Mutating Three Residues in the Bovine Rod Cyclic Nucleotide-Activated Channel Can Switch a Nucleotide from Inactive to Active

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ABSTRACT Cyclic nucleotide-gated (CNG) channels, which were initially studied in retina and olfactory neurons, are activated by cytoplasmic cGMP or cAMP. Detailed comparisons of nucleotide-activated currents using nucleotide analogs and mutagenesis revealed channel-specific residues in the nucleotide-binding domain that regulate the binding and channel-activation properties. Of particular interest are N^1-oxide cAMP, which does not activate bovine rod channels, and Rp-cGMPS, which activates bovine rod, but not catfish, olfactory channels. Previously, we showed that four residues coordinate the purine interactions in the binding domain and that three of these residues vary in the α subunits of the bovine rod, catfish, and rat olfactory channels. Here we show that both N^1-oxide cAMP and Rp-cGMPS activate rat olfactory channels. A mutant of the bovine rod α subunit, substituted with residues from the rat olfactory channel at the three variable positions, was weakly activated by N^1-oxide cAMP, and a catfish olfactory-like bovine rod mutant lost activation by Rp-cGMPS. These experiments underscore the functional importance of purine contacts with three residues in the cyclic nucleotide-binding domain. Molecular models of nucleotide analogs in the binding domains, constructed with AMMP, showed differences in the purine contacts among the channels that might account for activation differences.

INTRODUCTION

CNG channels are expressed in a diverse set of neurons in the retina, including rod (Fesenko et al., 1985; Kaupp et al., 1989) and cone photoreceptors (Bonigk et al., 1993; Yu et al., 1996), ganglion cells (Ahmad et al., 1994; Kawai and Sterling, 1999), and possibly bipolar cells (de la Villa et al., 1995; Nawy and Jahr, 1991; however, see Nawy, 1999). Olfactory sensory neuroepithelia (Dhallan et al., 1990; Ludwig et al., 1990; Nakamura and Gold, 1987), pineal (Bonigk et al., 1996; Dryer and Henderson, 1991; Sautter et al., 1997), and hippocampal neurons in the brain (Kingston et al., 1996) also express CNG channels (Parent et al., 1998). Furthermore, CNG channels are expressed in non-neuronal tissues including testis (Weyand et al., 1994), kidney (Ahmad et al., 1992; Distler et al., 1994; Karlson et al., 1995; McCoy et al., 1995), and muscle (Santi and Guidotti, 1996). In photoreceptor and olfactory neurons, CNG channels respond to changes in cytosolic cGMP or cAMP by transducing sensory information into localized cell membrane potential changes (Yau and Chen, 1995; Zufall et al., 1994). In other neuronal cells, the signaling pathways are not understood; however, CNG channels are believed to play a role in synaptic feedback and plasticity as well as development (Arancio et al., 1995, 1996; Savchenko et al., 1997; Wei et al., 1998; Zufall et al., 1997). CNG channels conduct both Na^+ and Ca^{2+} under physiological conditions. The nucleotide gating of CNG channels suggests that this channel family provides an important voltage-independent pathway for Ca^{2+} entry in cells expressing the channels.

CNG channels are tetramers with two major subunit types, denoted as α and β or 1 and 2, expressed in both rod and olfactory tissues (see reviews) (Kaupp, 1991; Zagotta and Siegelbaum, 1996; Zufall et al., 1994). The expression of two subunit types suggests that in situ channels are heteromeric. Heterologous expression of the cDNA encoding the α subunit of either the rod or olfactory CNG channel produces nucleotide-activated channels. The β subunits only express nucleotide-activated channels when co-expressed with an α subunit. The major functional differences between rod and olfactory CNG channels are seen with cAMP activation. In both native rods and heterologously expressed rod channels, cGMP activates currents at 15–20-fold lower concentrations than cAMP, and saturating concentrations of cAMP activate a small fraction of the maximal cGMP-activated current. In contrast, in olfactory channels, both cGMP and cAMP activate maximal currents. The concentration of nucleotides needed to activate olfactory channels differs among different species and depends on the exact subunit composition. We would like to better understand the structural basis of the nucleotide activation properties in rod and olfactory channels.

Presumably, differences in the nucleotide discrimination properties of rod and olfactory channels depend on residue differences in the nucleotide binding domain. Previous stud-

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Abbreviations used: CNG, cyclic nucleotide-gated; AMMP, *A*nother *M*olecular *M*odeling *P*rogram; CRP, catabolite repressor protein; PDE, cGMP phosphodiesterase; PET-cGMP, β-phenyl-1,N^2-etheno cGMP; PKA, cAMP-activated protein kinase; PKG, cGMP-activated protein kinase; Rp-cGMPS, guanosine-3′,5′-cyclic monophosphorothioate (Rp-isomer); Rp-8-CPT-cGMPS, 8-(4-chlorophenylthio) guanosine-3′,5′-cyclic monophosphorothioate (Rp-isomer); Sp-cGMPS, guanosine-3′,5′-cyclic monophosphorothioate (Sp-isomer).

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ies have revealed that, in addition to the highly conserved C-terminal nucleotide binding domain, a number of other regions of the CNG channel protein regulate the nucleotide-activated currents. These regions affect the channel opening probability and the channel conductance properties (Gordon and Zagotta, 1995; Goulding et al., 1994; Park and MacKinnon, 1995; Zong et al., 1998). For structure-function studies, homomeric channels offer the advantage of a defined subunit composition. Because cyclic nucleotides are small and relatively rigid molecules, they are good probes of the binding domain. Additionally, few contacts are expected to differ between rod and olfactory binding domains because the physiologically relevant nucleotides cGMP and cAMP differ only at the C^2 and C^6 positions of the purine.

Previous studies using nucleotide analogs have shown that the binding domain is remarkably tolerant of changes in the purine ring substituents. Analogs with a thio substituent as 6-thio-cGMP, a monobutyryl as N^6-monobutyryl cAMP, or rings as 1-N^6-etheno cAMP (Scott and Tanaka, 1995; Tanaka et al., 1989) and PET-cGMP (Wei et al., 1996), all activate currents in rod channels. Due to this tolerance for changes in both size and charge at various positions on the purine, the most interesting analogs are those that fail to activate CNG channels. Presumably, the purine alterations result in a loss of contacts with residues in the binding domain that are essential for either binding or channel opening. Notably, the only inactive purine-modified analogs identified to date are modified at the C^6 or N^1 positions. Two of these analogs have single atom changes: 2-aminopurine riboside 3′-5′-monophosphate (2-amino-cPNP), which has a hydrogen in place of the oxygen of cGMP at C^6 (Tanaka et al., 1989), and N^1-oxide cAMP with an oxygen instead of hydrogen at the N^1 position (Scott and Tanaka, 1995). Neither analog binds to rod channels, as shown by cGMP competition studies.

