Modulation by cyclic GMP of the odour sensitivity of vertebrate olfactory receptor cells

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SUMMARY

Recent evidence has indicated a significant role for the cGMP second messenger system in vertebrate olfactory transduction but no clear functions have been identified for cGMP so far. Here, we have examined the effects of 8-Br-cGMP and carbon monoxide (CO) on odour responses of salamander olfactory receptor neurons using perforated patch recordings. We report that 8-Br-cGMP strongly down-regulates the odour sensitivity of the cells, with a Ki of 460 nM. This adaptation-like effect can be mimicked by CO, an activator of soluble guanylyl cyclase, with a Ki of 1 μM. Sensitivity modulation is achieved through a regulatory chain of events in which cGMP stimulates a persistent background current due to the activation of cyclic nucleotide-gated channels. This in turn leads to sustained Ca2+ entry providing a negative feedback signal. One consequence of the Ca2+ entry is a shift to the right of the stimulus-response curve and a reduction in saturating odour currents. Together, these two effects can reduce the sensory generator current by up to twenty-fold. Thus, cGMP functions to control the gain of the G-protein coupled cAMP pathway. Another consequence of the action of cGMP is a marked prolongation of the odour response kinetics. The effects of CO/cGMP are long-lasting and can continue for minutes. Hence, we propose that cGMP helps to prevent saturation of the cell’s response by adjusting the operational range of the cAMP cascade and contributes to olfactory adaptation by decreasing the sensitivity of olfactory receptor cells to repeated odour stimuli.

1. INTRODUCTION

The primary response of vertebrate olfactory receptor neurons (ORNs) to many odour ligands is a rapid rise in cAMP (Pace et al. 1985; Sklar et al. 1986; Breer et al. 1990; Jaworsky et al. 1995) which directly activates cyclic nucleotide-gated nonspecific cation channels (CNG channels) (Nakamura & Gold 1987; Kurahashi 1990; Firsstein et al. 1991a, b; Zufall et al. 1991a) allowing Na+ and Ca2+ to enter the cell (for a review, see Zufall et al. 1994). Recent evidence from different converging experimental lines has indicated that cAMP is not the only odour-induced cyclic nucleotide but that cGMP also plays a role in odour transduction. Biochemical experiments have demonstrated that strong odour stimuli can cause additional formation of cGMP (Breer et al. 1992; Verma et al. 1993). Odour-induced cGMP formation occurs on a relatively slow timescale and remains elevated for much longer than the primary odour responses, sometimes outlasting the cAMP signal for minutes (Breer et al. 1992). The olfactory CNG channels can be gated by both cAMP and cGMP and even have a greater affinity for cGMP compared with cAMP (Nakamura & Gold 1987; Zufall et al. 1991a; Frings et al. 1992). This dualism extends to the level of the phosphodiesterase which also has high affinity for both cAMP and cGMP (Yan et al. 1995).

Further insights into the role of cGMP have been gained from studies of how the cGMP concentrations can be regulated in ORNs. Most if not all ORNs express a soluble guanylyl cyclase (sGC) in their cilia (Verma et al. 1993; Ingi & Ronnett 1995) and, at least in a distinct subpopulation of ORNs, a novel membrane receptor guanylyl cyclase has been identified (Fülle et al. 1995). Odour-induced cGMP formation is inhibited by haemoglobin providing a direct link between cGMP signals and the activity of diffusible messengers in this process (Breer et al. 1992; Verma et al. 1993). It has been proposed that carbon monoxide (CO) functions as an endogenous activator of olfactory sGC, based on the fact that the CO-producing enzyme heme oxygenase-2 (HO-2) exists in exceptionally high levels in olfactory cilia and that it is colocalized with sGC (Verma et al. 1993; Ingi & Ronnett 1995). In contrast, nitric oxide (NO) producing enzymes seem to be absent from adult ORNs (Kishimoto et al. 1993; Breit & Snyder 1994; Kulkarni et al. 1994; Roskams et al. 1994) arguing against a role of NO in odour transduction. Further support for CO as a regulator of sGC in adult ORNs comes from the findings that CO can be produced and released from ORNs (Ingi &
Ronnett 1995), that its production appears to occur in an odour-dependent manner (Verma et al. 1993), and that it acts as a potent activator of the CNG channels in intact ORNs via the cGMP pathway (Leinders-Zufall et al. 1995), allowing to establish a quantitative relation between CO and cGMP levels (Leinders-Zufall et al. 1995).

