12. DiFrancesco (*2*) reported a single-channel conductance of about 1 pS. Patch I_f was about 20 pA at −120 mV and had an extrapolated null potential of −20 mV. Assuming an open probability of 1.0, the channel density was about 200 per macropatch.
13. We have confirmed, in excised patches, the results of DiFrancesco and Tromba (*4*) in WC experiments that phosphorylation increases I_f. When a solution of ATP (2 mM), cAMP (100 μM), and cAMP-dependent protein kinase (1 μg/ml) was added to the bath, patch I_f was increased by 62.5 ± 15% (n = 4). This phosphorylating solution was ineffective when it was Mg^{2+}-free (n = 6).
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22. It is possible that two different subunits of I_f could each be regulated by a different G protein. In this case the allosteric changes on I_f would be produced by the subunit proteins rather than the G proteins themselves. Nonetheless, the changes on I_f would be allosteric.
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Odor Stimuli Trigger Influx of Calcium into Olfactory Neurons of the Channel Catfish

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Olfactory transduction is thought to be mediated by a G protein–coupled increase in intracellular adenosine 3′,5′-monophosphate (cAMP) that triggers the opening of cAMP-gated cation channels and results in depolarization of the plasma membrane of olfactory neurons. In olfactory neurons isolated from the channel catfish, *Ictalurus punctatus*, stimulation with olfactory stimuli (amino acids) elicits an influx of calcium that leads to a rapid increase in intracellular calcium. In addition, in a reconstitution assay a plasma membrane calcium channel has been identified that is gated by inositol-1,4,5-trisphosphate (IP_3), which could mediate this calcium influx. Together with previous studies indicating that stimulation with olfactory stimuli leads to stimulation of phosphoinositide turnover in olfactory cilia, these data suggest that an influx of calcium triggered by odor stimulation of phosphoinositide turnover may be an alternate or additional mechanism of olfactory transduction.

INTERACTION OF ODORS WITH RECEPTOR proteins on the membrane of olfactory cilia is the first of a sequence of biochemical events that leads to the firing of action potentials and conveys information to the olfactory bulb (*1, 2*). By coupling with G proteins (*3–6*), these receptors activate cAMP formation (*3, 7–10*) and phosphoinositide (PI) turnover (*11–13*). The discovery in excised patches of ciliary membrane of a conductance gated by cAMP (*14*) and the identification of an olfactory-specific G protein (G_{olf}) linked to adenylate cyclase (*6*) have led to the development of a model in which activation of receptors by odor stimuli causes an increase in intraciliary cAMP that acts directly to open cation channels, causing membrane depolarization. Although this cAMP hypothesis integrates many experimental observations, it is not clear how reports (*11, 12*) indicating that odors trigger an increase in intraciliary IP_3 fit within this hypothesis. To address this question, we have examined the role of Ca^{2+} in signal transduction in the olfactory system of the catfish.

Catfish have olfactory receptors capable of recognizing L-amino acids at concentrations as low as 10 nM (*15*). The electro-olfactogram (EOG), the integrated electrical response of the olfactory epithelium elicited by L-amino acids, was abolished by removal of Ca^{2+} from the medium bathing the epithelium (Fig. 1). This effect cannot be caused by damage to the epithelial structure because it was readily reversible. In addition, some, but not all, Ca^{2+} channel blockers inhibited the EOG (Fig. 1). These data confirm earlier observations (*16–18*) that suggested that Ca^{2+} is a necessary cofactor for the olfactory (EOG) response and that Ca^{2+} channels are probably involved.

Because odors elicit rapid activation of PI turnover leading to accumulation of ciliary IP_3 (*11, 12*), one possible explanation for the inhibition of the EOG response by removal of Ca^{2+} or by addition of Ca^{2+} channel blockers is that an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), resulting from Ca^{2+} influx triggered by the increase in PI turnover, plays a mediatory role in olfactory transduction. If this hypothesis is viable, (i) an increase in Ca^{2+} influx must occur upon stimulation with odorants and (ii) a mechanism must exist to link the increase in PI turnover to an increase in Ca^{2+} influx. To establish whether the first criterion is fulfilled, we measured $[Ca^{2+}]_i$ in isolated olfactory neurons with the fluorescent Ca^{2+} indicator fura-2 (*19*). In the presence of 1 mM extracellular Ca^{2+}, $[Ca^{2+}]_i$ was low (23 ± 19 nM, mean ± SD, n = 140) but increased rapidly to 0.8 to 1.5 μM after addition of 10 μM ionomycin, a Ca^{2+} ionophore (*20*). Five percent of the neurons (11 of a total of 219) responded to L-amino acids with a rapid increase in $[Ca^{2+}]_i$ (Fig. 2) (133 were stimulated with 100 μM L-alanine, L-arginine, and L-glutamate and 86 were stimulated with the above amino acids plus L-norleucine). Both the basal $[Ca^{2+}]_i$ of

