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Inositol 1,3,4,5-tetrakisphosphate-gated channels interact with inositol 1,4,5-trisphosphate-gated channels in olfactory receptor neurons

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ABSTRACT **Inositol 1,4,5-trisphosphate [InsP_3(1,4,5)] is a major second messenger regulating Ca^{2+} signaling in excitable and nonexcitable cells. InsP_3(1,4,5) is extensively metabolized through a network of phosphorylation and dephosphorylation steps to products with potential second messenger function. Inositol 1,3,4,5-tetrakisphosphate [InsP_4(1,3,4,5)], the direct metabolite of InsP_3(1,4,5), has also been associated with Ca^{2+} signaling, but whether InsP_4(1,3,4,5) acts in combination with InsP_3(1,4,5) or whether it regulates Ca^{2+} signaling directly and independently is unclear, particularly in neurons. We report that olfactory receptor neurons in the lobster (*Panulirus argus*) express an InsP_4(1,3,4,5) receptor in the plasma membrane that is a functional channel. The channel differs in conductance, kinetics, and voltage sensitivity from two plasma membrane InsP_3(1,4,5)-gated channels previously reported in these neurons. In close spatial proximity, the InsP_4(1,3,4,5)- and InsP_3(1,4,5)-gated channels interact reciprocally to alter the channels' open probabilities in what may be a novel mechanism for regulating Ca^{2+} entry in neurons.**

Stimulus-induced turnover of inositol phospholipid (IP) is a major intracellular signaling system that mediates the action of neurotransmitters, growth factors, and hormones (1). Odors also can induce IP turnover in olfactory receptor cells (2). In olfactory cilia, odors rapidly and transiently produce inositol 1,4,5-trisphosphate [InsP_3(1,4,5)] (3), which directly activates one or more plasma membrane InsP_3(1,4,5) receptors (4, 5). Since InsP_3(1,4,5) is extensively metabolized by cells, other IPs could be important intracellular signals in these neurons.

Isolation of receptors for inositol 1,3,4,5-tetrakisphosphate [InsP_4(1,3,4,5)] (6–12), as well as those for inositol hexakisphosphate (11, 12) and IP_xRec (13), suggests that phosphorylation products of InsP_3(1,4,5) may have signal function in other systems. InsP_4(1,3,4,5) has attracted particular attention as a putative second messenger associated with enhancing Ca^{2+} entry, sequestering or mobilizing Ca^{2+}, propagating Ca^{2+} waves, and altering membrane potential (1, 14). Still unclear is whether InsP_4(1,3,4,5) acts in combination with InsP_3 (15–19), or whether InsP_4 acts directly and independently as an intracellular signal (20–27). Recent evidence suggests that InsP_4(1,3,4,5) acts as a distinct intracellular signal by increasing the activity of an ion channel in endothelial cells (21), but whether InsP_4(1,3,4,5) or other metabolites of InsP_3(1,4,5) can directly gate ion channels in neurons is unknown.

To investigate the potential second messenger function of other inositol phospholipids in olfaction, we applied seven metabolites of the IP pathway to the inside face of cell-free patches of lobster olfactory receptor neurons (ORNs), a system in which plasma membrane InsP_3(1,4,5)-activated ion channels mediate excitatory transduction (5). We show that InsP_4(1,3,4,5) can directly and independently activate a different ion channel than those activated by InsP_3(1,4,5). At the same time, InsP_4(1,3,4,5)- and InsP_3(1,4,5)-activated channels can reciprocally interact to alter the channels' open probabilities (Pr_{open}) when they co-occur in the same patch of membrane. The InsP_4(1,3,4,5)-activated channels may be one arm of a dualistic, interactive mechanism by which IPs regulate signal detection in these neurons.

MATERIALS AND METHODS

Tissue Culture. Clusters of ORNs were dissected from the olfactory organs (lateral antennular filaments) of adult specimens of the Caribbean spiny lobster *Panulirus argus*, enzymatically dissociated, and sustained in primary culture as described (28).

