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Modulation of the Olfactory CNG Channel by Ptdlns(3,4,5)P_3

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Abstract. Recent data suggest that the 3-phosphoinositides can modulate cyclic nucleotide signaling in rat olfactory receptor neurons (ORNs). Given the ability of diverse lipids to modulate ion channels, we asked whether phosphatidylinositol 3,4,5-trisphosphate (PIP_3) can regulate the olfactory cyclic nucleotide-gated (CNG) channel as a possible mechanism for this modulation. We show that applying PIP_3 to the intracellular side of inside-out patches from rat ORNs inhibits activation of the olfactory CNG channel by cAMP. The effect of PIP_3 is immediate and partially reversible, and reflects an increase in the EC_{50} of cAMP, not a reduction in the single-channel current amplitude. The effect of PIP_3 is significantly stronger than that of PIP_2; other phospholipids tested have no appreciable effect on channel activity. PIP_3 similarly inhibits the recombinant heteromeric (A2/A4) and homomeric (A2) olfactory CNG channel expressed in HEK293 cells, suggesting that PIP_3 acts directly on the channel. These findings indicate that 3-phosphoinositides can be functionally important regulators of CNG channels.

Key words: Chemoreceptor — Cyclic nucleotide-gated channels — Phosphoinositides — Olfactory — Patch clamp — Sensory neurons

Introduction

Cyclic nucleotide-gated (CNG) ion channels are important effectors in diverse cellular systems, especially sensory systems (for a review *see* Kaupp & Seifert, 2002). In olfactory receptor neurons (ORNs), for example, ligand-bound olfactory receptors (Buck & Axel, 1991) activate a type-III adenylyl cyclase (Pace et al., 1985; Bakalyar & Reed, 1990) via the olfactory-specific G protein, G_{olf} (Jones & Reed, 1989). The resulting rise in intracellular cAMP generates a depolarizing inward current by activating a nonselective cyclic nucleotide-gated cation channel (Nakamura & Gold, 1987; Kurahashi, 1990; review: Zufall, Firestein & Shepherd, 1994) composed of three subunits (CNGA2, CNGA4, CNGB1b).

Like a number of types of ion channels, CNG channels have been reported in recent years to be regulated by the canonical membrane lipids phosphatidylinositol 4,5-bisphosphate [Ptdlns(4,5)P2, or PIP_2], DAG and retinal (Hilgemann, Feng & Nasuhoglu, 2001). Another class of membrane lipid, 3-phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3, or PIP_3], are also key second messengers in diverse cellular processes, including cell proliferation, membrane trafficking, and apoptosis (Cantley, 2002). The recent finding that phosphatidylinositol 3-kinase (PI3K)-dependent signaling can modulate the activation of rat ORNs by odors in a cyclic nucleotide-dependent manner (Spehr et al., 2002), suggests that 3-phosphoinositides and especially PIP_3, the main product of PI3-K activity in vivo (Vanhaesebroeck & Waterfield, 1999), may also be functionally important lipid regulators of CNG channels.

Here we report that exogeneous PIP_3 strongly inhibits the native CNG channel in rat ORNs, and does so to a significantly greater extent than previously reported effects of phospholipids on CNG channels. We also report that PIP_3 has a similar inhibitory effect on recombinant olfactory CNG channels expressed in HEK cells, suggesting that the lipid acts directly on the channel. Together with previously reported evidence for odor-stimulated PI3K in rat ORNs, these findings argue that 3-phosphoinositides are functionally important regulators of CNG channels and provide a potential

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cellular mechanism by which PI3K-dependent signaling can modulate the activation of mammalian ORNs by odors.

Materials and Methods

CNG channels were recorded in acutely dissociated ORNs and human embryonic kidney (HEK293) cells expressing recombinant homo- (CNGA2) or heteromeric (CNGA2/A4) CNG channels.

