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THE RESPONSE INDUCED BY INTRACELLULAR CYCLIC AMP IN ISOLATED OLFACTORY RECEPTOR CELLS OF THE NEWT

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SUMMARY

1. Responses induced by intracellular cyclic nucleotides were analysed in isolated olfactory receptor cells of the newt under a voltage-clamp condition by using the patch pipette in a whole-cell recording configuration. Cyclic nucleotides were applied by diffusion from the patch pipette.

2. Introduction of either cyclic AMP or cyclic GMP caused a transient inward current in cells held at −50 mV. The response amplitude was dose-dependent with the Hill coefficient of 3 and half-saturating concentration of 300 μM (concentration in the pipette) for both cyclic AMP and cyclic GMP. Cyclic CMP was less effective than those two nucleotides.

3. The response to intracellular cyclic AMP was seen in all cilia-bearing cells, but not in cells which lost the cilia during dissociation. The response latency was shorter when cyclic AMP was introduced into the ciliated terminal swelling (*ca* 0·2 s) rather than into the cell body (*ca* 1·4 s). These results suggest that the sensitivity to intracellular cyclic AMP is confined to the cilia.

4. The cyclic AMP-induced current was transient (half decay time, *ca* 2·3 s) despite the fact that cyclic AMP was continuously loaded from the patch pipette. The response time course was controlled by Ca^{2+}; the reduction of external Ca^{2+} concentration (replaced with Mg^{2+}) or loading the cell with 50 mM-EGTA prolonged the cyclic AMP-induced responses. The Ca^{2+}-induced suppression was reversible.

5. The reversal potential of the cyclic AMP-induced transient current was $-4{\cdot}8 \pm 3{\cdot}8$ mV, and that of the current re-induced by Ca^{2+} removal was $1{\cdot}5 \pm 2{\cdot}1$ mV, suggesting that both currents flowed through the same ionic channel. The channel permeates all alkali metal ions with the permeability ratios of $P_{Li}:P_{Na}:P_K:P_{Rb}:P_{Cs} = 0{\cdot}93:1:0{\cdot}93:0{\cdot}91:0{\cdot}72$, but not Cl^- or choline ions.

6. These results demonstrate that the cyclic AMP-induced response and the odorant-induced response of the isolated olfactory cell have nearly identical characteristics. The present study supports the notion that cyclic AMP is the internal messenger mediating olfactory transduction.

INTRODUCTION

Olfactory transduction starts at the ciliary membrane of receptor neurons located in the olfactory epithelium. Binding of odorants triggers slow and graded

depolarization of olfactory cells (Getchell, 1977; Suzuki, 1977; Trotier & MacLeod, 1983) by activating their cation-selective conductance (Kurahashi, 1989).

Several lines of evidence indicate that the olfactory transduction is mediated by a second messenger and that the most likely candidate for the internal messenger is cyclic AMP. Pace, Hanski, Salomon & Lancet (1985) have found that the ciliary membrane has an adenylate cyclase which is activated by several odorants. It has been briefly reported that the introduction of cyclic nucleotides into isolated olfactory cells causes a membrane response (Suzuki, 1986; Trotier & MacLeod, 1986). Nakamura & Gold (1987) have shown that the cyclic nucleotide increases a conductance of membrane patches excised from olfactory cilia when cyclic nucleotides are applied to the cytoplasmic side. Although the adenylate cyclase system in olfactory cilia is biochemically well characterized (mediation by G-protein, Pace *et al.* 1985; Pace & Lancet, 1986; activation by various kinds of odorants, Sklar, Anholt & Snyder, 1986; positive correlation between the amplitude of the electro-olfactogram (EOG) response and the activation of adenylate cyclase, Lowe, Nakamura & Gold, 1989), understanding of the cyclic nucleotide-gated channel is still limited. In the present study, the cyclic AMP-activated responses were further analysed in the isolated cell preparation dissociated from the newt olfactory epithelium and the response properties were compared with those of the odorant-induced responses (Kurahashi, 1989; Kurahashi & Shibuya, 1989, 1990). It was found that the cyclic AMP-induced response and the odorant-induced response have very similar characteristics (localization of the sensitivity at the cilia, ionic selectivity and Ca^{2+}-dependent adaptation). These observations strongly support the hypothesis that the cyclic AMP is the internal messenger mediating olfactory transduction.

Recently, Kurahashi & Shibuya (1990) have demonstrated that odorant-induced responses in isolated receptors show a strong adaptation to a prolonged stimulation, and that the adaptation is not observed in cells bathed in low-Ca^{2+}medium. They have suggested that the adaptation is caused by the Ca^{2+} influx that is a part of the odorant-induced current. Presumably, the olfactory transduction operates by a cascade of multiple steps (binding of odorant to the receptor molecule, activation of G-protein followed by adenylate cyclase activation, elevation of cyclic AMP concentration, opening of the cyclic AMP-gated channel), and it is possible that Ca^{2+} interacts with any step or steps of the cascade. However, the underlying mechanism of Ca^{2+} interaction is still open. The present study demonstrates that the cyclic AMP-induced response is transient, despite the fact that the cyclic AMP is continuously introduced to the cytoplasm. However, the response becomes sustained in low-Ca^{2+} medium. This observation presents a possibility that the mechanism of olfactory adaptation is accounted for by a Ca^{2+}-mediated modulation of cyclic AMP-gated channels or of cyclic AMP metabolism. Parts of this study have been presented at the Neuroscience Meeting of Japan in an abstract form (Kurahashi & Kaneko, 1990).

METHODS

Materials

Solitary receptor cells were dissociated enzymatically from the olfactory epithelium of the newt, *Cynops pyrrhogaster*. Dissociation protocols were similar to those previously reported (Kurahashi,

1989). In short, the animal was anaesthetized by cooling on ice, decapitated and pithed. The mucosae excised from the olfactory cavity were incubated for 5 min at 30 °C in a solution containing 0·1 % collagenase (Sigma, Type I, No. C-0130) and no added Ca^{2+} or Mg^{2+}. The tissue was then rinsed twice with a standard solution and triturated. Isolated cells were plated on the concanavalin A-coated glass cover-slip. Cells were maintained at 4 °C (up to 10 h) before use. Solitary receptor cells were identified by their characteristic morphology (cf. Fig. 1 of Kurahashi, 1989). Since the cilia are thought to be the transduction site (Kurahashi, 1989, and see below), cells having more than five cilia (up to about fifteen) were selected unless otherwise indicated.

Recording

Cells were superfused continuously by a rapid microsuperfusion system designed by Suzuki, Tachibana & Kaneko (1990). Solutions were supplied from a polyethylene nozzle (tip diameter 500 μm) positioned about 1 mm away from the recording cell. In this superfusion system a solution around the cell under study can be replaced within 100 ms (see Suzuki *et al.* 1990). Solutions used were identical to those of the previous study (Kurahashi, 1989). Ca^{2+} concentration of the solution to which neither Ca^{2+} salt nor EGTA was added was estimated to be about 10 μM from the experiment on the Ca^{2+}-dependence of the cyclic AMP-activated conductance (cf. Fig. 7). Solutions containing $\leqq 10$ μM-Ca^{2+} were prepared by using Ca^{2+}–EGTA buffer.

