Calcium/Calmodulin-activated Phosphodiesterase Expressed in Olfactory Receptor Neurons

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We show that calmodulin-dependent phosphodiesterase (CAM-PDE) is selectively expressed in mature olfactory receptor neurons within the olfactory mucosa. Immunocytochemical staining reveals neuronal immunoreactivity that is most pronounced within cilia, dendritic knobs, and axon bundles. Neither sustentacular cells nor basal cells display immunoreactivity. The extent of loss of neuronal immunoreactivity following bulbectomy resembles loss of the neuronal population. High-affinity CAM-PDE activity in olfactory cilia is fivefold greater than in brain, when assayed at low micromolar cAMP. This activity is depleted in turbinates following bulbectomy. Olfactory mucosal PDE activity is composed of a minimum of two major forms. In the absence of Ca^{2+} , rolipram-sensitive PDE comprises 65% of total activity. Following stimulation by Ca^{2+} , CAM-PDE activity is elevated sixfold to become the predominant form, thereby increasing total activity 300%, with half-maximal effect at $1 \mu\text{M}$ Ca^{2+} . We propose that Ca^{2+} stimulation of CAM-PDE may be necessary for termination of olfactory signals.

The initial events of olfaction occur in the ciliary processes of olfactory receptor neurons. These odorant-sensitive neurons are asymmetric bipolar cells that rest on a basal layer of proliferative stem cells and are intercalated between supportive sustentacular cells. Each neuron sends a long axon inward through the cribriform plate to synapse in the olfactory bulb, and an apical dendrite outward into the mucosal layer. This dendrite terminates in a knob from which originate the chemoreceptive cilia.

Cyclic nucleotides, especially cAMP, appear to play a crucial role in olfactory signal transduction. They mediate the activation of an odor-induced depolarization (Firestein and Werblin, 1989; Firestein et al., 1991), most likely by directly gating a channel in olfactory cilia (Nakamura and Gold, 1987; Frings and Lindemann, 1991; Lowe and Gold, 1991). Odorants stimulate adenylyl cyclase activity of olfactory cilia (Pace et al., 1985;

Sklar et al., 1986; Breer et al., 1990) and primary cultures of olfactory neurons (Ronnett et al., 1991a,b), effects that can occur rapidly and at low concentrations of odorants (Boekhoff et al., 1990; Ronnett et al., 1991b). The rapid decline in cAMP levels suggests an important regulatory role for phosphodiesterase (PDE). PDE activity has been previously demonstrated in cilia (Anholt and Rivers, 1990) and olfactory neurons (Margolis, 1977; Firestein et al., 1991), but the enzyme forms present have not yet been identified.

Evidence for a role of Ca^{2+} in olfaction suggests that a Ca^{2+} /calmodulin PDE (CAM-PDE) activity might be relevant. For instance, odorants stimulate the influx of Ca^{2+} into olfactory neurons (Restrepo et al., 1990), calmodulin antagonists alter the electro-olfactogram (Winegar et al., 1988), and odorants potently stimulate inositol 1,4,5-trisphosphate (IP_3) formation, which is associated with the release of sequestered Ca^{2+} (Boekhoff et al., 1990; Wood et al., 1990). Moreover, the adenylyl cyclase whose activity is enhanced by odorants in primary olfactory neuronal cultures is absolutely dependent upon Ca^{2+} (Ronnett et al., 1991b), while ciliary adenylyl cyclase is stimulated by Ca^{2+} /calmodulin (Anholt and Rivers, 1990).

At least five separate subtypes of PDE have been distinguished on the basis of substrate affinity, activation or inhibition by cGMP, effects of inhibitors, and the influence of Ca^{2+} and calmodulin (Thompson et al., 1972; Beavo, 1988; Kincaid and Manganiello, 1988). CAM-PDE is selectively stimulated by Ca^{2+} and calmodulin (Teo and Wang, 1973; Sharma and Wang, 1986). One form of PDE has high affinity for cAMP and is selectively inhibited by the antidepressant drug rolipram (Thompson et al., 1979; Wachtel, 1983; Nemoz et al., 1989). Another form with high affinity for cAMP is activated by cGMP (Beavo et al., 1971; Martins et al., 1982; Yamamoto et al., 1983), while a third form selective for cAMP is inhibited by cGMP (Weber and Appleman, 1982; Harrison et al., 1986; Simmons and Hartzell, 1988). The retina possesses a PDE with considerable selectivity for cGMP (Miki et al., 1973).

In the present study, we identify and localize a CAM-PDE in rats associated with ORNs.

