Cellular/Molecular

Phosphoinositide 3-Kinase-Dependent Antagonism in Mammalian Olfactory Receptor Neurons

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Phosphoinositide signaling, in particular, phosphoinositide 3-kinase (PI3K) signaling, has been implicated in mediating inhibitory odorant input to mammalian olfactory receptor neurons (ORNs). To better understand this phenomenon we investigated PI3K-dependent inhibition between single odorant pairs. The concentration-dependent inhibition of the response of native rat ORNs to octanol by citral is PI3K dependent; blocking PI3K activity with the β and γ isoform-specific inhibitors AS252424 (5-[5-(4-fluoro-2-hydroxyphenyl)-furan-2-ylmethylene]-thiazolidine-2,4-dione) and TGX221(7-methyl-2-(4-morpholinyl)-9-[1-(phenylamino)ethyl]-4*H*-pyrido [1,2-a]pyrimidin-4-one) eliminated or strongly reduced the inhibition. Interestingly, blocking PI3K also changed the apparent agonist strength of the otherwise noncompetitive antagonist citral. The excitation evoked by citral after blocking PI3K, could be suppressed by the adenylate cyclase III (ACIII) blockers MDL12330A (*cis-N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride) and SQ22536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine], indicating that citral could also activate ACIII, presumably through the canonical olfactory receptor (OR). The G-protein $G_{\beta}\gamma$ subunit blockers suramin (8,8′-[carbonylbis[imino-3,1-phenylen ecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid), gallein (3′,4′,5′,6′-tetrahydroxyspiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one), and M119 (cyclohexanecarboxylic acid [2-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)-(9CI)]) suppressed citral's inhibition of the response to octanol, indicating that the activation of PI3K by citral was G-protein dependent, consistent with the idea that inhibition acts via the canonical OR. Lilial similarly antagonized the response to isoamyl acetate in other ORNs, indicating the effect generalizes to at least one other odorant pair. The ability of methyl-isoeugenol, limonene, α-pinene, isovaleric acid, and isosafrole to inhibit the response of other ORNs to IBMX (3-isobutyl-1-methylxanthine)/forskolin in a PI3K-dependent manner argues the effect generalizes to yet other structurally dissimilar odorants. Our findings collectively raise the interesting possibility that the OR serves as a molecular logic gate when mammalian ORNs are activated by natural, complex mixtures containing both excitatory and inhibitory odorants.

Introduction

Organizational complexity is now appreciated to be inherent in the olfactory periphery of mammals. This includes not only the discovery of new functional subsystems within the mammalian nose (Ma, 2007; Munger, 2009; Kaupp, 2010), but also new classes of olfactory receptor neurons (ORNs) within the main olfactory epithelium (MOE) in addition to the canonical ORNs, for example cells expressing trace amine-associated receptors (TAARs) (Liberles and Buck, 2006) or guanylyl cyclase-D receptors (Hu et al., 2007; Leinders-Zufall et al., 2007). Long-standing evidence that odorants inhibit as well as excite ORNs, including mammalian ORNs suggests that organizational complexity in the olfactory periphery extends to individual ORNs (Ache and Young, 2005).

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In contrast to the well understood cyclic nucleotide-dependent excitation of canonical mammalian ORNs (Kaupp, 2010), it is unclear how odorants inhibit these cells although various mechanisms have been suggested over the years (Ache, 2010). Given that the olfactory receptors (ORs) in canonical mammalian ORNs are G-protein-coupled receptors (GPCRs) (Buck and Axel, 1991) and that ligand-induced selective signaling is rapidly becoming a generic theme for GPCRs (for review, see Rosenbaum et al., 2009; Millar and Newton, 2010), it is reasonable to consider the possible existence of odorant-specific modes of signaling in mammalian olfaction. Toward that end, increasingly compelling data suggest that phosphoinositide signaling may mediate inhibitory input to mammalian ORNs and play an important role in setting the output of the ORNs evoked by natural, complex odors (Ache, 2010). Phosphoinositide 3-kinase (PI3K), which generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) *in vivo*, is the key element through which cell-surface receptors regulate processes as diverse as proliferation, growth, survival, and intracellular trafficking (Fruman et al., 1998; Vanhaesebroeck et al., 2001), including the survival of mammalian ORNs (Moon et al., 2009). PI3K activity can also modulate odorant-activated intracellular Ca^{2+} response in acutely dissoci-

ated rodent ORNs (Spehr et al., 2002) and the rapid electrophysiological output of ORNs in the MOE (Ukhanov et al., 2010) consistent with the release of antagonism. PIP_3 negatively regulates the olfactory cyclic nucleotide-gated channel (CNGC) (Zhainazarov et al., 2004; Brady et al., 2006), suggesting the CNGC is a downstream target of PI3K-dependent antagonism. However, little is known about how odorants activate PI3K, i.e., the nature of the inhibitory input per se.

To address this question we identified native canonical ORNs which displayed PI3K-dependent antagonism within a pair of single odorants and then investigated the interaction between the two odorants in more detail. We show that for a specific pairing blocking PI3K changed the agonistic strength of the otherwise antagonistic odorant and that the resulting excitation was cyclic nucleotide dependent. Finding that both odorants have the potential to signal via the canonical cyclic nucleotide cascade suggests that the inhibitory odorant acts via the OR. We also show that our findings generalize to other PI3K-dependent inhibitory odorants, suggesting they are not unique to ORNs expressing a particular OR. Our findings raise the interesting possibility that the OR in canonical ORNs serves as a molecular logic gate when activated by complex natural odorant mixtures containing both excitatory and inhibitory odorants.

