

Phosphorylation of Mammalian Olfactory Cyclic Nucleotide-Gated Channels Increases Ligand Sensitivity

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In vertebrate olfactory sensory neurons, odorant receptors couple the sensory signal to the synthesis of the second messenger cAMP. Cyclic nucleotide-gated (CNG) channels are activated by binding of cAMP and conduct a depolarizing receptor current that leads to electrical excitation of the neuron. The sensitivity of olfactory CNG channels for cAMP can be significantly reduced by binding of calmodulin to a regulatory domain that resides within the N-terminus of the α -subunit of the channel. This regulatory domain also contains a consensus phosphorylation sequence for protein kinase C (PKC). We have investigated the effect of channel phosphorylation by PKC and

found that phosphorylation increases ligand sensitivity without counteracting modulation of the channel by calmodulin. We have identified the amino acid residue that is phosphorylated by PKC and have localized three isoforms of PKC in olfactory sensory cilia. The results of this study provide information about the control of ligand sensitivity in olfactory CNG channels by an intrinsic regulatory domain, representing both a calmodulin-binding site and a substrate for PKC.

Key words: cyclic nucleotide-gated channels; olfaction; sensory transduction; protein kinase C; protein phosphorylation; phorbol ester

Electrical excitation of olfactory sensory neurons (OSNs) in vertebrates is initiated by binding of odorants to receptor proteins in the plasma membrane of chemosensory cilia (Buck and Axel, 1991; Buck, 1992). The subsequent activation of adenylyl cyclase (Pace et al., 1985; Sklar et al., 1986; Lowe et al., 1989; Pfeuffer et al., 1989; Bakalyar and Reed, 1990; Boekhoff et al., 1990) causes an increase of the cAMP concentration within the ciliary lumen, and cyclic nucleotide-gated (CNG) cation channels are activated by binding of cAMP (Nakamura and Gold, 1987; Kurahashi, 1989; Firestein et al., 1991; Frings et al., 1992; Lowe and Gold, 1993a; Zufall et al., 1994). CNG channels are expressed at high density in the ciliary membrane (Kurahashi and Kaneko, 1991) and conduct a receptor current that leads to depolarization and electrical excitation of the neuron.

Native CNG channels form hetero-oligomeric complexes (Chen et al., 1993; Bradley et al., 1994; Liman and Buck, 1994; Körschen et al., 1995; Liu et al., 1996). The best studied olfactory channel polypeptide is the α -subunit, cloned from olfactory epithelia of various species (Dhallan et al., 1990; Ludwig et al., 1990; Goulding et al., 1992). Heterologously expressed α -homomeric channels resemble native olfactory CNG channels in many respects (sensitivity to cGMP, lack of ligand-induced desensitization, cation permeability, blockage by divalent cations, and modulation by calmodulin); however, they clearly differ in some functional properties (sensitivity to cAMP and channel gating). A number of key characteristics of olfactory CNG channels have been elucidated through studies of heterologously expressed α -subunits, and a particularly significant finding is that the ligand

sensitivity is reduced by calmodulin (Chen and Yau, 1994; Liu et al., 1994) and, possibly, other Ca^{2+} -binding proteins (Balasubramanian et al., 1996). The calmodulin-mediated modulation is expected to promote channel closure during adaptation when Ca^{2+} entry through CNG channels increases the ciliary Ca^{2+} concentration (Leinders-Zufall et al., 1997). Reduction of ligand sensitivity by binding of Ca^{2+} /calmodulin constitutes a negative feedback mechanism that terminates the receptor current (Kurahashi and Menini, 1997). Thus, regulation of ligand sensitivity is an important aspect for the role of CNG channels in olfactory signal transduction.

The activity of many ion channels is modulated through phosphorylation and dephosphorylation by a variety of protein kinases and phosphatases (for review, see Levitan, 1994). For CNG channels from rod photoreceptors, there is some evidence that phosphorylation modulates ligand sensitivity (Gordon et al., 1992). We investigated the expression pattern of protein kinase C (PKC) isoforms in rat OSNs and measured the ligand sensitivity of heterologously expressed α -homomeric olfactory CNG channels after stimulation of PKC. Here we show that the γ , δ , and λ isoforms of PKC are expressed in rat OSNs, and that activation of PKC leads to an increased ligand sensitivity of α -homomeric channels. The effect of PKC is mediated by a serine residue located within a regulatory domain in the N-terminal region of the channel, which also harbors the binding site for calmodulin.

MATERIALS AND METHODS

Immunohistochemistry. Adult Wistar rats were anesthetized with fluothane and decapitated. The olfactory epithelium was excised and immersion-fixed in 0.1 M phosphate buffer (PB), pH 7.4, containing 4% paraformaldehyde, for 1 hr. After several rinses in PB, the tissue was cryoprotected in PB containing 30% sucrose overnight, embedded in OCT compound (Miles, Elkhart, IN), and frozen at -20°C . Sixteen-micrometer-thick vertical sections (perpendicular to the mucosal surface) were cut on a cryostat and collected on gelatinized slides. Sections were air-dried, fixed in 4% paraformaldehyde for 5 min, washed in PB, and incubated in 10% normal goat serum (NGS; Sigma, St. Louis, MO) and 0.5% Triton X-100 in PB for 1 hr. Anti-PKC antibodies (Transduction

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Laboratories, Lexington, KY) were diluted in 5% NGS, 0.5% Triton X-100, and 0.05% NaN_3 in PB (α , 1:1000; β , 1:2000; γ , 1:4000; δ , 1:500; ϵ , 1:100; λ , 1:250; μ , 1:500; ν , 1:100; ζ , 1:200). Sections were incubated overnight at room temperature with primary antibodies. After several rinses in PB, sections were incubated in anti-mouse-biotin (Sigma, 1:80) diluted in 5% NGS and 0.5% Triton X-100 for 1.5 hr, washed in PB, and subsequently incubated with ExtrAvidin-HRP (Sigma, 1:300) diluted in PB for 1.5 hr. After several rinses in PB, antibodies were visualized using diaminobenzidine (DAB) as a chromogen (0.05% DAB and 0.01% H_2O_2 in PB). Sections were covered with Mowiol (Hoechst Pharmaceuticals, Frankfurt, Germany) and photographed using Nomarski optics.