Materials and Methods

Materials. 2,8-³H-cAMP (44.5 Ci/mmol) and 8,5-³H-cGMP (36.9 Ci/mmol) were obtained from NEN-Du Pont, Boston, MA. Rolipram was supplied by Schering AG, Berlin, Germany. Ro20-1724 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Denbutylline was supplied by Smith-Kline Beecham Pharmaceuticals (Betch-

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worth, England). Milrinone was supplied by Sterling-Winthrop Research Institute (Rensselaer, NY). CL-930 was supplied by Warner-Lambert/Parke-Davis (Anne Arbor, MI). *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) was purchased from GIBCO BRL (Gaithersburg, MD). Calmodulin, cAMP, cGMP, 5'-nucleotidase, Staph A, and all detergents and buffers were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise mentioned.

Immunohistochemistry. Adult male rats (150–200 gm) were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) (Steris Laboratories, Phoenix, AZ) and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brain and olfactory tissues were dissected and postfixed 1 hr in 4% PFA and then sunk in 15% (w/v) sucrose overnight. Blocks were embedded in Tissue-Tek (Miles, Elkhart, IN), stored at -70°C until use and then sectioned in a cryostat at 12 μm .

Staining was carried out using a modification of the method described by Ronnett et al. (1991a). Brain and olfactory tissue sections were rinsed three times in PBS with all subsequent rinses performed identically, and then permeabilized for 30 min in 0.5% (w/v) saponin in PBS or 0.1% (w/v) Triton X-100 in PBS, respectively. Slides were rinsed and incubated 1 hr in 10% (v/v) normal goat serum (NGS) (Jackson Labs, Westgrove, PA) or 10% (v/v) normal donkey serum (NDS) (Jackson Labs, Westgrove, PA), as appropriate to the secondary antibody, in PBS containing 1% (w/v) bovine serum albumin (BSA). Following removal of the block, slides were incubated with primary antibody made up in PBS containing 0.5% (w/v) BSA and 2% (v/v) NGS or NDS as appropriate. Slides were rinsed and incubated 25 min in PBS containing 0.3% (v/v) hydrogen peroxide to quench endogenous peroxidase. Slides were rinsed again and then blocked in 5% (v/v) NGS or NDS in PBS containing 0.5% BSA. After block removal, slides were incubated 45 min with the appropriate biotinylated secondary antibody in PBS containing 0.5% (w/v) BSA. Slides were rinsed, blocked 5 min in PBS containing 0.5% (w/v) BSA, and incubated 30 min with 2 $\mu\text{g}/\text{ml}$ peroxidase-conjugated streptavidin (Jackson Labs, Westgrove, PA). Slides were rinsed in PBS and then exposed 5 min to the chromagen 3,3'-diaminobenzidine tetrahydrochloride (GIBCO BRL). Slides were dehydrated with progressive ethanol gradations followed by xylene, and coverslipped.

Antisera were used as follows: mouse monoclonal antibody (MAb) ACC-A4C3 against Ca^{2+} /calmodulin-dependent phosphodiesterase (CAM-PDE) (Hansen and Beavo, 1986) at 1:250 dilution; affinity-purified sheep polyclonal antibody to calmodulin (Polysciences, Inc., Warrington, PA) at 1:10 dilution; goat anti-mouse IgG biotinylated secondary antibody (Jackson Labs) at 1:500 dilution; donkey anti-sheep biotinylated secondary antibody (Jackson Labs) at 1:500 dilution.

Specificity of the antibody reaction was established in several ways. Staining was negative when nonimmune ascites fluid was used in place of primary antibody. Similarly, no staining was obtained with only NGS or NDS. The ability of MAb ACC-A4C3 to immunoprecipitate PDE activity when coupled to formalin-fixed Staph A was examined using the method of Hansen and Beavo (1986). Antibody-conjugated Staph A and Staph A controls were incubated with soluble or microsomal tissue fractions overnight and then centrifuged 3 min at $10,000 \times g$. After extensive washing, the pellets were assayed for PDE activity. In both cases, the antibody precipitated threefold more PDE activity than Staph A alone. Precipitated PDE activity was doubled in the presence of Ca^{2+} and calmodulin.

Tissue preparation. Tissue was prepared either as cilia, using the method of Sklar et al. (1986), or as homogenates or subcellular fractions of olfactory turbinates or cerebral cortex. Rats were anesthetized with carbon dioxide, decapitated, and tissue dissected. Nasal turbinates were dissected intact and consisted of the entire olfactory mucosa, including endothelial tissue, olfactory epithelium, and overlying mucous. Tissue was immediately placed on ice in BHB buffer, which contained 50 mM *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) (Calbiochem, La Jolla, CA), 0.1 M EGTA, 0.08 mg/ml dithiothreitol (DTT), 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 0.5 $\mu\text{g}/\text{ml}$ antipain, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 0.6 $\mu\text{g}/\text{ml}$ chymostatin, 0.6 $\mu\text{g}/\text{ml}$ pepstatin, 0.06% dimethyl sulfoxide, and 0.1% ethanol, and homogenized 15 times up and down in a glass-Teflon homogenizer at setting 3.3 of the Wheaton overhead stirrer (Millville, NJ). For fractionation, tissue was first centrifuged at $500 \times g$ for 5 min in a Sorvall RC 2B superspeed refrigerated centrifuge (Du Pont, Wilmington, DE). The crude particulate (P_1) pellet was discarded and the supernatant (S_1) centrifuged 20 min at $28,000 \times g$. The resultant pellet (P_2) was washed in BHB, centrifuged, resuspended in 5 ml BHB, and designated the

