Protein Kinase C Sensitizes Olfactory Adenylate Cyclase

STEPHAN FRINGS

From the Department of Physiology, Universität des Saarlandes, D-6650 Homburg/Saar, Germany

ABSTRACT Effects of neurotransmitters on cAMP-mediated signal transduction in frog olfactory receptor cells (ORCs) were studied using in situ spike recordings and radioimmunoassays. Carbachol, applied to the mucosal side of olfactory epithelium, amplified the electrical response of ORCs to cAMP-generating odorants, but did not affect unstimulated cells. A similar augmentation of odorant response was observed in the presence of phorbol dibutyrate (PDBu), an activator of protein kinase C (PKC). The electrical response to forskolin, an activator of adenylate cyclase (AC), was also enhanced by PDBu, and it was attenuated by the PKC inhibitor Goe 6983. Forskolin-induced accumulation of cAMP in olfactory tissue was potentiated by carbachol, serotonin, and PDBu to a similar extent. Potentiation was completely suppressed by the PKC inhibitors Goe 6983, staurosporine, and polymyxin B, suggesting that the sensitivity of olfactory AC to stimulation by odorants and forskolin was increased by PKC. Experiments with deciliated olfactory tissue indicated that sensitization of AC was restricted to sensory cilia of ORCs. To study the effects of cell Ca^{2+} on these mechanisms, the intracellular Ca^{2+} concentration of olfactory tissue was either increased by ionomycin or decreased by BAPTA/AM. Increasing cell Ca^{2+} had two effects on cAMP production: (*a*) the basal cAMP production was enhanced by a mechanism sensitive to inhibitors of calmodulin; and (*b*) similar to phorbol ester, cell Ca^{2+} caused sensitization of AC to stimulation by forskolin, an effect sensitive to Goe 6983. Decreasing cell Ca^{2+} below basal levels rendered AC unresponsive to stimulation by forskolin. These data suggest that a crosstalk mechanism is functional in frog ORCs, linking the sensitivity of AC to the activity of PKC. At increased activity of PKC, olfactory AC becomes more responsive to stimulation by odorants, forskolin, and cell Ca^{2+}. Neurotransmitters appear to use this crosstalk mechanism to regulate olfactory sensitivity.

INTRODUCTION

The perception of odorants in vertebrates is mediated by at least two signal transduction pathways, which lead from binding of odorants at the sensory membrane of olfactory receptor cells (ORCs) to the generation of action potentials at the basal cell pole. Many odorants (e.g., eugenol and menthone) stimulate olfactory adenylate cyclase type III (AC), which is specific for the sensory membrane of ORCs

Address reprint requests to Dr. Stephan Frings, Institut für Biologische Informationsverarbeitung, Forschungszentrum Jülich, 5170 Jülich, Germany.

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(Pace, Hanski, Salomon, and Lancet, 1985; Sklar, Anholt, and Snyder, 1986; Lowe, Nakamura, and Gold, 1989; Pfeuffer, Mollner, Lancet, and Pfeuffer, 1989; Bakalyar and Reed, 1990; Boekhoff, Tareilus, Strotmann, and Breer, 1990; Asanuma and Nomura, 1991; Breer and Boekhoff, 1991; Menco, Bruch, Dau, and Danho, 1992). Other odorants (e.g., lyral and lilial) stimulate olfactory phospholipase C (PLC) (Boekhoff et al., 1990; Breer and Boekhoff, 1991). The molecular mechanisms by which cytosolic signal molecules of these pathways (adenosine 3:5-cyclic monophosphate [cAMP], inositol 1,4,5-trisphosphate [IP_3], diacylglycerol [DAG], and Ca^{2+}) induce electrical excitation of the sensory neurons are only partially understood. cAMP directly activates cation channels in the sensory membrane (Nakamura and Gold, 1987; Dhallan, Yau, Schrader, and Reed, 1990; Ludwig, Mavgalit, Eismann, Lancet, and Kaupp, 1990; Firestein, Zufall, and Shepherd, 1991; Frings, Lynch, and Lindemann, 1992), while IP_3 receptors in the sensory membrane of ORCs (Kalinoski, Aldinger, Boyle, Huque, Marecek, Prestwich, and Restrepo, 1992; Khan, Steiner, and Snyder, 1992) appear to be linked to IP_3-gated cation channels (Restrepo, Miyamoto, Bryant, and Teeter, 1990; Miyamoto, Restrepo, Cragoe, and Teeter, 1992). It is generally held that these ligand-activated ion channels conduct the receptor current.

Recently it was shown that a crosstalk mechanism exists in ORCs which gives rise to interaction between cAMP- and IP_3-mediated transduction chains: Ca^{2+} was shown to activate olfactory AC through a calmodulin-dependent mechanism (Anholt and Rivers, 1990; Choi, Xia, and Storm, 1992). Consequently, when cytosolic Ca^{2+} concentration increases after IP_3 generation in response to odorants or other stimuli, cAMP concentration is also expected to increase as a result of this interesting link between two signal transduction pathways.

In this report, further evidence is presented for the notion that the two olfactory signal transduction pathways involving AC and PLC do not function independently of each other. I have studied the effects of neurotransmitters on ORCs of the frog, and the results suggested that the activity of the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) in frog olfactory epithelium determined the responsiveness of AC to stimulation by odorants, forskolin, or cell Ca^{2+}. Increased PKC activity enhanced the sensitivity of AC, while reduced activity of PKC attenuated cAMP production in response to stimuli. Sensitivity of AC was also increased by the neurotransmitters carbachol and serotonin, but not by substance P. A model is proposed, explaining regulation of sensitivity of olfactory AC by neurotransmitters binding to PLC-coupled receptors. Neurotransmitters would cause activation of PKC through release of diacylglycerol and cell Ca^{2+}. Through the PKC-mediated sensitization of AC, neurotransmitters appear to modulate cAMP response to odorant stimulation, and to affect olfactory sensitivity.

METHODS

Tissue Preparation

Frogs (*Rana esculenta/ridibunda*) from Turkey were kept at 4°C in tap water during winter or in a terrarium at 20–30°C during summer where they were fed with crickets. Odorant responses could only be recorded regularly when animals were kept at room temperature. Frogs were killed by decapitation, the skin covering the nose was removed, and the two dorsal olfactory

mucosae were excised by cutting out the triangular plate of bone that supports the epithelium on each side of the septum. The epithelium was cut from the bone plates and washed in Ringer's solution (mM): 125 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 8 Na_2HPO_4, 1.5 KH_2PO_4, 5 glucose, and 5 pyruvate, pH 7.4, 265 mosM). The two ventral mucosae were carefully lifted from the bone with bent forceps and washed in Ringer's solution. Tissue from animals with damaged skin around the nares, and tissue with traces of haemorrhage were not used.

To obtain deciliated tissue, olfactory mucosa was first washed in Ringer's solution. The tissue pieces were then transferred to divalent cation-free solution (mM): 120 NaCl, 4 NaOH, 3 KCl, 10 HEPES, and 2 EDTA, pH 7.4) in which they were gently stirred for 15 min. After this, the Ca^{2+} concentration was abruptly raised to 10 mM (calcium shock) by adding 1 M $CaCl_2$ to the solution. In variance with the original method of deciliation (Chen and Lancet, 1984), ethanol was not used in our preparation in order to prevent damage to the deciliated tissue. The tissue was stirred in the high Ca^{2+} solution until a satisfactory degree of deciliation could be confirmed by microscope (30–50 min). Finally, the tissue was thoroughly washed and equilibrated in Ringer's solution for 30 min.

