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Activation State of the M3 Muscarinic Acetylcholine Receptor Modulates Mammalian Odorant Receptor Signaling

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Abstract

A diverse repertoire of heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) enables cells to sense their environment. Mammalian olfaction requires the activation of odorant receptors (ORs), the largest family of GPCRs; however, whether ORs functionally interact with other families of GPCRs is unclear. We show that the interaction of ORs with the type 3 muscarinic acetylcholine receptor (M3-R), which is found in olfactory sensory neurons (OSNs), modulated OR responses to cognate odorants. In human embryonic kidney-293T cells, ORs and the M3-R physically interacted, and the M3-R increased the potency and efficacy of odorant-elicited responses of several ORs. Selective M3-R antagonists attenuated odorant-dependent activation of OSNs, and, when the M3-R and ORs were expressed in transfected cells, OR activation was enhanced by muscarinic agonists and inhibited by muscarinic antagonists. Furthermore, M3-R-dependent potentiation of OR signaling synergized with that of receptor transporting protein 1S (RTP1S), an accessory factor required for the efficient membrane targeting of ORs. However, the M3-R did not enhance the abundance of ORs at the cell surface, suggesting that the M3-R acted through a distinct mechanism independent of RTP1S. Finally, the activation of ORs by cognate odorants transactivated the M3-R in the absence of its agonist. The cross talk between ORs and the M3-R suggests that the functional coupling of ORs and the M3-R is required for robust OR activation.

INTRODUCTION

Odor perception in mammals is a complex process that is mediated by the activation of odorant receptors (ORs) that are present on the cilia of millions of olfactory sensory neurons (OSNs) that line the olfactory epithelium (1,2). Upon activation by cognate odorants, ORs, which are class A heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs), interact with the G protein G_{olf} . Release of $G_{\alpha_{olf}}$ from its $\beta\gamma$ subunit enables it to activate type III adenylyl cyclase (ACIII), an enzyme that rapidly catalyzes the cyclization of adenosine monophosphate (AMP) to generate the second messenger cyclic AMP (cAMP) (3–6). An increase in the concentration of cytosolic cAMP triggers the activation of cAMP-gated Ca^{2+} channels, which ultimately results in membrane depolarization and the generation of action potentials. Although these pathways constitute the established paradigm for OR signaling, it has been proposed that some odorants activate a secondary signaling pathway that involves the secondary messenger inositol 1,4,5-

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trisphosphate (IP₃); however, the molecular mechanisms through which the IP₃ pathway is activated are not well understood (7,8), and these are complicated by the heterogeneity of ORs. This heterogeneity is partly a result of the existence of a large repertoire of mammalian ORs: More than 350 human and 1000 mouse ORs have been identified thus far. Although much is now known about the ligand specificity of other GPCRs, the odor discrimination and specificity of most ORs remain poorly characterized, despite having been discovered in 1991 (1).

The evidence thus far suggests that odor recognition in mammals depends on complex receptor-ligand interactions that result in the activation of a repertoire of ORs that are found on defined subsets of OSNs (9,10). Efforts toward understanding OR-ligand interactions have been impeded by the poor activation of ORs in non-OSNs, such as transfected cell systems. Several cofactors are now used, in combination, to improve the activation of ORs in transfected cells (11,12). For example, we demonstrated that the cotransfection of human embryonic kidney (HEK) 293T cells with plasmids encoding ORs and transmembrane, olfactory-specific receptor transporting proteins (RTPs) 1 and 2 substantially increases the abundance of ORs at the cell surface (13,14). ORs that are tagged at the N terminus with the first 20-amino acid residues of rhodopsin (Rho tag) are frequently used in transfected cell systems because the Rho-tag enhances the membrane targeting of some ORs (15).

Hague *et al.* found that the cell surface abundance of one OR, OR-M71, is enhanced when other GPCRs, including the β_2 -adrenergic receptor (β_2 AR) and the purinergic receptor P2Y1, are coexpressed in HEK 293 cells; however, this enhancement applies only to OR-M71 and a closely related receptor, but not to other ORs (16,17).

These findings have led to improvements in our ability to activate ORs in vitro, and they support the idea that ORs do not function alone, but require a number of accessory proteins. Given that the sensitivities of ORs in transfected cells remain poor compared to those of ORs in OSNs and that a large percentage of ORs are orphan receptors, we considered the possibility that additional cofactors might be required for OR functions that require receptor-receptor interactions.

Although GPCR heteromerization is not well understood, such interactions are thought to be essential for the function of a number of GPCRs through various mechanisms (18,19). For example, heterodimerization of the γ -aminobutyric acid type B (GABA_B) receptors GABA_BR1 and GABA_BR2 is required for the proper trafficking of GABA_B receptors in neurons (20). In other instances, however, the formation of heterodimers appears to regulate GPCR signaling. For example, heterodimerization is required for the reciprocal modulation of β_2 ARs and angiotensin II type 1 (AT1) receptors in cardiomyocytes. When the activity of β_2 AR is blocked with antagonists, the AT1 receptor is functionally decoupled from G α_q , independent of angiotensin binding. Conversely, when the AT1 receptor is blocked, the β_2 AR is decoupled from G α_s , which leads to the loss of cAMP signaling (21,22). However, apart from several other examples, the general physiological significance of GPCR heterodimers has been debated (18,23–25). Here, we found that the M3 muscarinic acetylcholine receptor (M3-R), which is a non-OR GPCR (26) that is found on OSNs, potentiated the function of ORs in transfected cells. In addition, we showed that when the M3-R was present with ORs on the same cells, M3-R agonists and antagonists respectively enhanced or inhibited OR signaling, suggesting that the activation state of the M3-R modulates OR function.