Acutely Dissociated Cells

ORNs were obtained from the nasal epithelium of Sprague Dawley rats (Harlan, Indianapolis, IN) anesthetized with CO_2 and sacrificed by decapitation. The septal bone with the olfactory epithelium was dissected from the head, the epithelium was immediately separated from the septal bone, cut into small pieces in mammalian Ringer's solution (RS, *see* Solutions) and transferred to divalent cation-free dissociation solution (DS, *see* Solutions) supplemented with papain (EC 3.4.22.2 from papaya latex, Sigma) at room temperature for 15 min without agitation. The tissue pieces were then transferred to normal RS and gently triturated with a fire-polished Pasteur pipette. Dissociated cells were plated on glass coverslips and stored in normal RS at 4°C until used within 4–6 hours. The coverslips with the cells were placed into an RS-filled culture dish (35 mm in diameter) mounted on the stage of an inverted microscope (Axiovert 200, Carl Zeiss) and viewed with phase-contrast optics at 320 × magnification. ORNs were identified by their morphology as described by Farbman (1992).

Cell Culture and Transfection

HEK293 cells were grown at 37°C in MEM medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum under a 5% CO_2 atmosphere. The cDNA clones of the rat olfactory CNG channel subunits CNGA2 and CNGA4, both in pCIS vectors (Dhallan et al., 1990; Wang & Reed, 1993), were kindly provided by Dr. R.R. Reed and Dr. J. Bradley (Johns Hopkins School of Medicine). Semiconfluent cells were transiently transfected in 35-mm dishes (Falcon) using the CaP-precipation technique and the plasmids pCNGA2 (7 μg), pCNGA4 (7 μg), and pGFP (1 μg) as described previously (Wetzel et al., 2001). Co-transfected cells were identified by GFP fluorescence 48 h post-transfection and selected for electrophysiological recording.

Recording from Acutely Dissociated ORNs

Inside-out patches were excised from the dendritic knob of acutely dissociated rat ORNs. Electrical recordings were made using the patch-clamp technique as described previously (Zhainazarov & Ache, 1999). Briefly, patch pipettes were pulled from borosilicate glass tubes (BF150-86-10; Sutter Instruments, Novato, CA) and fire-polished to a tip diameter less than 1 μm. The pipettes, when filled with Ca^{2+}-free pipette solution (*see* Solutions), had resistances of 6–10 MΩ and formed seals with resistances of 10–12 GΩ. A rotary perfusion system (RSC-100; Biologic, Claix, France) was used to perfuse isolated membrane patches with up to nine different solutions. After forming the patch in RS, the pipette was immediately placed into Ca^{2+}-free pipette solution that continuously flowed from one of nine tubes (100 μm inner diameter) and that completely engulfed the membrane patch. Switching between immediately adjacent tubes took less than 10 ms. Single-channel currents were recorded with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz (–3 dB; four-pole Bessel filter), digitized at 10 kHz (Interface, Digidata 1322A; software, pClamp 8; Axon Instruments), and stored on a computer hard disk for later analysis. The recordings were referenced to an Ag-AgCl wire electrode connected to the bath solution through a 3 M KCl-agar bridge. All recordings were made at room temperature (20–22°C).

Recording from HEK293 Cells

Integral currents were recorded in inside-out patches taken from the HEK293 cells using an EPC7 amplifier (List, Darmstadt, Germany) and PULSE software (HEKA, Lambrecht, Germany) running on a Macintosh Quadra 700 computer. Patch electrodes were pulled from borosilicate glass (Harvard Apparatus, Edenbridge, Kent, UK) using a horizontal pipette puller (Zeitz-Instruments, Augsburg, Germany) to pipettes with a resistance of 3–6 MΩ. Patch pipettes were filled with a Ca^{2+}-free solution. A theta-tubing delivery system (Wetzel et al., 2001) was used to expose excised patches to either cAMP-free or cAMP-containing Ca^{2+}-free solution.

Data Analysis

Single-channel current events were analyzed using pClamp 8 software. Patches typically contained more than one channel, so the open probability of a channel was determined using the equation $P_o = <I>/(Ni)$, where $<I>$ is the mean current over the interval of interest, N is the number of channels in the patch, and i is the single-channel current amplitude. When the number of channels in a patch was difficult to determine reliably, NP_o was used as a measure of the channel activity.