Recording of Spike Rate In Situ

To monitor electrical activity of single olfactory receptor cells in situ, action potential–induced signals were recorded from olfactory cilia as described earlier (Frings and Lindemann, 1990). Briefly, dorsal olfactory epithelia were mounted in a recording chamber such that cilia could be observed by microscope and could be pulled into a recording electrode by suction. Separate perfusion of serosal (interstitial) and mucosal (sensory) surfaces was achieved by folding the epithelium tightly around the serosal perfusion inlet and applying mucosal solutions directly to the area around the cells investigated. The recording electrode was voltage-clamped at 0 mV and the current was recorded at a gain of 200 mV/pA. Each action potential generated by the receptor cell gave rise to a capacitive current signal recorded by the electrode. Continuous records of the electrical activity of single ORCs lasting longer than an hour could thus be obtained, which allowed several successive experiments to be performed with the same cell. Since signals from more than one cell could be recorded simultaneously (Fig. 1 *A*), the recordings were processed digitally and the activity of each unit is displayed separately (Fig. 1 *B*). Each spike in the records shown represents one action potential, and the length of the spike depicts the amplitude of the first phase of the recorded signal (caused by the depolarizing phase of the action potential). Alternatively, plots of spike rate against time are presented (Fig. 1 *C*). To obtain rate plots, the time between each two spikes was measured. The inverses of these intervals are the rate values given in $seconds^{-1}$. The baseline of a rate plot indicates zero activity, and the solid lines were created by calculating and connecting the means of 10 consecutive rate values (cf. Frings and Lindemann, 1991).

Determination of cAMP Accumulation

The four pieces of olfactory epithelium (two dorsal and two ventral) obtained from each animal were used for two experiments: (*a*) dorsal test and dorsal control; and (*b*) ventral test and ventral control. Thus, every piece of tissue could be compared with a control tissue from the same animal. No significant differences in forskolin-induced cAMP production were observed between dorsal and ventral tissues. Accumulation of cAMP in whole tissue (not in isolated cilia) was measured in order to avoid disruption of cellular signal transduction chains. Each piece of epithelium was placed into a microtube (Eppendorf Inc., Fremont, CA) together with 1.5 ml Ringer's solution containing 0.3 mM isobutylmethylxanthine (IBMX) to prevent degradation of cAMP by phosphodiesterases, and was preincubated for 5 min with a test substance (e.g., activators or inhibitors of PKC). The epithelium was then stimulated (with forskolin or

ionomycin) for 10 min at room temperature. Control tissues were incubated for the same time in Ringer's solution containing IBMX. Solvent concentrations (ethanol or DMSO) were in the range of 0.03–0.3% and had no detectable effect on cAMP production. The microtubes were continuously rotated in a motor-driven device at 18 revolutions per minute. To stop stimulation, tissue pieces were transferred into 300 μl of ice-cold assay buffer (70 mM Na acetate, pH 6.1) and frozen at −70°C. To permeabilize cells and release the accumulated cAMP into the assay buffer, tissue was frozen and thawed twice, followed by centrifugation at 2,000 *g* for 3 min.

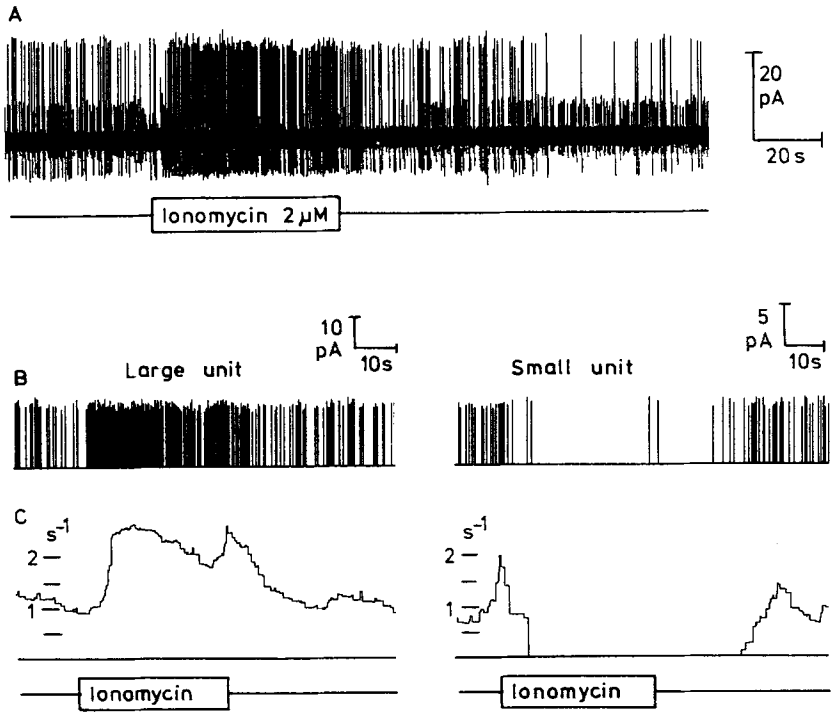

FIGURE 1. Presentation of results from ciliary recordings. (*A*) Spike recording from two ORCs in situ. Each unit is identified by one characteristic amplitude of its capacitive current signals. The amplitude of each individual unit stays more or less constant during the experiment, reflecting the area of sensory membrane pulled into the recording electrode. The record was low-pass filtered at 1 kHz. This trace shows the response to mucosal application of 2 μM ionomycin (see below). (*B*) After digital separation of signals originating from the two cells, single unit traces are presented indicating location and amplitude of each spike. (*C*) Plots of spike rates are created for each unit, showing inverse intervals between successive spikes, as explained in the text.

For cAMP radioimmunoassay (Striem, Naim, and Lindemann, 1991), the cAMP concentration of the supernatant was diluted to the most sensitive range of the assay (0.5–50 nM). Pellets served for the determination of protein content (Bradford, 1976) of the permeabilized tissue, which was used to calculate all AC activity values presented. All tests were carried out in duplicate. Results are given as cAMP production relative to control; that is, cAMP production (in picomoles cAMP/milligram protein per minute) of test tissue divided by cAMP production of the control tissue from the same animal (dorsal test divided by dorsal control, ventral test

divided by ventral control). This method of presentation was chosen because absolute values of cAMP production varied considerably between animals, whereas cAMP production relative to control, measured with each animal separately, was more consistent. Absolute values of cAMP production were 0.49 (±0.19, $n = 62$) in control tissues and 9.3 (±3.1, $n = 29$) pmol cAMP/mg protein per minute in the presence of 10 μM forskolin.

Materials

cAMP, carbamylcholine chloride (carbachol), compound R 24571 (calmidazolium), eugenol, forskolin, 5-hydroxytryptamine hydrochloride (serotonin), IBMX, mezerein, phorbol 12,13-dibutyrate (PDBu), polymyxin B sulfate, staurosporine, substance P, and trifluoperazine dihydrochloride were purchased from Sigma Chemie GmbH (Deisenhofen, Germany). 2-Isobutyl-3-methoxypyrazine (IBMP) and (−)-menthone were from Aldrich Chemie GmbH (Steinheim, Germany). [Bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid, tetra(acetoxymethyl)-ester] (BAPTA/AM) was from Calbiochem GmbH (Bad Soden, Germany). cAMP-2'-*O*-succinyl-3-^{125}I-iodotyrosine methylester for the radioimmunoassay was from Amersham Buchler GmbH (Braunschweig, Germany). cAMP antiserum and protein A bacterial adsorbent for the radioimmunoassay were purchased from Bio-Yeda, Ltd. (Rehovot, Israel). The PKC inhibitor Goe 6983 was kindly provided by Gödecke AG (Berlin).

RESULTS

Augmentation of Electrical Response by Neurotransmitters and Phorbol Ester

When in situ frog ORCs were stimulated with an odorant cocktail (containing 1 μM eugenol, 1 μM menthone, and 1 μM IBMP), 37% of the cells tested (22 of 58) responded with single bursts of action potentials lasting 2–10 s (Fig. 2 *A*). Applying 50 μM carbachol to the ciliated (mucosal) side of the olfactory epithelium did not change the basal spike rate, but odorant response was amplified in 7 of 16 cells (Fig. 2 *B*). Odorant response in the presence of carbachol showed increased maximal spike rate, increased response duration, or both. After washout of the transmitter, the cells resumed their control response pattern (Fig. 2 *C*). No effect on odorant response was observed when carbachol was added to the interstitial (serosal) solution.

The potentiating effect of carbachol could be mimicked by PDBu (0.5 μM), an activator of PKC. ORCs were first stimulated with 1 μM odorant (Fig. 3, left-hand traces), exposed to mucosal PDBu for 10 min, and subsequently challenged with the same odorant stimulus again (Fig. 3, right-hand traces). Odorant response after PDBu treatment was augmented in six out of nine cells tested.