Homology modeling was previously used to investigate the interactions of cyclic nucleotides with the CNG channel-binding domains. The models were based on the conservation of the channel binding domain structure with the cAMP binding domain of *Escherichia coli*, CRP (Kumar and Weber, 1992; Scott et al., 1996). The models predicted that residues at four positions in the binding domain could contact the C^2 and C^6 purine substituents (Scott and Tanaka, 1995; Scott et al., 1996). One residue, T560 in the bovine rod CNG channel α subunit, is conserved in all CNG channels (Altenhofen et al., 1991). The residues in the other three positions vary between the bovine rod and rat and fish olfactory channels. Mutating the three residues in the bovine rod channel confirmed their role in purine coordination and nucleotide activation (Scott and Tanaka, 1998; Varnum et al., 1995). The binding domain is less tolerant of changes in the ribofuranose moiety and, to date, only alterations of the phosphate group have been reported to activate currents (Tanaka et al., 1989; Zimmerman et al., 1985). This intolerance is consistent with the molecular models that show a tight packing of the ribofuranose with eight contacts between the binding domain and the ribofuranose (Kumar and Weber, 1992; Scott et al., 1996). Furthermore, the residues contacting the ribofuranose are conserved between CRP and all members of the CNG channel family. It is therefore interesting to note that the sulfur-substituted analog, Rp-cGMPS with sulfur in the equatorial position on the phosphate, activates rod photoreceptor channels (Zimmerman et al., 1985), but not catfish olfactory channels, where it competitively antagonizes cGMP (Kramer and Tibbs, 1996). However, Sp-cGMPS, with a sulfur in the axial position, activates both rod and olfactory channels (Kramer and Tibbs, 1996; Zimmerman et al., 1985).

To better understand differences between the ligand interactions with the rod and olfactory channels, we tested Rp-cGMPS and N^1-oxide cAMP on α homomeric rat olfactory channels and found that both analogs are agonists. We then asked whether differences in the residues of the binding domains could account for the selective activation of these analogs. Although the rat and catfish olfactory channels have highly conserved primary sequences, they differ at two of the three positions that coordinate the purine binding. To directly investigate the role of these residues, we constructed bovine rod channel α subunit mutants, substituting either rat or catfish olfactory channel residues at these positions. In the rat olfactory-like mutant channel, N^1-oxide cAMP was able to bind to the channel and activate a small current. Similarly, the catfish olfactory-substituted bovine rod mutant channel behaved like the catfish olfactory channel in that it was no longer activated by Rp-cGMPS. A preliminary report of this work was published in abstract form (Scott et al., 1999).

MATERIALS AND METHODS

Mutants of the bovine retina CNG channel α subunit cDNA were generated and sequenced as described previously (Scott and Tanaka, 1998). The cDNA was a gift from Dr. W. Zagotta and the cDNA encoding the rat olfactory α subunit was a gift from Dr. K-W. Yau. The CNG α subunit cDNAs were transiently expressed in cultured human tSA201 cells. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. Cells were passaged at ~90% confluency. Before transfection, ~2.5×10^5 cells were plated onto five glass coverslips (10 mm diameter) in six well dishes in 2.5 ml 10% fetal calf serum DMEM. Cells were incubated for 12–20 h before transfection. DNA-liposome complexes were formed using ~1.2 μg CNG channel cDNA in pCIS (Genentech, San Francisco, CA), ~0.4 μg green fluorescent protein cDNA marker in pRK7, and 0.4 μg p-AdVAntage cDNA (Promega, Madison, WI) with 100 μl 0% fetal calf serum growth media. The DNA was mixed with 12 μl Lipofectamine reagent (GIBCO BRL, Gaithersburg, MD) in a total of 200 μl 0% fetal calf serum growth media and incubated at room temperature for 30 min. This mixture was added to 0.8 ml 0% fetal calf serum growth media. The cell media in each well containing cells was replaced with the transfection media and the cells were returned to the incubator. After 5 h, 1.0 ml 20% fetal calf serum media was added to the wells to give a final concentration of 10% fetal calf serum. About 24 h after the start of transfection the media were replaced with 2.0 ml 10% fetal calf

serum growth media and patching was undertaken ~72 h after the start of the transfection.

Inside-out patches were excised from positive cells using fluorescence illumination as described elsewhere (Scott and Tanaka, 1998). Cyclic nucleotides including cGMP, cAMP, and N^1-oxide cAMP (Sigma Chemical Co., St. Louis, MO), Rp-cGMPS, and Rp-8-CPT-cGMPS (BioLog, La Jolla, CA) were applied to the bath. All solutions contained 120 mM NaCl, 5 mM HEPES solution, 2 mM EDTA, and 2 mM EGTA, at pH 7.2. Macroscopic currents were recorded with a patch clamp amplifier output (Dagan 8900), which was low-pass filtered at 1 kHz before digitization by an IBM 486 (5 kHz, 12-bit A/D). The patch pipette was positioned in the chamber inflow stream to measure cyclic nucleotide-activated currents. Solutions were superfused continuously over the cytoplasmic surfaces of excised patches to measure maximal nucleotide activated currents. Net activated currents were determined after subtraction of the bath current, measured in the absence of nucleotides.

The maximal response of each patch was determined from the maximal current activated with a saturating concentration of cGMP after subtraction of the bath current. Dose-response relations were determined by plotting the fraction of current activated as a function of the nucleotide concentration at +60 mV. The normalized currents were fitted as a function of the test concentration using the Hill equation:

$$\text{Fraction of current} = I_{nor}/[1 + (K_{0.5}/L)^n]$$

with a nonlinear Levenberg-Marquardt fitting routine in TableCurve (Jandel Scientific, Corte Madera, CA), where I_{nor} is the normalized maximal response, L is the ligand concentration, $K_{0.5}$ is the concentration at 50% of the I_{nor}, and n is the cooperativity index, or Hill coefficient.

Molecular modeling

Modeling of the phosphorothioate analogs of cGMP in the CNG channel binding domain was initiated using the coordinates of the previous models of bovine rod, catfish olfactory, and rat olfactory nucleotide binding domains (Scott et al., 1996). *Syn* and *anti* conformations of Rp-cGMPS and Sp-cGMPS were constructed from the *syn* and *anti* cGMP models by replacing the exocyclic oxygens with sulfur on the bound cGMP. The chemical structures of the nucleotides are shown in Fig. 1.

The external energy exerted on the ligand was used to determine the preferred ligand conformation; the total potential energy was computed using the program AMMP, available from Dr. R. Harrison (http://asterix.jci.tju.edu) (Harrison, 1993; Weber and Harrison, 1999). The models were minimized with the atoms sp4 parameter set, a modified UFF parameter set, in AMMP. Conjugate gradient minimization was performed, and model coordinates were written to a file every 20 iterations. The minimization was considered complete when the following criteria were met: 1) the total energy difference between two consecutive models was <1.5%, and 2) the test model had an L_{maxf} (absolute maximum force of any atom) <10% of its total potential energy. These criteria were usually met within 2500 iterative steps. The interaction energy between the ligand and binding domain was then computed using AMMP.

Unlike previous modeling, the amino acid side chains were not adjusted after the initial minimization except to position the water molecule between the T560 (bovine rod numbering) and the purine ring. The only constraint was placed on the water, not allowing it to move from its starting position. All other atoms, including the backbone, were allowed to move. The RMS deviation between cGMP and the Rp or Sp derivatives in the binding

