Distinct structural determinants of efficacy and sensitivity
in the ligand-binding domain of cyclic nucleotide-gated channels

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¶ Deceased July 29, 2003. This paper is dedicated to the memory of Natasha whose grace and resourcefulness were an example to all.

Running title: Gating determinants in CNG channel ligand-binding domain
SUMMARY

Cyclic nucleotide-gated (CNG) channels open in response to direct binding of cyclic nucleotide messengers. Every subunit in a tetrameric CNG channel contains a cytoplasmic ligand-binding domain (BD) that includes a \( \beta \)-roll (flanked by short helices) and a single C-terminal helix called the C-helix which was previously found to control efficacy (maximal open probability) and selectivity for cGMP vs. cAMP. We constructed a series of chimeric CNG channel subunits, each containing a distinct BD sequence (chosen from among six phylogenetically divergent isoforms) fused to an invariant non-BD sequence. We assayed these "BD-substitution" chimeras as homomeric CNG channels in *Xenopus* oocytes to compare their functions, and found that the most efficient activation by both cAMP and cGMP derived from the BD of the catfish CNGA4 olfactory modulatory subunit (fCNGA4). We then tested the effects of replacing subregions of the bovine CNGA1 BD with corresponding fCNGA4 sequence, and hence identified parts of the fCNGA4 BD producing efficient activation. For instance, replacing either the "hinge" that connects the roll and C-helix subdomains, or the BD sequence N-terminal of the hinge, greatly enhanced cAMP efficacy. Replacing the "loop-\( \beta \)8" region (the C-terminal end of the \( \beta \)-roll) improved agonist sensitivity for cGMP selectively over cAMP. Our results thus identify multiple BD elements outside the C-helix which control selective ligand interaction and channel gating steps by distinct mechanisms. This suggests the purine ring of the cyclic nucleotide may interact with both the \( \beta \)-roll and C-helix at different points in the mechanism.
INTRODUCTION

Cyclic nucleotide-gated (CNG) channels conduct mono- and divalent cations upon activation by the direct binding of the cytoplasmic messengers cAMP and cGMP. These channels are widespread in the nervous system and in a variety of other tissues, and most notably they are essential signaling components in visual and olfactory transduction, where their activation leads both to changes in membrane potential and to the influx of calcium into the cytoplasm (reviewed in references (1,2)). Functional CNG channels are tetramers (homomeric or heteromeric) of homologous subunits; vertebrates contain a family of six paralogous CNG channel subunit genes in two phylogenetic subfamilies(3), CNGA and CNGB. Distinct combinations of these paralogues are expressed in each tissue type to produce CNG channels whose response parameters are presumably adapted to the tissue's physiological requirements; these parameters include sensitivity and efficacy (maximal ligand-gated open probability) for cAMP and cGMP, and selectivity for one agonist over the other. Thus CNG channels hold promise as targets for tissue-specific pharmacological regulation of signaling through cyclic nucleotide-dependent, electrical, and calcium-dependent pathways. It is therefore important to understand how CNG channel function is determined by the sequences of individual subunits, and more generally how structural elements in the channel work together to control the quantitative properties of the ligand-gating mechanism.

All CNG channel subunits have a common modular architecture (reviewed in references (4,5)) incorporating recognized structural motifs, namely a "6TM" transmembrane domain joined at its cytoplasmic C-terminal end to a conserved "C-linker" region of ~80 residues, followed by a cyclic nucleotide-binding domain (BD). The 6TM domain is homologous to that of the voltage-gated potassium channel family, with six membrane-spanning segments (S1-S6) and a re-entrant "P-loop" between S5 and S6 that lines the aqueous pore. The BD is homologous to those of cAMP- and cGMP-dependent protein kinases, cAMP-dependent G-protein exchange factors, and the bacterial catabolite activator protein. BD sequences are also found (associated with 6TM and C-linker regions) in several ion channel families with homology to CNG channels, such as the HCN "pacemaker" and the EAG channels. Useful models for the CNG channel BD are based on known three-dimensional structures of homologous BDs(6-9), and
delineate a "roll subdomain" and "C-helix subdomain" connected by a conserved proline. The roll subdomain consists of an eight-stranded β-roll flanked by two short helices, called the "A-" and "B-helix". The β-roll itself contains (between its sixth and seventh β-strands) a short "P-helix" and "PB-loop" that constitute a conserved phosphate-binding cassette(10). The C-helix subdomain consists of a single long helix; one particular C-helix residue has been shown to control agonist selectivity in bovine CNGA1, presumably by forming a specific contact with the purine ring of the ligand(11).