Electrophysiological measurements of ligand sensitivity. Human embryonic kidney (HEK) 293 cells were transfected with cDNA encoding the olfactory CNG channel α -subunit of rat (Dhallan et al., 1990) or cattle (Ludwig et al., 1990) by calcium phosphate coprecipitation (Chen and Okayama, 1987) using the pcDNA1 vector (Invitrogen, San Diego, CA) as described previously (Baumann et al., 1994). During phorbol ester treatment and patch-clamp experiments (Hamill et al., 1981), cells were held in a solution containing (in mM): 120 NaCl, 5 NaOH, 3 KCl, 1 CaCl_2 , 3 MgCl_2 , 50 glucose, and 10 HEPES, pH 7.4. For patch-clamp experiments with rat channels (inside-out configuration), both the external (pipette) and the internal (bath) solutions contained (in mM): 110 NaCl, 12 NaOH, 2 EDTA, and 10 HEPES, pH 7.4. For experiments with bovine channels, the external (pipette) solution contained (in mM): 120 NaCl, 25 NaOH, 3 KCl, 10 EGTA, and 10 HEPES, pH 7.4. The internal (bath) solution contained (in mM): 120 KCl, 25 KOH, 5 NaCl, 10 EGTA, and 10 HEPES, pH 7.2. In the calmodulin experiments, the composition of the internal solution was (in mM): 140 KCl, 10 KOH, 5 NaCl, 0.3 CaCl_2 , and 10 HEPES, pH 7.2. Bovine brain calmodulin (Calbiochem, La Jolla, CA) was dissolved at 100 μM in distilled water and added to the internal solution containing 0.3 mM Ca^{2+} . Phorbol 12-myristate 13-acetate (PMA, 4- β isoform; Sigma) and 4- α -PMA (Alexis, San Diego, CA) were dissolved in DMSO at 1 mg/ml (1.62 mM) and used at a final concentration of 0.5 μM . Cells were incubated with phorbol ester for 30–60 min at 37°C, after which patch-clamp experiments were performed for up to 2 hr. cAMP and cGMP were obtained from Sigma, and 8-bromo-cAMP (8-Br-cAMP) was obtained from Biolog (Bremen, Germany). All cyclic nucleotide concentrations $>1 \mu\text{M}$ were measured spectrophotometrically ($\lambda = 260 \text{ nm}$; $\epsilon = 15,000 \text{ cm}^2/\text{mol}$ for all cyclic nucleotides). After current recordings were obtained with a series of cyclic nucleotide concentrations, leak currents (recorded in internal solution without cyclic nucleotides) were subtracted, and a dose–response relation was constructed for each patch by fitting to the data a Hill-type function, $I/I_{\text{max}} = c^n/[c^n + K_{1/2}^n]$, where I_{max} is the current at saturating concentrations of the ligand, c is the ligand concentration, n is the Hill coefficient, and $K_{1/2}$ is the concentration for half-maximal channel activation. The mean values for $K_{1/2}$ and n from all patches were used to construct the solid lines in the dose–response plots. The figures also show the mean values of I/I_{max} for each concentration with SDs. In the text, results are given as means \pm SD and numbers of experiments in parentheses. All dose–response relations were obtained at a membrane voltage (V_m) of +40 mV.

Construction of mutant bovine CNG channels. The truncated mutants MD30 and MP101 were constructed by PCR using pCHOLF102 (Altenhofen et al., 1991) as template and the following primers: a 5' adapter primer [containing an *EcoRV* restriction site, a consensus sequence for eukaryotic ribosomal-binding site (Kozak, 1984), an initiation codon, and 18 nucleotides following the initiation codon] and a gene-specific 3' primer. Using suitable restriction sites, the original sequence of pCHOLF102 was replaced by the truncated fragments. The point mutations S93A and S93E were introduced by PCR according to the method of Herlitz and Koenen (1990) using pCHOLF102 as template and oligonucleotides containing the desired nucleotide substitutions. All mutations were verified by sequencing the inserted PCR fragment, and the recombinant cDNA sequences were subcloned into the pcDNA1 vector.

RESULTS

Three isoforms of PKC are expressed in rat olfactory epithelium

A set of nine different monoclonal antibodies was used to localize PKC immunoreactivity in vertical sections of rat olfactory epithelium. The γ , δ , and λ isoforms are expressed in the sensory cilia of OSNs (Fig. 1A, λ ; B, γ ; D, δ). Labeling appears as a dark

reaction product within the mucociliary layer, which consists of olfactory mucus and the sensory cilia. No staining above background could be found in somata of OSNs, in supporting cells, or in the submucosal tissue. When the primary antibodies were omitted, no immunoreactivity was found in cilia (Fig. 1C). PKC δ was also found in dendritic knobs of OSNs. In the tissue section shown in Figure 1E, most of the cilia were lost during preparation, thus exposing the dendritic knobs, which can be seen at higher magnification. All knobs show strong immunolabeling (arrow). The localization of three PKC isoforms in chemosensory cilia and of PKC δ in dendritic knobs suggests a role of these enzymes in chemoelectrical signal transduction. With antibodies raised against the PKC α , β , ϵ , μ , ν , and ζ isoforms, no immunolabeling was observed, suggesting that these isoforms are not expressed at all or at levels below detection threshold in OSNs.

Phorbol ester treatment increases ligand sensitivity in olfactory CNG channels

Both PKC and olfactory CNG channels are expressed specifically in sensory cilia and dendritic knobs of OSNs. We, therefore, looked for effects of increased PKC activity on ligand sensitivity of olfactory CNG channels. For these studies, we used heterologously expressed α -homomeric CNG channels from rat (Dhallan et al., 1990) and bovine (Ludwig et al., 1990) olfactory epithelium, which display almost identical properties with regard to activation by cAMP and cGMP. HEK 293 cells expressing olfactory channels were incubated for 30–60 min with 0.5 μM PMA, a phorbol ester that specifically activates most PKC isoforms by substituting for diacylglycerol (Nishizuka, 1986). Cells were then transferred to PMA-free solution, and inside-out patches were taken and exposed to a series of solutions containing various concentrations of cAMP. The I – V_m relations depicted in Figure 2A show macroscopic currents activated by cAMP in a patch from an untreated cell expressing rat CNG channels. Figure 2B shows I – V_m relations from a cell treated with 0.5 μM PMA. The cAMP sensitivity of the channels was strongly increased so that lower cAMP concentrations were sufficient for activation. The dose–response relations in Figure 2C illustrate the shift of current activation to lower cAMP concentrations induced by PMA. The cAMP sensitivity of channels from control cells (circles) was characterized by an activation constant, $K_{1/2}$, of $75 \pm 12 \mu\text{M}$ cAMP, and a Hill coefficient, n , of 2.5 ± 0.4 (11 patches). These values are in good agreement with earlier reports (Dhallan et al., 1990; Altenhofen et al., 1991; Bradley et al., 1994; Liman and Buck, 1994). Fitting the Hill equation to the data from PMA-treated cells (triangles) yielded $K_{1/2}$ of $12.7 \pm 1.3 \mu\text{M}$ and n of 2.2 ± 0.16 (three patches). The PMA-induced increase of cAMP sensitivity persisted in excised patches and did not depend on the presence of Ca^{2+} , indicating that PMA treatment causes a stable, probably covalent modification of the channels.

Olfactory CNG channels display a much higher sensitivity to several analogs of cAMP, including 8-Br-cAMP and 8-chlorophenylthio-cAMP (Frings et al., 1992). We tested whether PMA treatment would also increase 8-Br-cAMP sensitivity and obtained from dose–response relations for control cells a $K_{1/2}$ of $13.3 \pm 1.7 \mu\text{M}$, with $n = 2.2 \pm 0.1$ (five patches) and for channels from PMA-treated cells a $K_{1/2}$ of $3.0 \pm 1.12 \mu\text{M}$, with $n = 1.70 \pm 0.17$ (five patches) (data not shown). Thus, PMA treatment increased the ligand sensitivity for cAMP and 8-Br-cAMP to a similar extent.