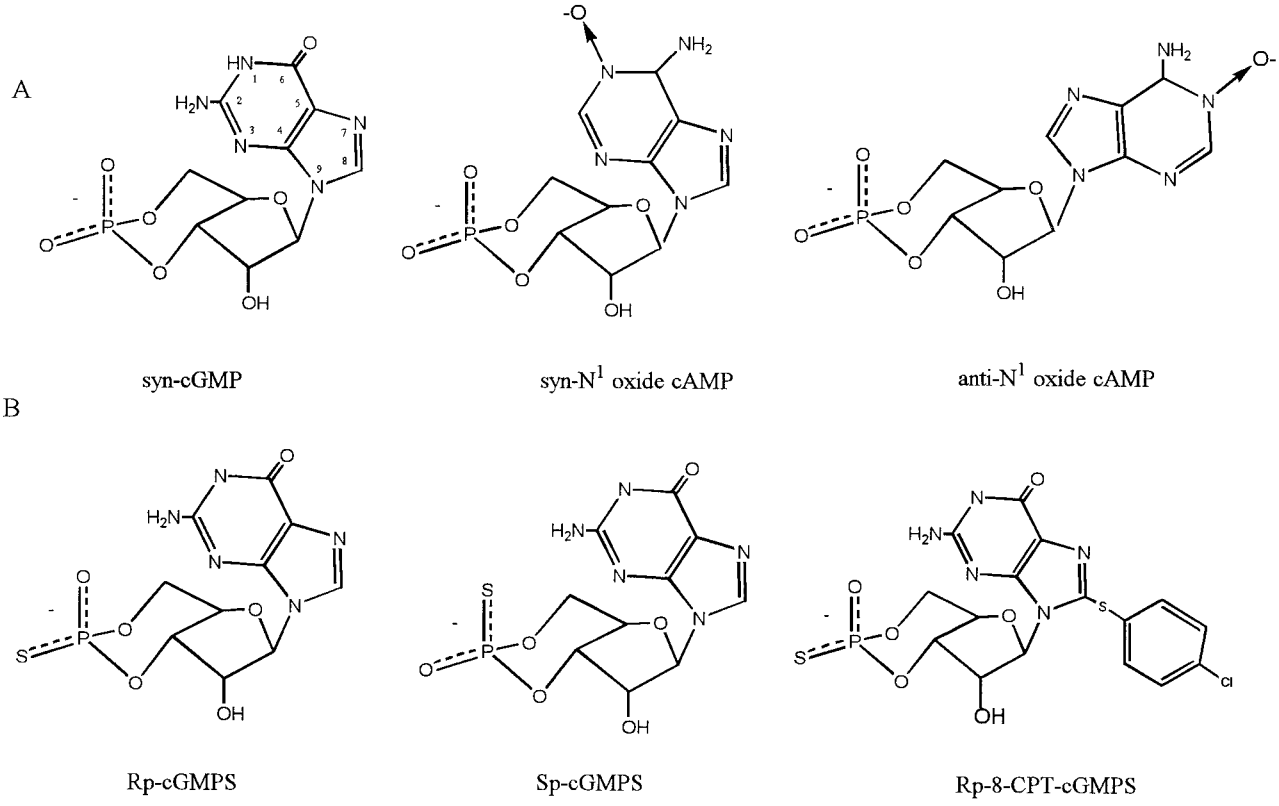

FIGURE 1 (*A*) The chemical structure of *syn* cGMP and *syn* and *anti* conformations of N^1-oxide cAMP. *Syn* cGMP shows the purine numbering. (*B*) The chemical structures of several phosphorothioate cGMP analogs: Rp-cGMPS, Sp-cGMPS, and Rp-8-CPT-cGMPS. Rp-cGMPS and Sp-cGMPS have alterations only in the exocyclic oxygen of the ribofuranose. Rp- and Sp-8-CPT-cGMP are modified at the C8 position on the purine. N^1-oxide cAMP has oxygen at the N^1 position of cAMP. These structures were drawn with CS ChemDraw Pro.

domain was compared to determine the effect of replacing the oxygen with sulfur using Insight II (MSI). The positions of selected atoms in the binding domain and the ligand were also compared. These vector measurements account for changes in the *x*, *y*, and *z* axis coordinates.

RESULTS

N^1-oxide cAMP activates rat olfactory CNG channels and binds to a rat olfactory-like bovine rod mutant

Inside-out patches were excised from fluorescent cells transfected with cDNAs encoding the α subunit of the rat olfactory CNG channel and green fluorescent protein as detailed in the Methods. A saturating concentration of cGMP was applied to the bath (cytoplasmic face) of the patch to activate maximal currents. For each patch, the maximal current is defined as the net current activated by a saturating concentration of cGMP. Dose-response curves for cGMP and cAMP are shown in Fig. 2 *A*. The $K_{0.5}$ and n_H values were determined by fitting the Hill equation and the values are given in the figure legends. The averaged $K_{0.5}$ values were 0.54 μM for cGMP and 54 μM for cAMP. In contrast to the rod homomeric channels where cAMP activates ~1% of the maximal current, cAMP activates ~100% of the maximal current in homomeric rat olfactory channels. These values are in the range of previous measurements on rat olfactory CNG channels, with $K_{0.5}$ values ranging from 0.5 to 1 μM cGMP and ~50 μM for cAMP (Frings et al., 1992; Zong et al., 1998).

N^1-oxide cAMP, shown in Fig. 1, does not activate rod CNG channels at 5 mM concentrations, nor does the addition of 1 mM N^1-oxide cAMP competitively antagonize cGMP activation (Scott and Tanaka, 1995). Clearly, then, N^1-oxide cAMP does not bind to native rod channels. Because the coordination of the purine depends on interactions with three residues in the binding domain that vary between the bovine rod and rat olfactory channels, we tested the ability of N^1-oxide cAMP to activate current in homomeric rat olfactory channel patches. As shown in Fig. 2 *A*, N^1-oxide cAMP activates ~80% of the maximal current in the rat olfactory CNG channel with an average $K_{0.5}$ of 656 μM. However, the >10-fold increase in the $K_{0.5}$, compared to that of the parent cAMP nucleotide, suggests that the addition of oxygen at N^1 on the purine leads to an unfavorable interaction with the purine in the rat olfactory binding site.

We further explored the role of these three residues using site-directed mutagenesis. Residues of the bovine rod channel were replaced with the residue occupying the equivalent position in the rat olfactory channel. Two mutants were examined: a double mutant, F533Y/K596R, and a triple mutant, F533Y/K596R/D604E, where the residue and position in the bovine rod α subunit are followed by the replacement residue of the rat olfactory α subunit (Scott and Tanaka, 1998). This approach should preserve the overall fold of the bovine rod channel and allows us to examine the influence of just the altered residues. Table 1 lists the residues in equivalent positions in various CNG channels. A comparison of the currents from these mutants is shown in Fig. 3. In this experiment, 2.5 mM N^1-oxide cAMP was tested for its ability to compete with cGMP. If N^1-oxide cAMP binds to the channel, the current activated by subsaturating concentrations of cGMP will be reduced. Our results show that N^1-oxide cAMP has no effect on currents activated by 20 μM cGMP in the F533Y/K596R mutant, but it inhibits ~35% of the 20 μM cGMP-activated current in the F533Y/K596R/D604E mutant. Similar results were seen in two other patches with current suppressions of 36%

