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Cyclic Adenosine Monophosphate Signaling in the Rat Vomeronasal Organ: Role of an Adenylyl Cyclase Type VI

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Abstract

The present study indicates that male rat urinary components in female rat vomeronasal organ microvillar preparations not only induce a rapid and transient IP_3 signal, but in addition, the level of cAMP decreases with a delayed and sustained time course. This decrease seems to be a consequence of the preceding activation of the phosphoinositol pathway rather than the result of an enhanced phosphodiesterase activity or an inhibition of adenylyl cyclase (AC) via $G\alpha_i$ or $G\alpha_o$. This notion is supported by the finding that activation of the endogenous protein kinase C suppresses basal as well as forskolin-induced cAMP formation. Furthermore, it was observed that elevated levels of calcium inhibit cAMP formation in rat VNO microvillar preparations. These properties of cAMP signaling in the VNO of rats may be mediated by a calcium- and protein kinase C-inhibited AC VI subtype, which is localized in microvillar preparations of the VNO.

Introduction

Most terrestrial vertebrates detect chemical signals by two anatomically and functionally distinct organs: the main olfactory epithelium (MOE) and the vomeronasal organ (VNO); whereas the main olfactory system is responsible for the detection of common odorants, the VNO appears to play a key role in the detection of pheromones (Keverne *et al.*, 1986; Halpern, 1987). In both sensory systems, the transduction of chemical information is mediated via G protein-coupled intracellular reaction cascades. In the main olfactory system, stimulation with odorants leads to an increase in the concentration of cyclic adenosine 3′-5′-monophosphate (cAMP) and/or 1,4,5-trisphosphate (IP_3) [for reviews see (Ache, 1994; Dionne and Dubin, 1994; Schild and Restrepo, 1998)], whereas in the VNO, pheromone application has been shown to increase the concentration of IP_3 (Luo *et al.*, 1994; Kroner *et al.*, 1996; Wekesa and Anholt, 1997; Krieger *et al.*, 1999; Sasaki *et al.*, 1999). However, the cAMP system also seems affected: in VNO preparations of the garter snake, stimulation with the chemoattractive protein ES20 not only induces an increase in IP_3 concentration but also causes a reduction of the cAMP level (Luo *et al.*, 1994). In addition, in female mouse VNO sensory tissue, two volatile pheromonal constituents of male mouse urine, DHB (dehydro-exo-brevicomin) and SBT (2-(sec-butyl)-4,5-dihydrothiazole), induced a decrease of the cAMP level (Zhou and Moss, 1997). Reduced cAMP concentrations could be either the result of an enhanced phosphodiesterase (PDE) activity or due to adenylyl cyclase (AC) inhibition, generally thought to be mediated by $G\alpha_i$ subtypes [for reviews see (Taussig and Gilman, 1995; Hurley, 1999)]. In the sensory epithelium of the VNO, it has been demonstrated that $G\alpha_{i2}$ and $G\alpha_o$ are highly expressed in distinct but non-overlapping areas (Halpern *et al.*, 1995; Berghard and Buck, 1996); in addition, stimulation of rat female VNO preparations with structurally different male urinary constituents led to a selective G_i- or G_o-controlled PLC activation (Krieger *et al.*, 1999). Since pertussis toxin-sensitive G proteins, like G_i and G_o subtypes, mediate phospholipase C (PLC) activation via $G\beta\gamma$ subunits (Rhee and Bae, 1997), one might suspect that the simultaneously released $G\alpha_i$ and $G\alpha_o$ subunits in the VNO could be responsible for the observed pheromone-induced decrease in the cAMP level due to AC inhibition. However, the AC subtype II, which is expressed in vomeronasal neurons of the mouse (Berghard and Buck, 1996) is unusual in that it is activated by $G\beta\gamma$ subunits (Tang and Gilman, 1991); furthermore, $G\alpha_i$-mediated inhibition of this AC isoform has been controversial (Chen and Iyengar, 1993; Taussig *et al.*, 1994). In the present study, attempts were made to explore the mechanism underlying the pheromone-induced decrease of the cAMP level in microvillar VNO preparations of female rats.

Materials and methods

Materials

Male and female adult Sprague–Dawley rats were purchased from Charles River (Sulzfeld, Germany). Hydroxyapatite Type I was obtained from BioRad (München, Germany), the centricon concentrators were purchased from Millipore (Eschborn, Germany) and enterokinase was from Roche (Mannheim, Germany). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), calphostin C and phorbol 12,13-dibutyrate (PDBu) were supplied by Calbiochem GmbH (Bad Soden, Germany). Antibodies against $G\alpha_o$ and adenylyl cyclase (AC) II and VI were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG-conjugated horseradish peroxidase and β-lactoglobulin were supplied from Sigma (Deisenhofen, Germany). The enhanced chemiluminescence system (ECL) for Western blots and the radioligand assay kits for cAMP (cyclic adenosine 3′–5′-monophosphate) and *myo*-[^{3}H]-inositol 1,4,5-trisphosphate determination were provided by Amersham (Braunschweig, Germany). Sources of other materials have been described previously (Löbel *et al.*, 1998). Unless otherwise specified, all reagents were from Sigma and had a purity of >99%.

Methods

Preparation of urinary ligands

Urine from fertile male rats (12–14 weeks old) was collected daily, pooled, centrifuged to remove cells (5 min, 5500 *g*) and stored in aliquots at –70°C until use.