RESULTS

Non-OR GPCRs increase the activities of ORs in transfected cells

To identify a non-OR GPCR that interacted with ORs and modulated their signaling, we screened potential receptors from a candidate library that consisted of nonchemosensory GPCRs that are found in the olfactory epithelium, as assessed by quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis (27). To determine whether these GPCRs affected the function of ORs, we cotransfected HEK 293T cells with plasmids encoding the non-OR GPCR together with one of three untagged or N-terminal Rho-tagged ORs—OR-S6, OR-EG, or Olfr62—in the presence of the OR-trafficking protein RTP1S and Ric-8B, an olfactory guanosine triphosphate (GTP)–guanosine diphosphate (GDP) exchange factor (12–14). We stimulated the transfected cells with the appropriate odorants and measured the activities of the ORs with a cAMP response element (CRE)–based luciferase reporter system (13,14,28,29). Of the 22 GPCRs that we cloned and tested (table S1), four significantly increased the responses to odorants of the three ORs that we tested ($P < 0.05$ after Bonferroni correction) (Fig. 1, A to F). Of these non-OR GPCRs, only the M3-R consistently increased the response of all untagged (Fig. 1, A to C) and Rho-tagged (Fig. 1, D to F) receptors tested. Similar results were obtained in experiments with Hana3A cells, which are HEK 293T–derived cells that stably express the appropriate accessory factors (fig. S1) (13).

Similar to mammalian ORs, muscarinic acetylcholine receptors are class A GPCRs and function in a number of physiological processes. To understand the role of all of the muscarinic acetylcholine receptors in modulating the activities of ORs, we tested the effects of the five muscarinic receptor family members on the signaling of OR-S6, OR-EG, and Olfr62 with the CRE-based luciferase assay. We found that whereas the odd-numbered, G_q -coupled subfamily members M1-R, M3-R, and M5-R enhanced the activation of the ORs tested, the even-numbered, G_i -coupled subfamily members M2-R and M4-R inhibited the function of the ORs (fig. S2).

The M3-R is found in OSNs

To determine whether *M3-R* messenger RNA (mRNA) was expressed by OSNs, we performed in situ hybridization assays on sections of the olfactory epithelium. We found that *M3-R* was expressed in the OSNs, which can be distinguished as the cell population that contains a large abundance of mRNA for olfactory marker protein (OMP) (Fig. 2, A to C). Furthermore, we performed immunohistochemical analysis of sections of the olfactory epithelium to assess whether the M3-R protein colocalized with the olfactory cilia in which ORs are localized. We found that the M3-R was localized at the cilia in the olfactory epithelium, similarly to ACIII (Fig. 2, D to G). In marked contrast, M1-R, M2-R, and M5-R proteins were low in abundance in olfactory cilia (Fig. 2, E and F, and fig. S3). Hence, although M1-R, M3-R, and M5-R potentiated OR signaling in vitro, only the M3-R was found in olfactory cilia, which suggests that M1-R and M5-R do not have a role in the potentiation of OR signaling and that the effect of M1-R and M5-R that we observed in vitro was likely a result of the sequence identity (> 50%) among the muscarinic acetylcholine receptor subfamily members, as seen when the peptide sequences for the two receptors are aligned against that of the M3-R.

The M3-R enhances the functions of various mammalian ORs

We addressed the question of whether the M3-R interacted with a few or many ORs by cotransfecting HEK 293T cells with plasmids encoding Rho-tagged ORs (from a set of mouse and human ORs for which ligands are known) and the accessory factor RTP1S with or without plasmid encoding the M3-R. In each case, we observed that the M3-R enhanced

the activities of ORs upon ligand stimulation by lowering the median effective concentration (EC_{50}) value of the odorant used, increasing the maximal OR response, or both (fig. S10). The degree of enhancement of signaling was OR-dependent; however, we observed a greater relative enhancement of signaling by the M3-R when it was co-expressed with those ORs that showed weaker responses to odorants in the absence of the M3-R. Thus, the effect of the M3-R on the signaling of those ORs that exhibited strong responses to their ligands was relatively small. That the M3-R interacted with a large number of diverse ORs further supports the idea that the M3-R could be physiologically important for olfactory signaling.

Potentiation of ORs by the M3-R in transfected cells is modulated by muscarinic receptor agonists and antagonists

Given that GPCRs that form heteromeric complexes often exhibit functional co-modulation, we wanted to determine whether altering the activity of the M3-R would directly affect the odorant-mediated activation of ORs. We cotransfected HEK 293T cells with plasmids encoding the M3-R and OR-S6, and stimulated the cells with nonanedioic acid (an OR-S6 agonist) in the presence of carbachol (a muscarinic receptor agonist) or atropine (a muscarinic receptor antagonist). Carbachol alone did not induce reporter activity at the concentration that we tested (100 nM) (fig. S4A). Costimulation of cells with nonanedioic acid and carbachol further enhanced the activity of OR-S6 compared to that in cells treated with nonanedioic acid alone. Conversely, the presence of atropine hindered the potentiation effect of the M3-R (Fig. 3). This inhibitory effect of atropine was only observed in the presence of the M3-R, consistent with the idea that the activation state of M3-R is an important factor in determining its effect on OR signaling.

