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Control of ligand specificity in cyclic nucleotide-gated channels from rod photoreceptors and olfactory epithelium

(site-directed mutagenesis/ion channels/signal transduction/photoreception/olfaction)

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ABSTRACT **Cyclic nucleotide-gated ionic channels in photoreceptors and olfactory sensory neurons are activated by binding of cGMP or cAMP to a receptor site on the channel polypeptide. By site-directed mutagenesis and functional expression of bovine wild-type and mutant channels in *Xenopus* oocytes, we have tested the hypothesis that an alanine/threonine difference in the cyclic nucleotide-binding site determines the specificity of ligand binding, as has been proposed for cyclic nucleotide-dependent protein kinases [Weber, I. T., Shabb, J. B. & Corbin, J. D. (1989) *Biochemistry* 28, 6122–6127]. The wild-type olfactory channel is ≈25-fold more sensitive to both cAMP and cGMP than the wild-type rod photoreceptor channel, and both channels are 30- to 40-fold more sensitive to cGMP than to cAMP. Substitution of the respective threonine by alanine in the rod photoreceptor and olfactory channels decreases the cGMP sensitivity of channel activation 30-fold but little affects activation by cAMP. Substitution of threonine by serine, an amino acid that also carries a hydroxyl group, even improves cGMP sensitivity of the wild-type channels 2- to 5-fold. We conclude that the hydroxyl group of Thr-560 (rod) and Thr-537 (olfactory) forms an additional hydrogen bond with cGMP, but not cAMP, and thereby provides the structural basis for ligand discrimination in cyclic nucleotide-gated channels.**

Cation channels that are directly gated by guanosine 3′,5′-cyclic monophosphate (cGMP) control the flow of ions across the surface membrane of vertebrate rod and cone photoreceptor cells (1, 2; for review, see refs. 3 and 4). A similar cation channel exists in vertebrate olfactory sensory neurons (5). Between the rod photoreceptor and the olfactory channel ≈74% of the aligned positions are occupied by identical or conserved amino acid residues (6–8). A single region near the C terminus of the channel polypeptides, comprising ≈80–100 amino acid residues, exhibits significant sequence similarity to both cGMP-binding domains of cGMP-dependent kinases (cGKs) (6). The sequence similarity is less pronounced between the corresponding regions of the channels and of cAMP-dependent kinases (cAKs) or of the catabolite gene activator protein of *Escherichia coli*. The comparison suggests that the channel polypeptides carry a ligand-binding site that is structurally similar to that of other cAMP- or cGMP-binding proteins.

A threonine residue is invariant in the two cGMP-binding domains of all cGKs but is exchanged for an alanine residue in 23 of 24 cAMP-binding sites in cAKs (9). The mammalian rod and olfactory cyclic nucleotide-gated channels contain a threonine residue at this particular position (6–8; Fig. 1). It has been proposed that this alanine/threonine difference might have been important in the evolutionary divergence of cyclic nucleotide-binding sites and that it provides the structural basis for discriminating between cAMP and cGMP (9). We tested the validity of this hypothesis for cyclic nucleotide-gated channels by mutagenesis and expression of wild-type and mutant channels from rod photoreceptors and olfactory epithelium.

MATERIALS AND METHODS

Construction of Recombinant pCHOLF102. PCR (13) was done with pCHOLF100 (8) as template and the following primers: a 5′ adapter primer [containing an *Eco*RV restriction site, a consensus sequence for eukaryotic ribosomal-binding sites (14), and the first nine nucleotides from the coding region of CHOLF100] and a gene-specific 3′ primer. The *Eco*RV/*Dra*III-digested PCR product replaced the corresponding fragment of pCHOLF100 to yield pCHOLF101. The insert of pCHOLF101 was subcloned into a pT7T3 vector to yield pCHOLF102.

Site-Directed Mutagenesis. The point mutations at positions 560 and 537 of the rod and olfactory channel polypeptides, respectively, were introduced by PCR procedure (13) with synthetic oligonucleotides containing the desired nucleotide substitutions.