To extract hydrophobic volatile odorants, 2 ml of pooled male urine was treated with 2 ml of dichlormethane; following separation of the organic and water phase by centrifugation (10 min, 6000 *g*), the dichlormethane urinary extract was collected and stored at –70°C.

Recombinant α_{2u}-globulin was expressed in *Escherichia coli* as described previously (Krieger *et al.*, 1999).

Isolation of microvillar fragments of the vomeronasal organ

Membrane fractions of the VNO were prepared as described previously (Kroner *et al.*, 1996). Briefly, VNOs removed from fertile female rats were washed twice in Ringer solution (120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO, 25 mM $NaHCO_3$, 7.5 mM glucose, pH 7.4) and subsequently frozen in liquid nitrogen. VNOs of 30–60 animals were thawed on ice, minced and subsequently subjected to Ringer solution containing 10 mM calcium chloride; after gently stirring for 10 min at 4°C, debris was removed by centrifugation (10 min, 3000 *g*); the resulting supernatant was collected and the pellet was resuspended again in Ringer solution containing $CaCl_2$ and processed as described above. The pooled supernatants were centrifuged for 30 min at 48 000 *g* and the resulting pellet containing the microvillar membrane fragments was resuspended in hypotonic TME buffer (10 mM Tris–HCl, 3 mM $MgCl_2$, 2 mM EGTA, pH 7.4) and stored in aliquots at –70°C.

Membrane fractions of rat cortex and female VNOs as well as cytosolic fractions of female VNOs were prepared as described previously (Krieger *et al.*, 1994). Protein concentrations were assayed by the Bradford method (Bradford, 1976).

SDS–PAGE and Western blot analysis

Protein samples, prepared as described previously (Krieger *et al.*, 1994), were mixed with 5× sample buffer (625 mM Tris–HCl, pH 6.8, 50% glycerol, 5% SDS, 7.5 mM Dithiothreitol, 0.05% bromphenol blue), boiled for 2 min and subsequently subjected to a 7% polyacrylamide gel using the Laemmli buffer system (Laemmli, 1970).

The separated proteins were transferred onto nitrocellulose using a semi-dry blotting system (Pharmacia, Freiburg, Germany). The blot was stained with Ponceau S, dried and stored at 4°C until use. For Western blot analysis, non-specific binding sites were blocked with 5% non-fat milk powder (Naturaflor, Dietmannsriel, Germany) in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20 (TBST); the blots were incubated overnight at 4°C with specific antibodies against AC VI (1:1000 in TBST, containing 3% non-fat milk powder). After three washes with TBST, a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10 000 dilution in TBST with 3% milk powder) was applied. Following three washes with TBST, the ECL system was used to visualize bound antibodies.

In situ hybridization

Freshly dissected vomeronasal organs of 2- to 3-week-old Sprague–Dawley rats (Charles River, Sulzfeld, Germany) were embedded in Tissue Tek (Miles Inc., Elkhart, IL) and rapidly frozen in a liquid N_2-cooled isopentane bath. Coronal sections of 10 µm were cut on a Leica cryostat (model CM 3000) at –30°C, thaw-mounted on silanated slides and air-dried for 3 h. Slides were subsequently treated with 4% formaldehyde in 50 mM phosphate-buffered saline for 5 min, 200 mM HCl for 10 min and 1% Triton X-100 for 2 min at room temperature. Sections were dehydrated in graded series of ethanol (60, 80, 95, 100, 100% for 1 min each) and stored in 95% ethanol at 4°C. For *in situ* hybridization, tissue sections were covered with 12 µl of hybridization solution (Amersham) containing 50% deionized formamide and 3–5 ng of digoxigenin-labeled antisense RNA of an AC VI partial cDNA clone representing nucleotides 1558–6036 of rat adenylate cyclase type VI (accession number L01115), then coverslipped. The antisense RNA probe was generated using the T3/T7 RNA transcription system according to the manufacturer's specifications (Boehringer, Mannheim, Germany). In brief, 2 µg of linearized vector was transcribed in the presence of 70 nmol of digoxigenin-11-uridine-5′-trisphosphate. Hybridization was carried out at 55°C for 16 h in closed humid boxes. Following incubation, sections were washed

twice for 30 min in 0.1 × SSC at 60°C. Hybridization was visualized using an anti-digoxigenin AP antibody (1:750, Boehringer) for 30 min at 37°C, followed by two washes in Tris-buffered saline (100 mM Tris–HCl, 150 mM NaCl, pH 7.0) for 15 min. Bound antibodies were visualized using nitro-blue tetrazolium and bromochloroindolyl phosphate (Biomol, Hamburg, Germany) as substrates. Subsequently, sections were mounted in Euparal (Roth, Karlsruhe, Germany) and examined under a Zeiss Axiophot microscope using Nomarski phase-contrast optics. In control experiments sense RNA was transcribed and hybridized to tissue sections as described for the antisense probe. No signals were observed in any of these controls.

Stimulation experiments and second messenger determination

To determine odorant-induced second messenger responses in the subsecond time range, stimulation experiments were performed with a rapid kinetic system (Biologic, Claix, France) at 37°C as described previously (Boekhoff *et al.*, 1990). Syringe I contained the stimulation buffer (200 mM NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% sodium cholate, 1 mM ATP and 4 μM GTP, pH 7.4) with free calcium concentrations as indicated. Syringe II was filled with the VNO preparation and syringe III contained the stop solution (7% perchloric acid). For the stimulation, 185 μl of stimulation buffer containing 50 μM recombinant α_{2u}-globulin were mixed with 40 μl of VNO microvillar membrane fragments and incubated for the indicated time periods (10–10 000 ms); at the appropriate time, the reaction was stopped by injection of perchloric acid.

