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Ion transport across the frog olfactory mucosa: the action of cyclic nucleotides on the basal and odorant-stimulated states

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The action of cyclic nucleotides on the short-circuit current across the isolated bullfrog olfactory mucosa was studied both in the absence and presence of odorants. 8-Bromo-cAMP applied to the ciliated side of the mucosa caused a concentration-dependent, reversible increase in the basal short-circuit current, but not when it was applied to the submucosal side. The current had a sigmoidal concentration dependence described by the Hill equation. The magnitude of the odorant-evoked current was enhanced after bathing the ciliated side with cAMP analogs or modulators of intracellular cAMP. GTPγS added to the ciliated side increased the odorant-evoked current, while GDPβS caused a decrease. Current transients induced by stimulating the ciliated side with either pulses of odorant or 8-bromo-cAMP were partially suppressed by amiloride, but only when amiloride and stimulant were presented simultaneously. Pulses of 8-bromo-cAMP and odorant presented simultaneously resulted in currents that added nonlinearly. In the absence of odorant, 8-bromo-cGMP caused a concentration-dependent decrease in net inward current that was reversed by 8-bromo-cAMP. Odorant-evoked currents were also reduced by 8-bromo-cGMP, and these could not be reversed by 8-bromo-cAMP. The results indicate that one type of olfactory transduction process involves the activation by cAMP of an inward current through an amiloride-sensitive apical ion channel and that this mechanism is mediated by a stimulatory G-protein.

Introduction

One type of olfactory transduction mechanism is believed to depend on the interaction of odorants with membrane-bound receptors [1–3]. Transduction is thought to result in an increase in ion channel conductance and the subsequent development of a depolarizing generator current across the apical membranes of the receptor neurons [2,4–7]. The discovery of odorant-stimulated, GTP-requiring, adenylate cyclase activity in isolated dendritic membranes of olfactory neurons, using biochemical techniques [8–11] and cyclic nucleotide-gated ion channel conductances in patch-clamped cilia from isolated olfactory cells [12] suggests that one way the generator current can be regulated is through cAMP. Testing this hypothesis on the intact olfactory mucosa has been difficult because no method of measuring the

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odorant-evoked transmucosal current was available. We recently developed whole tissue voltage-clamp methods for studying the ionic mechanisms associated with the unstimulated and odorant-stimulated currents across the physiologically intact olfactory mucosa [13]. We have now studied the effects of cyclic nucleotides on the transmucosal current under short-circuit conditions. cAMP analogues are here shown to be potent stimulators of inward short-circuit current. We present evidence that cAMP and agents that cause its generation in situ significantly increase the odorant-evoked short-circuit current and that cAMP analogues act additively with odorants in producing an inward current under appropriate conditions. Moreover, the short-circuit currents induced by 8-bromo-cAMP (BrcAMP) and odorants are each partially reduced by amiloride. This suggests that stimulation with cAMP or an odorant activates a common current source on the apical side of the olfactory mucosa. We further present evidence that a G-protein regulated adenylate cyclase is involved in the modulation of odorant-evoked current, consistent with inferences drawn from biochemical findings [8–11].

Materials and Methods

Tissue preparation

Tissue preparation, mounting and voltage-clamp measurement of transmucosal current were as previously described [13]. The dorsal olfactory mucosa of an anesthetized (0.4% MS222) bullfrog (*Rana catesbeiana*) was removed as a single sheet and mounted with the submucosal side supported by a nylon mesh backing (100 μm mesh) on a silicone rubber gasket. A lucite washer coated with cyanoacrylate adhesive was fitted over the tissue and formed a tight seal after curing. The exposed area of the mounted mucosa was 0.0755 cm^2. The apparatus is shown in Fig. 1. The tissue was secured between two Ussing chambers, with volumes of 0.5 ml on the ciliated side and 1.5 ml on the submucosal side. The smaller volume of the chamber on the ciliated side was optimal for rapid equilibration and removal of the changing media. The tissue was maintained in oxygenated amphibian Ringer's solution at room temperature (22°C). On the ciliated side, the solution was continually replaced at 0.2 ml/s using a constant pressure head.

Odorant or cyclic nucleotide presentation

Odorants and pharmacological agents dissolved in Ringer's solution were presented to the ciliated side by diverting the Ringer's solution through a charged sample loop via a 2-gang-3-way valve. In experiments where the mucosa was incubated with a solution for several minutes, valves for starting and stopping the flow of solution were inserted into the flow system. In experiments involving a brief stimulus pulse, the shape of the stimulus profile was measured by mounting a photodarlington transistor in place of the tissue and following the change in light absorbance when a blue dye was introduced as a sham stimulus.

For purposes of analysis a response was defined operationally as the change in current from its value at stimulus onset to peak stimulus-evoked current.

Chemicals and solutions

3′,5′-cAMP, 8-bromo-3′,5′-cAMP, 8-bromo-3′,5′-cGMP, 2′,3′-cAMP, 3-isobutyl-1-methylxanthine, theophylline and amiloride were from Sigma Chemical Co., St. Louis, MO. Forskolin, GTPγS and GDPβS were from Boehringer Mannheim Biochemicals, Indianapolis, IN. 1,8-Cineole was from Aldrich Chemicals, Milwaukee, WI, and 2-isobutyl-3-methoxypyrazine was from Pyrazine Specialities Inc., Atlanta, GA. All salts were reagent grade. Amphibian Ringer's solution contained 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, and 10 mM glucose (pH 7.3 at 22°C). In most experiments the tissue was maintained in this solution oxygenated with 100% O_2. However, ion replacement experiments of the solution bathing the ciliated side of the mucosa [13] as well as a recent measurement of the Na^+ ion concentration in frog olfactory mucus [14] indicate that 50 mM Na^+ on the ciliated side is a better approximation to normal values. Accordingly, in the later experiments, the ciliated side of the olfactory mucosa was bathed in a modified Ringer's solution containing 50 mM NaCl together with 50 mM *N*-methyl-D-glucammonium chloride to maintain the osmotic strength. Results obtained using modified