Despite this various evidence, no clear functions for cGMP in odour transduction have been identified thus far. Based on the previous results it has been suggested that cGMP may play a role in sensory adaptation of ORNs (Breer & Shepherd 1993; Leinders-Zufall et al. 1995), but experimental evidence for this hypothesis is lacking. We therefore have tested in the present work whether cGMP provides a molecular signal that can influence the odour responsiveness of the ORNs. Our results demonstrate that low concentrations of 8-Br-cGMP are sufficient to down-regulate the sensitivity of ORNs to their natural stimuli. This adaptation-like effect of 8-Br-cGMP can be mimicked by CO. A preliminary account of some of these data has appeared (Zufall et al. 1995).

2. METHODS

Current recordings under voltage-clamp (at —60 mV) were made from single freshly dissociated ORNs of the tiger salamander (Ambystoma tigrinum) while brief 100-ms long pulses of various concentrations of the odour molecule cineole (Firestein et al. 1993) were applied through micropuffusion to elicit odour responses. Procedures for the isolation of ORNs closely followed those described previously (Leinders-Zufall et al. 1995). To ensure the least possible disturbance of the internal milieu of the neurons and to prevent artificial Ca2+ buffering from influencing the results we have used here the perforated patch technique with amphoterin B as a membrane permeabilizing agent (Horn & Marty 1988; Rae et al. 1991). The methods for obtaining perforated patch recordings were as described in Leinders-Zufall et al. (1995). Current recordings, command potential sequences, data acquisition and on-line analysis were controlled by an EPC-9 patch clamp amplifier in combination with the Pulse software package (HEKA Electronic, Germany) and a Macintosh computer. Continuous and evoked currents were filtered at 300 Hz (—3 dB, 8-pole low-pass Bessel) and digitally sampled at 1 ms per point. The indifferent electrode consisted of an Ag-AgCl wire connected to the bath solution (see figure 1a). In virtually every experiment the membrane permeant cGMP analogue 8-Br-cGMP was added to the Ringer's solution and the remaining free Ca2+ concentration was verified by means of a Ca2+ selective electrode. For the experiment of figure 4b the external solution was buffered with EGTA to give a free Ca2+ level of ca. 1 μM. A concentration of 1 μM Ca2+ is at the limit of precision of our Ca2+ selective electrode and therefore should be regarded as an upper limit for the accurate value. An estimate for this value was calculated from the stability constants giving 0.6 μM free Ca2+. In all experiments, the patch pipettes were filled with the following solution (in mM): KCl, 17.7; KOH, 92.3; methanesulfonic acid, 82.3; EGTA, 5.0; HEPES, 10; pH 7.5 (KOH).

Odour solutions containing cineole (Sigma) were prepared in Ringer's solution with less than 0.1% DMSO. Focal stimulation of olfactory cilia was obtained by pressure ejecting the odourant solutions from multi-barrel glass pipettes which were placed within 5–10 μm from the cilia. Stimulus pipettes were located downstream from the cells. Under these conditions, the solution switching time was 30–40 ms as measured by the response to high K+ solutions. Odourant dose-response curves obtained with this method were in close agreement with previously described cineole responses in these cells (Firestein et al. 1993). To exclude a possible contribution of Ca2+-dependent desensitization mechanisms from our measurements (Kurahashi & Shibuya 1990; T. Leinders-Zufall & F. Zufall, unpublished observation) we used relatively long interstimulus intervals of ≥ 40 s for repetitive odour stimulation.

Tonic membrane currents were analysed using voltage ramps (slope: 0.35 mV ms-1) (Leinders-Zufall et al. 1995). Because of the very low level in baseline noise of the amphotericin B recordings (0.15–0.2 pA rms, at 300 Hz) we could reliably detect tonic shifts in the baseline current as low as 0.5 pA. These small currents are ca. six-fold below the resolution limit of previous whole-cell measurements (for comparison, see: Lowe & Gold 1993a). The seal resistance was continuously monitored to rule out that the observed currents resulted from small changes in the recording conditions. No standing ('basal') currents were observed in the absence of odor stimuli in our recordings (n = 15), which is in contrast to predictions made from experiments using the whole-cell recording technique (Lowe & Gold 1993a).