Electrophysiology. The cultured cells were viewed at 40× magnification with Hoffman optics for patch-clamp recording (29). Patch electrodes, fabricated from 1.8-mm o.d. borosilicate glass and fire polished to a tip diameter of ≈1.0 μm (bubble number 4.8) (30), produced seal resistances between 8 and 14 GΩ. Cell-free patches were excised from the soma membrane into serum-free, low glucose L15 medium. Patches containing channels activated by voltage or stretch were discarded without further analysis. Forty-two patches were studied by exposing them randomly to (symmetrical) patch solution, InsP_4(1,3,4,5), InsP_3(1,4,5), and one or more other phospholipids. Data are included from 142 additional patches exposed only to InsP_3(1,4,5). All solutions were "spritzed" on the inner face of the cell-free patches from a seven-barrel glass micropipette (Frederick haer) coupled to a single pressurized valve system (Picospritzer, General Valve, Fairfield, NJ) (28). Activating the valve for 550 ms at 20 psi (1 psi = 6.89 kPa) produced spherical boluses of solution (control or test ligand) that persisted around the patch for ≈2 s. The valve was activated (550 ms, 20 psi) at 1 Hz to maintain the solution around the patch at equilibrium for the duration of a recording. All patches were voltage-clamped at a holding potential of −60 mV unless noted otherwise.

Unitary currents were recorded with an integrating patch-clamp amplifier [Dagan Instruments (Minneapolis) model 3900], filtered at 2 kHz, and captured on videotape. On playback, the analog records were sampled every 100 μs and stored on an IBM-compatible computer, using PCLAMP software (Axon Instruments, Burlingame, CA). Mean unitary

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Abbreviations: InsP_4(1,3,4,5), inositol 1,3,4,5-tetrakisphosphate; InsP_3(1,4,5), inositol 1,4,5-trisphosphate; ORN, olfactory receptor neuron; IP, inositol phospholipid; Pr_{open}, open probability(ies).

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current was calculated based upon point-by-point amplitude histograms fit with gaussian distributions using χ^2 convergence (Levenberg-Marquardt fitting algorithm, pStat, Axon Instruments). The peak of the gaussian distribution at each membrane potential was used to construct the current–voltage relation, the slope of which was used to generate the conductance of the channel. The Pr_{open} was defined as the total time a channel spent in the open state divided by the length of the recording. Integration of the area under the gaussian curve in the amplitude histogram was used to calculate the proportional time a channel spent in a given state. For patches containing more than one channel, the Pr_{open} was calculated as a mean value across all channels in the patch: $Pr_{open} = [0(P_0) + 1(P_1) + 2(P_2) + 3(P_3) + \ldots]/N$, where N is the number of channels, P_0 is the closed state probability, P_1 is the probability of one channel open, and P_2 is the probability of two channels open, etc.

All results are presented as the mean ± SEM. Statistical significance was calculated at the 95% confidence level.

Chemicals and Solutions. The patch solution consisted of (in mM) 30 NaCl, 11 EGTA, 10 Hepes, 1 $CaCl_2$, 180 potassium acetate, and 696 glucose (pH 7.0). *Panulirus* saline consisted of (in mM) 458 NaCl, 13.4 KCl, 9.8 $MgCl_2$, 13.6 $CaCl_2$, 13.6 Na_2SO_4, 3 Hepes, and 2 glucose (pH 7.4). Low glucose L15 medium consisted of 50 ml of Liebowitz L15 medium, 50 ml of 1.6× normal concentration of *Panulirus* saline, 0.6 g of dextrose, 0.026 g of L-glutamine, 0.01% gentamicin, and 1% basic minimal essential vitamins. IP solutions were made up in patch solution as concentrated stock solutions and stored frozen at −20°C until use. Single