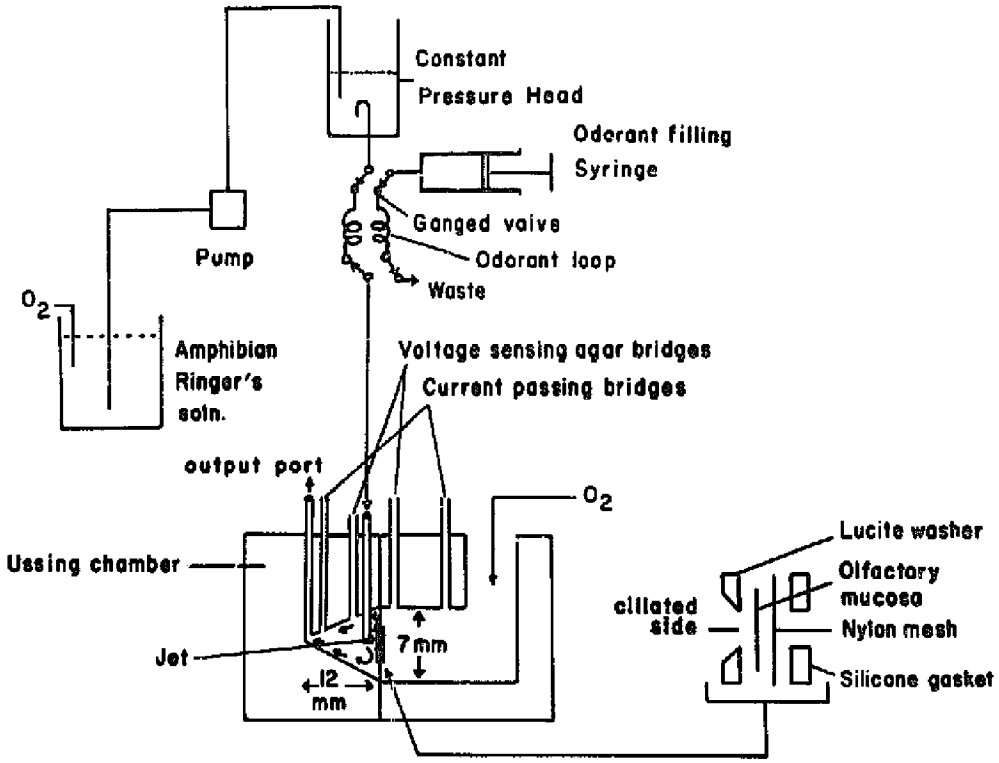

Fig. 1. Ussing preparation adapted for the bullfrog olfactory mucosa. The dorsal frog olfactory mucosa was mounted on the assembly shown in the inset. This consisted of a silicone gasket, a 100 μm nylon mesh backing and a lucite washer. The nylon mesh was attached to the silicone gasket with silicone adhesive. The olfactory mucosa was placed ciliated side up on the nylon mesh and was attached along the edges to the mesh with cyanoacrylate adhesive. A lucite washer, lightly coated with cyanoacrylate was placed over the olfactory mucosa so that the effective area exposed was 0.0755 cm^2. The mounted tissue was then assembled between two lucite Ussing chambers, a silicone gasket and screw brace providing the necessary sealing. The chamber on the ciliated side of the olfactory mucosa was conical in shape and 0.5 ml in volume to permit rapid and efficient changes of solution. That on the serosal side was 1.5 ml in volume. Solutions were introduced into the chamber on the ciliated side via a jet placed 1 mm from the tissue and at an incident angle of 45°, creating a rapid mixing vortex in that region. An exit port at the apex of the cone facilitated rapid removal. Voltage sensing and current-passing agar bridges (0.15 M NaCl) were inserted through sealed ports on the top of the chamber. Amphibian Ringer's solution was oxygenated with 100% O_2 and pumped to a constant pressure head reservoir. This was placed above the apparatus so that the flow rate of solution entering the Ussing chamber was 0.2 ml/s. Odorants were presented in aqueous solution to the ciliated side of the olfactory mucosa by switching an odorant-charged loop of Ringer's solution by means of a 2-gang-3-way FPLC valve. The stimulus profile was monitored by mounting a photodarlington transistor in the position where the tissue is normally seated and following the change in light absorbance when a blue dye (5,5′-indigosulfonic acid) was presented as a pulse instead of the odorant.

Ringer's solution are so indicated in the figure legends. In all cases the submucosal side was bathed in Ringer's solution containing 100 mM NaCl.

Stock solutions of the odorants 1,8-cineole and 2-isobutyl-3-methoxypyrazine (0.1 M) were made up in methanol. Working solutions were made up in Ringer's solution immediately before use, controls indicating no changes in short-circuit current due to the presence of methanol. The concentration of odorants used ranged from $5 \cdot 10^{-6}$ M to $4.8 \cdot 10^{-4}$ M.

Results

Cyclic nucleotides and the basal short-circuit current

Bullfrog olfactory mucosa bathed symmetrically with amphibian Ringer's solution had the ciliated side electronegative at about −3.6 mV, a transmucosal resistance of 67 ohm · cm^2, and a

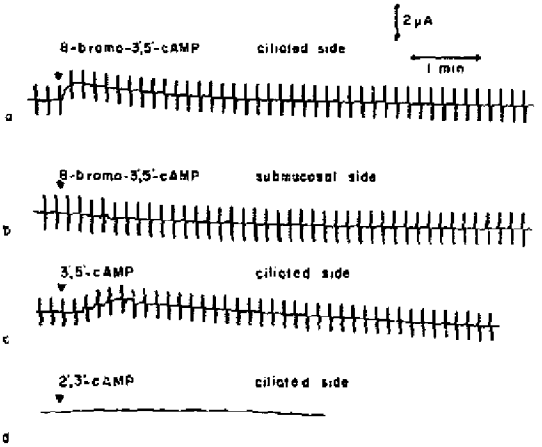

Fig. 2. Action of cyclic nucleotides on the basal short-circuit current. The arrowhead indicates the point at which 10^{-4} M solutions of each compound was introduced into the mucosal chamber. The bars represent the current excursions when 1 mV pulses were presented at regular intervals in order to measure the tissue resistance. Each trace shown is from a different tissue preparation and is representative of at least five preparations, with the exception of 2d where two preparations gave identical null results.

short-circuit current of about 53 $\mu A/cm^2$ [13]. Substituting a Ringer's containing 10^{-4} M 8-bromo-3',5'-cAMP (BrcAMP) on the ciliated surface elicited a net slow inward positive current transient that returned to baseline in about 2 min (Fig. 2a). When brief pulses of BrcAMP were presented to the ciliated side (see Fig. 5a for pulse

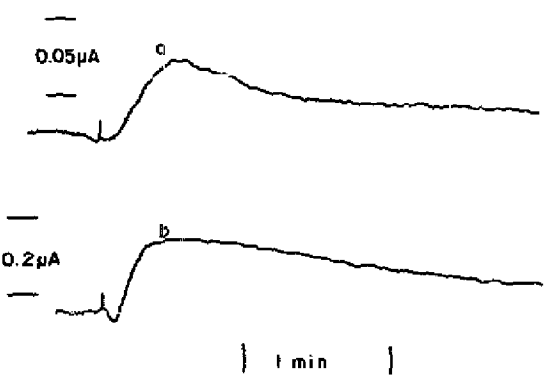

Fig. 3. (Curve a) Time course in short-circuit current resulting from a pulse of 10 μM BrcAMP. The artifact resulting from switching from Ringer's solution to Ringer's plus BrcAMP marks the start of BrcAMP presentation. (Curve b) Time course for 100 μM BrcAMP. This preparation shows a decreased current transient before an increase in current.