For stimulation experiments in the presence of IBMX, microvillar preparations were pretreated for 10 min on ice with different modulators (calphostin C, PDBu or antibodies); subsequently 40 μl of the preparation was mixed with 75 μl stimulation buffer and incubated for 2 min at 37°C in a shaking water bath before another 2 min stimulation period at 37°C was started by adding an additional 75 μl aliquot of stimulation buffer containing separated fractions of male rat urine or recombinant α_{2u}-globulin. The stimulation buffer and stop solution were the same as in the subsecond time range stimulation experiments, except that 1 mM IBMX was applied to the stimulation buffer and the microvillar preparations. Quenched samples stopped by the addition of PCA were stored on ice for 20 min and then analysed for second-messenger concentrations using the cAMP and IP_3 determination kits as described previously (Boekhoff *et al.*, 1997). The concentrations of the different modulators used are given in the result part and indicate concentrations during pretreatment of the microvillar preparations or ligand concentrations in the reaction buffer.

The concentration of free Ca^{2+} was calculated by the method described elsewhere (Pershadsingh and McDonald, 1979); magnesium and calcium present in the tissue was not included in the calculation.

Results

To explore whether lipophilic urinary components of male rat urine which stimulate PLCβ in female rat VNO preparations via a G_i-subtype (Krieger *et al.*, 1999) may also affect the cAMP level in the rat VNO, female VNO microvillar fragments were stimulated with different concentrations of the organic fraction of male urine in the presence of the phosphodiesterase (PDE) blocker IBMX. The results (Figure 1a) indicate that lipophilic urinary components of male rat urine caused a reduction in the cAMP level in the VNO preparation in a dose-dependent manner: application of 2% v/v of the organic urinary fraction reduced the cAMP level by ~25%. Experiments employing recombinant α_{2u}-globulin, a pheromonal urinary component of the lipocalin family (Flower, 1996) which activates PLCβ via G_o proteins (Krieger *et al.*, 1999), demonstrate that α_{2u}-globulin also induced a concentration-dependent inhibition of cAMP formation (Figure 1b): at 50 μM, the cAMP concentration was reduced by 35%. In contrast, application of β-lactoglobulin, a non-pheromonal lipocalin, did not affect the cAMP level, even at high ligand concentrations (Figure 1b).

Since activation of phospholipase C by pertussis toxin-sensitive G protein subtypes, like G_i and G_o, appears to be mediated by the βγ-subunits of trimeric G proteins [for review see (Rhee and Bae, 1997)], the reduction of the cAMP level may be realized by simultaneously released G_i or G_o α-subunits, leading to inhibition of AC [for review see (Simonds, 1999)]. This concept would imply a similar time course for the generation of IP_3 and the decrease of cAMP. To monitor the kinetics of the second messenger responses in the subsecond time range, a rapid quench device was used (Boekhoff *et al.*, 1990). Microvilli preparations were mixed with 50 μM α_{2u}-globulin and, after various periods of exposure, the levels of IP_3 as well as cAMP were determined. As illustrated in Figure 2, α_{2u}-globulin elicited a rapid increase in IP_3 concentration which reached a maximum after ~25 ms; thereafter, the IP_3 concentration decayed to nearly basal levels within 1000 ms. In contrast, the reduction of the cAMP concentration followed a different time course: the cAMP response was delayed but sustained, reaching a maximal reduction after ~2.5–5 s. These data indicate that α_{2u}-globulin-induced PLC activation and inhibition of AC do not occur simultaneously, suggesting that inhibition of AC may not be mediated by Gα subunits.

To explore whether the decrease in cAMP could be a consequence of the activated phosphoinositol pathway, VNO preparations were incubated with the selective PLCβ inhibitor U-73122 (Smith *et al.*, 1996) and subsequently exposed to α_{2u}-globulin. The results indicate that α_{2u}-globulin induced a 30% reduction of the cAMP level in control samples, whereas in the presence of 5 μM U-73122, only a minor inhibitory effect was observed (Figure 3).