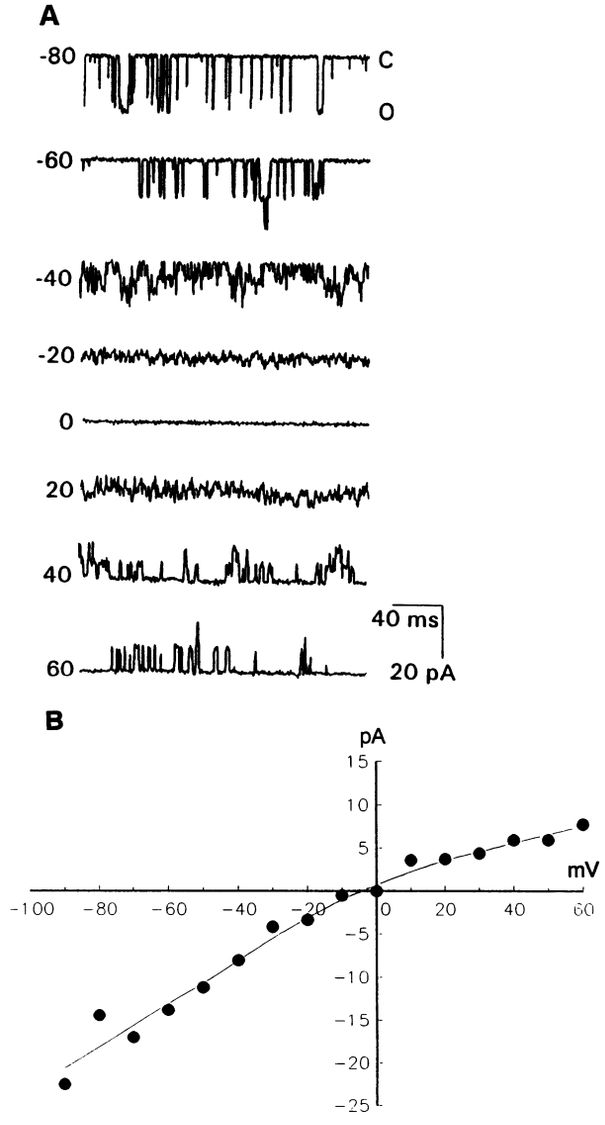

FIG. 1. (*A*) Unitary currents activated by applying 6.4 μM InsP_4(1,3,4,5) to the inside face of a cell-free patch from the soma of a cultured lobster ORN. The patch was voltage-clamped at various holding potentials. C, closed state; O, open channel. (*B*) Plot of the amplitude of the unitary currents shown in *A* as a function of holding potential. Each point represents the mean unitary current of at least 12 s of open channel events determined from a gaussian distribution fit of a point-by-point amplitude histogram at each holding potential. The data were fit by locally weighted regression (continuous line). The mean conductance, determined from the slope of a linear regression at negative holding potentials for 19 such experiments, was 193 ± 13.0 pS.