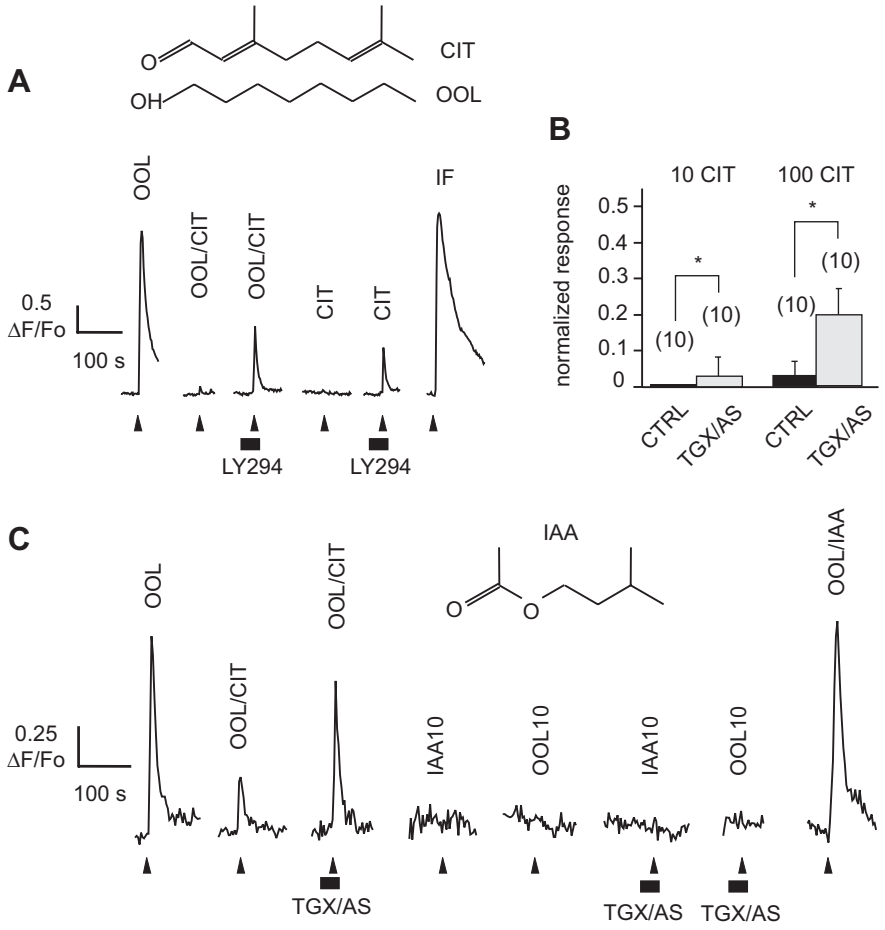

Figure 1. Blocking PI3K changes the antagonistic properties of single inhibitory odorant in mammalian ORNs. ***A***, Representative recording of the somatic Ca^{2+} response from one of 12 rat ORNs activated by OOL (50 μM) in which CIT (100 μM) evoked a weak response and substantially inhibited an almost saturating response to OOL. Pretreatment with LY294002 (LY294, 10 μM) relieved the CIT-dependent antagonism in all 12 cells. In 6 of the same 12 cells the response to CIT itself was also probed with LY294002 (10 μM) and showed a significant increase in the amplitude. All cells were stimulated with IBMX/forskolin (IF) (100/10 μM) to assess the maximal saturated response for normalization. Sensitivity to IF also proved the cells to be mature canonical ORNs. ***B***, Another 8 cells were stimulated with 10 and 100 μM CIT before (black bars) and after pretreatment with TGX221 and AS252424 (TGX/AS, 200 nM each, gray bars). Peak amplitude of Ca^{2+} response was normalized to the saturated IF response. The number of individual measurements is shown in parentheses (*$p < 0.05$). ***C***, To control for the specificity of the effect another 6 cells showing OOL/CIT antagonism were stimulated with OOL (10 μM) and a different odorant, IAA (10 μM), both at a reduced concentration to minimize possible risk of saturation. In none of the cells did a mixture of two PI3K-isoform-selective blockers TGX221 and AS252424 (TGX/AS, 200 nM each) increase or otherwise affect the very weak to no response to the odorants. IAA coapplied (OOL/IAA) at higher concentration (100 μM) with OOL (50 μM) did not affect the peak amplitude.

Materials and Methods

All experiments were performed on adult Sprague Dawley rats at least 6 weeks old. All procedures were performed in accordance with protocols approved by the University of Florida Institutional Animal Care and Use Committee. Rats were killed by inhalation of carbon dioxide and decapitated. All experiments were performed at room temperature (22–25°C).

Calcium imaging recording from acutely dissociated rat ORNs. Acutely dissociated rat ORNs were obtained and studied using standard approach described previously (Ukhanov et al., 2010). In brief, olfactory tissue was dissected in ice-cold modified artificial CSF (ACSF) saturated with 95% O_2 and 5% CO_2 that contained (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1.25 Na_2HPO_4, 1 $MgSO_4$, 1 $CaCl_2$, 10 glucose, 305 mOsm. The tissue was transferred in low-Ca^{2+} (0.6 μM free Ca^{2+} buffered with 5 mM EGTA) ACSF supplemented with 0.5 mg/ml papain (Sigma-Aldrich) and 10 units/ml DNase (Sigma-Aldrich). After incubation with enzymes for 20 min at 37°C in 5% CO_2, the tissue was gently washed with normal oxygenated ACSF several times, minced with a razor blade and accurately triturated with a large bore fire polished glass pipette. The resulting suspension was filtered through a 40 μm cell strainer (BD Biosciences) and stored at 4°C until needed. An aliquot of the suspension was mixed with 10 μM Fluo-3 containing 0.04% Pluronic F127 and placed on a glass coverslip coated with concanavalin A (Sigma-Aldrich) in a recording chamber (RC22, Warner Instruments). The volume of the chamber was 200 μl, allowing for complete exchange of the solution during application of odorant and/or inhibitors. The cells were illuminated at 500 nm and the emitted light was collected at 530 nm by a 12-bit cooled CCD camera (SensiCam, PCO). Both the illumination and image acquisition were controlled by Imaging Workbench 5.2 software (INDEC BioSystems). Each cell was assigned a region of interest (ROI) and changes in fluorescence intensity within each ROI were analyzed and expressed as the peak fractional change in fluorescent light intensity $\Delta F/F_0$ where F_0 is the baseline fluorescence before odorant application. We did not calculate the incidence rate for the odorants tested since our search was highly biased to identify cells with the required responsiveness as efficiently as possible. Typically, however, and as would be expected, of ~50 functional ORNs we find that are sensitive to 3-isobutyl-1-methylxanthine (IBMX)/forskolin, only few are activated by octanol (OOL), and only 10–15% of these cells show an antagonistic effect by citral (CIT) (Ukhanov et al., 2010). For quantitative comparison, the peak amplitude of the response of different cells was normalized to the saturated response elicited by application of a mixture of 100 μM IBMX (a phosphodiesterase inhibitor) and 10 μM forskolin (a unique diterpene agonist of adenylate cyclases) to robustly activate the cyclic nucleotide signaling pathway, and referred to in the text as relative units.