We reasoned that phylogenetically divergent CNG channel subunit isoforms should have accumulated mutations in many functionally important parts of the BD in the course of adaptation to different physiological settings, and analysis of these mutations could be useful in investigating structural determinants of channel activation. We previously assessed the importance of sequence polymorphism in the BD by constructing a series of chimeric CNG channel subunits, in which each chimera contained a distinct BD sequence, but all chimeras shared identical sequence outside the BD(12). These "BD-substitution" chimeras enabled direct functional comparisons between BD sequences. We have now expanded the previous set of three BD-substitution chimeras, to test BDs from a phylogenetically diverse range of CNG channel isoforms. This revealed a broad spectrum of response properties deriving from BD sequence polymorphism, and led to the identification of one particular sequence (from catfish CNGA4) which produced extremely efficient activation properties. This BD sequence was then dissected to identify specific subsequences in which polymorphism strongly influences efficacy or sensitivity. Our analysis shows that multiple BD regions (outside the previously studied C-helix ligand-contact) contribute to highly efficient ligand-gating; these regions moreover control distinct sets of molecular interactions during the activation process.
EXPERIMENTAL PROCEDURES

Molecular subcloning.

Subcloning in the oocyte expression vector pGEM-HE, and chimera construction and mutagenesis by PCR were done as described(12,13), using the following gene sequences: CNGA1 from cow(14), CNGA2 from catfish(15), CNGA4 from rat(16,17), CNGA4 from catfish (18; GenBank Accession #AF522297), TAX-4 from C. elegans(19), and TAX-2 from C. elegans(20). The C-terminal regions of these genes were aligned using ClustalW (Fig. 1C) to define homologous positions; then the BD sequence was defined as bCNGA1 Leu485-Ala614 and homologous sequences in the other subunits. Similarly, the putative ligand-contact position is defined as bCNGA1 Asp604 and homologous positions in other BDs. New X-chimeras were constructed by substitution of the BD in the chimera ROON-S2 previously studied(21,22); new RO-chimeras were constructed by BD-substitution in the chimera RO133 previously studied(13,21,23-25). X-bA1 and RO-bA1 in this work are synonymous with ROON-S2 and RO133, respectively. All sequences subjected to PCR were dideoxy sequenced.

Patch-clamp recording of channel currents and data analysis.

Procedures were essentially performed as described(12). In brief: Xenopus oocytes were injected with 0.25-25 ng RNA; 1-5 d later, inside-out patches were obtained with electrodes of resistance 1-5 MΩ (coated with Sylgard for single-channel recording). Pipette and bath solutions both contained (in mM): 67 KCl, 30 NaCl, 10 HEPES, 10 EGTA, 1 EDTA, pH 7.2 with KOH. Na-cAMP or Na-cGMP were included in the bath solution by iso-osmolar replacement of NaCl, and applied by gravity perfusion. Patch-clamp equipment, software, and data acquisition and analysis were as described(12).

Macroscopic currents elicited by cyclic nucleotide were recorded at -100 mV (filtered at 4 kHz, digitized at 1 kHz) after steady-state current level was reached, and were corrected by subtraction of leak currents recorded without agonist. Time stationarity of dose-response curves (i.e., completion of spontaneous runup or rundown(26)) was verified as described(12). For each curve, response current I at agonist concentration [A] was fitted (with weighting by 1/SD) with the Hill equation, \( I = I_{\text{max}} / (1 + (K_{1/2}/[A])^h) \), where \( K_{1/2} \) is concentration of A eliciting half-maximal activation, h is the Hill coefficient,
and $I_{\text{max}}$ is the maximal current amplitude. Dose-responses collected for cAMP and cGMP in the same patch were used to evaluate selectivity ratios ($K_{1/2,\text{cGMP}} / K_{1/2,\text{cAMP}}$ and $I_{\text{max, cAMP}} / I_{\text{max, cGMP}}$). Ratios significantly less than or greater than unity indicate selectivity for cGMP or cAMP respectively.

Single-channel currents in steady-state agonist concentrations were recorded at -80 mV (filtered at 4 kHz, digitized at 20 kHz), and open probability ($P_{\text{open}}$) evaluated from all-points current amplitude histograms as described(12). Maximal conductance and two prominent subconductances from proton block were characteristic of previously studied X-chimeras(12,22) and the RO-chimera, RO-bA1(24,25). $P_{\text{max}}$ was determined as $P_{\text{open}}$ from continuous stretches of data (>30s) in: 10 µM cAMP for X-fA4, 3 mM cAMP or cGMP for RO-fA4, 30 mM cAMP for RO-rA4, 3 mM cGMP or 30 mM cAMP for RO-fA4 Asp. To normalize dose-responses in terms of $P_{\text{open}}$ for Fig. 3, 4, and 6, data and Hill fits for both agonists were multiplied by $P_{\text{max}} / I_{\text{max}}$ of the agonist giving higher $I_{\text{max}}$.