Rod-channel mutant T560A was constructed by the method of Hemsley *et al.* (15). A circular plasmid with the wild-type rod-channel sequence from pRCG1 (6) was amplified by a pair of primers located "back-to-back" on opposite DNA strands. The resulting PCR product was recircularized and digested with *Nsi* I and *Sty* I. The corresponding *Nsi* I–*Sty* I fragment in pRCG1 was replaced by the mutated fragment to create pT560A. For the construction of rod-channel mutant T560S, we took advantage of a newly introduced *Cla* I restriction site near codon 560 in pT560A. A PCR fragment was produced by using a mutagenic and a complementary primer and linearized pT560A as template. The *Nsi* I–*Cla* I fragment containing the mutation was exchanged for the corresponding *Nsi* I–*Cla* I fragment of pT560A to generate pT560S.

Both olfactory-channel mutants T537A and T537S were constructed by combining two overlapping PCR fragments with the aid of newly introduced restriction sites at the locus of mutation (*Bss*HII for pT537A and *Rsr* II for pT537S) and other suitable restriction sites in the plasmid. pCHOLF102 was used as template. All mutations were verified by sequencing of the entire insert with the dideoxynucleotide chain-termination method.

Functional Expression. mRNA specific for the rod-photoreceptor channel, the olfactory channel, and the mutant channels was synthesized *in vitro* (16) by using the respective linearized plasmid cDNA as template. Transcription was primed with the cap dinucleotide 7-methylguanosine(5′)-triphospho(5′)guanosine (0.6 mM) (17). Macroscopic current measurements on excised inside-out patches (18–20) were made after injection of mRNA into *Xenopus* oocytes (mRNA

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Abbreviations: cAK, cAMP-dependent kinase; cGK, cGMP-dependent kinase; *I*, current; *I-V*, *I*–voltage; *n*, Hill coefficient; $K_{1/2}$, half-saturating concentration; I_{max}, maximum *I*.

A

cGMP-channel (rod)	(528)	DGITQFVVLSDGSYFGEISILNIKGSKAGNRRTANIKSIGYSDLFCLS	(575)
cAMP-channel (olf)	(505)	DGVTQYALLSAGSCFGEISILNIKGSKMGNRRTANIRSLGYSDLFCLS	(552)
cGK-bovine (1)	(153)	--GVKLCTMGPGKVFGELAILY------NCTRTATVKTLVNVKLWAID	(192)
cGK-bovine (2)	(275)	EDPVFLRTLGKGDWFGEKALQG------EDVRTANVIAAEAVTCLVID	(316)
cAK-bovine (1)	(186)	--NEWATSVGEGGSFGELALIY------GTPRAATVKAKTNVKLWGID	(225)

B

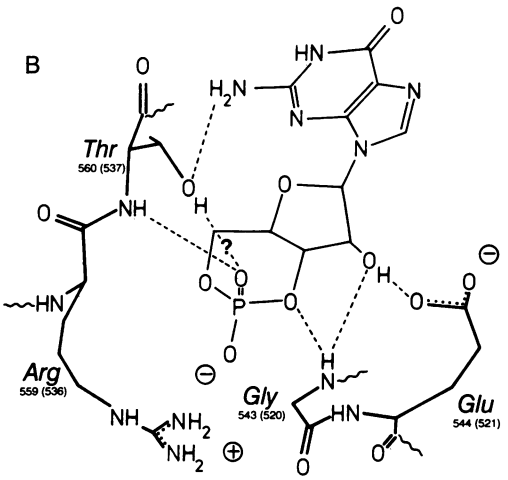

FIG. 1. (*A*) Comparison of the aligned amino acid sequences of parts of the cyclic nucleotide-binding site of several cyclic nucleotide-binding proteins (for details, see ref. 10). The position of the predicted structure of β-strands of the binding-site model in ref. 11 is indicated by bars and numbers. Arrowhead indicates position of the exchanged threonine residue, and asterisks indicate identical residues. Numbers in parentheses refer to binding domain 1 or 2. Sequences are from refs. 6, 8, 9, and 11. (*B*) Predicted sites of contact between cGMP and the binding site in cGKs and cyclic nucleotide-gated channels. Hypothetical hydrogen bonds are indicated by interrupted lines (adapted from ref. 12); numbers refer to position of amino acid residues in the sequence of the photoreceptor (olfactory) channel peptide. The hydrogen bond marked by a question mark most likely is not involved in cAMP or cGMP binding.