membrane fraction. The supernatant (S_2) was centrifuged 50 min at $230,000 \times g$ in Beckman L8-M ultracentrifuge (Fullerton, CA). The pellet (P_3) was washed in BHB, recentrifuged in an ultracentrifuge, and resuspended in 2 ml of BHB as the microsomal fraction. The supernatant (S_3) was saved as the soluble fraction and visually inspected for absence of cloudiness or lipid droplets. In all cases, protein was assayed by the Bradford method using Coomassie protein assay reagent (Pierce, Rockford, IL) and BSA as standard.

PDE. PDE assays employed the method of Thompson et al. (1972) as modified by Bauer and Schwabe (1980), with standard reaction mixture adapted from Kincaid and Maganiello (1988). Before use, ^3H -cAMP was purified over QAE-Sephadex (Pharmacia, Piscataway, NJ). Reaction mixture (200 μl) containing 60,000–80,000 cpm ^3H -cAMP was added to test tubes and prewarmed to 37°C . Tissue was warmed to 37°C , and 100 μl aliquots containing 1–2 μg of protein were added to initiate the assay, which was conducted for 20 min at 37°C . Final concentrations in the incubation mixture were 50 mM BES, 5 mM MgCl_2 , 0.2 mg/ml BSA, 0.78–200 μM unlabeled cAMP, and 0.1 mM EGTA. For experiments at 100 μM free Ca^{2+} , the reaction mix included 0.2 mM CaCl_2 and 50 nM calmodulin where appropriate. Low Ca^{2+} conditions were achieved by addition of 2.0 mM EGTA. Ca^{2+} levels were measured against standards using a conductivity meter and Ca^{2+} electrode (Bio-medical Instrumentation Group, Philadelphia, PA). Following incubation, reactions were stopped by boiling for 1.5 min and cooling on ice, whereupon 100 μl aliquots of 5'-nucleotidase (10 U/ml in 0.1 M Tris, pH 8.1) were added and samples incubated for 30 min at 37°C . Incubation mixtures were applied to QAE-Sephadex columns preequilibrated with 30 mM ammonium formate, with flow through and eluate from two 1 ml washes collected in plastic scintillation vials. Ten milliliters of formula 963 (Du Pont, Wilmington, DE) were added, and radioactivity was assessed in Beckman LS 3801 counter (Irvine, CA). Assays of cGMP PDE were performed similarly, except that cGMP was used in place of cAMP and columns were eluted with 3 ml wash volume.

Bulbectomy. Destruction of the olfactory bulb causes retrograde degeneration of olfactory receptor neurons in the olfactory mucosa (Margolis et al., 1974). Bulbectomies were performed unilaterally permitting comparison of normal and denervated sides. Adult male rats, 150–200 gm, were anesthetized with chloral hydrate (0.5 g/kg, i.p.) and placed in a stereotaxic head holder. A single 2 cm midline incision was made starting midway between the eyes. The dura on the right side was scraped to bare the bone. A hole was drilled through the bone directly above the olfactory bulb. A narrow glass pipette was placed in the hole and suction was gently applied. In a successful bulbectomy, the bulb could be seen as a white mass during aspiration. After reaching bone, aspiration was complete. The hole was packed with Gelfoam and the skin was closed with surgical staples. Rats were kept warm while recovering, allowed to awaken naturally, and then returned to animal holding facilities until needed 10–11 d later.

Cell culture. Primary cultures of olfactory receptor neurons were prepared as described previously (Ronnett et al., 1991a).

Results

Biochemical characterization of PDE activity in olfactory tissue

In initial experiments, we explored the biochemical properties of PDE activity in homogenates of olfactory mucosa (Table 1). Using 1 μM cAMP or 1 μM cGMP in the presence of 100 μM Ca^{2+} , basal PDE activity in mucosal homogenates is threefold higher than with 100 μM EGTA. Calmodulin further enhances the stimulation of enzyme activity by Ca^{2+} , although to a greater extent in cilia than in mucosal homogenates.

