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The Cellular and Molecular Basis of Odor Adaptation

Frank Zufall and Trese Leinders-Zufall

Department of Anatomy and Neurobiology and Program in Neuroscience, University of Maryland, Baltimore, MD 21201

Correspondence to be sent to: Frank Zufall, Department of Anatomy and Neurobiology, University of Maryland, 685 West Baltimore Street, Baltimore, MD 21201, USA. e-mail: fzufa001@umaryland.edu

Abstract

An important recent advance in the understanding of odor adaptation has come from the discovery that complex mechanisms of odor adaptation already take place at the earliest stage of the olfactory system, in the olfactory cilia. At least two rapid forms and one persistent form of odor adaptation coexist in vertebrate olfactory receptor neurons. These three different adaptation phenomena can be dissected on the basis of their different onset and recovery time courses and their pharmacological properties, indicating that they are controlled, at least in part, by separate molecular mechanisms. Evidence is provided for the involvement of distinct molecular steps in these forms of odor adaptation, including Ca^{2+} entry through cyclic nucleotide-gated (CNG) channels, Ca^{2+}-dependent CNG channel modulation, Ca^{2+}/calmodulin kinase II-dependent attenuation of adenylyl cyclase, and the activity of the carbon monoxide/cyclic GMP second messenger system. Identification of these molecular steps may help to elucidate how the olfactory system extracts temporal and intensity information and to which extent odor perception is influenced by the different mechanisms underlying adaptation.

Introduction

Odor perception not only depends on the chemical structure and strength of an odor stimulus but also on the previous experience of the olfactory neurons, via the process of odor adaptation. In general terms odor adaptation can be viewed, therefore, as a form of neuronal plasticity (Colbert and Bargmann, 1995). In the context of sensory processing, odor adaptation refers to the ability of the olfactory system to adjust its sensitivity at different stimulus intensities, an operation that is likely to be essential for preventing saturation of the cellular transduction machinery and allowing the retention of high sensitivity during continuous or repetitive odor stimulation. Manifestations of adaptation can be seen as a time-dependent, reversible reduction in sensitivity due to prior odor exposure or during steady stimulation with odors, effects that are expected to be associated with a shift in the stimulus–response curve to higher concentrations.

Because of the time-dependence of both the onset of odor adaptation and its recovery (disadaptation), both processes must also play critical roles in determining the temporal response properties of the olfactory system (Moore, 1994). This may be particularly important in olfaction-mediated orientation behaviors. How an odor response changes with time provides important information which is used by animals to help locate the odor source (Christensen *et al.*, 1998). For example, in moths continuous or intermittant stimulation with the same odors elicits two distinct flight behaviors [see (Christensen *et al.*, 1998) and references therein]. This provides evidence that temporal information may be an important part of the chemosensory code. By studying the cellular and molecular basis of odor adaptation, we may be able to understand better how the olfactory system extracts temporal and intensity information from odor signals and to what extent odor perception is influenced by the various mechanisms underlying adaptation.

In this review, we discuss the steps leading to odor adaptation in intact vertebrate olfactory receptor neurons (ORNs) of the main olfactory epithelium, based on the results from our own studies and those of others. The results provide evidence that complex mechanisms of odor adaptation already take place at the earliest stage of the olfactory system, within the olfactory cilia. Odor adaptation depends on feedback signaling causing modulation of the signal transduction machinery present in the olfactory cilia. Ca^{2+}, entering the cilia via the transduction channels, plays a crucial role as a feedback signal. We also summarize the evidence for the coexistence of three different forms of odor adaptation in single ORNs, short-term adaptation–desensitization–long-lasting adaptation, and how these different forms of adaptation can be dissected experimentally.

Potential sites for feedback regulation of odor transduction

It is now known that the odor response of many vertebrate ORNs is generated by a G-protein-coupled transduction

Table 1 Potential sites for feedback regulation of odor transduction in vertebrate ORNs