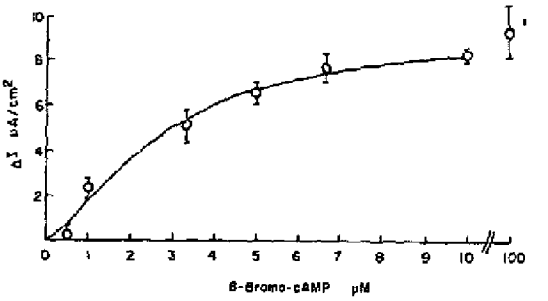

Fig. 4. Concentration dependence of the inward current induced by 8-bromo-cAMP. Ascending concentrations of BrcAMP were applied to the ciliated side of the olfactory mucosa. The maximum increase in current was taken as the response. The change in the short-circuit current was fitted to the Hill equation by nonlinear least-squares minimization criteria to obtain values for the half-maximal response of $2.75 \cdot 10^{-6}$ M and a Hill coefficient of 1.47 ± 0.03 (mean ± S.E., $N = 11$).

profile) and the responses observed at higher gain, the rise in current was seen to be either delayed (Fig. 3a) or preceded by a transient decreased current (Fig. 3b). Similar changes in the basal short-circuit current were not observed when BrcAMP was added to the submucosal side (Fig. 2b). In view of the fact that BrcAMP can itself elicit an inward current, the effect of preincubating the mucosa with BrcAMP on the responses to odorant pulses was determined only after the current had returned to basal levels following incubation with BrcAMP. The ability of BrcAMP to stimulate an inward current when added to the ciliated side was concentration-dependent (see below) and usually near maximal at 10^{-4} M. There was no change in the transmucosal resistance during BrcAMP-stimulated current transients. 3',5'-cAMP gave a similar current transient to that of BrcAMP at the same concentration, but the response was slower and of lower amplitude (Fig. 2c). The physiologically inactive derivative, 2',3'-cAMP, caused no change in the unstimulated short-circuit current (Fig. 2d).

With ascending concentrations of BrcAMP applied in turn to the ciliated side of the mucosa, there was a concentration-dependent increase in slow inward short-circuit current. After a transient increase, the current typically reached a broad maximum then declined slowly (cf. Fig. 2a). The current maximum following each addition of

BrcAMP was plotted as a function of BrcAMP concentration (Fig. 4) and fitted to the Hill equation to obtain values for the half maximal response of $2.75 \cdot 10^{-6}$ M and a Hill coefficient (n) of 1.47 ± 0.03 (S.E., $N = 11$). The average maximum increase in current caused by BrcAMP was 9.7 ± 1.6 $\mu A/cm^2$ (mean $\pm$ S.E., $N = 15$).

The results show that BrcAMP induces a reversible concentration-dependent inward current transient only when applied to the ciliated side of the olfactory mucosa.

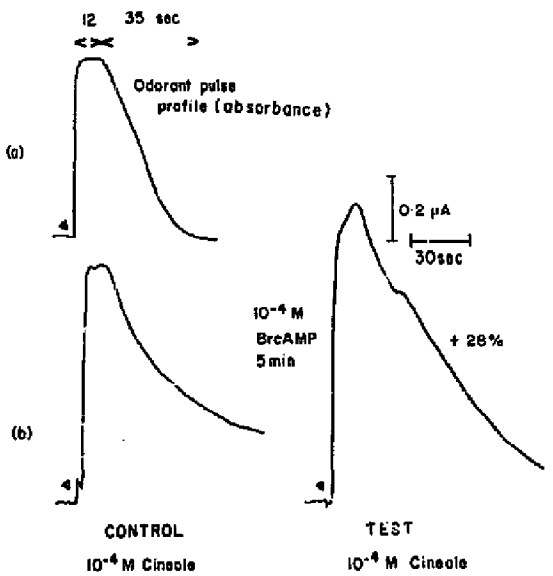

Fig. 5. Interaction of 8-bromo-cAMP and the odorant-evoked current. (a) The time profile of the pulse of odorant solution introduced into the reservoir facing the ciliated side of the mucosa. The mucosa was replaced with a photodarlington transistor and a pulse of blue dye was introduced into the reservoir in place of the odorant solution. The trace shows the current change across the phototransistor which is proportional to dye absorbance. The peak was reached in 1 s and maintained this amplitude for 12 s before declining to baseline in about 35 s. (b) The left trace shows the odorant-evoked current transient recorded when a pulse of 10^{-4} M 1,8-cineole was presented to the ciliated side of the olfactory mucosa. The ciliated side was bathed with 10^{-4} M BrcAMP for 5 min, returned to Ringer's solution and when basal current had again been restored the response to 1,8-cineole was again measured (right trace). The magnitude of the odorant-evoked current was increased by 28% in the case shown. Arrowheads indicate the point at which the stimulus was introduced. Table I shows the effects seen with higher odorant concentration and BrcAMP concentration.

TABLE I

EFFECT OF CYCLIC NUCLEOTIDES OR CYCLIC NUCLEOTIDE MODULATORS ON THE ODORANT RESPONSE

The ciliated side of the frog olfactory was incubated with the required concentrations of each compound for 5 min. The magnitude of the odorant-evoked current transients were recorded before and after each incubation. The magnitude of the odorant-evoked current transients after each incubation are shown relative to the control values and are presented as the mean ± S.D. where applicable or mean (range) where the range is the difference between the largest and smallest values. IBMX, 3-isobutyl-1-methylxanthine.

Compound	% Odorant response [a]	No. of tissue preparations tested
10^{-4} M BrcAMP	136 ± 3.8	4
10^{-4} M BrcAMP [b]	135 (36.8)	2
10^{-3} M BrcAMP	163 ± 2.4	4
10^{-3} M cAMP	116 (10.4)	2
10^{-6} M forskolin	123 (16.2)	2
10^{-5} M forskolin	153 ± 17.5	3
10^{-5} M GTPγS	143 ± 30.2	3
10^{-5} M GDPβS	61 (10.4)	2
10^{-4} M IBMX	139 (8.4)	2
10^{-6} M BrcGMP	58 ± 6.0	3
10^{-5} M BrcGMP	33 (9.6)	2
10^{-4} M BrcGMP	15.2 (11.6)	2

[a] Relative to control value.

[b] $2.4 \cdot 10^{-4}$ M 2-isobutyl-3-methoxypyrazine used as a test odorant. In all other cases $2.4 \cdot 10^{-4}$ M 1,8-cineole was used.