Selective antagonists of the M3-R attenuate the activation of OSNs

If the activation state of the M3-R influenced odor responses as a result of direct receptor-receptor interactions in vivo, then it is likely that antagonists of the M3-R would inhibit the activation of OSNs. Because the stimulation of OSNs by odorants leads to transient increases in the concentration of intracellular calcium ions (Ca^{2+}), we used Ca^{2+} imaging to monitor the activation of OSNs. We compared the responses of individual, acutely dissociated OSNs from mice to a mixture of 10 odorants or to the same mixture containing either darifenacin or pfHHSiD, two M3-R-selective antagonists. When OSNs were stimulated with odorants containing darifenacin, the Ca^{2+} responses of each OSN were substantially attenuated compared to those of OSNs exposed to odorants in the absence of darifenacin (Fig. 4, A and B, and fig. S5). To ensure that the attenuation of the Ca^{2+} response in OSNs that was observed when darifenacin was administered with odorants was not affected by OR attenuation, we showed that changing the order in which we administered either the odorant mixture or the odorant mixture with darifenacin yielded similar results (Fig. 4B). Consistent with observations made in transfected cells (fig. S10), the degree of inhibition was different in different neurons, which we assume expressed different ORs (fig. S5). We observed similar findings in experiments with pfHHSiD, another M3-R-selective antagonist (Fig. 4, C and D, and fig. S5). Together, these results support the idea that the M3-R enhances the function of ORs and that its activation state affects the odorant-mediated activation of ORs in OSNs.

The M3-R does not increase the cell surface abundance of ORs

Because the cofactors RTP1S and RTP2 and the receptors β_2 AR and P2Y1 promote the cell surface trafficking of ORs (13,16,17), we hypothesized that the M3-R might play a similar role. However, live-cell immunostaining and flow cytometric analysis of transfected HEK 293T cells expressing Rho-tagged ORs alone or in the presence of the M3-R revealed that the M3-R did not increase the relative density of OR-S6 at the cell surface, thus precluding the possibility that the M3-R functions by supporting OR trafficking (Fig. 5, A and B).

The M3-R synergizes with RTP1S but does not affect the ligand specificity of ORs

To determine whether the potentiation of OR signaling by the M3-R was dependent on the presence of other accessory factors, we cotransfected HEK 293T cells with plasmids encoding Rho-tagged or untagged ORs (OR-S6 or Olfr62) with a combination of plasmids encoding the M3-R, RTP1S, and Ric-8B, and we measured OR responses. We found that whereas untagged ORs showed relatively poor responses to odorants in the presence of the M3-R or RTP1S alone, their responses were enhanced when both M3 and RTP1S were present (Fig. 6, A and B). When we performed this experiment in cells with Rho-tagged ORs, we observed a similar synergism between the M3-R and RTP1S; however, the responses of the tagged ORs were substantially enhanced relative to those of the untagged receptors (Fig. 6, C and D). Ric-8B did not substantially affect OR responses in our assay. These results suggest that the M3-R and RTP1S synergistically enhanced the function of ORs, indicating that the M3-R acted by enhancing the signaling of ORs that were targeted to the membrane by RTPs and further supporting the idea that the functional role of the M3-R is different from that of previously identified accessory proteins.

To determine whether functional interactions between the ORs and the M3-R altered their signaling, we stimulated HEK 293T cells that were cotransfected with plasmids encoding an OR, RTP1S, and the M3-R with forskolin, an activator of AC. We observed enhanced responses only when OR-S6, RTP1S, and the M3-R were all present in cells, suggesting that the OR and M3-R together potentiated the activation of AC in the absence of OR ligands (fig. S4B). This is consistent with the idea that the M3-R facilitates efficient signal transduction. When we stimulated cells with ionomycin, a Ca^{2+} ionophore, we did not detect any reporter activity, indicating that ionomycin had no effects on OR activation (fig. S4C). Finally, when we stimulated cells with the M3-R agonist carbachol, we observed a synergistic effect when cells were stimulated with more than 1 μM carbachol, but only when OR, RTP1S, and the M3-R were all present. This suggests that at high concentrations of carbachol, activation of the M3-R could lead to the transactivation of ORs (fig. S4A). To determine whether the M3-R altered the ligand specificity of ORs, we stimulated transfected cells that had ORs in the presence or absence of the M3-R with a number of known OR ligands or with compounds that are not OR ligands. We found that for both OR-S6 and Olfr62, the M3-R potentiated each odor-specific response without altering their relative responses to the odors, suggesting that the M3-R plays little or no role in OR ligand selectivity (fig. S6).