Chemicals such as 8-Br-cGMP, cadmium, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) or LY83583 ([3-phenylamino]-5,8-quinolinedione; Research Biochemicals International) were prepared in external solution as previously described (Leinders-Zufall et al. 1995). Likewise, the procedures for CO generation were as in a previous publication (Leinders-Zufall et al. 1995). The gas was obtained in research quality (Matheson, Gloucester, Massachusetts) and a small amount of CO stock solution was prepared by bubbling the gas until saturation in distilled water. This solution was immediately diluted to the desired concentration in Ringer's solution and was injected directly into the recording chamber.

All data analysis and calculations were done using the Igor Pro software package (WaveMetrics Inc., Oregon, U.S.A.) running on Macintosh computers. Through this program user-defined functions in combination with an iterative Levenberg-Marquardt nonlinear, least-squares fitting routine were applied to the data. Results are expressed as means ± s.d. and number of observations (n).

3. RESULTS

To determine whether cGMP would alterate the odour responsiveness of individual ORNs, we added the membrane permeant cGMP analogue 8-Br-cGMP to the bath solution (see figure 1a). In virtually every cell tested, the presence of 8-Br-cGMP led to several coordinated changes in the whole-cell responses.

First, the peak amplitude of transient inward currents elicited by fixed odour concentrations was markedly reduced during application of 8-Br-cGMP (figure 1a). This effect was concentration dependent. Results from seven experiments were plotted to give a
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500 nM 8-Br-cGMP 2.0 μM 8-Br-cGMP

Figure 1. (a) Effect of 8-Br-cGMP on the odour response of an isolated olfactory receptor cell. Perforated patch recording, voltage-clamped to —60 mV. Exposure of the cell to 500 nM 8-Br-cGMP reduces peak odour response amplitudes from 34.1 ± 1.8 pA (n = 4) to 17.8 ± 0.7 pA (n = 4) and leads to the activation of a small sustained inward current of 0.5 pA. A higher 8-Br-cGMP level (2 μM) results in an increased sustained inward current (1.9 pA) and further decreases odour responses to 10.7 ± 1.2 pA (n = 4). (b) Normalized dose response curve for the effect of 8-Br-cGMP on the peak amplitude of odour responses (100 μM cineole, seven cells) giving the following results: 0.85 ± 0.03 (300 nM), 0.55 ± 0.04 (500 nM), 0.31 ± 0.09 (1 μM), 0.24 ± 0.05 (2 μM), 0.18 ± 0.02 (5 μM). The solid line is a least squares fit of the data to equation (1) giving the following parameters: \( K_I = 460 \text{nM}, n = 2.2 \). (c) Current-voltage relations obtained from voltage ramps demonstrating a conductance increase associated with the sustained inward current induced by 8-Br-cGMP (different cell as in a). Control ramp currents have been subtracted. The membrane conductance increases by 27.1 pS (500 nM 8-Br-cGMP) and 43.7 pS (1 μM 8-Br-cGMP), respectively. Line fit of noisy traces is by regression analysis. (d) Effect of 8-Br-cGMP (500 nM and 1 μM) on the kinetics of odour responses demonstrating the standing current induced by cGMP at the beginning of the trace and the concentration-dependent prolongation of the odour response kinetics. The stimulus trace indicates the timing of the odour pulse. Responses are averaged currents each from four individual measurements. Data are taken from the same cell as in (c). (e) To facilitate viewing of the kinetic changes induced by 8-Br-cGMP, increment odour responses from (d) are rescaled to give the same peak current amplitudes. Time-to-peak is 0.62 s (control), 0.75 s (500 nM) and 0.86 s (1 μM). The decay phase of the currents can be fitted with single exponential functions of the following time constants: 0.4 s (control), 0.7 s (500 nM) and 1.1 s (1 μM).

The normalized dose-response curve (figure 1 b) and fitted with the Hill equation:

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E = E_{\text{min}} + (E_{\text{max}} - E_{\text{min}})/(1 + (K_I/C)^n),
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with \( E = I/I_{\text{control}} \) where \( I \) is the incremental odour-stimulated peak inward current (referred to as odour response), \( I_{\text{control}} \) is the odour response in the absence of 8-Br-cGMP, \( C \) is the 8-Br-cGMP concentration and \( n \) is the Hill coefficient. The halfmaximal effect of 8-Br-cGMP occurred at 460 nM and the Hill coefficient (n) of the fitted curve was 2.2, suggesting cooperativity. Response amplitudes were maximally reduced by about five-fold (at 5 μM 8-Br-cGMP) as compared with the control values. Interestingly, the concentration range of the steep portion of the dose-response curve (300 nM to 2 μM) closely matched the previously reported range of cGMP concentrations resulting from stimulation of soluble guanylyl cyclase (sGC) by carbon monoxide (CO) in these neurons (Leinders-Zufall et al. 1995).

