J. Membrane Biol. 127, 173–183 (1992)

The Journal of
Membrane Biology

IP_3- and cAMP-Induced Responses in Isolated Olfactory Receptor Neurons from the Channel Catfish

Takenori Miyamoto*, Diego Restrepo, Edward J. Cragoe, Jr.**, and John H. Teeter
Monell Chemical Senses Center, Philadelphia, Pennsylvania 19104, and Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-3308

Summary. Olfactory receptor neurons enzymatically dissociated from channel catfish olfactory epithelium were depolarized transiently following dialysis of IP_3 or cAMP (added to the patch pipette) into the cytoplasm. Voltage and current responses to IP_3 were blocked by ruthenium red, a blocker of an IP_3-gated Ca^{2+}-release channel in sarcoplasmic reticulum. In contrast, the responses to cAMP were not blocked by extracellularly applied ruthenium red, nor by L-*cis*-diltiazem or amiloride and two of its derivatives. The current elicited by cytoplasmic IP_3 in neurons under voltage clamp displayed a voltage dependence different from that of the cAMP response which showed marked outward rectification. A sustained depolarization was caused by increased cytoplasmic IP_3 or cAMP when the buffering capacity for Ca^{2+} of the pipette solution was increased, when extracellular Ca^{2+} was removed or after addition of 20–200 nM charibdotoxin to the bathing solution, indicating that the repolarization was caused by an increase in $[Ca_i]$ that opened Ca^{2+}-activated K^+ channels. The results suggest that different conductances modulated by either IP_3 or cAMP are involved in mediating olfactory transduction in catfish olfactory receptor neurons and that Ca^{2+}-activated K^+ channels contribute to the termination of the IP_3 and cAMP responses.

Key Words olfactory receptor neuron · IP_3 · cAMP · olfactory transduction · second messengers

Introduction

Studies of odorant-stimulated cAMP[1] formation in olfactory cilia membranes (Pace et al., 1985; Sklar, Anholt & Snyder, 1986; Breer, Boekhoff & Tarelius, 1990; Bruch & Teeter, 1990), patch-clamp studies of cAMP-modulated conductances in isolated olfactory neurons, excised olfactory cilia and reconstituted ciliary membranes (Nakamura & Gold, 1987; Suzuki, 1989; Bruch & Teeter, 1990; Kolesnikov, Zhainazarov & Kosolapov, 1990; Kurahashi, 1990; Kurahashi & Kaneko, 1991) and the recent cloning and functional expression of an olfactory-specific cyclic nucleotide-gated channel (Dhallan et al., 1990; Ludwig et al., 1990; Goulding et al., 1991) indicate that a cascade of biochemical reactions leading to opening of a cAMP-gated channel is a mechanism that mediates olfactory transduction for a variety of odor stimuli in vertebrates. However, recent biochemical (Huque & Bruch, 1986; Breer et al., 1990; Breer & Boekhoff, 1991) and electrophysiological (Restrepo et al., 1990; Fadool, Michel & Ache, 1991; Suzuki, 1991) studies suggest that, for certain olfactory stimuli in vertebrates and invertebrates, an odorant-induced increase in intracellular levels of inositol-1,4,5-triphosphate and subsequent opening of an IP_3-gated cation channel present in the ciliary membrane may be an alternate or additional mechanism for olfactory transduction. In addition, channels directly gated by odorants have been reported in olfactory cilia of rats (Vodyanoy & Murphy, 1983) and frogs (Labarca, Simon & Anholt, 1988), and whole-cell patch-clamp studies in the lobster indicate that conductances with different ionic selectivities are involved in the responses of olfactory neurons to stimulation with mixtures of odorants (Schmiedel-Jakob et al., 1990). Taken together, these studies suggest that multiple second-messenger pathways are involved in mediating olfactory transduction.

Studies of odorant regulation of IP_3 and cAMP formation in rat (Breer & Boekhoff, 1991) and catfish (Huque & Bruch, 1986; Bruch & Teeter, 1990) indi-

* *Present address:* Department of Physiology, Nagasaki University School of Dentistry, Nagasaki, Japan.
** *Present address:* PO Box 631548, Nacogdoches, Texas 75963-1548.

[1] *Abbreviations:* ATP, adenosine 5′-triphosphate; BAPTA, (bis-(o-aminophenoxy)-ethane-*N*-*N*-*N*′-*N*′-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; CTX, charybdotoxin; DCB, 3′,4′-dichlorobenzamil; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenglycol-bis-(b-aminoethyl)-*N*-*N*-*N*′-*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IP_3, inositol-1,4,5-triphosphate; NMDG, *N*-methyl-D-glucamine.

cate that both pathways are expressed in the same species. In addition, specific olfactory stimuli activate either IP_3 or cAMP formation exclusively in the rat (Breer & Boekhoff, 1991). However, little is known about how these pathways are expressed in individual olfactory receptor cells. We have used the whole-cell patch-clamp technique to study the response of isolated olfactory neurons from catfish to intracellularly applied IP_3 and cAMP, and we have performed an initial characterization of the pharmacological properties of the conductances mediating these responses. The experiments indicate that catfish olfactory neurons express both IP_3- and cAMP-gated conductances and suggest colocalization of these two conductances in individual cells. A preliminary account of some of this work has been published (Restrepo et al., 1990).

Materials and Methods

Cell Isolation

Experiments were performed on olfactory receptor neurons dissociated from channel catfish (*Ictalurus punctatus*) olfactory epithelia. Catfish were obtained from commercial suppliers and maintained in 250-gal aquaria at 17°C. The animals were euthanized, and the olfactory epithelium was quickly removed from the nasal cavity, cut into pieces of approximately 2 × 2 mm, and incubated for 15 min at room temperature in divalent cation-free Ringer solution containing L-cysteine-activated papain (15 U/ml) and 2 mM EDTA. The tissue was rinsed with normal Ringer solution and gently triturated with a pasteur pipette. Following dissociation, cells were stored at 4°C. This procedure results in the dissociation of large numbers of olfactory neurons, respiratory cells and round cells with varying morphologies as described in detail in Restrepo and Teeter (1990). Olfactory cilia and pieces of axons were often observed under phase-contrast optics. Only cells with the characteristic bottle shape of catfish olfactory neurons were employed for recordings. For the electrophysiological measurements, aliquots of the cell suspension were placed on glass coverslips which acted as the bottom of the experimental chamber. The cells did not attach to the glass.

Electrophysiology

Membrane current and voltage were measured in isolated cells using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 1B amplifier (Axon Instruments, Burlingame, CA). The current signal was filtered at 1 kHz, digitized at 20–40 kHz and stored on an AT-compatible microcomputer running pCLAMP software (Axon Instruments, Burlingame, CA), which was also used to control the D/A converter for generation of clamp protocols. Patch electrodes with resistances of 7–20 MΩ were fabricated from borosilicate glass capillaries using a Sutter P-80/PC electrode puller (Sutter Instrument, San Raphael, CA). Gigaohm seals were easily obtained with most cells by applying weak negative pressure. The whole-cell configuration was achieved by application of brief voltage pulses and additional negative pressure. Input resistances were from 1 to 14 GΩ. The cells displayed a variety of voltage-dependent and odorant-activated currents described in detail elsewhere (Miyamoto, Restrepo & Teeter, 1991).

Materials and Media

1,4,5-IP_3, 1,3,4-IP_3 (Calbiochem, La Jolla, CA) and cAMP (Sigma, St. Louis, MO) were dissolved in deionized water at a concentration of 1 mM and were added to pipette solutions by dilution to the appropriate final concentrations. Amiloride (Sigma, St. Louis, MO), phenamil, DCB (synthesized by the method of Cragoe et al., 1967), ruthenium red (Serva, Westbury, NY), *l-cis*-diltiazem (Tanabe Seiyaku, Osaka, Japan), charybdotoxin (Alomone Laboratories, Jerusalem, Israel) and apamin (Sigma) solutions were prepared immediately before experiments from stock solutions stored at −20°C.