Similar to the reaction to odorant stimulation, responses to mucosal pulses of forskolin, an activator of AC, were enhanced in the presence of 0.5 μM PDBu (Fig. 4 *A*) in 9 of 11 cells. Thus, activation of PKC by PDBu could increase the response of olfactory AC to stimulation by both odorants and forskolin. When PKC was inhibited by superfusion of the ciliary surface with the specific PKC inhibitor Goe 6983,[1] the transient increase of spike rate caused by a forskolin pulse was reduced (Fig. 4 *B*).

[1] Goe 6983 is a PKC inhibitor developed by Goedecke AG, Freiburg, Germany. Concentrations of Goe 6983 for half-maximal inhibition of various protein kinases are (μM): 0.02 PKC, 34 PKA, 16 PKG, 5.7 myosin light chain kinase, and >100 tyrosine-specific protein kinase (Dr. C. Schächtele, Goedecke AG, personal communication).

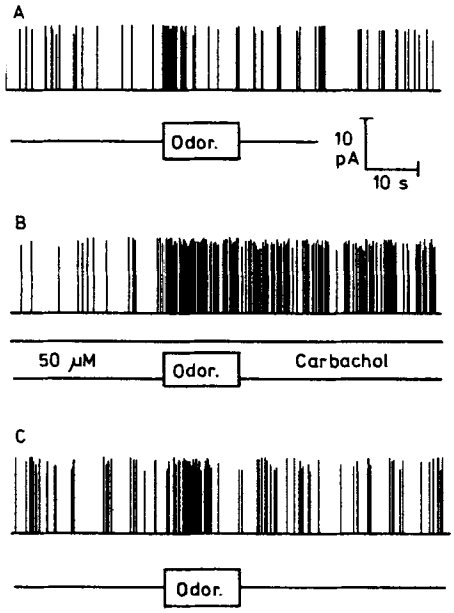

FIGURE 2. Sensitization of an ORC by carbachol. (*A*) Ciliary recording from an ORC challenged with a 15-s pulse of odorant mix (1 μM menthone, 1 μM eugenol, 1 μM IBMP). The pulse elicited a burst of action potentials lasting 4 s. (*B*) After a 3-min washout of odorants, the mucosal superfusate was switched to Ringer's solution containing 50 μM carbachol. 5 min later, an odorant pulse was again applied, this time increasing spike rate for 55 s. (*C*) 10 min after washout of carbachol, the odorant-induced spike burst lasted 8 s.

After washout of Goe 6983, forskolin response returned to the control pattern (all of four cells). Goe 6983 was also effective in the presence of phorbol ester. Fig. 4 *C* shows recordings from an ORC challenged repeatedly with pulses of 1 μM mucosal forskolin (5 s). After exposing the mucosal surface to 0.5 μM PDBu for 10 min, forskolin-induced spike rate was increased (second trace) compared with control (first trace). Adding 1 μM Goe 6983 to the PDBu solution attenuated the response to forskolin significantly (third trace), and this inhibitory effect of Goe 6983 was reversed after a 10-min washout of the PKC blocker (fourth trace). These observations suggested that activation of PKC increased the responsiveness of olfactory AC to forskolin stimulation, while inhibition of PKC decreased the responsiveness of AC.

Augmentation of cAMP Production by Neurotransmitters and Phorbol Ester

To further investigate the effects of neurotransmitters and phorbol ester on olfactory AC, forskolin-induced cAMP production was measured by radioimmunoassay. Accu-

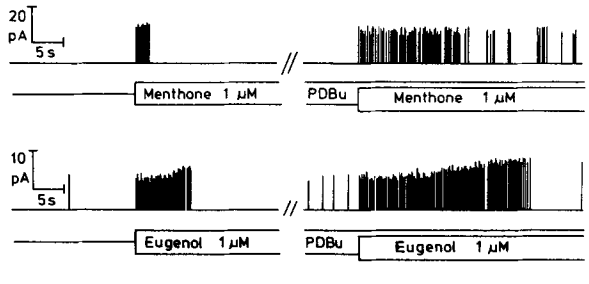

FIGURE 3. Amplification of odorant response by phorbol ester. Ciliary recording showing the response of two ORCs to sustained stimulation with 1 μM odorant (*left-hand traces*). After a 10-min superfusion of the ciliated surface of olfactory epithelium with 0.5 μM PDBu, the same stimuli were reapplied, causing longer responses than before PDBu application.

mulation of cAMP was measured in intact frog olfactory epithelium to preserve the function of cellular signal transduction processes, and, in particular, to avoid interference with cytosolic Ca^{2+} signals. Degradation of cAMP was inhibited by addition of 0.3 mM IBMX, an effective blocker of olfactory phosphodiesterase activity (Borisy, Ronnett, Cunningham, Juilfs, Beavo, and Snyder, 1992), to all samples. This method did not allow reliable measurements of odorant-induced cAMP production because the amount of cAMP generated during the brief transient activation of

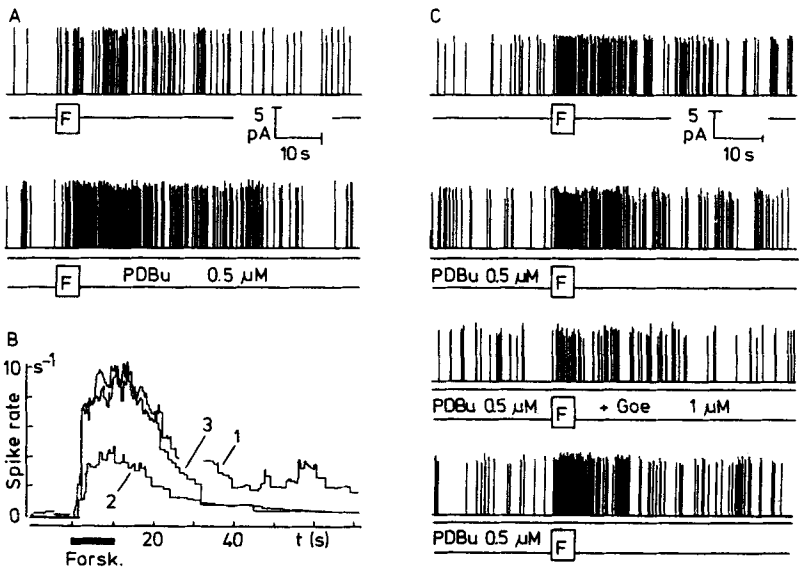

FIGURE 4. Effects of PKC modulators on electrical response to forskolin. (*A*) Ciliary recording from an ORC showing response to a 5-s pulse of 1 µM mucosal forskolin, indicated as *F* (*upper trace*). After a 10-min superfusion with 0.5 µM PDBu, response to a similar pulse was augmented (*lower trace*). (*B*) Time courses of spike rate recorded from a single ORC in response to 10-s pulses with 1 µM mucosal forskolin. (*1*) control response; (*2*) response after a 10-min superfusion with 1 µM Goe 6983; (*3*) recovery of response after a 10-min washout of Goe 6983. (*C*) Inhibitory effect of Goe 6983 in the presence of phorbol ester. All traces are from the same cell. Traces from top: control response to a 5-s pulse with 1 µM forskolin; enhanced forskolin response after a 10-min superfusion with 0.5 µM PDBu; attenuation of forskolin response after a 10-min superfusion with 0.5 µM PDBu and 1 µM Goe 6983; partial recovery of forskolin response after a 10-min washout of Goe 6983.

adenylate cyclase by odorants (50–100 ms; Breer, Boekhoff, and Tareilus, 1990) was too small to be accurately assessed in the intact tissue preparation. The sustained, tonic stimulation of AC by forskolin, however, gave rise to cAMP accumulation that could easily be detected.