Olfactory CNG channels can also be activated by cGMP. In fact, the cGMP sensitivity of α -homomeric channels is much

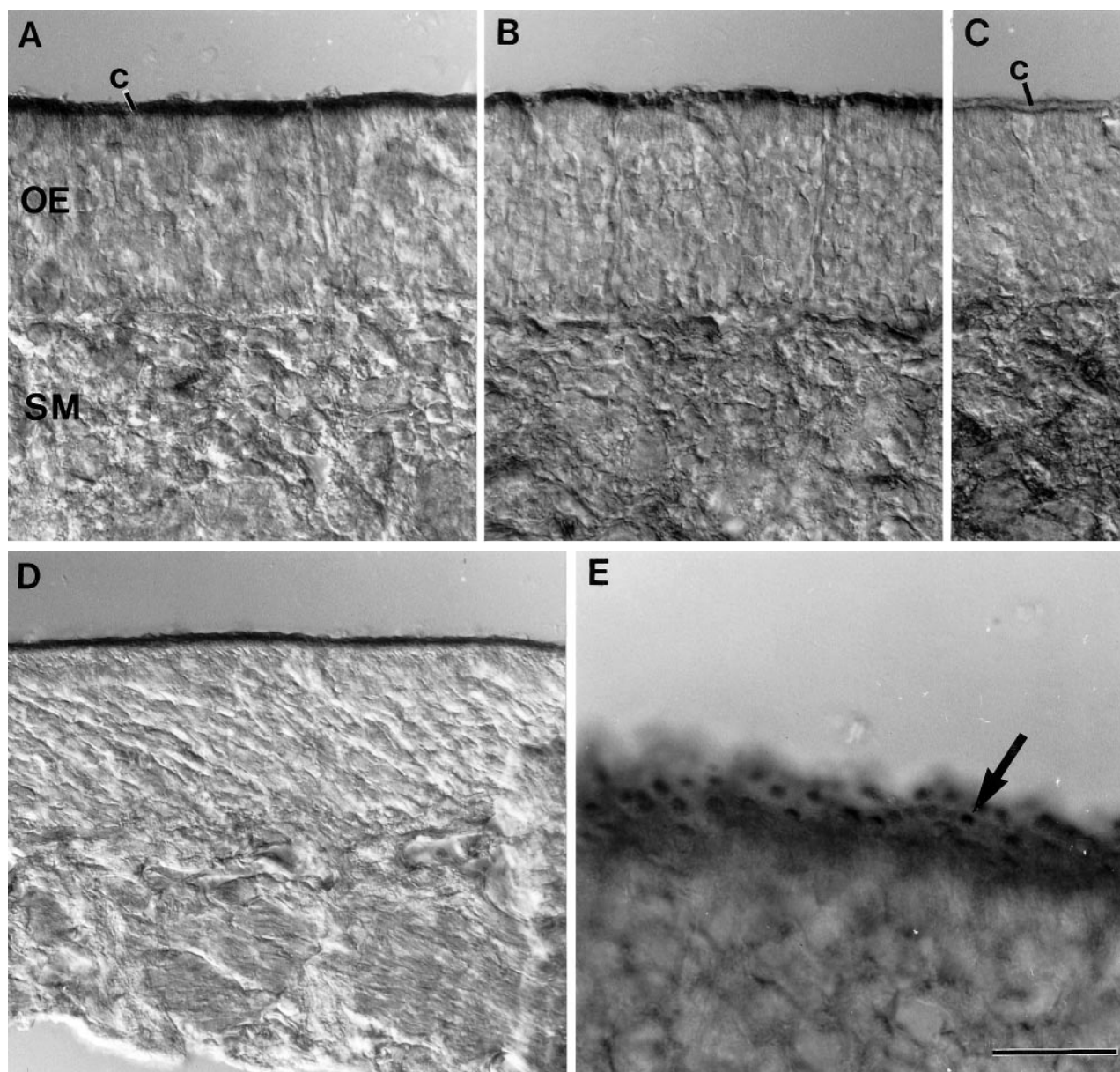


Figure 1. PKC-like immunoreactivity in vertical sections of the rat olfactory epithelium. All photomicrographs were taken with Nomarski optics. *A*, A monoclonal antibody against PKC α strongly labeled olfactory cilia (*c*). *OE*, Olfactory epithelium; *SM*, submucosal layer. *B*, PKC γ -like immunoreactivity in cilia. *C*, Omission of the first antibody abolished immunostaining in cilia. *D*, *E*, PKC δ -like immunoreactivity is strong in cilia (*D*) but also in dendritic knobs (*E*). In *E*, the tissue was sectioned slightly oblique. Most of the cilia were lost during preparation of this tissue, enabling clear identification of immunolabeled dendritic knobs (*arrow*). Scale bar: *A–D*, 50 μ m; *E*, 20 μ m.

higher than the sensitivity to cAMP and closely resembles the cGMP sensitivity of the native channel (Dhallan et al., 1990; Altenhofen et al., 1991; Frings et al., 1992). We investigated whether the sensitivity for cGMP is affected by PMA treatment. Figure 3*A* shows a family of $I-V_m$ relations for cGMP-activated current obtained from a control cell expressing bovine CNG channels (without PMA), and Figure 3*B* shows $I-V_m$ relations from a PMA-treated cell. The corresponding dose–response relations in Figure 3*C* were fitted with $K_{1/2}$ of $1.45 \pm 0.36 \mu$ M; $n = 2.23 \pm 0.33$ (seven patches) for control currents (*filled circles*) and with $K_{1/2}$ of $0.30 \pm 0.1 \mu$ M; $n = 2.03 \pm 0.24$ (18 patches) for currents from PMA-treated cells (*triangles*), indicating similar effects on the sensitivities for cGMP and cAMP.

To distinguish stimulation of PKC by PMA from unspecific

effects of the phorbol ester, we repeated the experiments using 4- α -PMA, a PMA isomer that does not activate PKC (Van Duuren et al., 1979). As shown in Figure 3*C* (*open circles*), the cGMP sensitivity of the channels was not significantly changed by 4- α -PMA ($K_{1/2} = 1.48 \pm 0.13 \mu$ M; $n = 2.4 \pm 0.24$; six patches). We, therefore, conclude that the PMA effect on ligand sensitivity is specifically mediated by PKC, and that the activation of endogenous PKC in HEK 293 cells leads to phosphorylation of the olfactory channel protein, resulting in increased sensitivity of the channel to its ligand.

Most patches maintained high ligand sensitivity after patch excision. However, 3 of 18 patches showed a partial or complete reset of cGMP sensitivity to control values 15–25 min after excision (data not shown). In one patch, the current activation