Cells were voltage clamped by a patch pipette in the whole-cell recording configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The tip diameter of the pipette was about 1 μm and resistance was 10 MΩ. The patch membrane was fully ruptured at the instant of the brief application of negative pressure to the pipette. The rupture was signalled by a transient artifact on the current trace. In most experiments the recording pipette was filled with a K^+ solution (in mM: 122, K^+; 120, Cl^-; 0, EGTA). Cs^+ pipette solution (in mM: 132, Cs^+; 120, Cl^-; 5, EGTA) was also used to eliminate the voltage- and Ca^{2+}-activated K^+ currents. Cyclic AMP or one of the related substances was dissolved in the pipette solution and was allowed to diffuse into cells from the tip opening of the recording pipette through the ruptured hole of the plasma membrane. Membrane currents were recorded with a patch-clamp amplifier (Nihon Kohden, CEZ-2200), stored on an FM magnetic tape (SONY, NFR-3715) and sampled at 0·2–1 kHz by a 12-bit analog-to-digital converter connected to a micro-computer (NEC, PC9801VX). The data presented in this paper were corrected for the junction potentials at the pipette tip and at the indifferent electrode in a similar manner as described before (Kurahashi, 1989). All experiments were carried out at room temperature (23–25 °C).

RESULTS

Transient inward current induced by cyclic AMP and cyclic GMP

Introduction of cyclic AMP induced an inward current in cells voltage-clamped at their resting potentials (*ca.* −50 mV). Figure 1*A* illustrates an example. First, the gigaseal was formed with a patch pipette containing 0·5 mM-cyclic AMP. At time zero, the patch membrane was ruptured. The instant of the rupture was signalled by a transient artifact in the current trace (arrowhead). An inward current started approximately 0·1 s after the rupture of the patch membrane, and reached a peak in about 0·6 s. A nearly identical response was induced when the pipette contained 0·5 mM-cyclic GMP (Fig. 1*B*). Since no response was induced by establishing the whole-cell configuration when the pipette did not contain either cyclic nucleotide (Fig. 1*C*), it is inferred that the inward current was induced by the intracellularly introduced cyclic nucleotide.

Cyclic AMP-induced response was transient. Despite the continuous diffusion of cyclic AMP from the patch pipette, the current amplitude gradually diminished to a small sustained level which was approximately 4 % of the peak amplitude. The time to half-decay from the response peak was $2·3 \pm 1·4$ s (mean $\pm$ S.D.; $n = 6$) when

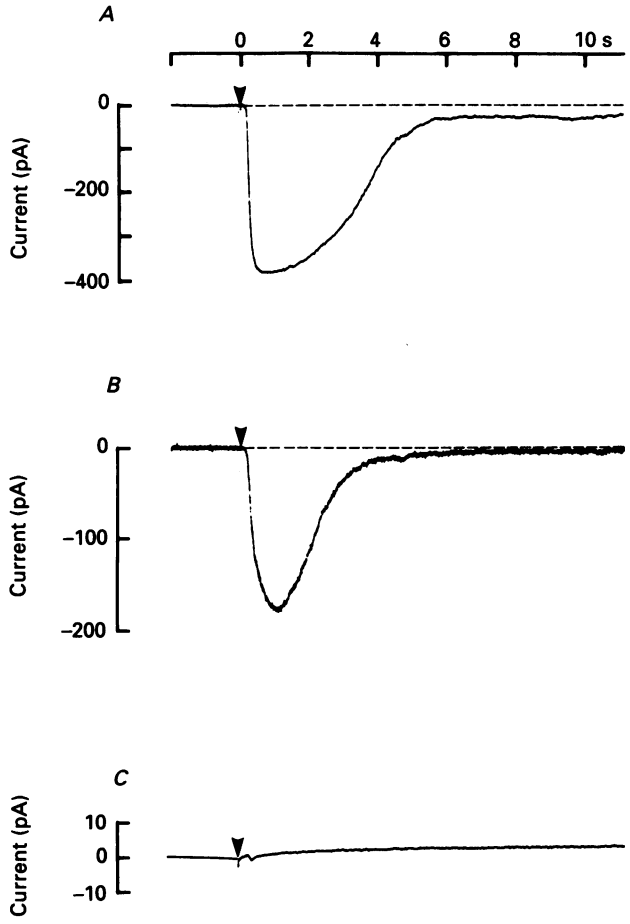

Fig. 1. Transient inward currents induced in isolated olfactory receptor cells by an introduction of cyclic nucleotide from the patch pipette. First, a gigaseal was formed on the terminal swelling (apical dendritic portion). The plasma membrane at the pipette tip was ruptured by a brief application of negative pressure at the time zero indicated by a downward arrow-head. The cyclic nucleotide was allowed to diffuse from the pipette into the cell through the ruptured hole. Holding potential, −50 mV. Cells were bathed in the standard solution. *A*, the pipette contained 0·5 mM-cyclic AMP. *B*, the pipette contained 0·5 mM-cyclic GMP. *C*, the pipette contained no cyclic nucleotide. All three records were obtained from different cells. Difference in time course or in amplitude between *A* and *B* is within the variation among cells recorded under the identical conditions.

0·5 mM-cyclic AMP was introduced from the pipette. Response with a slower decay was seen when the 1 mM-cyclic AMP was introduced (compare Fig. 1 and Fig. 7).

Among various nucleotides cyclic AMP, cyclic GMP and cyclic CMP were all effective, but neither 5′-AMP, ATP nor GTP were effective (tested at 1 mM). Mean response amplitudes evoked by various cyclic nucleotides at 0·5 mM (saturating concentration for cyclic AMP and cyclic GMP) were 311 ± 128 pA ($n = 11$) to cyclic AMP, 322 ± 130 pA ($n = 5$) to cyclic GMP and 88 ± 10 pA ($n = 4$) to cyclic CMP.

Since cyclic AMP and cyclic GMP produced similar responses, detailed studies were carried out mainly by using cyclic AMP.

Generation site of the cyclic AMP-induced response

Two lines of evidence suggest that the site of response generation to the internally applied cyclic AMP or cyclic GMP is the ciliary membrane. First, the sensitivity to

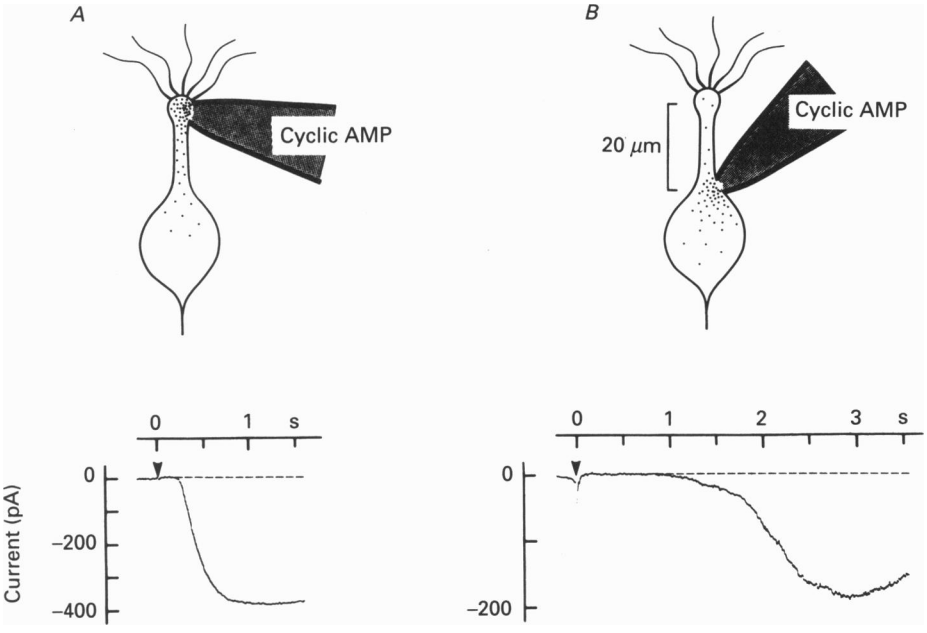

Fig. 2. Relation between the response time course and the site of cyclic AMP introduction. *A*, 0·5 mM-cyclic AMP was introduced to the terminal swelling. *B*, 0·5 mM-cyclic AMP was introduced to the proximal part of dendrite about 20 μm long. The pipettes were filled with K^+ solution. Cells were bathed in standard solution. Arrow-heads indicate the timing of the rupture of patch membrane. Holding potential, −50 mV.