The M3-R increases the amount of cAMP produced upon OR activation

To verify that the M3-R did not increase the activity of the luciferase reporter gene by mechanisms independent of OR-mediated production of cAMP, we quantified the changes in the amount of cAMP in odorant-stimulated cells that contained OR-S6 and RTP1S with or without the M3-R. When the OR, RTP1S, and the M3-R were all present, the basal amount of cAMP was slightly greater than that in other cells. After we stimulated the cells with the OR-S6 agonist nonanedioic acid, we observed an increase in the amount of cAMP in cells that had the M3-R, mirroring the results obtained from the luciferase gene reporter assays (Fig. 7A). This result confirmed that the M3-R modulated the OR signal transduction pathway at the level of or upstream of the production of cAMP. With this same assay, we also showed that the increase in the concentration of cAMP after OR stimulation was even greater in the presence of carbachol (100 nM) (Fig. 7B).

Activation of ORs leads to transactivation of the M3-R

Because the M3-R potentiated signaling by ORs, we considered whether OR activation might enhance the extent of M3-R signaling. We transfected HEK 293T cells with plasmids encoding the M3-R with or without OR-S6 and its accessory factors, and we monitored Ca^{2+}

release by these cells after their stimulation with carbachol or odorant. As expected, we did not observe a Ca^{2+} response when HEK 293T cells expressing OR-S6 alone were stimulated with nonanedioic acid. As a positive control for OR expression, we showed that cells containing ORs and $\text{G}\alpha_{15\text{olf}}$, a promiscuous, chimeric G protein, generated a Ca^{2+} response after stimulation with nonanedioic acid (Fig. 8) (14). In contrast to OR-S6, the M3-R is a G_q -coupled receptor, and stimulation of cells that expressed the M3-R with carbachol resulted in a robust Ca^{2+} response. No response was observed when these cells were stimulated with nonanedioic acid. However, when the cells contained OR-S6 and its cofactors together with the M3-R, stimulation with nonanedioic acid alone elicited a strong Ca^{2+} response. This response required activation of the M3-R, because the response was abolished by stimulating the same cells with odorant and the M3-R antagonist atropine. These results suggest that odor activation of ORs can lead a Ca^{2+} response mediated through the M3-R.

The M3-R physically interacts with ORs

Because of the functional interaction between the M3-R and the ORs, we hypothesized that they might interact to form stable heteromers. To assess this, we performed coimmunoprecipitation studies with HEK 293T cells cotransfected with plasmids encoding N-terminal hemagglutinin (HA)-tagged M3-R and FLAG-tagged OR-S6. FLAG-tagged OR-S6 was present on the cell surface and responded to nonanedioic acid (figs. S7 and S8). When we immunoprecipitated FLAG-tagged M3-R from cell extracts with antibody against the FLAG tag, HA-tagged OR-S6 proteins were copurified, as assessed by Western blot analysis. Conversely, when we performed immunoprecipitations with cell extracts and antibodies against the HA tag, we detected FLAG-tagged S6 proteins (Fig. 9). We could not coimmunoprecipitate HA-tagged CD28, a negative control, with FLAG-tagged OR-S6 proteins in these cells (Fig. 9A). When FLAG-tagged OR-S6 was coexpressed in cells with other HA-tagged GPCRs, the M3-R and M2-R could be coimmunoprecipitated. In contrast, other GPCRs were not efficiently copurified with ORs. Thus, the M3-R and OR-S6 likely interacted to form stable, functional complexes in HEK 293T cells. The interaction between the M2-R and the OR may explain the inhibition of OR activation by the M2-R that we observed earlier (fig. S1). Because we did not observe M2-R immunoreactivity in the olfactory cilia (fig. S3), we assume that the M2-R does not have a physiological role in olfactory transduction in vivo.

DISCUSSION

Here, we provide evidence suggesting that a non-OR GPCR may play a broad, yet functionally relevant, role in mammalian olfaction. We showed that the muscarinic acetylcholine receptor M3-R specifically potentiated the activation of a diverse range of ORs. The M3-R lowered the EC_{50} value and increased the maximum responses of ORs in transfected cells. In addition, activation of the M3-R further enhanced the responses of ORs, whereas inhibition of M3-R activity abolished these effects. Although Liberles and Buck showed that the trace amine-associated receptors (TAARs), which are individually expressed in non-OR-expressing OSNs, are non-OR GPCRs that play an important role in the olfactory recognition of amine compounds (30), we have shown the first instance in which a non-OR GPCR that is coexpressed with an OR can substantially alter the potency and efficacy of a large number of ORs.

The muscarinic acetylcholine receptors are activated by the physiological ligand acetylcholine, a key neurotransmitter that is found in both the peripheral and the central nervous systems. These receptors are responsible for a broad range of functions, including the contraction of skeletal muscle and secretion by tracheal mucosa. On the basis of our findings, the M3-R also appears to have a unique, and unexpected, role in olfactory signal

transduction. Given the extensive innervation of the olfactory mucosa by cholinergic parasympathetic neurons, it is possible that secreted acetylcholine could activate muscarinic receptors on OSNs (31,32). As we have shown in our transfected cell system, 100 nM carbachol was adequate to substantially potentiate OR function; hence, even a low concentration of acetylcholine, whether constitutively available or released upon OR stimulation, could improve the olfactory response. Indeed, Hedlund and Shepherd provided the first evidence for the presence of muscarinic acetylcholine receptors in the olfactory epithelium of the salamander; moreover, they showed the presence of both choline acetyltransferase and acetylcholine esterase, further supporting the idea that acetylcholine plays a physiological role in the olfactory epithelium (33).