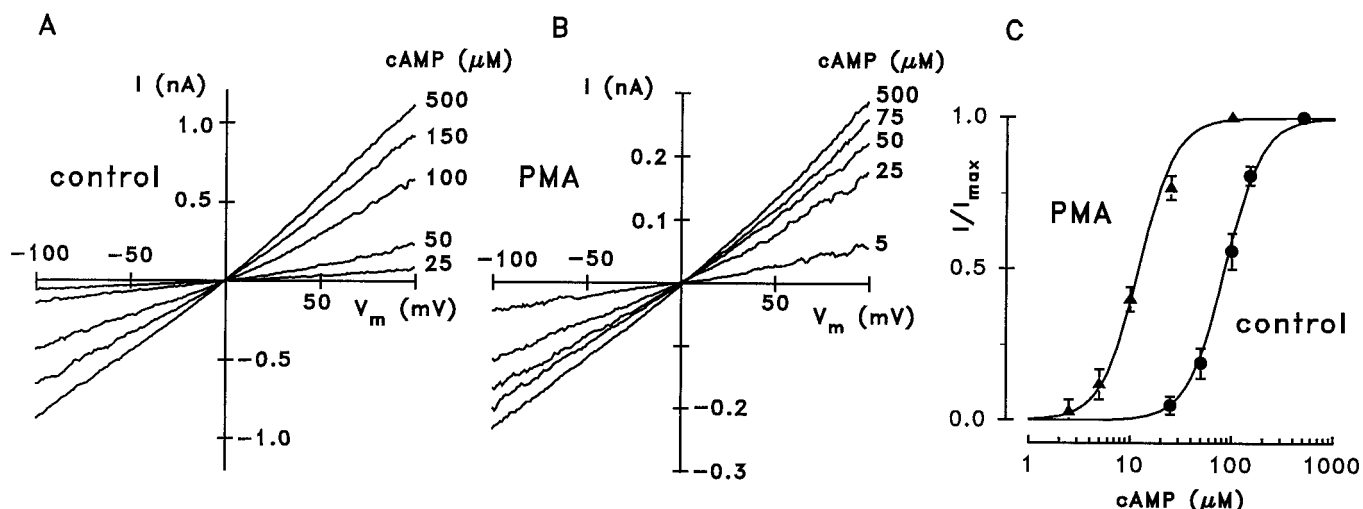


Figure 2. Modulation of cAMP sensitivity of rat α -homomeric CNG channels by treatment with $0.5 \mu\text{M}$ PMA. *A*, I - V_m relations recorded from an inside-out patch from an untreated HEK 293 cell expressing rat olfactory channels. The respective cAMP concentrations are indicated at each trace. *B*, I - V_m relations obtained from a cell after treatment with $0.5 \mu\text{M}$ PMA, showing increased cAMP sensitivity of CNG channels. *C*, Dose-response relations for current activation by cAMP at $V_m = +40$ mV in control (circles) and PMA-treated (triangles) cells. Solid lines were constructed by fitting to the normalized current a Hill-type function, as described in Materials and Methods. Fitting parameters were $K_{1/2} = 75 \pm 12 \mu\text{M}$; $n = 2.5 \pm 0.4$ (11 patches) for control and $K_{1/2} = 12.7 \pm 1.3 \mu\text{M}$; $n = 2.2 \pm 0.16$ (3 patches) for PMA-treated channels.

shortly after excision from a PMA-treated cell was characterized by $I_{\text{max}} = 745$ pA, $K_{1/2} = 0.3 \mu\text{M}$, and $n = 2.2$ ($V_m = +40$ mV). Twenty-five minutes later, I_{max} was 720 pA, whereas $K_{1/2}$ was increased to $\sim 2 \mu\text{M}$, indicating that the ligand sensitivity had been reset to the control value. We cannot explain this observation, but, possibly, some phosphatase activity was preserved in the excised patch configuration, sufficient to dephosphorylate the channels in these patches.

To test for possible effects of phosphorylation on channel conductance in cGMP-activated channels, we obtained single-channel recordings from control cells (Fig. 3*D*) and from PMA-treated cells (Fig. 3*E*). The recordings from control cells were obtained at $2 \mu\text{M}$ cGMP and the indicated values of V_m . The voltage dependence of single-channel current derived from these recordings is shown in Figure 3*F* (circles) and indicates a conductance of 36.8 ± 2.7 pS (11 patches) for control channels. Single-channel traces from PMA-treated cells were recorded at $0.5 \mu\text{M}$ cGMP to obtain a comparable open probability of the channels. The conductance of phosphorylated channels (38.2 ± 3.2 pS; four patches) is not significantly different from the value observed in untreated channels (Fig. 3*F*, triangles). Thus, phosphorylation enhances the ligand sensitivity of olfactory CNG channels by increasing their open probability without affecting their conductance.

The PKC effect is mediated by a single amino acid residue

Recently it was shown (Liu et al., 1994) that the ligand sensitivity of olfactory CNG channels is controlled by a stretch of amino acid residues within the cytoplasmic N-terminal region of the α -subunit (Fig. 4*A*, *CaM*, *FR*). This domain exhibits a high degree of sequence homology among all cloned olfactory α -subunits (Fig. 4*B*). It contains a binding site for calmodulin (Fig. 4, *CaM*) and a flanking region (Fig. 4, *FR*) that extends in the C-terminal direction from the calmodulin-binding site. Both regions control the ligand sensitivity of the channel. If either of the two regions is deleted, the ligand sensitivity is reduced. Binding of calmodulin

causes a similar decrease of ligand sensitivity as deletion of either of the two regions (Chen and Yau, 1994; Liu et al., 1994).

Because this domain is crucial for modulating the ligand sensitivity, we investigated its involvement in the PKC effect. We first constructed a truncated version of the bovine olfactory α -subunit, which did not contain the calmodulin-binding site and the adjacent 21 amino acid residues of the flanking region. This construct, MP101 (truncated until Gly¹⁰⁰), was expressed in HEK 293 cells and subjected to the same PMA treatment as the wild-type channels. In accordance with results obtained in the rat α -subunit (Liu et al., 1994), MP101 displayed a reduced cGMP sensitivity ($K_{1/2} = 8.2 \pm 1.07 \mu\text{M}$; $n = 2.76 \pm 0.29$; seven patches) (Fig. 5*A*, circles). The cGMP sensitivity of MP101 did not increase after treatment with PMA (triangles) but was slightly reduced ($K_{1/2} = 11.9 \pm 2.2 \mu\text{M}$; $n = 2.42 \pm 0.32$; five patches). Figure 5*B* shows that the truncated channel is not sensitive to calmodulin. As control experiments, inside-out patches were perfused with internal solution containing 0.3 mM Ca^{2+} but no calmodulin (circles). The $K_{1/2}$ was $14.6 \pm 3.1 \mu\text{M}$, with $n = 2.2 \pm 0.32$ (five patches) under control conditions. (All olfactory channels tested show a slightly reduced ligand sensitivity in the presence of elevated $[\text{Ca}^{2+}]_i$; cf. Table 1.) After exposure of the patches to $0.5 \mu\text{M}$ calmodulin for 3 min, the cGMP sensitivity was virtually unchanged ($K_{1/2} = 15.5 \pm 3.6 \mu\text{M}$; $n = 1.88 \pm 0.13$; five patches) (Fig. 5*B*, triangles). Under the same experimental protocol, calmodulin caused a sevenfold increase of $K_{1/2}$ in the wild-type channel (see below). Thus, both PKC-mediated increase and calmodulin-induced decrease of ligand sensitivity are absent in the truncated channel MP101. A second truncated channel polypeptide, MD30, which contained both the calmodulin-binding site and the flanking region (truncated until Lys²⁹) showed responses to calmodulin and to PMA treatment similar to the wild-type channels (Table 1). These experiments show that the PKC-induced modulation of ligand sensitivity in olfactory channels involves a segment of the N-terminal region between Lys²⁹ and Gly¹⁰⁰.