the cyclic nucleotides was found only in cells equipped with cilia. Of 114 cells tested, those having cilia (109 cells) all responded to either cyclic AMP or cyclic GMP at 0·5–1 mM concentration, but the remaining five cells which lost cilia during dissociation did not show any response.

Second, the response amplitude and the time course differed markedly depending on the site of cyclic-AMP introduction (Fig. 2). When cyclic AMP was introduced to the terminal swelling (close to the cilia, Fig. 2*A*), it evoked a response of large amplitude, short latency (0·2±0·04 s, $n = 6$) and rapid rising time (the time from the onset to the peak, 0·9±0·2 s). However, when introduced into a proximal part of the dendrite (Fig. 2*B*), cyclic AMP evoked a response of small amplitude (approximately 1/6 on average, $n = 4$), long latency (1·4±0·4 s) and slow rising time (2·8±0·5 s).

The long latency of the response by cyclic AMP introduction away from the ciliated terminal can be accounted for by the time needed for diffusion of the nucleotide. The diffusion coefficient of cyclic AMP was estimated from its molecular

size (*ca* 10 Å in diameter, cf. Watenpaugh, Dow & Jensen, 1968) as being $4{\cdot}4 \times 10^{-6}$ cm^2 s^{-1}. The time needed for one-dimensional diffusion of cyclic AMP for 25 μm was then estimated to be 0·7 s. The value agrees with the observed response latency when cyclic AMP was introduced into the proximal part of the dendrite (Fig. 2*B*).

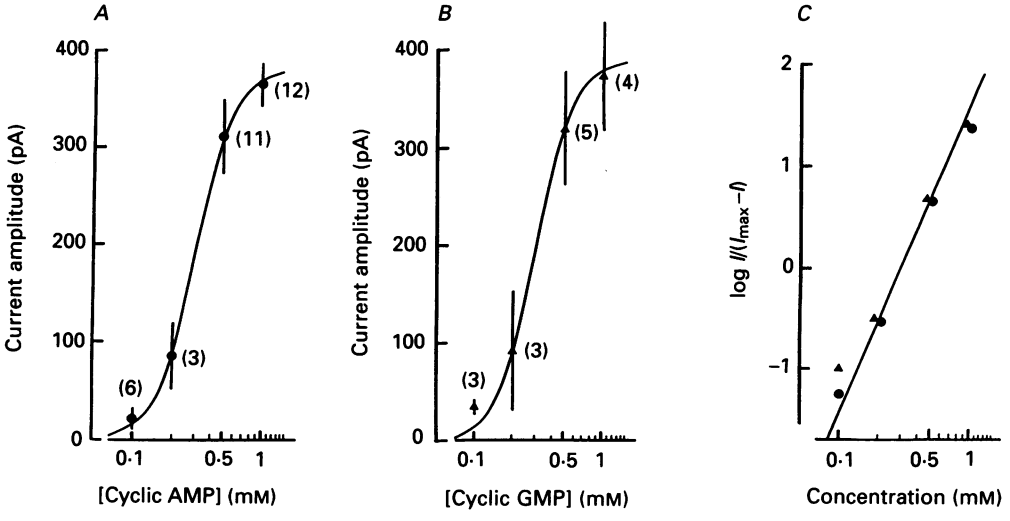

Fig. 3. Dose-dependence of the response induced by cyclic AMP (*A*) and cyclic GMP (*B*). Abscissae represent the concentration of the cyclic nucleotide in the pipette solution (in log scale). Cyclic nucleotides were introduced to the terminal swelling. Continuous lines represent the Hill equation, $I = I_{max} \times C^n/(C^n + K_m^n)$ with $n = 3$ and $K_m = 302$ μM (*A*) or 295 μM (*B*). Mean (filled symbols) and S.E.M. (vertical line) values are plotted with their sampling numbers (in parentheses). Cells were bathed in the standard solution. Holding potential, −50 mV. *C*, Hill plot of data, for cyclic AMP (from *A*, ●) and for cyclic GMP (from *B*, ▲). The straight line was fitted by eye.

Dependence of response amplitude on the cyclic AMP concentration

The amplitude of the cyclic AMP-induced response was dose-dependent. Although the present method of nucleotide application allowed only a single dose to each cell, cumulative results from a group of cells indicate the dose-dependence of the response amplitude (Fig. 3*A*, *B*). The least effective dose of cyclic AMP was estimated to be in the order of 100 μM, since a small response (< 100 pA) was seen in two out of six cells tested. Hill plot of the mean response amplitude ($I/I_{max} - I$ plotted against the concentration on double logarithmic coordinates) could be well fitted by a straight line with a slope of 3 (Fig. 3*C*). Consequently, the cooperativity of cyclic AMP was estimated to be 3. Data points in the dose–response curve were fitted by a Hill equation, $I = I_{max} C^3/C^3 + K_m^3$, where C is the concentration. The curve gave the best fit with a K_m value of approximately 300 μM (Fig. 3*A*). Similar cooperativity and K_m values were obtained for cyclic GMP (Fig. 3*B*).

Reversal potential

The reversal potential of cyclic AMP-induced current was measured by applying ramps under the voltage clamp. While ramp command voltages were given