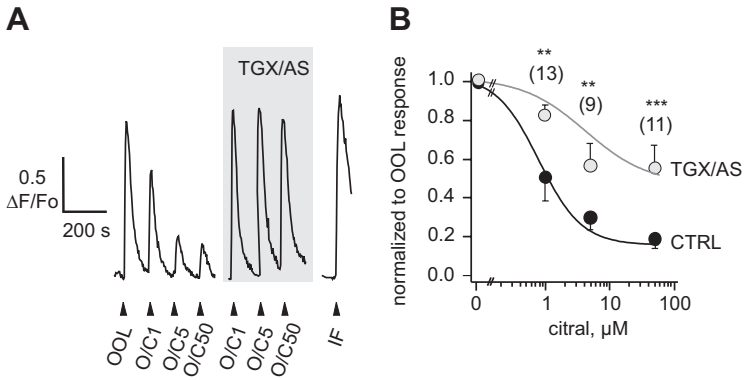

Figure 2. PI3K activity controls concentration-dependent inhibition in antagonistic odorant pair. ***A***, Increasing concentrations of CIT (1, 5, 50 μM) inhibited the response to OOL (50 μM) in a concentration-dependent manner. Odorants were presented as a 5 s pulses with a minimum of 100 s interval to allow for recovery. Preincubation of cells with TGX221 and AS252424 (TGX/AS) (200 nM each) rescued the CIT-induced inhibition (traces in shaded box). ***B***, Plot of the peak Ca^{2+} response to each concentration of OOL/CIT mixture normalized to the response to OOL and plotted as a function of concentration and fit to the Hill equation (black line). The PI3K blockers (TGX/AS) reversed the CIT antagonism and significantly shifted the inhibition curve to the right (gray line). The Hill coefficients were as follows: $IC_{50} = 0.76$ μM and $k_H = 1.3$ (control) compared with $IC_{50} = 3.78$ μM and $k_H = 0.9$ (TGX/AS). The half-maximal concentrations, IC_{50}, were significantly different ($p < 0.01$). The number of measurements made from 9 different cells is shown in parentheses (**$p < 0.01$, ***$p < 0.001$).

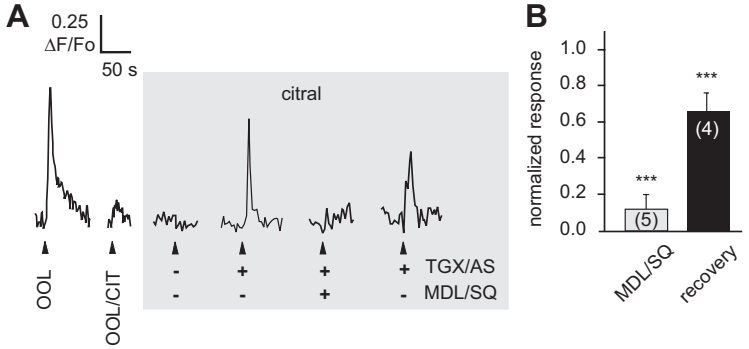

Figure 3. PI3K-dependent antagonism is mediated through the canonical cyclic nucleotide pathway. ***A***, Representative recording of the somatic Ca^{2+} response in a cell showing OOL/CIT antagonism (first and second traces). Gray box denotes the actual experiment. The response to CIT was negligible yet increased by pretreatment with the PI3K blockers (forth trace). Preincubation for 5 min with a mixture of two inhibitors of ACIII, MDL12330A and SQ22536 (MDL/SQ, both at 300/200 μM), significantly reduced the enhanced response to CIT (fifth trace). The response partially recovered after 15 min of the inhibitors' removal (last trace). Application of the odorant is marked by triangles. Addition or omission of the blockers is noted with plus or minus signs under the application mark. ***B***, Peak response amplitude was normalized to the control value measured before adding the inhibitors. The number of cells (same as number of individual measurements) used in this experiment is shown in parentheses (***$p < 0.001$).

Reagents and solution application. Odorants were delivered as solutions prepared in freshly oxygenated ACSF. Henkel-100, a complex odorant mixture was dissolved 1:1 in anhydrous DMSO as a stock. Single odorants were of highest purity obtained from Sigma-Aldrich and were prepared as 0.5 M stock solution in anhydrous DMSO. ACSF supplemented with 0.1% DMSO as a carrier for odorants served as the control solution. For calcium imaging odorant solutions were delivered from independent gravity-fed supply lines connected to the perfusion chamber through a multichannel Teflon manifold (Warner Instruments). Odorant or IBMX/forskolin solutions were applied for 5 s to provide sufficient volume to replace the solution in the recording chamber. Pharmacological inhibitors of PI3K were applied for 10–30 s before odorant stimulation. A pan-specific PI3K blocker LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was prepared as 50 mM stock in DMSO. PI3K-isoform-specific blockers TGX221 (7-methyl-2-(4-morpholinyl)-9-[1-(phenylamino)ethyl]-4*H*-pyrido[1,2-a] pyrimidin-4-one) and 5-[5-(4-fluoro-2-hydroxy-phenyl)-furan-2-ylmethylene]-thiazolidine-2,4-dione (AS252424; Cayman Chemical) were supplied as 3.3 mM solution in pure ethanol. 8,8′[Carbonylbis[imino-3,1-phenylen ecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid (Suramin; Acros Organics) was prepared as 100 mM stock in distilled water. Inhibitors of $G_\beta\gamma$ signaling, 3′,4′,5′,6′-tetrahydroxyspiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one (gallein; Tocris Bioscience) and cyclohexanecarboxylic acid [2-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)-(9CI)] (M119; Developmental Therapeutics Program catalog #NSC 119910), kindly provided by the Developmental Therapeutics Program (National Cancer Institute-National Institutes of Health, http://dtp.nci. nih.gov), were prepared as 20 mM stocks in anhydrous DMSO. Adenylate cyclase III (ACIII) blockers MDL12330A (*cis-N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride) (Sigma-Aldrich) and SQ22536 [9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine, 9-THF-Ade] (Biomol) were prepared as 100 mM stocks in DMSO.