Solutions

RS consisted of (mM): 137 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.6 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4 adjusted with 1 M NaOH. DS contained (mM): 137 NaCl, 5 KCl, 2 EDTA, 10 glucose, and 10 HEPES, pH 7.4 adjusted with 1 M NaOH. Ca^{2+}-free patch pipette solution (native cells) consisted of (in mM): 137 NaCl, 1 $CaCl_2$, 10 EGTA, and 10 HEPES, pH 7.4 adjusted with 1 M Tris. Bath solution for the HEK cells during recording contained (mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.3). Ca^{2+}-free patch pipette solution (HEK cells) consisted of (mM): 120 NaCl, 25 NaOH, 10 EGTA, 10 HEPES (pH 7.4). PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (Matreya, State College, PA) stock solutions (1 mM) were prepared by dispersing the phosphoinositides in water with 30 min sonication on ice, aliquoted, and stored at −20°C until use within 3 days. Stock solutions were diluted to working solutions of the desired concentration and sonicated for an additional 30 min on ice just before use. Monoclonal antibodies against PtdIns(4,5)P_2 were purchased from Assay Designs (Ann Arbor, MI) and diluted 200-fold into the working solution as described below. All inorganic salts were obtained from Fisher Scientific (Houston, TX). All other chemicals were obtained from Sigma (St. Louis, MO).

Results

Exogenous PIP_3 Lowers the cAMP Sensitivity of the Native Olfactory CNG Channel

Single-channel recordings from inside-out patches excised from the dendritic knob of acutely dissociated

rat ORNs were used to examine the possible effect of PIP_3 on the olfactory CNG channel (Fig. 1*A*). PIP_3 (8 μM) in the presence of 8-Br-cAMP (1 μM), a hydrolysis-resistant cAMP analog, strongly inhibited CNG channel activity ($n = 3$) over 20–40 sec ($n = 3$, Fig. 1*B*). The CNG channel remained inhibited immediately after washout of PIP_3, but the effect partially reversed on prolonged (30–45 min) washout. Patches typically contained more than one CNG channel such that the number of channels in the patch was difficult to determine reliably, so we used NP_o as a measure of the channel activity. PIP_3 (4 μM) did not alter the unitary current amplitude of the CNG channel, which was −0.8 ± 0.1 pA (mean ± SEM; $n = 26$) and −0.8 ± 0.1 pA ($n = 12$) at −40 mV in the presence and absence of PIP_3, respectively (Fig. 2*A–C*). PIP_3 (4 μM) did, however, shift the concentration-activity relationship of the CNG channel to the right along the concentration axis relative to the control from $K_{1/2} = 2.3 \pm 0.1$ μM ($n = 4$–5) to $K_{1/2} = 25.3 \pm 6.1$ μM ($n = 6$–8), respectively (Fig. 2*D*). In presence of PIP_3 (4 μM), the channel activity was measured within at least 1 min after the start of the phosphoinositide application. We applied various concentrations of cAMP in both ascending and descending orders. We did not observe any significant difference in the concentration-activity relationships for these two modes of ligand application.

Exogenous PIP_2 Had a Significantly Weaker Effect on the cAMP Sensitivity of the Native Olfactory CNG Channel than Did PIP_3

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2 or PIP_2] (4 μM), another membrane phosphoinositide (Czech, 2003) that has emerged as a potent regulator of many ion channels and transporters, including the mammalian rod CNG channel (Hilgemann et al., 2001), had a significantly weaker effect on the olfactory channel than did PIP_3. PIP_2 typically took 3–4 min to fully inhibit the channel (Fig. 3*A*), and increased the half-effect concentration of cAMP only to 11.2 ± 0.7 μM ($n = 3$–11) (Fig. 3*B*). A monoclonal anti-PIP_2 antibody (Fukami et al., 1988) (1:200 dilution) had no substantial effect on the channel ($n = 3$), suggesting that constitutive PIP_2 does not have a strong regulatory effect on CNG channel activity in rat ORNs (Fig. 3*B*). The same anti-PIP_2 antibody (1:200 dilution) was active in blocking the effect of PIP_2 on Na^+-gated nonselective cation channels in lobster ORNs (Zhainazarov & Ache, 1999). Other membrane phospholipids, including phosphatidylinositol, phosphatidylcholine, phosphatidylserine, and phosphatylethanolamine (all 8 μM) had no appreciable effect on channel activity ($n = 3$ in each instance, *data not shown*). Thus, exogenous PIP_3, and to a lesser extent PIP_2, decrease the apparent affinity of the CNG channel for cAMP in rat ORNs.