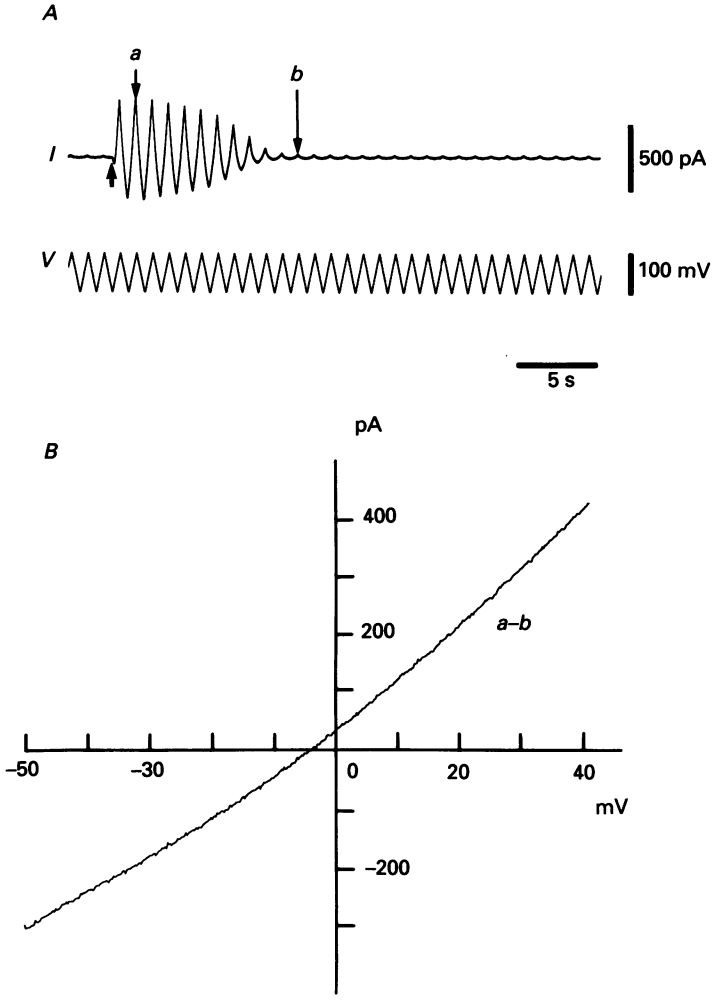

Fig. 4. Current–voltage relation of the cyclic AMP-induced response. *A*, measurement of the *I–V* relation during a cyclic AMP-induced transient response. Repetitive ramp command voltages (*V*) varying from −50 to +46 mV at a rate of 195 mV s^{-1} were applied throughout recording, and the resulting current (*I*) was measured. Cyclic AMP (0·5 mM) was introduced at the time indicated by the upward arrow to the terminal swelling. The cell was bathed in a solution containing (in mM): 85, NaCl; 3, $CaCl_2$; 35, TEA-Cl; 3, $CoCl_2$; 2, NaOH to block the voltage-gated currents. *B*, *I–V* relation of the cyclic AMP-activated response. Cyclic AMP insensitive component was removed by subtracting the *I–V* curve measured after the response decay (Fig. 4*A*, arrow '*b*') from that at the response peak (arrow '*a*').

repetitively, cyclic AMP was introduced into an isolated olfactory receptor cell (Fig. 4*A*). The voltage-gated channels of the cell had been blocked to reduce those currents which were not affected by cyclic AMP. When the patch membrane was ruptured, the excursion of the current trace was increased suggesting an increase in membrane conductance. After reaching the peak in about 1 s, the current excursion gradually decreased. The *I–V* relation of the cyclic AMP-activated response was almost linear

within the voltage range between −50 and +50 mV. A weak outward rectification was seen.

The reversal potential of the cyclic AMP-induced current was −4·8±3·1 mV (n = 4; $[Na^+]_o$ = 86·9 mM, K^+ pipette). The observed value of the reversal potential can be accounted for by either the cationic channel with low selectivity or the Cl^- channel, but the latter possibility should be discarded, since the cyclic AMP-activated channel does not permeate Cl^- (see later). The reversal potential of the cyclic AMP-induced current is close to the reversal potential of the cyclic nucleotide-gated channels in the patch membrane excised from the cilia of the frog (Nakamura & Gold, 1987).

The effect of Ca^{2+} on the response time course

As mentioned earlier, the cyclic AMP-induced response was transient. A similar time course was also found in odorant-induced responses, and this phenomenon was considered to be equivalent to the olfactory adaptation. The adaptation of odour response became less prominent in the medium containing low Ca^{2+} or by intracellular loading of EGTA (Kurahashi & Shibuya, 1990). To compare the characteristics of the cyclic AMP-induced response and of the odorant-induced response, the effect of the extracellular Ca^{2+} and intracellular EGTA on the cyclic AMP-induced response was studied.

Low extracellular Ca^{2+}

Introduction of cyclic AMP into cells bathed in the medium with zero added Ca^{2+} induced a sustained response (in nine cells tested), as exemplified in Fig. 5. Presence of Mg^{2+} was irrelevant. In the solution containing 3 mM-Mg^{2+} (with 0 added Ca^{2+}), the introduction of cyclic AMP induced a maintained current similar to that illustrated in Fig. 5. Re-application of standard medium containing 3 mM-Ca^{2+} gradually suppressed the maintained current, indicating that the maintained inward current was not due to the damage in the low Ca^{2+} solution.

Intracellular loading of EGTA

Intracellular loading of EGTA slowed down the decay time course of the cyclic AMP-induced response. Figure 6 shows the response when 0·5 mM-cyclic AMP and 50 mM-EGTA were simultaneously introduced into a cell bathed in the Ca^{2+}-containing solution. Introduction of EGTA alone did not cause any detectable response. In comparison with the response evoked by the cyclic AMP alone (see Fig. 1), the response in Fig. 6 was maintained for a longer period of time. In cells to which no EGTA was introduced, the current amplitude at 10 s after the rupture of the patch membrane (0·5 mM-cyclic AMP) was 4±4% of the peak value (six cells; holding potential = −50 mV). In EGTA-loaded cells, the amplitude of the induced current (0·5 mM-cyclic AMP) at 10 s was 70±26% of the peak (n = 5).

Maintained current induced by removal of external Ca^{2+} in cyclic AMP-loaded cells

As has been described above, the cyclic AMP-induced response was transient. However, reduction of $[Ca^{2+}]_o$ re-induced an inward current after the response was once suppressed (Fig. 7*A*). The re-induced current was maintained as long as the cell

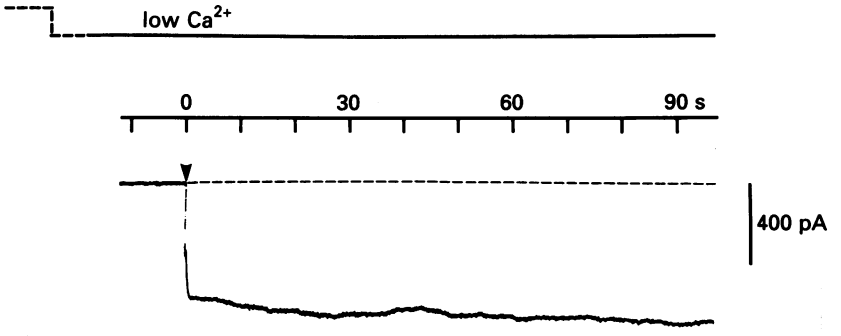

Fig. 5. Response elicited by intracellular introduction of cyclic AMP to a cell bathed in 0 Ca^{2+}, 0 Mg^{2+} solution. Cyclic AMP (1 mM) was introduced to the terminal swelling at the timing indicated by the arrow-head. Holding potential, −40 mV.