concentration, 0.4 μg/μl; average volume injected per oocyte, ≈50 nl) and incubation for 2–3 days in Barth's medium. Oocytes were prepared for recording by standard techniques (20, 21). The solution in the pipette, and the perfusion medium contained 100 mM KCl, 10 mM EGTA·KOH, and 10 mM Hepes·KOH (pH 7.2). Pipettes (2–4 MΩ) gave seal resistances of 0.2–90 GΩ. Macroscopic currents across excised inside-out patches were recorded under voltage-clamp conditions. Voltage ramps were delivered as a triangle (±80 mV, 50–80 mV/sec). Membrane current was low-pass filtered (50 Hz, 8-pole Bessel filter, Frequency Devices, Haverhill, MA) and continuously recorded on-line (pClamp 5.5, Axon Instruments, Burlingame, CA).

The cytoplasmic face of the excised membrane patch was superfused by using one or two concentric arrays of seven fused glass pipettes (i.d., 0.8 mm; Hilgenberg, Malsfeld, F.R.G.) each having an opening diameter of 30–70 μm. Solutions flowed from each port independently (1 ml/min), and a membrane patch could be exposed to a particular solution within ≈100 msec by moving the mouth of the port with a computer-controlled micromanipulator to the patch pipette. The bath temperature was 18–23°C.

Current was measured with a virtual ground current-to-voltage converter (L/M-EPC-7, List Electronics, Darmstadt, F.R.G.). Current–voltage relations (*I–V*) were recorded in the control (leak current) and different test solutions containing ligand intermittently. Leak currents [typically 0.3–20% of maximum current (I_{max})] recorded without ligand were subtracted from currents measured with ligand. Each *I–V* curve represents the average of three consecutive voltage ramps.

RESULTS

Expression of Wild-Type Channel. Photoreceptor channel-specific mRNA derived by *in vitro* transcription from clone pRCG1 of ref. 6 gave rise to cGMP-stimulated channel activity after injection into *Xenopus* oocytes (Fig. 2*A*, *Inset*). cGMP-stimulated currents were not observed in excised patches of uninjected or water-injected control oocytes. mRNA specific for the olfactory channel derived from clone pCHOLF100 of ref. 8 did not produce functional cyclic nucleotide-gated channels in oocytes. However, mRNA derived from recombinant clone pCHOLF102 that lacked the complete 5′ noncoding region and contained a consensus sequence for a eukaryotic ribosomal-binding site (see *Materials and Methods*) gave rise to cyclic nucleotide-stimulated currents (Fig. 2*B*, *Inset*).

Maximum currents at saturating cGMP concentrations typically varied between 2 nA and 13 nA at +80 mV and were largely similar for both channel species. Occasionally, amplitudes of I_{max} could be as low as a few picoamperes, depending mainly on the seasonal or temporal variations in the quality of oocytes. In the rod channel, saturating cAMP concentrations activated a current that was ≤17% of the maximum amplitude of cGMP-stimulated currents, whereas in the olfactory channel both cyclic nucleotides produced the same I_{max} amplitudes. This difference between the rod and olfactory channel with respect to activation by cAMP was qualitatively preserved in all mutant channels. Similar observations have been previously reported for excised patches of amphibian rod-photoreceptor and olfactory channel (22, 23). This result suggests that the open state of the cAMP-liganded rod channel is different from that of the olfactory channel.

Fig. 2 shows the relation between I/I_{max} and cGMP or cAMP concentration for the rod and olfactory wild-type channel. For comparison, half-saturating concentration ($K_{1/2}$) values for cAMP and cGMP of the rod channel are indicated by small arrows in Fig. 2*B*. Mean values of $K_{1/2}$ and Hill coefficient (n) for wild-type and mutant channels are summarized in Table 1. The expressed olfactory channel is ≈22- to 24-fold more sensitive to both cyclic nucleotides than the rod channel; and both channel types are ≈40-fold less sensitive to cAMP than to cGMP (see Table 1). We consistently found a higher degree of cooperativity for the olfactory channel (n_{cGMP} = 2.4, n_{cAMP} = 2.6) than for the rod-photoreceptor channel (n_{cGMP} = 1.7, n_{cAMP} = 1.8). The difference is statistically significant on the 5% level.