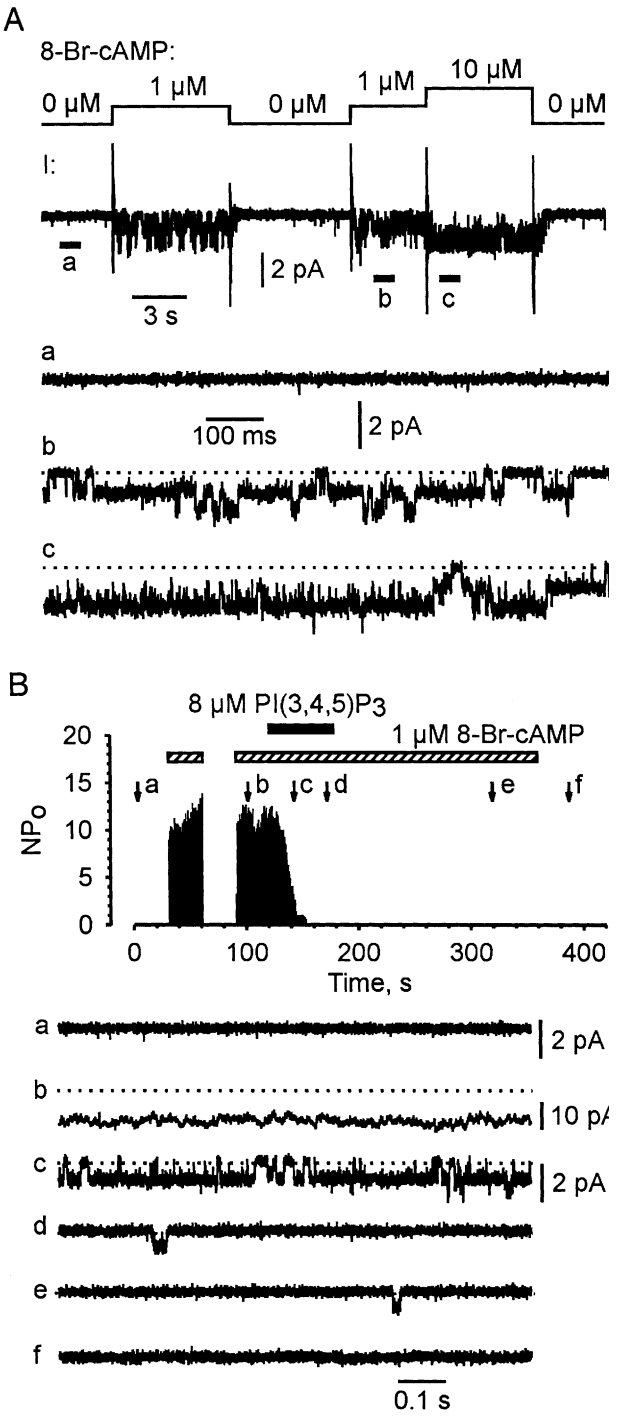

Fig. 1. PtdIns(3,4,5)P_3 (PIP_3) inhibits cyclic nucleotide-gated (CNG) channel activity in rat olfactory receptor neurons (ORNs). (*A*) Single-channel currents (second trace from top activated by 8-Br-cAMP (1–10 μM) applied to the intracellular face of an inside-out patch excised from the dendritic knob. Top trace, time course of 8-Br-cAMP application. Bottom traces (*a*, *b*, and *c*), fragments taken where noted from the primary record (second trace) and shown on an expanded time scale. (*B*) Plot of the channel open probability as a function of time (*top*) during treatment with 8 μM PIP_3 (*black bar*). *Dashed bar*, duration of 8-Br-cAMP (1 μM) application. *Bottom*, fragments of single-channel current traces taken at time points indicated by the arrows (*a–f*). Membrane potential, −40 mV.