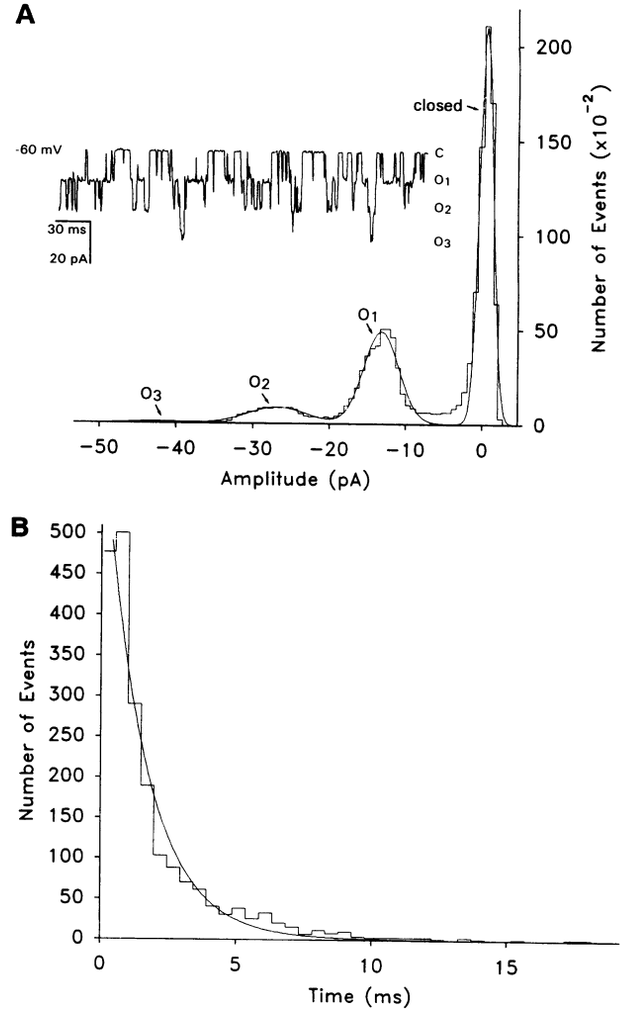

FIG. 2. (*A*) Histogram of the point-by-point amplitude of a digitized record containing three 193-pS channels activated by 6.4 μM InsP_4(1,3,4,5) in a cell-free patch from a lobster ORN; 36,432 open channel events were fit by gaussian distributions (continuous line). C, closed state; O_1, one open channel; O_2, two open channels; O_3, three open channels. The mean Pr_{open}, calculated from the area under the distributions, for 14 such ORNs at −60 mV was 0.11 ± 0.02. (*Inset*) Portion of the record used to generate the histogram. The density of the 193-pS channel was relatively high: patches containing three to nine channels were not uncommon. Given an average surface area of 380.13 μm^2 for the soma of cultured ORNs (28), the calculated average density of the 193-pS channel was 0.60–10.80 channels per μm^2. (*B*) Histogram of the open dwell time (t_o) of 2052 openings of a single 193-pS channel activated by 6.4 μM InsP_4(1,3,4,5) in a cell-free patch from a lobster ORN. The distribution was best fit by a single exponential (continuous line). Mean t_o for 11 such experiments was 5.03 ± 0.97 ms.

aliquots were thawed and diluted with patch solution to the stated working concentration. The working concentration of InsP_4(1,3,4,5) was fixed at 6.4 μM, slightly below that reported to evoke channel activity in nonexcitable cells (10–100 μm) (21). Greater concentrations frequently yielded high Pr_{open} of up to 10 channels per patch, while lower concentrations increased the probability of underestimating the true number of channels present and therefore of overestimating the Pr_{open}. IPs of the highest purity available were obtained from Sigma and Boehringer Mannheim. Salts were obtained from Sigma. Sources for the culture media and supplements have been cited previously (28).

RESULTS

InsP_4(1,3,4,5) (6.4 μM) activated a novel unitary current in 25 of the 42 patches studied (Fig. 1*A*). The average slope conductance of this channel was 193 ± 13.0 pS (n = 19) (Fig. 1*B*), three to six times that of two InsP_3(1,4,5)-activated channels (30 pS, 74 pS) in these neurons (5). The channel showed slight inward rectification at more positive membrane potentials. The average Pr_{open} for the 193-pS channel at −60 mV was 0.11 ± 0.02 (n = 14) (Fig. 2*A*), roughly one-half the mean Pr_{open} of InsP_3(1,4,5)-gated channels held at −60 mV and stimulated with micromolar InsP_3(1,4,5) (Pr_{open} = 0.26 ± 0.01; n = 50). The mean open time (t_o) for the channel was best fit by a single exponential, 5.03 ± 0.97 ms (n = 11) (Fig. 2*B*), indicating a single open state. The Pr_{open} of the channel was voltage dependent; the voltage function described a bell-shaped curve that decreased at hyperpolarizing and depolarizing extremes (Fig. 3*A*). These gating properties further distinguish the 193-pS channel from the two InsP_3(1,4,5)-gated channels, which have two open states and either decrease their Pr_{open} throughout the depolarizing range or are relatively voltage independent (Fig. 3*B*).

InsP_4(1,3,4,5) (6.4 μM) activated the 193-pS channel in 25 (86%) of 29 patches that contained an InsP_4(1,3,4,5)-activated channel. In the remaining 4 (14%) patches, InsP_4(1,3,4,5) activated unitary currents with slope conductances similar to those of the InsP_3(1,4,5)-activated channels (29.9 ± 4.8 pS, n = 3; 90.5 pS, n = 1). Like the InsP_3(1,4,5)-activated channels, these channels also demonstrated two mean open times (t_o) (21.27 ± 5.42 ms and 2.69 ± 0.44 ms). InsP_3(1,4,5) (0.24 μM) also activated the channels, but with a ~2-fold greater Pr_{open} than did InsP_4(1,3,4,5), indicating that InsP_4(1,3,4,5) was a less effective ligand (data not shown).