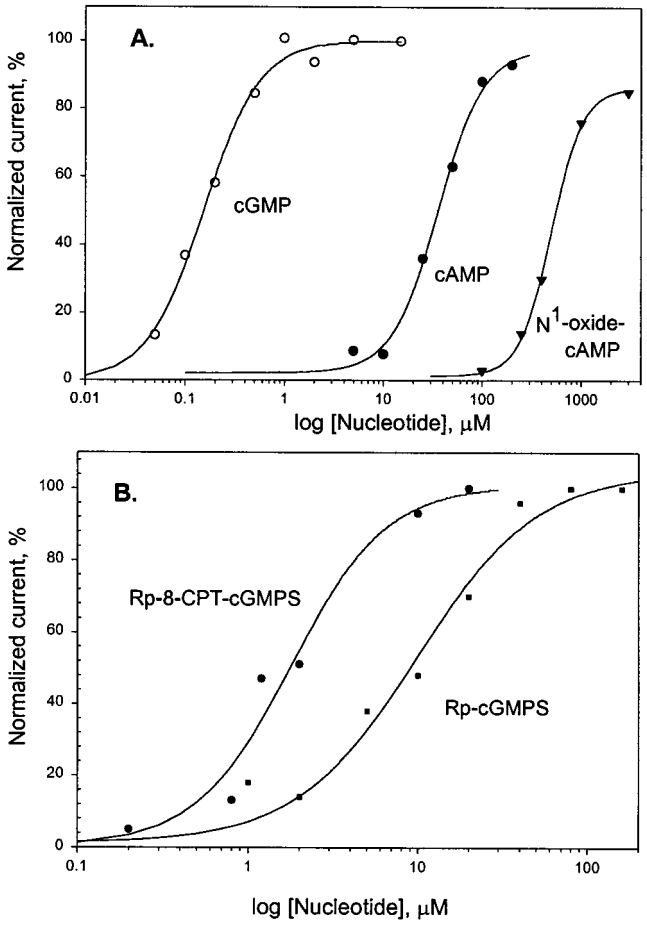

FIGURE 2 (*A*) Activation of the rat olfactory CNG channel by cGMP, cAMP, and N^1-oxide cAMP. Normalized currents for cGMP (○), cAMP (●), and N^1-oxide cAMP (▼) are plotted at +60 mV with the Hill fits to the data indicated by the solid line. The $K_{0.5}$ for cGMP was 0.14 μM with n_H = 1.5, the $K_{0.5}$ for cAMP was 35.6 μM with n_H = 1.9, and the values for N^1-oxide cAMP were 619 μM and 3.9, respectively. Averaged values for the $K_{0.5}$ and n_H were 0.54 μM and 1.14 (n = 4) for cGMP, 54 μM and 2.0 (n = 3) for cAMP, and 656 μM and 2.5 (n = 3) for N^1-oxide cAMP. (*B*) Activation of the rat olfactory CNG channel by Rp-cGMPS isomers. Normalized currents were plotted as a function of nucleotide concentration. The solid lines indicate the Hill fits. Rp-cGMPS (●) had a $K_{0.5}$ of 10.6 μM with an n_H of 1.2, and Rp-8-CPT-cGMPS (▼) had a $K_{0.5}$ of 1.8 μM with an n_H of 1.6.

TABLE 1 Predictions of agonist or antagonist property of Rp-cGMPS based on the residues in three positions

CNG channel	β5 strand	Cα helix	Cα helix	Rp-cGMPS	Reference
Bovine rod	F533	K596	D604	Agonist	Kaupp et al., 1989
Chick rod	F487	K550	D558	Agonist	Bonigk et al., 1993
Dog rod	F534	K597	D605	Agonist	Veske et al., 1997
Human rod	F531	K594	D602	Agonist	Dhallan et al., 1992
Mouse rod	F526	K589	D597	Agonist	Pittler et al., 1992
Rat rod	F526	K589	D597	Agonist	Ding et al., 1997
Bovine cone	F557	K620	D628	Agonist	Weyand et al., 1994
Chick cone	F580	K643	D651	Agonist	Bonigk et al., 1993
Human cone	F538	K602	D610	Agonist	Yu et al., 1996
Bovine olfactory	Y510	R573	E581	Agonist	Ludwig et al., 1990
Catfish olfactory	F503	R566	Q574	Antagonist	Goulding et al., 1992
Rat olfactory	Y512	R575	E583	Agonist	Dhallan et al., 1990
Bovine testis	F557	K620	D528	Agonist	Weyand et al., 1994
Chick pineal	F580	K643	D651	Agonist	Bonigk et al., 1996
Mouse kidney	F527	K590	D598	Agonist	Karlson et al., 1995
Rabbit aorta	Y578	R641	E649	Agonist	Biel et al., 1993
Drosophila	L485	R548	D556	Agonist	Baumann et al., 1994
Tax4 *C. elegans*	F549	K612	D620	Agonist	Komatsu et al., 1996

and 37% at +80 mV. These results illustrate the importance of the side chain of residue 604 in current activation. We conclude, then, that the presence of Glu at position D604 on the putative C helix permits N^1-oxide cAMP to bind to the rat olfactory channel.

We then asked whether N^1-oxide cAMP could activate the F533Y/K596R/D604E mutant. Results from a typical patch activated with cGMP, cAMP, and N^1-oxide cAMP are shown in Fig. 4. In the presence of 5 mM N^1-oxide cAMP we observe channel openings that are not present in the bath trace. These channels appear to have longer openings than those activated with cAMP, although we did not characterize the single channel properties. Similar results were seen in two other patches. In contrast, no channel activity was seen in three patches excised from the F533Y/K596R mutants.

In previous experiments with the rat olfactory-like F533Y/K596R/D604E mutant, saturating concentrations of cAMP activated ~8% of the maximal cGMP-activated current (Scott and Tanaka, 1998). This fraction of current represents an eightfold increase over the 1% fraction of maximal current activated by saturating concentrations of cAMP in the homomeric α bovine rod channel. Although the N-terminus (Gordon and Zagotta, 1995; Goulding et al., 1994) and C-linker regions (Zong et al., 1998) of the rat olfactory channel are important regulators of nucleotide gating in CNG channels, the activation of the rat olfactory-like mutant by N^1-oxide cAMP and the increase in the relative cAMP-activated currents seen with this mutant underscore the role of these three residues in current activation.

Rp-cGMPS activates rat olfactory CNG channels but not a catfish olfactory-like rod mutant

Rp-cGMPS and Sp-cGMPS are altered only at the phosphate position of the ribofuranose (Fig. 1). Rp-cGMPS is a competitive antagonist of native and homomeric catfish olfactory channels (Kramer and Tibbs, 1996) although it is a full agonist of both native and homomeric rod channels, with a $K_{0.5}$ value of ~1200 μM (Zimmerman et al., 1985). Sp-cGMPS, however, activates both rod and olfactory channels. We asked whether Rp-cGMPS activates the rat olfactory channel. Rp-cGMPS is a full agonist in this olfactory channel, in contrast to that of the catfish, as shown in Fig. 2 *B*. The average $K_{0.5}$ for Rp-cGMPS was 20.3 μM. We also tested Rp-8-CPT-cGMPS, a membrane-permeant analog of Rp-cGMPS. The addition of the C8 substituent increases the apparent affinity, as shown in Fig. 2 *B*, and the average $K_{0.5}$ value was 1.4 μM for this analog.

Activation of the rat, but not the catfish, olfactory channel by Rp-cGMPS is quite surprising because the degree of homology between these channels is very high. Even more perplexing is the realization that the contacts of the ribofuranose are conserved in all the CNG channels. Our previous modeling suggested that differences in the ribofuranose packing would alter the purine contacts in these channels. Because the rat and catfish olfactory channels differ at two of the three positions contacting the purine, we examined the effect of mutating the bovine rod α subunit with the catfish olfactory channel residues at these positions. A double-mutant K596R/D604Q was tested, but we