Furthermore, humans and animals show an increase in acuity in odor detection by learning. This olfactory perceptual learning is modulated by acetylcholine (34). Although previous studies suggested the importance of changes by acetylcholine in neurons of the olfactory bulb and the piriform cortex, our results raise the possibility that acetylcholine might have direct effects in the activation of ORs, thus contributing to olfactory learning at the receptor level. In the future, it will be interesting to determine whether acetylcholine is secreted in the nasal mucus and, if so, whether modulating the amount of acetylcholine could be a mechanism through which the native functions of ORs might be regulated by the activity of the M3-R and whether changes in OR function by acetylcholine might affect olfactory learning.

The ability of GPCRs to act not simply as monomers but also as dimers or even higher-order oligomers has been reported in a number of systems; however, whether these observations are physiologically relevant has been debated in some cases (18,23–25). Although M3-R signaling has never been proposed to play a role in olfaction, others have postulated that neurotransmitters act to modulate OR activation. Firestein and Shepherd showed that the application of atropine, a muscarinic receptor antagonist, substantially reduced the activation of ORs in salamander OSNs (35), consistent with the idea that the activation state of the M3-R is important for OR function. It is possible that other non-OR GPCRs modulate the functions of ORs. As mentioned earlier, β_2 AR and P2YRs enhance the functions of specific ORs. Other GPCRs may also alter the functions of other subsets of ORs. Our data appear to support this idea, because several other GPCRs from our screen appeared to enhance the function of some of the ORs that we tested (Fig. 1). This implies that there may be inherent heterogeneity and complexity in OR–non-OR interactions. In the future, it will be necessary to examine how the M3-R and other OR-modulating proteins affect OR function and what the molecular mechanism of this interaction might be.

How does the M3-R enhance OR function? It is clear that no single paradigm can account for the diverse interactions observed between GPCR homomers and heteromers (18). In contrast to the ability of β_2 AR and P2YRs to increase the surface abundance of OR-M71, the M3-R did not increase the amount of ORs at the cell surface. This is consistent with results presented by Bush *et al.*, in which the M3-R was not identified as a potential OR modulator, precisely because they also found that co-expressing the M3-R did not increase the cell surface abundance of ORs (17). Our screen directly assessed OR activation, which enabled us to identify the M3-R.

In contrast to instances in which GPCR heteromers promote receptor trafficking, the stable interaction between ORs and the M3-R might lead to a change in OR conformation or ligand-induced changes in OR conformation that improve ligand binding. Another possibility is that M3-R and OR heteromers may couple with G proteins more efficiently than do monomeric receptors. Indeed, it has been proposed that two receptor molecules are required to fully bind with each G protein, because structural studies have shown that

GPCRs form multiple points of contact with the α and $\beta\gamma$ subunits of G proteins, which require different GPCR conformations (36,37). However, general mechanisms for GPCR cross talk may not apply to this particular instance, because the action of the M3-R is likely specific. Although we tested a diverse set of 22 non-OR GPCRs, including multiple G_q -coupled receptors, no other G_q -coupled receptor showed properties similar to those of the M3-R. This suggests that the presence of G_q -coupled GPCRs is not sufficient to potentiate OR signaling and that a more specific interaction is likely required.

Finally, we showed that the interaction between the ORs and the M3-R was bidirectional. Activated ORs directly elicited an M3-R-dependent Ca^{2+} response, which was abolished in the presence of the M3-R antagonist atropine. Transactivation has been documented for other GPCR heteromers. For example, in the case of G_i -coupled A1 adenosine receptor (A1R) and the G_q -coupled P2Y1 receptor, the purinergic agonist adenosine 5'-diphosphate (ADP) $_{\beta S}$, which normally does not affect cAMP production, promotes a reduction in the amount of cAMP through receptor heteromers, when A1R and P2Y1 are present (38). Likewise, heteromers of ORs and the M3-R may be able to couple to G_q as well as to $G_{olf/s}$. We do not yet know the molecular mechanism of the interaction between the ORs and the M3-R. As a first step, it will be important to identify the receptor domains that are required for this interaction. Although we showed that ORs and the M3-R formed stable heteromers, we cannot exclude the possibility that the modulatory effects might be explained by complex interactions among signaling pathways that are independent of these physical interactions.

Our findings raise the possibility that the M3-R not only modulates OR activity but also plays a role in olfactory signal transduction through its own downstream targets. One possibility is that the odor-specific activation of ORs might lead to the activation of the M3-R in olfactory cilia, which may then activate IP₃ signaling. Although cAMP is the predominant secondary messenger system in olfaction (39), certain odors elicit an IP₃ response in OSNs (7,40–42). Although G_q is not abundant in olfactory cilia (43,44), the M3-R may couple with other G_q family G proteins or to G_o to induce the production of IP₃ in OSNs. Future work is necessary to examine this possibility. In summary, our results suggest that the M3-R acts specifically to modulate the responses of a number of ORs to odorants. Some ORs and the M3-R appear to form specific, heteromeric interactions that support robust activation of these ORs, extending the concept of functional interaction to the largest class of GPCRs. Because weak activation of ORs in transfected cell systems has been one of the major barriers in understanding olfactory signaling, this discovery is likely to lead to advances in identifying active ligands for many, thus far, orphan ORs.