Selective inhibitors of PDE were used to distinguish isoforms. Hydrolysis of both cAMP and cGMP in olfactory mucosa and cilia was assayed under Ca^{2+} chelating conditions or in the presence of 100 μM Ca^{2+} (Fig. 1). When PDE activity in olfactory mucosa was assayed in the presence of 100 μM EGTA and no added Ca^{2+} (Fig. 1A,B), 10 μM rolipram, 10 μM Ro20-1724, and 10 μM denbufylline inhibited cAMP hydrolysis by 57%, 55%, and 65%, respectively. Neither 10 μM rolipram nor 10 μM Ro20-

Figure 1. Effect of selective pharmacological agents on phosphodiesterase of olfactory turbinates and cilia. PDE activity in homogenates of olfactory turbinates was assayed using $1 \mu\text{M}$ cAMP or $1 \mu\text{M}$ cGMP as substrate as described in Materials and Methods. *MIX* was made initially as 500 mM stock in 0.1 M NaOH and used at 1:1000 dilution at 500 μM final concentration. All other inhibitors were made initially as 10 mM stocks in 60% EtOH and used at 1:1000 dilution at 10 μM final concentration. Final ethanol concentration was 0.06%. Assays were performed with 100 μM Ca^{2+} (open bars) or with 100 μM EGTA (solid bars). Abbreviations: *Ro20*, Ro20-1724; *ROL*, rolipram; *DEN*, denbufylline; *MIL*, milrinone; *CL9*, CL-930; *MIX*, 1-methyl-3-isobutyl xanthine. Error bars indicate SEM. *A*, Olfactory mucosa, cAMP PDE activity, assayed at $1 \mu\text{M}$ cAMP. Data are means of three experiments performed in duplicate. *B*, Olfactory mucosa, cGMP PDE activity, assayed at $1 \mu\text{M}$ cGMP. Data are means of two experiments performed in duplicate. *C*, Olfactory cilia, cAMP PDE activity, assayed at $1 \mu\text{M}$ cAMP. Data are means of four experiments performed in duplicate. *D*, Olfactory cilia, cGMP PDE activity, assayed at $1 \mu\text{M}$ cGMP. Data are means of two experiments performed in duplicate.

1724 inhibited cGMP hydrolysis, while 10 μM denbufylline inhibited cGMP hydrolysis only about 25%. These three drugs have been reported to inhibit selectively the high-affinity cAMP-selective form of PDE (Epstein et al., 1982; Wachtel, 1983; Nicholson et al., 1989). The cardiotonic drugs milrinone (10 μM) and CL 930 (10 μM), selective for cGMP-inhibited PDE (Harrison et al., 1986; Kincaid and Manganiello, 1988), reduced PDE activity about 20% whether in the presence of 100 μM

EGTA or with 100 μM Ca^{2+} . In cilia (Fig. 1C,D), neither rolipram (10 μM) nor Ro20-1724 (10 μM) inhibited PDE, whether assayed in the presence or absence of Ca^{2+} . Denbufylline (10 μM) inhibited both ciliary cAMP and cGMP PDE about 15% in the presence or absence of Ca^{2+} . Less than 10% inhibition occurred with 10 μM milrinone or 10 μM CL-930 in cilia regardless of assay conditions.

We also examined the effect of $1 \mu\text{M}$ cGMP on cAMP PDE

Table 1. Effects of Ca^{2+} and calmodulin on olfactory PDE activity

	Cilia		Olfactory mucosa	
	cAMP	cGMP	cAMP	cGMP
Control	1.8 ± 0.2 (6)	1.5 ± 0.2 (5)	0.42 ± 0.12 (4)	0.20 ± 0.05 (4)
100 μM Ca^{2+}	3.1 ± 0.04 (4)	2.4 ± 0.1 (3)	1.3 ± 0.5 (3)	0.62 ± 0.15 (3)
100 μM Ca^{2+} and 50 nM calmodulin	5.7 ± 0.6 (6)	4.3 ± 0.5 (5)	1.4 ± 0.5 (4)	0.79 ± 0.15 (4)

Data are PDE specific activity (nmol/mg/min) in cilia and homogenates of olfactory mucosa determined as described in Materials and Methods using $1 \mu\text{M}$ cAMP or $1 \mu\text{M}$ cGMP as substrate. Control conditions were assayed in the presence of 100 μM EGTA. Where appropriate, 200 μM Ca^{2+} was added alone or in combination with 50 nM calmodulin. Results are expressed as mean $\pm$ SEM of n independent experiments performed in duplicate, with n indicated in parentheses.

Figure 2. Comparison of PDE activities in olfactory cilia and homogenates of olfactory epithelium, respiratory epithelium, and cerebral cortex. *A*, Direct plot of enzyme activity versus cAMP concentration for olfactory mucosa, cilia, cerebral cortex, and respiratory epithelium. *B*, Eadie-Hofstee analysis of enzyme activity (nmol/mg/min) versus activity divided by substrate concentration (μM). This experiment is representative of three experiments performed in duplicate.

activity. In both cilia and olfactory mucosa, cGMP stimulated cAMP hydrolysis to a limited extent, with a maximal 20–30% enhancement at 10 μM cAMP, in the presence of 100 μM EGTA. This effect diminished markedly when Ca^{2+} and calmodulin were added, suggesting that cGMP-stimulated PDE is much less abundant than CAM-PDE.