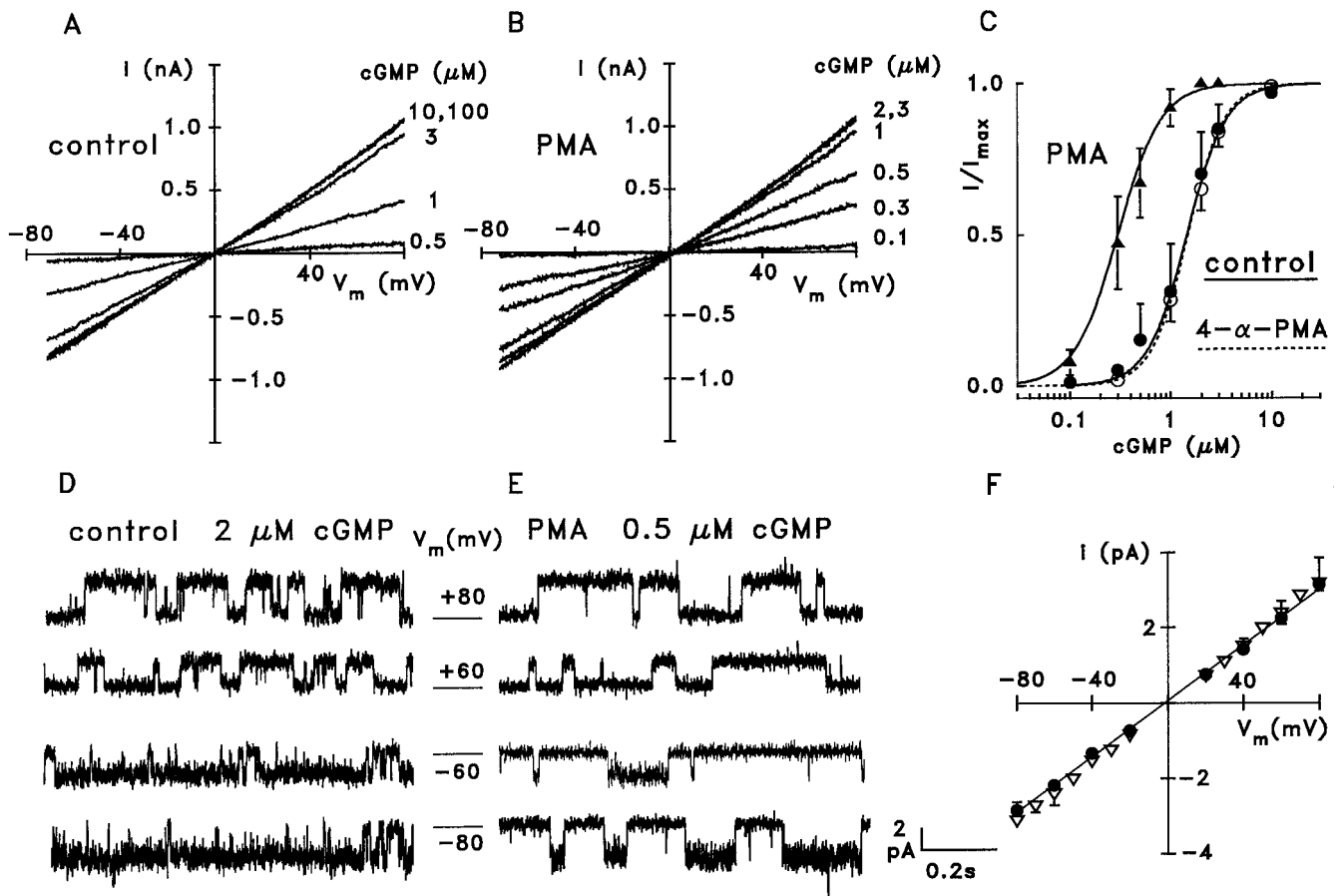


Figure 3. Increase of cGMP sensitivity in bovine α -homomeric CNG channels after PMA treatment. *A*, I - V_m relations recorded from an inside-out patch of untreated HEK 293 cells expressing bovine olfactory CNG channel α -subunits. *B*, I - V_m relations from a patch after treatment of the cell with PMA showing activation of channel current by lower cGMP concentrations. *C*, Dose-response relations for channel activation by cGMP obtained from patches without (filled circles) and with (triangles) PMA treatment ($V_m = +40$ mV). Fitting parameters are given in Table 1. The PMA isomer 4- α -PMA, which does not activate PKC, does not modulate sensitivity in olfactory CNG channels (open circles). *D*, Single-channel currents recorded from an α -homomeric olfactory CNG channel. The recordings were obtained from an inside-out patch of an untreated cell with 2 μ M cGMP, at the indicated values of V_m . Sample rate, 3 kHz; low-pass filter, 1 kHz. *E*, Single-channel recordings from a PMA-treated cell with 0.5 μ M cGMP. *F*, Voltage dependence of the single-channel current of control (circles) and PMA-treated (triangles) channels. The slope conductances are 36.8 ± 2.7 pS (11 patches) for control, and 38.2 ± 3.2 pS (4 patches) for PMA-treated channels.

The flanking region (Fig. 4, *FR*) contains a consensus phosphorylation sequence (Arg⁹⁰-Ser⁹³ in the bovine channel) that may form a recognition site for PKC (Pearson and Kemp, 1991). To test whether phosphorylation of Ser⁹³ mediates the PMA effect on ligand sensitivity, we replaced Ser⁹³ with alanine (mutant S93A) and measured the cGMP sensitivity with and without PMA treatment (Fig. 5C). Under control conditions (circles), S93A showed a cGMP sensitivity similar to that of the wild type ($K_{1/2} = 1.93 \pm 0.15$ μ M; $n = 2.03 \pm 0.35$; five patches). After PMA treatment (triangles), the cGMP sensitivity was not changed significantly ($K_{1/2} = 2.2 \pm 0.56$ μ M; $n = 2.06 \pm 0.27$; six patches). This lack of PMA effect in S93A strongly suggests that a PKC-mediated phosphorylation of Ser⁹³ is responsible for the increase of ligand sensitivity.

The effect of phosphorylation can sometimes be mimicked by replacing the amino acid residue serving as the kinase substrate by glutamate or aspartate. These residues can substitute for the negative charge otherwise provided by phosphate and, thereby, can produce the phenotype corresponding to the phosphorylated state of the protein (e.g., Smith and Goldin, 1996). We generated an S93E mutant of the olfactory CNG channel to test whether

replacement of Ser⁹³ by a glutamate residue would produce a channel with constitutively increased cGMP sensitivity. However, S93E displayed the same properties as S93A (Table 1). Thus, the modulation of ligand sensitivity by phosphorylation of Ser⁹³ cannot be explained by the introduction of a negative charge into the region flanking the calmodulin-binding site.

Calmodulin-induced modulation is preserved in the phosphorylated channel

We examined whether the PKC-mediated increase of ligand sensitivity interferes with the antagonistic modulation of the channel by calmodulin. Inside-out patches from transfected cells were first washed in Ca²⁺-free solution to remove any endogenous Ca²⁺-binding proteins of the HEK 293 cells. To obtain dose-response relations for channel activation under control conditions, I - V_m curves were first recorded in internal solution containing 0.3 mM Ca²⁺ and various concentrations of cGMP. Patches were then exposed for 3 min to the same solution containing 0.5 μ M calmodulin and no cGMP to saturate the channels with calmodulin. Subsequently, I - V_m relations were again recorded at increasing cGMP concentration in the presence of 0.3