$P_{\text{max,cAMP}}$ values in Table III were determined as $P_{\text{max,cAMP}} = I_{\text{max,cAMP}} / I_{\text{max,cGMP}}$ measured at -100 mV in macroscopic current patches. This assumes that open channel conductance is the same in saturating cAMP and cGMP, and that $P_{\text{max,cGMP}}$ is near unity. If the latter assumption is omitted, $I_{\text{max,cAMP}} / I_{\text{max,cGMP}}$ is an upper limit on $P_{\text{max,cAMP}}$. However, both assumptions were validated for RO-bA1 previously(13,21) and for RO-fA4 Asp in this study, so likely hold for RO-chimeras derived from these original two chimeras. In addition, $P_{\text{max,cGMP}} > 0.95$ was verified directly in single-channel recordings for at least two patches each (>3 mM cGMP) of: RO-bA1{4}, RO-bA1{4.1}, RO-bA1{4.2}, RO-bA1{1-3}. We assumed a fully liganded channel has a single closed and a single open state, so the free energy of opening in saturating cAMP is $\Delta G_{\text{sat,cAMP}} = -RT \ln [P_{\text{max,cAMP}} / (1 - P_{\text{max,cAMP}})]$. The change in $\Delta G_{\text{sat,cAMP}}$ associated with converting RO-bA1 to a subregion chimera was calculated by subtracting the mean $\Delta G_{\text{sat,cAMP}}$ for RO-bA1 from the mean $\Delta G_{\text{sat,cAMP}}$ for the new chimera, that is, $\Delta \Delta G_{\text{sat,cAMP}} = \Delta G_{\text{sat,cAMP}}[\text{new}] - \Delta G_{\text{sat,cAMP}}[\text{RO-bA1}]$.

Unless otherwise noted, means are reported ± SD with n the sample size, and unpaired t-test was used to assess significance in population differences.
RESULTS

Agonist sensitivities of diverse BD sequences directly compared using BD-substitution chimeras.

Our previously studied "X-chimeras" (12) all share identical sequence outside the BD region; differences between X-chimeras can thus be attributed to BD sequence differences. The invariant non-BD sequence ("X-") consists of bovine CNGA1 (bCNGA1) sequence with two sequence regions replaced by corresponding residues from catfish CNGA2 (fCNGA2) for technical advantages (see Fig. 1A). Replacement of the P-loop increases ion conductance through the open channel(23), facilitating current detection in both macroscopic current and single-channel recordings. Replacement of the "N-S2" region favors the intrinsic opening transition, enhancing the response to any agonist(21,27). These two replacements increase the chances of observing cyclic nucleotide-activated currents from X-chimeras containing BDs of unknown functionality that might work poorly in activation.

{Figure 1 here}

The previous study tested BDs from bCNGA1, fCNGA2 and rat CNGA4 (rCNGA4); this study included BDs from more phylogenetically diverse isoforms, namely CNGA4 recently cloned from catfish olfactory epithelium (fCNGA4), and TAX-4 and TAX-2 from the nematode C. elegans (Fig. 1B, C). Whereas bCNGA1, fCNGA2, and TAX-4 are classed as "conventional" type because they can form functional homomeric CNG channels, rCNGA4, fCNGA4 and TAX-2 are classed as "modulatory" type because they cannot form functional homomeric CNG channels but do coassemble with conventional subunits in heteromers. Nonetheless, X-chimeras derived from BDs of either conventional or modulatory subunits were equally capable of expression alone in Xenopus oocytes, forming channels that responded to cyclic nucleotide when assayed in excised inside-out membrane patches using voltage-clamp recording. Thus like the previously studied rCNGA4 BD, the BDs of the modulatory subunits fCNGA4 and TAX-2 can support channel activation in a homomeric channel without relying on any residues of a conventional subunit BD.

Sensitivity of the X-chimeras to low agonist concentrations was quantified by $K_{1/2}$, the concentration eliciting half-maximal activation (Table I). The chimera X-fA4, containing the fCNGA4
BD, stands out because of its extreme sensitivity to cAMP. Single-channel records (Fig. 2A) show that 10 µM cAMP is sufficient to increase the open probability (P_{\text{open}}) of X-fA4 to >0.99 (only rare brief channel closures detected). X-fA4 is also sensitive to micromolar cGMP, but this property is not unique, appearing also in X-bA1, X-TAX4, and X-TAX2. However, these latter chimeras have K_{1/2,cAMP} more than an order of magnitude higher than K_{1/2,cGMP}. Thus the fCNGA4 BD in X-fA4 is unique in that its high sensitivity applies similarly to both agonists.