Normal Ringer solution contained (in mM): 100 NaCl, 3.5 KCl, 1.0 $CaCl_2$, 1.6 $MgCl_2$ and 10 HEPES-NaOH (pH = 7.5) unless otherwise indicated. $NMDG^+$ was substituted for Na^+ in Na^+-free Ringer solution, and Ca^{2+}-free Ringer solution contained 1 mM EGTA and no added Ca^{2+}. Patch pipettes were filled with a KCl internal solution containing (in mM): 100 KCl, 0.1 $CaCl_2$, 2.0 $MgCl_2$, 1.1 EGTA and 10 HEPES-KOH (pH = 7.5) or CsCl internal solution where Cs^+ was substituted for K^+ in the KCl internal solution, unless otherwise indicated. Occasionally, 5–30 mM KCl or CsCl in the pipette solution were replaced with equimolar amounts of KF (KF internal solution) or CsF (CsF internal solution) to minimize ion channel "run down" (Matteson & Armstrong, 1986). To increase the buffering capacity for Ca^{2+} in the pipette solution without modifying the free calcium concentration, the concentrations of EGTA and $CaCl_2$ in the internal solutions were increased by 10-fold. In some experiments, BAPTA (Calbiochem) was employed instead of EGTA.

Statistical Analysis

Errors quoted are SD unless otherwise noted. Significance of the difference between means was tested using a two-tailed *t* test.

A key question resulting from these studies is whether some olfactory cells possess both cAMP and IP_3-gated conductances. If it is assumed that IP_3- and cAMP-regulated conductances are not present in the same cell (*null hypothesis*), and if the frequencies for expression of IP_3 (f_{IP_3})- and cAMP (f_{cAMP})-gated conductances in the whole population of neurons are known, it is possible to test the difference between the experimentally observed values ($fexp_{IP_3}$ of 0.90, $n = 20$ and $fexp_{cAMP}$ of 1.0, $n = 10$) against the actual frequencies using a chi-squared test (Steel & Torrie, 1960). The number of degrees of freedom are two because, under the null hypothesis, a cell can either have IP_3, cAMP, or no conductances. Since we do not know the actual frequencies (f_{IP_3} and f_{cAMP}) for the entire neuron population, we have minimized the value of chi squared for any combination of f_{IP_3} and f_{cAMP} subject to the constraint $f_{IP_3} + f_{cAMP} < 1$. We find that this minimum value of chi squared is 13.3 for $f_{IP_3} = 0.52$ and $f_{cAMP} = 0.44$. The probability of obtaining this value of chi squared is $P < 0.005$, indicating with high certainty that the null hypothesis must be discarded. That is, cAMP- and IP_3-mediated responses occur in the same cell.

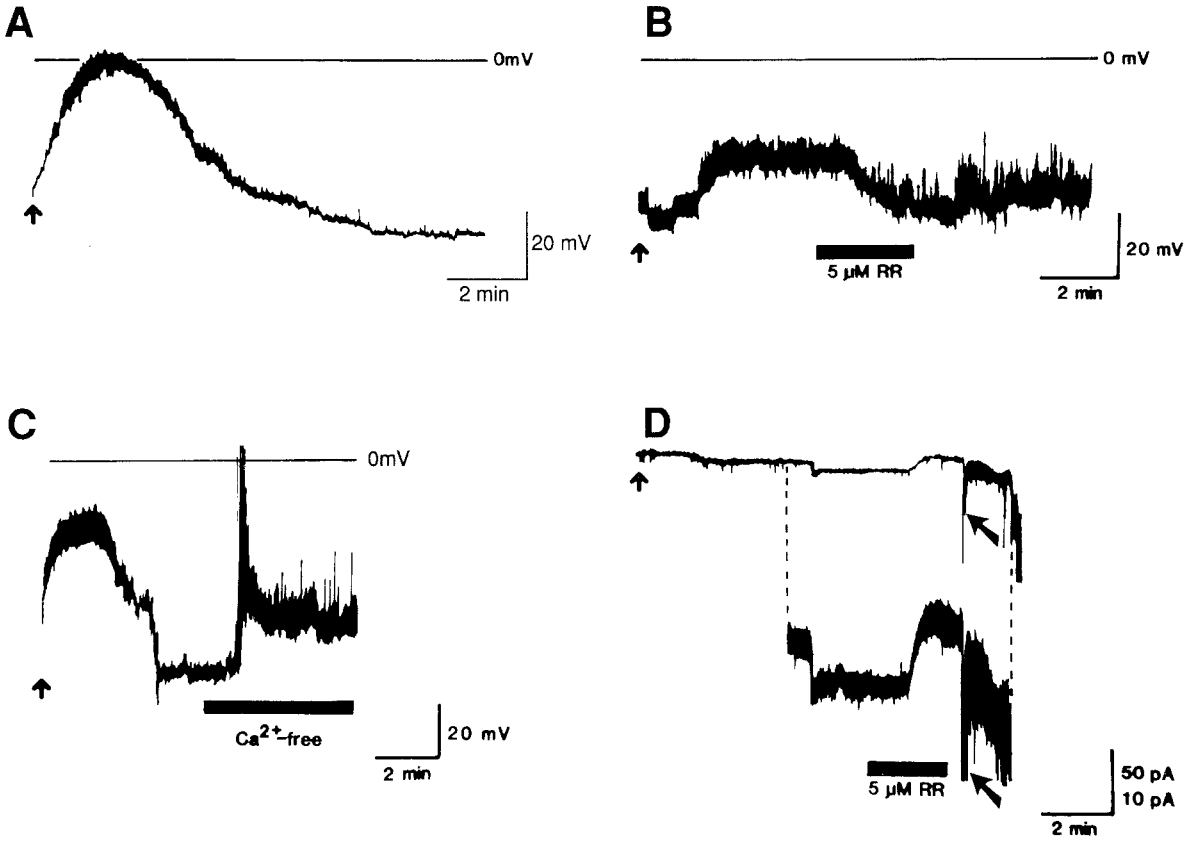

Fig. 1. IP_3-induced response. (*A*) Transient depolarization obtained after brief delay upon rupture of patch membrane (small arrow) when 8 μM 1,4,5-IP_3 was added to the pipette solution (30 mM KF internal solution). (*B*) A sustained depolarizing response was obtained by addition of 10 μM 1,4,5-IP_3 to the pipette in the presence of 11 mM BAPTA (5 mM KF internal solution). Ruthenium red (*RR*) at a concentration of 5 μM blocked this sustained response. (*C*) Transient depolarization obtained by addition of 10 μM IP_3 to the pipette (10 mM KF medium). Removal of extracellular calcium (Ca^{2+}-free Ringer) results in a sustained depolarization. (*D*) A sustained current response obtained by addition of 11 mM BAPTA as well as 10 μM 1,4,5-IP_3 to the pipette solution (5 mM KF internal solution). The sustained current response was blocked by 5 μM ruthenium red (*RR*) applied during a period indicated by black bars. Blocking of IP_3-induced current response was followed by a train of transient inward current spikes (large arrows) and followed by development of a large sustained inward current. Bottom trace in *D* is a magnification of the portion of the record shown in the top trace during the period enclosed by the two broken lines. Cells were under zero current clamp for Figs. 1*A–C* and under voltage clamp with a holding potential of −60 mV for Fig. 1*D*.