Interaction of BrcAMP and odorant-evoked current

(a) Preincubation of the tissue with BrcAMP. Responses to odorants were characterized before and after incubating the ciliated side of the olfactory mucosa with 10^{-4} M BrcAMP for 5 min. Fig. 5a shows the time profile of the dye sham stimulus introduced into the Ussing chamber. It had a sharp, rapid rise to a constant level of 12 s duration, then an exponential decay to baseline within 35 s. Fig. 5b (left) shows the odorant-evoked current response to a pulse of 10^{-4} M 1,8-cineole. The current transient elicited to odorant by the tissue lagged behind the stimulus pulse measured by the pulse monitor. The ciliated side was then bathed with 10^{-4} M BrcAMP for 5 min, returned to Ringer's solution and the response to 1,8-cineole again measured after the current returned to basal level (Fig. 5b right). The odorant-induced current transient was increased by an average of 36% with

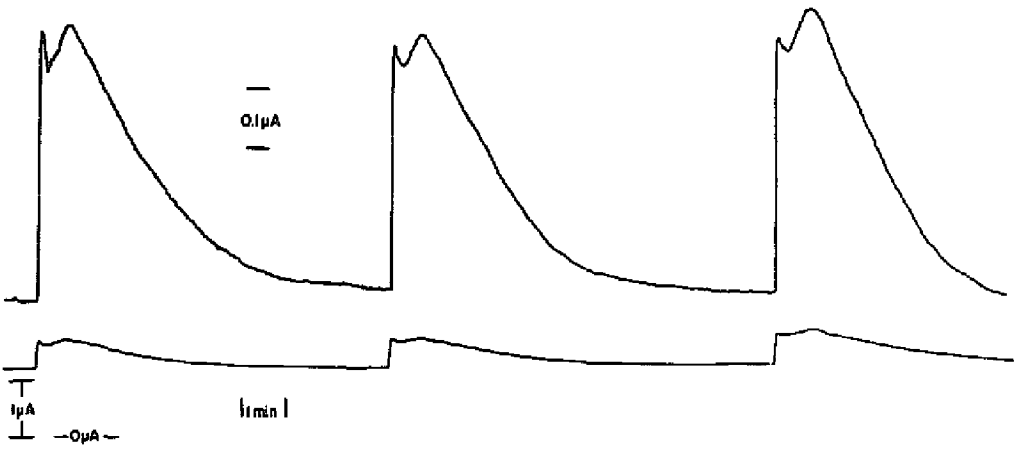

Fig. 6. (Lower trace) Continuous trace of the short-circuit current during three successive stimulations with pulses of 10^{-4} M cineole. The odorant response is superimposed on a standing short-circuit current (baseline) of 1.24 μA. (Upper trace) The same responses at 10-fold higher gain. An odorant-evoked response is measured relative to the standing short-circuit current as baseline. Responses are in modified Ringer's containing 50 mM NaCl.

10^{-4} M BrcAMP and 63% with 10^{-3} M BrcAMP (Table I). This effect of BrcAMP was observed with other odorants including the bell-pepper smelling odorant, 2-isobutyl-3-methoxypyrazine (Table I). Thus the effect was not specific for the single odorant cineole which is consistent with the postulated role for cAMP in a general mechanism for olfactory transduction [8,10,11].

(b) Effect of BrcAMP and odorant presented simultaneously. The ability of BrcAMP to increase the magnitude of the current transient due to a subsequently applied odorant provides biophysical evidence that cAMP may be an intermediate in olfactory transduction as has been suggested by the in vitro biochemical studies of odorant-stimulated adenylate cyclase activity [8–11]. To explore this further in a physiologically intact preparation, we examined the current transients produced when BrcAMP and 1,8-cineole were presented as a mixture to the ciliated side of the mucosa. As shown in Fig. 4, the BrcAMP-evoked current was described by a sigmoidal function of the BrcAMP concentration. The cineole dose-response is also sigmoidal [13]. If both odorants and BrcAMP can activate the same current generating pathway, then it should be possible to demonstrate that certain mixtures of BrcAMP and 1,8-cineole can give enhanced response magnitudes, purely additive re-

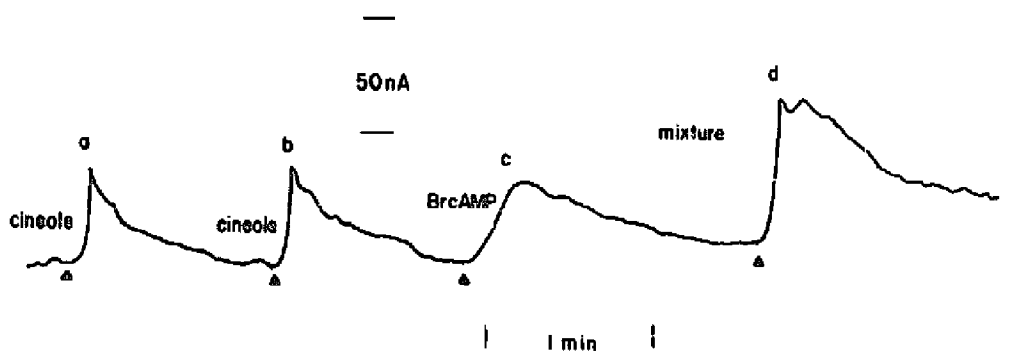

Fig. 7. Effect of BrcAMP and odorant presented simultaneously. Traces a and b are consecutive responses to 5 μM cineole. Trace c is the response to 1 μM BrcAMP, and trace d is the response to the mixture of 5 μM cineole and 1 μM BrcAMP. Responses are in modified Ringer's containing 50 mM NaCl.

sponses, or partially additive responses, these reflecting the nonlinear, saturable, character of the BrcAMP-current relation. All three response types can be observed at appropriate concentrations.

In each experiment a stable response pattern to 10^{-4} M cineole was first established. Fig. 6 shows three successive stimulations with pulses of 10^{-4} M cineole. The lower set of traces illustrates the relationship between the stimulated current and the basal current, i.e. the odorant response current is superimposed on the standing short-circuit current. In a stable preparation responses are reproducible to within 10%. The upper trace shows the same responses at 10-fold higher gain which allows us to focus attention on the odorant response apart from the standing current. Thus as stated earlier a response is always defined as a relative change in current above standing (baseline) levels. Thus small variations in the standing current, that occur in times that are long compared to the time of an odorant response, will not have an effect on the response.

Figs. 7a and 7b show responses to pulses of 5 μM cineole. This is followed by a response to 1 μM BrcAMP (Fig. 7c). The latter response follows the pattern shown in Fig. 3a. Fig. 7d shows the response to a mixture of 5 μM cineole and 1 μM BrcAMP. These data are analyzed in Fig. 8. Here the first 30 s are shown of the response to BrcAMP, the response to cineole (data of Fig. 7b), the numerically summed responses of cineole, and BrcAMP, and the response to the mixture of cineole and BrcAMP. The mixture initially gave a response that was greater than the sum of the responses to cineole and BrcAMP presented separately. This was observed in three preparations and is consistent with the sigmoidal character of the dose-response curves for BrcAMP and cineole. Both cineole and BrcAMP concentrations used here are well below their respective concentrations