Since PLC activity generates two second messengers, the

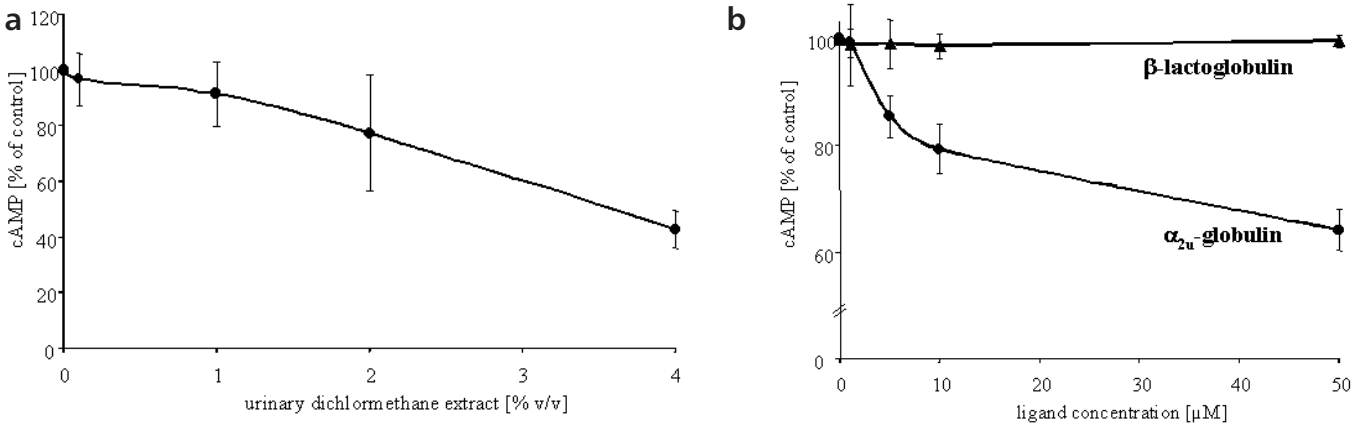

Figure 1 Dose–response curve of the male rat urinary constituents-induced decrease in the cAMP level in microvillar VNO preparations of female rats. Microvillar preparations were stimulated in the presence of 1 mM IBMX with different concentrations of either male rat dichlormethane-extracted urine **(a)** or with recombinant α_{2u}-globulin **(b)**, and subsequently, cAMP concentration was determined. Basal level of cAMP (458 ± 77 pmol cAMP/mg protein) was not affected upon treating samples with the highest dilution of pure dichlormethane (4% v/v: 448 ± 81 pmol cAMP/mg protein). Data are calculated as % of basal cAMP and are the mean values of three independent experiments with duplicate determinations ± SD.

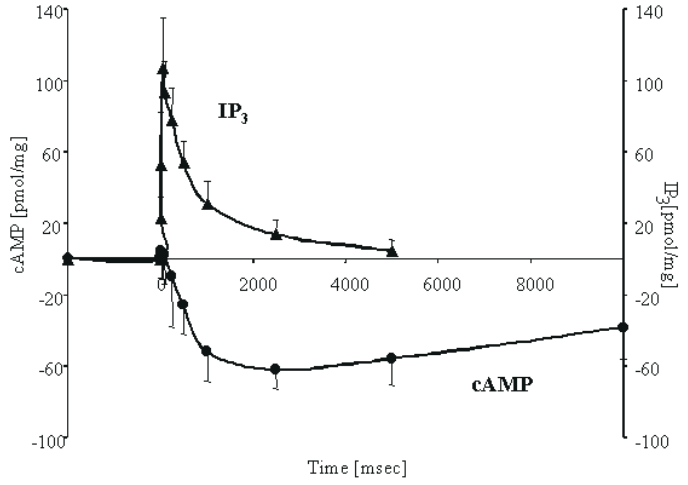

Figure 2 Time course of α_{2u}-globulin-induced second messenger signaling in female rat VNO preparations. Samples of female rat microvillar preparations were stimulated for different time periods with 50 µM recombinant α_{2u}-globulin, followed by the determination of the concentration of IP_3 and cAMP. The *x*-axis represents time (ms) at which quenching of samples occurred. The results are expressed as α_{2u}-globulin-induced second messenger concentration. Basal IP_3 was 188 ± 37 pmol/mg protein; basal cAMP was 170 ± 73 pmol/mg protein. Data are the mean values of 3–4 independent experiments with triplicate determinations ± SD.

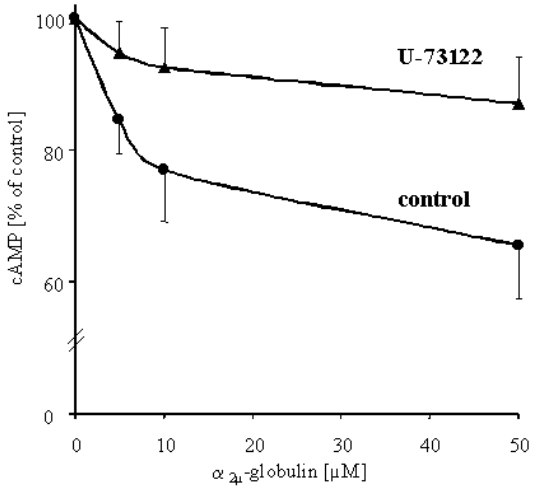

Figure 3 Effect of the PLC-inhibitor U-73122 on α_{2u}-globulin-induced decrease in the cAMP level. Microvillar VNO preparations of female rats were pretreated for 10 min on ice either with hypotonic TME buffer, used to dilute the PLC inhibitor, or with 5 µM U-73122, and subsequently stimulated with different concentration of α_{2u}-globulin before cAMP formation was determined. Basal levels of cAMP (434 ± 118 pmol/mg protein) were not affected by U-73122 (468 ± 97 pmol/mg protein). Data are calculated as % of basal cAMP with or without U-73122 and are the mean values of three independent experiments with duplicate determinations ± SD.

Ca^{2+} mobilizing IP_3 (Berridge, 1993) and the protein kinase C (PKC) activator diacylglycerol (DAG) (Nishizuka, 1992), it is conceivable that AC activity may be modulated by calcium and/or PKC [for review see (Mons *et al.*, 1998)]; thus experiments were performed to analyze the effect of a broad concentration range of free calcium concentrations and PKC inhibitors/activators. Since all AC subtypes appear to be inhibited by very high (100–1000 µM) concentrations of calcium as a result of competition for the AC cofactor magnesium (Taussig and Gilman, 1995), calcium dependence of cAMP formation was analyzed at a several thousand fold higher free magnesium than calcium concentration. The results summarized in Table 1 indicate that an increase of the calcium concentration reduces the cAMP level in a dose-dependent manner; at 100 nM free calcium, the cAMP concentration was reduced by ~60%. To evaluate if calcium may affect the AC in VNO preparations, the effect of increasing calcium concentrations was monitored upon activation of AC by forskolin (Table 1): high calcium also inhibited forskolin-induced cAMP for-

mation; at 100 nM free calcium, it was reduced to 40%. To explore a possible role of PKC in governing cAMP signals in the VNO, microvillar fragments were incubated with the PKC activator PDBu (Hannun *et al.*, 1986), and subsequently the basal as well as the forskolin-induced cAMP formation was determined; PKC activation led to both an attenuated basal and forskolin-induced cAMP formation in VNO preparations. This inhibitory effect of PKC was synergistically potentiated by high calcium concentrations; whereas at low calcium (12 nM), activated PKC reduced basal and forskolin-induced cAMP formation by ~30%, at high (100 nM) calcium, inhibition exceeded ~70%.