These results suggest the presence of at least three forms of PDE in the olfactory mucosa, the two predominant ones being CAM-PDE and the rolipram-inhibitable PDE with high affinity for cAMP. PDE forms regulated by cGMP may be present to a lesser extent. In washed cilia, CAM-PDE appeared to be the predominant PDE.

We examined PDE activity with a wide range of cAMP concentrations in olfactory cilia, olfactory mucosa, respiratory epithelium, and brain homogenates (Fig. 2, Table 2). Olfactory cilia displayed the highest affinity for cAMP, as is evidenced in a direct plot of enzyme activity versus cAMP concentration (Fig. 2*A*) as well as in an Eadie-Hofstee analysis (Fig. 2*B*). By contrast, the highest maximal velocity occurred in brain tissue, with a V_{max} more than five times that of cilia. At low micromolar concentrations of cAMP, on the other hand, ciliary PDE activity was three to five times greater than in the brain. Maximal enzyme activity in olfactory mucosa was more than double that

of respiratory epithelium. The presence of multiple enzymes with similar K_m values is suggested by the slight curvatures of the Eadie-Hofstee plots. In studies of primary cultures of olfactory receptor neurons, which possess odorant-stimulated adenylyl cyclase activity (Ronnelt et al., 1991b), the apparent K_m for cAMP was 2.2 μM , about the same as in olfactory mucosa, while the V_{max} was 1.25 nmol/mg/min, similar to that of respiratory epithelium.

Ca^{2+} and calmodulin produced their greatest stimulation of PDE activity at low (1 μM) cAMP levels with 3.5–4.0-fold enhancement in olfactory epithelium, cilia, and brain and somewhat lesser stimulation in respiratory epithelium and primary olfactory neuronal cultures. Unlike the low-affinity brain CAM-PDE, which had a K_m of approximately 40 μM , olfactory CAM-PDE appeared to be a high-affinity enzyme, with a K_m of 1.4 μM in cilia. These results suggest that the olfactory CAM-PDE may be a different enzyme than that found in brain.

PDE activity in cilia was markedly stimulated by calmodulin (Fig. 3). At 1 μM cAMP, half-maximal stimulation by calmodulin is apparent at 10 nM. The calmodulin antagonist W-7 substantially reduced stimulation by calmodulin, while raising the concentration of calmodulin from 0.1 nM to 1 μM progressively augmented both cAMP and cGMP PDE activity, consis-

Table 2. Kinetic constants of PDE activity in nasal and brain tissue

	Olfactory mucosa	Olfactory cilia	Cerebral cortex	Respiratory epithelium
K_m (μM)	2.0 ± 0.3	1.4 ± 0.1	39.6 ± 4.8	3.7 ± 1.3
V_{max} (nmol/mg/min)	4.9 ± 0.2	15.8 ± 2.8	66.2 ± 10.4	1.7 ± 0.6
Hill coefficient	0.51 ± 0.06	0.42 ± 0.10	0.89 ± 0.04	0.44 ± 0.10
V (at 1 μM cAMP)	1.5 ± 0.1	6.6 ± 1.1	1.6 ± 0.2	0.35 ± 0.11

PDE activity was compared in olfactory cilia and homogenates of olfactory mucosa, respiratory epithelium, and cerebral cortex. PDE assay was performed with 100 μM Ca^{2+} and 50 nM calmodulin as described in Materials and Methods. K_m and V_{max} were calculated from Eadie-Hofstee plots based on the best-fit lines and represent the average values determined for a mixture of PDE isozymes. Hill coefficients were calculated from plot of $\log (V/(1 - V))$ versus $\log [\text{cAMP}]$. Results are expressed as mean $\pm$ SEM of three independent experiments performed in duplicate.

Figure 3. PDE activity in cilia as a function of calmodulin concentration. PDE activity was assayed using 1 μ M cAMP as substrate. Calmodulin concentration was varied from 10^{-10} M to 5×10^{-7} M. Three conditions were compared: 100 μ M EGTA (open circles), 100 μ M Ca^{2+} (solid circles), or 100 μ M Ca^{2+} in the presence of 100 μ M W-7 (solid squares). This experiment is representative of two experiments performed in duplicate.

tent with competition between calmodulin and W-7. Ca^{2+} potentially stimulated ciliary PDE activity, as shown using 1 μ M cAMP (Fig. 4). Free Ca^{2+} was measured in the presence of 200 μ M EGTA and 50 nM calmodulin with increasing concentrations of added CaCl_2 . Stimulation was half-maximal at about 1 μ M Ca^{2+} and saturated by approximately 100 μ M Ca^{2+} .

