (see *Discussion*). The open probability of the channel appeared to be voltage independent (Fig. 4*D*), but the possibility of substates (see *Discussion*) prevented normalizing the mean probability of the channel being open to a defined number of channels. The open dwell-time distribution could be fit by a single exponential, with $\tau_1 = 0.83 \pm 0.08$ msec ($n = 5$). The closed dwell-time distribution could best be fit by a double exponential, with $\tau_1 = 2.8 \pm 0.4$ msec and $\tau_2 = 22.4 \pm 2.4$ msec ($n = 4$). In two of the patches in which cAMP evoked unitary currents, 1 mM cAMP activated a channel with a rectifying current–voltage relationship at positive potentials, but with a slope conductance of 54 ± 2 pS in the negative range. This channel was almost always in the open state when activated by 1 mM cAMP, in contrast to the apparent lower sensitivity of the smaller conductance channel to the ligand. The low incidence of this channel prevented further characterization of its properties.

The cAMP-activated channel could be discriminated clearly from InsP_3-activated channels at positive membrane potentials where the probability of the InsP_3-activated channels being open was greatest and the cAMP-activated channels showed strong rectification (Fig. 5). We took advantage of this fact to demonstrate that both cAMP and InsP_3-activated channels could colocalize to the same patch of membrane. Of 17 instances in which both cAMP and InsP_3 could be tested on the same patch, both cAMP and InsP_3-activated channels clearly occurred together in three patches and with reasonable certainty in two others.

DISCUSSION

Finding distinct cAMP and InsP_3-activated ion channels in the outer dendritic membrane of lobster ORNs *in situ* is

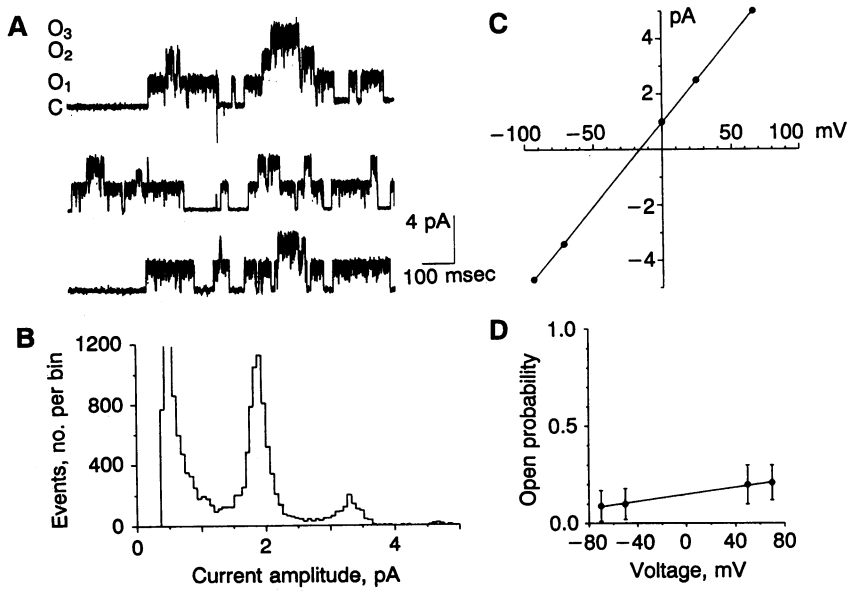

FIG. 3. Unitary currents at 64 pS activated by applying 2 μM InsP_3 to the inner face of cell-free patches from outer dendritic membrane vesicles of lobster ORNs. (*A*) Records after exposure to the ligand. Membrane potential was +30 mV. Records were filtered at 2 kHz. (*B*) Amplitude histogram of 7980 open-time events from the membrane patch shown in *A*. (*C*) Plot of the current (ordinate)–voltage (abscissa) relationship of the channel shown in *A*. Line was fit by eye. (*D*) Plot of the open probability of the channel (ordinate) as a function of membrane voltage (abscissa). Line was fit by linear regression. Note the weak voltage dependence of this channel, compared to that shown in Fig. 2*D*.