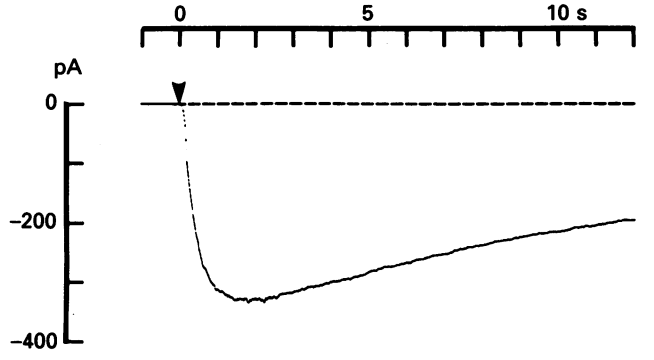

Fig. 6. Effect of intracellular loading of EGTA on the cyclic AMP-induced response. Cyclic AMP (0·5 mM) and EGTA (50 mM) were filled together in the patch pipette (124 mM-K^+) and introduced into a cell at the timing indicated by the arrow-head. The cell was bathed in the standard solution. Holding potential, −50 mV.

was bathed in the low Ca^{2+} medium. Furthermore, the current was reversibly suppressed by superfusing with the Ca^{2+}-containing solution. Since cells to which no cyclic AMP was loaded did not show a detectable current in response to the removal of $[Ca^{2+}]_o$ (examined at the holding potential of −90 mV, not illustrated), the current developed by the reduction of $[Ca^{2+}]_o$ seems to be the response induced by the pre-loaded cyclic AMP. Similar enhancement of the cyclic AMP-induced current by low $[Ca^{2+}]_o$ was also observed in the inside-out patch preparation excised from the ciliary membrane of the frog (Nakamura & Gold, 1987).

The amplitude of the Ca^{2+}-sensitive current was dependent on $[Ca^{2+}]_o$ (Fig. 7*B*). In cyclic AMP-loaded cells, the current was developed when $[Ca^{2+}]_o$ was reduced to less than 100 μM. The amplitude of Ca^{2+}-sensitive current became larger than the initial transient current recorded in 3 mM $[Ca^{2+}]_o$ (Fig. 1) when $[Ca^{2+}]_o$ was reduced to 1 μM (625 ± 131 pA, $n = 5$, holding potential, −50 mV). Similar $[Ca^{2+}]_o$ dependence was observed in all cells tested ($n = 5$).

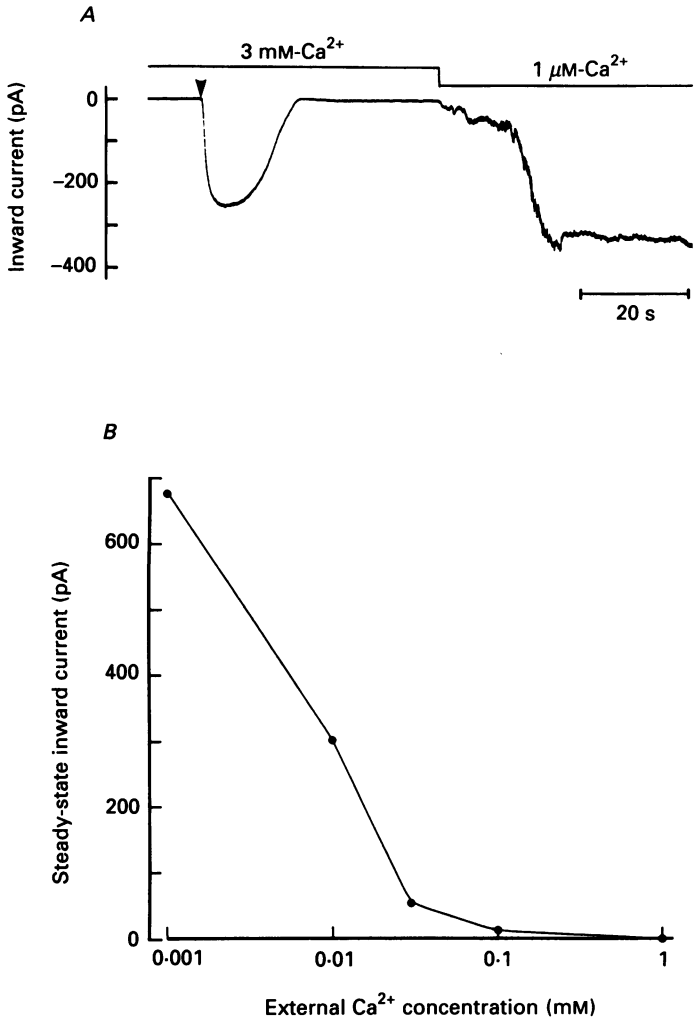

Fig. 7. *A*, inward current elicited by reduction of $[Ca^{2+}]_o$ in a cell pre-loaded with cyclic AMP. First, cyclic AMP (1 mM in the pipette) was introduced to the cell bathed in the standard solution at the time indicated by the arrow-head. The initial response was transient. After the inactivation of the transient current (40 s), $[Ca^{2+}]_o$ was lowered to 1 μM. Holding potential, −90 mV. *B*, the relation between the sustained current amplitude and $[Ca^{2+}]_o$ in a cyclic AMP-loaded cell. Holding potential, −50 mV. Pipette was filled with 0·5 mM-cyclic AMP in the K^+ solution. At about 60 s after the rupture of the patch membrane, each of the solutions containing various Ca^{2+} concentrations was applied for 15 s with approximately 50 s interval in an increasing sequence, and finally recovery was tested by an application of 0·001 mM-Ca^{2+} solution. In this cell, 72% recovery was seen. Different cell from *A*.

The *I–V* curve of the current that is re-induced by lowering $[Ca^{2+}]_o$ was measured by applying the ramp command voltage (Fig. 8). Again, the two *I–V* curves were recorded in the presence and absence of Ca^{2+}, and the former was subtracted from the latter to obtain the *I–V* curve of the current re-induced by lowering $[Ca^{2+}]_o$. The

reversal potential was $+1{\cdot}5 \pm 2{\cdot}1$ mV (superfusate, 122 mM-Na^+ solution; pipette, 120 mM-Cs^+ solution; $n = 6$). This reversal potential was close to that of the initial transient current induced by the introduction of cyclic AMP in the presence of Ca^{2+} ($-4{\cdot}8$ mV).

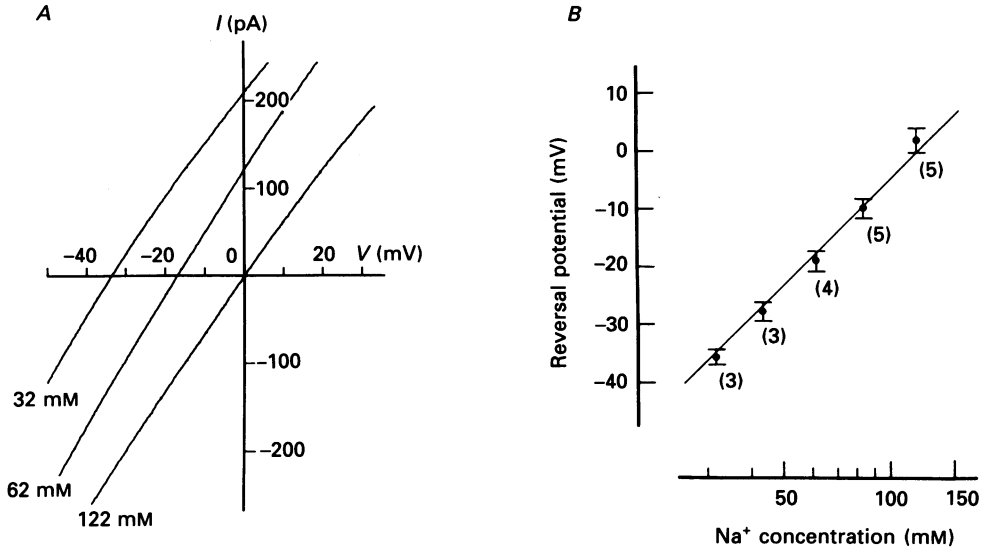

Fig. 8. Dependence of the cyclic AMP-activated conductance on $[Na^+]_o$. *A*, *I–V* relation of the sustained response induced by Ca^{2+} removal (no EGTA was used, cf. Fig. 7) in cyclic AMP (0·5 mM in the pipette) loaded cells under the three different $[Na^+]_o$. Choline ions were substituted for Na^+. The *I–V* curves were obtained by applying a voltage ramp (ramp rate = 195 mV s^{-1}) and were corrected for the cyclic AMP-insensitive components. Co^{2+} (3 mM) in the bathing solution and Cs^+ in the pipette were used to block the voltage-gated currents. *B*, relation between the reversal potential of the cyclic AMP-induced current and $[Na^+]_o$. Filled circle and vertical bars represent the average and S.D. of studied cells (number in parentheses). The straight line has a slope of 58 mV per ten fold change of $[Na^+]_o$.