Second messenger	Target	Mediator	Effect of an increase in second messenger concentration	References
Ca^{2+}	CNG channel	calmodulin or other yet unidentified Ca^{2+} binding proteins	decreases affinity for cAMP; reduces the overall gain of transduction	(Zufall *et al.*, 1991a) (Kramer and Siegelbaum, 1992) (Lynch and Lindemann, 1994) (Chen and Yau, 1994) (Liu *et al.*, 1994) (Balasubramian *et al.*, 1996) (Kurahashi and Menini, 1997) (Kleene, 1999)
	adenylyl cyclase	CaM kinase II	decreases cAMP production; reduces the overall gain of transduction	(Wei *et al.*, 1996, 1998) (Leinders-Zufall *et al.*, 1999a)
	phosphodiesterase	VILIP calmodulin	decreases cAMP production stimulates cAMP/cGMP hydrolysis	(Boekhoff *et al.*, 1997) (Borisy *et al.*, 1992) (Yan *et al.*, 1995)
CO/cGMP	CNG channel		increases Ca^{2+} entry; reduces the overall gain of transduction	(Ingi and Ronnett, 1995) (Leinders-Zufall *et al.*, 1996, 1997) (Zufall and Leinders-Zufall, 1997)
cGMP	protein kinase G		decreases cAMP production; increases cAMP production	(Kroner *et al.*, 1996) (Moon *et al.*, 1998)
NO	CNG channel		stimulates Ca^{2+} entry; inhibits Ca^{2+} entry	(Broillet and Firestein, 1997) (Lynch, 1998)
cAMP	protein kinase A		increases desensitization	(Boekhoff and Breer, 1992)
	G-protein receptor kinase		increases desensitization; reduces cAMP generation	(Dawson *et al.*, 1993) (Schleicher *et al.*, 1993) (Peppel *et al.*, 1997)

cascade that contains a number of sites for feedback regulation (see Table 1). Activated odor receptors couple to a G-protein (G_{olf}), which in turn stimulates type III adenylyl cylase [for a review see (Reed, 1992)]. The resulting cAMP formation causes cyclic nucleotide-gated (CNG) ion channels in the olfactory cilia to open (Nakamura and Gold, 1987; Zufall *et al.*, 1994). The substantial Ca^{2+} permeability of the CNG channels (Zufall and Firestein, 1993; Frings *et al.*, 1995; Dzeja *et al.*, 1999) then causes a rapid Ca^{2+} increase in the lumen of the olfactory cilia (Leinders-Zufall *et al.*, 1997, 1998). This Ca^{2+} signal controls both excitation and adaptation. By activating Ca^{2+}-dependent Cl^- channels conducting a depolarizing Cl^- current, it serves to increase the gain of transduction (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993). The Ca^{2+} rise also mediates adaptation. This latter role for Ca^{2+} was first supported by experiments that showed that the desensitization by steady odor exposure is almost completely absent by exposure to external solutions that eliminate an odor-evoked rise in intracellular Ca^{2+} (Kurahashi and Shibuya, 1990; Zufall *et al.*, 1991a).

These observations stimulated interest in identifying the molecular basis of the action of Ca^{2+} in adaptation. The principal molecular targets of elevated Ca^{2+} are listed in Table 1 and include the CNG channels, adenylyl cyclase and phosphodiesterase. There is now substantial evidence that Ca^{2+} acts on at least two of these components, CNG channels and adenylyl cyclase, to mediate short-term adaptation and desensitization in intact ORNs (see below).

In parallel to these investigations, biochemical studies have shown that cAMP is not the only odor-induced cyclic nucleotide, but that odor stimulation can also lead to an elevation of cGMP (Breer *et al.*, 1992; Kroner *et al.*, 1996; Moon *et al.*, 1998). cGMP has also been proposed to act as a feedback messenger in odor adaptation (Breer and Shepherd, 1993), stimulating interest in examining the precise function of cGMP in ORNs (Zufall and Leinders-Zufall, 1998). There is now considerable evidence for an involvement of cGMP in a form of adaptation that operates on a much slower time scale than short-term adaptation and desensitization (see below). Finally, several Ca^{2+}-independent mechanisms have also been proposed to mediate odor adaptation, including odor receptor phosphorylation by protein kinase A (Boekhoff and Breer, 1992) and G-protein-coupled receptor kinase 3 (GRK3) (Dawson *et al.*, 1993; Schleicher *et al.*, 1993; Peppel *et al.*, 1997), but as yet there is no evidence for a role of these mechanisms in adaptation in intact ORNs.

Short-term adaptation is mediated by Ca^{2+} entry through CNG channels

For our initial efforts to investigate the mechanisms underlying odor adaptation in intact ORNs, we have used ORNs that were freshly dissociated from salamander olfactory epithelium. The neurons were voltage-clamped by means of the perforated patch-clamp technique. The simplest form of odor adaptation obtained under these experimental conditions is depicted in Figure 1. When a brief (100 ms) odor stimulus is presented twice within a limited period of time, less neural activity is evoked by the second presentation. As the interpulse interval is increased, the amplitude of the second response becomes larger and eventually, with an interval ≥10 s, matches the first (conditioning) response. Because of this relatively rapid recovery, which has been observed in both salamander and newt (Kurahashi and Shibuya, 1990; Kurahashi and Menini, 1997; Leinders-Zufall *et al.*, 1998, 1999a), we termed this phenomenon short-term adaptation (STA). The rate of recovery from STA was derived from single exponential fits of a plot of the amplitude of the second response versus the interpulse interval. This analysis revealed that amphibian ORNs recover from STA with a time constant of 4–5 s (Leinders-Zufall *et al.*, 1998, 1999a).