To examine an association of olfactory mucosal PDE with olfactory neurons, we evaluated the effects of olfactory bulbectomy (Table 3). The efficacy of bulbectomy was ensured in several ways. When the site of bulbectomy was directly inspected, only about 10% of olfactory bulb tissue remained. Histochemical examination of the olfactory epithelium revealed a marked depletion of olfactory neurons (see Fig. 6). Moreover, staining for olfactory marker protein (Margolis et al., 1974) was depleted about 80% (data not shown).

In olfactory epithelium from control animals, about 75% of total PDE activity measured in the presence of EGTA occurred in the soluble supernatant fraction, with 13% each in the mi-

Figure 4. PDE activity in cilia as a function of free Ca^{2+} concentration. PDE activity and Ca^{2+} concentrations were measured in the presence of 1 μ M cAMP and 50 nM calmodulin. Free Ca^{2+} concentrations were determined using a conductivity meter and Ca^{2+} electrode. Ca^{2+} at 10^{-7} M to 10^{-3} M was prepared in the presence of 200 μ M EGTA with increasing concentrations of CaCl_2 added as appropriate. Ca^{2+} at 1.8×10^{-8} M was prepared in the presence of 2 mM EGTA. PDE activity is expressed in units of activity normalized to activity at 16 nM Ca^{2+} . Data are means of three experiments performed in triplicate, $\pm$ SEM.

croosomal (P_3) and denser particulate (P_2) fractions. Stimulation by Ca^{2+} and calmodulin was greatest in the P_3 and P_2 fractions. In the presence of Ca^{2+} and calmodulin, only 50% of PDE activity was soluble, while 20%–25% occurred in each of the P_3 and P_2 fractions. Following bulbectomy, PDE specific activity was depleted about 90% and 80% in the P_2 and P_3 fractions, respectively, while soluble specific activity was diminished only about 50%. Total soluble activity was not significantly changed following bulbectomy. Depletion was greatest for Ca^{2+} /calmodulin stimulated activity but still pronounced when measured in the presence of EGTA (Table 3).

Immunohistochemical localization of CAM-PDE in olfactory mucosa

We have conducted immunohistochemical studies utilizing an MAb with high-affinity for CAM-PDE of rat brain and 100 times

Table 3. Subcellular distribution of PDE activity in control and bulbectomized olfactory mucosa

	Dense particulate (P_2)		Microsomal (P_3)		Soluble (S_3)	
	Specific activity (nmol/mg/min)	Units (pmol/min)	Specific activity (nmol/mg/min)	Units (pmol/min)	Specific activity (nmol/mg/min)	Units (pmol/min)
Control						
Ca^{2+}	1.78 ± 0.35	182 ± 62	1.16 ± 0.87	148 ± 45	0.96 ± 0.39	368 ± 83
EGTA	0.46 ± 0.02	48 ± 11	0.41 ± 0.24	38 ± 8	0.65 ± 0.32	222 ± 46
Bulbectomized						
Ca^{2+}	0.15 ± 0.02	36 ± 8	0.15 ± 0.07	51 ± 20	0.52 ± 0.28	404 ± 150
EGTA	0.08 ± 0.02	19 ± 3	0.09 ± 0.04	28 ± 11	0.18 ± 0.06	172 ± 71

Bulbectomy and subcellular fractionation were performed as described in Materials and Methods. PDE assays were performed using 1 μ M cAMP in the presence of 50 nM calmodulin and 100 μ M Ca^{2+} or 2 mM EGTA. Results are expressed as mean $\pm$ SEM of three to four independent experiments performed in duplicate. Units of activity (pmol/min) in individual fractions were calculated from the specific activity and protein concentration in the given fractions.

Figure 5. Immunohistochemical localization of CAM-PDE in olfactory turbinates. Immunohistochemistry was performed as described in Materials and Methods. *A*, Immunoreactivity (IR) of MAb ACC-A4C3 against CAM-PDE is shown at 200 $\times$ magnification, using Nomarski optics. IR is present in olfactory neuron cell bodies, dendritic knobs, cilia, and olfactory nerve bundles. No IR is present in either the sustentacular or basal cell layers. *B*, IR of sheep polyclonal antibody against calmodulin is shown at 200 $\times$ magnification, using Nomarski optics. IR is present in olfactory neuron cell bodies, dendritic knobs, and nerve bundles, and to a lesser extent in the ciliary layer. *C*, Oil immersion phase image (1000 $\times$ magnification) of CAM-PDE IR shows dense staining in dendritic knobs and cilia as well as soma. *Arrowheads* indicate dendritic knobs and cilia exhibiting positive IR. *O.N.*, olfactory neuron. *D*, Control photomicrograph at 200 $\times$ magnification, using Nomarski optics, shows very little IR. MAb ACC-A4C3 against CAM-PDE was preabsorbed with a fivefold molar excess purified CAM-PDE from bovine brain (61 kDa and 63 kDa forms) for 1 hr prior to immunostaining. Almost all IR was eliminated.

less affinity for calmodulin itself or other calmodulin-binding proteins (Hansen and Beavo, 1986). No staining is apparent with several ascites control preparations. The specificity of the antibody was also confirmed by its ability to immunoprecipitate Ca^{2+} /calmodulin-activated PDE activity (data not shown).