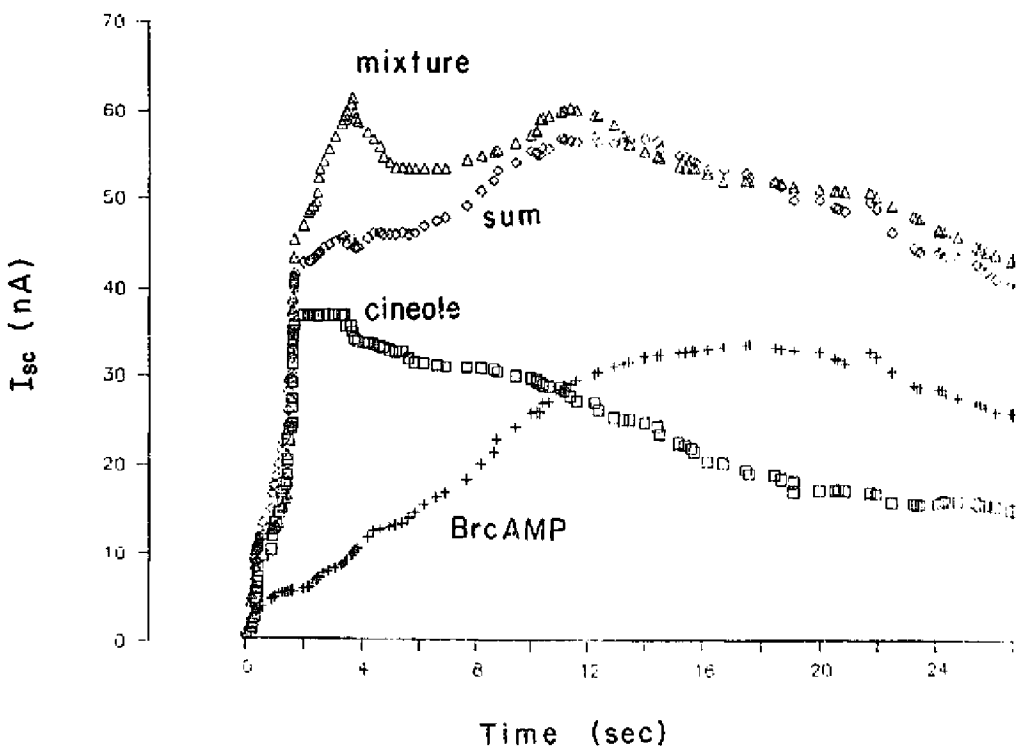

Fig. 8. Traces b, c, and d of Fig. 7 were digitized and superimposed. The responses shown are: to BrcAMP (+); to cineole (□); the numerical sum of traces of BrcAMP and cineole and b (◇); and the mixture of cineole and BrcAMP (△). Note that for early times the response of the mixture is greater than the sum of the individual responses. Responses are in modified Ringer's containing 50 mM NaCl.

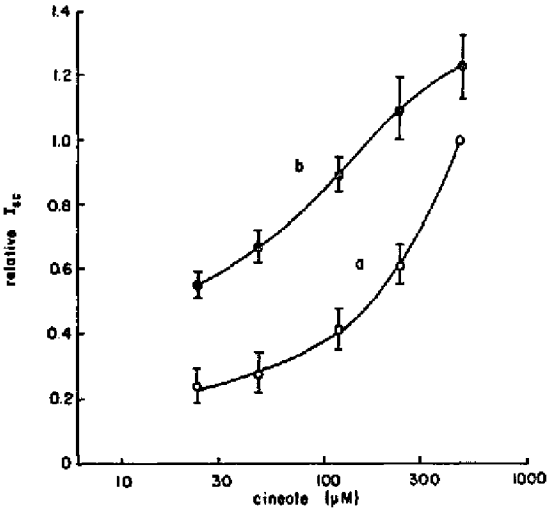

Fig. 9. Additive effect of BrcAMP and odorant on the current response. The interaction of BrcAMP and the odorant in evoking a current transient was investigated by presentation of pulses of increasing concentrations of 1,8-cineole while keeping the concentration of BrcAMP constant at $5 \cdot 10^{-6}$ M. (a) Concentration-response profile measured with odorant alone (○) and (b) odorant plus BrcAMP (●). All currents in each experiment were expressed as a fraction of that observed with $4.8 \cdot 10^{-4}$ M cineole in the absence of BrcAMP. Under these conditions the addition of BrcAMP displaced the cineole points by a nearly constant amount except for the highest concentration of cineole for which the mixture produced a response that was less than additive. Each point represents the mean ± S.D. for three preparations.

giving half-maximal response. At 1 μM the BrcAMP concentration is 0.36 of the value that gives half-maximal response (2.75 μM). From the concentration-response relation for cineole [13] the concentration giving half-maximal response is 75 μM. A cineole concentration of 5 μM is therefore 0.067 of the cineole concentration giving half-maximal response. Under these conditions the cineole responses are in the early sigmoidal part of the concentration-response curve, a necessary condition for a supra-additive result.

At higher cineole and BrcAMP concentrations responses were strictly additive. This is shown in Fig.9 using a range of cineole concentrations between 24 μM and 480 μM (Fig. 9a), and the same cineole concentrations containing 5 μM BrcAMP (Fig. 9b). At intermediate odorant concentrations the concentration-response relation of cineole-BrcAMP mixtures was displaced upward on the current axis by an amount equal to the response due to 5 μM BrcAMP alone. However, a limit on the total current output was evident at high cineole concentrations. At a cineole concentration of 480 μM, the short-circuit current evoked by cineole and 5 μM BrcAMP presented together was less than the sum of the currents evoked by cineole and BrcAMP separately. In this case, BrcAMP pushes the concentration-response relation to saturation. This is consistent with the established saturability of the current as a function of the cineole concentration [13].

The results of these experiments show that when mixtures of odorant and BrcAMP are presented simultaneously, an enhanced current transient results due to the addition of the components of the currents induced by each compound.

Modulators of cAMP and the odorant-induced current

Compounds that modify the intracellular cAMP concentration in situ can also modify the basal and odorant-evoked currents in a concentration-dependent way. High concentrations (> 1 mM) of cAMP, its analogs or reagents that cause its generation increased the basal short-circuit current by about 20–30% for as long as 30 min. During this time, odorant-evoked current transients were substantially reduced in amplitude. Partial recovery of the odorant-evoked current was observed only as the short-circuit current returned to the original baseline. In light of their effects on the basal current at high concentration, we employed low concentrations of cyclic nucleotide modulators to investigate their effects on the odorant-evoked response. These compounds produced comparable effects on the in vitro preparation as reported for the biochemical preparation [8]. Concentrations less than 10^{-5} M of forskolin, GTPγS, and GDPβS, and 10^{-4} M 3-isobutyl-1-methylxanthine caused little change in the basal short-circuit current when delivered to the ciliated side. The responses to odorants were characterized before and after the application of forskolin and guanine nucleotides at concentrations below 10^{-5} M. The tissue was preincubated with them for 5 min prior to presentation of odorant. Forskolin, a powerful stimulant of adenylate cyclase activity [15], caused a 23% increase in the odorant response at a con-