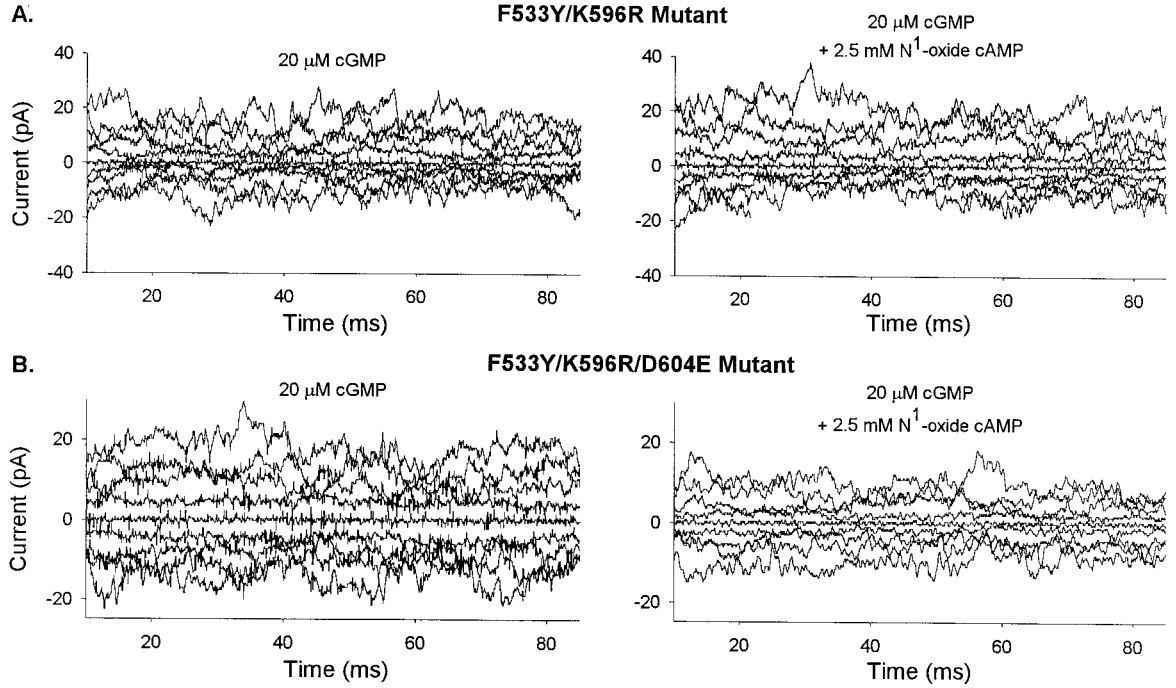

FIGURE 3 N^1-oxide cAMP binds to the triple-mutant F533Y/K596R/D604E (*B*), but not the double-mutant F533Y/K596R (*A*) of the bovine rod channel. Rat olfactory-like bovine rod mutant channels were heterologously expressed as described in the Methods. Currents were recorded at 20 mV intervals from −80 mV to 80 mV. Currents were sampled at 20 MHz and double-pole active-filtered at 1 KHz. Recorded currents with leak current subtracted are shown from 10 to 85 ms for each voltage. The left side of *A* shows the current in the F533Y/K596R mutant elicited by 20 μM cGMP, and the right side shows 20 μM cGMP plus 2.5 mM N^1-oxide cAMP. Currents in the absence of ligand were subtracted from all traces. No change is seen in the currents. At holding potentials of −80 and +80 mV, the raw (un-subtracted) currents averaged −22.2 ± 3.5 pA and 31.2 ± 4.2 pA for cGMP alone, and −22.5 ± 3.4 pA and 35.0 ± 4.5 pA with cGMP and N^1-oxide cAMP both present. *B* shows currents recorded from the F533Y/K596R/D604E mutant with the same solutions as in *A*. In this mutant, the net currents decreased in the presence of the N^1-oxide cAMP. Raw currents at −80 mV and +80 mV were −11.8 ± 3.2 and 23.6 ± 3.0 pA with cGMP alone, and −7.4 ± 2.2 and 15.1 ± 2.7 pA in the presence of cGMP and N^1-oxide cAMP.

were unable to record cGMP-activated currents in excised patches. We then examined a triple-mutant F533Y/K596R/D604Q, which replaced F533 with tyrosine in addition to the two catfish olfactory channel replacements. The rat olfactory channel has a Tyr, Y512, at the corresponding position. As shown in Fig. 5, this mutant expressed cGMP-activated currents and, consistent with previous reports on a singly substituted D604Q mutant (Varnum et al., 1995), cAMP activated large currents relative to the currents activated by cGMP. In three patches from this mutant, 1 mM cAMP activated the same or more current than was activated with 500 μM cGMP. When Rp-cGMPS concentrations as high as 5 mM were tested on these mutants, there was less than a 2% increase in the mean current and a small increase in the current noise. These results are identical to those reported by Kramer and Tibbs (1996) for catfish olfactory channels. We conclude, therefore, that substituting the bovine rod α subunit residues that contact the purine with those of the olfactory α subunit converts the response to Rp-cGMPS from a rod-type to a catfish olfactory-type channel. Furthermore, because the F533Y/K596R/D604Q mutant has a Tyr at position 533 and Arg in position 596, similar to the rat olfactory channel, the major determinant of the loss of Rp-cGMPS activation is the Gln in position 604.

Molecular modeling of several CNG binding domains provides insight into selective ligand activation

We constructed molecular models to address how N^1-oxide cAMP and Rp-cGMPS might selectively activate rat olfactory channels. The models were based on earlier models (Scott et al., 1996) constructed from the coordinates of the cAMP binding domain of the *E. coli* CRP (McKay et al., 1982; Weber and Steitz, 1987). The overall architecture of the binding domain is an eight-stranded β barrel with an N-terminal α helix and two C-terminal α helices (Fig. 6 *A*). The ribofuranose interacts with a number of conserved residues in the β barrel of CRP, and presumably these contacts are preserved in the channel-binding domains. It seems possible, then, that nonconserved residues in the surrounding region may change the overall packing in different proteins.

Activation of the channel is dependent on contacts between the purine and the binding pocket, particularly contacts located on the putative Cα helix and β5 strand. Based on the homology modeling, the communication between these two secondary structural elements is facilitated by the binding of the ligand (Scott and Tanaka, 1998). However,