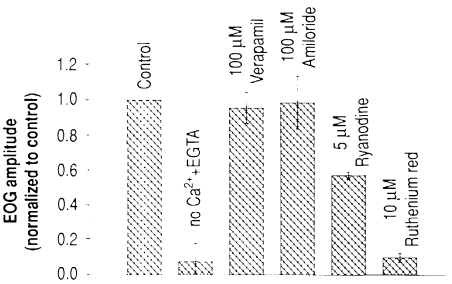

Fig. 1. (**A**) Effect of removal of apical Ca^{2+} or addition of Ca^{2+} channel blockers on EOG responses to 100 μM L-norleucine [mean ± SD; n = 3 for no Ca^{2+}, verapamil, and amiloride; n = 2 for ryanodine and ruthenium red (ammoniated ruthenium oxychloride)]. Each response in the presence of a drug or EGTA was preceded by its own control. Responses were normalized by dividing by control. Responses for no Ca^{2+}, ryanodine, and ruthenium red were significantly different from control by a t test ($P < 0.05$). Similar results were found with L-arginine as the stimulus. Catfish (150 to 250 g) were immobilized with Flaxedil (gallamine triethiodide; 0.1 mg per 100 g of body weight) and anesthetized with MS-222 in the respiratory water. Underwater EOGs (*32*) were recorded according to the method of Caprio (*33*). The epithelium was perfused with artificial pond water (0.2 mM $CaCl_2$, 0.02 mM KCl, 0.3 mM NaCl, and 3 mM $NaHCO_3$, pH 8.3). We modified the artificial pond water by removing the $CaCl_2$ and adding 0.5 mM Na_2EGTA, pH 8.3.

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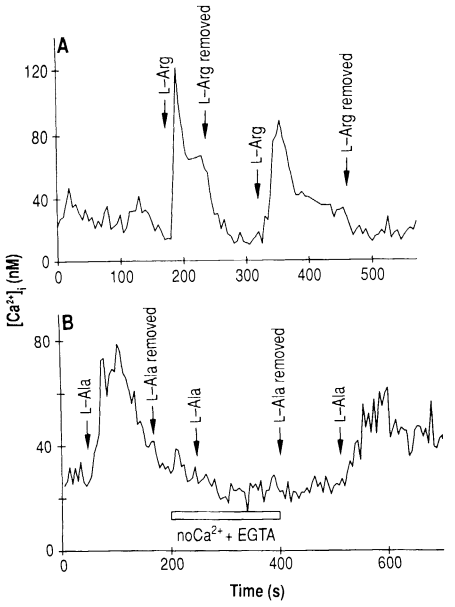

Fig. 2. Effect of L-amino acids on $[Ca^{2+}]_i$ in isolated olfactory neurons. (**A**) Stimulation with 100 μM L-arginine. (**B**) Addition of 100 μM L-alanine in the presence of 1.5 mM EGTA and absence of $CaCl_2$. Cells were isolated by dissociation with papain in divalent cation–free medium (*20*). $[Ca^{2+}]_i$ was measured with fura-2 (*20*). Fish Ringer's: 110 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH 7.6.

cells responding to odorants (18 ± 12 nM, mean ± SD, n = 11) and the increase in Ca^{2+} (range, 60 to 1400% over basal, 449 ± 387%, n = 11) were variable. Phasic and tonic components of the response could be discerned in some traces (Fig. 2A), and stimulation could be repeated several times. Five cells responded specifically to a single amino acid (one each for L-alanine, L-arginine, and L-norleucine and two for L-glutamate), one cell responded to all four amino acids, and the rest of the cells were only tested with one amino acid or were tested with a mixture of all four amino acids. The increase in Ca^{2+} triggered by L-amino acids is mediated by influx of Ca^{2+} since the response could not be elicited in the absence of extracellular Ca^{2+} (Fig. 2B) (n = 4).