In contrast, InsP_3(1,4,5) (0.24 μM) only activated 30- and 74-pS channels in the 42 patches studied. In a larger sample, InsP_3(1,4,5) (0.24 μM) activated the 193-pS channel, but only in 6 (5%) of 110 patches that contained an InsP_3(1,4,5)-activated channel. Inositol hexakisphosphate (0.01 mM) activated the 193-pS channel in 1 of 13 patches. Inositol (n = 7, 1 mM), inositol 1-monophosphate (n = 8, 0.01 mM), inositol cyclic 1,2-monophosphate (n = 9, 0.01 mM), inositol 1,4-bisphosphate (n = 7, 0.1 mM), and inositol 1,3,4-trisphosphate (n = 9, 0.01 mM) failed to gate channel activity. The inactivity of InsP_3(1,3,4) excluded the possibility that the 193-pS channel was activated by membrane-associated 3-phosphatases generating InsP_3(1,3,4) from InsP_4(1,3,4,5) (31).

The lower and upper channel density of the InsP_4(1,3,4,5)-activated channel (Fig. 2*A*) approximated earlier density measurements of the InsP_3(1,4,5)-gated channels in these cells (0.9–5.3 channels per μm^2; ref. 32) and suggests that, consistent with our observation that 18 of the 42 patches (43%) contained both types of IP-activated channels, the estimated channel densities permit channel colocalization. When they occurred in the same patch of membrane, the InsP_4(1,3,4,5)- and InsP_3(1,4,5)-gated channels interacted. Interaction altered the Pr_{open} but not the conductance of the channels (Fig. 4*A*), suggesting that interaction altered the gating mechanism without interfering with the pore region. In patches containing both types of channels, the Pr_{open} of the InsP_4(1,3,4,5)-activated channel significantly increased when both ligands were copresented, compared to that with InsP_4(1,3,4,5) stimulation alone (n = 16, paired t test, Fig. 4*B*). Interaction required that the InsP_3(1,4,5)-activated channel be not only present, but gated, since the Pr_{open} of the InsP_4(1,3,4,5)-activated channel was not significantly different in patches with and without an unstimulated InsP_3(1,4,5)-gated channel (n = 8, Student's t test, Fig. 4*B*). The InsP_4(1,3,4,5)-activated channel, in turn, decreased the Pr_{open} of both InsP_3(1,4,5)-activated channels. This interaction only required that the InsP_4(1,3,4,5)-activated channel be present, not gated. Copresentation of both ligands had no effect on the Pr_{open} of the InsP_3(1,4,5)-activated channels (n = 10, paired t test, Fig. 4*B*), but the Pr_{open} of the InsP_3(1,4,5)-activated channel was significantly increased in patches that only contained InsP_3(1,4,5)-activated channels, compared to patches that contained both types of IP-activated channels (n = 6, Student's t test, Fig. 4*B*).

DISCUSSION

That InsP_4(1,3,4,5) was able to initiate and sustain unitary currents without observable rundown in cell-free patches of plasma membrane implies that InsP_4(1,3,4,5) directly gated the channels it activated. The gating properties of the channel activated by InsP_4(1,3,4,5) differed from those described