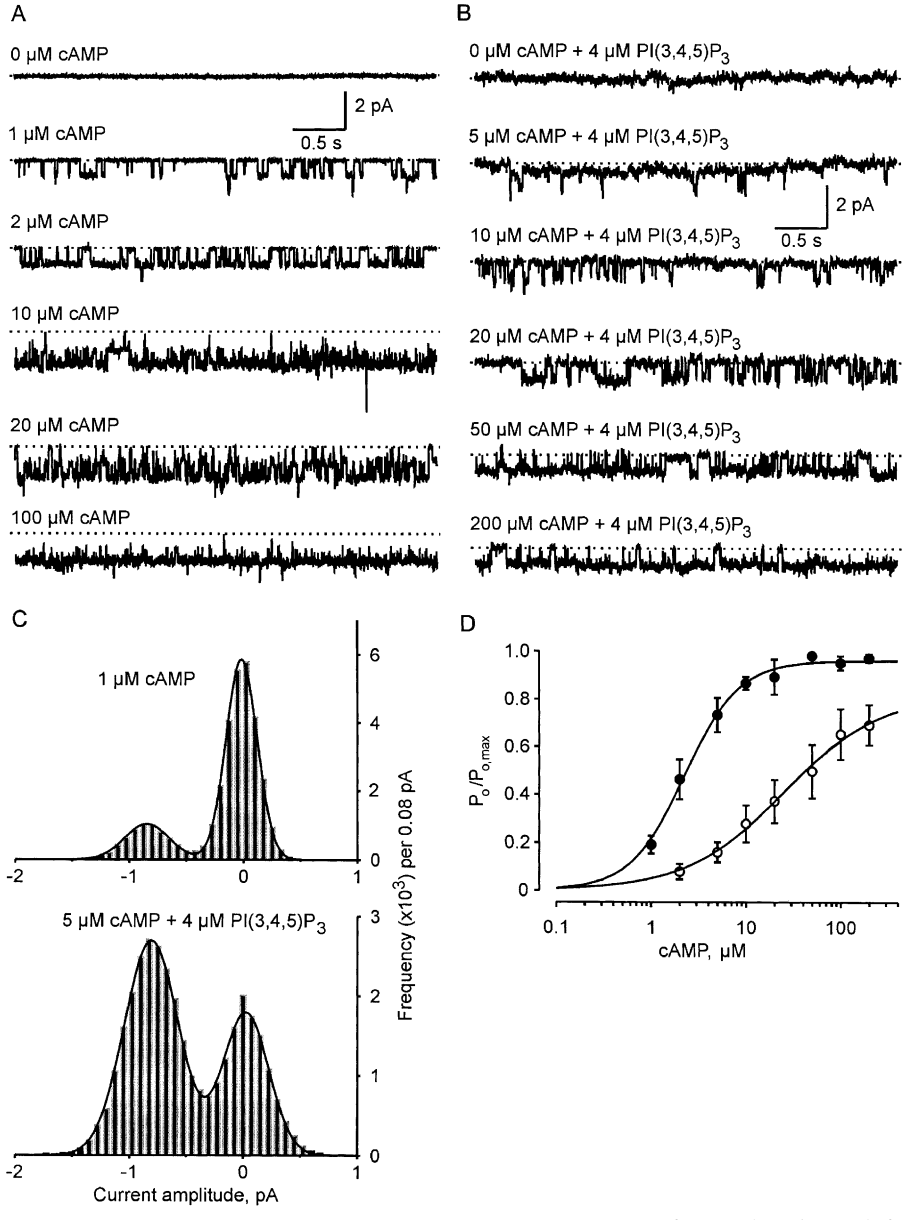

Fig. 2. PIP_3 right-shifts the concentration-response relationship of the olfactory CNG channel with no effect on the single-channel current amplitude. Representative recordings of single-channel activity evoked by the concentrations of cAMP indicated before (*A*) and during (*B*) exposure to 4 μM PIP_3. Traces in *A* and *B* were obtained from two different cells. (*C*) All-points amplitude histograms of single-channel current events evoked by 1 μM cAMP (*top*) and 5 μM cAMP plus 4 μM PIP_3 (*bottom*). The amplitude histograms were calculated from short (1 s) stretches of 1-min long recordings selected in order to maximize a number of channel openings. Solid lines, best fit of the sum of two Gaussian functions with mean values of −0.8 pA and 0.0 pA for both *A* and *B*. (*D*) Concentration-response relationship in the absence of (*filled circles*) and presence of 4 μM PIP_3 (*empty circles*). Open probabilities (P_o) were normalized to the open probability at a 200 μM concentration of cAMP before exposure to PIP_3. The continuous lines are the best fits of the Hill equation ($P_o/P_{o,max} = A{\cdot}[cAMP]^{\beta}/([cAMP]^{\beta} + K_{1/2}{}^{\beta})$) to the data with the following parameters: $K_{1/2}$ (in μM), β, *A*: 2.3 ± 0.1, 1.5 ± 0.1, 0.96 ± 0.01 (*filled circles*; n = 4–5), and 25.3 ± 6.1, 0.8 ± 0.1, 0.82 ± 0.06 (*empty circles*; n = 6–8). Membrane potential, −40 mV.