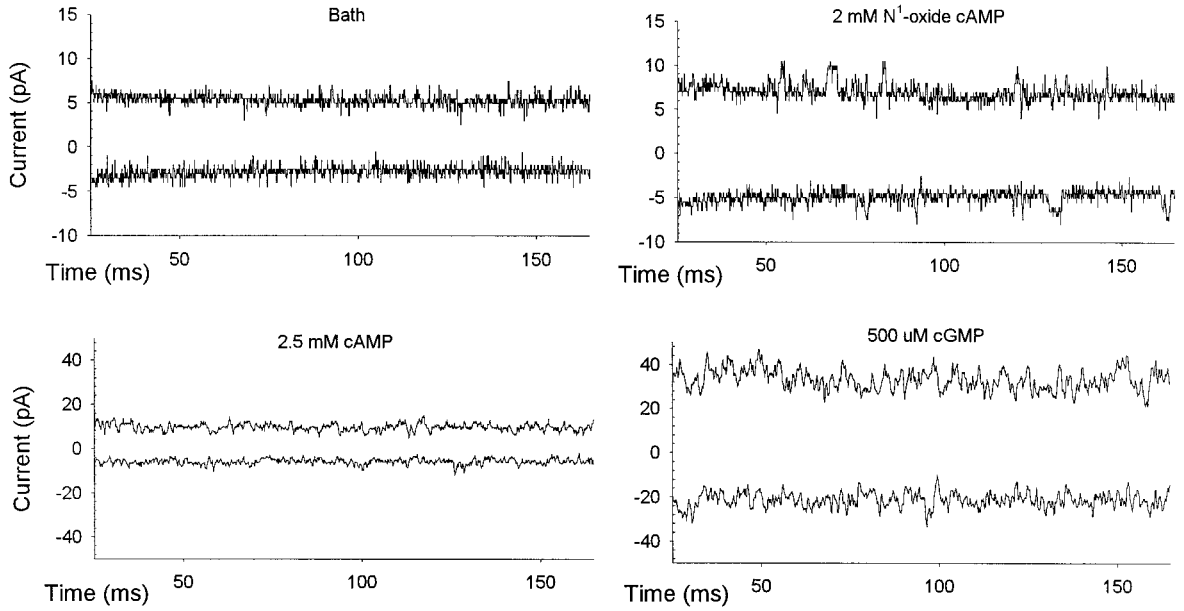

FIGURE 4 Activation by 2 mM N^1-oxide cAMP in the triple mutant F533Y/K596R/D604E. Currents are displayed for ~150 ms at a holding potential of −80 mV and +80 mV. No activated currents are seen in the bath traces, but in the presence of N^1-oxide cAMP, infrequent channel-like openings are seen. Currents activated with saturating concentrations of cAMP and cGMP are also shown for this patch. The mean current activated by 500 mM cGMP was 38 ± 0.53 pA at +80 mV. Mean current for 2.5 mM cAMP activation was 5.7 ± 1.6 pA, and for the bath and N^1-oxide cAMP activation, both mean currents were 5.3 pA (± 0.53 pA for bath and ± 1.0 pA for N^1-oxide cAMP). Similar N^1-oxide cAMP activity was seen in this mutant in three other patches.

the contacts will differ depending on whether the nucleotide is bound in the *syn* or the *anti* conformation (see Fig. 1). Using AMMP, the energetically favored conformation of the bound ligand was calculated for the rat and catfish olfactory channels and the bovine rod channel-binding domain. As discussed in detail previously (Scott et al., 1996), the energetically favored conformation is determined from the absolute energy difference between the *syn* and *anti* conformations of the bound ligand (Fig. 6 *B*). In the rat olfactory binding domain, R575 on the Cα helix selects for the *anti* conformation of the cAMP (Scott et al., 1996). The models show that in this conformation, the N^1-oxide is positioned away from Y512, on the β5 strand of the model, and E583, on the Cα helix. In contrast, the rod binding domain models show no energetically favored conformation for cAMP (Scott et al., 1996) and with the *syn* conformation of N^1-oxide cAMP, the highly negative oxygen would likely disrupt the communication between the purine and the β5 strand and Cα helix. The experimental results with the rat olfactory-like mutants show that the acidic residue (D604 in bovine rod), presumed to lie on the Cα helix, is an important determinant of whether N^1-oxide cAMP binds to the channel. The additional length of the Glu in the mutant, compared to the Asp in the bovine rod channel, may provide the required flexibility so that the negatively charged side chain can avoid electrostatic repulsion from the N^1-oxide.

An explanation of why Rp-cGMPS does not activate catfish olfactory channels must take into account the fact that the residues contacting the ribofuranose are conserved in all members of the channel family, and that Sp-cGMPS is a full agonist in the catfish olfactory channel. Our models show that the equatorial sulfur of Rp-cGMPS, but not the axial sulfur of Sp-cGMPS, is buried within the β barrel. By using AMMP we calculated the binding energies of both *syn* and *anti* cGMP, Rp-cGMPS, and Sp-cGMPS. We used these energies to predict preferred configurations in Table 2. Differences smaller than ~7 kcal/mol are not considered selective. Clearly, the *syn* conformation is favored for cGMP in the bovine rod and the rat olfactory α subunit binding domains, but the *anti* conformation is preferred in catfish olfactory channels.

Replacing the exocyclic oxygen of cGMP with sulfur altered some of the contacts between the ribofuranose and the binding domain, but the actual displacements were small. From models of Rp- and Sp-cGMPS, bound in the preferred configuration, we computed the displacements of three atoms relative to their positions when cGMP is bound. The atoms were located on the side chains of conserved residues E544, R559, and T560 (bovine rod sequences), all of which contact the ribofuranose. The results are presented in Table 3. Inspection of the *anti* conformation of Rp-cGMPS in the catfish olfactory binding domain shows that the largest displacement is seen with the conserved Glu on the β6 strand.

The important insight to emerge from the modeling of the phosphorothioate analogs is that the sulfur induces changes in the nucleotide packing that propagate through the linked ring systems, causing a tilt in the bound purine. The purine

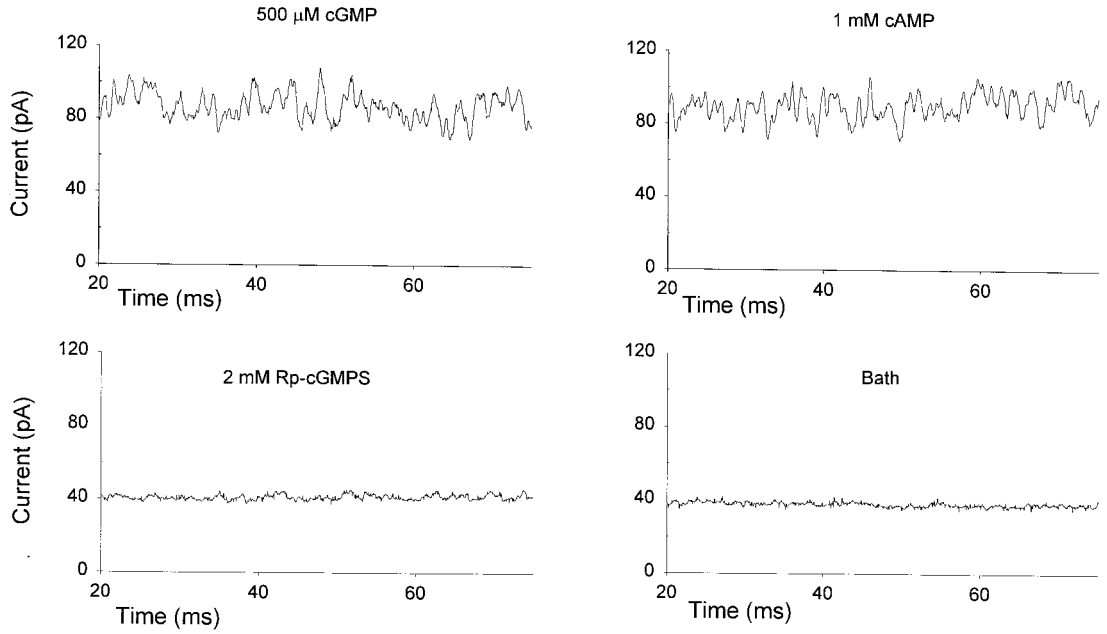

FIGURE 5 A catfish olfactory-like mutant, F533Y/K596R/D604Q, no longer responds to Rp-cGMPS. The bovine rod triple-mutant F533Y/K596R/D604Q was expressed in tSA201 cells. Current activation by 500 μM cGMP (*upper left*) and 1 mM cAMP (*upper right*) are shown. The mean current for cGMP was 87 $\pm$ 7.1 pA; that for cAMP was 90 $\pm$ 6.5 pA. The lower traces show current responses to 2 mM Rp-cGMPS (*lower left*) and bath (*lower right*). The Rp-cGMPS mean current was 41 $\pm$ 1.6 pA; the bath current was 38 $\pm$ 1.1 pA. All traces were held at 0 mV and stepped to +80 mV for 80 ms. See the text for a description of the catfish olfactory wild-type channel to Rp-cGMPS.

displacement, which differs among the models, alters the purine contacts with residues on the β5 strand and the C helix as shown by the displacements of C^2, C^6, and N^9 purine atoms in Table 3. The models of the rat olfactory channel show strong interactions between the Rp-cGMPS purine and residues Y512 (residue F533 of bovine rod) and E583 (residue D604 of bovine rod). In the catfish olfactory binding domain, the purine of *anti* Rp-cGMPS is unable to contact the conserved T530, which lies on the β7 strand (residue T560 of bovine rod). Furthermore, the ligand tilt results in less favorable purine contacts with F503 on the β5 strand and Q574 on the Cα helix. The loss of Rp-cGMPS activation in the catfish olfactory-like mutant of the bovine rod channel provides experimental support for the modeling predictions. There is no equivalent purine displacement in the *syn* conformation of Rp- or Sp-cGMPS in the rat olfactory or bovine rod binding domain, or with *anti* Sp-cGMPS in the catfish olfactory binding domain. The modeling is therefore consistent with Sp-cGMPS acting as a universal agonist.