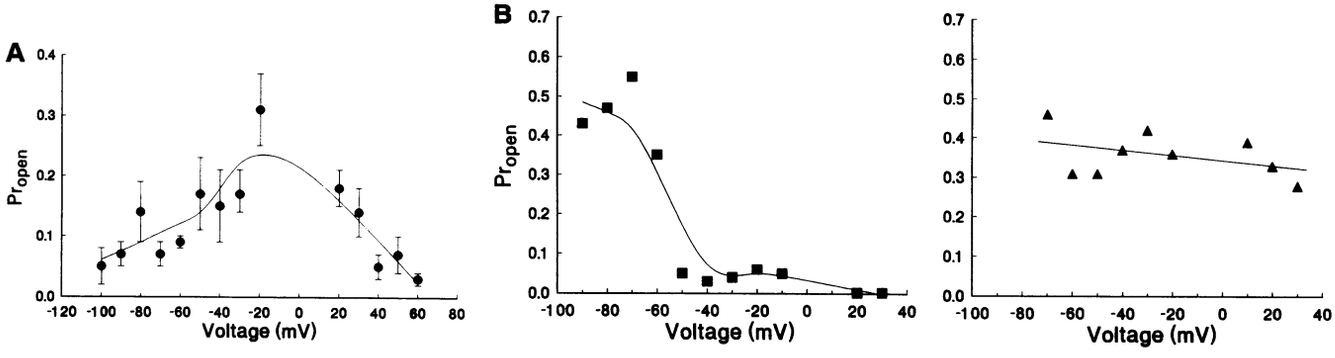

FIG. 3. (*A*) Plot of the Pr_{open} of the 193-pS channel activated by InsP_4(1,3,4,5) in cell-free patches from lobster ORNs as a function of holding potential. The data were fit by a locally weighted regression (continuous line). Values at and bordering the reversal potential (−10 to +10 mV) could not provide a meaningful Pr_{open} calculation and were excluded from the fit. Each point represents the mean ± SEM for five experiments. (*B*) Plot of the Pr_{open} of an InsP_3(1,4,5)-gated 30-pS (left) and 74-pS (right) channel from lobster ORNs as a function of holding potential. The data were fit by a locally weighted or linear regression (continuous lines). Data are representative of five such experiments for each type of channel.