Second, 8-Br-cGMP caused the activation of small, sustained net inward currents (figure 1 a). Several observations led us to conclude that they too, like the odour currents, resulted from activation of cyclic nucleotide-gated (CNG) channels (see also figure 4 a). The amplitude of the tonic background currents increased with increasing concentrations of 8-Br-cGMP (figure 1 a). Current-voltage analysis (figure 1 c) revealed that the persistent cGMP-induced currents were caused by a conductance increase, ranging from 27 pS (at 500 nM 8-Br-cGMP) to 104 pS (at 5 μM 8-Br-cGMP). Furthermore, the persistent currents as well as the primary odour responses were reversibly abolished by pharmacological agents known to act as blockers of CNG channels such as Gd³⁺, the calmodulin inhibitor W-7 (Kleene 1994; Leinders-Zufall et al. 1995), and LY83583 (Leinders-Zufall & Zufall 1995) (data not shown). Because tonic 8-Br-cGMP-induced currents displayed relatively negative reversal potentials (around —28 mV in the depicted example)
and their overall amplitudes were much smaller than under whole-cell recording conditions (Leinders-Zufall et al. 1995) it appeared that they were counterbalanced by outward currents, most likely caused by Ca²⁺-activated K⁺ channels (Morales et al. 1995). Consistent with this view, the reversal potential of the tonic currents shifted toward 0 mV upon removal of external Ca²⁺ (not shown).

As a third effect of 8-Br-cGMP, the kinetic properties of odour-stimulated currents were significantly altered (figure 1d, e). 8-Br-cGMP caused a profound concentration-dependent prolongation of both the rising and the decaying phase of the odour responses (n = 4).

Because 8-Br-cGMP is a hydrolysis-resistant analogue of cGMP, it was of interest to examine whether cGMP itself would produce an equivalent effect. Earlier studies have provided evidence that cGMP levels in ORNs can be regulated by CO suggesting that CO may serve as an endogenous activator of soluble guanylyl cyclase in these neurons (Verma et al. 1993; Ingi & Ronnett 1995; Leinders-Zufall et al. 1995). We therefore tested whether CO would simulate the effects of 8-Br-cGMP. Figure 2 illustrates that low sub-micromolar amounts of CO were sufficient to completely mimic the effects of 8-Br-cGMP. In the cell depicted in figure 2 exposure of CO (800 nM) caused a marked reduction of the odour response which was accompanied by the generation of a small sustained inward current and a prolongation of the odour response kinetics (figure 2a). While the effect of CO on the odour response amplitude was fully reversible, we obtained only partial recovery of the kinetic changes of the odour responses during the course of our recordings (figure 2b). Complete recovery of the sustained inward current occurred 2.2 min after removal of CO (not shown). The time course of the CO-mediated reduction in odour responsiveness is plotted in figure 2c. The odour responses remained reduced for much longer than the time during which CO was presented usually outlasting the CO application for 2–3 min. The final recovery back to control levels likewise was on the timescale of minutes (n = 5). Other data demonstrated that the effect of CO on peak odour responses was concentration-dependent, with $K_i = 1 \mu$M (figure 2d).