Permeability of the cyclic AMP-activated channel to alkali metal ions

In the previous study (Kurahashi, 1989), the ionic selectivity of the odorant-sensitive channel was measured under 0 Ca^{2+} condition. The ionic selectivity of the cyclic AMP-activated channel was estimated under similar conditions. Since the response induced by cyclic AMP introduction was transient, the measurements were made on the conductance developed by Ca^{2+} removal in cyclic AMP-loaded cells.

The reversal potential of the cyclic AMP-induced current depended strongly on $[Na^+]_o$. Figure 8*A* shows the *I–V* relation of the current under three different $[Na^+]_o$. The reversal potential was 0 mV in the cell bathed with a solution containing 122 mM-Na^+, -17 mV in 62 mM $[Na^+]_o$ and -34 mV in 32 mM $[Na^+]_o$. Figure 8*B* illustrates the relation between $[Na^+]_o$ and the reversal potential. The data points fell very close to the theoretical line that has a slope of 58 mV per 10-fold change of $[Na^+]_o$. Good agreement between the data points and the theoretical line indicates that the cyclic AMP-activated channel is highly permeable to Na^+, but not to

choline. The observation also indicates that the channel is impermeable to Cl^-, since its concentration was kept constant throughout the experiments.

To estimate the ionic selectivity of the cyclic AMP-activated channel, the *I–V* relations of the cyclic AMP-induced current were measured under the approximate

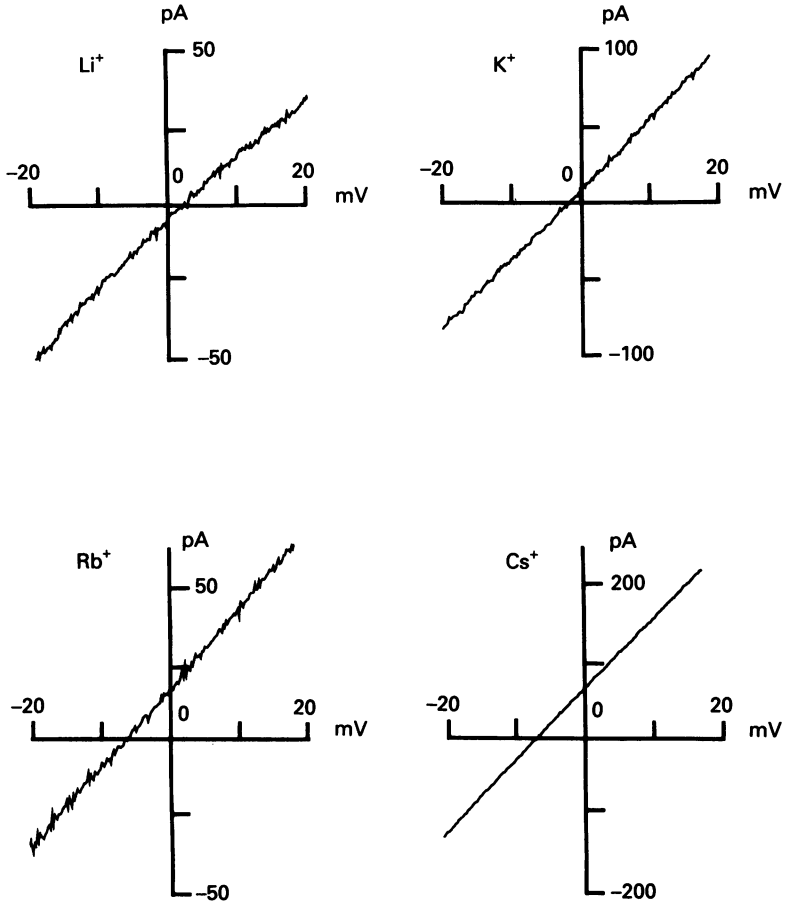

Fig. 9. *I–V* relations of the cyclic AMP-induced current recorded under four different ionic conditions (120 mM-Li^+, 120 mM-K^+, 120 mM-Rb^+ and 120 mM-Cs^+). *I–V* curves were obtained by subtracting the current at the Ca^{2+}-containing solution from the current at the low Ca^{2+} solution (the same method as Fig. 8). Pipette was filled with 0·5 mM-cyclic AMP dissolved in the Cs^+ solution.

bi-ionic conditions. The extracellular medium contained one of the alkali metal ions, Li^+, K^+, Rb^+, or Cs^+ (each at 120 mM, Fig. 9). The recording pipette contained 132 mM Cs^+ throughout the experiments. The reversal potential obtained under each of these conditions was $-0{\cdot}2 \pm 2{\cdot}2$ mV for Li^+ ($n = 4$), $1{\cdot}5 \pm 2{\cdot}1$ mV for Na^+ ($n = 5$), $-0{\cdot}2 \pm 1{\cdot}5$ mV for K^+ ($n = 6$), $-0{\cdot}7 \pm 1{\cdot}8$ mV for Rb^+ ($n = 4$) and $-6{\cdot}6 \pm 2{\cdot}2$ mV for Cs^+ ($n = 3$). Since the cyclic AMP-activated channel was not permeable to Cl^-, the relative permeability was derived from the Goldman–Hodgkin–Katz equation as follows;

$$P_{Li} : P_{Na} : P_K : P_{Rb} : P_{Cs} = 0{\cdot}93 : 1 : 0{\cdot}93 : 0{\cdot}91 : 0{\cdot}72.$$

These values are very close to the permeability ratios of odorant-sensitive channel to alkali metal ions (Kurahashi, 1989).

DISCUSSION

The present study has demonstrated that the intracellular introduction of cyclic nucleotide induces an inward current in isolated olfactory receptor cells. The inward current was carried by ionic current through the elevated cationic conductance with low selectivity.

The site of cationic conductance activated by cyclic AMP is located probably on the ciliary membrane, firstly because the introduction of cyclic AMP close to the ciliated dendritic terminal induced responses of larger amplitude and of shorter latency than the response induced by cyclic AMP injection into the cell body, and secondly because the cells that do not have cilia were insensitive to the internal cyclic AMP. The present finding agrees with the previous report of Nakamura & Gold (1987) that membrane patches excised from the ciliary membrane show a high sensitivity to cyclic AMP. The density of cyclic AMP-gated channels in the ciliary membrane was estimated to be about $10000\,\mu m^{-2}$ (from the single channel conductance of about 30 pS observed under 0 Ca^{2+}/0 Mg^{2+} condition, Nakamura & Gold, 1988). In contrast, the channel density in dendrite or cell body was estimated to be only $2–3\,\mu m^{-2}$ (T. Kurahashi, unpublished observation; N. Suzuki, personal communication). The odorant-binding site of the fish (Rhein & Cagan, 1980) or odorant-activated adenylate cyclase (Pace *et al.* 1985; Pfeuffer, Mollner, Lancet & Pfeuffer, 1989) are also localized at the ciliary membrane. The localization of all elements of the transduction system is consistent with the hypothesis that the ciliary membrane is the site at which odorant-induced responses are generated (Adamek, Gesteland, Mair & Oakley, 1984; Kurahashi, 1989).