We have hypothesized that the Ca^{2+} transients present in the olfactory cilia following cAMP-mediated gating of the CNG channels not only provide a key signal for triggering STA, but also determine the rate of recovery from STA. To test this, we have used high resolution confocal Ca^{2+} imaging to visualize localized, odor-induced Ca^{2+} changes in individual olfactory cilia. This approach has enabled us for the first time to analyze the time courses of the ciliary Ca^{2+} signals in response to brief odor stimuli (Figure 2A,B). The ciliary Ca^{2+} transients probably depend entirely on Ca^{2+} entry through the CNG channels but not on other sources such as voltage-operated Ca^{2+} channels and Ca^{2+} stores (Leinders-Zufall *et al.*, 1997, 1998; Zufall *et al.*, 2000). Superposition of the recovery time course of the Ca^{2+}

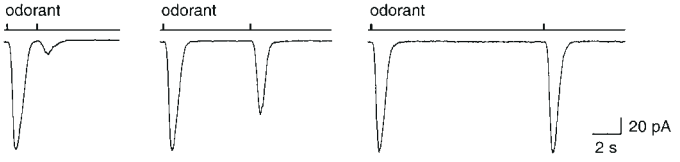

Figure 1 STA of the odor response of an intact ORN. An ORN was stimulated with two identical 100 ms pulses of cineole (300 μM) with varying interpulse intervals, separated by 40 s rest periods. The cell was voltage-clamped to –60 mV and the resulting membrane currents were monitored. For an interpulse interval of 2 s (left trace), the second pulse elicited a strongly declined peak response reflecting odor adaptation. This effect lasted only for several seconds. As the interpulse interval was increased to 6 s (middle trace), partial recovery from adaptation was evident. With an interpulse interval of 10s (right trace), the amplitude of the second response matched the first one, indicating that recovery from adaptation was complete. Modified from (Leinders-Zufall *et al.*, 1998).