(Table I and Fig. 2 here)

A given subsaturating concentration of cGMP reliably elicited larger X-fA4 currents than did the same concentration of cAMP (Fig. 2B, upper traces). This cGMP selectivity was unexpected because the fCNGA4 BD has a methionine residue in its putative C-helix ligand contact position(11), where other cGMP-selective X-chimeras (X-bA1, X-TAX4, and X-TAX-2) have aspartate. In fact, in the rCNGA4 BD, a methionine ligand-contact imparts cAMP selectivity(28,29), as confirmed in X-rA4(12). The cGMP selectivity of X-fA4 might thus suggest that the methionine in the fCNGA4 C-helix cannot form the ligand interaction required for cAMP selectivity, perhaps because of a different structure in this region. We disproved this possibility by constructing a new X-chimera, named X-rA4_{R}/fA4_{C}, which contains the roll subdomain of rCNGA4 (indicated by subscript R) and the C-helix of fCNGA4 (indicated by subscript C); this chimera shows cAMP selectivity at low agonist concentrations (Fig. 2B, C). Fig. 2D summarizes K_{1/2} measurements for cAMP and cGMP in individual patches of X-fA4, X-rA4, and X-rA4_{R}/fA4_{C}, which all have the methionine ligand-contact. Even though the absolute K_{1/2} values observed for a chimera varied from patch to patch, the K_{1/2} selectivity of X-fA4 was always in favor of cGMP, whereas both X-rA4 and X-rA4_{R}/fA4_{C} always favored cAMP. This shows that the fCNGA4 C-helix has cAMP-selective determinants (presumably including the ligand-contact methionine) similar to those of the rCNGA4 C-helix. We propose that the intact fCNGA4 BD exhibits cGMP selectivity because the fCNGA4 roll subdomain contains some cGMP-selective elements, whose energetic contributions to gating properties outweigh those of the cAMP-selective C-helix.
Efficacies compared using a new series of BD-substitution chimeras.

Comparisons of X-chimeras clearly show differences in BD sensitivity, but the $K_{1/2}$ parameter in isolation is poorly informative of microscopic physical processes such as ligand binding or channel opening(30). It is more valuable to compare the efficacy (maximal open probability, $P_{\text{max}}$) in saturating agonist concentrations where every channel should have uniform (i.e., maximal) BD occupancy. Unfortunately, several X-chimeras have extremely high efficacy (e.g., X-fA4 in Fig. 2A), and differences in their respective equilibrium constants for channel opening at saturating agonist concentration would not be easily detectable, because the numerical difference in their $P_{\text{max}}$ values would be too small. We predicted that BD-substitution chimeras using a design similar to the X-chimeras, but containing a different N-S2 sequence that disfavored intrinsic channel opening, might exhibit efficacies that were significantly less than unity; efficacy differences between chimeras would then be more readily apparent.

We therefore incorporated the BDs from modulatory subunits (rCNGA4, fCNGA4, TAX-2) into a new series of chimeras, called "RO-chimeras", in which the N-S2 sequence was that of intact bCNGA1 (see Fig. 3A). The RO-chimeras still contain the P-loop of fCNGA2 to facilitate single-channel recording, but all sequence N-terminal of the P-loop matches exactly that of bCNGA1. All of the RO-chimeras were found to be capable of forming functional homomeric CNG channels, repeating our success with the X-chimeras. Table II summarizes the properties of the new RO-chimeras, and includes also RO-bA1 containing the bCNGA1 BD, which was called RO133 in previous studies(13,21,23-25).

The $K_{1/2}$ selectivity properties of RO-chimeras are similar to those of corresponding X-chimeras (compare Tables I and II); however, the absolute $K_{1/2}$ values are higher in the RO-chimeras than in corresponding X-chimeras, for any BD and any agonist. Besides this sensitivity difference, RO-chimeras have lower efficacy (Fig. 3A) than corresponding X-chimeras. RO-rA4, with the rCNGA4 BD, has mean $P_{\text{max,cAMP}} = 0.468 \pm 0.090$ (n = 3), whereas X-rA4 was previously(12) found to have a much higher $P_{\text{max,cAMP}}$ of 0.980 $\pm$ 0.025 (n = 5). RO-fA4, with the fCNGA4 BD, exhibits a high mean $P_{\text{max,cAMP}}$ of 0.974.
± 0.034 (n = 5), but this efficacy is nonetheless lower than that of X-fA4, as evinced by far more frequent channel closures (compare Figs. 3A and 2A). Thus multiple observations show that introduction of the bCNGA1 N-S2 in RO-chimeras reliably reduced activation efficiency compared to the corresponding X-chimeras; nevertheless, each chimera retains the ability to form functional homomeric channels with particular agonist selectivity properties controlled by the BD sequence.

