Commentary

The Relative Contributions of cAMP and $InsP_3$ Pathways to Olfactory Responses in Vertebrate Olfactory Receptor Neurons and the Specificity of Odorants for Both Pathways

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One of the recent controversies in olfaction has been the relative contribution of the cAMP and $InsP_3$ (IP_3) second messenger pathways in olfactory transduction, especially in vertebrates, and whether individual odorants specifically act via the two pathways. This controversy has been profitably addressed in a recent study by Takeuchi et al. (2003, in this issue).

Odorants are known to bind to highly selective G protein–coupled receptors, heavily concentrated on the cilia and dendritic knob of each olfactory receptor neuron (ORN). From genetic studies it has been suggested that there are ~500–1,000 different odorant receptors (e.g., in mouse) and that each ORN probably only expresses one type of receptor (Buck, 1996). Furthermore, the evidence suggests that ORNs of a particular type converge to the same glomerulus in the olfactory bulb (Sullivan and Dryer, 1996).

It was proposed by Sklar et al. (1986), on the basis of measurements of adenylyl (adenylate) cyclase (AC) activity, that there are two separate subgroups of odorants that mediate their response via two different transduction mechanisms. In one, the odorant response is mediated via adenosine 3′,5′-cyclic monophosphate (cAMP) and in the other, the response was considered to be mediated by another separate transduction system. They found that the responses of a large number of odorants fell into two groups: (a) in which olfactory AC was clearly stimulated (the "cAMP-dependent" subgroup) and (b) where it was not (the "cAMP-independent" subgroup). Generally, those in the more pleasant (fruity, floral, minty, and herbaceous) categories tended to fall into the first subgroup, whereas those in the more unpleasant (putrid and odorous chemical solvent) categories tended to fall into the second subgroup. However, there were examples from all the various categories that fell into the second subgroup, which failed to obviously stimulate AC. These included (the more pleasant) limonene, lyral, lilial and (the more unpleasant) isovaleric acid, triethylamine, and pyrazine. It was later reported that some of the odorants in the second subgroup, which did not seem to increase AC activity, produced inositol 1,4,5,-trisphosphate ($InsP_3$ or IP_3) instead (e.g., pyrazine in rat olfactory cilia, Boekhoff et al., 1990; lilial and lyral, Breer and Boekhoff, 1991; cited by Takeuchi et al., 2003). It was then suggested that both pathways were important in the olfactory response to odorants (e.g., Restrepo et al., 1996).

In addition, Kashiwayanagi et al. (1996) showed in a whole-cell patch-clamp study that a single bullfrog ORN could be stimulated by odorants from both subgroups (e.g., hedione and/or citralva from the "cAMP-dependent" subgroup and lyral from the other "cAMP-independent" subgroup). They then used a cross-adaptation procedure to try and determine whether these were different odorant receptors. After the inward current response to the first subgroup odorant (e.g., 10 μM hedione) had adapted to its baseline, the second subgroup odorant (e.g., 10 μM lyral) was applied and another inward current was induced. They concluded that single ORNs could carry more than one type of receptor and that these could be mediated by dual second messenger pathways.

However, it has also been suggested that the cAMP pathway is the only pathway mediating olfactory transduction in vertebrates (e.g., Gold, 1999).

In the case of the cAMP pathway the response to odorants has been well established (e.g., in amphibia, Kurahashi and Yau, 1993; and in rat, Lowe and Gold, 1993a). It is now accepted that the G protein, G_{olf}, couples the odorant receptor to adenylyl (adenylate) cyclase, which converts cytoplasmic ATP to cAMP, which is then able to activate a cyclic nucleotide–gated (CNG) channel, allowing cations, particularly Na^+ and Ca^{2+}, to flow down their electrochemical gradients into the cell, depolarizing the ORN. Furthermore, the Ca^{2+} entering the cell is able to activate a Ca^{2+}-activated Cl^- channel (e.g., Kleene and Gesteland, 1991; Lowe and Gold, 1993b), which because of the relatively high internal Cl^- within these cells, would allow Cl^- to flow out of the cell, thus further increasing the depolarization. In addition, the internal free Ca^{2+} can also have a negative feedback effect by binding to calmodulin and a membrane-attached calcium-binding protein to reduce the