Basal cAMP production in the presence of 0.3 mM IBMX was 0.49 (±0.19, $n = 62$) pmol cAMP/mg protein per min. Forskolin (10 µM) enhanced cAMP production by a factor of 24.7 (±4.6, $n = 36$) of basal values. After 5-min preincubation with

neurotransmitters (50 μM), basal cAMP production was not changed significantly, but the forskolin effect was enhanced by 131% (±8.5%, n = 3) with serotonin and by 135.2% (±15.8%, n = 3) with carbachol, reaching values 70–80 times that of basal production (Fig. 5). The lack of effect on basal cAMP production, together with the potentiation of the forskolin response, suggests that neurotransmitters increased the sensitivity of AC to stimulation by forskolin. Since this sensitization was completely suppressed by Goe 6983 (1 μM), preincubated together with neurotransmitters for 5 min (Fig. 5), a participation of PKC in the neurotransmitter effect should be expected. No sensitization of AC was observed with substance P (50 μM; Fig. 5), a neurotransmitter involved in the regulation of secretory processes in olfactory mucosa (Getchell, Zielinski, and Getchell, 1988).

Forskolin response was also potentiated by phorbol ester. After a 5-min preincubation with 0.5 μM PDBu, forskolin-induced cAMP production was enhanced by 127.6% (±22.4%, n = 3). PDBu did not affect basal cAMP production (see below).

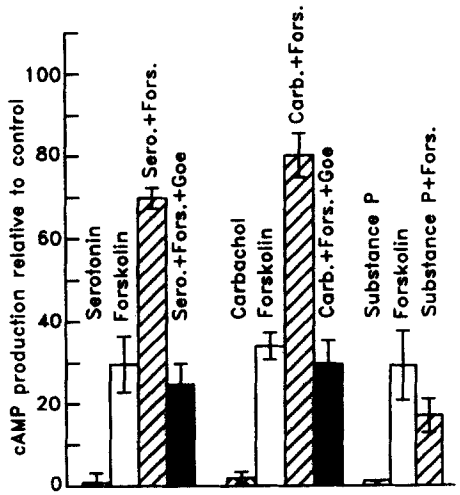

FIGURE 5. Potentiation of forskolin response by neurotransmitters. Forskolin-induced cAMP production with 10 μM forskolin alone (*open bars*), in the presence of 50 μM neurotransmitter (*hatched bars*), and with neurotransmitters plus 1 μM Goe 6983 (*filled bars*). Neurotransmitters alone had no effect on cAMP production (*cross-hatched bars*). Each data point in this and the following diagrams represents six tests from three animals unless stated otherwise. Error bars indicate SEM.

The potentiating effect of PDBu on cAMP accumulation was fully suppressed by 1 μM Goe 6983 (Fig. 6 *A*). This substance, in fact, reduced forskolin response below control values, indicating a basal, PKC-sustained sensitivity of olfactory AC. Two other PKC inhibitors also blocked sensitization of AC by PDBu. Staurosporine (0.5 μM), an inhibitor of PKC and other protein kinases (Ruegg and Burgess, 1989), and polymyxin B (200 μM), a more specific PKC inhibitor (Mazzei, Katoh, and Kuo, 1982), suppressed potentiation of the forskolin effect by PDBu (Fig. 6 *A*).

After preincubation with PDBu, cAMP production was potentiated at all effective forskolin concentrations tested (in the range of 0.3–100 μM forskolin) by 172.9% (±10.7%, n = 22; Fig. 6 *B*). Saturation of AC activity could not be achieved due to the limited solubility of forskolin in aqueous solutions (cf. Seamon and Daly, 1986). Again, application of 1 μM Goe 6983 inhibited cAMP production below control values. This figure also shows that PDBu did not have any detectable effect on cAMP production at forskolin concentrations <0.3 μM.

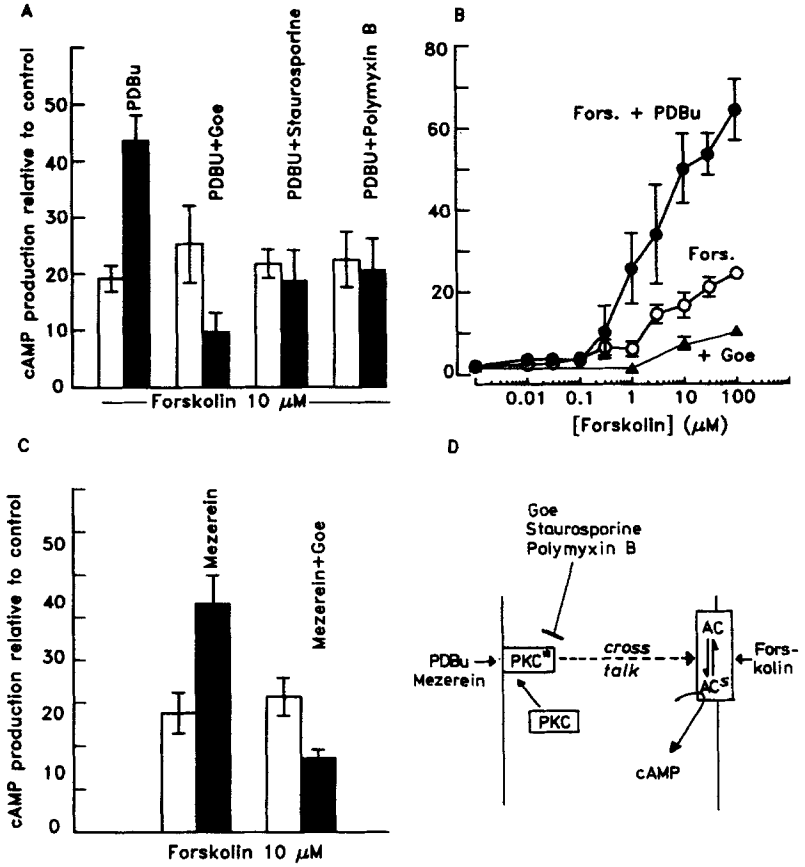

FIGURE 6. Sensitization of AC by activators of PKC. (*A*) Forskolin-induced cAMP production is shown with 10 μM forskolin alone (*open bars*), and with forskolin after a 5-min preincubation with 0.5 μM PDBu (*filled bars*). The response to forskolin was potentiated in the presence of PDBu. When PKC inhibitors (1 μM Goe 6983, 0.5 μM staurosporine, 200 μM polymyxin B) were added during preincubation with PDBu, no potentiation was observed. (*B*) Dose–response relationships of forskolin-induced cAMP production with forskolin alone (*open circles*), with forskolin after a 5-min preincubation with 0.5 μM PDBu (*filled circles*), and with forskolin after preincubation with both 0.5 μM PDBu and 1 μM Goe 6983 (*filled triangles*). (*C*) Augmentation of forskolin-induced cAMP production by mezerein. The response to stimulation with 10 μM forskolin is shown without (*open bars*) and with 0.5 μM mezerein (*filled bars*). Mezerein-induced potentiation was suppressed by 1 μM Goe 6983 (*filled bar on the right*). (*D*) Scheme of proposed crosstalk mechanism showing PKC, its activators, and inhibitors, as well as AC that is converted to a sensitized state (A^S) by PKC.

Phorbol esters activate PKC because they substitute for diacylglycerol, the physiological activator of PKC, with a diacylglycerol-like structure in their molecules (Nishizuka, 1986). The diterpene mezerein, a compound without structural similarity to diacylglycerol, has also been shown to activate PKC (Miyake, Tanaka, Tsuda, Kaibuchi, Kikkawa, and Nishizuka, 1984), and can, therefore, be used as an

additional probe for the involvement of PKC in biochemical reactions. In frog olfactory epithelium, 0.5 μM mezerein potentiated forskolin-induced cAMP accumulation by 93% (±14%, n = 3), an effect sensitive to 1 μM Goe 6983 (Fig. 6 *C*). Taken together, these data indicate an interaction between olfactory PKC and AC, probably a crosstalk mechanism by which the activated PKC converts AC into a sensitized form (AC^s, Fig. 6 *D*).