From our previous results this amount of CO is roughly equivalent to a free cGMP concentration of 450 nM in these cells (Leinders-Zufall et al. 1995). The data are
Figure 3. Effects of 8-Br-cGMP (1 μM) on stimulus-response curves elicited by various amounts of cineole. (Closed circles) control currents, (closed triangles) after exposure to 8-Br-cGMP. Data points with mean ± s.d. represent at least three independent measurements. Dose-response curves from two individual cells are shown as absolute currents (a, c) and in normalized form (b, d) where both axes are normalized values. The ordinate is the peak current normalized to the saturating peak current. The abscissa is the cineole concentration C normalized to the control $K_i$ value in each cell. All peak responses are measured as increment currents $\Delta I$ without taking the sustained background response by cGMP into account. Continuous lines are best fits (obtained with a Levenberg-Marquardt algorithm) by using the Hill equation (equation (1)). Because each individual cell has a characteristic Hill coefficient and $K_i$ value for a given odour molecule stimulus, data are not displayed in collected form. Data are fitted with the following parameters: $\Delta I_{sat} = 81 \text{ pA}$, $K_i = 88 \text{ μM}$, $n = 1.89$ for control (circles); $\Delta I_{sat} = 32 \text{ pA}$, $K_i = 160 \text{ μM}$, $n = 1.87$ for 8-Br-cGMP (triangles). $\Delta I_{sat} = 34.5 \text{ pA}$, $K_i = 44 \text{ μM}$, $n = 3.14$ for control (circles); $\Delta I_{sat} = 13.8 \text{ pA}$, $K_i = 54 \text{ μM}$, $n = 3.04$ for 8-Br-cGMP (triangles). Dotted lines indicate $K_i$ values.

Thus in close agreement with the results of figure 1b. Furthermore, they indicate that the CO/cGMP system, because of its relatively long-lasting activity, can affect responses to subsequent, repeated odour stimuli thus priming the cells for later exposure to odour ligands.

To examine some of the mechanisms by which cGMP elicited the described effects, stimulus-response curves of odour currents in the presence and absence of 8-Br-cGMP were generated. Figure 3a, c show two different cells in which the absolute peak currents of odour responses were plotted versus the cineole concentration. The data are well fitted with the Hill equation (equation 1) and, consistent with earlier results (Firestein et al. 1993), demonstrate that each individual cell has a characteristic Hill coefficient and $K_i$ value for a given odour molecule stimulus. Under control conditions, Hill coefficients for cineole responses ranged from 1.9 to 4.7 and $K_i$ values ranged from 42 μM to 88 μM ($n = 6$). The presence of 8-Br-cGMP (1 μM) caused two profound changes in the dose-response curves. First, there was a strong reduction in the maximum currents and this effect could not be overcome with higher odour concentrations (figure 3a, c). Second, there was a decrease in the apparent affinity of the cell for that odour stimulus. To facilitate viewing of this shift in apparent affinity, dose-response curves were plotted in normalized form (figure 3b, d). The results show that the $K_i$ values shifted to the right during the presence of 8-Br-cGMP while the slope of the curves remained nearly unchanged. Data from five different cells (at 1 μM 8-Br-cGMP) revealed an average $K_i$ shift of 1.7-fold (range 1.2–1.9) and a reduction in saturating current by 2.5 to 3-fold. This sensitivity reduction is significant given that the entire response range to odour stimuli of an individual cell covers only approximately one logarithmic unit.

Previous work has shown that activation of the CNG channels by cAMP is reduced by elevated internal Ca$^{2+}$ (Zufall et al. 1991b; Kramer & Siegelbaum 1992; Chen & Yau 1994; Lynch & Lindemann 1994) and our finding that the apparent affinity to odour stimuli can be shifted to higher odour concentrations is in part reminiscent of the $K_i$ shift of CNG channels by Ca$^{2+}$-calmodulin (Chen & Yau 1994). The results of figure 3 therefore pointed to the possibility that cGMP was not the final mediator of the described effects but rather suggested that it was Ca$^{2+}$ influx resulting from...
CNG channel activation by cGMP that provided a negative feedback signal causing decreased odour responsiveness. If Ca\(^{2+}\) is part of a feedback system, then lowering the external Ca\(^{2+}\) concentration and thus reducing movements of Ca\(^{2+}\) across the cellular membrane should alter the effect of cGMP on odour responses. The experiments depicted in figure 4 demonstrate that this is the case. In figure 4a, the amount of external Ca\(^{2+}\) strongly influenced both the rate and the extent of decreased odour responsiveness resulting from cGMP exposure. Under control conditions with 1 mM Ca\(^{2+}\) in the external solution (figure 4a, upper trace) decreased odour responsiveness was complete at the first test pulse after exposure to 8-Br-cGMP. In contrast, it took about 1 min to reach a new equilibrium of reduced odour responses with Ca\(^{2+}\) lowered to 50 \(\mu\)M (figure 4a, middle trace). Further reduction of Ca\(^{2+}\) to \(\leqslant 1 \mu\)M fully prevented the decline of odour responses caused by 8-Br-cGMP (figure 4a, lower trace). Because the external Mg\(^{2+}\) concentration remained high in these experiments Ca\(^{2+}\) alone is sufficient to account for the effect. The increase in the peak odour responses under low Ca\(^{2+}\) conditions is at least partly caused by a decrease of the Ca\(^{2+}\) block of the CNG channel pore resulting in greater currents (Zufall & Firestein 1993; Zufall et al. 1994; Frings et al. 1995; Kleene 1995). Consistent with this result, the amplitude of the sustained cGMP-activated currents was also increased at lower Ca\(^{2+}\) concentrations (in the example shown to \(-21 \mu\)A) thus showing the expected behavior of CNG channels.