Exogenous PIP_3 also Lowers the cAMP Sensitivity of Recombinant Olfactory CNG Channels

As with the native olfactory CNG channel, PIP_3 (5–10 μM) also inhibited both the recombinant heteromeric (A2/A4 subunits) and homomeric (A2 subunits) olfactory CNG channels (Kaupp & Seifert, 2002) heterologously expressed in HEK293 cells. PIP_3 (10 μM) shifted the concentration-activity relationship of the recombinant channel to the right along the concentration axis from $K_{1/2}$ = 6.8 ± 0.3 μM (n = 8) to 35.7 ± 8.0 μM (n = 4–11) (Fig. 4*A*) for the heteromeric channel, and from $K_{1/2}$ = 30.6 ± 0.1 μM (n = 5–9) to 177.9 ± 14.7 μM (n = 3–25) (Fig. 4*B*)

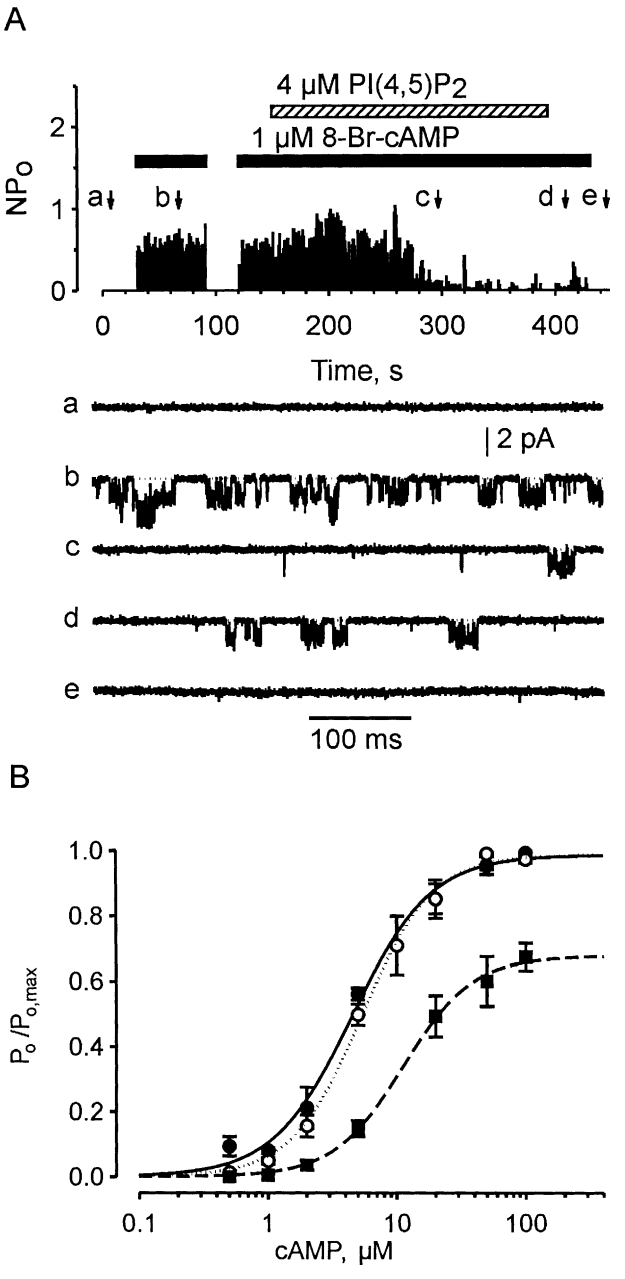

Fig. 3. PIP_2 has a smaller effect than PIP_3 on the olfactory CNG channel (*A* and *B*). (*A*) Plot of the channel open probability as a function of time (*top*) during treatment with 4 μM PIP_2 (*hatched bar*). *Solid bars*, periods of 8-Br-cAMP (1 μM) application. Bottom, fragments of single-channel current traces taken at time points indicated by the arrows (*a–e*). (*B*) Concentration-response relationship in the absence (*filled circles*) and presence of either 4 μM PIP_2 (*filled squares*) or monoclonal anti-PIP_2 antibody (1:200 dilution; *empty circles*). Open probabilities (P_o) were normalized to the open probability at a 200 μM concentration of cAMP before exposure to PIP_2. The lines are the best fits of the Hill equation to the data with the following parameters: $K_{1/2}$ (in μM), β, *A*: 4.5 ± 0.5, 1.1 ± 0.2, 0.99 ± 0.03 (*filled circles*; n = 3), 5.3 ± 0.3, 1.6 ± 0.1, 0.99 ± 0.02 (*empty circles*; n = 3), and 11.2 ± 0.7, 1.6 ± 0.1, 0.68 ± 0.02 (*filled squares*; n = 3–11). Membrane potential in *A* and *B*, −60 mV.