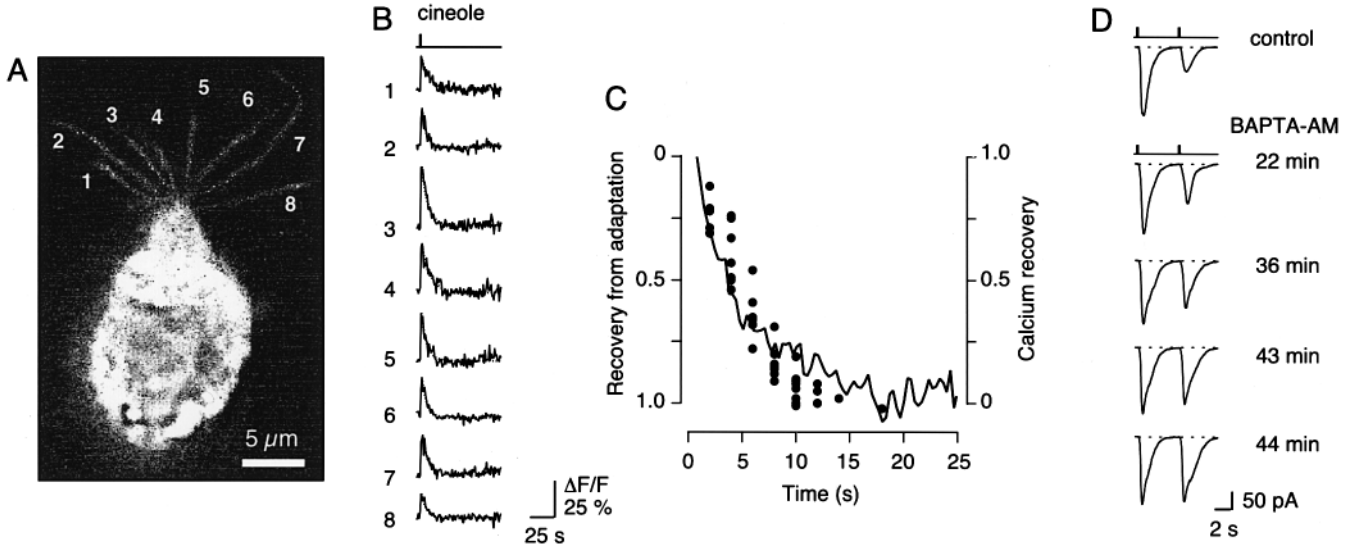

Figure 2 Evidence that ciliary Ca^{2+} transients are critical for STA. **(A)** Fluorescence image (grey scale) of an ORN taken at peak fluorescence intensity following a 1 s pulse of cineole (300 μM). Eight individual cilia (indicated by the numbers 1–8) are clearly seen. **(B)** Time course of odor-induced ciliary Ca^{2+} transients. All cilia produced synchronized Ca^{2+} transients with remarkably uniform kinetic properties following exposure to odor ligand. **(C)** The recovery from STA matches the decay time course of the ciliary Ca^{2+} transients. Shown are normalized, averaged Ca^{2+} responses (cineole, 300 μM) from five ORNs (23 cilia) (solid line). The decay time course was fitted with a single exponential function giving a time constant $\tau = 5.1$ s. Superimposed in this graph is the recovery from STA measured in eight individual ORNs (closed circles) using the protocol shown in Figure 1. The responses are expressed as a percentage of the fully recovered amplitude. Employing a nonlinear least squares fitting routine, the data points were fitted with a single exponential function of a time constant $\tau = 5.6$ s (not shown). **(D)** STA is disrupted after bath-application of the membrane-permeant intracellular Ca^{2+} chelator BAPTA-AM (100 μM). Paired odor responses (cineole, 300 μM) are elicited at a fixed interval of 4 s. As the BAPTA-AM is taken up by the cell and cleaved by endogenous esterases, the response to the second pulse becomes larger until it eventually matches the first response. Modified from (Leinders-Zufall *et al.*, 1998).

signals (Figure 2C, continuous line) onto the STA recovery curve (Figure 2C, closed circles) revealed a close match between both curves, indicating that the dynamics of the Ca^{2+} signal indeed determine the rate of recovery from STA. If this hypothesis is correct then STA should be eliminated in the absence of a Ca^{2+} signal. To demonstrate this, we applied BAPTA-AM, a membrane-permeant intracellular Ca^{2+} chelator (Figure 2D). Ca^{2+} buffering resulted in a nearly complete loss of STA. This was demonstrated by the time-dependent increase of the odor current caused by the second pulse, eventually reaching the same response magnitude as the first current (Leinders-Zufall *et al.*, 1998). Together, these two results provide evidence that odor-induced ciliary Ca^{2+} transients are essential for mediating negative feedback regulation underlying STA, and that their recovery time course is sufficient to explain the time course of recovery from STA. Furthermore, when Na^+-dependent Ca^{2+} extrusion is abolished by exposing ORNs to a solution in which choline$^+$ has been substituted for Na^+, recovery from adaptation is prevented (Reisert and Matthews, 1998). This indicates that the restoration of Ca^{2+} to basal levels plays a major role in restoring ORN sensitivity after stimulation.

Ca^{2+} modulation of the CNG channels as a mechanism for short-term adaptation

How does elevated Ca^{2+} in the ciliary lumen lead to odor adaptation? We have hypothesized that the CNG channel itself is a target of a Ca^{2+}-mediated negative feedback loop causing odor adaptation (Zufall *et al.*, 1991a). Perhaps the best evidence that STA occurs by down-regulation of the cAMP-gated channels comes from a study by Kurahashi and Menini (Kurahashi and Menini, 1997). These authors compared the properties of adaptation induced by brief odor pulses with the adaptation induced by photolysis of caged cAMP and found no significant difference. Because second messenger production in the case of caged cAMP was determined by the light pulse and not by G-protein-coupled activation of adenylyl cyclase, it was concluded that adaptation occurs entirely downstream from adenylyl cyclase. The data also provided some evidence that phosphodiesterase was not involved in STA, leaving the CNG channels as the only possible locus of adaptation (Kurahashi and Menini, 1997).

Much interest has focused on the precise mechanisms by which Ca^{2+} can down-regulate CNG channel activity. Initially, we found that elevated intracellular Ca^{2+} could decrease the open probability of single CNG channels, but the underlying mechanism remained unclear (Zufall *et al.*, 1991a). This was followed by a paper concluding that an unknown endogenous Ca^{2+}-binding protein decreases the binding affinity for cAMP at the CNG channel (Kramer and Siegelbaum, 1992). Other studies showed that added Ca^{2+}/calmodulin can serve to modulate CNG channels (Chen and Yau, 1994; Liu *et al.*, 1994). More recently, new evidence has been presented suggesting that either calmodulin or another, unknown endogenous factor, or both, mediates this Ca^{2+}-induced modulation (Balasubramian *et al.*, 1996; Kleene, 1999). Meanwhile, a variety of Ca^{2+}-binding proteins, including calmodulin, calretinin, calbindin-D28k, neurocalcin, recoverin, p26olf and visinin-like protein, have been identified in ORNs (Bastianelli *et al.*, 1995; Boekhoff *et al.*, 1997; Miwa *et al.*, 1998) but, except for calmodulin (Liu *et al.*, 1994), it is unclear whether any of these molecules interacts with CNG channels. Thus, definitive evidence on the nature of the endogenous molecule(s) mediating Ca^{2+}-dependent CNG channel modulation and STA is still lacking.