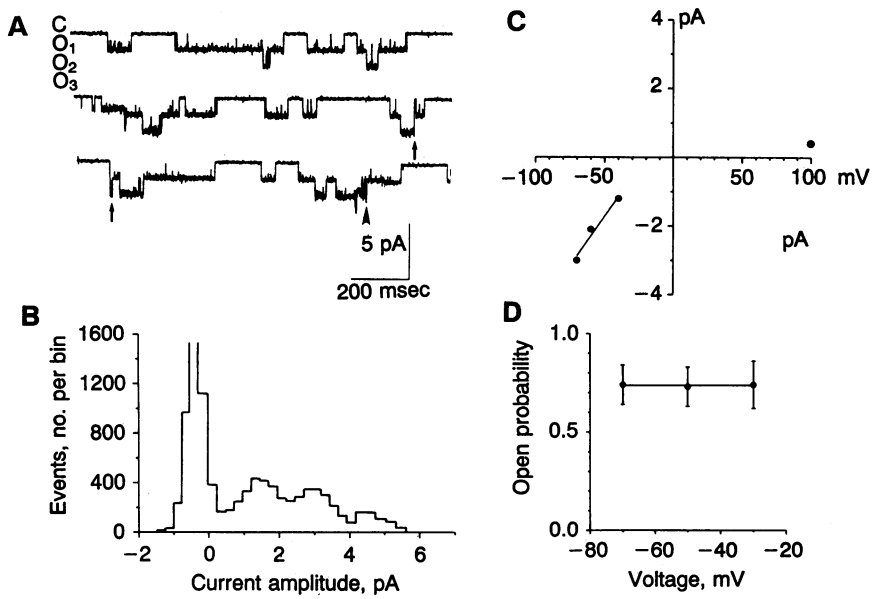

FIG. 4. Unitary currents at 28 pS activated by applying 1 mM cAMP to the inner face of cell-free patches from outer dendritic membrane vesicles of lobster ORNs. (*A*) Records after exposure to the ligand. Membrane potential was −60 mV. Records were filtered at 2 kHz. Note instances of apparent smooth transitions between two distinct open levels (arrows) and subconductance states (arrowhead) in these records. (*B*) Amplitude histogram of 7890 open-time events from the membrane patch shown in *A*, showing three peaks corresponding to the three open levels. (*C*) Plot of the current (ordinate)–voltage (abscissa) relationship of the channel shown in *A*. Line was fit by eye. (*D*) Plot of the open probability of the channel (ordinate) as a function of membrane voltage (abscissa). Line was fit by linear regression. Note the absence of voltage dependence of this channel(s).

consistent with the recent finding that odors transiently elevate both cAMP and InsP_3 in the outer dendrite and do so sufficiently fast to account for these ligands acting as olfactory second messengers (14). The channels we report here presumably serve as the respective targets for the two second messengers. These findings collectively provide direct evidence that InsP_3 and cAMP mediate dual transduction pathways in lobster ORNs *in situ*.

That InsP_3 initiated and sustained unitary currents without observable run down in cell-free patches of membrane implies that InsP_3 directly gated the channels it activated. Several lines of evidence indicate that the InsP_3-gated channels in the outer dendrite are similar if not identical to the InsP_3-gated channels from the soma of cultured lobster ORNs (12). The slope conductances of the two types of InsP_3-gated channels are remarkably similar in both preparations (27 vs. 30 pS and 64 vs. 74 pS) and each demonstrates characteristic flickering behavior in the open state. At least the larger conductance channel can be blocked reversibly by heparin in both types of preparations. The open probability of the smaller conductance channel in both preparations is extremely voltage-dependent, in contrast to the weaker voltage dependence of the larger conductance channel. Although we did not characterize the ionic permeability of the channels in the outer dendrite, the fact that the current–voltage relationship of the two channels reverses polarity at different potentials argues that the channels have different ionic selectivities, as do the InsP_3-gated channels in the cultured ORNs (27). The presence of InsP_3-gated channels in the outer dendrites strengthens the contention (21) that lobster ORNs in culture express the normal transduction machinery in the soma. Plasma membrane InsP_3 receptors have also been implicated in olfactory transduction in catfish (9, 28) and mammals (29, 30).

That cAMP also initiated and sustained unitary currents without observable run down in cell-free patches of membrane implies that it, too, directly gated the channels it activated. The presence of cyclic nucleotide-gated channels in the outer dendrite confirms preliminary evidence for the existence of such channels obtained from patch-clamp data from the soma membrane of cultured ORNs (23). It cannot be determined whether the multiple open states in our recordings reflect more than one type of cAMP-gated channel or substates of a single type of channel. The original records show some evidence of substates (Figs. 4 and 5) and in several instances rapid transitions across two levels without any inflection (verified at faster sweep, Figs. 4 and 5) suggest