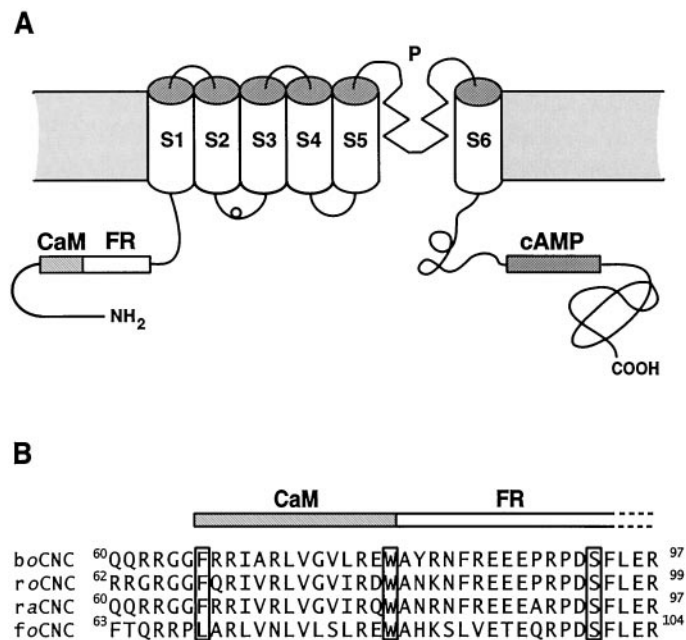


Figure 4. *A*, Schematic representation of the transmembrane topology of the olfactory CNG channel α -subunit according to Henn et al. (1995); S1–S6, Transmembrane regions; P, pore region. The N-terminus harbors a regulatory domain consisting of a calmodulin-binding site (CaM; Phe⁶⁶–Trp⁷⁹ in boCNC) and its flanking region (FR). The border of the FR region toward the S1 segment is not known. The C-terminus contains the cyclic nucleotide-binding site (cAMP; Gly⁴⁶¹–Leu⁵⁷⁸ in boCNC). *B*, Alignment of the amino acid sequence of the NH₂-terminal regulatory domain of bovine olfactory α -subunits (boCNC; Ludwig et al., 1990) with the corresponding domains of rat (roCNC; Dhallan et al., 1990), rabbit (raCNC; Biel et al., 1993), and catfish (foCNC; Goulding et al., 1992). Boxed amino acid residues are the hydrophobic residues that represent positions 1 and 14 of the calmodulin-binding site, as well as a serine residue (Ser⁹³ in boCNC) that is part of a consensus site for protein phosphorylation (RXXS).

mM Ca²⁺ (Fig. 6A). The dose–response relations in Figure 6B demonstrate a calmodulin-induced shift of cGMP sensitivity from the control ($K_{1/2} = 1.76 \pm 0.26 \mu\text{M}$; $n = 2.47 \pm 0.38$; seven patches) (Fig. 6A, circles) to the calmodulin-bound state of the channel ($K_{1/2} = 12.0 \pm 2.8 \mu\text{M}$; $n = 2.8 \pm 0.9$; 14 patches) (Fig. 6A, triangles). A similar calmodulin-induced increase of $K_{1/2}$ was reported earlier for α -homomeric channels of the rat (Chen and Yau, 1994). The calmodulin effect was readily reversible; washing the patch for 2–5 sec in Ca²⁺-free internal solution caused dissociation of calmodulin from the channel and the consequent reset of control cGMP sensitivity (data not shown). When patches from PMA-treated cells were subjected to the same calmodulin treatment, we observed a strong decrease of the cGMP sensitivity; recordings from phosphorylated channels without calmodulin (Fig. 6C, circles) yielded a $K_{1/2}$ of $0.35 \pm 0.03 \mu\text{M}$ and $n = 1.85 \pm 0.19$ (six patches). After exposure to calmodulin (triangles), activation was characterized by a $K_{1/2}$ of $14.4 \pm 2.3 \mu\text{M}$ and $n = 2.35 \pm 0.7$ (six patches). $K_{1/2}$ was reversed to the control value by brief exposure to Ca²⁺-free solution. These results show that the PKC-mediated phosphorylation of Ser⁹³ induces an increase of ligand sensitivity only if the calmodulin-binding site is not occupied. They also demonstrate that the dynamic range of the calmodulin-dependent modulation is much larger when Ser⁹³ is phosphorylated; the value of $K_{1/2}$ increases during binding of calmodulin 40-fold in phosphorylated channels but only sevenfold

in channels from untreated cells. Mutation of Ser⁹³ did not change the sensitivity of the channel for calmodulin; application of calmodulin to mutant S93A reduced the cGMP sensitivity from control ($K_{1/2} = 2.19 \pm 0.48 \mu\text{M}$; $n = 2.34 \pm 0.19$; seven patches) to the calmodulin-bound state ($K_{1/2} = 17.3 \pm 6.1 \mu\text{M}$; $n = 1.8 \pm 0.47$; five patches) by a similar extent as observed with wild-type channels (eightfold).

DISCUSSION

Recent results from several groups indicate that the ligand sensitivity of olfactory CNG channels can be regulated through binding of calmodulin or other cytosolic components. During odorant stimulation, olfactory channels can carry substantial Ca²⁺ currents into the cilia (Frings et al., 1995), causing the ciliary Ca²⁺ concentration to increase (Lowe and Gold, 1993b; Tareilus et al., 1995; Leinders-Zufall et al., 1997). At elevated ciliary Ca²⁺ concentration, calmodulin and, possibly, other Ca²⁺-binding proteins present in OSN (Bastianelli et al., 1995; Balasubramanian et al., 1996; Boekhoff et al., 1997) reduce ligand sensitivity, promote closure of the channels, and terminate the excitatory phase of the odorant response (Kurahashi and Menini, 1997).

The purpose of the present study was to investigate a second regulatory mechanism of ligand sensitivity in olfactory CNG channels: phosphorylation of the α -subunit by PKC. There is ample evidence for an involvement of PKC in olfactory signal transduction. PKC was first detected in ciliary preparations of amphibian OSNs by Anholt et al. (1987), and, more recently, PKC was shown to participate in adaptation by inactivating odorant receptors (Boekhoff and Breer, 1992; Boekhoff et al., 1992). Moreover, activation of PKC appears to increase the odorant-stimulated cAMP synthesis in amphibian olfactory epithelium (Frings, 1993), suggesting a modulatory role of this enzyme also in the excitation of the neuron.

We have shown that the γ -, δ -, and λ isoforms of PKC are expressed in the sensory cilia of rat OSNs. This set of enzymes covers the entire range of known PKC isoforms (for review, see Dekker, 1997). The γ isoform belongs to the group of “classic” PKCs that are Ca²⁺-dependent, activated by diacylglycerol, and exhibit comparably low substrate specificity. The δ isoform is a “novel” and the λ isoform an “atypical” PKC, both characterized by Ca²⁺-independent activity and a more restricted substrate profile. Interestingly, PKC λ is not sensitive to diacylglycerol or phorbol esters and may be activated by arachidonic acid or phosphatidylinositol 3,4,5-trisphosphate (Akimoto et al., 1994). Regulation of the three PKC isoforms in OSNs as well as their cellular targets has yet to be identified. Our data suggest that the olfactory CNG channel could be the target of PKC-mediated phosphorylation. Activation of PKC in HEK 293 cells leads to an increase of ligand sensitivity of heterologously expressed α -homomeric channels. This effect is brought about by phosphorylation of Ser⁹³, an amino acid residue situated in the intracellular N-terminal region of the channel polypeptide. Ser⁹³ is located near the calmodulin-binding site. But while calmodulin binding decreases ligand sensitivity, phosphorylation of Ser⁹³ has the opposite effect. Thus, the regulatory domain within the N-terminal region (consisting of the calmodulin-binding site and the flanking region that contains Ser⁹³) can serve two opposing functions. Interestingly, binding of calmodulin reduces the ligand sensitivity regardless of the phosphorylation state of Ser⁹³, suggesting that the conformational transition induced by binding of calmodulin is the main determinant of sensitivity. On the other