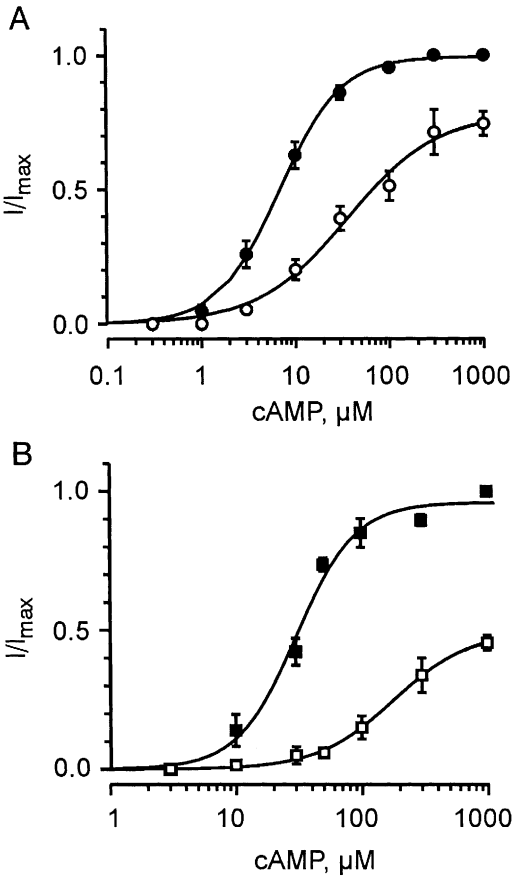

Fig. 4. PIP_3 right-shifts the concentration-response relationship for cAMP of the heterologously expressed recombinant olfactory CNG channel. Effect of 10 μM PIP_3 (*empty symbols*) compared with control (*filled symbols*) on the cAMP dependence of the integral current through heteromeric A2/A4 (*A*) and homomeric A2 (*B*) CNG channels in inside-out patches excised from HEK293 cells. Currents (*I*) were normalized to the maximal current (I_{max}) at a saturating concentration of cAMP (1 mM) before exposure to PIP_3. Membrane potential, −50 mV. Continuous lines are the best fits of the Hill equation to the data with the parameters $K_{1/2}$ (in μM) and β: 6.8 ± 0.3, 1.3 ± 0.1 (*filled circles*; n = 8), 35.7 ± 8.0, 0.9 ± 0.1 (*empty circles*; n = 4–11), 30.6 ± 0.1, 1.8 ± 0.3 (*filled squares*; n = 5–9), and 177.9 ± 14.7, 1.4 ± 0.1 (*empty squares*; n = 3–25).

for the homomeric channel. PIP_3 also decreased the maximum integral current through the channel in the patch (Fig. 4). PIP_2 (10 μM) had no measurable effect on the homo- or heteromeric CNG channel activated by 3 μM (n = 4), 10 μM (n = 11), or 1 mM (n = 7) cAMP (*data not shown*). The effect of PIP_3 on the recombinant CNG channel suggests that PIP_3 acts directly on the channel itself.