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sensitivity of the CNG channels to cAMP (Balasubramanian et al., 1996). The basic molecular organization of these CNG channels, now also cloned, is well understood (e.g., Zufall et al., 1994; Zagotta and Siegelbaum, 1996) and their electrophysiological properties are being thoroughly investigated by many groups using site-directed mutagenesis and patch clamping.

In the case of the $InsP_3$ pathway, a different subset of receptor proteins is considered to be activated by a different subset of odorants, which are coupled to phospholipase C (PLC) by a different G protein (G_q). PLC cleaves the head group of the membrane phospholipid, phosphatidylinositol, to produce diacylglycerol and water soluble $InsP_3$ (e.g., Restrepo et al., 1996). $InsP_3$ can then directly open a Ca^{2+} channel and a nonselective cation channel, which will predominantly let both Na^+ and Ca^{2+} enter the ORN (K^+ would only have a very small electrochemical gradient). In addition, it is also suggested that the increase in Ca^{2+} might activate a calcium-activated K^+ channel, if present. The first two channels would depolarize the ORN, but the last channel would hyperpolarize it. Although $InsP_3$-gated channels have been demonstrated to be present in invertebrates since the early 1990s (e.g., catfish, Restrepo et al., 1990; lobster, Fadool and Ache, 1992), the same was previously not so obvious for vertebrates (e.g., Lowe and Gold, 1993a; Nakamura et al., 1996).

It therefore seemed that the major second messenger system in vertebrates was the cAMP-gated one. Furthermore, a series of elegant mouse "knockout" experiments by Gold and colleagues strongly suggested that the cAMP pathway was the predominant, if not the only, second messenger olfactory transduction pathway in these animals. "Knockout" mice were bred with either dysfunctional CNG channels (Brunet et al., 1996), dysfunctional G_{olf} proteins (Belluscio et al., 1998), or dysfunctional type 3 adenylyl cyclase (Wong et al., 2000), to see how the targeted gene mutations affected olfactory transduction. For the dysfunctional CNG animals, the pore-forming region between the fifth and sixth membrane-spanning regions in the α subunit of the CNG channels was deleted. The density of ORNs, the expression patterns of odorant receptor genes, the presence of adenylyl cyclase, and the other electrophysiological properties of the ORNs did not appear to be affected by the procedure (Brunet et al., 1996). Brunet et al. (1996) tested each of the two subgroups of odorants to see whether they could get any odorant response using an electro-olfactogram (EOG) to measure the summated electrical response of the olfactory epithelium to both cAMP and "$InsP_3$"[1] odorants. Four examples of odorants in each subgroup (cAMP odorants: 2-hexylpyridine, isomenthone, citralva, geraniol; "$InsP_3$ odorants": pyrazine, lilial, ethylvanillin, isovaleric acid) together with three more complex and "natural" substances (mouse urine, coyote urine, and peanut butter) all failed to produce a significant response in the mutant mice, compared with clear strong negative-going electrical responses in the wild-type (WT) mice with normal CNG channels, suggesting that the knockout mice were completely anosmic. In the G_{olf} knockout experiment a null mutation in G_{olf} was employed. It was shown that whereas the expression of olfactory marker protein (OMP), G_s (another G protein, normally of very much lower concentration than G_{olf}), CNG channels and adenylyl cyclase III was essentially unchanged, there was now no distinguishable expression of G_{olf}. The response to all the odorants was essentially insignificant, again supporting the hypothesis that the G_{olf} knockout mice were anosmic (Belluscio et al., 1998). In the dysfunctional adenylyl cyclase (AC3) experiments, in spite of the knockout mice still displaying a normal distribution of G_{olf} and OMP, there was again no significant response in the knockout mice to either subgroup of odorants, again in contrast to the large responses of the WT mice. Furthermore, the AC3 knockout mice failed some olfaction-based behavioral tests suggesting that both the AC3 and the cAMP pathway are critical for olfactory-dependent behavior (Wong et al., 2000).