The newt olfactory cell responded to both cyclic AMP and cyclic GMP with almost identical sensitivity. Cyclic CMP was much less potent. Similar effectiveness of cyclic nucleotides has been reported for the membrane patches excised from olfactory cilia (Nakamura & Gold, 1987) and for the whole-cell preparation of the frog (Suzuki, 1989). Olfactory cells have the adenylate cyclase system, but no guanylate cyclase (Pace *et al.* 1985; Pace & Lancet, 1986; Shirley, Robinson, Dickinson, Aujla & Dodd, 1986; Sklar *et al.* 1986). Cyclic GMP has been shown to gate the ionic channel in the rod and cone outer segment membrane (Fesenko, Kolesnikov & Lyubarsky, 1985; Haynes & Yau, 1985), but the photoreceptor cells are insensitive to cyclic AMP. It is puzzling why cyclic nucleotide-gated channels of olfactory cells respond similarly both to cyclic AMP and to cyclic GMP. Since these cyclic nucleotides have similar molecular conformation, it might not be so unreasonable to think that the channels of olfactory cells can not segregate the two cyclic nucleotides. Perhaps, the channels of photoreceptors require more strict conformational matching. A definite answer to this puzzle is expected to be given by determining the three dimensional conformation of the channel proteins of olfactory and photoreceptors.

The cooperativity of cyclic AMP for gating the channel was about 3. The observed value agrees well with those found in the excised patches of either the frog olfactory cilia (Nakamura & Gold, 1987) or the rod outer segment (Fesenko *et al.* 1985, Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986; Matthews & Watanabe, 1988;

Nakatani & Yau, 1988*a*). However, the K_m value obtained in the present experiment is approximately two orders of magnitude higher than that reported for the excised patch membrane of the olfactory cilia (Nakamura & Gold, 1987). In the whole-cell preparation, it is likely that the endogenous phosphodiesterase remains active in the cytoplasm, and decomposes the introduced cyclic nucleotides. Also the cyclic nucleotide has to diffuse through a narrow ciliary cytoplasm (0·2 μm in diameter). Consequently, the concentration of the cyclic nucleotide in the vicinity of the ciliary membrane could be equilibrated at a much lower level than the concentration in the pipette. Experiments are being made to examine whether the phosphodiesterase inhibitor could lower the K_m value.

The cyclic nucleotide-induced response in the olfactory cell decayed rapidly under the control condition, despite the fact that the cyclic-nucleotide was continuously supplied from the pipette. The response decay does not seem to be induced by the agonist desensitization, since the response became more sustained when either the extracellular concentration of Ca^{2+} was lowered or the intracellular concentration of Ca^{2+} was maintained at a lower level by intracellular loading of Ca^{2+} chelating agent. No desensitization to the cyclic nucleotide has been seen in the excised patch of olfactory cells (T. Nakamura, personal communication) and of rod photoreceptor cells (Fesenko *et al.* 1985; Matthews, 1986; Karpen, Zimmerman, Stryer & Baylor, 1988).

It seems probable that cytoplasmic Ca^{2+} is important, since EGTA loading prolonged the response time course. The main source of cytoplasmic Ca^{2+} is probably the Ca^{2+} that flowed as a constituent of the inward current. Two possibilities can be considered as the underlying mechanism of Ca^{2+} action. First, as has been shown for the excised patch membrane, the cyclic nucleotide-gated channels could be blocked directly by cytoplasmic Ca^{2+} (Nakamura & Gold, 1987). Second, Ca^{2+} could facilitate cyclic AMP hydrolysis by activating phosphodiesterase, as has been reported for rods (Robinson, Kawamura, Abramson & Bownds, 1980). If the increased Ca^{2+} accelerated hydrolysis of the cyclic AMP that diffused to the cilia through the narrow ciliary cytoplasmic space, the response decays despite the continuous supply of cyclic AMP from the pipette. Ca^{2+}-induced suppression of cyclic AMP synthesis (Shirley *et al.* 1986; Sklar *et al.* 1986) need not be considered under the present experimental condition.

The cyclic AMP-induced response has very similar characteristics to the odorant-induced response of isolated newt olfactory cells (Kurahashi, 1989; Kurahashi & Shibuya, 1990). Cilia are the most sensitive site both to intracellularly applied cyclic AMP and to the extracellularly applied odorants. To either stimulation of a long duration, responses show a rapid decay to a very small level, and the decay is strongly dependent on the influx of Ca^{2+}. Reversal potentials of the two kinds of responses are close (approximately 0 mV, cf. Trotier, 1986; Firestein & Werblin, 1989), and the ionic selectivity is similar. All these similarities between the cyclic AMP-induced responses and the odorant-induced responses strongly support the notion that the olfactory transduction is mediated by the adenylate cyclase–cyclic AMP system (Pace *et al.* 1985; Nakamura & Gold, 1987).

It has been suggested that the elevation of the cytoplasmic Ca^{2+} induces odour adaptation (Kurahashi & Shibuya, 1990). Since the cyclic AMP-induced response is

also suppressed by the cytoplasmic Ca^{2+}, it seems highly likely that odour adaptation is mediated by the interaction between Ca^{2+} and cyclic AMP. Although the precise molecular mechanisms of this interaction remains still open, it may be worth noting that light adaptation in rods is also regulated by modification of cyclic GMP metabolism by Ca^{2+} (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988*b*).

Perhaps the Ca^{2+}-induced odour adaptation can account for the strong outward rectification of the *I–V* curve of the odorant-induced current in the voltage region more negative than the resting potential (Fig. 4 of Kurahashi, 1989). Such outward rectification was not seen in the *I–V* curve of the cyclic AMP-induced conductance. Since the amplitude of the odorant-induced response was measured at the peak (approximately 1 s after the response onset), it seems reasonable to assume that the responses induced in cells held at negative voltages were inactivated more strongly by a larger Ca^{2+} influx than the responses induced at more positive holding voltages. The *I–V* relation of the present study was obtained by ramp voltage command immediately after the response onset, when the suppressive mechanism was presumably not affecting the response yet.

It has been shown that each olfactory cell has different sensitivity to various odorants (Mathews, 1972; Sicard & Holley, 1984; Kurahashi, Kaneko & Shibuya, 1990). The present study has demonstrated that intracellularly applied cyclic AMP induced responses in all receptor cells studied that are supposed to include cells responding selectively to different odorants. While the specific odour-binding molecule have not yet been identified in the receptive membrane, the present findings indicate that the transduction mechanism is common to all olfactory cells, and that the discrimination of odorants is entirely dependent on the odour-binding molecule.

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REFERENCES

ADAMEK, G. D., GESTELAND, R. C., MAIR, R. G. & OAKLEY, B. (1984). Transduction physiology of olfactory receptor cilia. *Brain Research* **310**, 87–97.