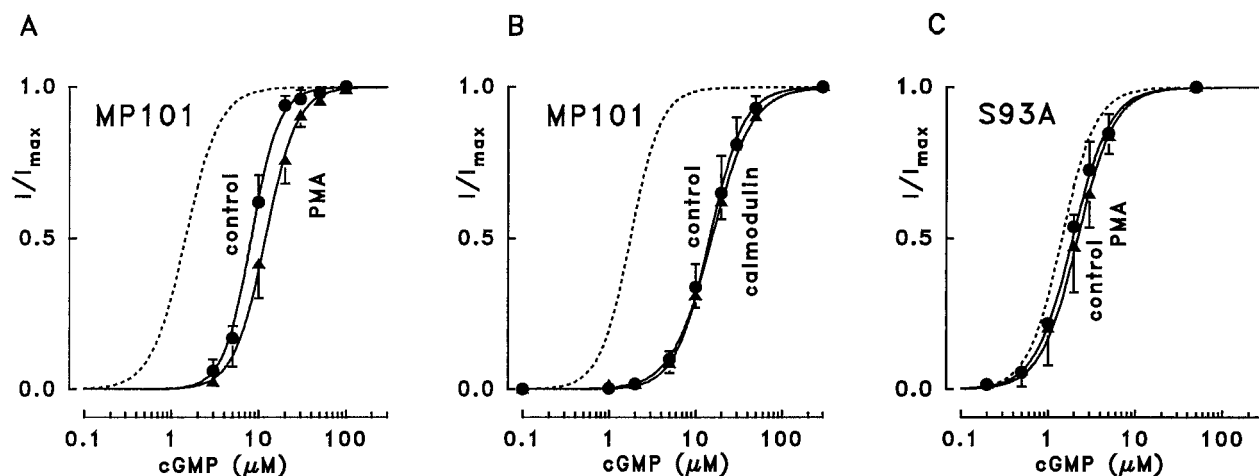


Figure 5. Localization of the amino acid residue that mediates the PMA-induced effect on ligand sensitivity. *A*, Loss of PMA effect in the mutant channel MP101 (truncated N-terminally until Gly¹⁰⁰). Dose-response relations were obtained at $V_m = +40$ mV in Ca^{2+} -free internal solution from cells expressing mutant channels without (circles) and with (triangles) PMA treatment. The phorbol ester treatment did not increase but slightly decreased the cGMP sensitivity of the channel. For comparison, a dose-response relation of the untreated wild-type channel is shown (dotted line). *B*, Loss of calmodulin sensitivity in MP101. Dose-response relations in the presence of internal solution containing 0.3 mM Ca^{2+} before (circles) and after (triangles) exposure of patches to 0.5 μ M calmodulin for 3 min. The same protocol causes a sevenfold increase of $K_{1/2}$ in wild-type channels (see Fig. 6). The dotted line shows the dose-response relation of the wild-type channel in internal solution containing 0.3 mM Ca^{2+} without calmodulin. *C*, Replacement of Ser⁹³ abolishes the effect of PMA. Dose-response relations of S93A mutant channels without (circles) and with (triangles) PMA treatment show that the mutant does not respond to activation of PKC, whereas its cGMP sensitivity under control conditions is only slightly reduced with respect to the wild type (dotted line).

hand, deleting only the flanking region renders the channel insensitive to calmodulin (Liu et al., 1994), although calmodulin presumably still binds. It thus appears that both parts of this regulatory domain are involved in channel opening, and that both can contribute to the determination of ligand sensitivity.

Table 1. cGMP sensitivity of wild-type (WT) and mutant bovine olfactory CNG channel α -subunits

	Ca^{2+} -free		0.3 mM Ca^{2+}	
	Control	+PMA	Control	+Calmodulin
WT				
$K_{1/2}$	1.45 \pm 0.36	0.30 \pm 0.10	1.76 \pm 0.26	12.0 \pm 2.8
n	2.23 \pm 0.33	2.03 \pm 0.24	2.47 \pm 0.38	2.80 \pm 0.90
Exp	7	18	7	14
MP101				
$K_{1/2}$	8.20 \pm 1.07	11.9 \pm 2.2	14.6 \pm 3.10	15.5 \pm 3.60
n	2.76 \pm 0.29	2.42 \pm 0.32	2.20 \pm 0.32	1.88 \pm 0.13
Exp	7	5	5	5
MD30				
$K_{1/2}$	1.48 \pm 0.20	0.35 \pm 0.03	1.62 \pm 0.24	21.8 \pm 4.80
n	2.15 \pm 0.43	1.85 \pm 0.15	2.01 \pm 0.12	2.20 \pm 0.17
Exp	6	6	5	7
S93A				
$K_{1/2}$	1.93 \pm 0.15	2.20 \pm 0.56	2.19 \pm 0.48	17.3 \pm 6.10
n	2.03 \pm 0.35	2.06 \pm 0.27	2.34 \pm 0.19	1.80 \pm 0.47
Exp	5	6	7	5
S93E				
$K_{1/2}$	2.11 \pm 0.48	2.27 \pm 0.57		
n	1.97 \pm 0.24	1.73 \pm 0.12		
Exp	7	4		

Concentration for half-maximal activation ($K_{1/2}$), Hill coefficient (n), and numbers of experiments (Exp) for channel activation by cGMP at $V_m = +40$ mV. Obtained from inside-out recordings in either Ca^{2+} -free internal solution (PMA experiments) or with 0.3 mM internal Ca^{2+} (calmodulin experiments).

These findings give further evidence for a critical allosteric role of the N-terminal region in the gating of CNG channels, which was demonstrated previously in mutagenesis studies (Goulding et al., 1994; Tibbs et al., 1997). It appears that the N-terminus determines the ease with which the binding of cyclic nucleotides to the C-terminus is converted into channel opening. Most recently, Varnum and Zagotta (1997) showed that the N- and C-termini of olfactory CNG channels interact. The calmodulin-binding site and part of the flanking region in the N-terminus bind to an identified domain in the C-terminus. This interdomain interaction facilitates channel opening, and it may be that phosphorylation of Ser⁹³ increases gating efficacy of the channel by stabilizing this interdomain complex.

Does phosphorylation modulate the native olfactory CNG channel?

Phosphorylation of Ser⁹³ profoundly affects the ligand sensitivity of the α -homomeric channel. However, the native olfactory CNG channel most probably consists of at least two different subunits, and we do not know whether modulation by PKC is conserved in the heteromeric protein. Direct evidence can only be obtained from intact OSNs or, alternatively, from heterologously expressed CNG channels with correct subunit composition.

To measure ligand sensitivity of native channels, OSNs must be isolated from the olfactory epithelium, and patch-clamp recordings can then be obtained from the membrane of dendritic knobs (Frings et al., 1992; Balasubramanian et al., 1995, 1996). Because of the small size of sensory cilia (diameter, 0.2 μ m), ciliary CNG channels are not accessible for such recordings. We measured the cAMP sensitivity of native rat channels in membrane patches from dendritic knobs. We obtained a $K_{1/2}$ of 4.13 \pm 1.9 μ M; $n = 1.65 \pm 0.1$ (four patches) for control channels and a $K_{1/2}$ of 3.50 \pm 0.9 μ M; $n = 1.50 \pm 0.2$ (eight patches) for channels measured after OSNs had been treated with 1 μ M PMA and 0.6 μ M okadaic acid (to inhibit phosphatase activity) before, during, and after the