Around this time, Lischka et al. (1999) showed that there were two groups of $InsP_3$-gated channels, which could be measured in excised patches of soma plasma membrane of rat ORNs. These could be classified into small-conductance (~16 pS) and large-conductance (~64 pS) nonspecific cation-selective channels. Then Kaur et al. (2001) were able to measure the relative abundance of $InsP_3$-gated channels relative to CNG channels in the soma and dendritic knob of rat olfactory neurons. They showed that in the dendritic knobs, the $InsP_3$-gated channel density was only 85 channels/μm^{-2} (with 36% of patches responding) compared with a CNG channel density of 1,000 channels/μm^{-2} (with 83% of patches responding), giving an effective proportion of ~3.6% only of the channels being activated by $InsP_3$. The proportion of $InsP_3$-gated channels at the soma, with much smaller channel densities, was larger (~7%) and it could possibly be even smaller on the cilia, where the channel densities are expected to be the greatest. Hence, even though there were a finite proportion of $InsP_3$-gated channels in these ORNs, the relative density of channels is consistent with the cAMP pathway being absolutely dominant.

The paper by Takeuchi et al. (2003, in this issue) has now reassessed the specificity of odorant receptors in the newt for the two different subgroups of odorants by again exploring the response of a single ORN to both

[1] In order to keep open the possibility that such odorants may still be produced via a cAMP mediated system, an odorant from this subgroup of will be referred to as an "$InsP_3$" odorant.

cAMP odorants and "$InsP_3$" odorants, and by investigating the cross-adaptation between the two subgroups of odorants. Using two independent puffer pipettes to deliver odorants to a single ORN under whole-cell voltage clamp conditions, they measured the induced inward current responses to both subgroups of odorants. They generally found that the currents produced by the "$InsP_3$" odorants (e.g., lilial and lyral) were about half of the magnitude of those produced by the cAMP odorant (cineole) and that the probability of obtaining a response from the "$InsP_3$" odorants was also much smaller than that obtained with the cAMP odorant. In a large number of ORNs, 26% of cells responded to cineole, while only 3.4% and 1.7% responded to lilial and lyral, respectively. By applying odorants of both subgroups (e.g., cineole and lilial) at a concentration of 1 mM to the same cell, they showed that of a group of 1,256 ORNs in which they tested both odorants, 24% responded to cineole (cAMP), 3.4% responded to lilial, and 2.1% responded to both odorants. It was also interesting that there was a positive correlation in cell sensitivity to both odorants, so that there was a higher response to cineole in the lilial-responding cells than in those cells which were insensitive to lilial. Similarly, there was a higher response to lilial in the cineole-responsive cells than in those that were insensitive to cineole. Likewise the time dependence of the response of the same cell to both odorants (shape, latency, time-to-peak, and half decay times) was extremely similar. As in the case of cAMP-producing odorants (e.g., Kurahashi, 1989), Takeuchi et al. (2003) showed that the cell sensitivity for the "$InsP_3$ odorants" was strongly localized to the apical dendrite where the sensory cilia were situated. The current-voltage curve and reversal potential for the "$InsP_3$" odorant-induced currents, and the relationship between the odorant-induced current responses and the pressure and duration of odorant perfusion, were very similar to those previously obtained for the cAMP-producing odorants (e.g., Kurahashi, 1989; Lowe and Gold, 1993a).