At low magnification, staining for CAM-PDE is most prominent in the ciliary layer but is also apparent in neuronal cell bodies and axon bundles (Fig. 5*A*). At higher power, we can determine that staining of the ciliary layer is due to immunoreactivity within the cilia as well as the dendritic knobs (Fig. 5*C*). Staining with an antibody that specifically recognizes calmodulin (Dedman et al., 1978) also shows immunoreactivity in cell bodies, axon bundles, and dendritic knobs; however, staining in the ciliary layer is less pronounced (Fig. 5*B*). Specificity of the CAM-PDE antibody is demonstrated by preabsorption with the 61 kDa and 63 kDa forms of bovine CAM-PDE eliminating almost all staining (Fig. 5*D*). Antisera to the cGMP-inhibited and the retinal rod PDE enzymes gave no staining. Antiserum to the cGMP-stimulated PDE did not stain olfactory receptor neurons but specifically stained goblet cells.

We observed marked differences in olfactory epithelial staining between normal and bulbectomized sides (Fig. 6). The intense staining of the ciliary layer in the intact side (Fig. 6*A*) is absent in the bulbectomized side (Fig. 6*B*). By contrast, in the bulbectomized side, neuronal cell bodies are stained to a greater extent than in the intact side, presumably reflecting cells surviving bulbectomy or regenerating cells. The dendritic processes of the stained cells in the bulbectomized side tend not to reach the ciliary layer, suggesting that these represent regenerating olfactory neuronal cells. CAM-PDE immunoreactivity appears to be selective for olfactory neurons. By comparison, respiratory epithelial cells and respiratory cilia are not stained by this antibody (Fig. 7).

CAM-PDE has been previously localized by immunocytochemistry in the brain (Kincaid et al., 1987). To ensure that our antibody selectively recognizes PDE, we conducted immunocytochemical examination in the cerebellum (data not shown). As observed previously by Balaban et al. (1989), CAM-PDE staining was highly localized to Purkinje cells and their processes. Calmodulin staining was also greatest in Purkinje cells

Figure 6. Comparison of immunohistochemical localization of CAM-PDE in control and bulsectomized turbinates. Bulbectomy and immunohistochemistry were performed as described in Materials and Methods. CAM-PDE immunoreactivity was visualized by Nomarski optics and magnified $620\times$. *A*, Control epithelium. Solid arrowheads indicate positive immunoreactivity in clumps of cilia, while open arrows show immunoreactivity in olfactory nerve bundles. *B*, Bulbectomized epithelium. Cilia cannot be easily seen. Solid arrowheads indicate positive immunoreactivity in dendritic knobs that have not yet reached the epithelial surface. Open arrows show immunoreactivity in olfactory nerve bundles.

but appeared to a lesser extent in granule cells. Preabsorption with PDE eliminated staining by the CAM-PDE antibody.

Discussion

The principal findings of this study are that within olfactory mucosa a CAM-PDE appears to be the major PDE and is selectively localized to olfactory neurons. Our biochemical assays suggest that olfactory CAM-PDE may be a novel isoform of this enzyme and the predominant form in this tissue. Moreover, we observed negligible staining of rat olfactory mucosa with antisera to the cGMP-activated, the cGMP-inhibited, and the retinal rod PDE enzymes.

Biochemical properties of the olfactory epithelial CAM-PDE are also consistent with prominent activity under physiological concentrations. The enzyme is stimulated by physiologic submicromolar concentrations of Ca^{2+} . Also, the olfactory epithelial enzyme has higher affinity for cAMP than the brain enzyme. We have calculated that the physiological cAMP concentration in olfactory neurons is about 10^{-6} M, based on measurements

Figure 7. Immunohistochemical localization of CAM-PDE at region of overlap between olfactory and respiratory epithelium. Immunohistochemistry was performed as described in Materials and Methods. CAM-PDE immunoreactivity was visualized by Nomarski optics and is shown at $250\times$ magnification. Only olfactory receptor neurons exhibit immunoreactivity to MAb ACC-A4C3 against CAM-PDE. Respiratory epithelial cells and respiratory cilia remain unstained. Areas of olfactory epithelium and respiratory epithelium are marked by arrows. The open arrow indicates an isolated olfactory receptor neuron (ORN).