Results

IP_3-Induced Response

As previously reported (Restrepo et al., 1990), when 8–10 μM IP_3 was added to the pipette solution, current-clamped olfactory receptor neurons responded with a transient depolarization after rupture of the patch membrane (Fig. 1*A*). The plasma membrane repolarized within 2–5 min. Responses to IP_3 were observed consistently (18 of 20 trials) when 5 to 30 mM KF was added to the pipette solution and less frequently (30% of trials, $n = 13$) in the absence of F^-. Membrane depolarization was substantially smaller under control conditions when 30 mM KF was included in the pipette solution in the absence of IP_3 (4.7 ± 3 mV, $n = 3$) (Restrepo et al., 1990). The magnitude of the depolarization elicited by 8–10 μM 1,4,5-IP_3 with KF in the pipette was 36.3 ± 15.1 mV ($n = 18$) at a resting potential of −50.3 ± 11.6 mV ($n = 18$). The latency of the response was highly variable but decreased as the concentration of IP_3 in the pipette was increased (the Table). In addition, the response to IP_3 was stereospecific: the depolarization induced by 8 μM 1,3,4-IP_3 in the presence of 30 mM KF at a resting potential of −39 ± 1.2 mV ($n = 3$) was significantly smaller (11.3 ± 5.0 mV, $n = 3$) than the response to 1,4,5-IP_3 ($P < 0.05$).

In some cells, when the concentrations of EGTA (or BAPTA) and of $CaCl_2$ in the pipette were raised proportionally by 10-fold in order to increase buffering capacity for Ca^{2+} while keeping the free Ca^{2+}

Table 1. Latency and time to peak for the transient depolarization caused by cAMP and IP_3 in neurons under zero current clamp

Second-messenger concentration (μM)	Latency (sec)	Time to peak (sec)
8–10 IP_3	25 ± 33 (21)[1,5]	85 ± 62 (21)[3,6]
80 IP_3	6 ± 2 (4)[1]	45 ± 18 (4)[3]
10 cAMP	73 ± 84 (6)[2,5]	115 ± 79 (6)[4,6]
100 cAMP	25 ± 15 (3)[2]	45 ± 23 (3)[4]

Results shown are mean ± SD (n). cAMP and IP_3 were introduced in the cytoplasm by dialysis from the patch pipette. *See* Figs. 1 and 3 for representative traces. Significance of differences using a two-tailed t test: [1]$P < 0.001$, [2]$P < 0.05$, [3]$P < 0.001$, [4]$P < 0.02$, [5]$P < 0.001$, [6]$P < 0.01$.

concentration constant, the IP_3-induced depolarization became sustained (Fig. 1*B*). This occurred in 33% of the trials with 11 mM EGTA ($n = 15$) and in 50% of the trials with 11 mM BAPTA ($n = 6$). In addition, removal of extracellular Ca^{2+} following the transient IP_3-induced depolarization resulted in a sustained depolarization (Fig. 1*C*, $n = 2$). In control experiments we found that increasing the buffering capacity for Ca^{2+} of the pipette solution or removal of extracellular calcium did not induce sustained depolarization in the absence of IP_3. These observations indicate that, at least in some cells, the repolarization which followed the IP_3-induced depolarization was Ca^{2+} dependent and that an influx of Ca^{2+} contributed to this process.

Inhibition of IP_3-Induced Response by Ruthenium Red

The sustained response produced by IP_3 with increased Ca^{2+}-buffering capacity in the pipette solution was completely inhibited by 10 μM ruthenium red (Fig. 1*B*), which has been shown to block an IP_3-gated cation channel in ciliary plasma membranes reconstituted into phospholipid bilayers (Restrepo et al., 1990). Under voltage clamp, and in the presence of increased Ca^{2+} buffer in the pipette or in the absence of extracellular Ca^{2+}, intracellular IP_3 (10 μM) induced a sustained inward current (Fig. 1*C*) whose magnitude varied from 10 to 70 pA at a holding potential of −60 mV (21.1 ± 27.4 pA, $n = 10$) (5 to 30 mM CsF internal solution). The current induced by IP_3 was of the same order of magnitude as the current elicited by stimulation with odorant amino acids (Miyamoto et al., 1991). The inward current elicited by internal IP_3 was also blocked by ruthenium red (Fig. 1*C*).

Although ruthenium red blocks the IP_3-induced inward current, it appears to affect other conductances as well. Prolonged application of ruthenium red occasionally resulted in a train of transient inward currents followed by an irreversible decrease in membrane resistance under voltage clamp (Fig. 1*D*) or a train of spikes followed by irreversible depolarization above 0 mV under current clamp (*see* Figs. 1*B* and 5*A*). In addition, Fig. 2 shows that, in the absence of internal IP_3, ruthenium red potentiated the amplitude of the voltage-dependent inward current (Fig. 2*B*) and made the time course (decay time constant) more prolonged (*not shown*). This suggests that ruthenium red facilitates opening of channels mediating a transient inward current (possibly the voltage-gated Na^+ channel), resulting in vigorous spike discharge and cell death. However, inhibition of the IP_3-induced response by ruthenium red always preceded the initiation of a train of transient inward currents. Therefore, ruthenium red appears to have at least two different sites of action in catfish olfactory neurons.

cAMP-Induced Response

Catfish olfactory cells under whole cell current clamp responded with a transient depolarization when cAMP was included in the pipette solution (Fig. 3*A*). All cells tested with 10 or 100 μM cAMP responded ($n = 10$). The cAMP-induced depolarization declined within a few minutes after rupture of the patch membrane. The amplitude of the depolarization evoked by 10 μM cAMP was 41.2 ± 7.2 mV ($n = 6$) at a resting potential of −49.0 ± 13.2 mV ($n = 6$) with KF in the pipette. The time course of the response, as characterized by the latency and time to peak, was highly variable (the Table). Similar to the IP_3-induced depolarization, the cAMP-induced response became sustained when external Ca^{2+} was removed (Fig. 3*B*) or when the buffering capacity for Ca^{2+} in the pipette solution was increased (Fig. 3*C*). Interestingly, the sustained responses to cAMP were more easily obtained by increasing the Ca^{2+}-buffering capacity of the internal solution than those to IP_3: 90% of the responses to

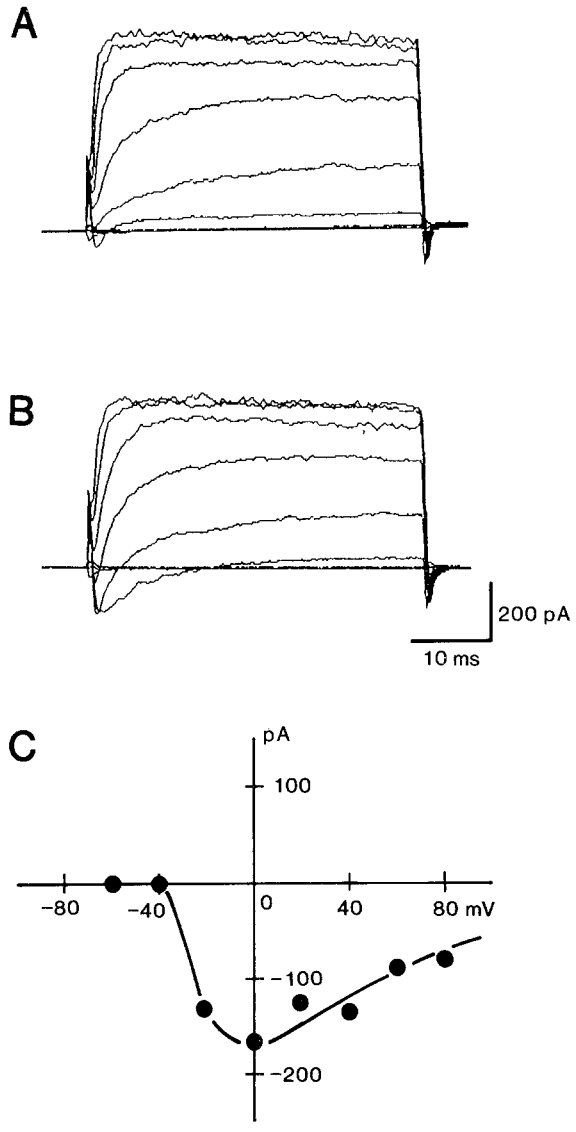

Fig. 2. Effect of ruthenium red on voltage-gated inward current. Current responses evoked by 40-msec voltage steps between −60 and 80 mV in 20-mV increments from a holding potential of −80 mV in normal (*A*) and in 2 μM ruthenium red (*B*) Ringer solution. (*C*) Difference of peak inward current between *A* and *B* plotted as a function of voltage. Pipette: K internal solution.