Table 1 Effect of different free calcium concentrations and PKC activation on basal and forskolin-induced cAMP formation in microvillar VNO preparations of female rats

Free Ca^{2+} [μM]	Control	Control + PDBu	Forskolin	Forskolin + PDBu
0	1773 ± 188	1320 ± 226	3304 ± 241	3126 ± 280
0.001	1657 ± 98	1186 ± 176	3059 ± 190	2843 ± 230
0.012	1214 ± 71	854 ± 189	2558 ± 231	2084 ± 185
0.050	877 ± 110	537 ± 148	1682 ± 223	1211 ± 111
0.100	707 ± 63	380 ± 54	1342 ± 176	872 ± 147
1	313 ± 70	148 ± 89	472 ± 92	226 ± 99

Microvillar VNO fractions were pretreated for 10 min on ice either with TME buffer or 5 μM PDBu and subsequently incubated for 4 min at 37°C with stimulation buffer or stimulation buffer supplemented with 5 μM forskolin. Free calcium concentrations varied from 1 nM to 1 μM; to ensure that the observed inhibition of calcium is not due to the competition of calcium to the AC cofactor magnesium, incubation was performed in the presence of 12.5 mM $MgCl_2$. Data are presented as cAMP (pmol/mg protein) and are the mean values of three independent experiments with duplicate determination ± SD.

In order to analyze if the α_{2u}-globulin-induced cAMP decrease was affected by PKC, VNO preparations were pretreated with the selective PKC inhibitor calphostin C (Svetlov and Nigam, 1993). As shown in Figure 4a, the cAMP decrease was completely prevented by inhibiting PKC. This notion was confirmed in experiments activating PKC with PDBu; activation of endogenous PKC reduced basal cAMP by 50%, which was not further suppressed upon α_{2u}-globulin stimulation (Figure 4b).

Recent molecular cloning approaches have identified a novel AC subtype (AC_{VN}), which is expressed abundantly in bipolar neurons of the sensory epithelium of the VNO of the garter snake, and which shows a high degree of identity to AC type VI of the rat and the mouse (Liu *et al.*, 1998). The AC subtype VI is inhibited by PKC phosphorylation (Lai *et al.*, 1997) and high concentrations of calcium (Ishikawa *et al.*, 1992; Katsuskila *et al.*, 1992; Yoshimura and Cooper, 1993), thus reflecting the functional characteristics of AC in rat VNO preparations. To explore whether this AC subtype is expressed in vomeronasal sensory cells, *in situ* hybridization experiments were performed under high stringency conditions using a digoxigenin-labeled antisense RNA probe corresponding to a partial cDNA AC VI clone. As illustrated in Figure 5a, labeled cells are restricted to a subset of cells within the sensory epithelium of the VNO; no hybridization signals were detected in the non-sensory epithelium.