Replacement of Thr-560 (Rod) and Thr-537 (Olfactory) by Alanine. Both channel mutants T560A (rod) and T537A (olfactory) were functional when expressed in *Xenopus* oocytes. The mean of the maximum cGMP-stimulated current amplitude in the T560A [I_{mean} = 2831 ± 2232 pA (number of experiments, x = 9)] and T537A [I_{mean} = 2037 ± 1035 pA (x = 8)] mutant channels was ≈2-fold smaller than that seen for wild-type channel [I_{mean} = 5203 ± 3064 pA (x = 11) (rod) and 5614 ± 1168 pA (x = 15) (olfactory)]. The *I–V* relationships of the mutant channels were not significantly different

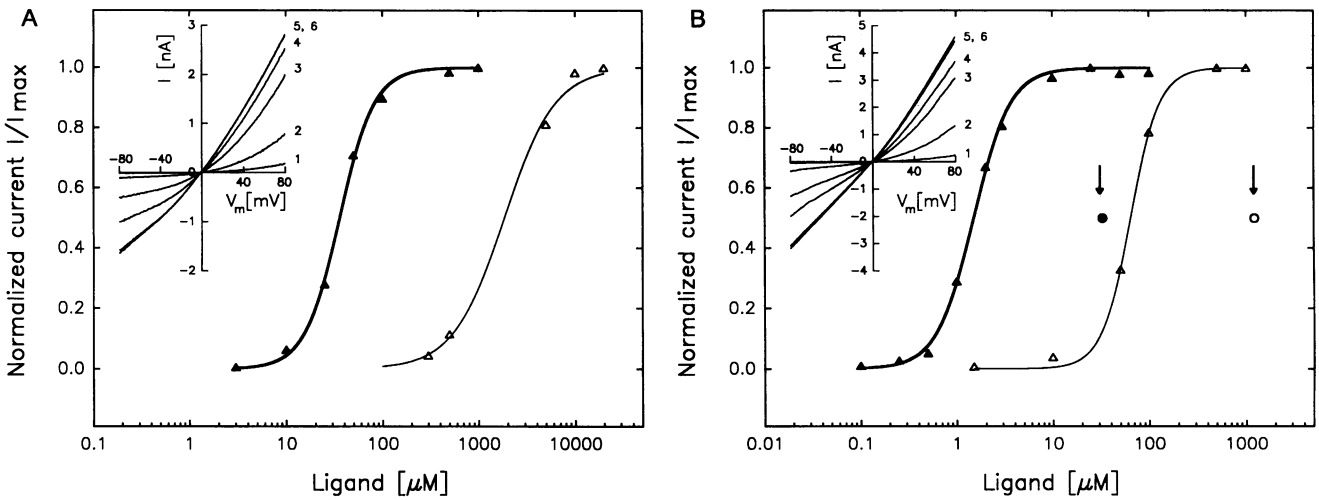

FIG. 2. Ligand sensitivity of normalized currents I/I_{max} of wild-type channels. Smooth curves represent a least-square-fit of the Hill equation $I/I_{max} = c^n/(c^n + K_{1/2}^n)$ to the experimental data, where c = concentration of the ligand. Normalized currents I/I_{max} were determined from current amplitudes at +80 mV of *I–V* curves similar to those shown in *Insets*. (*A*) Rod-photoreceptor channel; ▲, cGMP; △, cAMP. $K_{1/2}$ (cGMP) = 28.0 ± 1.0 μM; n = 2.0 ± 0.1; $K_{1/2}$ (cAMP) = 1849 ± 200 μM; n = 1.64 ± 0.1; data points for cAMP come from two different patches. (*B*) Olfactory channel; ▲, cGMP; △, cAMP; $K_{1/2}$ (cGMP) = 1.5 ± 0.03 μM; n = 2.2 ± 0.1; $K_{1/2}$ (cAMP) = 64 ± 1.8 μM; n = 2.9 ± 0.2. Arrows indicate the $K_{1/2}$ constants for cGMP (●) and cAMP (○) of the rod channel. (*Insets*) Series of *I–V* recordings in the presence of different cGMP concentrations. (*A*) Trace 1, 10 μM; trace 2, 25 μM; trace 3, 50 μM; trace 4, 100 μM; trace 5, 500 μM; and trace 6, 1000 μM; pipette resistance 4 MΩ. (*B*) Trace 1, 0.5 μM; trace 2, 1 μM; trace 3, 2 μM; trace 4, 3 μM; trace 5, 10 μM; and trace 6, 25 μM; pipette resistance, 3.5 MΩ.

from those of the wild-type channels (Fig. 3*B*, *Inset*), suggesting that the electrical and the underlying structural properties of the channels are preserved by these mutations. However, we have not yet tested the electrical properties on a single-channel level.