Even though RO-fA4 exhibits less efficient activation than X-fA4, its efficacy is nevertheless unusually high compared to other RO-chimeras. The mean $P_{\text{max,cAMP}}$ of RO-fA4 is more than double that of RO-rA4, and $P_{\text{max,cGMP}}$ for RO-fA4 (not measured directly) must be similar to $P_{\text{max,cAMP}}$ since $I_{\text{max,cAMP}} / I_{\text{max,cGMP}}$ was essentially unity in macroscopic current experiments. Thus efficacy of RO-fA4 significantly surpasses that of RO-rA4, for both agonists (Fig. 3B). Moreover, the cAMP efficacy of RO-fA4 far surpasses those of RO-chimeras with bCNGA1 and TAX-2 BDs, whose extremely low $I_{\text{max,cAMP}} / I_{\text{max,cGMP}}$ values indicate low $P_{\text{max,cAMP}}$. Thus, comparative analysis of the RO-chimera series confirms the previous analysis of the X-chimera series: the BD of the modulatory subunit fCNGA4 is unique in mediating extremely efficient activation by both cGMP and cAMP.

*Identification of regions of the fCNGA4 BD responsible for high cAMP efficacy.*

The unusually efficient cAMP activation of RO-fA4 must derive from residues in the fCNGA4 BD not conserved in the other BDs studied — but does this include the C-helix ligand-contact? To test the importance of this methionine residue in RO-fA4 we mutated it to aspartate; this should introduce the same mechanism for strong cGMP selectivity used in RO-bA1. This expectation is borne out in the mutated chimera, RO-fA4 Asp. Relative to RO-fA4, the aspartate mutation dramatically decreases the $K_{1/2}$ selectivity ratio more than 100-fold to $(3.29 \pm 0.42) \times 10^{-3}$ (n = 7), because $K_{1/2,cGMP}$ is lower and $K_{1/2,cAMP}$ is higher (Fig. 4A: compare RO-fA4 Asp data in black circles, with RO-fA4 curves in gray). The efficacy selectivity ratio is also decreased: macroscopic RO-fA4 Asp current experiments showed $I_{\text{max,cAMP}} / I_{\text{max,cGMP}} = 0.71 \pm 0.18$ (n = 10), and single-channel recordings (Fig. 4B) show extremely high
The ratio \( \frac{I_{\text{max,cAMP}}}{I_{\text{max,cGMP}}} \) from macroscopic experiments is higher than the ratio \( \frac{P_{\text{max,cAMP}}}{P_{\text{max,cGMP}}} \) from single-channel experiments, possibly because spontaneous "runup" drifts in activation properties(26) were more complete in macroscopic experiments. Thus the strong cGMP selectivity of RO-fA4 Asp confirms our conclusion from X-rA4p/fA4c, that the predicted ligand-contact position of the fCNGA4 C-helix is indeed a selectivity determinant, just as it is in the other BDs studied.

Although mutating the ligand-contact in RO-fA4 from methionine to aspartate did decrease cAMP efficacy, the cAMP efficacy of RO-fA4 Asp is still much higher than that of RO-bA1 which also has an aspartate ligand-contact (Fig. 4A: compare RO-fA4 Asp, solid circles, with RO-bA1, solid triangles). Thus while the highly efficient cAMP activation mediated by the fCNGA4 BD is partly due to the presence of a cAMP-selective methionine ligand-contact, it is also due in significant part to residues distinct from the ligand-contact which are not conserved with the bCNGA1 BD. To locate residues responsible for the functional difference between RO-fA4 Asp and RO-bA1, we constructed a number of new RO-chimeras by substituting selected subsequences of the fCNGA4 BD one at a time into the bCNGA1 BD of RO-bA1. The C-helix ligand-contact was aspartate in every new chimera, and we used the resultant high cGMP efficacy to our advantage, estimating \( P_{\text{max,cAMP}} \) rapidly from macroscopic current measurements of \( \frac{I_{\text{max,cAMP}}}{I_{\text{max,cGMP}}} \). From \( P_{\text{max,cAMP}} \) we calculated the Gibbs free energy of channel opening in saturating cAMP, \( \Delta G_{\text{sat,cAMP}} \), with a negative value indicating favorable opening (see Experimental Procedures). The baseline reference for the "subregion replacement" chimeras is RO-bA1, with \( \Delta G_{\text{sat,cAMP}} = +9.4 \pm 1.3 \text{ kJ/mol} \) (n = 17); this value was subtracted from each chimera's \( \Delta G_{\text{sat,cAMP}} \) value to calculate \( \Delta \Delta G_{\text{sat}} \), the net energetic effect of the sequence substitution.