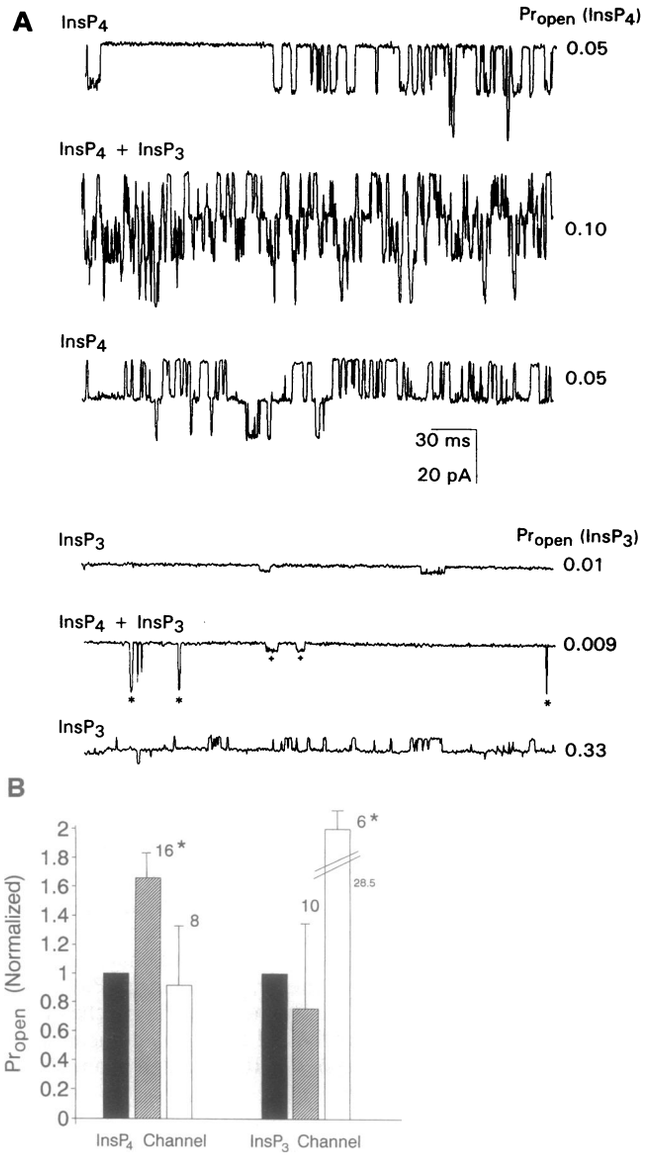

FIG. 4. (*A*) Representative records of unitary currents activated by applying 6.4 μM InsP_4(1,3,4,5) and 0.24 μM InsP_3(1,4,5) to the inner face of cell-free patches of lobster ORNs. InsP_4(1,3,4,5) (trace 1) and InsP_3(1,4,5) (trace 4) activated the 193-pS and the 74-pS channel, respectively, in the same patch. Presenting both ligands simultaneously to the same patch as in traces 1 and 4 increased the Pr_{open} of the InsP_4(1,3,4,5)-activated channel (trace 2 vs. trace 1), but not the InsP_3(1,4,5)-activated channel (trace 5 vs. trace 4). Trace 5 was selected to allow the InsP_3(1,4,5)-activated channel (+) to be distinguished from the InsP_4(1,3,4,5)-activated channel (*). In this and all records, thousands of channel transitions were fit to determine the mean Pr_{open}. The Pr_{open} of the InsP_4(1,3,4,5)-activated channel in a patch that contained only this type of channel (trace 3) did not differ from that when both types of channels were present (trace 1). The Pr_{open} of the InsP_3(1,4,5)-activated channel, however, was greater in the absence of an InsP_4-activated channel (trace 6 vs. trace 4). Pr_{open}, C, O_1, and O_2 as in Fig. 2. (*B*) Bar graph comparing the normalized Pr_{open} for all channels tested as in *A*. Solid bar, Pr_{open} for that channel when both channels were contained in the same patch in response to its own ligand. Striped bar, Pr_{open} for that channel when then copresented with both ligands. *, Significant difference, $P \leq 0.05$; arcsin transformation of percentage data followed by paired *t* test. Open bar, Pr_{open} for that channel activated by its own ligand in patches known to contain only that type of channel. *, Significant difference, $P \leq 0.05$; arcsin transformation of percentage data followed by Student's *t* test.

earlier for channels gated by InsP_3(1,4,5) in these cells (5). The slope conductance of the channel gated by InsP_4(1,3,4,5) (193 pS) is 3- to 6-fold greater than that reported for the InsP_3(1,4,5)-gated channels (30, 74 pS), suggesting notably different permeability. The channel gated by InsP_4(1,3,4,5) slightly rectifies at more positive potentials. Such a conductance change occurs in InsP_3(1,4,5)-gated channels, but only in the presence of high (10–30 mM) internal Ca^{2+}, and, then, does so more typically across the entire voltage range (33). The bell-shaped voltage dependence of the 193-pS channel contrasts with the voltage independence (74-pS channel) and linearly decreasing voltage sensitivity with depolarization (30-pS channel) of the InsP_3(1,4,5)-gated channels, suggesting a differential effect of charge. Both the open and closed dwell times of the 193-pS channel could be best fit by a single exponential, implying a simple $C \underset{\beta}{\overset{\alpha}{\rightleftarrows}} O$ Markov process regulating channel state transitions vs. the more complex, two-open and two-closed states of each of the InsP_3(1,4,5)-gated channels. We assume, therefore, that InsP_4(1,3,4,5) activates a distinct plasma membrane channel rather than modifying the properties of either or both of the InsP_3(1,4,5)-gated channels.

This contention is supported by the selectivity of the two types of channels for their respective ligands. As the solutions tested free of contaminating IP metabolites, we presume that the relatively low incidence with which InsP_3(1,4,5) activated the 193-pS channel and InsP_4(1,3,4,5) activated the 30- and 74-pS channels reflects low-affinity cross-receptor binding. Other inositol metabolites bind InsP_4(1,3,4,5) and InsP_3(1,4,5) receptors in mammalian brain only at high concentrations and with low affinity (8, 11, 12, 34). InsP_4(1,3,4,5)-sensitive channels in endothelial cells (21) are also specific to their ligand; InsP_3(1,4,5) does not gate or potentiate these channels.