MATERIALS AND METHODS

DNA and vector preparation

The open reading frames of 22 GPCRs and all of the muscarinic family members (Fig. 1) were amplified by PCR with primers flanked by Mlu I and Not I restriction sites at the 5' and 3' ends, respectively, with Phusion polymerase (Finnzymes) according to the manufacturer's protocols. A complementary DNA (cDNA) template of these receptors was prepared from C57BL/6 mice, as described previously (13). Amplified fragments were subcloned into the plasmid pCI (Promega) with Mlu I and Not I restriction sites and sequence-verified.

Dual Glo luciferase assay and chemicals

HEK 293T and Hana3A cells were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS, M10) with penicillin, streptomycin, and amphotericin. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the

manufacturer's protocols. The Dual-Glo Luciferase Assay System was performed as described previously (29). Briefly, we used CRE-luciferase (Stratagene) to measure the activation of ORs. *Renilla* luciferase driven by a constitutively active simian virus 40 (SV40) promoter (pRL-SV40; Promega) served as an internal control to determine cell viability and transfection efficiency. Cells were plated on poly-D-lysine-coated 96-well plates (NUNC) 2 days before the assay. HEK 293T or Hana3A cells were transfected with plasmids encoding ORs and various combinations of RTP1S, Ric-8B, the M3-R, *CRE*-luciferase, and pRL-SV40. Eighteen to 24 hours after transfection, cells were rinsed with 50 μ l of CD293 (chemically defined medium; Invitrogen) and stimulated with odorant solution (25 μ l) dissolved in CD293. Cells were incubated for 4 hours at 37°C and 5% CO₂ before luciferase assays were performed. Protocols for measuring luciferase and *Renilla* luciferase activities were performed as described by the manufacturer (Promega). We calculated the normalized luciferase activity with the formula $(L_N - L_{\min}) / (L_{\max} - L_{\min})$, where L_N is the luminescence of firefly luciferase in response to the odorant, $L_{\min}$ is the minimum luciferase value on a plate or set of plates, and $L_{\max}$ is the maximum luciferase value on a plate or set of plates. We analyzed the data with Microsoft Excel and GraphPad Prism 5. All odorants and muscarinic agonists and antagonists were from Sigma, except for darifenacin, which was purchased from Santa Cruz Biotechnology.

Immunohistochemistry and in situ hybridizations

The procedure for in situ hybridization was previously described (13). Briefly, digoxigenin (DIG)-labeled complementary RNA probes were used to hybridize target mRNAs in frozen tissues. Hybridization signals were detected with an alkaline phosphatase (AP)-conjugated antibody against DIG (Roche) and with the AP chromogen BCIP-NBT (bromo-chloroindolyl phosphate-nitro blue tetrazolium) (Promega). For immunohistochemical analysis, frozen sections were incubated with antibodies against the M3-R (Sigma), M1-R (Sigma), M5-R (Sigma), and ACIII (Santa Cruz Biotechnology). After washing, tissues were incubated with Cy-3-conjugated antibody against rabbit immunoglobulin G (IgG; Jackson Immunologicals). We used a Zeiss Axioskop fluorescence microscope and a CCD (charge-coupled device) camera to visualize the stained tissue.

Calcium imaging

To image Ca²⁺ in acutely dissociated OSNs, we followed a previously described protocol (9,45), with modifications. Dissected adult mouse olfactory epithelium was treated with 0.025% trypsin (Invitrogen) for 12 min at 37°C. Minced tissues were then "printed" on poly-D-lysine-coated coverslips. Cells were loaded with Fluo-4 (4 μ M; Invitrogen) and Fura red (7 μ M; Invitrogen) for 60 min at room temperature. Odorant stimulation and data acquisition were performed as described previously (46). Briefly, we used the "Live Imaging" mode of a Leica confocal microscope to record Ca²⁺-dependent cell fluorescence. Cells were exposed to a constant flow of bath solution (Hank's buffer containing 10 mM Hepes). Odor solution [a mix of 10 odorants: isoeugenol, vanillin, coumarin, nonanoic acid, heptanal, benzyl acetate, nonanediol, acetophenone, octanol, and (–)-carvone at 10 μ M each] was applied for ~8 s by changing the bath solution with a peristaltic pump. For data analysis, we first counted neurons by identifying those cells that showed a clear Ca²⁺ response to KCl. Of these, cells that showed a response to the odor mix, to the odorant mix and antagonist, or to both were marked for further analysis. Each field that we recorded typically contained 40 to 200 KCl-responding cells and 1 to 5 odor-responding cells. We used Euler's trapezoidal method to calculate and compare the responses of each cell to the odorant mix with or without antagonist (fig. S9) (47). Specifically, the area (A_{total}) under the total response curve between time zero (t_0) and the time of interest, defined for a total of 30 s before and after the response maxima, was calculated by adding the net response r_n and r_{n+1} and multiplying by the duration of the response. We added the responses in increments of 1 s so that the

trapezoid method summed the response underneath the curve between any two consecutive seconds [that is, between t_1 and t_2 , the area A_1 would equal $(r_1 + r_2) \times t$, where $t = 1$]. To normalize A_{total} against the background fluorescence of each cell, we drew a baseline curve with the average baseline response (r_{base}) of the first four of the 20 r_n values, which were the net response values before cell stimulation. We then multiplied r_{base} by 20 s, which was the total time interval monitored for each cell to determine the A_{base} . This baseline area (A_{base}) was subtracted from the net response of each cell that was calculated with the trapezoid method, leaving what is an A_{net} value that defines only the area underneath the response curve. The A_{net} values for odorants with and without antagonist were compared using the Student's paired t test. A total of 34 and 36 cells were used for each t test analysis for the two different antagonists, respectively. All data analysis was done with ImageJ, Igor Pro, and Microsoft Excel. Ca^{2+} imaging with HEK 293T cells was performed as described previously (46).