Ca^{2+}/calmodulin kinase II is critical for adaptation to maintained odor stimuli

It is now clear that odor adaptation is more complex than previously thought and that Ca^{2+} modulation of the CNG channels is not the only mechanism for adaptation. When an odor stimulus is presented for a prolonged period (8 s in our experiments), there is a decline in the sensory response despite the continued presence of the odorant, an effect that was termed odor response desensitization (Figure 3, control). This desensitization, which was first observed by Ottoson (Ottoson, 1956) using EOG recordings, is thought to represent the main manifestation of odor adaptation (Getchell and Shepherd, 1978). Like STA, desensitization is triggered by Ca^{2+} entry through the transduction channels

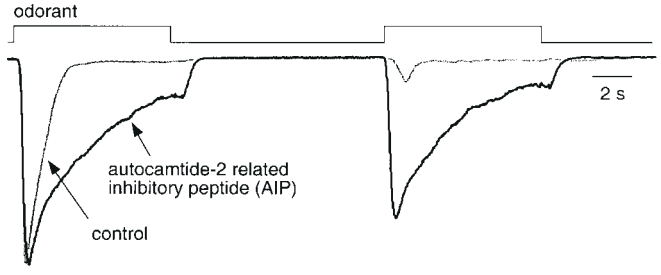

Figure 3 Adaptation to sustained odor pulses (desensitization) depends on CaMKII. An ORN was stimulated with two identical 8 s odor pulses (50 μM cineole) separated by an interpulse interval of 11 s. Under control conditions (control, grey trace) the current to the first stimulus decreased from a peak value of 330 pA to a plateau value of ~3 pA during odor exposure. Recovery from the adaptation induced by an 8 s stimulus was much slower than from the adaptation caused by a 100 ms stimulus (compare with Figure 1). This is evident in the response to the second stimulus whose peak amplitude was still strongly attenuated, even after an interval of 11 s. To test for an involvement of CaMKII in desensitization, ORNs were pretreated with a membrane-permeant form of a specific CaMKII inhibitor, AIP (1 μM). With CaMKII function disrupted, odor adaptation was strongly impaired. It can be seen that the onset rate of adaptation was markedly reduced and that the peak amplitude of the second response was much greater than in untreated ORNs (AIP, black trace). This provides evidence that CaMKII controls both the onset and recovery rate from adaptation to sustained odor pulses. Modified from (Leinders-Zufall *et al.*, 1999a).

(Kurahashi and Shibuya, 1990; Zufall *et al.*, 1991a; Leinders-Zufall *et al.*, 1999a) and does not require odor receptor activation (Leinders-Zufall *et al.*, 1999a). However, when we compared the properties of desensitization and STA in more detail, we found that both depended on distinct sets of molecular mechanisms, leading us to conclude that desensitization and STA represent different forms of odor adaptation (Leinders-Zufall *et al.*, 1999a).

There are three principal differences between STA and desensitization. First, the recovery rate from adaptation to sustained odor pulses is significantly slower (five- to sixfold) than from adaptation to brief (100 ms) pulses. On average, it took ~1–1.5 min to fully recover from the adaptation induced by an 8 s stimulus under our experimental conditions. This recovery time is much longer than the recovery of the measured Ca^{2+} transients in the olfactory cilia, suggesting that the kinetics of the ciliary Ca^{2+} signal alone are not sufficient to account for the recovery from desensitization (Leinders-Zufall *et al.*, 1999a).

Second, desensitization involves distinct changes in the odor response kinetics that are not seen with STA. Specifically, the slope of the initial rising phase of the sensory current, which reflects the activity of adenylyl cyclase, is reduced after adaptation to a sustained stimulus. At the same time, the rate of desensitization is increased relative to the control values. This indicates that ORNs must use a variety of Ca^{2+}-dependent steps for adaptation, depending on the exact conditions of odor stimulation. In particular, the data suggest that Ca^{2+}, in regulating odor adaptation, not only modulates the activity of cAMP-gated channels but also reduces the rate of adenylyl cyclase activation (Leinders-Zufall *et al.*, 1999a).

Third, to test directly whether Ca^{2+}-mediated attenuation of adenylyl cyclase is involved in desensitization, we attempted to disrupt this molecular step. Previously, it was reported that Ca^{2+}/calmodulin kinase II (CaMKII) is abundantly expressed in olfactory cilia and that CaMKII-mediated phosphorylation can attenuate the activity of olfactory adenylyl cyclase type III (Wei *et al.*, 1998). We therefore applied a potent and selective inhibitor of CaMKII, autocamtide-2-related inhibitory peptide (AIP, 1 μM), which does not affect other protein kinases (Ishida *et al.*, 1995). With CaMKII function disrupted, the onset rate of desensitization was markedly reduced (by threefold) and the peak amplitude of the second response was much greater than in untreated ORNs, indicating that recovery from adaptation occurred at a faster rate (Figure 3, AIP) (Leinders-Zufall *et al.*, 1999a). Using a paired-pulse paradigm, we found that recovery from desensitization was more than sixfold faster after AIP-treatment. At the same time, the properties of STA remained unchanged (Leinders-Zufall *et al.*, 1999a). Thus, only adaptation induced by sustained odor pulses was impaired after AIP-treatment whereas adaptation induced by brief odor pulses was not, providing evidence that STA and desensitization depend differentially on CaMKII.