To confirm further the hypothesis that Ca\(^{2+}\) entry into the cells mediated the above described effects of cGMP, stimulus-response curves were generated in low Ca\(^{2+}\) solution (see figure 4b). The results demonstrate that both the cGMP-induced sensitivity shift and the reduction in saturating odour current by cGMP could be abolished by lowering external Ca\(^{2+}\) to \(\leqslant 1 \mu\)M (\(n = 4\)). The decreased odour responsiveness caused by cGMP can therefore be attributed wholly to Ca\(^{2+}\) entry through the sustained activation of CNG channels.

4. DISCUSSION

The main finding of the present study is that 8-Br-cGMP strongly down-regulates the sensitivity of olfactory receptor cells to odour stimuli. This modulatory action of 8-Br-cGMP occurs at relatively low concentrations, with a \(K_I\) of 460 nM. This concentration range closely matches the previously reported range of cGMP concentrations resulting from stimulation of soluble guanylyl cyclase by the gaseous messenger CO in these neurons (Leinders-Zufall et al. 1995). In agreement with these data, we found that the effects of 8-Br-cGMP on odour responses can be mimicked by submicromolar amounts of CO providing.
further support to the notion that CO acts as a potent activator of the cGMP second messenger system in olfactory neurons (Verma et al. 1993; Ingi & Ronnett 1995; Leinders-Zufall et al. 1995). Sensitivity modulation by cGMP was achieved by regulation of both the gain and the kinetics of the cAMP-mediated odour transduction cascade, which is consistent with the view that both parameters are important determinants of the overall sensitivity of the olfactory system (Zufall et al. 1994). The observation that the effects of cGMP on odour responses were completely abolished by lowering the external Ca²⁺ concentration to levels ≤ 1 μM suggests that cGMP was not the final mediator of the described effects but that Ca²⁺ entry into cells was mainly responsible for sensitivity modulation. This finding makes it unlikely, though not impossible, that the cGMP-induced sensitivity regulation as reported here was mediated by a cGMP-dependent protein kinase.

Our results thus indicate that cGMP functions to modulate the cAMP system primarily by persistent activation of the CNG channels leading to long-lasting Ca²⁺ entry. It has been shown earlier that the olfactory CNG channels are not only gated by cAMP but also by cGMP, with a Kᵢ of 1.3–4 μM in salamander (Zufall et al. 1991a; Leinders-Zufall et al. 1995). Further studies showed that the principal effect of low micromolar amounts of cGMP on ORNs is a tonic activation of the CNG channels (Leinders-Zufall et al. 1995). Because of the extraordinary Ca²⁺ permeability of the olfactory CNG channels (Frings et al. 1995) it is reasonable to assume that tonic CNG channel activity leads to significant accumulation of Ca²⁺ in ORNs, even if the overall current amplitudes are relatively small. It will be particularly interesting to determine the precise molecular sites for the action of Ca²⁺ in the CNG channels. It has been shown earlier that the olfactory CNG channels are not only gated by cAMP but also by cGMP, with a Kᵢ of 1.3–4 μM in salamander (Zufall et al. 1991a; Leinders-Zufall et al. 1995). Further studies showed that the principal effect of low micromolar amounts of cGMP on ORNs is a tonic activation of the CNG channels (Leinders-Zufall et al. 1995). Because of the extraordinary Ca²⁺ permeability of the olfactory CNG channels (Frings et al. 1995) it is reasonable to assume that tonic CNG channel activity leads to significant accumulation of Ca²⁺ in ORNs, even if the overall current amplitudes are relatively small. It will be particularly interesting to determine the precise molecular sites for the action of Ca²⁺ in the CNG channels.