Data analysis. All data are expressed as mean ± SEM. Statistical significance was assessed with one-way ANOVA or *t* test using SigmaStat 3.11 (Systat Software). For the concentration dependence functions data were fitted to the Hill equation yielding Hill coefficient (k_H) and half-effective concentration value (EC_{50}) using built-in function in IgorPro 4.09 (Wavemetrics).

Results

The antagonistic effect of citral on the response to octanol is PI3K dependent

We focused initially on the odorant CIT since we earlier showed it can inhibit the response to OOL in some ORNs in a PI3K-dependent manner (Ukhanov et al., 2010). Again in the present study, coapplication of 100 μM CIT reduced the peak Ca^{2+} response to 50 μM OOL >3-fold [0.50 ± 0.11 to 0.15 ± 0.04, normalized to the saturated response elicited by IBMX/forskolin (100/10 μM), 12 cells, $n = 16$] (Fig. 1*A*, first two traces). Preincubation of the cells with the pan-specific PI3K blocker LY294002 (10 μM) rescued the antagonism, returning the normalized peak response to OOL/CIT back to the control level (0.58 ± 0.10, 12 cells, $n = 16$) (Fig. 1*A*, third trace). Blocking PI3K was never observed to have an effect on the strong agonist OOL, including when it was tested at reduced nonsaturating concentrations (Fig. 1*C*, fifth and seventh traces). The ability of all cells to be activated by IBMX/forskolin (100/10 μM) denoted them as canonical ORNs (Fig. 1*A*, last trace).

Interestingly, in ORNs that showed a PI3K-dependent OOL/CIT antagonism, the pan-specific PI3K blocker LY294002 (10 μM) dramatically increased the otherwise weak normalized peak response to CIT alone, increasing it from 0.069 ± 0.09 to 0.438 ± 0.247 ($n = 9, 6$ cells) (Fig. 1*A*, fourth vs fifth trace). In 8 other cells in which CIT antagonized the response to OOL, blocking PI3K with a mixture of the PI3K β and γ isoform-specific inhibitors, TGX221 and AS252424 (200 nM each), increased the peak excitatory response to 10 μM CIT from 0.003 ± 0.005 to 0.05 ± 0.04

($n = 10$), and that to 100 μM CIT from 0.03 ± 0.04 to 0.19 ± 0.14 ($n = 10$) (Fig. 1*B*, gray bars). Again, the ability of all cells to be activated by IBMX/forskolin (100/10 μM) denoted them as canonical ORNs (Fig. 1*A*, last trace).

Blocking PI3K with a mixture of the PI3K isoform-specific inhibitors, TGX221 and AS252424 (200 nM each), did not increase the response to a non-saturating concentration of OOL (10 μM), or to a different odorant, isoamyl acetate (IAA) (10 μM), in 6 other ORNs in which CIT inhibited the response to OOL (Fig. 1*C*, sixth and seventh traces). At this lower odorant concentration, cells were unable to generate any measurable Ca^{2+} signal. We conclude that the PI3K-dependent change in the agonistic properties of CIT was odorant specific in these cells and not the result of a PI3K-dependent increase in the overall sensitivity of the cells. Similarly, the antagonizing effect of CIT on the response to OOL was also specific to that odorant pairing in these cells since 100 μM IAA did not reduce the response to 50 μM OOL but rather increased it by 26 ± 8% ($n = 5$, 4 cells) (Fig. 1*C*, last trace).

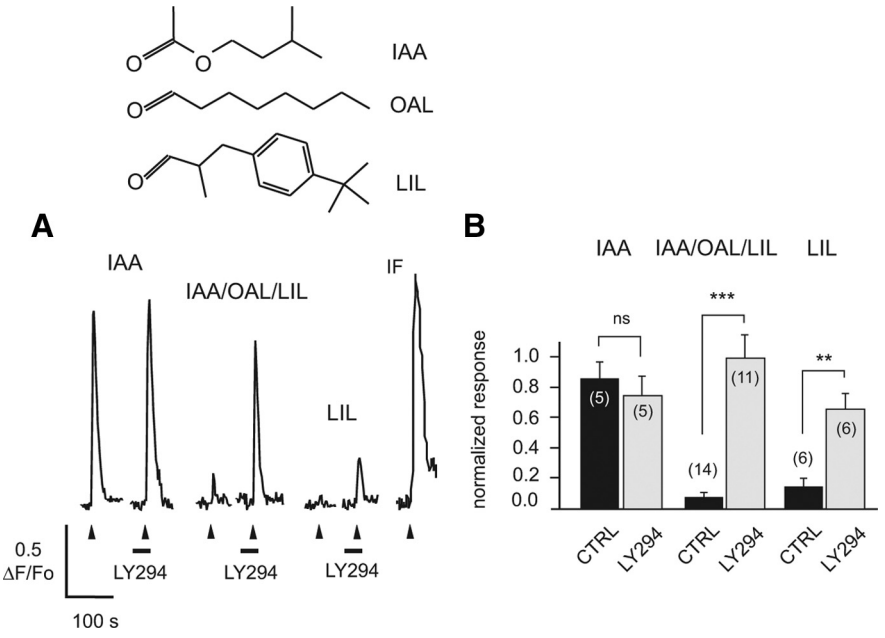

Figure 4. Other odorant pairs can show the same PI3K-dependent antagonism seen with OOL/CIT. ***A***, Two potential inhibitory odorants, OAL (195 μM) and LIL (140 μM), were identified by the ability of their mixture to inhibit the response to IAA (230 μM). Representative Ca^{2+} response from one of 8 cells in which pretreatment with the pan-specific PI3K blocker LY294002 (10 μM) rescued the inhibited response to the mixture (IAA/OAL/LIL). "Dissection" of the mixture showed the effect of the blockade was on LIL, but not on IAA. The remaining odorant (OAL) was not tested for its sensitivity to the PI3K blockade. Cells were canonical ORNs, as demonstrated by their response to stimulation with IF. ***B***, Bar graph presenting the response data from the same cells used in **A**, normalized to the response to IF. Response to IAA was measured in 4 cells, response to the mixture IAA/OAL/LIL in 8 cells, response to LIL in 3 cells. The number of individual measurements is shown in parentheses (ns, not significant, $^{**}p < 0.01$, $^{***}p < 0.001$).