Immunocytochemistry and flow cytometric analysis

For cell surface staining and flow cytometry, we followed a previously described protocol (29). Briefly, cells were incubated in MEM with 10% FBS containing a mouse monoclonal antibody against rhodopsin (4D2; a gift from R. Molday) (48) at 4°C for 30 min. After washing, cells were incubated with Cy3-conjugated donkey antibody against mouse IgG (Jackson Immunologicals), washed, and mounted for observation. For flow cytometric analysis, HEK 293T cells transfected with plasmids encoding Rho-tagged ORs were labeled with phycoerythrin (PE)-conjugated antibody against mouse IgG (Jackson Immunologicals). Cells were cotransfected with plasmid encoding green fluorescent protein (GFP) as a positive control marker. Quantification of the intensity of staining of cells with antibody against cell surface ORs was taken as the ratio of PE to GFP fluorescence. 7-Amino-actinomycin D (Calbiochem) was used to stain the cells before flow cytometric analysis to mark dead cells, which were excluded from the analysis.

cAMP assays

We used the AlphaScreen cAMP assay (PerkinElmer) to measure changes in the amount of cAMP in cells. Cells were plated on 96-cell plates (coated with poly-D-lysine; NUNC). Twenty-four hours after transfection, cells were washed in Hank's buffer containing 5 mM Hepes, 0.1% bovine serum albumin (BSA), and 500 μM isobutylmethylxanthine (IBMX) for 30 min. Cells were stimulated with Hank's buffer containing 5 mM Hepes, 0.1% BSA, and IBMX (500 μM) and the appropriate ligands. The stimulated cells were incubated for 10 min at room temperature before lysis. We followed the manufacturer's protocol to measure the amount of cAMP.

Immunoprecipitations

Immunoprecipitations were performed as previously described (13). Briefly, HEK 293T cells were plated and transfected with plasmids encoding ORs, RTP1S, and the M3-R. Cells were incubated for 24 hours at 37°C in 5% CO_2 and then incubated with lysis buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, and 1% NP-40] containing protease inhibitor mix (Complete, Mini; Roche). The lysates were incubated with affinity gel containing the M2 antibody against the FLAG tag (Sigma) or an affinity matrix containing antibody against the HA tag (Roche) for 2 hours at 4°C and washed with lysis buffer. Bound proteins were eluted by incubation with SDS sample buffer at room temperature for 2 hours. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed according to the manufacturer's instructions for the Mini-Protean 3 Cell system (Bio-Rad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The M3-R increases the activity of ORs. (**A to C**) Untagged ORs. (**D to F**) Rho-tagged ORs. Lysates of Hana3A cells that were cotransfected with plasmids encoding the indicated ORs and individual GPCRs from a panel of 22 receptors were subjected to luciferase assays under the following conditions: (**A and D**) Cells expressing OR-S6 were stimulated with nonanedioic acid (3 μ M); (**B and E**) cells expressing OR-EG were stimulated with vanillic acid (30 μ M); and (**C and F**) cells expressing Olfr62 were stimulated with 2-coumaronone (300 μ M). Asterisks indicate those GPCRs that significantly increased the activity of each OR relative to that in cells transfected with a vector control, $P < 0.05$ after Bonferroni correction. Averages were obtained from triplicate assays, and the experiment was repeated four times.

Fig. 2.

M3-R mRNA is found in OSNs, and M3-R protein is localized to the cilia of the olfactory epithelium. (A to C) In situ hybridization analysis of the olfactory epithelium demonstrating that M3 mRNA is abundant in olfactory neurons. OMP is a marker for mature OFNs. IL-8RB, which does not hybridize to the OSNs, is used as a control GPCR probe. (D to G) Immunohistochemical analysis with antibodies against the M1-R, M3-R, and M5-R. The M3-R, but not M1-R and M5-R, is abundant at the cilia of the olfactory epithelium. A probe to ACIII was used as a positive control. The experiment was repeated at least three times.

Fig. 3.

Potential of OR responses by the M3-R is enhanced by muscarinic receptor agonists but inhibited by muscarinic receptor antagonists. (A to D) Nonanedioic acid dose-response curves for luciferase assays performed in HEK 293T cells expressing (A) the M3-R alone, (B) the M3-R and Rho-tagged OR-S6, (C) Rho-tagged OR-S6 and RTP1S, and (D) the M3-R, Rho-tagged OR-S6, and RTP1S, and the M3-R in the presence of the muscarinic receptor agonist carbachol (100 nM, purple), the muscarinic antagonist atropine (1 μ M, green), and carbachol and atropine (black), or in the absence of additional treatment (red). Error bars indicate the SEM; the assays were performed in triplicate.

Fig. 4.