The first five subregion chimeras, called RO-bA1\{1\} through RO-bA1\{5\}, together constitute a complete screen of the BD residues of RO-fA4 Asp (see Fig. 5A). In each of these five chimeras, the bCNGA1 subsequence replaced contains many residues that are conserved between RO-bA1 and RO-fA4...
Asp; the number of unconserved residues ranges from four to nine (Fig. 1C). Fig. 5B and Table III shows that of these five chimeras, the highest cAMP efficacy (lowest $\Delta G_{\text{sat,cAMP}}$) is found in RO-bA1{4}, with $\Delta \Delta G_{\text{sat}} = -9.0 \pm 1.9$ kJ/mol ($n = 12$), a large (>3kT) energetic change in favor of opening. The subregion responsible for this phenotype starts at the beginning of the B-helix and ends five residues into the C-helix; this "hinge" structure bridges the junction between the two major subdomains of the BD. Within the hinge, might there be one amino acid difference that alone accounts for the difference between RO-bA1{4} and RO-bA1? For instance, a previous study (31) found that gating was affected by a mutation just before the conserved hinge proline, which is tyrosine 586 in bCNGA1 but phenylalanine in fCNGA4. We constructed two new chimeras, RO-bA1{4.1} and RO-bA1{4.2}, containing replacements of portions of the hinge, either N- or C-terminal to the dividing proline (see Fig. 5A). Both of these exhibit enhanced cAMP efficacy relative to RO-bA1, with $\Delta \Delta G_{\text{sat}}$ of about -5.0 kJ/mol (about 2kT). We conclude that the enhanced cAMP efficacy of RO-bA1{4} derives from multiple fCNGA4 residues in the hinge region, including one or more residues distinct from the previously studied position 586.

Replacing the hinge of RO-bA1 to make RO-bA1{4} did not reconstitute the extremely low $\Delta G_{\text{sat,cAMP}}$ value of RO-fA4 Asp. We noticed that replacements of sequences N-terminal of the hinge region (the A-helix and $\beta$-roll) produced small but detectable enhancements of cAMP efficacy. We combined all the substitutions of RO-bA1{1}, {2}, and {3} in a new chimera, RO-bA1{1-3}, with a large $\Delta \Delta G_{\text{sat}} = -8.6 \pm 1.6$ kJ/mol ($n = 11$), which is >3kT. This shows that fCNGA4 residues in different parts of the A-helix+$\beta$-roll sequence make a significant collective contribution (similar to that of the hinge) to the high efficacy of RO-fA4 Asp. We went further to test the effects of replacing the entire roll subdomain (A-helix, $\beta$-roll, and B-helix), a substitution analogous to those made previously in X-chimeras. The chimera RO-fA4$/$bA1$\_C$, combining the substitutions from RO-bA1{1}, {2}, {3}, and {4.1}, has $\Delta G_{\text{sat,cAMP}}$ lower than that of any of the initial subregion chimeras, and in fact slightly lower than that of RO-fA4 Asp itself. In complementary fashion we replaced the C-helix of RO-bA1 with that of RO-fA4.
Asp to produce the chimera RO-bA1/fA4 C Asp, which combines the substitutions of RO-bA1{4.2} and {5}. This chimera has poor efficacy, similar to that of RO-bA1. Therefore the high cAMP efficacy of RO-fA4 Asp can be largely explained by the roll subdomain residues found in fCNGA4 and not in bCNGA1.

It is notable that when several subregion replacements are combined in one chimera, a summation of their ΔΔG_{sat} values approximates the ΔΔG_{sat} for the resultant combination chimera. For instance, the ΔΔG_{sat} values for swapping the two major BD subdomains separately (in RO-fA4/bA1 C and RO-bA1{4}/fA4 C Asp) can be summed to make -12.2 ± 2.6 kJ/mol, which closely matches the effect of a complete BD swap (ΔΔG_{sat} = -11.9 ± 2.5 kJ/mol in RO-fA4 Asp). Likewise, the sum of ΔΔG_{sat} values for RO-bA1{1}, RO-bA1{2}, and RO-bA1{3} is -6.1 ± 3.7 kJ/mol, which is within kT of the ΔΔG_{sat} value -8.6 ± 1.6 kJ/mol of RO-bA1{1-3}. Although summing multiple ΔΔG_{sat} values introduces some imprecision in our assessment, it appears that several small subregions (mostly in the roll subdomain) make individually small contributions, which combine their energetic effects independently (i.e., additively) in the intact fCNGA4 BD to produce exceptionally high efficacy.