We conclude, therefore, that Ca^{2+} entry through CNG channels leads to feedback regulation at multiple sites in the cAMP signaling cascade, depending on the exact conditions of odor stimulation, and that this feedback regulation is an essential component of odor adaptation (see Figure 4). Our studies provide the first evidence that CaMKII function is necessary for determining the temporal response properties of ORNs during odor adaptation. Although CaMKII most likely mediates its effect by attenuation of adenylyl cyclase, additional targets for CaMKII function cannot be ruled out. Based on our results and those of Kurahashi and Menini (Kurahashi and Menini, 1997), one can estimate quantitatively the contribution of two distinct Ca^{2+}-dependent mechanisms to odor adaptation: modulation of CNG channels and adenylyl cyclase. If ORNs are exposed to a brief odor pulse, there seems to be no contribution of adenylyl cyclase. In this case, all adaptation appears to depend on Ca^{2+} modulation of the CNG channels because CaMKII disruption has no effect and all aspects of adaptation can be mimicked by photolysis of caged cAMP. If

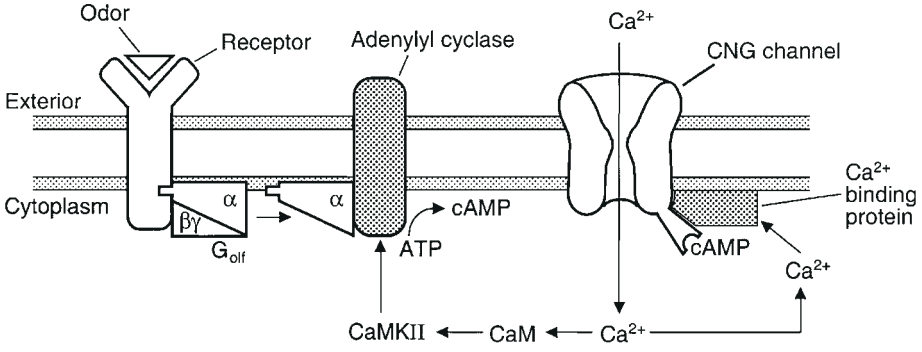

Figure 4 Schematic diagram of olfactory signal transduction and its Ca^{2+}-dependent feedback regulation during STA and desensitization. Note that, within the olfactory cilia, Ca^{2+} entry occurs via activated CNG channels. Elevated intracellular Ca^{2+} causes adaptation by at least two different molecular steps: inhibition of the activity of adenylyl cyclase via CaMKII-dependent phosphorylation and down-regulation of the affinity of the CNG channel to cAMP. The Ca^{2+} binding protein involved in this reaction may be calmodulin or another, as yet unidentified protein, or both. See text for additional details. This is an extension and modification of two other models (Kramer and Siegelbaum, 1992; Balasubramanian *et al.*, 1996).