The M3-R-selective antagonists darifenacin and pfHHSiD attenuate odor-mediated responses of OSNs. **(A)** Kinetics of changes in the ratio of Fluo-4 and Fura-red fluorescence for a representative OSN stimulated by a mixture of 10 odorants (10 μ M each) with or without darifenacin. **(B)** Darifenacin (100 nM) attenuated odor-mediated responses of OSNs. (P values shown are from paired t tests; $n = 36, 18$, and 18 OSNs for each pair of treatments.) **(C)** Kinetics of changes in the ratio of Fluo-4 and Fura-red fluorescence for a representative OSN stimulated by a mixture of odorants with or without pfHHSiD. **(D)** pfHHSiD (1 μ M) attenuated odor-mediated responses of OSNs. (P values shown are from paired t tests; $n = 34, 15$, and 19 OSNs for each pair of treatments.)

Fig. 5.

(**A** and **B**) The M3-R does not increase the cell surface abundance of ORs. HEK 293T cells were cotransfected with plasmids encoding Rho-tagged OR-S6 together with plasmids encoding RTP1S, the M3-R, or both, and the cell surface of OR-S6 was determined by immunostaining (**A**) or was quantified by flow cytometric analysis (**B**). The cell surface abundance of ORs increased substantially in cells co-transfected with plasmid encoding RTP1S alone, but not in cells transfected with plasmid encoding the M3-R alone. Cotransfecting cells with plasmids encoding both the M3-R and the RTP1S did not substantially increase the cell surface abundance of either OR when compared to that in cells transfected with plasmid encoding RTP1S alone. Results are from a single experiment; experiments were replicated at least three times (**A**) or twice (**B**).

Fig. 6.

Potential of OR activity by the M3-R is synergistic with that of RTP1S and is independent of tag. (A to D) Dose-response curves of the indicated OR ligands for luciferase assays performed in HEK 293T cells that were transfected with a combination of plasmids encoding RTP1S, Ric-8B, and the M3-R and (A) untagged OR-S6, (B) untagged Olfr62, (C) Rho-tagged OR-S6, or (D) Rho-tagged Olfr62. Error bars indicate the SEM, and assays were performed in triplicate.

Fig. 7.

M3-R increases the amount of cAMP produced upon activation of ORs. **(A)** Dose-response curves of nonanedioic acid for the production of cAMP were performed in HEK 293T cells that were cotransfected with plasmids encoding OR-S6 alone, OR-S6 and the M3-R, OR-S6 and RTP1S, or OR-S6, RTP1S, and the M3-R. There was a substantial increase in the concentration of cAMP produced in cells expressing OR-S6, the M3-R, and RTP1S relative to that in cells expressing OR-S6 and either in comparison to cotransfection with either alone (purple curve versus orange and blue, respectively). Data were obtained from triplicate samples, and the experiment was replicated four times. **(B)** cAMP production is further increased upon costimulation of cells with the nonspecific M3-R agonist carbachol. Dose-response curves of nonanedioic acid for the production of cAMP were performed in HEK 293T cells that were co-transfected with plasmids encoding OR-S6, RTP1S, or the M3-R. Co-stimulation of cells with carbachol (100 nM) further stimulated the production of cAMP in cells cotransfected with plasmids encoding the M3-R and RTP1S. Data were obtained from triplicate samples and the experiment was repeated twice.

Fig. 8.

Activation of OR-S6 leads to Ca^{2+} release mediated by activation of the M3-R. **(A)** Nonanediolic acid-induced coupling of OR-S6 to the promiscuous G protein $\text{G}_{\alpha 15\text{olf}}$ enables odorants to increase the intracellular Ca^{2+} concentration. **(B)** Ca^{2+} influx of cells expressing the M3-R when treated with carbachol (100 nM). **(C)** Treating cells that express both S6 and the M3-R (in the absence of RTP1S) with nonanediolic acid did not affect a Ca^{2+} response triggered by carbachol. **(D)** In the absence of the M3-R, treating cells expressing OR and RTP1S did not generate a Ca^{2+} response. **(E)** Increases in intracellular Ca^{2+} in cells expressing OR-S6, RTP1S, and the M3-R in the presence of nonanediolic (100 μM) acid, similar to the response elicited by carbachol (100 nM). **(F)** Ca^{2+} release is blocked when non-anediolic acid and atropine (1 μM) are simultaneously used to stimulate cells expressing S6, RTP1S, and the M3-R. Data shown here were taken from one experiment; experiments were repeated three times.

Fig. 9.

The M3-R and S6 stably interact in HEK 293T cells. Coimmunoprecipitation studies of HA-tagged M3 and FLAG-tagged OR-S6 in HEK 293T cells. (**A to D**) When cell extracts were used in immunoprecipitation assays with antibody against the HA tag, we could detect FLAG-tagged S6 proteins. (**B**, lane 3). No coimmunoprecipitation was detected when FLAG-tagged CD28, a negative control, was used (**B**, lane 4). Likewise, when cell extracts were used in immunoprecipitation assays with antibody against the FLAG tag, we detected HA-tagged M3-R protein (**C**, lane 5). (**E and F**) When FLAG-tagged OR-S6 was coexpressed with various HA-tagged GPCRs, OR-S6 could be coimmunoprecipitated with either HA-tagged M2-R or HA-tagged M3-R; however, other GPCRs were not coimmunoprecipitated (**F**). Data are from single experiments.