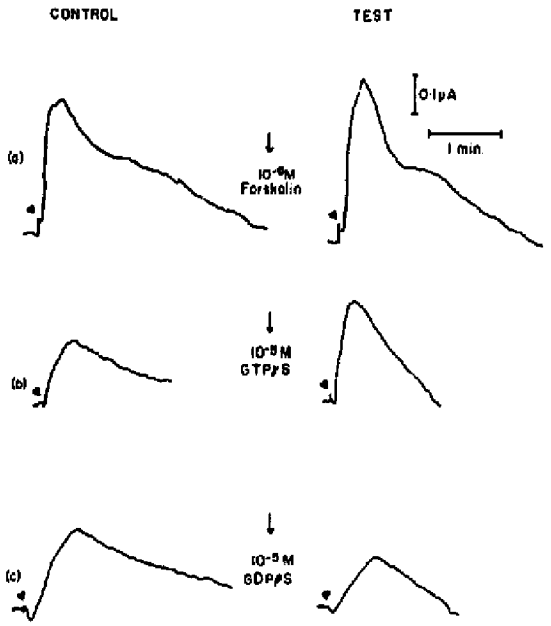

Fig. 10. Action of cAMP modulators on the odorant-evoked current. The traces show the odorant-evoked current transients obtained when a pulse of $2.4 \cdot 10^{-4}$ M 1,8-cineole was presented (left to right) before and after bathing the ciliated side of the mucosa with (a) 10^{-6} M forskolin, (b) 10^{-5} M GTPγS, (c) 10^{-5} M GDPβS for 5 min. Each procedure was carried out on different tissue preparations and Table I shows the number of preparations tested with each compound. Arrowheads indicate the point at which stimuli were introduced into the Ussing chamber. In this case, the flow rate of the solution was 0.11 ml/s.

centration of 10^{-6} M (Fig. 10a) and a 53% increase at 10^{-5} M (Table I). Similarly, increasing the endogenous cAMP concentration with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine at 10^{-4} M enhanced the odorant responses by 39% (Table I). Theophylline (10^{-4} M) had a similar effect. These results are consistent with the observations of increased magnitudes of the odorant responses when BrcAMP was applied externally on the ciliated side of the mucosa (Fig. 4). Furthermore, they indicate that intracellular cAMP levels can modulate an odorant-dependent current source on the ciliated side of receptor cells, and that exogenously added BrcAMP probably acts by entering the intracellular cAMP pool of the receptor neurons.

GTP-binding proteins have been identified biochemically in olfactory cilia and have been suggested to be components in olfactory transduction [8–11]. The effects of the nonhydrolysable guanine nucleotides, GTPγS and GDPβS, on the odorant-evoked current were investigated. GTPγS at 10^{-5} M caused an enhancement of the odorant response (Fig. 10b and Table I) while GDPβS at the same concentration caused suppression (Fig. 10C and Table I). This is a result consistent with the involvement of a stimulatory G-protein in increasing endogenous cAMP levels in the physiologically intact olfactory mucosa.

We showed that the odorant-evoked current was due to the activation of amiloride-sensitive current sources located on the ciliated side of the olfactory mucosa [13]. This could be distinguished readily from the basal short-circuit current that was amiloride-insensitive and blocked by furosemide. For these nucleotide experiments we reestablished that 10^{-4} M amiloride causes no change to the standing short-circuit current when applied for 5 min. To determine whether BrcAMP-evoked current and the odorant-evoked current activate a common pathway, we presented mixtures of 10^{-5} M BrcAMP and 10^{-4} M amiloride or 10^{-4} M 1,8-cineole and 10^{-4} M amiloride, as pulses to the ciliated side of the olfactory mucosa and recorded the current transients evoked. There were major differences in the properties of the current transients but the overall effect of amiloride was the same (Fig. 11). In the first series (11 a–c) the odorant-evoked response consisted of a rapid influx of positive current that slowly returned to baseline, the response magnitude was reduced substantially by amiloride (Fig. 11b) and was reversible to within 70% (Fig. 11c). In contrast, the current transient elicited by low BrcAMP concentration consisted of a slower response in which the inward positive short-circuit current was preceded by an initial current decrease (Fig. 11d, see also Fig. 3b). The effect of amiloride was similar to that on the odorant-evoked response in that the inward current transient was reduced (Fig. 11e) reversibly (Fig. 11f).

Taken together, these experiments are consistent with the hypothesis that odorant-induced current is the end result of a sequence of steps leading to the production of cAMP. The results with forskolin, GTPγS and GDPβS confirm the biochemical evidence that suggests that the inter-

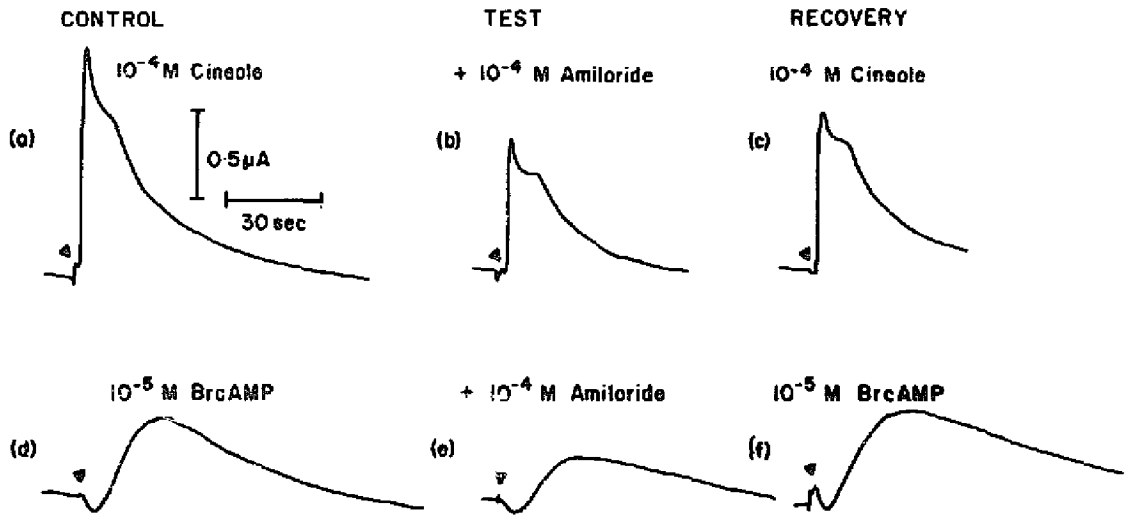

Fig. 11. Effect of amiloride on the current-transients evoked by odorant or BrcAMP. Mixtures of 10^{-4} M amiloride with 10^{-5} M BrcAMP or 10^{-4} M 1,8-cineole were presented as pulses to the ciliated side of the olfactory mucosa. The traces show the current transients recorded. (a) Control response to 1,8-cineole. (b) Reduced magnitude of response to a mixture of amiloride and cineole. (c) Response to cineole alone showing 70% recovery of response. (d) Control response to BrcAMP. (e) Reduced magnitude of response to a mixture of amiloride and BrcAMP. (f) Response to BrcAMP alone showing 100% recovery of response.

mediate steps involve a G_s-protein coupled adenylate cyclase. The fact that the currents evoked by BrcAMP and cineole can be supra additive in certain mixtures, and that exogenously added BrcAMP and odorant both activate an amiloride-sensitive current source is further indication that cAMP production may be a key factor in the normal response to some odorants.