The finding that the apparent affinity to odour stimuli is shifted to the right is reminiscent of the Kᵢ shift of CNG channels by Ca²⁺-calmodulin (Chen & Yau 1994). However, Chen & Yau did not find a Ca²⁺-calmodulin mediated reduction of saturating current in native rat CNG channels whereas cloned homomeric CNG channels (subunit 1) did exhibit this feature if activated by cAMP (Chen & Yau 1994). This suggests that either the salamander CNG channels behave differently from their rat counterparts in this respect (for comparison see: Zufall et al. 1991b) or that additional Ca²⁺-dependent mechanisms contribute to the reduced odour responsiveness. As the activity of the adenylyl cyclase appears to increase with elevated intracellular Ca²⁺ (Anholt & Rivers 1990; Choi et al. 1992; Jaworsky et al. 1995) and Ca²⁺-activated chloride channels rather tend to amplify than to decrease odour responses (Kleene 1993; Kurahashi & Yau 1993; Lowe & Gold 1993b; Zhainazarov & Ache 1995) both are unlikely to mediate the reduced sensitivity of transduction because of elevated intracellular Ca²⁺. Future experiments should examine the relative contribution of each of the possible sites to the observations shown here.

An essential requirement for tight regulation of the gain of olfactory signal transduction is suggested by the large number of ionic channels available for activation. Odour stimuli potentially can generate up to 1 nA of net inward current in salamander ORNs (Firestein et al. 1993). However, because of the high input resistance of the cells, only a few picoamperes of inward current are sufficient for action potential generation (Lynch & Barry 1989; Leinders-Zufall et al. 1995) suggesting that only a small fraction of the available channels need to be active during an odour response. Hence, without effective gain control mechanisms such as those initiated by cGMP it appears that even moderate stimuli would saturate the transduction apparatus and put the cells in danger of extensive cationic influx.

Together with previous biochemical evidence demonstrating that cGMP formation can be promoted by odour stimuli of a given strength (Breer et al. 1992; Verma et al. 1993) our results thus support a model in which cGMP is part of a parallel regulatory cascade activation of which leads to decreased sensitivity of olfactory transduction. We found that 1 μM cGMP on average reduced the apparent affinity of a given cell to odour stimuli by about 1.7-fold and also resulted in a strong reduction in maximum current. In terms of current gain, these two effects can produce up to a 12 to 20-fold reduction in activation of the sensory generator current as calculated from the Hill coefficients, depending on the effective cGMP concentration and the slope of the dose-response curve of a given cell for the odour stimulus. This modulation is therefore likely to play an important role in olfactory adaptation. The main advantage for the cell of having a distinct, parallel pathway mediating adaptation is to impose an adaptational time course that is independent of the timecourse of the primary odour response. In the case of cGMP this effect would be relatively long-lasting and could continue for minutes after recovery of the cAMP signal, thus affecting the responses to subsequent, repeated odour stimuli. That slow and long-lasting forms of olfactory adaptation do indeed exist at the systems level has been demonstrated in humans (Ekman et al. 1967; Murphy 1987) and in invertebrates (Colbert & Bargmann 1995). Recently, our own experiments demonstrated that pre-exposure of individual ORNs to specific odour stimuli gave the same adaptation effect as exogenous cGMP or CO, and that this effect could be selectively reversed and uncoupled from the primary excitation process by heme oxygenase inhibitors but not by blockers of nitric oxide synthase (Zufall & Leinders-Zufall 1996), giving further support to the proposal presented above.

5. CONCLUSION

In summary, the described results are consistent with a functional role for cGMP in sensory adaptation of vertebrate ORNs. We propose that cGMP, most
likely produced via the CO second messenger pathway, mediates a long-lasting form of odour response adaptation and our preliminary data (Zufall & Leinders-Zufall 1996) seem to support this hypothesis. Our observations may help to explain earlier results reporting inhibitory effects of 8-BrcGMP on odour-induced short circuit currents across the intact bullfrog mucosa (Persaud et al. 1988). They also provide an explanation for the long-standing puzzle that the olfactory CNG channels can be activated by both cAMP and cGMP. The fact that these channels have a greater affinity for cGMP may reflect the very low concentrations of cGMP that underlie the mechanism of adaptation revealed in the present study.

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