The forskolin-induced cAMP accumulation in intact olfactory tissue is generated only partly by odorant-sensitive AC type III residing in the sensory cilia. A different isoform of AC, which is not stimulated by odorants (Chen, Pace, Heldman, Shapiro, and Lancet, 1986), generates ~60% of the total AC activity in olfactory tissue. This AC activity is located outside of sensory cilia in the mucosal and submucosal tissue, where it is not expected to contribute directly to the primary events of olfactory signal transduction (Chen and Lancet, 1984; Pace et al., 1985). To distinguish between effects of PKC on ciliary AC and its effects on AC in the residual tissue, frog olfactory mucosa was deciliated by the Ca^{2+} shock method, and the response to forskolin and PDBu was studied with deciliated tissue. The deciliated preparation was chosen for

TABLE I

Sensitization of AC in Intact and Deciliated Olfactory Epithelium

	Intact tissue	Deciliated tissue
Forskolin*	18.1 ± 2.8	22.8 ± 2.1
Forskolin + PDBu*	40.9 ± 4.0	26.7 ± 4.1
PDBu-induced sensitization‡	126%	17%
No. of observations	9	10

*Cyclic AMP production: Forskolin-induced AC activity (in picomoles cAMP/milligram per minute) relative to basal activity.

‡Percent increase of cAMP production caused by 0.5 μM PDBu.

these experiments because it was assumed that cellular signal transduction chains were still functional in the various cell populations of the olfactory tissue after removal of sensory cilia. PKC-induced sensitization mechanisms in supporting cells, basal cells, or deciliated ORCs should, therefore, be detectable. In contrast, investigation of crosstalk effects in isolated membrane preparations of sensory cilia would require functional reconstitution of all molecular components involved in such reactions, a considerably more difficult problem that will be addressed in the future.

Intact and deciliated tissues were stimulated with 10 μM forskolin. Through deciliation the protein content of tissue pieces was reduced by ~15% (intact: 1.05 ± 0.17 mg, n = 9; deciliated: 0.89 ± 0.07 mg, n = 10), while forskolin-induced absolute AC activity (not weighted by protein content) decreased by 40% (intact: 9.5 ± 2.4 pmol cAMP/min, n = 9; deciliated: 5.7 ± 1.25 pmol cAMP/min, n = 10). This indicates that ~40% of olfactory AC activity resides within the cilia, a result in good accordance with the data from Chen et al., 1986.

After equilibration of the deciliated tissue in Ringer's solution, the effect of PDBu on forskolin-induced cAMP production was measured. The results of these experiments are shown in Table I. While AC activity in intact and deciliated tissue was

stimulated by 10 μM forskolin to a similar degree (18.1 and 22.8 times basal production), only a very weak enhancement of forskolin response was observed in the deciliated preparation after PDBu (0.5 μM) treatment. PDBu enhanced forskolin-induced cAMP production by 126% in the intact tissue and by 17% in the deciliated tissue, indicating that sensitization of AC by PKC appears to be restricted to the sensory cilia.

Cell Calcium

PKC is activated by diacylglycerol and cell Ca^{2+}. It was, therefore, interesting to study effects of intracellular Ca^{2+} concentration on the sensitivity of olfactory AC. Intracellular Ca^{2+} concentration can be increased by addition of the Ca^{2+} ionophore ionomycin to the extracellular medium, thereby increasing the Ca^{2+} permeability of the cell membrane. Cell Ca^{2+} can be decreased by application of BAPTA/AM, a nonchelating, membrane-permeant ester of BAPTA that becomes an active Ca^{2+} chelator after hydrolysis by cytosolic esterases (Tsien, 1981). The following observations were obtained with such alterations of cell Ca^{2+} activity in intact frog olfactory epithelium.

During application of ionomycin to the ciliated side of frog olfactory epithelia, the spike rate of individual ORCs either increased or decreased, depending on the concentration used. At 1–2 μM ionomycin, the predominant response of ORCs was an increase of spike rate (10 of 12 cells; Fig. 7 *A*), while 5 μM ionomycin inhibited spiking activity reversibly (all of six cells; Fig. 7, *B* and *C*). The decrease of spike rate at 5 μM ionomycin was accompanied by an increase in cAMP production (Fig. 7 *D*), indicating that cell Ca^{2+} activity was not high enough to cause inhibition of AC (olfactory AC is inhibited by cell Ca^{2+} with 50% inhibition at ~200 μM; Sklar et al., 1986; Anholt, Farmer, and Karavanich, 1989). These results show that, in the presence of high concentrations of cell Ca^{2+}, cells were hyperpolarized although intracellular cAMP concentration was high.

In further studies of Ca^{2+} effects on cAMP production, 2 μM ionomycin showed two distinct effects: (*a*) Ionomycin increased the basal cAMP accumulation by a factor of 4.03 (±1.1, $n = 18$). This stimulation was sensitive to inhibitors of calmodulin (50 μM trifluoperazine or 100 μM calmidazolium; Gietzen, Wüthrich, and Bader, 1981; Van Belle, 1981) but not to the PKC inhibitor Goe 6983 (1 μM; Fig. 8 *A*), and it probably reflected direct activation of AC by Ca^{2+}/calmodulin, as described before by Anholt and Rivers (1990). Ionomycin-induced cAMP production was potentiated by activation of PKC. Preincubation of olfactory tissue with 0.5 μM PDBu increased the subsequent ionomycin response by 431.0% (±59.2%, $n = 3$). When 100 μM calmidazolium was added during preincubation with PDBu, ionomycin-induced cAMP production was almost completely suppressed (Fig. 8 *B*). These data suggested that Ca^{2+} activation of olfactory AC, both in the control state and in the sensitized state of the enzyme, involved calmodulin.

(*b*) Elevated levels of cell Ca^{2+} also affected the response to forskolin. In six of eight animals, ionomycin potentiated forskolin-induced cAMP accumulation by 95.9%

(±8.9%, n = 6). Potentiation was not reduced by calmidazolium, but was sensitive to Goe 6983 (Fig. 8 *C*). These ionomycin results resemble observations with phorbol ester or mezerein, and they indicate activation of PKC by cell Ca^{2+}, with resulting sensitization of AC to forskolin.

When cell Ca^{2+} was lowered by loading the cells with 0.1 mM BAPTA/AM for 30 min, cAMP production after subsequent application of ionomycin or forskolin was suppressed and sensitization of AC was no longer observed (Fig. 8 *D*). In these experiments, cellular Mg^{2+} concentration was much less affected than cell Ca^{2+}, since the affinity of BAPTA is 10^5-fold higher for Ca^{2+} (K_M = 110 nM) than for Mg^{2+} (K_M = 17 mM; Tsien, 1980).

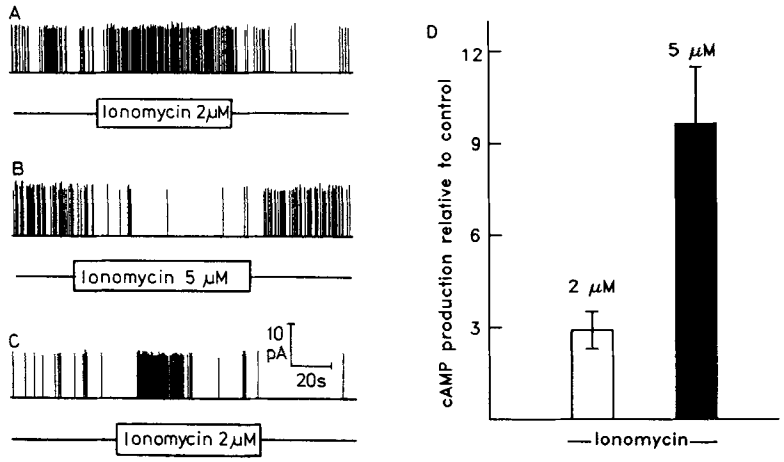

FIGURE 7. Effects of different concentrations of mucosal ionomycin on spike rate and cAMP production. (*A*) Increase of spike rate of an ORC recorded during mucosal superfusion with 2 μM ionomycin. (*B*) Decrease of spike rate of the same cell during application of 5 μM ionomycin. (*C*) 6 min after washout of ionomycin, the cell was challenged again with 2 μM ionomycin. The response was a transient increase of spike rate. (*D*) Increased cAMP production in intact olfactory epithelium caused by application of ionomycin. The effects of two different concentrations of ionomycin on tissues from the same animal were compared, showing more cAMP production at the higher ionomycin concentration. Means of five animals.