As the PI3K dependence of the antagonism was suggestive of a noncompetitive mechanism of interaction, we next explored the nature of the PI3K-dependent interaction between OOL and CIT by probing another group of 9 ORNs activated by a fixed concentration of OOL (50 μM) with increasing concentrations of CIT (1–50 μM) (Fig. 2*A*). The resulting normalized concentration dependence of the inhibition was fit by a Hill equation with a half-maximal inhibitory concentration $IC_{50} = 0.76$ μM (Fig. 2*B*, black curve). Preincubation with a mixture of the PI3K isoform-specific inhibitors, TGX221 and AS252424, respectively (200 nM each), significantly shifted the inhibition concentration curve to the right, yielding an $IC_{50} = 3.78$ μM (9 cells, $p < 0.01$) (Fig. 2*B*, gray curve). However, the inhibition could not be overcome with increasing concentrations of agonist, as is the hallmark of classical competitive inhibition.

CIT signals as a strong agonist through the cyclic nucleotide pathway

To determine whether the excitatory response to CIT that results from blocking PI3K is mediated through the canonical cyclic nucleotide pathway, we treated 5 ORNs that showed PI3K-dependent OOL/CIT antagonism with a mixture of two ACIII blockers, MDL12,330A and SQ22536 (300/200 μM). Blocking ACIII activity for at least 5 min significantly reduced the enhanced response to CIT in the absence of OOL to 11.7 ± 5.8% of the control level ($n = 5$, $p < 0.001$) (Fig. 3*A*, fifth trace; Fig. 3*B*, first bar). Recovery was slow; recovering in amplitude to only 62.1 ± 8.1% of the control level ($n = 4$) within 15 min (Fig. 3*B*, second bar). Inhibiting ACIII eliminated the response to OOL/CIT in 3 other such ORNs (cf. Fig. 1) where application of PI3K blockers relieved the antagonism (data not shown). We conclude that the OR mediating PI3K-dependent antagonism of OOL signaling by CIT is also upstream of the canonical cyclic nucleotide pathway.

PI3K-dependent antagonism generalizes to other ORNs and odorants

To determine whether the behavior of CIT was specific to the particular OOL/CIT odorant pairing or perhaps just cells responsive to OOL, we probed ORNs with a mixture of IAA (250 μM), octanal (OAL, 190 μM) and lilial (LIL, 150 μM), which were shown earlier to reduce the response of rat ORNs to bourgeonal in a PI3K-dependent manner (Spehr et al., 2002). In the course of searching with this mix of 3 odorants, we found cells in which an otherwise very weak excitatory response was strongly enhanced when PI3K was blocked with LY294002 (10 μM) (Fig. 4*A*, fourth trace vs third trace), increasing the normalized response from 0.08 ± 0.03($n = 14$, 8 cells) to 0.97 ± 0.15 ($n = 11$, 8 cells) (Fig. 4*B*, fourth bar vs third bar). On further analysis, we found that IAA was a strong agonist (Fig. 4*A*, first trace; Fig. 4*B*, first bar) and LIL a relatively weak agonist (Fig. 4*A*, fifth trace; Fig. 4*B*, fifth bar) for these cells. Potential interaction between IAA and OAL or OAL and LIL was not investigated any further since we assumed that OAL was not an effective odorant for these cells. As expected blocking PI3K with LY294002 (10 μM) did not significantly change the normalized response to IAA (Fig. 4*A*, second trace); the normalized response was 0.84 ± 0.11 (control) vs 0.73 ± 0.12 following blockade ($n = 5$, 4 cells) (Fig. 4*B*, second bar). However, blocking PI3K significantly increased the normalized response to LIL (Fig. 4*A*, sixth trace), which increased from 0.14 ± 0.05 to 0.64 ± 0.10 following blockade ($n = 6$, 3 cells) (Fig. 4*B*, sixth bar). These findings extend PI3K-dependent antagonism to at least one other pair of structurally different odorants.