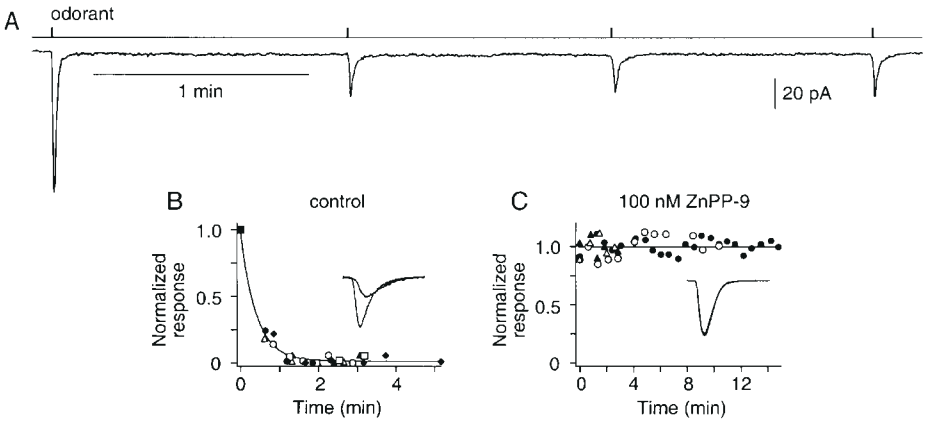

Figure 5 LLA of the odor response of an intact ORN. **(A)** Response of an individual ORN to four repeated 100 ms odor pulses (cineole, 100 μM) plotted at low time resolution. Note that, despite the fact that the interpulse interval was >1 min, which is sufficient to allow for recovery from early forms of adaptation, the response to the second stimulus nonetheless was strongly reduced compared with the response to the first pulse. There was no further decrement in the responses to subsequent stimuli. This adaptive effect was fully reversible after 6 min in odor-free solution (not shown). **(B)** Plot of the onset time course of LLA under control conditions. Data are from six cells (shown with different symbols). Cells are stimulated with 300 μM cineole. Odor responses of a given cell are normalized to $(I - I_{LLA})/(I_{control} - I_{LLA})$, so that the incremental peak response under control conditions ($I_{control}$) is set to the value one and the mean equilibrium peak response after producing LLA (I_{LLA}) is given the value zero. The continuous line is a best fit of the data with a single exponential function giving a time constant $\tau = 24$ s. Insets are representative waveforms of transient odor-induced currents taken before LLA (large response) and after LLA was established (small response). **(C)** Effect of ZnPP-9 (100 nM, $n = 4$) on LLA demonstrating that LLA is abolished after ZnPP-9 treatment in all tested cells. The solid line was computed by regression analysis. Insets demonstrate that the characteristic properties of the cAMP-mediated response remain unchanged during ZnPP-9 treatment and that there is no response decrement over time (the second response was taken 12 min after the first). Modified from (Zufall and Leinders-Zufall, 1997).

ORNs are exposed to sustained odor pulses, however, CaMKII inhibition of adenylyl cyclase becomes rate-limiting for recovery from adaptation and also contributes significantly to the onset rate of adaptation.

Long-lasting adaptation depends on the CO/cGMP second messenger system

There is mounting evidence from a variety of vertebrate and invertebrate animal models that, in addition to these rapid forms of odor adaptation, ORNs can also undergo much slower and longer-lasting changes in sensitivity to odors (Getchell, 1986; Voigt and Atema, 1990; Marion-Poll and Tobin, 1992; Colbert and Bargmann, 1995), but the molecular basis of these forms of adaptation remained unclear. In our experiments, we noted that the odor sensitivity of the cells sometimes was reduced after only a single brief odor stimulus and that this effect was more persistent than the adaptation phenomena described above, as if the cell would switch to a different sensitivity mode (Figure 5A). There is now considerable evidence that this adaptive effect, at least in salamander ORNs, depends on the carbon monoxide (CO)/cGMP second messenger system because it can be uncoupled from excitation and completely eliminated by inhibitors of CO synthesis (Zufall and Leinders-Zufall, 1997).

A starting point for our investigations into the mechanisms underlying persistent forms of adaptation was the question of whether olfactory cilia contain signaling pathways other than the cAMP cascade that also lead to Ca^{2+} influx into the cilia. Given that Ca^{2+} is critical for feedback regulation, any mechanism that causes a Ca^{2+} elevation within the olfactory cilia potentially could be significant for odor adaptation. Currently, there is only one other second messenger besides cAMP that is known to cause a ciliary Ca^{2+} rise, namely cGMP. This was demonstrated by the result that application of a membrane-permeant cGMP analog caused a sustained Ca^{2+} rise due to the gating of CNG channels that was confined spatially to the cilia and the knob (Leinders-Zufall *et al.*, 1997). Because of the high sensitivity of the cyclic nucleotide binding site of native salamander CNG channels (Zufall *et al.*, 1991b), micromolar doses of cGMP are sufficient to cause tonic Ca^{2+} elevations in the cilia. These localized Ca^{2+} elevations can persist for time spans of several minutes, depending on the time course of the cGMP signal (Leinders-Zufall *et al.*, 1997). These results fit well with biochemical studies indicating that odor-induced cGMP signals can outlast the cAMP transient for min (Breer *et al.*, 1992; Kroner *et al.*, 1996; Moon *et al.*, 1998). We also found that a Ca^{2+} rise caused by micromolar amounts of cGMP is capable of influencing the responsiveness of the ORNs: at this low concentration, cGMP produces only a low-level, subthreshold inward current that is not sufficient to excite the neurons but, due to the persistant leakage of Ca^{2+}, the odor responses are attenuated and the stimulus–response relation is shifted to the right, by perhaps as much as 20-fold (Leinders-Zufall *et al.*, 1996). CO, a diffusible messenger that has been proposed to act as an endogenous stimulator

Table 2 Distinct forms of odor adaptation in vertebrate olfactory receptor neurons