DISCUSSION

N^1-oxide cAMP and Rp-cGMPS are cyclic nucleotide analogs that selectively activate rat olfactory CNG channels. N^1-oxide cAMP does not bind to the rod channel and Rp-cGMPS is a competitive antagonist of the catfish olfactory CNG channel. We show with site-specific mutations that the rat olfactory channel is preferentially activated by these analogs because of contacts with the purine at Y512, R575, and E583. The power of site-directed mutagenesis in studying ligand recognition is that the overall fold of the protein should be conserved, and all changes in the behavior of the mutant can be attributed to the amino acid substitutions. These substitutions may lead to changes in the overall protein folds and changes in ligand contacts, but this issue is of much more concern with chimera constructs where a functional channel is formed from different regions of the bovine rod and rat olfactory channels.

Molecular modeling provides insights about the channel-selective activation by these analogs. First, small shifts in the position of the ribofuranose are caused by changes in the packing caused by the addition of the sulfur in the analogs. These shifts are larger in the catfish olfactory channel and they propagate through the coupled ring system of the nucleotide and alter the purine contacts with residues on the β5 strand and the Cα helix that influence binding and activation. Second, the modeling suggests that the selective activation of the rat olfactory channel by N^1-oxide cAMP is largely due to the preference for the *anti* conformation of the ligand in this binding site compared to the *syn* conformation in the bovine rod channel. The conformation of cAMP is determined, in part, by R575 of the rat olfactory channel, which is long enough to interact with the purine. The conformation is also influenced by the acidic residue on the Cα helix. In the catfish olfactory channel, the Gln at

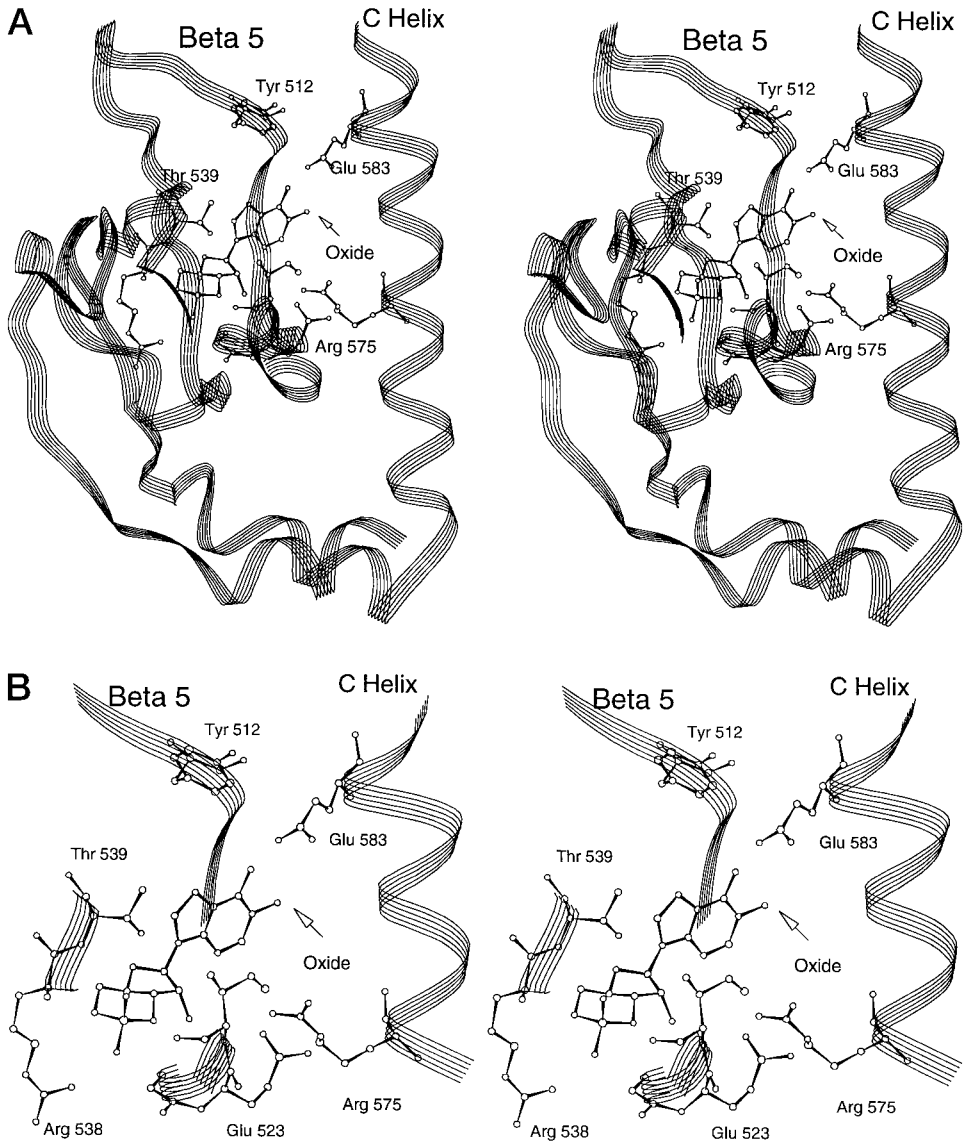

FIGURE 6 (*A*) Predicted secondary structure of the cyclic nucleotide binding domain is represented by the rat olfactory CNG channel with *anti* N^1-oxide cAMP. The cyclic nucleotide binding domain surrounds the ligand. The purine ring lies between the β5 strand and the Cα helix, and the ribofuranose lies buried within the other β strands. (*B*) A close view of the contacts shown in *A*. The ribofuranose binding pocket is formed, in part, by E523, R538, and T539. The purine ring interacts with Y512, R575, and E583.

position 604 forces cGMP into the *anti* conformation. This unfavorable interaction of Gln with cGMP is likely the reason that cGMP is a poor agonist in our F533Y/K596R/D604Q mutant and in the D604Q mutant of Varnum et al. (1995). In both of these mutants, cGMP is a much weaker agonist than in the wild-type bovine rod channel, with an increased $K_{0.5}$ value and a relative current similar to that activated by cAMP.

The activation properties of N^1-oxide cAMP and Rp-cGMPS strengthen the hypothesis that the β5 strand and the Ca helix communicate in the presence of an activating ligand

The first clue that protein contacts with the N^1 and C^6 positions of the purine are required for ligand activation of CNG channels was seen with 2-amino-cPNP. This cyclic nucleotide analog, which is identical to cGMP except for the replacement of the C^6 oxygen with hydrogen, does not bind to rod channels (Tanaka et al., 1989). Subsequently, two other inactive cyclic nucleotide analogs were identified: N^1-oxide-cAMP and N^6-monosuccinyl-cAMP (Scott and Tanaka, 1995). More recently, PET-cGMP was shown to be a competitive antagonist of rod channels, capable of binding, but not activating, these channels (Wei et al., 1996). The important and shared feature of all of these analogs is that they are all altered at either the N^1 or C^6 on the purine. Coupled with the recognition that changes in other positions of the purine are well tolerated by CNG channels, this result implies that contacts with the binding domain at the N^1/C^6 positions of the purine are crucial for channel activation.