**Distinct effects of BD subregions on cAMP and cGMP activation.**

The favorable activity determinants identified in the fCNGA4 BD might in principle enhance cGMP activation as well as cAMP activation. Since RO-bA1 already has high cGMP efficacy (13,21) of P_{\text{max},\text{cGMP}} = 0.94 ± 0.03, any enhancement of cGMP activation from a fCNGA4 subregion replacement would be detectable as a decreased K_{1/2,cGMP}. Fig. 5C shows that most of our subregion chimeras did exhibit lower K_{1/2,cGMP} than RO-bA1, but only replacement of the entire roll subdomain replicated the extremely low K_{1/2,cGMP} of RO-fA4 Asp. Thus efficient cGMP activation in RO-fA4 Asp, like efficient cAMP activation, arises from multiple fCNGA4 BD elements which are clearly distinct from the C-terminal region of the C-helix around the ligand-contact residue. A correlation between enhanced cAMP activation and enhanced cGMP activation in subregion chimeras is reminiscent of that observed earlier in comparisons of RO-chimeras with X-chimeras. This might suggest that the different replacements had
essentially the same mechanism as an N-S2 replacement(21,27), namely to stabilize the open state in an agonist-independent manner, without discriminating between cAMP and cGMP. However, this cannot be correct, because as we argue below, selected chimeras provide exceptions to the general rule of correlated effects on cAMP and cGMP activation, and lead us to conclude that individual subregions control distinct structural features of the ligand-gating mechanism.

Some chimeras in our study have mutations in entirely different subregions but by coincidence have similar cAMP efficacy. For instance, fCNGA4 A-helix+β-roll replacement and fCNGA4 hinge replacement (in RO-bA1{1-3} and RO-bA1{4}, respectively) both produce similar ΔΔG_{sat} values. Strikingly however, RO-bA1{4} exhibits significantly weaker agonist sensitivity. Table III and Fig. 6A show that for either cGMP or cAMP, the K_{1/2} for RO-bA1{4} (Fig. 6A, triangles) is 10-fold higher (P<10^-6) than that for RO-bA1{1-3} (circles). In fact, the K_{1/2,cAMP} of RO-bA1{4} (solid triangles) is even higher (P<0.02) than that of RO-bA1 (gray solid line). This means, for instance, that a 10 mM concentration of cAMP is not high enough to saturate the binding sites of RO-bA1{4}, as it would be for RO-bA1 or for RO-bA1{1-3}. Thus hinge replacement and A-helix+β-roll replacement both enhance the energetics of opening a channel that is fully liganded by cAMP, but hinge replacement additionally has a paradoxically deleterious effect on the channel's overall ability to bind cAMP. Hence these particular mutation sets in the hinge and A-helix+β-roll represent two different modifications to the ligand-gating energetics. While their effects on cAMP efficacy happen to be identical, their full mechanistic consequences (including those relevant to cGMP activation and cAMP binding) are in fact not identical.

Comparison of RO-bA1{4} and RO-bA1 with another subregion chimera, RO-bA1{3}, further illustrates divergent mechanistic effects and moreover supports the notion that subregions outside the C-helix can govern agonist selectivity. The sequence replacement producing RO-bA1{3} starts with the PB-loop and ends with the last (eighth) strand of the β-roll, so we call this sequence the "loop-β8" region. The cGMP dose-responses of RO-bA1{3} and RO-bA1{4} are nearly coincident (Fig. 6B, up- and down-
point open triangles), with no significant difference in mean $K_{1/2,cGMP}$ ($P>0.1$). Yet whereas hinge replacement results in greatly enhanced cAMP efficacy compared to RO-bA1, loop-β8 replacement leaves cAMP sensitivity unchanged ($P>0.6$) and has minimal effects on cAMP efficacy ($P<0.03$, but $\Delta\Delta G_{sat}$ is only -1.3 kJ/mol). Therefore loop-β8 replacement and hinge replacement represent two distinct ways to enhance cGMP sensitivity (either by facilitating channel opening or cGMP binding, or both) which are distinguishable by their different effects on cAMP activation. The molecular interactions governing cAMP activation and cGMP activation must differ at one or more steps, and it is at such a step that the loop-β8 region must interact with agonist to produce its cGMP-selective effect.
DISCUSSION

This study advances our understanding of structural features that control the energetics of the CNG channel mechanism and thereby tune the channel's overall response profile (sensitivity and efficacy for given agonists). First, we used the BD-substitution strategy for comparative analysis of highly divergent BD sequences, to identify a particular BD sequence which mediated unusually efficient channel activation and therefore deserved closer attention. Next, we showed that sizable energetic contributions to efficacy derived from sequences outside the previously studied C-helix ligand-contact, specifically the "hinge" sequence bridging the roll and C-helix subdomains, as well as the A-helix and β-roll sequences N-terminal of the hinge. Finally, we showed that multiple BD regions contributing to efficient function (such as the hinge or loop-β8) govern different features of the ligand-gating mechanism, since sequence changes in these regions affect efficacy, sensitivity, and selectivity in a differential manner.