Discussion

We show that PIP_3 inhibits activation of the olfactory CNG channel by downregulating the sensitivity of the channel to cAMP without altering the single-

channel current amplitude. The ability of micromolar PIP_3 to shift the sensitivity to cAMP approximately an order of magnitude (EC_{50}, 2.3 to 25.3 μM) argues that PIP_3 is a potent regulator of channel activity. While phosphoinositides, in particular PIP_2, have been previously reported to inhibit CNG channels (Hilgemann et al., 2001), this is the first report that 3-phosphoinositides can regulate these channels. Our finding that micromolar PIP_3 inhibited the channel significantly more than did micromolar PIP_2 (EC_{50}, to 25.3 vs to 11.2 μM) argues that 3-phosphoinositides may be especially potent phospholipid regulators of this channel. The inability of the other phospholipids tested to shift the sensitivity of the channel to cAMP argues that the ability of phosphoinositides to regulate the channel is specific.

The similar relative shift in the sensitivity of the recombinant olfactory CNG channel to that seen in the native olfactory CNG channel to cAMP suggests that the CNG channel is the direct target of PIP_3's action and is not mediated through one or more *specific* intermediary proteins. Since the relative shift in the efficacy of cAMP to activate the channel is roughly the same for both the homo- and heteromeric CNG channels, the A2 subunit appears to be the primary target of PIP_3's action.

One obvious difference in the effect of PIP_2 on the native and recombinant channels allows the suggestion that phospholipids may act in part through one or more *ubiquitous* intermediary proteins. Nor did PIP_2 have any substantial effect on the recombinant CNG channel either here or when the recombinant olfactory CNG channel was expressed in oocytes (Womack et al., 2000). This functional difference may potentially reflect differences in the specific protein-lipid environment in which the CNG channel functions in rat ORNs versus that in HEK293 cells. As with other types of channels (Tillman & Cascio, 2003), CNG channels target to lipid rafts, disruption of which dramatically alters the function of the A2 homomer (Brady et al., 2004). The functional differences could also reflect differences in the subunit composition of the native and recombinant CNG channels since it is generally assumed that a third subunit (CNGB1b) comprises the native olfactory CNG channel (Kaupp & Seifert, 2002). Further experiments will be required to resolve these alternatives.

The nature of the effect of PIP_3 on the channel is consistent with the finding that blocking PI3K in rat ORNs increases the magnitude of the odor-evoked intracellular calcium signal in some cells in response to stimulation with a complex odor mixture (Spehr et al., 2002). This suggests that the regulation of the olfactory CNG channel by PIP_3 we observed in cell-free patches is functionally important. The intracellular calcium signal reflects cyclic nucleotide-dependent activation of the cell (Zufall, Leinders-Zufall & Greer, 2000), so the increased calcium signal on blocking PI3K presumably reflects release from PIP_3-mediated inhibition induced by one or more components of the odor mixture. PIP_3's ability to downregulate the sensitivity of the olfactory CNG channel to cAMP would effectively inhibit the output of the cell and could explain how activation of PI3K inhibits the odor-evoked output of rat ORNs in vivo. The CNG channel would not necessarily be the sole target of PIP_3. RGS2, GTPase-activating protein (GAPs) for G protein α subunits (Druey, 2001), regulates signal transduction in rat ORNs by attenuating the activation of adenylyl cyclase (Sinnarajah et al., 2001). PIP_3 can bind to the RGS domain of RGS4 and thus inhibit the GAP activity of the protein (Popov et al., 2000). PIP_3-mediated inhibition of RGS proteins also plays a crucial role in the physiological regulation of the voltage-dependent behavior of G protein-gated K^+(K_G) channels in cardiac atrial myocytes (Ishii, Inanobe & Kurachi, 2002). It also will be interesting to explore whether PIP_3 also targets the Ca^{2+}-activated Cl^- channel thought to secondarily amplify the CNG channel-mediated receptor current in vertebrate ORNs (Kleene, 1993, Kurahashi & Yau, 1993, Lowe & Gold, 1993, Zhainazarov & Ache, 1995).

Overall, our findings argue that PIP_3 is a functionally important regulator of the olfactory CNG channel, and provide a potential cellular mechanism by which PI3K-dependent signaling can modulate the activation of mammalian ORNs by odorants. Although we explored the regulatory potential of PIP_3 in relation to the olfactory CNG channel, evidence that PIP_3 can activate and modulate the Na^+ sensitivity of a Na^+-activated non-selective cation channel in lobster ORNs (Zhainazarov et al., 2001) suggests our findings may generalize to other classes of ion channels.

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