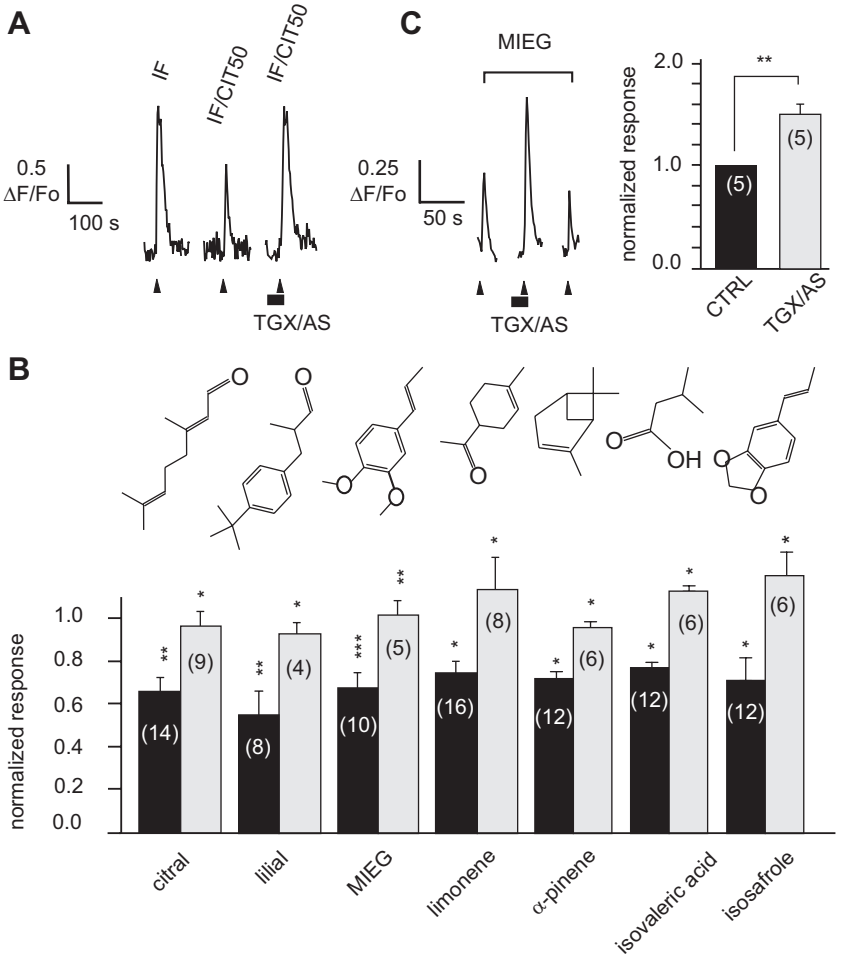

Figure 5. Identification of additional potentially PI3K-dependent inhibitory odorants. ORNs were activated by application of forskolin (2 μM) and IBMX (IF) (60 μM) to bypass the need to identify a cognate excitatory odorant, and structurally dissimilar odorants were coapplied to identify potential inhibitory odorants. ***A***, Representative recording of the somatic Ca^{2+} response of a cell in which the response to IF was diminished by coapplication of CIT (50 μM) and then was rescued following pretreatment with TGX221 and AS252424 (200 nM each). ***B***, MIEG, CIT, lilial, limonene, α-pinene, isovaleric acid and isosafrole (all at 50 μM) inhibited the response to IF in ~2% of cells that responded to IF (see Results). Blocking of PI3K with TGX221 and AS252424 rescued the response to IF (gray bars). ***C***, A representative Ca^{2+} response from one of 5 cells activated by MIEG. TGX221 and AS252424 (200 nM each) reversibly increased the response to MIEG (middle trace). Peak response amplitude was normalized to the control value of the response measured before adding the inhibitors. The number of measurements used to build the graph is shown in parentheses (*$p < 0.05$, **$p < 0.01$).

To more effectively identify other potential PI3K-dependent inhibitory odorants, we screened odorants (50 μM) using ORNs activated by a nonsaturating concentration of IBMX/forskolin (60/2 μM) before and after preincubation with the PI3K isoform-specific inhibitors TGX221 and AS252424 (200 nM each), as shown for CIT in Fig. 5*A*. This screen identified 5 more candidate odorants: methyl isoeugenol (MIEG), limonene, isovaleric acid, isosafrole, and α-pinene, each of which inhibited the response to a subsaturating concentration of IBMX/forskolin in a PI3K-dependent manner, with a slightly different incidence: 2.4% (4/166 cells) for MIEG, 3% (6/208 cells) for CIT, 1.4% (3/174 cells) for LIL, 3.3% (4/120 cells) for limonene, 2% (4/203 cells) for α-pinene, 3.8% (3/79 cells) for isovaleric acid and 2.5% (3/119 cells) for isosafrole (Fig. 5*B*). Further analysis of one of these candidate odorants, MIEG, showed the same PI3K-dependent effect seen with CIT. Preincubation with the PI3K isoform-specific inhibitors TGX221 and AS252424 (200 nM each) enhanced the agonistic strength of MIEG (50 μM) (Fig. 5*C*, left) increasing the response by 49.7 ± 10.1% ($n = 5$, $p < 0.05$) (Fig. 5*C*, right). These findings suggest that PI3K-dependent antagonism generalizes to other inhibitory odorants.

CIT antagonism on the response to OOL is G-protein dependent

As the PI3K β and γ isoforms are known to be activated by GPCRs in other systems (Hirsch et al., 2000; Guillermet-Guibert et al., 2008), and as a mixture of PI3K β- and γ-specific blockers reversed CIT's antagonism of OOL, we explored possible G-protein mediation of the effect. Accordingly, we tested whether blocking G-protein activity would disrupt the antagonism in dissociated ORNs in which CIT inhibited the response to OOL (Fig. 6*A*, first two traces). Pretreatment of 8 of 16 cells with suramin (20 μM), which disrupts interaction between the Gα and G$\beta\gamma$ subunits (Chung and Kermode, 2005), significantly reversed the inhibitory effect of CIT on the response to OOL (Fig. 6*A*, third trace). Similar results were obtained when 4 of the cells were tested with the novel small molecule inhibitor of $G_\beta\gamma$ signaling, gallein (10 μM) (Fig. 6*A*, fourth trace; Fig. 6*B*, fifth bar), and when 8 cells were tested with the structurally related compound M119 (10 μM) (Fig. 6*B*, sixth bar). In all 14 of ORNs tested, CIT's antagonism of the response to OOL could be rescued by the isoform-specific blockade of PI3K with TGX221 and AS252424 (200 nM each) (Fig. 6*A*, fifth trace; Fig. 6*B*, third bar). Different pairings of the three $G_\beta\gamma$ inhibitors acted similarly. Both suramin and gallein, for example, had a similar effect in the cell shown in Figure 6*A*. As would be expected if the inhibitors block signaling through $G_\beta\gamma$ rather than enhance signaling through $G_{\alpha olf}$, the $G_\beta\gamma$ inhibitors did not enhance all odorant responses, only those showing PI3K-dependent inhibition (data not shown). Although these pharmacological probes implicate the involvement of $G_\beta\gamma$ subunit signaling in PI3K-dependent antagonism of CIT on the response to OOL, they do not identify the specific Gα isoform involved.