To explore further whether AC VI is localized to the

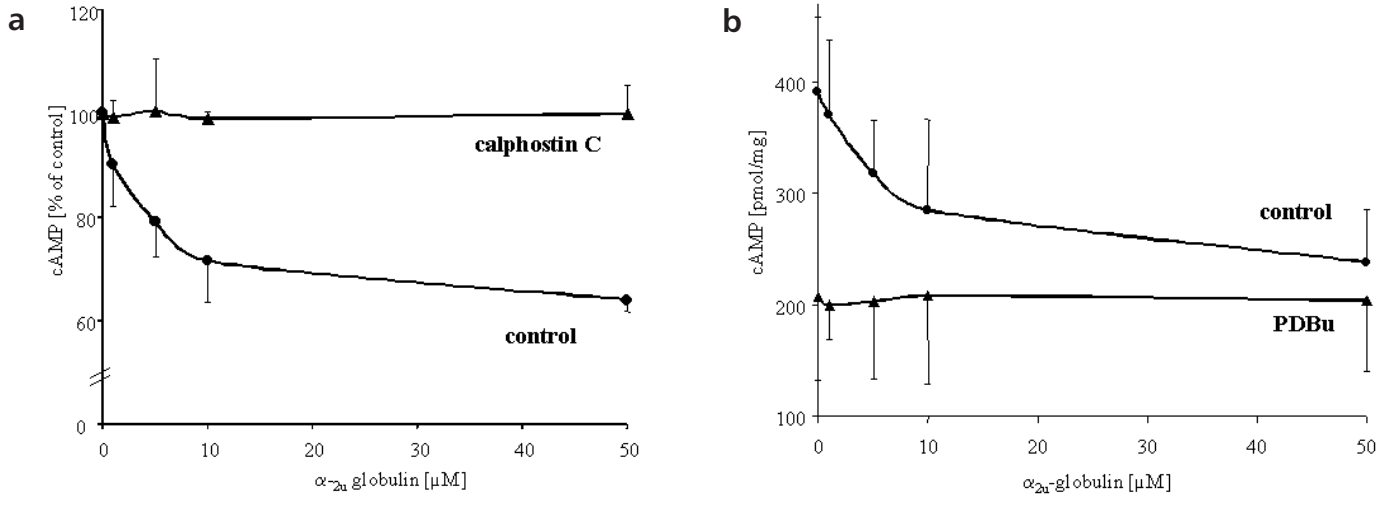

Figure 4 Effect of PKC modulators on α_{2u}-globulin-induced decrease in the cAMP level in VNO preparations. **(a)** Inhibition of PKC prevents a α_{2u}-globulin-induced decrease in cAMP. Microvillar VNO preparations were pretreated for 10 min on ice with either TME buffer or 5 μM calphostin C, and subsequently incubated with different concentrations of recombinant α_{2u}-globulin as described in Materials and methods. The basal level of cAMP (407 ± 119 pmol/mg protein) was slightly increased upon PKC inhibition (487 ± 87 pmol/mg protein). Data are calculated as % of basal cAMP either under control conditions or upon pretreatment with calphostin C. Data are the mean values of three independent experiments with duplicate determinations ± SD. **(b)** Effect of the PKC activator PDBu on pheromone- induced cAMP decreases in VNO preparations. Female microvillar VNO preparations were pretreated either with TME buffer (control) or with 5 μM PBDu for 10 min on ice; α_{2u}-globulin concentrations ranged from 1 to 50 μM. Data are presented as cAMP (pmol/mg protein) and are the mean values of three independent experiments with duplicate determination ± SD.

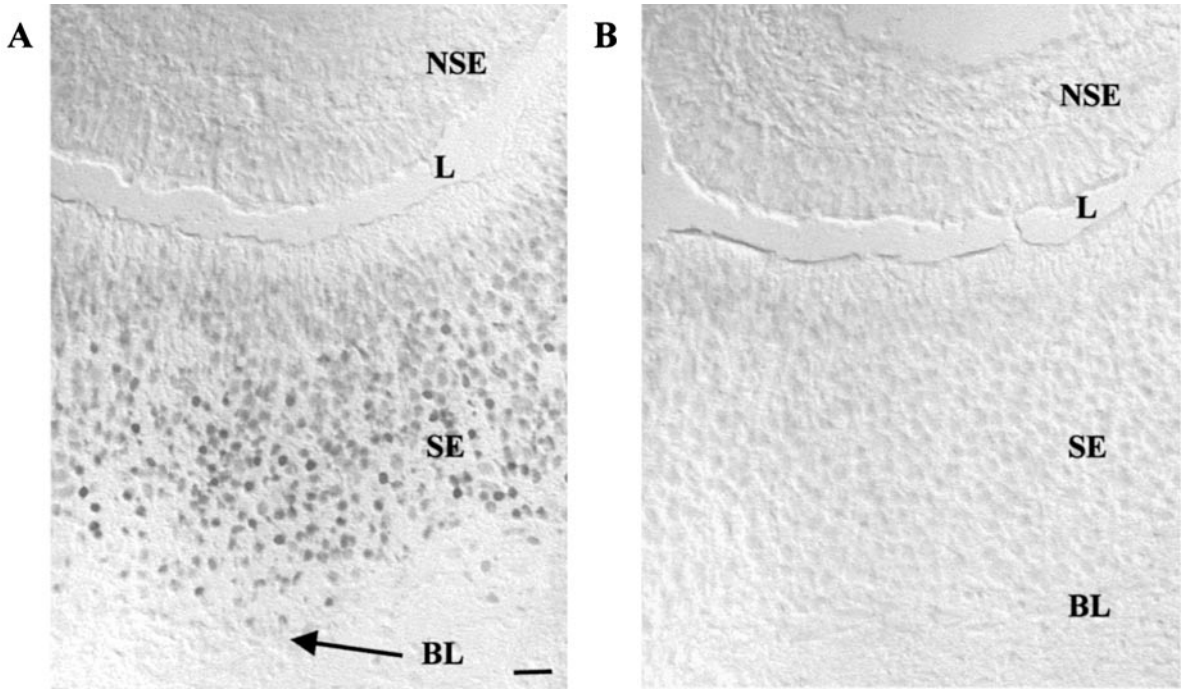

Figure 5 Cellular localization of AC VI expression in the VNO of rat. Expression of AC VI was examined using digoxigenin-labeled antisense **(A)** or sense **(B)** RNA probes against a conserved region of AC VI. Visualization was achieved with an AP-conjugated anti-digoxigenin antibody. The arrow in (A) marks the basal lamina. Positive signals are restricted to cells in the sensory epithelium of the VNO whereas no reactivity was detected in the nonsensory epithelium (scale bar: 20 μm). SE, sensory epithelium; NSE, non-sensory epithelium; L, lumen; BL, basal lamina.

microvilli, the proposed site of pheromone sensory transduction, Western blot analysis with a subtype-specific AC VI antibody was performed (Figure 6); since all isoforms of AC are predicted to be transmembrane glycoproteins (Taussig and Gilman, 1995), equal amounts of isolated VNO microvillar fragments (Mv), cytosolic (S_2) and membrane (P_2) fractions of the VNO as well as rat brain cortical membranes (Co) were separated by SDS–PAGE and analyzed on Western blots. In cortical membranes, a band at ~200 kDa is strongly labeled, as previously described for bovine brain cortical membranes [for review see (Choi *et al.*, 1993)]. Comparing the labeling in the different VNO fractions, no reactivity was detected in the cytosolic fraction; in the membrane fraction, the antibody recognized a faint band ~200 kDa; however, in the isolated microvillar fragments, labeling of a band with the same molecular size was much stronger; furthermore, after preabsorption with the synthetic peptide, no labeling was detectable, emphasizing the specificity of the observed reaction.