cAMP (n = 10) were sustained when 11 mM EGTA was included in the pipette solution, whereas sustained responses to IP_3 were obtained in only 33% of the trials in the presence of 11 mM EGTA (n = 15) and in 50% of the trials with 11 mM BAPTA (n = 6). This suggests that, in many of the cells that were stimulated with internal IP_3, a large amount of Ca^{2+}, capable of overcoming the Ca^{2+}-buffering action of EGTA, may have entered the neurons. In contrast, the Ca^{2+} influx that takes place upon stimulation with internal cAMP is of a smaller magnitude and does not exceed the Ca^{2+}-buffering capacity of the EGTA.

Ruthenium red (7 μM), which inhibited the response to IP_3, *did not* suppress the sustained response to cAMP (Fig. 3*C*, n = 10). In addition, DCB (10 μM), which has been shown to inhibit the cAMP-dependent channel in excised membrane patches from frog olfactory cells when applied to the cytoplasmic side (Kolesnikov et al., 1990), had no effect on the cAMP-induced responses when applied extracellularly in catfish olfactory neurons (Fig. 3*C*, n = 3). Amiloride (100–500 μM) (Fig. 3*C*, n = 10) and phenamil (10 μM, n = 2, *not shown*) also had no effect on cAMP-induced responses. Amiloride appeared to depolarize the cell slightly. In contrast, Na^+-free Ringer solution suppressed the response of neurons under current clamp to cytosolic cAMP or IP_3 (*not shown*). *l-cis*-diltiazem (20 μM), which has been reported to inhibit the cAMP-gated channel in the frog when added internally (Kolesnikov et al., 1990), had no effect on the cAMP-induced responses in the catfish when added to the bath (n = 2, *not shown*).

Voltage Dependence of IP_3 and cAMP-Induced Responses

In some cases the onset of the IP_3 and cAMP currents was delayed, presumably because dialysis of IP_3 or cAMP into the cytoplasm was slow. In these cells, direct comparison of whole-cell *I-V* relationships before and after the addition of the second messengers was possible. Figure 4 shows the sustained current responses induced by 10 μM IP_3 (Fig. 4*A*) and 5 μM cAMP (Fig. 4*C*) at a holding potential of −60 mV when the buffering capacity for Ca^{2+} in the pipette was increased by addition of 11 mM BAPTA to the pipette solution (5 mM CsF internal solution). In both cases the response began 3–4 min after rupture of the patch membrane (arrows). The steady-state *I-V* relationships obtained from currents evoked by voltage ramps before (*a*) and during (*b*) the responses to IP_3 (Fig. 4*B*) and cAMP (Fig. 4*D*) are also shown in Fig. 4. The effect of addition of cytoplasmic cAMP or IP_3 was to increase membrane conductance. However, the shapes of the *I-V* relationships during the responses to IP_3 and cAMP were clearly different from each other: the *I-V* relationship of the IP_3-induced response (Fig. 4*B*) displayed only slight rectification, while marked outward rectification was observed for the cAMP-induced response (Fig. 4*D*). These results indicate that two distinct second messenger (IP_3 and cAMP)-modulated conductances are present in channel catfish olfactory receptor cells.

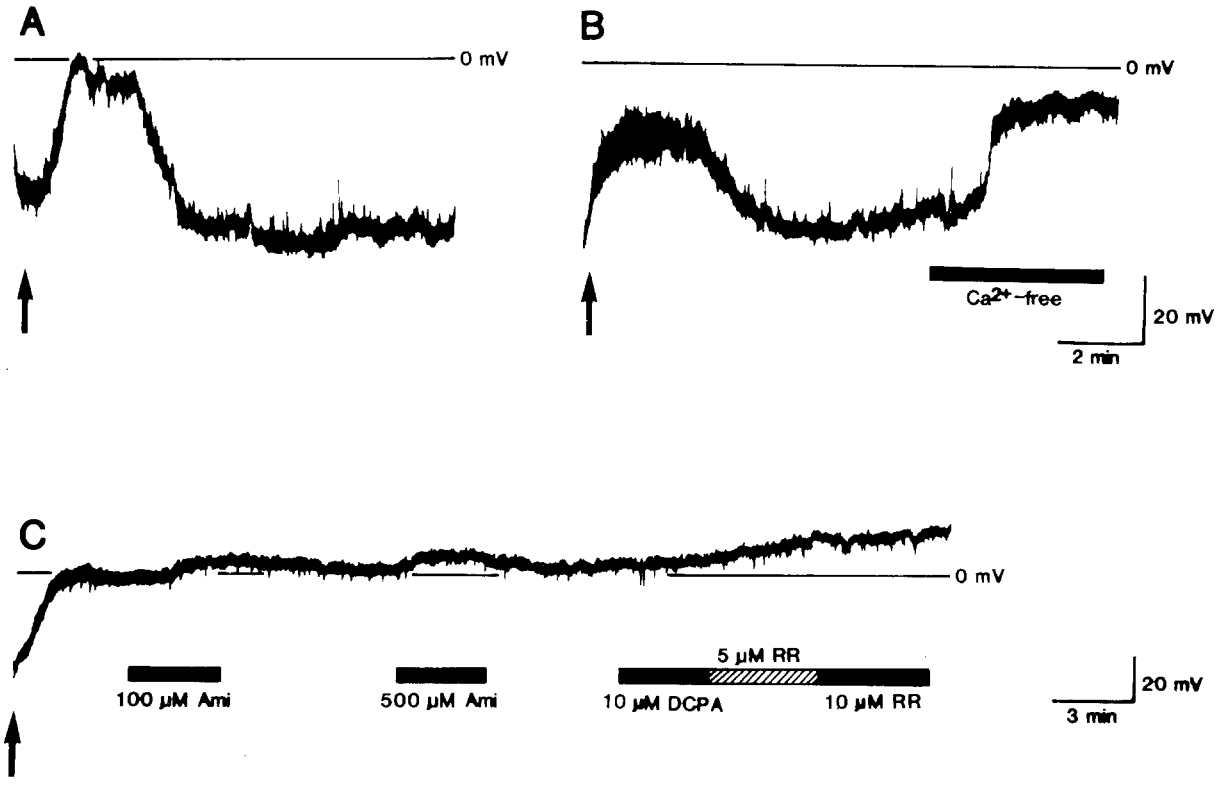

Fig. 3. cAMP-induced response. (*A*) A transient depolarization obtained after brief delay upon rupture of patch membrane (large arrow) when 100 μM cAMP was present in the pipette solution (KF internal solution). (*B*) A transient depolarization was also elicited by 10 μM cAMP. When Ca^{2+}-free Ringer solution was perfused in the bath during the period indicated by a black bar after repolarization, the cell depolarized again. Pipette: 5 mM KF internal solution. (*C*) A sustained depolarizing response was obtained by addition of 10 μM cAMP in the presence of 11 mM EGTA in the pipette solution (5 mM KF internal solution). The sustained depolarization was not blocked by 100–500 μM amiloride (*Ami*), 10 μM DCB or 5–10 μM ruthenium red (*RR*). The drugs were applied during the period indicated by black or striped bars. Holding potential was −60 mV.