Taken together, the experiments involving perturbations of the cellular Ca^{2+} activity suggested that increasing cell Ca^{2+} had three different effects: (*a*) AC was activated by a Ca^{2+}/calmodulin-dependent mechanism in both the control (AC) and sensitized (AC^S) states of the enzyme (Fig. 8 *E*, right-hand pathway); (*b*) AC was sensitized by Ca^{2+} through a PKC-mediated, but calmodulin-independent mechanism (Fig. 8 *E*, left-hand pathway); and (*c*) high concentrations of cell Ca^{2+} hyperpolarized ORCs, even in the presence of elevated levels of cAMP. The results further indicate that the responsiveness of olfactory AC is critically dependent on cell Ca^{2+}, pointing to a prominent role of Ca^{2+} in the modulation of cAMP-mediated olfactory responses.

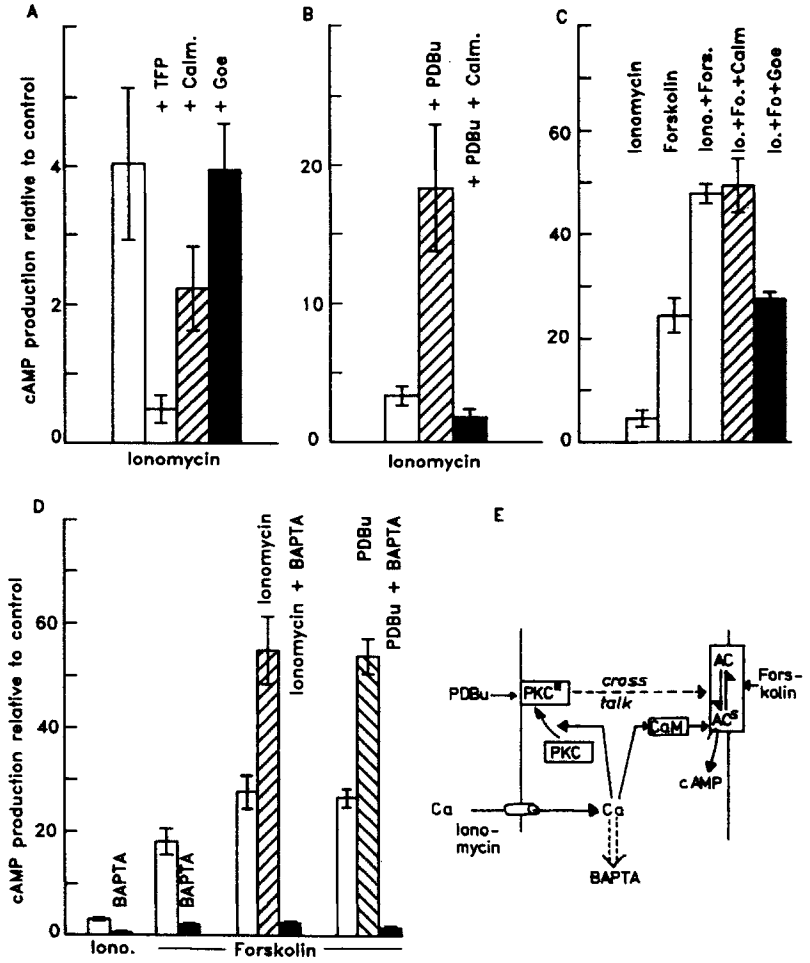

FIGURE 8. Effects of cell Ca^{2+} on cAMP production. (*A* and *B*) Activation of AC by Ca^{2+}/calmodulin. (*A*) Ionomycin-induced cAMP production with ionomycin (2 μM) alone, and after a 5-min preincubation with 50 μM trifluoperazine (*TFP*), 100 μM calmidazolium (*Calm.*), or 1 μM Goe 6983. The ionomycin effect was reduced by calmodulin inhibitors but not by the PKC inhibitor. (*B*) Potentiation of ionomycin response by phorbol ester. Note different scaling of *y*-axis. Epithelia were stimulated with 2 μM ionomycin alone (*open bar*) or after a 5-min preincubation with 0.5 μM PDBu (*hatched bar*). Including 100 μM calmidazolium (*Calm.*) during preincubation with PDBu suppressed ionomycin response (*filled bar*). (*C*) Sensitization of AC by Ca^{2+}. Ionomycin potentiates forskolin response. Production of cAMP induced by 10 μM forskolin was increased after a 5-min preincubation with 2 μM ionomycin. Including 1 μM Goe 6983 suppressed potentiation of forskolin response (*filled bar*), whereas adding 100 μM calmidazolium (*Calm.*) had no effect (*hatched bar*). (*D*) Ca^{2+} enables AC activity. Effects of intracellular Ca^{2+} chelation on responses to forskolin and ionomycin. cAMP production induced by forskolin (10 μM) and ionomycin (2 μM), as well as forskolin (10 μM) responses potentiated by, respectively, ionomycin (2 μM) or PDBu (0.5 μM) are shown under control conditions (*open and hatched bars*) and after chelation of cell Ca^{2+} with 100 μM BAPTA/AM (*filled bars*). The extracellular Ca^{2+} concentration was 1 mM. (*E*) Proposed mechanisms of interaction of cell Ca^{2+} with olfactory AC and PKC. Ca^{2+} enters the cytosol via ionomycin and is chelated by BAPTA. *Right-hand pathway*, Ca^{2+} activates AC through calmodulin (*CaM*). If Ca^{2+}/CaM activates AC^S, cAMP production is much stronger than during activation of AC (cf. *B*). *Left-hand pathway*, Ca^{2+} activates PKC, leading to sensitization of AC to stimulation by forskolin (cf. *C*).

DISCUSSION

Sensitization of Adenylate Cyclase

It is now generally accepted that cAMP acts as a second messenger for signal transduction in olfactory receptor cells (reviewed by Lancet, 1986; Snyder, Sklar, and Pevsner, 1988; Anholt, 1989). ORCs contain a specific AC (adenylate cyclase type III; Pfeuffer et al., 1989; Bakalyar and Reed, 1990), which is restricted to the sensory cilia (Pfeuffer et al., 1989; Asanuma and Nomura, 1991; Menco et al., 1992). This enzyme is activated through the G protein G_{olf} (Pace et al., 1985; Anholt, 1988; Jones and Reed, 1989) after binding of odorant molecules to receptor proteins in the sensory membrane (Buck and Axel, 1991). Activation of AC causes a fast, transient increase of cAMP in the ORC (Breer et al., 1990), leading to activation of apical cation channels (Nakamura and Gold, 1987; Firestein et al., 1991), and to a transient inward current (Firestein and Werblin, 1989; Kurahashi, 1990; Lowe and Gold, 1991) that depolarizes the ORC membrane and increases action potential rate (Frings, Benz, and Lindemann, 1991; Frings and Lindemann, 1991). In addition to AC type III, sensory cilia of ORCs also possess PKC (Anholt, Mumby, Stoffers, Girard, Kuo, and Snyder, 1987), PLC (Huque and Bruch, 1986; Boyle, Park, Huque, and Bruch, 1987; Boekhoff et al., 1990), and calmodulin (Anholt and Rivers, 1990; Biffo, Goren, Khew-Goodall, Miara, and Margolis, 1991), the three proteins mediating activation and sensitization of AC proposed in this report.

Olfactory AC type III differs from other AC isozymes by a remarkably low basal activity, a high turnover number, and a high molar concentration in the sensory membrane (Pfeuffer et al., 1989; Bakalyar and Reed, 1990). These properties enable the sensory cells to keep a low cAMP level when unstimulated, in addition to reacting to odorant stimulation with a rapid and substantial production of the second messenger. My results suggested that the activity of AC in frog olfactory tissue is subject to modulation by PKC, and that this PKC-sensitive AC activity is localized mainly, if not exclusively, in the sensory cilia. Since nonciliary AC was not sensitized by PDBu, the sensitization factors measured in the intact tissue underestimate the extent to which ciliary activity is potentiated by the crosstalk mechanism. Taking into account that only 40% of total AC generated 126% sensitization in intact tissue (Table I), it is expected that the response of a purified cilia preparation to 10 μM forskolin will be augmented by at least 300% in the presence of 0.5 μM PDBu.