Discussion

Under normal conditions the inhibitory odorants we identified (CIT, LIL) were also weak agonists, eliciting on average only 10–20% of the peak Ca^{2+} response at 100 μM that the strong agonists (OOL and IAA, respectively) elicited at 50 μM, potentially allowing them to competitively antagonize the stronger agonists OOL and IAA, respectively, when present in mixture. Indeed, systematic study of the rat I7, mouse eugenol ORs and human OR17-40 using quantitative Ca^{2+} imaging, functional analysis of site-directed mutants, and computational reconstruction of the putative docking pocket support the idea of competitive inhibition between structurally similar odorants (Oka et al., 2004; Katada et al., 2005; Jacquier et al., 2006; Peterlin et al., 2008). Our findings, however, suggest a different mechanism, at least for inhibitory

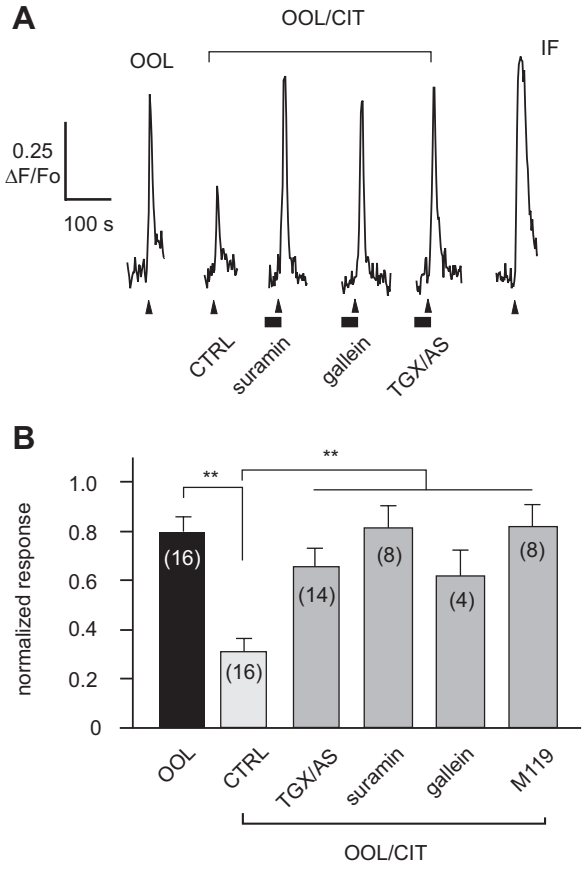

Figure 6. PI3K-dependent antagonism is dependent on the $G_{\beta}\gamma$ subunit signaling. ***A***, Representative Ca^{2+} responses from one of 10 cells showing a PI3K-blockade-dependent increase of the response to OOL/CIT following application of TGX221 and AS252424 (200 nM each). Preincubation with suramin (20 μM, 6 cells), gallein (20 μM, 4 cells) or M119 (20 μM, 8 cells) essentially replicated the effect in this cell which was still able to elicit a saturated response to IF. ***B***, Pooled data normalized to the response to IF show a similar and significant increase of the peak amplitude of the control response to OOL/CIT (CTRL, second bar) induced by TGX221 and AS252424 or $G_{\beta}\gamma$ subunit blockers. The number of individual measurements is shown in parentheses (**$p < 0.01$).

odorant pairs such as OOL/CIT and IAA/LIL with little structural similarity, which is PI3K-dependent noncompetitive antagonism. This interpretation is consistent with our ability to demonstrate the expected nonsaturating concentration-response function of the effect using single odorant pairs. Studying single odorant pairs also allows us to provide new insight into the nature of the increase in responsiveness to OOL/CIT (or IAA/LIL) following blockade of PI3K. The logical assumption is that this would reflect reduced antagonism of CIT and LIL on OOL and IAA, respectively, but we now show it can also reflect a change in the agonistic properties of the antagonist (CIT, LIL) itself.

The effect of PI3K-dependent inhibition of CIT and LIL were specific to those ligands for the ORNs being tested and did not reflect a generalized PI3K-dependent increase in receptor sensitivity. Blocking PI3K did not alter the response to OOL or IAA, the respective strong agonists, where an effect was seen on CIT and LIL. Control experiments showed that the cells still had reserve capacity to respond, i.e., they were not saturated, and therefore could have increased their response to their respective strong agonists (OOL, IAA) following blocking PI3K. Moreover, a different odorant (IAA) was unable to mimic the inhibition of CIT in the same cell. The specificity of the effect of blocking PI3K on the antagonistic odorant establishes that the inhibitory odorant is signaling through activation of PI3K.

While we only tested two single odorant pairs, and in both cases inhibition was imposed by aldehydes, we would predict such PI3K-dependent noncompetitive antagonism generalizes to odorants belonging to other structural classes. We show that five other, structurally dissimilar odorants inhibited the activation of different ORNs by IBMX/forskolin in a PI3K-dependent manner. Definitive conclusion as to the breadth of PI3K-dependent noncompetitive antagonism across the population of ORNs, however, requires testing a considerably broader range of odorants and cells expressing defined ORs.

It would appear, therefore, that at least two different processes regulate the response of ORNs to odorant mixtures, competitive antagonism and PI3K-dependent noncompetitive antagonism. Supporting this argument is a recent attempt to model the experimentally measured response of mammalian ORNs to binary odorant mixtures showing that almost 50% of the cells did not fit a model in which the odorants compete for a common binding site but rather were better fit by a model in which noncompetitive interactions could account for the observed mixture interaction (Rospars et al., 2008). Evidence that odorants can directly inhibit a number of ion channels expressed in ORNs, including the CNGC (Kawai et al., 1997; Chen et al., 2006), which has been implicated in general odor masking (Takeuchi et al., 2009), suggests other mechanisms potentially could regulate the response of ORNs to odorant mixtures, although this mechanism fails to explain the specificity of odorant inhibition in any one cell. Clearly more remains to be learned about the organizational complexity of individual mammalian ORNs.

The PI3K-dependent antagonism of CIT (or other odorants) has important implications for the receptor mediating inhibition. Since blocking ACIII impairs the PI3K-dependent antagonism of CIT, the receptor for CIT presumably also couples to the cyclic nucleotide pathway. As canonical ORs expressed by mature mammalian ORNs also signal through the cyclic nucleotide pathway, this finding can be most conservatively interpreted to suggest that both CIT and OOL (or IAA and LIL) signal through the same OR, consistent with the generally accepted view that mature mammalian ORNs express a single OR (Mombaerts, 2004; Serizawa et al., 2004). There is clear precedent for the same odorant ligand to activate two different signaling pathways through GPCRs, as are canonical mammalian ORs (Buck and Axel, 1991). In other cell type stromal cell-derived factor SDF-1α, for example, can activate both G_q and G_{i2} through the chemokine receptor CXCR4, each coupled to independent pathways that drive chemotaxis in T cells in an opponent manner (Molon et al., 2005; Ngai et al., 2009).