Figure 4 also shows that the IP_3-induced response was suppressed by 5 μM ruthenium red but not by 100 μM amiloride (Fig. 4*A*), whereas the cAMP-induced response was not affected by 5 μM ruthenium red, 40 μM amiloride or 20 μM *l-cis*-diltiazem added to the bath.

Effects of Charybdotoxin and Apamin on the Response to IP_3

Sustained responses to IP_3 and cAMP were obtained by increasing the intracellular-buffering capacity for Ca^{2+} or by removal of external Ca^{2+} (Figs. 1*B* and *C* and 3*C*). These results suggest that the decline of the depolarizing response is mediated by Ca^{2+} influx as reported for retinal receptor cells (Fain & Matthews, 1990) and olfactory receptor neurons of the frog (Suzuki, 1989) and the newt (Kurahashi, 1990; Kurahashi & Shibuya, 1990). The presence of a Ca^{2+}-activated K^+ current has been reported in most of vertebrate olfactory neurons examined by the patch-clamp technique (Trotier, 1986; Firestein & Werblin, 1987; Maue & Dionne, 1987; Schild, 1989; Suzuki, 1989; Miyamoto et al., 1991). Because Ca^{2+} passes both through IP_3- (Restrepo et al., 1990) and cAMP-gated channels (Suzuki, 1989; Kolesnikov et al., 1990; Kurahashi, 1990), it is likely that an increase in internal Ca^{2+} activates a Ca^{2+}-dependent K^+ conductance following activation of either the IP_3- or cAMP-gated conductances.

To test this hypothesis, we employed two specific blockers of Ca^{2+}-activated K^+ channels, CTX and apamin (Castle, Haylett & Jenkinson, 1989). When 20–200 nM CTX was added to the bathing solution, a sustained response to IP_3 was sometimes, but not always, obtained (Fig. 5*A*, four out of nine trials resulted in a sustained response). The resting membrane potential was not affected by 20 to 200 nM CTX (Fig. 5*B*). Addition of 50–250 nM apamin to the bathing medium had no effect on either the IP_3 response or the resting membrane potential. These results indicate that, at least in some cells, the transient nature of the IP_3 response could result from

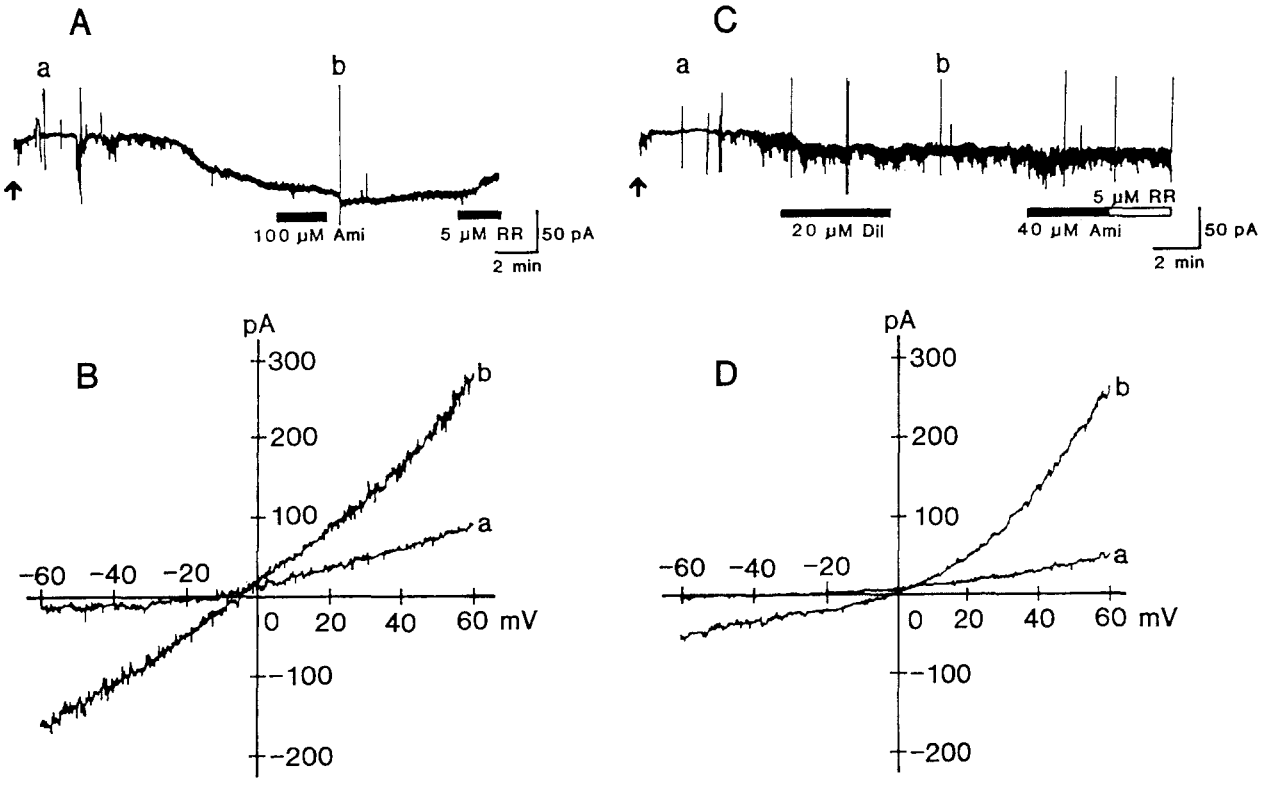

Fig. 4. Comparison between IP_3- and cAMP-induced currents. (*A*) and (*C*) Sustained current induced by 10 μM IP_3 (*A*) and 5 μM cAMP (*C*). Arrows show the rupture of patch membrane. Pipette: 5 mM CsF internal solution with 11 mM BAPTA. The holding potential was −60 mV. (*B*) and (*D*) Voltage dependence of currents induced by IP_3 (*B*) and cAMP (*D*). The *I-V* relationships were obtained from currents induced by a voltage ramp where the membrane potential was changed from −60 to 60 mV with a constant rate of 0.12 V/sec. Currents before (*a*) and during (*b*) response to IP_3 or cAMP are shown. Amiloride (*Ami*), *l-cis*-diltiazem (*Dil*) and ruthenium red (*RR*) were applied during the period indicated by filled and open bars. Pipette solutions did not contain Mg^{2+}.

opening of CTX-sensitive Ca^{2+}-dependent potassium channels.

Discussion

Multiplicity of Second-Messenger-Activated Conductance

The present results clearly show that independent conductances, activated by either IP_3 or by cAMP, are present in channel catfish olfactory receptor cells. We have previously reported that stimulation of isolated catfish olfactory neurons with L-amino acids results in responses which differ from cell to cell in terms of their current-voltage relationships and pharmacology (Miyamoto et al., 1991). One group of cells displayed L-amino acid-induced currents that showed marked outward rectification and were resistant to ruthenium red and amiloride. These properties resemble those of the cAMP-induced currents in the present study, which, in agreement with other studies of cyclic nucleotide-gated channels in olfactory neurons (Nakamura & Gold, 1987; Dhallan et al., 1990; Kurahashi, 1990) and retinal rods (Stern, Kaupp & MacLeish, 1986; Nicol et al., 1987), displayed marked outward rectification and that, in addition, were not blocked by external ruthenium red or amiloride.

Activation of different second-messenger-mediated conductances could explain the diversity of responses to odorant amino acids (Restrepo et al., 1990; Miyamoto et al., 1991). Taken together with the demonstration in catfish isolated olfactory cilia of L-amino acid-stimulated formation of IP_3 (Huque & Bruch, 1986) and cAMP (Bruch & Teeter, 1990), albeit with different time courses and concentration dependencies, the present results are consistent with the parallel action of two second messengers (cAMP and IP_3) in catfish olfactory receptor neurons.