To approach the question of whether the AC VI subtype or the AC II isoform, previously demonstrated to be expressed in the VNO of the mouse (Berghard and Buck, 1996), may be involved in the α_{2u}-globulin-induced decrease in cAMP formation, VNO preparations were pretreated with different concentrations of either specific AC subtype II or subtype VI antibodies. Figure 7 indicates that pretreatment of VNO microvillar preparations with AC II antibodies did not affect the decrease in cAMP; in contrast,

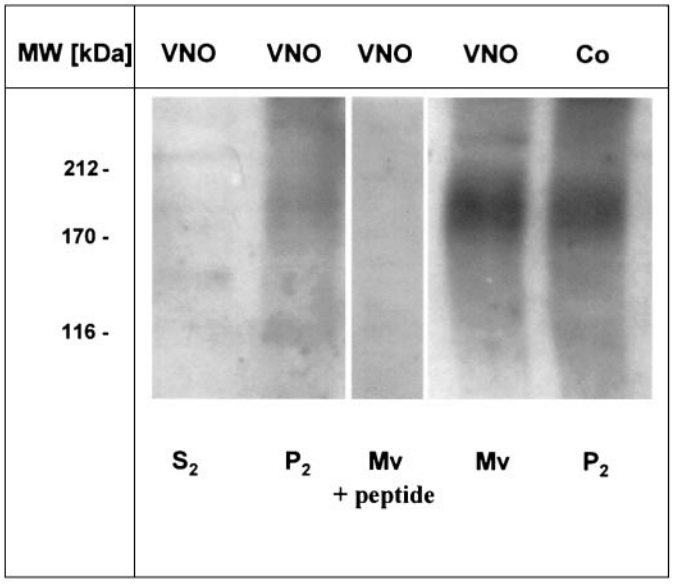

Figure 6 Western blot analysis of AC subtype VI in rat tissue. Protein (25 μg) from different VNO fractions as well as from rat brain cortical membranes was subjected to SDS–PAGE (7% acrylamide), transferred to nitrocellulose and probed with an affinity-purified antibody (1:1000) to AC VI. Antibodies labeled a polypeptide band with a molecular mass of ~200 kDa in microvillar fragments of the VNO (Mv), in membrane fractions of the VNO (P_2) and in membranes of the cortex (Co). In the cytosolic fraction (S_2) as well as microvillar fractions where the antibody was neutralized with the synthetic peptide (Mv + peptide), no immunoreactivity was detected. The positions of the high molecular mass markers (Pharmacia) are shown in the left.

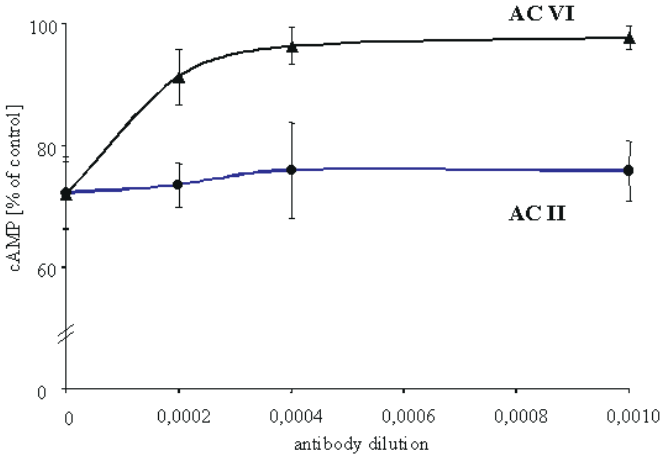

Figure 7 AC subtype VI antibodies prevent α_{2u}-globulin-induced decrease in the cAMP level in VNO preparations. Aliquots of microvillar VNO fractions of female rats were pretreated for 10 min on ice with different concentrations of specific antibodies for either AC subtype II or subtype VI, and subsequently stimulated with 50 μM α_{2u}-globulin before the concentration of cAMP was determined. Data are calculated as % of basal cAMP for each concentration of the applied antibodies. Basal cAMP (419 ± 101 pmol/mg protein) was not affected upon antibody treatment, even at high antibody concentrations (1:1000 dilution of the AC II antibody, 394 ± 76; 1:1000 dilution of the AC VI antibody, 380 ± 89 pmol/mg protein). Data are the mean values of 4–5 independent experiments with duplicate determinations ± SD.

AC VI-specific antibodies prevented cAMP decrease in a dose-dependent manner; at 1:2500 dilution of the antibody, inhibition was almost completely abolished.