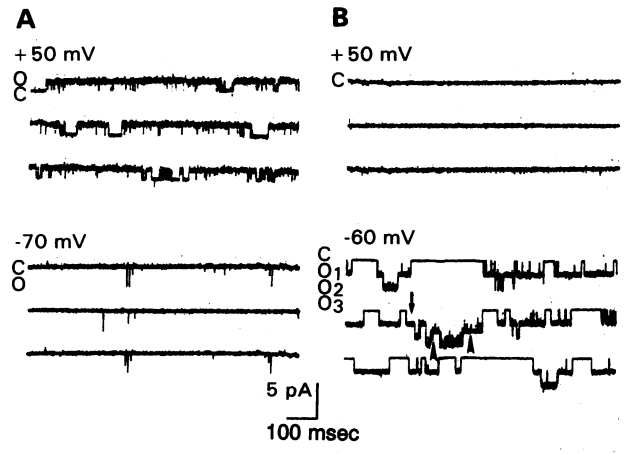

FIG. 5. Unitary currents activated by applying 2 μM InsP_3 (*A*) and 1 mM cAMP (*B*) to the inner face of the same cell-free patch from outer dendritic membrane vesicles of lobster ORNs at positive and negative membrane potentials. Records were filtered at 2 kHz, except those for cAMP at +50 mV, which were filtered at 5 kHz to maximize the possibility of observing any brief transitions. Note the strong rectification of the cAMP-activated channel under our recording conditions truncates the amplitude of the channel at positive membrane potentials, where the open probability of the InsP_3-activated channels is maximal. The opposite situation exists at negative membrane potentials, making it possible to discern both types of channels in the same patch of membrane. Note instances of apparent smooth transitions between two distinct open levels (arrow) and subconductance states (arrowheads) in the cAMP records.

that the cAMP-gated channel may have multiple conductance states. This idea would be consistent with the smoothing observed in the amplitude histograms for cAMP-gated channel (Fig. 4*B* vs. Figs. 2*B* and 3*B*). Thus, the larger conductance channel observed in two instances and the more common smaller conductance channel may represent subconductance states of a single type of cAMP-gated channel, but our limited data do not allow resolution of this point.

The strong rectification of the current–voltage relationship of the cAMP-gated channel imposed by our asymmetric ionic conditions at positive voltages is consistent with the channel being K^+ selective. Outward current through the patch at positive membrane potentials would oppose the electrochemical gradient of K^+, which has a calculated equilibrium potential of +66 mV in our recording configuration. The hypothesized K^+ permeability of these channels would be consistent with earlier evidence that membrane-permeant probes for cAMP selectively evoke an outward K^+ current that hyperpolarizes these cells (11). Cyclic nucleotide-gated channels occur in vertebrate ORNs but are nonselective for cations and their activation depolarizes the cell (e.g., ref. 7). However, cyclic nucleotide-gated K^+ channels occur on insect skeletal muscle (31) and, together with the lobster olfactory channel, may represent evolutionary precursors of cyclic nucleotide-gated channels in phylogenetically higher animals that are hypothesized to have evolved from voltage-activated K^+ channels (32).

The ability to record channels activated by cAMP and $InsP_3$ in the same patch of membrane strongly implies that channels of both types occur on the same outer dendritic branch and, therefore, in the same ORN. The outer dendrites were not induced to vesiculate under any of the extreme conditions that typically are required for membranes to fuse (e.g., ref. 33). Although we used mild osmotic shock (80% lobster saline) and reduced calcium to speed up the rate of vesiculation, the vesicles would also form in normal lobster saline. The outer dendritic branches extend to the tip of the hair in an intermolt animal (25), so a tip section that was, say, 40% of the length of a hair should contain 300-μm-long branches. There would be sufficient membrane in such a branch to form, for example, two vesicles, each 4 μm in diameter. Therefore, we assume that any one vesicle was derived from the membrane of an individual outer dendritic branch and, thus, that channels occurring in a single patch came from the same ORN.

The single recording of a channel activated by $InsP_4$ is difficult to interpret by itself but is significant in light of the recent characterization of an $InsP_4$-gated channel of similar conductance (199 vs. 193 pS) in the soma membrane of cultured lobster ORNs (27). We never observed that $InsP_3$ activates a channel of this conductance in the vesicle preparation, thereby increasing the probability that the channel *in situ* was activated by $InsP_4$ and not by $InsP_3$ or some other metabolite that may have contaminated the $InsP_4$, even though we were unable to test the specificity of the channel for the ligand in the vesicle preparation. Plasma membrane $InsP_4$ receptors occur in nonexcitable cells, where they regulate Ca^{2+} entry (34). Finding a presumptive $InsP_4$ receptor in the outer dendrite of lobster ORNs suggests that these channels can also have a functional role in excitable cells.

While we can demonstrate cAMP- and $InsP_3$-gated ion channels can occur in the same ORN, how the two types of $InsP_3$-gated channel and the $InsP_4$-gated channel are distributed across cells is unknown. Determining the extent to which multiple effectors are expressed by the same or by different ORNs will open up another level of understanding as to how primary receptor cells encode information about odors.

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