The latencies of the responses to cAMP or IP_3 were relatively slow (the Table). As shown by Pusch

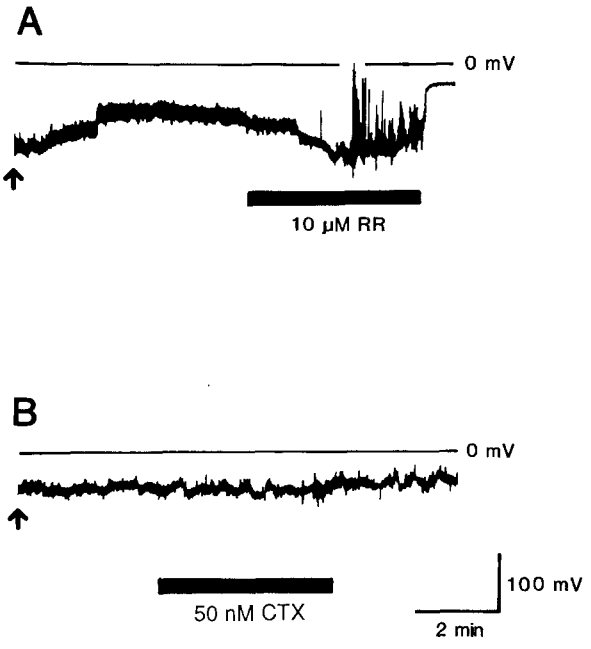

Fig. 5. Effect of CTX on IP_3-induced response and the resting membrane potential. (*A*) Sustained depolarizing response obtained by addition of 10 μM 1,4,5-IP_3 to the pipette solution (5 mM KF internal solution) when the cell was pretreated with Ringer solution containing 100 nM CTX for 10 min. The sustained response was suppressed by 10 μM ruthenium red applied during the period indicated by the black bar, and the suppression was followed by vigorous spike initiation and irreversible depolarization. The arrow indicates the time when the patch membrane ruptured. (*B*) CTX (50 nM) applied during the period indicated by the black bar did not affect the resting membrane potential. Pipette: K internal solution containing 2 mM Na_2ATP.

and Neher (1988) this is not unexpected since, for a pipette resistance of 7–20 MΩ, the estimated time constants for diffusion of substances with the molecular weight of cAMP and IP_3 are in the range from 30 to 90 sec, in agreement with the values in the Table. Additional variability in this value is expected to arise from the substantial differences in cell geometry displayed by the population of isolated catfish olfactory neurons. The response to cAMP in amphibian olfactory neurons under whole cell patch clamp is considerably faster than the responses we measure in catfish (*compare* the Table with Kurahashi, 1990; Suzuki, 1989; Trotier, Rosin & MacLoed, 1990). This probably results from the high concentration of cAMP (500 μM) used in the amphibian studies, which is 50 times larger than the concentration of cAMP ordinarily used in this study. Also, we introduced the second messengers at the soma, which, as shown by Kurahashi (1990), introduces a significant latency compared to introduction of the second messenger at the proximal end of the dendrite. More importantly, as shown in the Table, when it is taken into account that latency and time to peak are dependent on the concentration of second messenger, there is no apparent discrepancy between the time courses of cAMP and IP_3 responses.

Based on the positive correlation between the magnitude of adenyl cyclase stimulation in isolated olfactory cilia with the magnitude of the electroolfactogram elicited by various odorants in frog, Lowe, Nakamura and Gold (1989) have suggested that adenyl cyclase exclusively mediates olfactory transduction. However, recent biochemical work with stopped-flow measurements of second-messenger formation in isolated olfactory cilia from rat indicate that phosphatidyl inositol turnover is involved in olfactory transduction for a variety of odorants that do not stimulate cAMP formation (Breer & Boekhoff, 1990). IP_3 has also been implicated in olfactory transduction in catfish (Huque & Bruch, 1986; Restrepo et al., 1990), frog (Suzuki, 1991) and lobster (Fadool et al., 1991). In addition, in catfish olfactory cilia there is no correlation between cAMP formation and the magnitude of the electroolfactogram elicited by stimulation with various amino acids (Bruch & Teeter, 1990). In the lobster, different ionic conductances with different ionic sensitivity are activated when different olfactory neurons are stimulated with a complex mixture of odorants (Schmiedel-Jakob et al., 1990). Together with the present studies, these results support the hypothesis that different transduction mechanisms are stimulated in response to different olfactory stimuli.

Virtually all cells responded to cAMP or IP_3 (when assayed in the presence of internal KF or CsF medium). A statistical analysis of the data indicates, with high certainty ($P < 0.005$), that *both* cAMP- and IP_3-regulated conductances are present in some olfactory neurons (*see* Materials and Methods). The percent of olfactory neurons possessing both cAMP and IP_3 conductances (90%) can be estimated from the percent of cells responding to IP_3 (90%) or cAMP (100%) alone. This raises the possibility of interactions in some cells between the two pathways, such as stimulation of adenyl cyclase by IP_3-mediated changes in Ca^{2+} (Anholt & Rivers, 1990).

Pharmacological Properties of the cAMP- and IP_3-Modulated Conductances

To isolate and characterize the particular conductances that mediate responses to various odorants in individual olfactory neurons it is important to find pharmacological agents that inhibit one pathway specifically. We found that extracellular ruthenium red, a blocker of a high conductance (100-pS) IP_3-gated Ca^{2+}-release channel in sarcoplasmic re-

ticulum (Suarez-Isla et al., 1988) and of an IP_3-gated cation channel from catfish olfactory cilia (Restrepo et al., 1990), blocked the whole-cell current elicited by intracellular IP_3 but did not alter the cAMP-induced current. Although the effect of ruthenium red was pathway specific, it is important to note that ruthenium red had at least one other effect in catfish olfactory neurons because it facilitated transient inward voltage-gated current (Fig. 2).

In contrast, amiloride and the amiloride derivatives phenamil and DCB, which inhibit the olfactory (Kolesnikov et al., 1990, in frog) and visual (Nicol et al., 1987) cyclic nucleotide-gated channels when added to the intracellular side and are potent inhibitors of epithelial Na^+ channels and of the Na^+/Ca^{2+} exchanger (Kleyman & Cragoe, 1988), did not have an effect on the cAMP (or the IP_3)-stimulated conductance in catfish when added to the bath. It is possible that extracellularly added amiloride was not able to diffuse into the cytoplasm in high enough amounts to block the channel. In frog olfactory neurons Frings, Benz and Lindemann (1991) have reported that amiloride does not block the response of cells to forskolin when added at high concentration (0.5 mM) to the medium bathing the epithelium. Frings et al. (1991) did find that *l-cis*-diltiazem inhibits the response of frog olfactory neurons to forskolin stimulation, but the onset of inhibition is slow (1 min), suggesting that *l-cis*-diltiazem must diffuse into the cell to block the response. It is possible that *l-cis*-diltiazem did not block the cAMP-modulated conductance in the present study because transport of *l-cis*-diltiazem across the plasma membranes of catfish olfactory neurons is slow. However, we cannot eliminate the possibility that the cAMP-gated channel of catfish olfactory neurons may not be sensitive to amiloride or to *l-cis*-diltiazem. The results suggest that special attention should be given to the interpretation of the effects of these drugs.