Although the InsP_3(1,4,5)- and InsP_4(1,3,4,5)-gated channels in lobster ORNs are selective for their respective ligands and can act independently, the channels can interact when in spatial proximity. As noted in the Introduction, InsP_4(1,3,4,5) and InsP_3(1,4,5) receptors interact in nonexcitable cells, where several divergent, proposed mechanisms of interaction recently have been integrated into a unified model (31, 35). In this model, the intracellular (endoplasmic reticulum) InsP_3 receptor is locked into an inactive conformation when its InsP_3 binding site is bound by a plasma membrane InsP_4 receptor; InsP_4 binding dissociates the coupled receptors to allow Ca^{2+} entry and free access of InsP_3 to its receptor. This model does not superficially generalize to our system, where both types of IP-gated channels are plasma membrane bound, and our data suggest further, mechanistic differences. As in the model, activation of an InsP_4 channel in lobster ORNs alters the activity of an InsP_3 channel, suggesting that receptors in suitably close proximity in the same membrane might also block cytoplasmic binding sites. However, InsP_4(1,3,4,5)-activated channels *decreased*, not increased, the InsP_3(1,4,5) open channel activity. Interestingly, just spatial proximity of the unactivated InsP_4 channel appears sufficient to down-regulate InsP_3(1,4,5)-gated activity. Across a large population (n = 352) of lobster InsP_3(1,4,5)-gated channels held near normal resting membrane potentials (−60 to −80 mV), 37% had an Pr_{open} of <0.05 (unpublished results), as might be predicted if some patches contained an unstimulated, but interactive, InsP_4(1,3,4,5)-gated channel. In lobster ORNs, InsP_3(1,4,5)-gated channels can be stimulated both in the absence of InsP_4(1,3,4,5) and in patches that do not contain InsP_4(1,3,4,5)-gated channels, whereas in the model, InsP_4(1,3,4,5) is necessary for activation of InsP_3 receptors. Finally, it is InsP_3(1,4,5) that potentiates the activity of InsP_4(1,3,4,5)-gated channels in lobster ORNs, not vice versa, and does so only in patches containing both types of IP channels—i.e., only through interaction. Thus, interaction of these neuronal plasma membrane InsP_3- and InsP_4-gated

channels appears to differ from that proposed for the interaction of IP receptors in nonexcitable cells.

The InsP_4(1,3,4,5)-gated channel potentially functions in olfactory transduction, even though it was characterized in the soma of the cells. Cultured lobster ORNs express other elements of the transduction cascade (5, 28, 29, 33, 36) in the soma. All three types of IP-gated channels occur in the outer dendrite, the site of signal transduction in the mature cell (37). Odors rapidly and transiently elevate InsP_3(1,4,5) (38), the metabolic precursor of InsP_4(1,3,4,5), in the outer dendrite. InsP_3(1,4,5) would be expected to increase Ca^{2+} influx through the InsP_3(1,4,5)-activated channels (35) and potentially activate a Ca-dependent 3-kinase to produce InsP_4(1,3,4,5). Although the role of InsP_4(1,3,4,5) as an intracellular signal in olfactory receptor neurons remains to be determined, the InsP_4(1,3,4,5)-gated channel, through its ability to interact with the InsP_3(1,4,5)-gated channels, could enhance detection of the odor signal by accentuating the onset of the receptor current. The interaction of the two IP channels could additionally or alternately be responsible for termination of the odor signal since InsP_4(1,3,4,5) decreases the total current flow through InsP_3(1,4,5)-gated channels by decreasing channel Pr_{open}. The InsP_4(1,3,4,5)-gated channel itself would independently inactivate at depolarized potentials given its voltage-dependent properties.

If, as in other systems, the InsP_4(1,3,4,5)-gated channel in lobster ORNs is calcium permeable, as preliminary experiments suggest ($n = 2$, data not shown), then at least two types of IP channels could mediate Ca^{2+} entry. Dual receptors mediating Ca^{2+} flux have been implicated in controlling fertilization (39, 40) and two Ca^{2+} releasing channels have been colocalized immunocytochemically in brain (41). Having more than one Ca^{2+}-mobilizing channel may be a fundamental property of neurons, if not all cells, and interaction among the channels may provide fine control of neuronal output.

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