Upon replacement of Thr-560 by alanine in the rod channel, the $K_{1/2}$ constant for the activation by cGMP increased ≈30-fold (from 32.6 to 939 μM), whereas the activation by cAMP remained almost constant (Fig. 3*A*). The n values of activation were not significantly changed. The T537A mutant of the olfactory channel behaved similarly: the cGMP sensitivity of channel activation was decreased ≈40-fold (from 1.4 to 53.0 μM), whereas the cAMP sensitivity increased 2- to 3-fold (Fig. 3*B*). Again, the cooperativity of the channel activation was not influenced by the threonine/alanine exchange (see Table 1). The $K_{1/2}$ constants for activation by cGMP in the T537A (olfactory) mutant and by cAMP in the wild-type channel became identical (compare thick line/filled symbols with thin line/no symbols in Fig. 3*B*).

Replacement of Thr-560 (Rod) and Thr-537 (Olfactory) by Serine. If the hydroxyl group of the 560 or 537 residue is of critical importance, replacement of Thr-560 or Thr-537 by a serine residue should yield mutant channels with a ligand specificity similar to that of the respective wild-type channels. Surprisingly, the T560S mutant of the rod channel was activated at 5- to 6-fold lower cGMP concentrations ($K_{1/2}$ = 6.4 μM) than the wild-type rod channel (see Table 1). The T537S mutant of the olfactory channel was also 2-fold more sensitive to cGMP than the wild-type channel. The cAMP sensitivity of T560S was slightly decreased, and that of T537S was slightly increased. These results support the notion that a hydroxyl function in the amino acid residue at position 560 (and 537) is important for recognition of cGMP but not of cAMP.

DISCUSSION

The rod-photoreceptor channel is more sensitive to cGMP than to cAMP and contains a threonine residue at a particular

Table 1. $K_{1/2}$ and n of wild types and mutants

	Rod-photoreceptor channel		Olfactory channel	
	$K_{1/2}$, μM	n	$K_{1/2}$, μM	n
WT				
cGMP	32.63 ± 13.32 [15.67–53.57 (11)]	1.66 ± 0.56 [0.95–2.65]	1.36 ± 0.37 [0.89–2.39 (15)]	2.40 ± 0.64 [1.42–3.43]
cAMP	1210 ± 300 [850–1580 (3)]	1.81 ± 0.93 [1.05–3.12]	53.73 ± 15.05 [28.24–72.78 (6)]	2.59 ± 0.87 [1.81–4.23]
Thr → Ala				
cGMP	940 ± 140 [755–1265 (9)]	1.73 ± 0.44 [1.35–2.78]	53.03 ± 16.04 [33.38–77.74 (8)]	1.98 ± 0.56 [1.25–2.89]
cAMP	2750 ± 910 [1980–4030 (3)]	1.60 ± 0.27 [1.28–1.94]	16.29 ± 3.69 [10.90–25.94 (15)]	2.07 ± 0.54 [1.36–3.44]
Thr → Ser				
cGMP	6.40 ± 1.01 [4.68–8.23 (8)]	2.29 ± 0.19 [1.94–2.58]	0.69 ± 0.15 [0.55–0.95 (6)]	2.59 ± 0.54 [1.46–3.07]
cAMP	2240 ± 330 [1900–2690 (3)]	1.93 ± 0.10 [1.79–2.02]	13.87 ± 3.73 [10.17–18.98 (3)]	1.51 ± 0.37 [1.23–2.03]

Data are presented as means ± SDs. Ranges are in brackets; number of experiments are in parentheses.