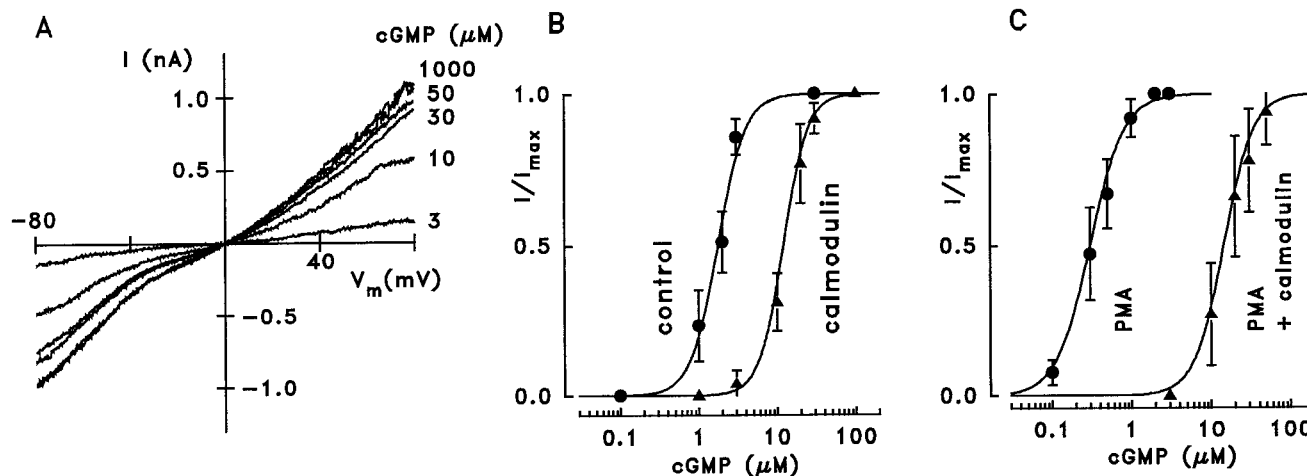


Figure 6. The calmodulin effect on ligand sensitivity is not counteracted by PKC-mediated phosphorylation. *A*, I - V_m relations recorded from an inside-out patch of a cell expressing wild-type channels after exposure to $0.5 \mu\text{M}$ calmodulin for 3 min. The patch was held in internal solution containing 0.3 mM Ca^{2+} , and the indicated concentrations of cGMP were applied. Before calmodulin application, and also after removal of calmodulin in Ca^{2+} -free internal solution, the patch showed almost maximal channel activation by $3 \mu\text{M}$ cGMP (data not shown). The nonlinear shape of the I - V_m relations results from voltage-dependent channel blockage by internal Ca^{2+} . *B*, Dose-response relations for channel activation obtained from untreated wild-type channels before (circles) and after (triangles) exposure to calmodulin. Both relations were derived at $V_m = +40 \text{ mV}$ with internal solution containing 0.3 mM Ca^{2+} . *C*, Calmodulin-induced reduction of cGMP sensitivity in wild-type channels after PMA treatment. Inside-out patches from PMA-treated cells were exposed to internal solution containing 0.3 mM Ca^{2+} and various concentrations of cGMP before (circles) and after (triangles) incubation with $0.5 \mu\text{M}$ calmodulin. The dose-response relations show a 40-fold increase of $K_{1/2}$ for channel activation caused by the exposure of the phosphorylated channels to calmodulin.

dissociation procedure (data not shown). Thus, PMA treatment does not significantly increase ligand sensitivity in CNG channels from dendritic knobs of isolated OSNs. However, several aspects have to be considered for the interpretation of this observation: (1) OSNs may be damaged during dissociation, may lose the ability to phosphorylate the channel, or may allow dephosphorylation during cell isolation; (2) channels in dendritic knobs analyzed here are colocalized only with PKC δ , which may not recognize Ser⁹³ as a substrate, whereas Ser⁹³ of channels expressed in the ciliary membrane could be phosphorylated by the γ and λ isoforms; (3) phosphorylation of native channels may be specifically mediated by PKC λ , which is not activated by phorbol esters; (4) channels may be constitutively phosphorylated so that activation of PKC does not lead to a change of ligand sensitivity; and (5) additional channel subunits may obstruct phosphorylation of the α -subunit or may prevent the change of ligand sensitivity in the phosphorylated channel. Because these problems cannot be investigated in isolated OSNs, the most promising approach is the heterologous coexpression of different channel subunits and phosphorylation experiments with channels of the correct subunit composition. This will probably be feasible in the near future, because the subunits that contribute to the native olfactory channel of the rat have been identified recently (F. Sesti, W. Bönigk, J. Bradley, F. Müller, S. Frings, and U. B. Kaupp, unpublished data).

Indirect evidence supporting a role of phosphorylation in the modulation of ligand sensitivity comes from studies of rod photoreceptor CNG channels. Gordon et al. (1992) have demonstrated that the cGMP sensitivity of CNG channels in membrane patches excised from the outer segment of isolated photoreceptors can be changed by application of protein phosphatases. This observation suggests that CNG channels in these cells are phosphorylated, and that the phosphorylation state determines ligand sensitivity. Thus, native photoreceptor CNG channels seem to be modulated by phosphorylation much like other ligand-gated chan-

nels. Modulation by protein phosphorylation is well documented for receptors of acetylcholine, GABA, glycine, and glutamate (for review, see Swope et al., 1992). To understand the role of phosphorylation in the regulation of olfactory channels, it will be crucial to identify physiological activators of PKC in OSNs. Promising candidates may be agents that activate phospholipase C, such as neurotransmitters (Frings, 1993) and certain types of odorants (Boekhoff et al., 1990), or activators of PKC λ that use other pathways of lipid metabolism.

A possible physiological role for phosphorylation of olfactory CNG channels

In an earlier report (Frings, 1993), we have shown that frog OSNs *in situ* respond more strongly to odorant stimulation when the olfactory epithelium is incubated with phorbol ester, an effect that is probably mediated by PKC. Under conditions of enhanced PKC activity, the stimulus-induced synthesis of cAMP is potentiated so that more CNG channels can be activated, resulting in an increased receptor current. If such a neurotransmitter-controlled PKC activity is also functional in mammalian OSNs, it affords the neurons with a way of optimizing the transduction efficiency. At increased PKC activity, both cAMP synthesis by the olfactory adenylyl cyclase and the cAMP sensitivity of CNG channels would be high, and weak odorant stimuli are sufficient to elicit electrical excitation. In this speculative concept, phosphorylation of the CNG channel α -subunit contributes to an increase of olfactory sensitivity. Moreover, the Ca^{2+} -dependent adaptation of OSNs may also be affected by channel phosphorylation, because $K_{1/2}$ in phosphorylated channels shifts 40-fold on binding of Ca^{2+} /calmodulin, instead of sevenfold in control channels. Interestingly, native olfactory CNG channels respond to calmodulin with a 20-fold (Chen and Yau, 1994) to 60-fold (Balasubramanian et al., 1996) increase of $K_{1/2}$. Whether this extended range of modulation reflects a modification of the calmodulin effect by other subunits or is attributable to constitutive phosphorylation of

the α -subunit needs to be clarified in further experiments. Taken together, our results suggest that phosphorylation of the α -subunit may influence both excitation and adaptation of OSNs.

In addition to the olfactory epithelium, the olfactory CNG channel α -subunit is also expressed in other tissues, including aorta (Biel et al., 1993) and various parts of the brain (El-Husseini et al., 1995; Kingston et al., 1996; Bradley et al., 1997). Because this channel readily conducts Ca^{2+} , it is expected to function as a Ca^{2+} entry pathway that couples the cyclic nucleotide metabolism to cytosolic Ca^{2+} homeostasis in neuronal somata and synaptic terminals. Activation of CNG channels is currently discussed as a factor contributing to synaptic plasticity, neuronal growth, and axon guidance (for review, see Zufall et al., 1997). Considering the substantial increase of ligand sensitivity described here, it is tempting to speculate that phosphorylation of the olfactory CNG channel α -subunit in hippocampus, cerebellum, and olfactory bulb contributes to the regulation of neuronal growth and plasticity.

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