FESENKO, E. E., KOLESNIKOV, S. S. & LYUBARSKY, A. L. (1985). Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature* **313**, 310–313.

FIRESTEIN, S. & WERBLIN, F. S. (1989). Odor-induced membrane currents in vertebrate-olfactory receptor neurons. *Science* **244**, 79–82.

GETCHELL, T. V. (1977). Analysis of intracellular recordings from salamander olfactory epithelium. *Brain Research* **123**, 275–286.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HAYNES, L. W., KAY, A. R. & YAU, K.-W. (1986). Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane. *Nature* **321**, 66–70.

HAYNES, L. W. & YAU, K.-W. (1985). Cyclic GMP-sensitive conductance in outer segment membrane of catfish cones. *Nature* **317**, 61–64.

KARPEN, J. W., ZIMMERMAN, A. L., STRYER, L. & BAYLOR, D. A. (1988). Molecular mechanics of the cyclic GMP-activated channel of retinal rods. *Cold Spring Harbor Symposia on Quantitative Biology* **53**, 325–332.

KURAHASHI, T. (1989). Activation by odorants of cation-selective conductance in the olfactory receptor cell isolated from the newt. *Journal of Physiology* **419**, 177–192.

KURAHASHI, T. & KANEKO, A. (1990). Cyclic AMP-induced current in isolated olfactory receptors of the newt. *Neuroscience Research Supplement* (in the Press).

KURAHASHI, T., KANEKO, A. & SHIBUYA, T. (1990). Ionic mechanisms of the olfactory transduction studied on isolated receptor cells of the newt. *Neuroscience Research Supplement* (in the Press).

KURAHASHI, T. & SHIBUYA, T. (1989). Membrane responses and permeability changes to odorants in the solitary olfactory receptor cells of newt. *Zoological Science* **6**, 19–30.

KURAHASHI, T. & SHIBUYA, T. (1990). Ca^{2+}-dependent adaptive properties in the solitary olfactory receptor cell of the newt. *Brain Research* **515**, 261–268.

LOWE, G., NAKAMURA, T. & GOLD, G. H. (1989). Adenylate cyclase mediates olfactory transduction for a wide variety of odorants. *Proceedings of the National Academy of Sciences of the USA* **86**, 5641–5645.

MATHEWS, D. F. (1972). Response patterns of single neurons in the tortoise olfactory epithelium and olfactory bulb. *Journal of General Physiology* **60**, 166–180.

MATTHEWS, G. (1986). Comparison of the light-sensitive and cyclic GMP-sensitive conductances of the rod photoreceptor: Noise characteristics. *Journal of Neuroscience* **6**, 2521–2526.

MATTHEWS, G. & WATANABE, S.-I. (1988). Activation of single ion channels from toad retinal rod inner segments by cyclic GMP: concentration dependence. *Journal of Physiology* **403**, 389–405.

MATTHEWS, H. F., MURPHY, R. L. W., FAIN, G. L. & LAMB, T. D. (1988). Photoreceptor light adaptation is mediate by cytoplasmic calcium concentration. *Nature* **334**, 67–69.

NAKAMURA, T. & GOLD, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325**, 442–444.

NAKAMURA,T. & GOLD, G. H. (1988). Single channel properties of the ciliary cyclic nucleotide-gated conductance. In *Abstract book of Association for Chemoreception Sciences*, p. 193.

NAKATANI, K. & YAU, K.-W. (1988*a*). Guanosine 3′,5′-cyclic monophosphate-activated conductance studied in a truncated rod outer segment of the toad. *Journal of Physiology* **395**, 731–753.

NAKATANI, K. & YAU, K.-W. (1988*b*). Calcium and light adaptation in retinal rods and cones. *Nature* **334**, 69–71.

PACE, U., HANSKI, E., SALOMON, Y. & LANCET, D. (1985). Odorant-sensitive adenylate cyclase may mediate olfactory reception. *Nature* **316**, 255–258.

PACE, U. & LANCET, D. (1986). Olfactory GTP-binding protein: Signal-transducing polypeptide of vertebrate chemosensory neurons. *Proceedings of the National Academy of Sciences of the USA* **83**, 4947–4951.

PFEUFFER, E., MOLLNER, S., LANCET, D. & PFEUFFER, T. (1989). Olfactory adenylyl cyclase. *Journal of Biological Chemistry* **264**, 18803–18807.

RHEIN, L. D. & CAGAN, R. H. (1980). Biochemical studies of olfaction: Isolation, characterization, and odorant binding activity of cilia from rainbow trout olfactory rosettes. *Proceedings of the National Academy of Sciences of the USA* **77**, 4412–4416.

ROBINSON, P. R., KAWAMURA, S., ABRAMSON, B. & BOWNDS, M. D. (1980). Control of the cyclic GMP phosphodiesterase of frog photoreceptor membranes. *Journal of General Physiology* **76**, 631–645.

SHIRLEY, S. G., ROBINSON, C. J., DICKINSON, K., AUJLA, R. & DODD, G. H. (1986). Olfactory adenylate cyclase of the rat. *Biochemical Journal* **240**, 605–607.

SICARD, G. & HOLLEY, A. (1984). Receptor cell responses to odorants: similarities and differences among odorants. *Brain Research* **292**, 283–296.

SKLAR, P. B., ANHOLT, R. H. & SNYDER, S. H. (1986). The odorant-sensitive adenylate cyclase of olfactory receptor cells. *Journal of Biological Chemistry* **261**, 15538–15543.

SUZUKI, N. (1977). Intracellular responses of lamprey olfactory receptors to current and chemical stimulation. In *Food Intake and Chemical Senses*, ed. KATSUKI, Y., SATO, M., TAKAGI, S. F. & OOMURA, Y., pp. 13–22. University of Tokyo Press, Tokyo, Japan.

SUZUKI, N. (1986). Cyclic nucleotide-induced conductance increase in solitary olfactory receptor cells. In *Proceedings of the 20th Japanese Symposium on Taste and Smell*, pp. 37–40. Japanese Association for the study of Taste and Smell, Asahi University, Gifu, Japan.

SUZUKI, N. (1989). Voltage- and cyclic nucleotide-gated currents in isolated olfactory receptor cells. In *Chemical Senses*, ed. BRAND, J. G., TEETER, J. H., CAGAN, R. H. & KARE, M. R., pp. 469–493. Marcel Dekker, New York.

SUZUKI, S., TACHIBANA, M. & KANEKO, A. (1990). Effects of glycine and GABA on isolated bipolar cells of the mouse retina. *Journal of Physiology* **421**, 645–662.

TROTIER, D. (1986). A patch-clamp analysis of membrane currents in salamander olfactory receptor cells. *Pflügers Archiv* **407**, 589–595.

TROTIER, D. & MACLEOD, P. (1983). Intracellular recordings from salamander olfactory receptor cells. *Brain Research* **268**, 225–237.

TROTIER, D. & MACLEOD, P. (1986). cAMP and cGMP open channels and depolarize olfactory receptor cells. *Chemical Senses* **11**, 674.

WATENPAUGH, K., DOW, J. & JENSEN, L. H. (1968). Crystal and molecular structure of adenosine 3′,5′-cyclic phosphate. *Science* **159**, 206–207.

ZIMMERMAN, A. L. & BAYLOR, D. A. (1986). Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores. *Nature* **321**, 70–72.