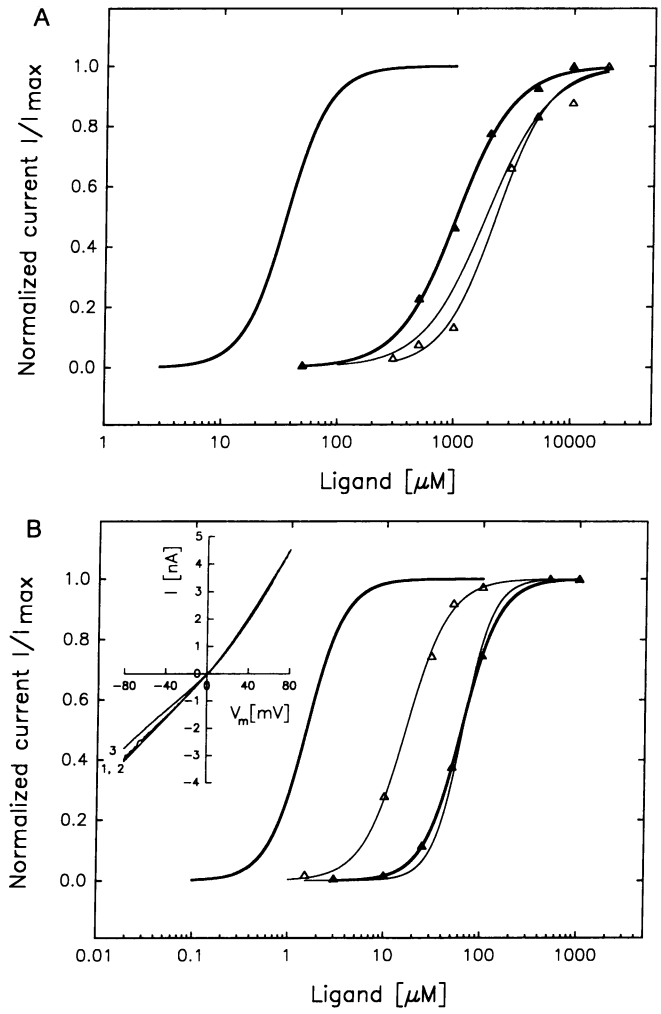

FIG. 3. Ligand sensitivity of normalized currents I/I_{max} of mutant channels. The smooth lines without symbols represent the fitted dose–response curves of wild-type channels for cGMP (thick lines) and cAMP (thin lines). (*A*) T560A rod mutant: ▲, cGMP; △, cAMP. $K_{1/2}$ (cGMP) = 1038 ± 30 μM, n = 1.8 ± 0.1; $K_{1/2}$ (cAMP) = 2250 ± 150 μM, n = 1.9 ± 0.2. (*B*) T537A olfactory mutant: ▲, cGMP; △, cAMP. $K_{1/2}$ (cGMP) = 62.2 ± 0.3 μM, n = 2.3 ± 0.03; $K_{1/2}$ (cAMP) = 16.4 ± 1.3 μM, n = 2.0 ± 0.3. (*Inset*) *I–V* curves from olfactory wild type (trace 1), T537A mutant (trace 2), and T537S mutant (trace 3) at saturating cGMP concentrations (scaled to *I–V* of wild type at +80 mV).

position in the cyclic nucleotide-binding site that is also conserved in cGKs but not in cAKs. Thus, the rod-photoreceptor channel by these criteria is a cGMP-gated channel. Surprisingly, the expressed olfactory channels from rat (7) and bovine tissue (this paper) are also much more sensitive to cGMP than to cAMP and exhibit a higher degree of sequence similarity to the cGMP-binding region of the rod channel and of cGKs than to the respective cAMP-binding domain of cAKs. Thus, by these criteria the olfactory channel has also a cGMP-specific binding site, even though cAMP most likely represents the physiologic ligand of the olfactory channel (24, 25). The available evidence does not support a similar role for cGMP in olfactory transduction (26).

The hypothesis by Weber *et al.* (9) predicts that a threonine residue enhances cGMP binding by forming a hydrogen bond with the guanine 2-amino group of cGMP, whereas no such interaction can occur with cAMP. Specifically, the $K_{1/2}$ constants for cGMP and cAMP should become similar in the threonine/alanine mutant channels if the difference in binding affinity is caused by the threonine–ligand interaction alone. The experimental results described in this paper are consistent with these basic features of the hypothesis. The similar cAMP sensitivity of wild-type and mutant channels argues against the possibility that the decrease in cGMP sensitivity in the threonine/alanine mutants might be caused by some unintended structural perturbations that propagate through the entire binding site.