Experiments with activators and inhibitors of PKC indicated that PKC controlled the sensitivity of olfactory AC to stimulation by odorants, forskolin, and cell Ca^{2+}. Such crosstalk regulation of AC by PKC has already been demonstrated in a number of tissues (reviewed by Houslay, 1991). The results presented here, however, do not explain the molecular mechanism underlying sensitization of AC. Different experiments have to be designed to reveal the substrate of olfactory PKC that mediates sensitization. Since the response to forskolin was modulated by PKC, phosphorylation is not expected to occur at the level of odorant receptors. Substrates for phosphorylation by PKC could be the GTP-binding protein G_{olf}, inhibitory G proteins, or the catalytic subunit of adenylate cyclase itself. Finally, phosphodiesterases could be substrates for PKC, and cAMP degradation could be modulated by their phosphorylation.

In rat olfactory cilia, odorant receptors that couple to AC seem to be phosphorylated by cAMP-dependent protein kinase (PKA), but not by PKC (Boekhoff and Breer, 1992). Stimulatory G proteins like G_{olf} are not known to be substrates for PKC (Houslay, 1991). In contrast, phosphorylation of AC-associated inhibitory G proteins was demonstrated in several cell types. Phosphorylation of the inhibitory G protein may remove a tonic inhibition from AC (e.g., Bell and Brunton, 1986; Choi and Toscano, 1988; Gordeladze, Björo, Torjesen, Osterberg, Haug, and Gautvik, 1989; Bushfield, Lavan, and Houslay, 1991), and, while modulating G_i-mediated receptor effects, this phosphorylation may either leave the response to forskolin unchanged (e.g. Katada, Gilman, Watanabe, Bauer, and Jakobs, 1985; Gordeladze et al., 1989), or profoundly affect the cells' response to forskolin (e.g., Darfler, Mahan, Koachman, and Insel, 1982; Sibley, Jeffs, Daniel, Nambi, and Lefkowitz, 1986; Rozengurt, Murray, Zachary, and Collins, 1987). In ORCs there is, however, no indication that G_i proteins participate in the transduction process at all. Although G_i proteins have been identified in frog olfactory cilia (Pace et al., 1985; Anholt et al., 1987), they occurred at much lower concentration than the stimulatory G_{olf}, and no inhibitory receptors have been identified. Finally, degradation of cAMP by phosphodiesterases was already severely reduced by IBMX in our preparation. It was recently found that 0.5 mM IBMX inhibited phosphodiesterase activity in olfactory epithelium by $>95\%$ (Borisy et al., 1992). Amplification of the cAMP signal through further inhibition of phosphodiesterase is, therefore, not likely to have contributed much to the sensitization effects.

Consequently, the possibility remains that, as in other cell types, the catalytic subunit of AC itself was phosphorylated by PKC (cf. Yoshimasa, Sibley, Bouvier, Lefkowitz, and Caron, 1987; Simmoteit, Schulzki, Palm, Mollner, and Pfeuffer, 1991), and that, as a result of phosphorylation, its sensitivity to stimulation by forskolin, G_{olf}, or cell Ca^{2+} increased.

Cell Calcium

The concentration of cytosolic Ca^{2+} appears to have a pivotal role in olfactory signal transduction. Decreasing cell Ca^{2+} below control levels effectively prevented activation of AC, indicating a sustaining function of Ca^{2+} in the generation of the cAMP signal. Similarly, Ronnett, Parfitt, Hester, and Snyder (1991) have shown that odorant response in cultured rat ORCs was suppressed at Ca^{2+} concentrations <0.1 μM. Maximal response required 10–100 μM cell Ca^{2+}. It appears, therefore, that Ca^{2+} enables olfactory AC to respond to odorants and forskolin.

Raising cell Ca^{2+} above basal levels allowed the following observations: (*a*) Spike rates increased at low ionomycin concentrations, while cAMP production was enhanced. Anholt and Rivers (1990) have already shown that frog olfactory AC in vitro is activated by Ca^{2+}/calmodulin. Similarly, Choi et al. (1992) showed that cloned AC type III from rat olfactory cilia was activated by Ca^{2+}/calmodulin in the presence of forskolin or GTP, showing a half-maximal effect at 5 μM Ca^{2+}. These results are in good agreement with the finding of Eliot, Dudai, Kandel, and Abrams (1989), who proposed that the vast majority, if not all, neural forms of AC are Ca^{2+}/calmodulin sensitive. Our results point to the same mechanism, since Ca^{2+} activation of AC was sensitive to calmodulin inhibitors. This direct activation of olfactory AC by Ca^{2+}/

calmodulin is expected to induce rapid and considerable cAMP production when ciliary Ca^{2+} concentration increases after activation of IP_3-gated, Ca^{2+}-permeable ion channels in the sensory membrane. It constitutes a link between odorant-activation of ciliary PLC and the cAMP-gated channels: for instance, an odorant like isovaleric acid that liberates IP_3 in isolated cilia preparations, where crosstalk mechanisms are not functional (Breer and Boekhoff, 1991), but which also stimulates cAMP production in intact cells (Ronnett et al., 1991) may use the Ca^{2+}/calmodulin pathway to translate PLC activation into a cAMP signal. Furthermore, cAMP-gated channels in frog olfactory cilia also conduct Ca^{2+} (Frings, S., and B. Lindemann, manuscript in preparation). Opening of such channels as a consequence of an increasing ciliary cAMP concentration causes inflow of Ca^{2+} and a further increase of cAMP production. Such positive feedback is expected to decrease the time lag between stimulation and response, and to amplify the olfactory signal. Finally, my results show that the response of AC to stimulation with Ca^{2+} was potentiated by phorbol ester, possibly by the same crosstalk mechanism that sensitized AC to stimulation by forskolin and odorants. Thus, ciliary Ca^{2+} concentration, increased by IP_3-gated or cAMP-activated ion channels, will have a particularly strong effect on AC when olfactory PKC is stimulated.

(*b*) At higher ionomycin concentrations spike rate of ORCs decreased, while cAMP production showed a further increase. This effect may be explained by the hyperpolarizing effect of K^+ channels opened by increasing cell Ca^{2+}. Ca^{2+}-activated K^+ channels have been demonstrated to be functional in olfactory sensory membrane of frog (Labarca, Simon, and Anholt, 1988) and mouse (Maue and Dionne, 1987).

(*c*) Ionomycin caused activation of PKC and, thereby, sensitization of AC to forskolin stimulation in most but not all animals. The lack of sensitivity to calmodulin antagonists suggested that this effect was brought about by an additional molecular mechanism, different from the calmodulin-mediated activation of AC. Ca^{2+} can activate PKC by promoting its translocation from cytosol to the plasma membrane, where the enzyme becomes active (Nishizuka, 1986). Ca^{2+} activation of PKC shows half-maximal effects at 0.2–0.6 μM (Huang, 1990). Thus, only slightly elevated levels of cell Ca^{2+} may be expected to enhance sensitivity of the cAMP-generating system in ORCs by increasing the activity of olfactory PKC, while higher Ca^{2+} concentrations are needed to activate olfactory AC directly (K_M = 5 μM; Choi et al., 1992).

Modulation of Olfactory Sensitivity by Neurotransmitters

Carbachol enhanced the odorant response only when added to the mucosal solution. This may indicate that receptors for this transmitter are located close to the apical surface of the tissue, and that, in our experiments, carbachol had to gain access to its receptors through the tight junctions. Insufficient permeability of tight junctions to carbachol may also account for the relatively small fraction (< 50%) of sensitized cells observed by spike recording. The assumption that carbachol receptors are located on the distal part of the dendrite rests on the following consideration: diffusional access to binding sites close to the ciliary knob is restricted from both aspects of the olfactory epithelium. We have earlier observed that TTX, when added to the serosal solution, takes ~1 min to reach the basal pole of ORCs through the submucosal tissue (Frings et al., 1991). Molecules diffusing from there towards the ciliary knob

have to overcome a layer of densely packed tissue (about 150 μm thick), with dendrites of ORCs often tightly embedded in membrane folds of supporting cells (Costanzo and Morrison, 1989). Access to binding sites near the ciliary knob may, therefore, be easier through tight junctions than from the basal side of the epithelium. And failure to elicit neurotransmitter effects from the submucosal side can be interpreted as reflecting a distal location of receptors on the ORCs.