In the present experiments, the IP_3-induced responses were more frequently observed using pipettes containing F^- (5–30 mM). Because F^- is known to induce cAMP formation through direct G-protein stimulation in catfish olfactory cilia (Bruch & Teeter, 1990), a simultaneous increase in cAMP and IP_3 could in principle be induced by simultaneous injection of IP_3 and F^-. However, this is unlikely because no ATP was added to the pipette solution containing IP_3 and because control experiments show that internal addition of F^-, in the absence of IP_3, causes only a slight depolarization (4.7 ± 3 mV, $n = 3$, *see* Restrepo et al., 1990), significantly smaller than the depolarization produced by addition of internal IP_3 (36.3 ± 15.1 mV, $n = 18$). Other mechanisms could also account for the increased response frequency with IP_3 in the presence of F^-. For example, the IP_3 5-phosphatase that dephosphorylates 1,4,5-IP_3 to 1,4-IP_2 displays high activity in catfish olfactory cilia (Kalinoski et al., 1991). Considering that diffusion of IP_3 into the cilia is probably constrained, high activity by this phosphatase could lower the concentration of IP_3 near the ciliary membrane. This enzyme is inhibited by millimolar amounts of F^- (Storey et al., 1984), suggesting that the effect of F^- could be due to a local increase in IP_3 concentration in an area near the plasma membrane where the IP_3-gated channels are located.

Termination of the IP_3- and cAMP-Induced Responses

Both the IP_3- and cAMP-induced responses displayed a gradual decline within a few minutes when the pipette solution had a low buffering capacity for Ca^{2+}. When the buffering capacity for internal Ca^{2+} was increased or when Ca^{2+} was removed from the bath solution, sustained responses were obtained. Similar phenomena have been reported in frog (Suzuki, 1989) and newt (Kurahashi, 1990) olfactory receptor neurons. These observations suggest that Ca^{2+}, passing through IP_3- and cAMP-gated channels, may play a role in the termination of the second-messenger voltage and current responses which has usually been attributed to regulation of adenylate cyclase or phosphodiesterase activities by Ca^{2+} (Suzuki, 1989; Kurahashi, 1990) in a manner analogous to desensitization in the visual system (*see* review by Fain & Matthews, 1990). However, if Ca^{2+} induces termination of the responses to both IP_3 and cAMP, Ca^{2+} may regulate a process common to both second-messenger systems.

One possibility would be opening of Ca^{2+}-activated K^+ channels that would tend to repolarize the cell membrane. The fact that the IP_3-induced response was sustained after application of CTX, a specific blocker of large and intermediate Ca^{2+}-activated K^+ channels (Castle et al., 1989), supports the hypothesis that Ca^{2+}-activated K^+ channels play a role, at least in some cells, in the repolarization phase of the IP_3-induced response. Neither CTX nor apamin affected the resting membrane potential itself, suggesting that the toxin-sensitive Ca^{2+}-activated K^+ channels are inactive in the resting state. This is consistent with the observation that catfish olfactory neurons maintain $[Ca_i]$ below 90 nM (Restrepo & Boyle, 1991). Thus, the Ca^{2+}-activated K^+ current may be activated only when Ca^{2+} influx is evoked by membrane depolarization and activation of voltage-dependent Ca^{2+}

channel or by opening of transduction channels through which Ca^{2+} can enter the cell.

CTX did not always elicit a sustained IP_3-induced response, suggesting the possibility that other mechanisms also contribute to the repolarization phase of the response. Data from Kurahashi (1990) suggests that the cAMP-gated conductance is shut-down in a Ca^{2+}-dependent manner in the presence of a constant concentration of intracellular cAMP. This could be mediated by a direct effect of Ca^{2+} on the cAMP-gated channel (Firestein, 1991). On the other hand, opening of Ca^{2+}-activated Cl^- channels (Kleene, Pun & Gesteland, 1991) could mediate the repolarization in some of the cells.

The cAMP-induced response became sustained upon increasing the intracellular-buffering capacity for Ca^{2+} more frequently (90%) than the IP_3-induced response (30%). This may be due to the fact that the IP_3-gated cation channel, whose permeability for divalent cations is somewhat higher than the permeability for monovalent cations (Restrepo et al., 1990), may mediate a larger influx of Ca^{2+} than the cAMP-gated channel which, although displaying a higher selectivity for Ca^{2+} than for Na^+, is blocked by extracellular Ca^{2+} (Nakamura & Gold, 1987; Kolesnikov, 1990). In addition, IP_3 may evoke Ca^{2+} release from internal stores when injected into the cell body as in the present experiments. Therefore, the evidence suggests that Ca^{2+}-dependent processes in the olfactory receptor neurons may be more powerfully activated by IP_3 than by cAMP. The fact that BAPTA, which is a more effective chelator of Ca^{2+} than EGTA, induced sustained responses with a higher frequency than EGTA is consistent with this hypothesis.

We would like to thank the Tanabe Seiyaku Co., Ltd., for their gift of *l-cis*-diltiazem. This work was supported by National Institutes of Health grants DC00566 and BRSG S07RR05825.

References

Anholt, R.R., Rivers, A.M. 1990. Olfactory transduction: Cross-talk between second-messenger systems. *Biochemistry* **29:**4049–4054

Breer, H., Boekhoff, I. 1991. Odorants of the same odor class activate different second messenger pathways. *Chem. Senses* **16:**19–29

Breer, H., Boekhoff, I., Tarelius, E., 1990. Rapid kinetics of second messenger formation in olfactory transduction. *Nature* **345:**65–68

Bruch, R.C., Teeter, J.H. 1990. Cyclic AMP links amino acid chemoreceptors to ion channels in olfactory cilia. *Chem. Senses* **15:**419–430

Castle, N.A., Haylett, D.G., Jenkinson, D.H. 1989. Toxins in the characterization of potassium channels. *Trends Neurosci.* **12:**59–65

Cragoe, E.J., Jr., Woltersdorf, O.W., Bicking, J.B., Kwong, S.W., Jones, J.H. 1967. Pyrazine diuretics: II. N-amidino-3-amino-5-substituted-6-halopyrazinecarboxamides. *J. Med. Chem.* **10:**66–75

Dhallan, R.S., Yau, K.-W., Schrader, K.A., Reed, R.R. 1990. Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* **347:**184–187

Fadool, D.A., Michel, W.C., Ache, B.W. 1991. G-proteins and inositol-phospholipid metabolism implicated in odor response of cultured lobster olfactory neurons. *Chem. Senses.* **10:**518 (*Abstr.*)

Fain, G.L., Matthews, H.R. 1990. Calcium and the mechanism of light adaptation in vertebrate photoreceptors. *Trends Neurosci.* **13:**378–384

Firestein, S. 1991. Physiology of transduction in the single olfactory receptor neuron. *J. Gen. Physiol.* **98:**2*a*

Firestein, S., Werblin, F.S. 1987. Gated currents in isolated olfactory receptor neurons in the larval tiger salamander. *Proc. Natl. Acad. Sci. USA* **84:**6292–6296

Frings, S., Benz, S., Lindemann, B. 1991. Current recording from sensory cilia of olfactory receptor cells *in situ*. II. Role of mucosal Na^+, K^+ and Ca^{2+} ions. *J. Gen. Physiol.* **97:**725–748

Goulding, E.H., Ngai, J., Kramer, R.H., Colicos, S., Axel, R., Siegelbaum, S., Chess, A. 1992. Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* **8:**45–58

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Huque, T., Bruch, R.C. 1986. Odorant- and guanine nucleotide-stimulated phosphoinositide turnover in olfactory cilia. *Biochem. Biophys. Res. Commun.* **137:**36–42

Kalinoski, D.L., Aldinger, S., Boyle, A.G., Huque, T., Restrepo, D. 1991. Characterization of an inositol-1,4,5-trisphosphate (IP_3) receptor in isolated olfactory cilia. *Biophys. J.* **59:**526*a*

Kleene, S.J., Gesteland, R.C. 1991. Calcium-activated chloride conductance in frog olfactory cilia. *J. Neurosci.* **11:**3624–3629