Optimal hydrogen bonding requires the collinear alignment of the donor and acceptor group -O-H· · ·N (27). The methyl group at C_β in threonine might restrict rotational freedom around the C_α—C_β axis of the amino acid and thereby prevent optimal alignment of the atoms that form the hydrogen bond. Serine contains a hydrogen atom instead of a methyl group at C_β, and formation of a collinear hydrogen bond might be facilitated.

From the ratio of half-saturating cGMP concentrations $K_{1/2}$ between a binding site that can form an "optimal" hydrogen bond (T560S mutant) and a binding site that lacks this possibility altogether (T560A mutant), a difference in the incremental Gibbs free energy ΔG_b of ≈−3.0 kcal/mol is calculated according to the equation:

$$\Delta G_b = RT \ln[K_{1/2}\ (\text{T560S})/K_{1/2}\ (\text{T560A})].$$

This difference is quantitatively similar to the free energy of hydrogen bond formation (>3–6 kcal/mol), supporting the notion, but not proving it, that the higher cGMP sensitivity results from an additional hydrogen bond.

Experiments similar to those presented here have not yet been performed in other cGMP-binding proteins—for example, cGK. Shabb *et al.* (12), however, reported an increase in cGMP binding by introducing a threonine for an alanine residue in one of the two cyclic nucleotide-binding sites of the regulatory subunit of cAK—i.e., the reverse of the mutations introduced into the channel proteins. Our results are qualitatively similar to those obtained in cAK but differ in some quantitative aspects. For example, a mutant of cAK in which an alanine was replaced by serine, does not bind cGMP as strongly as the respective alanine/threonine mutant (12), and the alanine/threonine mutant still prefers binding of cAMP over cGMP.

Functional expression of mutant cyclic nucleotide-gated channels is particularly suitable for the study of structural features of the ligand-binding site. These channels are homo-oligomers (28) that contain only one cyclic nucleotide-binding domain per monomer (6), whereas the regulatory subunits of cAK and cGK contain two kinetically different ligand-binding sites. The cyclic nucleotide sensitivity and specificity of mutant binding sites in channels can be accurately determined *in situ* in excised membrane patches, whereas the regulatory subunit-cAK mutants need to be purified after expression for a functional or a binding assay (12).

The threonine/alanine difference is only one among several factors that determine the absolute binding affinity. Apparently, there are at least two different levels of structural organization of cyclic nucleotide-binding sites. The first level involves an invariant sequence pattern of amino acids shared by all cyclic nucleotide-binding sites (10): [NDEQRK]G[DEA]X[AG]XXX[FY]XXXXG{15–35}GE{5–20}-R[ATSQ]A. X can be any amino acid, letters in brackets denote alternative amino acid residues at the relevant position, and numbers indicate the variable number of intervening residues. We define this sequence motif as the "core" of the binding site. The highly conserved arginine and glutamate residues (see Fig. 1) have been shown in catabolite gene activator protein to interact with the cyclic phosphodiester and the ribose moiety, respectively. At this level, a threonine residue increases cGMP affinity by formation of an additional hydrogen bond. In contrast, binding of cAMP to cAK does not involve hydrogen bonds with the adenine ring but differ-

ent mechanisms such as stacking interactions or van der Waals forces (for review, see ref. 29). Depending on the relative contribution of each interaction mechanism, it is conceivable that cyclic nucleotide-binding sites might exist that contain a threonine residue but which are more sensitive to cAMP than to cGMP. In this respect it will be interesting to determine the amino acid sequence of cAMP-specific channels (30, 31). For the rod-photoreceptor and olfactory channel, however, we were able to show that the threonine/alanine exchange is sufficient to establish the physiological range of nucleotide selectivity.

At a second level, interaction between the core of the ligand-binding site and other parts of the same or a neighboring subunit mediates activation of the channel. It affects the activation constant independently of the ligand species and might account for the absolute difference in sensitivity between the rod-photoreceptor and the olfactory channel. In fact, the $K_{1/2}$ constant of a chimeric rod channel that contains the binding region of the olfactory channel is identical with that of the wild-type rod channel. An analogous result was obtained with the respective olfactory channel chimera (unpublished observation). The importance of interactions between the binding site and other parts of the polypeptide for binding and activation has been also demonstrated in cAK, cGK, and catabolite gene activator protein (11, 32–35).

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