of total endogenous cAMP in cultured olfactory receptor neurons and employing an assumption that tissue is approximately 10% protein. These levels are similar to those in liver and brain (Gilman, 1970). Nakamura and Gold (1987) detected two types of cyclic nucleotide-gated channels in olfactory receptor neurons, with $K_{1/2}$ values of 10^{-6} and 10^{-5} M. Given such a highly active conductance, odorant stimulation mediated via elevation of cyclic nucleotides would require basal levels of cAMP and cGMP to be approximately 10^{-7} M or less. Such a system might also require the presence of a high-affinity PDE. Brain CAM-PDE is one of the most active in the body (Butcher and Sutherland, 1962). At the low micromolar physiological concentrations of cAMP, enzyme activity in olfactory epithelium is four to five times greater than in brain. In most mammalian tissues, the predominant CAM-PDE is the low-affinity enzyme (Beavo, 1988) so that olfactory epithelium is notable in having a CAM-PDE with apparent high affinity for cAMP.

The Hill coefficient for PDE activity is almost 0.9 in brain, but 0.4–0.5 in cilia, olfactory mucosa, and respiratory epithelium.

lium. These low Hill coefficients most likely reflect the presence of multiple populations of PDE with differing affinities for cAMP rather than negative cooperativity of the enzyme. Rolipram and Ro20-1724 are selective inhibitors of a PDE with high affinity for cAMP and not linked to Ca^{2+} and calmodulin (Schwabe et al., 1976; Wachtel, 1983; Kaulen et al., 1989; Nemoz et al., 1989). In the absence of Ca^{2+} , olfactory mucosal PDE is inhibited about 60% by rolipram, but only about 20% in the presence of Ca^{2+} . Our assays most likely are detecting a mixture of at least two enzymes, with the rolipram-sensitive PDE being dominant in the absence of Ca^{2+} and the CAM-PDE becoming the predominant form in response to Ca^{2+} stimulation.

Our immunocytochemical studies reveal a selective localization of CAM-PDE to olfactory neurons, with strong immunoreactivity in the olfactory cilia. These observations further support a role for CAM-PDE in olfactory transduction, as all the major elements of olfactory transduction, such as a selective G-protein (Jones and Reed, 1989), olfactory-specific adenylyl cyclase (Sklar et al., 1986; Bakalyar and Reed, 1990), and a cyclic nucleotide-gated ion channel (Lowe and Gold, 1991), are localized to these cilia. Calmodulin mRNA is also expressed in olfactory receptor neurons (Biffo et al., 1991).

The Ca^{2+} sensitivity of olfactory neuronal PDE fits with a variety of evidence supporting a role for Ca^{2+} in olfaction. Odorants potently and rapidly stimulate the formation of IP_3 in olfactory cilia (Boeckhoff et al., 1990; Breer et al., 1990) and primary olfactory neuronal cultures (Wood et al., 1990). IP_3 acts by releasing Ca^{2+} . IP_3 gates plasma membrane Ca^{2+} channels in human T-lymphocytes (Kuno and Gardner, 1987) and catfish olfactory cilia (Restrepo et al., 1990). In brain and some peripheral tissues, IP_3 receptors are localized to endoplasmic reticulum, providing the principal source of intracellular Ca^{2+} released by IP_3 (Ross et al., 1990). However, in lymphocytes (A. Khan, J. Steiner, and S. H. Snyder, unpublished observations) and olfactory cilia (A. Cunningham, D. Ryugo, R. Reed, A. Sharp, G. Ronnett, and S. H. Snyder, unpublished observations), immunohistochemical studies by confocal and electron microscopy have localized IP_3 receptor to the plasma membrane, suggesting that in olfaction IP_3 regulates Ca^{2+} entry into the cilium. In primary olfactory neuronal cultures, phosphoinositide turnover is enhanced by odorants more rapidly than adenylyl cyclase, and the adenylyl cyclase of these cultures is absolutely dependent upon Ca^{2+} for activity (Wood et al., 1990; Ronnett et al., 1991b). Ciliary adenylyl cyclase is also potently stimulated by Ca^{2+} and calmodulin (Anholt and Rivers, 1990). While odorant enhancement of adenylyl cyclase in olfactory cilia has been reported with media containing low Ca^{2+} concentration, these cilia were first exposed to high levels of Ca^{2+} (Boeckhoff et al., 1990). These findings suggest that odorants initially stimulate IP_3 formation, with the released Ca^{2+} then activating adenylyl cyclase. The prominence of CAM-PDE in olfactory cilia suggests that CAM-PDE is activated at the same time to degrade the newly formed cAMP. Coordinated control of adenylyl cyclase and phosphodiesterase activity has been previously observed in guinea pig brain (Piascik et al., 1980). Such a model accords with the rapid decline in levels of cAMP formed following odorant stimulation (Boeckhoff et al., 1990; Ronnett et al., 1991b).

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