Discussion

The results of the present study indicate that stimulation of female VNO preparations with male urinary ligands not only elicit generation of IP_3 (Krieger *et al.*, 1999), but in addition induce a decrease of the cAMP level. This observation is in line with previous studies on the VNO of the garter snake, where stimulation with the chemoattractive protein ES20 also caused an increase in IP_3 and a reduction in the cAMP level (Luo *et al.*, 1994; Wang *et al.*, 1997). The pheromone-induced cAMP response in the VNO is different from cAMP signaling in the main olfactory system where odorant stimulation led to increases of cAMP concentrations (Sklar *et al.*, 1986; Breer and Boekhoff, 1991; Boekhoff *et al.*, 1994). In addition, the kinetics of second messenger signaling in both chemosensory systems differ significantly: in the main olfactory system, where both second messengers are supposed to be involved in the primary transduction process (Schild and Restrepo, 1998), cAMP and/or IP_3 concentrations rapidly elevate upon stimulation with appropriate odorants (Boekhoff *et al.*, 1990; Dawson *et al.*, 1993; Restrepo *et al.*, 1993; Boekhoff *et al.*, 1994). In the VNO, IP_3 and cAMP signaling are not equally fast: stimulation with α_{2u}-globulin induces a rapid pulse in IP_3 generation resembling the kinetics of second messenger signaling in the main olfactory system, whereas the decrease in cAMP occurs with a delayed and persistent time course (Figure 2). A variety of studies have demonstrated that pheromones induce an increase in IP_3 levels in VNO preparations (Luo *et al.*, 1994; Wekesa and Anholt, 1997; Krieger *et al.*, 1999; Sasaki *et al.*, 1999); furthermore, the observation that pheromone-induced IP_3 generation is fast enough to cause membrane permeability changes responsible for the electrical response of olfactory sensory neurons (OSNs) in the VNO (Kroner *et al.*, 1996) point to the concept of an IP_3-dependent pathway for the chemo-electrical signal transduction process in the VNO. This notion is supported by electrophysiological experiments demonstrating that dialysis of IP_3 into rat VNO neurons induces inward currents (Inamura *et al.*, 1997a), whereas inhibitors of PLC block the increase of impulse frequency generated by stimulation with urinary fractions (Inamura *et al.*, 1997b). In addition, a specific 'transient receptor potential' channel, strictly localized to the sensory microvilli of OSNs in the VNO (Liman *et al.*, 1999), might be a possible downstream target of the IP_3 cascade in the VNO.

The delayed cAMP response to pheromones suggests that cAMP is not a primary messenger in the chemo-electrical transduction process of rodent vomeronasal sensory neurons; this view is supported by the observation that upon injection of cAMP no depolarizing current was observed (Liman and Corey, 1996); in addition, only the olfactory CNG-channel subunit oCNC2 has been identified in VNO neurons, which does not form active channels when expressed in heterologeous systems (Bradley *et al.*, 1994; Liman and Buck, 1994; Berghard and Buck, 1996; Wu *et al.*, 1996). Even in the absence of CNG channels, changes in the cAMP level can have myriad effects through phosphorylation reactions mediated by protein kinase A (PKA). In photoreceptor cells, where the primary sensory transduction occurs via a rapid light-activated cGMP-enzyme cascade (Yau, 1994), illumination also causes a decrease in the cAMP level (Blazynski and Cohen, 1984; Cote *et al.*, 1984), which subsequently leads to a reduction in PKA activity (Lee *et al.*, 1990). It has been demonstrated that phosducin, a specific PKA substrate, which in its unphosphorylated form tightly binds to Gβγ subunits (Lee *et al.*, 1987), serves as a negative feedback regulator of the transduction process and contributes to light adaptation (Lee *et al.*, 1992; Yoshida *et al.*, 1994; Wilkins *et al.*, 1996). Moreover, it has been found that phosducin controls the responsiveness to odorants in the MOE, where phosducin serves as a PKA-regulated inhibitor of Gβγ-dependent membrane targeting of a receptor-specific kinase subtype 3, thereby controlling phosphorylation of odorant receptors (Boekhoff *et al.*, 1997). Although it has not been shown that phosducin is present in the VNO, it is conceivable that phosducin may be involved in regulating the responsiveness of VNO neurons. The decrease in the cAMP level may cause a dephosphorylation of phosducin which subsequently

results in formation of phosducin–Gβγ-complexes. This scavenge of Gβγ subunits would diminish or stop pheromone-induced PLC activation in VNO preparations, as has been described previously for other Gβγ-controlled effector enzymes (Hawes *et al.* 1994; Hekmann *et al.*, 1994).

The results of the present study indicate that a pheromone-induced decrease in the cAMP level is not the result of enhanced PDE activity, or due to inhibition of AC by $G\alpha_i$ or $G\alpha_o$, but rather seems to be a consequence of the preceding activity of the phosphatidylinositol cascade. This view is based on the observation that calcium and PKC attenuate cAMP signaling in the VNO and is further supported by the discovery of an AC VI subtype, which is highly enriched in microvillar preparations from the VNO, thus resembling the conditions in the VNO of the garter snake (Liu *et al.*, 1998). In addition, it has previously been reported that an AC II subtype is also expressed in the VNO of the mouse (Berghard and Buck, 1996); however, AC II is insensitive to calcium [for reviews see (Cooper *et al.*, 1998; Mons *et al.*, 1998)] and is activated rather than inactivated by PKC (Zimmermann and Taussig, 1996; Bol *et al.*, 1997; Ebina *et al.*, 1997), suggesting that this AC isoform is not involved in the pheromone-induced reduction of the cAMP level. This view is supported by the fact that AC II is stimulated by Gβγ subunits (Tang and Gilmann, 1991), thus leading to an increase in cAMP. However, pheromone application did not elicit generation in cAMP (Kroner *et al.*, 1996; Krieger *et al.*, 1999; Sasaki *et al.*, 1999); furthermore, PLC activation in VNO microvillar preparations is mediated by Gβγ subunits (A. Schmitt, in preparation). Thus, the exact role of the AC II subtype remains to be established.

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