Takeuchi et al. (2003, in this issue) also compared self and cross-adaptation between the two types of odorants. First of all, using the "$InsP_3$" odorant, lilial, they used a double pulse protocol (with a pulse duration of 100 ms), giving a conditioning pulse of lilial followed by a second pulse of lilial at different interstimulus intervals. They obtained a typical convex curve, with the second response varying in magnitude from ~37 ± 14% when applied immediately after the first response was terminated, and with full recovery being established after an interval of ~10–20 s. They noted that these were fairly similar parameters to those reported previously for self-adaptation produced by cAMP producing odorants (Kurahashi and Shibuya, 1990; Kurahashi and Menini, 1997). Takeuchi et al. (2003) then

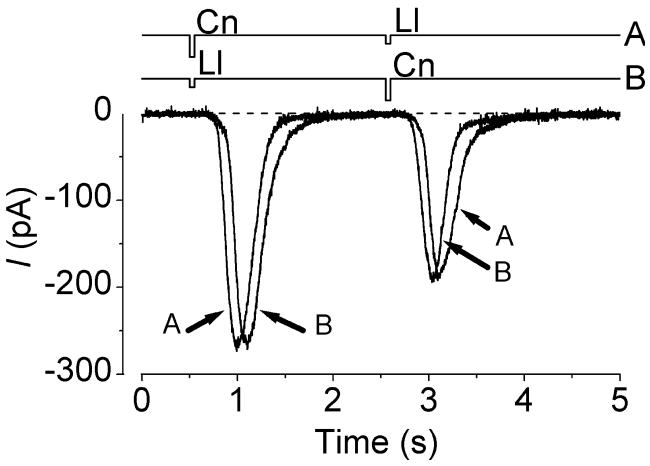

FIGURE 1. A composite figure showing the virtually identical superposition of two interchanged cross-adaptation responses of an "$InsP_3$" odorant t (lilial; Ll) and a cAMP-producing odorant (cineole; Cn) (from Fig. 7 C of Takeuchi et al., 2003, in this issue). The top stimulation trace A indicates the pressure conditioning pulse of cineole and a test pulse of lilial occurring 2 s later, whereas the bottom trace A shows the inward currents generated by the two odorant pulses. The B traces show the effect of a lilial conditioning pulse on a test cineole pulse.

looked at cross-adaptation between the "$InsP_3$" odorant, lilial, and the cAMP-producing odorant, cineole, for ORNs that responded to both odorant types. They adjusted the pressure of the two puffer pipettes to produce the same odorant-induced response on its own. In the first part of their experiment, they gave a conditioning pulse of cineole, following this by a second pulse of lilial 2 s later. They then reversed the sequence, with a conditioning pulse of lilial followed 2 s later by a second pulse of cineole. Their results showed (a) that the conditioning pulse of the first odorant produced an adaptation to the second odorant, even though they belonged to different odorant subgroups, and (b) that the amount of cross-adaptation produced by cineole on the lilial response was virtually identical to that produced by lilial on the cineole response (see Fig. 1).

They then did the same interchange experiment over varying interstimulus intervals and found that the rate of recovery was the same, no matter which odorant was used for the conditioning pulse, with the other being used for the test pulse. The simplest conclusion, which they reasonably drew both from the above cross-adaptation results and their other results, showing very similar response waveforms for both odorant subgroups, was that ". . . both types of odorants activate the same transduction machinery" (Takeuchi et al., 2003, in this issue). Their conclusion also supports the previous work of Chen et al. (2000), who showed that two specific and selective antagonists of adenylyl cyclase blocked the responses of "$InsP_3$" odorants in both single salamander

ORNs and blocked field potentials in the main mouse olfactory epithelium.

While there has been good evidence for the presence of $InsP_3$-gated channels even in mammals and evidence of the generation of $InsP_3$ by some odorants, the weight of evidence seems to suggest that olfactory transduction, particularly in vertebrates, is almost exclusively mediated by the cAMP pathway. The evidence (Takeuchi et al., 2003) also suggests that even the "$InsP_3$" odorants, which have a much smaller probability of inducing a response in ORNs, are still themselves mediating their response via the cAMP transduction machinery. The precise role of any $InsP_3$ generated, and of the relatively small number of $InsP_3$-gated channels, still remains to be elucidated.

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