	Short-term adaptation (induced by a brief odor pulse)	Desensitization (induced by a sustained odor pulse)	Long-term adaptation (cumulative adaptation, brief repetitive odor pulses)
Onset time constant	?	1–4 s	25 s
Recovery time constant	5 s	25 s	a few minutes
Adaptation-induced changes in odor response kinetics	no changes	prolonged rising phase; accelerated decay phase	prolonged rising phase; prolonged decay phase
Activation threshold	no	no	yes
Odor receptor activation necessary	no	no	?
Ca^{2+} dependence (BAPTA-AM)	abolished	abolished	abolished
CaMKII phosphorylation (AIP, 1 μM)	no effect	impaired	?
CO/cGMP pathway (ZnPP-9, 100 nM)	no effect	no effect	abolished
NO/cGMP pathway (L-NOARG, 100 μM)	no effect	no effect	no effect

of cGMP formation in ORNs (Ingi and Ronnett, 1995; Ingi *et al.*, 1996; Leinders-Zufall *et al.*, 1995), mimicked the effects of cGMP, indicating that sufficient amounts of cGMP were synthesized within the olfactory cilia (Leinders-Zufall *et al.*, 1996).

Based on these results, we hypothesized that the CO/cGMP second messenger system is part of an endogenous signaling cascade in ORNs that is involved in slower and more persistent sensitivity changes to odors. To test this, we searched for long-lasting forms of odor adaptation with characteristics similar to the effects of exogenous CO/cGMP and identified a form of adaptation that operates on the time scale of minutes (Figure 5A,B), a phenomenon that was termed long-lasting adaptation (LLA) (Zufall and Leinders-Zufall, 1997). Under our experimental conditions, LLA occurred only in a subset of the experiments making it possible to study early forms of adaptation without interference from LLA. Because the properties of LLA were indistinguishable from the effects of exogenous cGMP or CO, we tested whether it was caused by endogenous cGMP formation. This was done by investigating the effects of pharmacological inhibitors of second messengers that act as upstream stimulators of the cGMP-synthesizing enzyme guanylyl cyclase. LLA was completely abolished by agents that are known to inhibit the function of the CO-producing enzyme heme oxygenase type 2 (HO-2), such as zinc(II) protophorphyrin IX (ZnPP-9) and zinc(II) deuteroporphyrin IX 2,4-bisglycol (ZnBG) (Figure 5C). By contrast, two inhibitors of nitric oxide (NO) production, N^G-nitro-L-arginine and N^G-monomethyl-L-arginine, were without effect. These results indicated that endogenously formed CO, but not NO, is essential for LLA (Zufall and Leinders-Zufall, 1997). Meanwhile, we have also tested whether inhibitors of CO or NO formation would inhibit the two early forms of adaptation described above. However, this was not the case (see Table 2). Thus, the HO-2 inhibitors used here selectively block one particular form of odor adaptation in single ORNs, whereas the NO synthase inhibitors are ineffective. As a whole, these data provide the first clear evidence for a distinct function of the CO/cGMP second messenger pathway in the nervous system, but more work is needed to fully understand how CO synthesis is coupled to odor receptor activation and through which steps an odor-induced cGMP elevation is translated into long-lasting changes in odor sensitivity.

Concluding remarks

The work summarized in this review shows that odor adaptation at the level of a single ORN requires complex mechanisms of second messenger signaling. Our results provide evidence that vertebrate ORNs express at least two rapid forms and one persistent form of odor adaptation. These three different adaptation phenomena can be distinguished on the basis of their different onset and recovery time courses and their pharmacological properties, indicating that they are controlled, at least in part, by separate molecular mechanisms (Table 2). We have also provided evidence for the involvement of multiple molecular steps in odor adaptation including Ca^{2+} entry through CNG channels, Ca^{2+}-dependent CNG channel modulation, CaMKII-dependent phosphorylation and the activity of the CO/cGMP second messenger system. These results challenge an earlier notion stating that odor adaptation is a simple process and consists essentially of a single molecular step, Ca^{2+} modulation of the CNG channels (Gold and Pugh, 1997; Kurahashi and Menini, 1997).

One important future goal will be to relate the different molecular and cellular events identified here to odor perception. An unexpected result of our work is that odor adaptation essentially includes mechanisms that are not unlike those involved in other use-dependent forms of cellular plasticity in the central nervous system (Malenka and Nicoll, 1999). Given these considerations, we believe

that the best approach for studying the relation between molecular, cellular and behavioral odor adaptation is to use similar strategies as those developed for the investigation of synaptic plasticity in learning and memory, namely to conduct experiments with transgenic animals in which specific candidate molecules have been rendered nonfunctional [see, for example (Silva *et al.*, 1992)]. It seems unlikely that this approach can be conducted in salamander in the near future, but rather, may be performed in animal models that are more accessible to genetic manipulation, such as the mouse. We therefore have begun to develop physiological preparations that are more amenable to this goal (Leinders-Zufall *et al.*, 1999b, 2000).

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