Interaction of BrcGMP with the basal and odorant-evoked currents

In contrast to BrcAMP, the effect of BrcGMP was a concentration-dependent decrease in the basal short-circuit current (Fig. 12). The concentration of BrcGMP giving half-maximal suppression was $2.55 \cdot 10^{-7}$ M and the Hill coefficient (n) was 0.8 ± 0.02 (S.E., $N = 11$). The average diminution of current at 10^{-4} M BrcGMP was 3.96 ± 0.96 $\mu A/cm^2$ (S.E., $N = 11$). This decrease was 5–10% of the basal short-circuit current. Following washout of BrcGMP with Ringer's, the current remained low. However, the subsequent addition of BrcAMP caused an immediate increase in current. The 10-fold lower concentration of BrcGMP giving half-maximal suppression of current compared to that giving half-maximal increase with BrcAMP may imply differences in affinities between the two cyclic nucleotides for their sites of action. It is presently unknown

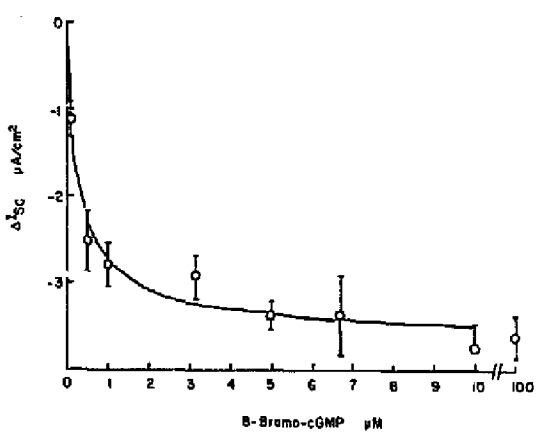

Fig. 12. Effect of 8-bromo-cGMP on the basal current. The ciliated side of the olfactory mucosa was incubated with increasing concentrations of BrcGMP. The plot shows the change in basal current with concentration fitted to the Hill equation. The concentration of BrcGMP giving half-maximal suppression was $2.55 \cdot 10^{-7}$ M and the Hill coefficient was 0.8 ± 0.02 (mean $\pm$ S.E., $N = 11$). The suppression of basal current by BrcGMP could be overcome by subsequent addition of BrcAMP to the solution bathing the ciliated side.

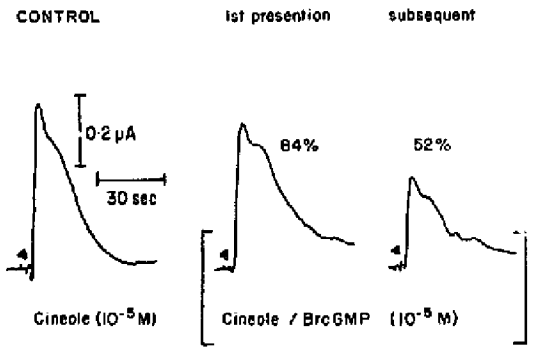

Fig. 13. Interaction of 8-bromo-cGMP with the odorant-evoked current transient. The traces show the responses to 10^{-5} M 1,8-cineole alone (left), a mixture of 10^{-5} M cineole and 10^{-5} M BrcGMP (center) and a subsequent presentation of this mixture (right). The magnitude of the current transient was reduced to 52% of the control and this effect was not reversible in the time scale of the preparation.

whether BrcAMP and BrcGMP act on a single site or different sites with overall antagonistic effects.

BrcGMP was a potent inhibitor of the odorant-evoked current. Bathing the olfactory mucosa with 10^{-6} M BrcGMP for 5 min reduced the magnitudes of subsequent odorant responses by 42%, while higher concentrations caused further diminution (Table I). Reduced odorant-evoked current may be the result of the summation of two opposing currents – that due to BrcGMP induced efflux of cations from the cells together with the inward positive current due to odorant stimulation. The reduction of the odorant response could not be overcome when BrcAMP was applied subsequently.

When a mixture of 10^{-5} M 1,8-cineole and 10^{-5} M BrcGMP was applied as a pulse to the ciliated side of the mucosa, the amplitude of the evoked current was initially only slightly diminished, but subsequent responses to this mixture were reduced by approx. 50% (Fig. 13). There was no recovery of response to odorant alone for the duration of the preparation. This is in contrast to the reversible effect of BrcAMP or amiloride on the odorant-evoked current (cf. Fig. 11). The results of these experiments indicate that cGMP must have its primary effect on an outward current source which contrasts with the apparent action of cAMP.

Discussion

cAMP induced inward currents

Olfactory mucosa short-circuited in vitro had a mean steady state inward current of 53 $\mu A/cm^2$ in the absence of odorant stimulation. Our previous study. [13] indicated that this basal current is due mainly to a furosemide sensitive chloride-dependent ion transport pathway. Superimposed on this standing current are odorant-evoked current transients that are partially amiloride-sensitive but not affected by furosemide. The maximum magnitude of the odorant-evoked currents usually ranges from 0.1 to 1 μA or 2.5% to 25% of the standing short-circuit current. Earlier attempts to elucidate the effects of cyclic nucleotides on the electrical properties of the olfactory mucosa relied on measuring a voltage transient called the electro-olfactogram [16,17]. It was recorded on a floating baseline, so interpretations of the results of the action of cyclic nucleotides on transduction were ambiguous, when 3′,5′-cAMP and its bromo derivative were applied to the ciliated side of the voltage-clamped mucosa in our studies, a slow inward positive current transient developed. BrcAMP was more effective than 3′,5′-cAMP presumably due to a higher membrane permeability. Both were ineffective when applied to the submucosal side of the preparation, indicating that the site of action is on the ciliated side. The inward current was not accompanied by changes in transmucosal resistance. Therefore, the dominant transmucosal current pathways, which are likely to be the paracellular shunts, are not affected by cAMP. The changes in basal current induced by different concentrations of BrcAMP were described by a sigmoidal concentration-response relationship that fit the Hill equation. The Hill coefficient of 1.47 ± 0.03 may imply a cooperative effect in the activation of a single channel type or a heterogeneous population of channels. This value and the half-maximal value of 2.75 μM agree well with the parameters describing the concentration-dependent ciliary conductance induced by direct application of cAMP (1.82 and 2.38 μM, respectively) [12]. This correspondence also suggests that BrcAMP can fully equilibrate between the external and internal cell compartments. Our results demonstrate for the first time that increas-

ing cAMP levels is accompanied by a slow inward current transient in a physiologically intact preparation of olfactory mucosa.