Based on the modeling and previous mutagenesis showing the importance of the communication between the β5 strand and the Cα helix (Scott and Tanaka, 1998), we

TABLE 2 Energetically favored purine ring conformation determined from the overall energy differences between the models

CNG channel model	Ligand	E_{syn} (kcal/mol)	E_{anti} (kcal/mol)	$\|\Delta E\|$	Conformation	Activity
Bovine rod	cGMP	−83	−76	7	Syn	Agonist
	Sp-cGMPS	−82	−76	6	Syn	Agonist*
	Rp-cGMPS	−78	−67	11	Syn	Agonist*
Catfish olfactory	cGMP	−89	−100	11	Anti	Agonist*
	Sp-cGMPS	−90	−104	14	Anti	Agonist*
	Rp-cGMPS	−89	−103	14	Anti	Antagonist*
Rat olfactory	cGMP	−119	−88	31	Syn	Agonist
	Sp-cGMPS	−122	−96	26	Syn	Agonist†
	Rp-cGMPS	−121	−109	22	Syn	Agonist

*Taken from Kramer and Tibbs, 1996.
†Predicted.

suggest that N^1-oxide cAMP in the rod channel is not able to bind because the negative oxide prevents the C^6 amino substituent from interacting with F533 on the β5 strand and D604 on the Cα helix. The contrasting situation with the rat olfactory channel is due to the preferred *anti* conformation of N^1-oxide cAMP and the additional flexibility of the Glu side chain. Here, the N^1-oxide faces away from Y512 on the β5 strand and E583 on the Cα helix, which allows the C^6 amino to interact with both residues. Similar reasoning holds for Rp-cGMPS in the catfish olfactory channel. With this analog, the large tilt of the purine in the catfish olfactory channel prevents the C^6 oxygen from interacting with F503 on the β5 strand.

Predicting whether Rp-cGMPS will be an agonist or antagonist for other homomeric CNG channels

The ability of Rp-cGMPS to activate a particular CNG channels depends on the strength of the purine interactions with four residues (Altenhofen et al., 1991; Scott and Tanaka, 1998; Varnum et al., 1995). The threonine on the β7 strand is conserved in all CNG channels, but residues at the other positions vary among the family members. The experiments and the modeling with Rp-cGMPS suggest that the rat olfactory β5 strand interacts with the purine through Y512, whereas the equivalent F503 in the catfish olfactory channel is unable to interact with the purine. The other important residue on the C helix is the basic residue, K596 in the bovine rod channel and R575 in the rat olfactory channel. Arginine, but not the lysine, is capable of interacting with the purine ring (Scott and Tanaka, 1998). The N^1-oxide cAMP activation of the rat olfactory channel, but not the bovine rod channel, supports the idea that this residue is a determinant of the ligand conformation. Using this information, we predicted the response of other CNG channels to Rp-cGMPS. Notably, despite wide species and tissue diversity, only the catfish olfactory CNG channel lacks an acidic residue on the Cα helix. We predict, therefore, that Rp-cGMPS will activate the other CNG channels listed in Table 1. This prediction is also based on the conservation of aromatic residues on the β5 strand, except for the *Drosophila* CNG channel. The *Drosophila* channel also has L485 in the position of the aromatic in the other channels (F533 in bovine rod), making it difficult to predict whether Rp-cGMPS will activate these channels. Although co-expression of the β subunit could modify our predic-

TABLE 3 Atomic position displacements, relative to their positions with cGMP in the binding domain, caused by binding of phosphorothioate cGMP analogs in the binding domain models of the bovine rod, catfish olfactory, and rat olfactory

Channel	Ligand Conformation	Ligand	Glu: CD1*	Arg: NH1	Thr: OG1	Nucleotide Atomic Displacements†				RMS
						P	C^2	C^6	N^9	
Bovine rod‡	Syn	Sp-cGMPS	0.23	0.18	0.14	0.05	0.13	0.09	0.10	0.178
		Rp-cGMPS	0.07	0.15	0.07	0.11	0.24	0.19	0.24	0.366
Catfish olfactory	Anti	Sp-cGMPS	2.26	0.49	0.11	0.63	0.12	0.26	0.30	0.681
		Rp-cGMPS	1.70	0.31	0.58	0.65	0.47	0.52	0.51	0.385
Rat olfactory§	Syn	Sp-cGMPS	0.60	0.14	0.21	0.33	0.15	0.16	0.12	0.213
		Rp-cGMPS	0.40	0.12	0.11	0.45	0.15	0.16	0.16	0.152

All displacements are in Å. All ligands are bound in their preferred conformation (from Table 2).
*Glu:CD1 refers to the carboxyl oxygen of the δ carbon, Arg:NH1 refers to a terminal nitrogen in the arginine side chain, and Thr:OG1 refers to the γ oxygen in the threonine side chain.
†P is the phosphate atom on the ribofuronose; C^2, C^6, and N^9 are the atoms in the purine.
‡Bovine rod sequences: E544 on the β6 strand, R559 and T560 on the β7 strand. Catfish olfactory sequences: E514, R529, T530.
§Rat olfactory sequences: E523, R538, T539.

tions, studies with Rp-cGMPS showed that homomeric rod and olfactory channels display similar properties to the native channels (Kramer and Tibbs, 1996; Zimmerman et al., 1985).

Why are the phosphorothioate cGMP analogs important?

CNG channels and PKG are major players in signal transduction schemes involving changes in cytosolic cGMP. The recognition of guanylate cyclase as a target for the gaseous neurotransmitters nitric oxide (NO) and carbon monoxide, reviewed by Zufall et al., 1997 and Mancuso et al., 1997, suggests that neuronal feedback loops could involve the activation of CNG channels or PKG or both. One way to dissect these pathways in cells is to use cGMP analogs that selectively inhibit one or the other effector. For example, hippocampal long-term potentiation (LTP), which is involved in learning and memory, has been shown to involve stimulation of guanylate cyclase by NO (Arancio et al., 1996). Although it has not yet been possible to trace the effector pathways involved in hippocampal LTP, CNG channels might be involved because some hippocampal neurons express CNG channels (Bradley et al., 1997; Kingston et al., 1996; Leinders-Zufall et al., 1995; Parent et al., 1998). It was also recently observed that an estimated 50% of retinal ganglion cells express CNG currents presumably activated through an NO pathway (Kawai and Sterling, 1999). Molecular dissection of the signal transduction cascade in these cells will be greatly aided by pharmacological agents specific for one of the effectors. Rp-cGMPS is a potent selective inhibitor of PKG types Iα, Iβ, and II (Butt et al., 1990, 1995) and prudent use of this compound in studying signal transduction pathways requires knowledge of how the analog affects CNG channels.

In the initial report showing that Rp-cGMPS was a competitive antagonist of cGMP activation in catfish olfactory channels, Rp-cGMPS was proposed to discriminate between "olfactory-type" and "rod-type" channels (Kramer and Tibbs, 1996). Such a discriminating pharmacological agent would be useful to rapidly categorize newly identified channels in the absence of sequence information. Our finding that Rp-cGMPS activates rat, but not catfish, olfactory channels rules out this simple characterization scheme of CNG channel family members. The different patterns of ligand activation among highly homologous family members from the same tissue type suggest caution, in general, with regard to typing CNG channels. Clearly, differences in one or two residues at key positions can have significant physiological consequences.

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