We have also used the patch-clamp technique (*21*) to study the voltage-dependent and odor-induced currents in olfactory neurons. All neurons displayed voltage-dependent inward and outward currents (Fig. 3A). Inward currents activated and inactivated rapidly and were blocked by addition of 1 μM tetrodotoxin, indicating that they were mediated by voltage-dependent Na^+ channels. Outward K^+ currents activated slowly and were nearly completely blocked by replacement of intracellular K^+ by Cs^+ or by addition of external Ba^{2+} (10 mM). Spontaneous action potentials were frequently recorded in the cell-attached configuration. Resting potentials (measured as zero current potentials) varied from −25 to −56 mV (−40 ± 8 mV, mean ± SD, n = 20). Stimulation with a mixture of L-amino acids (100 μM each of L-alanine, L-arginine, L-norleucine, and L-glutamate) induced an inward current at negative holding potentials in 11 of 43 cells tested (Fig. 3B). These electrical properties are similar to those reported for olfactory neurons isolated from other species (*22–26*). IP_3 injected via the patch pipette elicited a large depolarization in neurons under zero current clamp, suggesting that IP_3 (directly or indirectly) modulates a plasma membrane conductance (Fig. 3C). The depolarization induced by IP_3 was transient but could be prolonged if the Ca^{2+} buffering capacity of the pipette solution was increased while free Ca^{2+} concentration was kept constant. Under these conditions, the resulting sustained depolarization was blocked by addition of 10 μM ruthenium red to the bath (Fig. 3D).

Our data indicate that increases in $[Ca^{2+}]_i$, caused by stimulation with odorants, are mediated by an influx of Ca^{2+} and that intracellular IP_3 causes plasma membrane depolarization. Because IP_3 is known to modulate the opening of a Ca^{2+} channel associated with the endoplasmic reticulum (*27*), we searched for the presence of an analogous channel in the ciliary plasma membrane of olfactory neurons. We observed that bursts of channel activity could be induced by addition of IP_3 in otherwise silent lipid bilayers into which ciliary membrane vesicles had been incorporated (Fig. 4, A and B). Like the IP_3-gated channels of the sarcoplasmic reticulum (*28*), these channels had high conductance for Ba^{2+} (79 ± 5 pS, mean ± SEM, n = 5 for 55 mM Ba^{2+}) (Fig. 4C). In addition, they were selective for Ba^{2+} over K^+ and *N*-methyl-D-glucamine ($NMDG^+$) [minimum permeability ratios from extrapolated reversal potentials (*29*) are 7- and 54-fold for Ba^{2+} over K^+ and $NMDG^+$]. Nimodipine (5 μM), an inhibitor of L-type voltage-dependent Ca^{2+} channels in olfactory cilia (*20*), as well as in other cell systems (*30*), did not inhibit the IP_3-gated channel. Ruthenium red (10 μM), which blocked the EOG response (Fig. 1) and the depolarization induced by intracellular IP_3 under whole-cell patch clamp (Fig. 3D), also blocked the IP_3-gated channel. Although the channel density in the ciliary plasma membrane cannot be es-