The existence of a cAMP-gated conductance in the olfactory mucosa does not by itself demonstrate a role for cAMP in olfactory transduction. However, we have shown in Table I that preincubation of the tissue on the ciliated side with compounds known to increase intracellular levels of cAMP as well as externally applied BrcAMP result in a reversible enhancement of odorant-evoked responses. This is evidence suggesting that the modulation of intracellular cAMP levels is linked to modulation of odorant-evoked currents. Previously reported biochemical evidence demonstrated that odorants stimulate adenylate cyclase activity in olfactory cilia [8,10,11]. A potent stimulator of adenylate cyclase activity, forskolin, substantially enhanced the magnitude of the odorant-evoked current transient in our studies. This is evidence in support of the hypothesis that the level of adenylate cyclase activity is coupled to changes in conductance induced by the odorant. The biochemical results also indicate a role for the coupling of a G_s protein between the adenylate cyclase and the odorant-receptor [8]. In view of the known effects of GTP analogs on G-protein activity in other receptor systems [18,19,21], our results showing enhancement of the odorant-evoked currents by GTPγS and their inhibition by GDPβS further confirm that adenylate cyclase activity is regulated by a G_s-protein. It should be noted that a 5-min preincubation period with the GTP and GDP analogues is sufficient for an effect to be observed. This is presumably enough time for entry by diffusion across the large surface area presented by the olfactory cilia. Liscia et al. have recently investigated the role of cAMP on the chemosensory system of the blowfly [18]. Their work demonstrates the effectiveness of extracellular addition of GDPβS.

If exogenous BrcAMP and odorant-induced cAMP are activating the same current source their effects should be additive. Fig. 4 shows that the short-circuit current increases with an increasing external concentration of BrcAMP. The resulting current is a nonlinear function of BrcAMP concentration. The concentration response function for cineole is similar [13]. Therefore, the effects of the simultaneous addition of odorant and BrcAMP will be constrained by the nonlinear properties of both concentration-response functions. If cineole-evoked currents were independent of BrcAMP-evoked currents, mixtures of cineole and BrcAMP would produce additive responses at all concentrations. However, the data in Figs. 7 and 8 show experimentally induced enhancement at low odorant concentrations. This presumably results from the addition of an external source of BrcAMP to the endogenous cAMP production evoked by the odorant. The combined effect of cAMP from the two sources produces a current greater than the sum of those currents produced by each separately. Also, the data in Fig. 9 show additivity at intermediate concentrations and partial additivity at the highest odorant concentration. This results from the linear relationship at the midrange in the concentration-response curve and saturability at higher concentrations.

The odorant-evoked current is partially blocked by amiloride [13]. Our results also show that amiloride partially suppresses the current evoked by exogenous BrcAMP. Thus, both odorant stimulation and exogenously applied BrcAMP appear to converge on a common current source distinct from that regulating the basal current. In view of the known action of amiloride [13,20], this is probably a cation channel with some sodium selectivity. It is only blocked by amiloride when an odorant is presented simultaneously, indicating that the amiloride action is quite reversible and most effective on the activiated state of the channel [20]. This conclusion is supported by the fact that simultaneous application of amiloride and BrcAMP is required to suppress the effect of BrcAMP alone.

The odorant- and BrcAMP-evoked currents have markedly different onset times. With addition of BrcAMP there is a lag or current decrease just prior to the influx of positive current. In some cases the current may reach a maximum several seconds after BrcAMP has been washed out of the chamber. The lag could be due, in part, to diffusion limitations. However, prior to the influx of positive current many preparations show an initial downward deflection in the current trace. This suggests that BrcAMP can also activate a small transient outward current. In contrast to

BrcAMP, an odorant induces a rapid initial influx of positive current. The difference in the response kinetics is further indication that BrcAMP is not itself an odorant, but rather an intermediate in normal olfactory function. This conclusion is strengthened by the fact that BrcGMP does not give responses similar to BrcAMP as might have been expected were the two acting as olfactory stimuli.

cGMP inhibition of short-circuit current

In contrast to the effects of BrcAMP on the basal short-circuit current, BrcGMP caused a concentration-dependent decrease. The cyclic nucleotide, BrcAMP, did not reverse the effects of BrcGMP on the basal short-circuit current. BrcGMP was effective at a 10-fold lower concentration than BrcAMP. This evidence suggests the existence of a higher affinity site for cGMP. It is presently not known if the small outward current often seen following BrcAMP addition represents action at the cGMP site.

The odorant-evoked short-circuit current was also suppressed by BrcGMP and its action could not be reversed by BrcAMP within the time course of the experiment. In contrast, BrcAMP reversibly enhanced the amplitude of the odorant-evoked current transient. These observations suggest that cAMP and cGMP have independent actions in the intact tissue. The reduced amplitudes of the odorant-evoked currents after simultaneous presentation of BrcGMP and odorant may, therefore, be the result of parallel activation of opposing

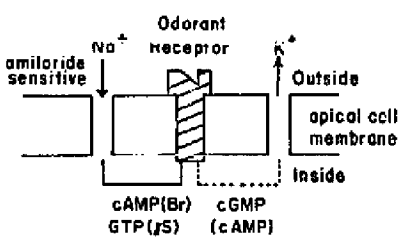

Fig. 14. Tentative assignment of the interaction of cyclic nucleotides with the membranes of the ciliated side of the olfactory mucosa. The odorant receptor is coupled to an amiloride-sensitive ion channel via a GTP-modulated, cAMP mediated mechanism. It is possible that cGMP may inhibit this process. The effects of BrcGMP also suggest the activation of parallel potassium channels which may also be odorant activated.

membrane currents as shown in Fig. 14. This would suggest that in the intact preparation the net effects of cAMP and cGMP can be quite dissimilar. On the basis of our study cAMP has two effects: the production of a small outward current, followed by a larger inward current. At similar concentrations cGMP appears to mediate only outward currents. The qualitative similarity between cAMP and cGMP that emerges from conductance measurements on isolated membrane patches [12] may indicate that both activate a common channel mediating outward current. However, as shown herein, cAMP has an additional function not shared with cGMP, that is, the mediation of an inward current.

Interdependent roles of cyclic nucleotides

These studies provide the first biophysical evidence for the role of adenylate cyclase and a GTP binding protein in the intermediate steps in olfactory transduction. The results confirm the biochemical studies of adenylate cyclase in ciliary membrane fragments and extend them to the cellular level in a physiologically intact preparation. In addition, we have demonstrated that cAMP and GTP modulate a cation conductance in an amiloride-sensitive channel that is activated by odorants. The small transient outward current induced by BrcAMP prior to initiation of an inward current may indicate that cAMP, like cGMP, can also activate a site for outward current. Potassium channels have been identified in olfactory neurons using patch-clamp techniques [5,6,21–23] and implications for their function in odorant responses have been suggested from reconstituted membrane experiments [24–26]. In addition LaBarca et al. [26] have presented evidence suggesting that odorants can also directly activate channel conductances. In view of the large K^+ concentration gradient between the inside and outside of the cell, it is possible that the current decrease induced by BrcGMP may be as a result of K^+ ion efflux. cGMP and cAMP may therefore also regulate the $[K^+]_i/[K^+]_o$ in the basal and odorant-stimulated states of the olfactory mucosa. The occurrence of a parallel ionic mechanism for inward and outward currents may be an important on the sensitization-desensitization cycle and on the adaptive properties in olfactory receptor neurons.

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