Kleyman, T.R., Cragoe, E.J., Jr. 1988. Amiloride and its analogs as tools in the study of ion transport. *J. Membrane Biol.* **105:**1–21

Kolesnikov, S.S., Zhainazarov, A.B., Kosolapov, A.V. 1990. Cyclic nucleotide-activated channels in the frog olfactory receptor plasma membrane. *FEBS Lett.* **266:**96–98

Kurahashi, T. 1990. The response induced by intracellular cyclic AMP in isolated olfactory receptor cells of the newt. *J. Physiol.* **430:**335–371

Kurahashi, T., Kaneko, A. 1991. High density cAMP-gated channels at the ciliary membrane in the olfactory receptor cell. *Neuroreport* **2:**5–8

Kurahashi, T., Shibuya, T. 1990. Ca^{2+}-dependent adaptive properties in the solitary olfactory receptor cell of the newt. *Brain Res.* **515:**261–268

Labarca, P., Simon, S.A., Anholt, R.R.H. 1988. Activation by odorants of a multi-state cation channel from olfactory cilia. *Proc. Natl. Acad. Sci. USA* **85:**944–947

Lowe, G., Nakamura, T., Gold, G.H. 1989. Adenylate cyclase mediates olfactory transmission for a wide variety of odorants. *Proc. Natl. Acad. Sci. USA* **86:**5641–5645

Ludwig, J., Margalit, T., Eismann, E., Lancet, D., Kaupp, U.B. 1990. Primary structure of cAMP-gated channel from bovine olfactory epithelium. *FEBS Lett.* **270:**24–29

Matteson, D.R., Armstrong, C.M. 1986. Properties of two types of calcium channels. *J. Gen. Physiol.* **87:**161–182

Maue, R.A., Dionne, V.E. 1987. Patch clamp studies of isolated mouse olfactory receptor neurons. *J. Gen. Physiol.* **90:**95–125

Miyamoto, T., Restrepo, D., Teeter, J.H. 1992. Voltage-dependent and odorant-regulated currents in isolated olfactory receptor neurons of the channel catfish. *J. Gen. Physiol. (in press)*

Nakamura, T., Gold, G.H. 1987. A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325:**442–444

Nicol, G.D., Schnetkamp, P.P., Saimi, Y., Cragoe, E.J., Jr., Brownds, M.D. 1987. A derivative of amiloride blocks both the light-regulated and cyclic GMP-regulated conductances in rod photoreceptors. *J. Gen. Physiol.* **90:**651–669

Pace, U., Hanski, E., Salomon, Y., Lancet, D. 1985. Odorant-sensitive adenylate cyclase may mediate olfactory reception. *Nature* **316:**255–258

Pusch, M., Neher, E. 1988. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pfluegers Arch.* **411:**204–211

Restrepo, D., Boyle, A.G. 1991. Stimulation of olfactory receptors alters regulation of $[Ca_i]$ in olfactory neurons of the catfish (*Ictalurus punctatus*). *J. Membrane Biol.* **120:**223–232

Restrepo, D., Miyamoto, T., Bryant, B., Teeter, J.H. 1990. Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science* **249:**1166–1168

Restrepo, D., Teeter, J.H. 1990. Olfactory neurons exhibit heterogeneity in depolarization-induced calcium changes. *Am. J. Physiol.* **258:**C1051–C1061

Schild, D. 1989. Whole-cell current in olfactory receptor cells of *Xenopus laevis*. *Exp. Brain Res.* **78:**223–232

Schmiedel-Jakob, I., Michel, W.C., Anderson, P.A.V., Ache, B.W. 1990. Whole cell recording from lobster olfactory receptor cells: Multiple ionic bases for the receptor potential. *Chem. Senses* **15:**397–405

Sklar, P.B., Anholt, R.R.H., Snyder, S.H. 1986. The odorant-sensitive adenylate cyclase of olfactory receptor cells: Differential stimulation by distinct classes of odorants. *J. Biol. Chem.* **261:**15538–15543

Steel, R.G., Torrie, J.H. 1960. Principles and procedures of statistics. p. 352. McGraw-Hill, New York

Stern, J.H., Kaupp, V.B., MacLeish, P.R. 1986. Control of the light-induced current in rod photoreceptors by cyclic GMP, calcium and *l-cis*-diltiazem. *Proc. Natl. Acad. Sci. USA* **83:**1163–1167

Storey, D.J., Shears, S.B., Kirk, C.J., Michell, R.H. 1984. Stepwise enzymatic dephosphorylation of inositol 1,4,5-triphosphate to inositol in liver. *Nature* **312:**374–376

Suarez-Isla, B.A., Irribarra, V., Oberhauser, A., Larralde, L., Bull, R., Hidalgo, C., Jaimovich, E. 1988. Inositol (1,4,5)-trisphosphate activates a calcium channel in isolated sarcoplasmic reticulum membranes. *Biophys. J.* **54:**737–741

Suzuki, N. 1989. Voltage- and cyclic nucleotide-gated currents in isolated olfactory receptor cells. *In:* Chemical Senses. Vol. 1, pp. 461–494. J.G. Brand, J.H. Teeter, R.H. Cagan, and M.R. Kare, editors. Marcel-Dekker, New York—Basel

Suzuki, N. 1992. Multipathway transduction in vertebrate olfaction. *Chem. Senses (in press) (Abstr.)*

Trotier, D. 1986. A patch clamp analysis of membrane currents in salamander olfactory receptor cells. *Pfluegers Arch.* **407:**589–595

Trotier, D., Rosin, J.F., MacLeod, P. 1990. Channel activities in *in vivo* and isolated olfactory receptor cells. *In:* Chemical Senses. Vol. 1, pp. 427–448. J.G. Brand, J.H. Teeter, R.H. Cagan and M.B. Kare, editors. Marcel Dekker, New York—Basel

Vodyanoy, V., Murphy, R.B. 1983. Single-channel fluctuations in bimolecular lipid membranes induced by rat olfactory epithelial homogenates. *Science* **220:**717–719

Received 27 June 1991; revised 13 November 1991

Note Added in Proof

After this paper was *in press*, a description of the molecular and single-channel properties of a cyclic nucleotide-gated channel from catfish olfactory cilia was published (Goulding et al., *Neuron* **8:**45–58, 1992). Although this class of channels very likely mediated the responses of isolated catfish olfactory receptor neurons to intracellular addition of cAMP reported in the present paper, the precise role of cAMP-gated channels in olfactory signal transduction in the catfish remains to be established. Amino acids, which are potent and well-characterized odorants for the catfish (Caprio and Byrd, *J. Gen. Physiol.* **84:**403–422, 1984; Bruch and Rulli, *Comp. Biochem. Physiol.* **91B:**535–540, 1988), do not stimulate accumulation of cAMP within the time frame necessary to mediate olfactory transduction, even at high concentrations (Bruch and Teeter, *Chem. Senses* **15:**419–430, 1990). In contrast, amino acid odorants produce a rapid, transient increase in inositol 1,4,5-triphosphate (IP3) in catfish olfactory cilia (Huque and Bruch, *Biochem. Biophys. Res. Commun.* **137:**36–42, 1986). Micromolar concentrations of IP3 have been shown to directly activate cation channels in cilia membranes, distinct from those gated by cAMP (Restrepo et al., *Science* **240:**1166–1168, 1990; this paper). In addition, a novel protein that binds IP3 with micromolar affinity has recently been identified in catfish olfactory cilia (Kalinoski et al., *Biochem. J.* **281:**449–456, 1992). These observations indicate that amino acid receptors in the catfish olfactory system are coupled to generation of IP3. Odorants acting through accumulation of cAMP (or cGMP) remain to be identified.