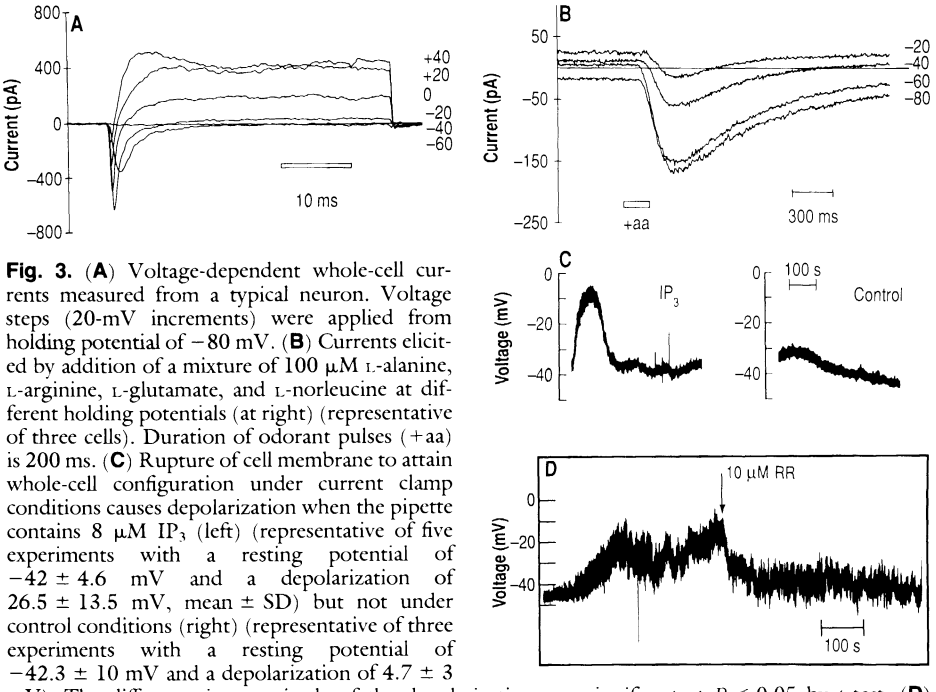

Fig. 3. (**A**) Voltage-dependent whole-cell currents measured from a typical neuron. Voltage steps (20-mV increments) were applied from holding potential of −80 mV. (**B**) Currents elicited by addition of a mixture of 100 μM L-alanine, L-arginine, L-glutamate, and L-norleucine at different holding potentials (at right) (representative of three cells). Duration of odorant pulses (+aa) is 200 ms. (**C**) Rupture of cell membrane to attain whole-cell configuration under current clamp conditions causes depolarization when the pipette contains 8 μM IP_3 (left) (representative of five experiments with a resting potential of −42 ± 4.6 mV and a depolarization of 26.5 ± 13.5 mV, mean ± SD) but not under control conditions (right) (representative of three experiments with a resting potential of −42.3 ± 10 mV and a depolarization of 4.7 ± 3 mV). The difference in magnitude of the depolarization was significant at $P < 0.05$ by t test. (**D**) Addition of IP_3 (10 μM) in the presence of high EGTA (11 mM) and 1 mM $CaCl_2$ (same internal free Ca^{2+} concentration as in (C) produces a sustained depolarization. This depolarization is blocked by 10 μM ruthenium red (RR). Gigaohm seals were obtained with patch pipettes of more than 10 megohms resistance. Whole-cell configuration was attained by application of brief current and pressure pulses. Voltage-clamp recordings were performed with an Axopatch 1-B amplifier (Axon Instruments). Signals were digitized at 40 kHz and stored in an IBM AT compatible computer running pClamp software. A computer-controlled Picospritzer (General Valve) was used to apply stimulus pulses. Bathing solution: 110 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1.6 mM $MgCl_2$, 5 mM Hepes, pH 7.5; pipette solution: 95 mM KCl, 5 mM KF, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1.1 mM EGTA, 5 mM Hepes, 2 mM sodium adenosine triphosphate, pH 7.5. For IP_3 experiments, the pipette solution had 60 mM KCl, 30 mM KF, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, and 5 mM Hepes, pH 7.5.