Localization of neurotransmitter receptors in the sensory (apical) membrane cannot be ruled out but appears unlikely, because the possible sources of carbachol and serotonin, terminal varicosities of trigeminal nerves (see below), are situated near the distal part of the dendrites, and have not been found on the apical surface. Furthermore, since neurotransmitters alone increased neither cAMP production nor spike rate significantly, a direct effect on odorant receptors in the sensory membrane seems improbable. More likely, they cause augmentation of stimulus-evoked responses, as demonstrated for other cell types (e.g., Choi, Wong, Hinds, and Storm, 1992). The potentiating effect of carbachol and serotonin on cAMP production was apparently mediated by PKC, being sensitive to the PKC inhibitor Goe 6983. Could neurotransmitters in vivo modulate olfactory sensitivity through the crosstalk mechanism described above?

Binding studies have shown that ORCs do indeed bind carbachol and other muscarinic agonists (Hedlund and Shepherd, 1983). The olfactory mucosa is innervated by the ophthalmic branch of the trigeminal nerve. Trigeminal fibers project to the surface layer of the epithelium, where their endings, resembling presynaptic nerve terminals, are located close to the proximal side of tight junctions, next to supporting cells and dendritic endings of ORCs. These fibers occur at a density of 1 per 30 ORCs in frog olfactory mucosa (Zielinski, Getchell, and Getchell, 1989). Terminal varicosities of such fibers contain substance P, but possibly also cholinergic and serotoninergic vesicles (Zielinski et al., 1989). These trigeminal fibers are known to participate in the sensory perception of irritants, chemicals, and odorants (e.g., Silver and Moulton, 1982), and they are also involved in the regulation of secretion (Getchell et al., 1988). But ORCs are affected as well: antidromic stimulation of the ophthalmic branch of frog trigeminal nerve elicited an electrical potential transient reminiscent of the electro-olfactogram, and it influenced the spiking pattern of ORCs (Bouvet, Delaleu, and Holley, 1987). These effects could be mimicked by application of acetylcholine or substance P (Bouvet, Delaleu, and Holley, 1988). Thus, there appears to be an interaction between neurotransmitters originating from autonomic nerve endings and ORCs. The results presented here suggest that such an interaction may influence the ORCs' electrical properties and modulate their response to odorant stimulation.

Assuming a dendritic localization of carbachol and serotonin receptors, one has to consider the distance between neurotransmitter binding site and ciliary AC, which may be as much as 300 μm in frog. Ca^{2+} waves travel with propagation velocities of 20–100 μm/s (e.g., Cornell-Bell, Finkbeiner, Cooper, and Smith, 1990; Cornell-Bell and Finkbeiner, 1991; Wier and Blatter, 1991; Lechleiter and Clapham, 1992), implying that a Ca^{2+} signal generated in the distal dentritic region of an ORC will take 3–15 s to reach the tips of sensory cilia. Because of this delay, it is not likely that neurotransmitter signals have significant effects within the time range of a single

odorant response (< 10 s). But it is conceivable that a tonic stimulation of dendritic neurotransmitter receptors generates sufficient DAG and Ca^{2+} to adjust the level of ciliary PKC activity, hence setting the odorant sensitivity of the neuron. In conclusion, the two effects of Ca^{2+} discussed in this report are distinguished both by the Ca^{2+} sensitivity of their target proteins (K_M (AC) = 5 μM; K_M (PKC) = 0.2–0.6 μM), and by

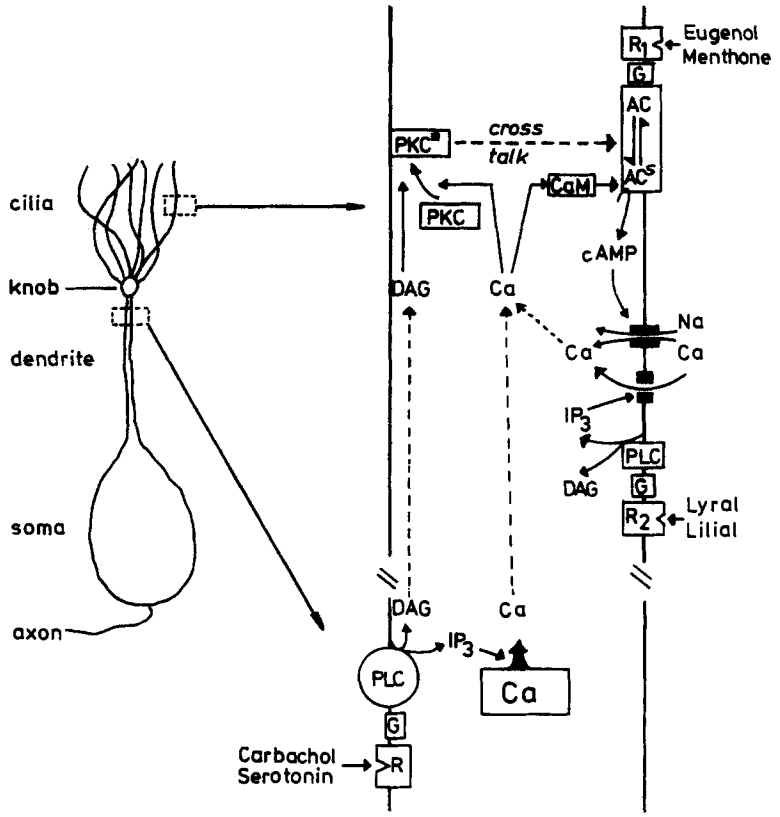

FIGURE 9. Schematic representation of a frog ORC and of the hypothetical model summarizing interactions of cAMP- and IP_3-generating signal transduction pathways as explained in the text. Eugenol and menthone are odorants known to activate AC, while lyral and lilial activate PLC. *AC*, adenylate cyclase type III, state of low sensitivity; AC^S, adenylate cyclase type III, state of high sensitivity; *CaM*, calmodulin; *DAG*, diacylglycerol; *G*, GTP-binding protein; *PKC*, inactive, cytosolic protein kinase C; *PKC**, active, membrane-bound protein kinase C; *PLC*, phospholipase C; *R*, receptor protein binding neurotransmitters in the dendritic membrane; R_1, odorant receptor protein coupled to AC; R_2, odorant receptor protein coupled to PLC.

differences in the expected time course of their response. While a fast, phasic increase of ciliary Ca^{2+} follows the transient activation of ligand-operated ion channels in the sensory membrane, a tonic adjustment of the Ca^{2+} level will be sustained by neurotransmitter-induced Ca^{2+} release in the dendrite.

Fig. 9 summarizes the proposed molecular mechanisms involved in sensitization

and activation of olfactory AC. Two aspects of crosstalk interaction are illustrated: (*a*) The binding of neurotransmitters to PLC-coupled receptors in the distal part of the dendrite causes release of Ca^{2+} and DAG, the physiological activators of PKC. A continuous generation of Ca^{2+} and DAG in the dendrite supports a tonic activity of ciliary PKC. Through a crosstalk mechanism, ciliary PKC increases the sensitivity of AC, amplifying its response to stimulation by odorants or Ca^{2+}/calmodulin. The enhanced production of cAMP causes increased receptor currents, conducted by cAMP-gated ion channels. Thus, neurotransmitters sustain and enhance the responsiveness of ORCs through sensitization of olfactory AC. (*b*) Ciliary Ca^{2+} concentration can increase rapidly, following activation of cAMP-gated cation channels or IP_3-gated Ca^{2+} channels in the ciliary membrane. This will activate olfactory AC through the calmodulin-mediated pathway, resulting in a rapid increase of ciliary cAMP. According to this model, odorants (like lyral and lilial) that activate PLC and release IP_3 in the cilia are also expected to cause cAMP generation, thus recruiting an additional depolarizing current to induce excitation of the neuron.

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