The argument that the inhibitory odorant, e.g., CIT, signals through the OR receives further support from our evidence that the signaling is G-protein dependent, since inhibitors implicated in $G_{\beta}\gamma$ signaling, suramin (Freissmuth et al., 1996; Chung and Kermode, 2005), and gallein and its structural analog M119 (Bonacci et al., 2006; Lehmann et al., 2008), mimic the effect of blocking in ORNs the PI3K activity. Suramin competes with $G_{\beta}\gamma$ binding to the effector and disrupts interaction between the Gα and $G_{\beta}\gamma$ dimer (Freissmuth et al., 1996; Chung and Kermode, 2005), while gallein and M119 disrupt $G_{\beta}\gamma$-dependent activation of PI3Kγ in neutrophiles (Lehmann et al., 2008) and M119 can efficiently prevent activation of PLCβ3 *in vivo* (Bonacci et al., 2006). The effect of disrupting $G_{\beta}\gamma$ subunit signaling is consistent with earlier evidence that the two isoforms of PI3K implicated in inhibitory odorant signaling, PI3K β and γ (Brunert et

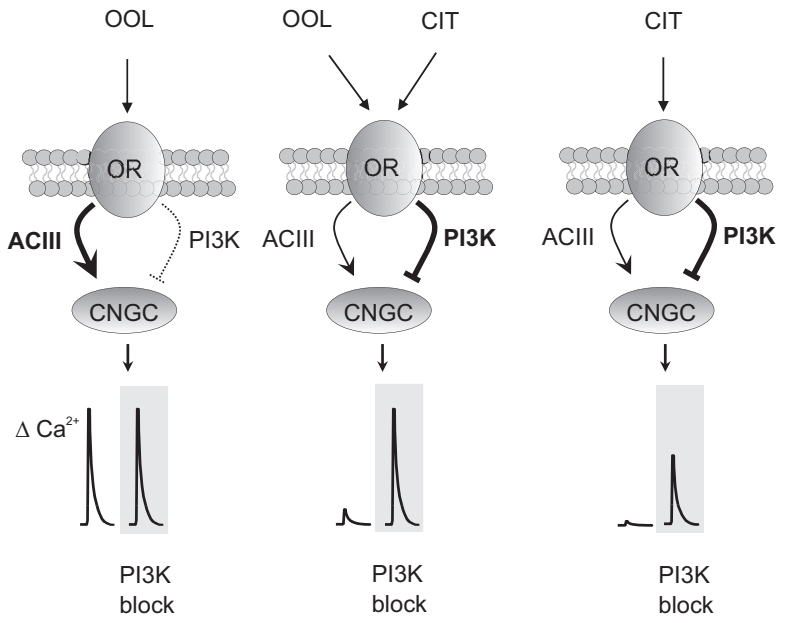

Figure 7. Diagram summarizing our findings to suggest how odorant selective signaling could occur in a mammalian ORN with a single OR. Left, An excitatory odorant for a cell, e.g., OOL, primarily activates ACIII and excites the cell through the canonical CNGC, elevating intracellular Ca^{2+} (ΔCa^{2+}), with little or no activation of PI3K. Blocking PI3K (shaded box), therefore, does not alter the ΔCa^{2+} evoked by OOL. Right, An inhibitory odorant for that cell, e.g., CIT, primarily activates PI3K, with little or no activation of ACIII and change in ΔCa^{2+}. Blocking PI3K (shaded box) reveals the potential of the inhibitory odorant to activate ACIII. Earlier findings suggest PI3K potentially inhibits the cell by opposing activation of the CNGC. Middle, OOL and CIT together primarily activate ACIII and PI3K, respectively, in proportion to the relative concentration of OOL and CIT, evoking a smaller ΔCa^{2+} than OOL alone. Blocking PI3K (shaded box) again reveals the potential of the inhibitory odorant to activate ACIII.

al., 2010; Ukhanov et al., 2010), are activated through $G_\beta\gamma$ in other systems (Maier et al., 1999). As we never observed an effect of inhibiting $G_\beta\gamma$ on the response to OOL except in cells showing OOL/CIT antagonism, we assume disrupting the interaction between the G_α and $G_\beta\gamma$ subunits of G_{olf} did not activate $G_{\alpha olf}$, which would be predicted to affect all canonical ORNs.

Our data do not allow us to distinguish whether the inhibitory odorant is signaling through the $G_\beta\gamma$ subunit of G_{olf}, the G-protein mediating the canonical ACIII signaling cascade (Sklar et al., 1986), or a different G-protein. However, the ability of ORs to couple through different G_α proteins and the ability to modify the ligand specificity of heterologously expressed ORs by changing the associated G_α (Shirokova et al., 2005), together with evidence for multiple G_α proteins in olfactory cilia (Schandar et al., 1998; Gorojankina et al., 2007; Mayer et al., 2008), favors the latter alternative.

Our results could potentially be interpreted to indicate that PI3K activity constitutively regulates the OR such that blocking PI3K would alter the binding site to allow formerly weak agonists greater access to the binding pocket. However, basal PIP_3 levels are generally considered to be low in resting cells and only increase in response to external stimuli (Vanhaesebroeck et al., 2001). Second, exogenous PIP_3 blocks the forskolin-activated response in rat ORNs (Spehr et al., 2002), arguing that PIP_3 primarily targets downstream of the OR. Consistent with this interpretation, we show here that odorants inhibit mammalian ORNs in a PI3K-dependent manner that are activated by IBMX/forskolin independent of the OR. Thus, we suggest that CIT stabilizes the OR, perhaps allosterically, in a specific conformation that selectively interacts with PI3K and opposes excitation downstream of the OR. Such ligand-induced selective signaling has become a common theme for GPCRs (Millar et al., 2004). Rather than viewing ligand binding as consistently eliciting a specific intracellular signal, it has become increasingly clear that the nature of the ligand and the dynamically changing intracellular environment alter the manner of signaling for many different GPCRs (Rosenbaum et al., 2009; Millar and Newton, 2010). In this sense, our findings suggest the OR should be viewed as serving as a molecular logic gate (Kompa and Levine, 2001) and not just a simple throughput to the brain (Fig. 7).

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