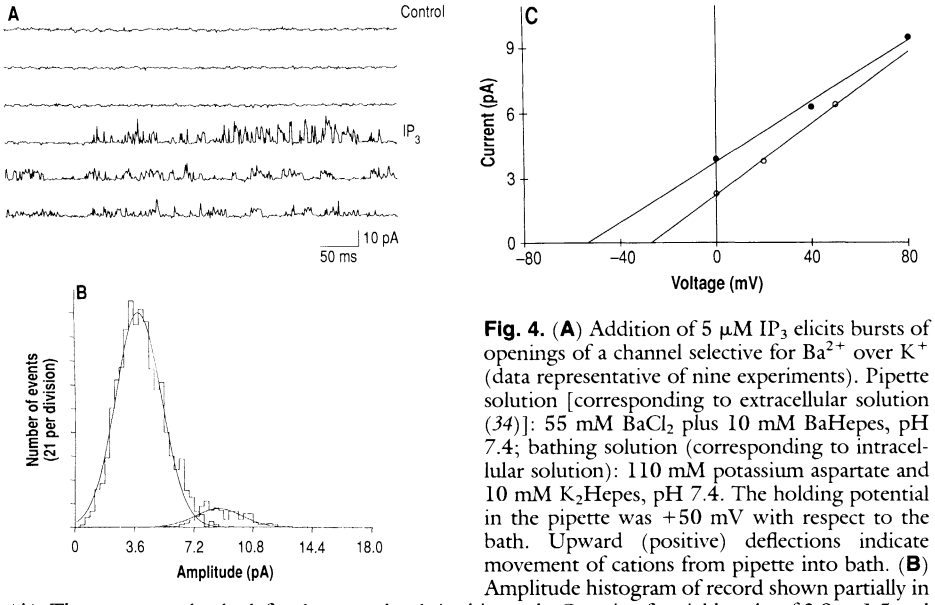

Fig. 4. (**A**) Addition of 5 μM IP_3 elicits bursts of openings of a channel selective for Ba^{2+} over K^+ (data representative of nine experiments). Pipette solution [corresponding to extracellular solution (*34*)]: 55 mM $BaCl_2$ plus 10 mM BaHepes, pH 7.4; bathing solution (corresponding to intracellular solution): 110 mM potassium aspartate and 10 mM K_2Hepes, pH 7.4. The holding potential in the pipette was +50 mV with respect to the bath. Upward (positive) deflections indicate movement of cations from pipette into bath. (**B**) Amplitude histogram of record shown partially in (A). There are two clearly defined current levels in this patch. Gaussian fits yield peaks of 3.9 ± 1.5 and 8.7 ± 1.4 pA. (**C**) Current-voltage relation for IP_3-gated channel with 55 mM $BaCl_2$ in the pipette and either 110 mM potassium aspartate (open circles) or 110 mM *N*-methyl-D-glucamine chloride (NMDGCl, closed circles) in the bath. Slope conductances and extrapolated reversal potentials are 82 ± 3 pS and −27 ± 2 mV for potassium aspartate in the bath and 70 ± 6 pS and −54 ± 8 mV for NMDGCl in the bath (parameters ± SEM). Current and voltage sign conventions are as in (A). Olfactory cilia were isolated as described (*35*) and incorporated into azolectin bilayers formed at the tip of a patch pipette (*36, 37*) as in (*20*). The pipette was removed to a well containing 110 mM potassium aspartate or 110 mM NMDGCl plus 10 mM Hepes, pH 7.4. IP_3 was added to final concentrations of 1 to 15 μM. Single-channel currents were recorded with conventional patch-clamp techniques (*21*).

tablished by the reconstitution method, IP_3-gated channels were observed in approximately 40% of the bilayers (9 of 23 trials), compared to a frequency of 50% for cAMP-gated channels, which can also be detected in catfish ciliary plasma membranes by the same reconstitution protocol (*9, 10*). These proportions suggest that the abundance of the two types of channels is roughly the same. In addition, in over 20 trials we have been unable to detect IP_3-gated channels when olfactory microsome membranes (*31*) were reconstituted into artificial bilayers, suggesting that the IP_3-gated channel is exclusively located in the plasma membrane.

We propose that, in at least some olfactory neurons in the catfish, odor stimuli act via G protein–coupled receptors to enhance the formation of intraciliary IP_3, which in turn directly gates plasma membrane Ca^{2+} channels that produce cell depolarization. According to this hypothesis, Ca^{2+} influx would play a mediatory role in olfactory transduction. This hypothesis accounts for several findings that are difficult to integrate within the context of the cAMP hypothesis of olfactory transduction: (i) the presence of odor-stimulated PI turnover (*11–13*) and Ca^{2+} influx (Fig. 2), (ii) the inhibition of EOG by removal of Ca^{2+} and by Ca^{2+} channel blockers (*16–18*) (Fig. 1), (iii) depolarization caused by intracellular IP_3 (Fig. 3C), and (iv) the presence of an IP_3-gated Ca^{2+} channel in the plasma membrane (Fig. 4). There is no reason why the two second messenger pathways (IP_3 and cAMP) could not be stimulated in parallel in cilliary membranes. Exposure of catfish olfactory cilia to relatively large concentrations of odor stimuli does stimulate cAMP formation, and catfish olfactory cilia membranes have a cAMP-gated channel similar to that in toad cilia (*9, 10*). The relative importance of cAMP and IP_3 for the generation of the electrical responses to different odor stimuli at different concentrations is unknown. Although our results were obtained in catfish, a recent demonstration of rapid stimulation of PI turnover by odor stimuli that produce poor stimulation of adenylate cyclase in rat olfactory cilia (*12*) suggests that PI turnover may also play a mediatory role in olfactory transduction for some stimuli in mammals.

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