*BD-substitution chimeras enable systematic comparison of divergent BDs.*

The success of our first study introducing BD-substitution chimeras(12) has been generalized here: functional homomeric CNG channels are formed by X-chimeras containing BDs of widely divergent subunits, including even TAX-2, a modulatory subunit in the B-subfamily. Moreover, both conventional and modulatory subunit BDs can accommodate the unfavorable channel opening energetics imparted by the bCNGA1 N-S2 region in RO-chimeras. BD-substitution chimeras thus represent a simple method (the only one available) for studying modulatory subunit BDs in the absence of conventional subunit BD sequence, and for making direct functional comparisons of a diverse pool of BD sequences.

The catfish CNGA4 BD produced extremely strong ligand sensitivity and high efficacy which were obviously exceptional among the six BDs surveyed, and its sequence may prove useful for engineering sensitive cyclic nucleotide biosensors for applications such as patch-cramming(32). Although the rat and fish CNGA4 orthologues both occur in olfactory epithelium, their BDs differ markedly in function, consistent with the numerous sequence differences that arose since the divergence of mammals from fishes (see Fig. 1B and C). Notably, the catfish olfactory conventional subunit (fCNGA2) is
expected to coassemble with fCNGA4 in vivo, but has a BD whose function is inefficient compared to related mammalian BDs such as bCNGA1(12). We speculate that during evolution, gain-of-function mutations accumulated in the fCNGA4 BD as an adaptive compensation for deleterious mutations in the fCNGA2 BD. These mutations did not radically change the protein fold of the fCNGA4 BD, which retains the C-helix ligand-contact mechanism for agonist selectivity found in the bCNGA1 and rCNGA4 BDs. We believe that the fCNGA4 BD preserves the essential features of structure and mechanism typical of CNG channels, but fortuitously contains numerous particular amino acids favorable for activation.

Multiple subregions contribute to efficient BD function by distinct mechanisms.

We identified RO-fA4 Asp and RO-bA1 as a pair of chimeras with dramatically different function, and then sought elements responsible for this difference, which must lie within the BD region. This could include not only BD tertiary structure differences but also differences in functionally important interactions between the BD and non-BD regions. We need not assume that every interaction between BD and non-BD regions will be replicated in every chimera; on the contrary, since the non-BD regions are invariant, a failure of one particular BD sequence to form such a required interaction would lead to the identification of the missing structural element of the BD as important for efficient activation.

Our systematic piecewise scan of the entire BD sequence is complementary to many previous studies of cyclic nucleotide-activated proteins which depended on homology models of the BD structure for the initial identification of particular residues with likely functional relevance. Several BD residues identified in this way were previously studied by mutagenesis(11,22,26,31,33-35), but they are all either conserved between the two BDs examined here or else (bCNGA1 Tyr586) were shown here not to be sufficient to explain the observed functional differences. Another previous study(8) compared the BD structures of EPAC (a G-protein nucleotide exchange protein) and cAMP-dependent protein kinase (PKA) in unliganded and liganded form respectively, and predicted that the hinge region might control cAMP activation since its conformation differed in the two structures. That study then validated its prediction by mutagenesis of the hinge of EPAC, but did not compare the hinge's contributions with those from other parts of the BD which might have been of equal importance. Our study provides an important
confirmation that the hinge is a prominent control element in CNG channels, without making assumptions about the (unknown) BD structure; yet we also establish that the energetic contributions of the A-helix+β-roll match those of the hinge region in magnitude.

We found that fCNGA4 sequences for different BD subregions influence efficacy without any marked synergistic effect, i.e., their energetic effects are additive, with no added advantage derived from being present in combination. Neither do the individual effects of distinct replacements occlude one another significantly, which would imply that both modified the same set of molecular interactions(36). Additivity also applies to the roll and C-helix subdomains, which have been envisioned(10,35) as independently folded units separated by a flexible proline pivot. This contrasts with the previous finding(12) that synergistic subdomain interaction was a key factor enabling the rCNGA4 BD to produce higher efficacy than the fCNGA2 BD. The likely explanation is that some roll-C-helix interactions do contribute to efficacy, but these are all conserved in the fCNGA4/bCNGA1 BD pair and not conserved in the rCNGA4/fCNGA2 BD pair. The high efficacy of the fCNGA4 BD relative to the bCNGA1 BD does not depend on roll-C-helix interactions, but can be attributed to fCNGA4 roll subdomain residues working independently of the C-helix, i.e., directly contacting a part of the agonist that does not also contact the C-helix, or else interacting with non-BD residues.

Additivity of cAMP efficacy effects implies that subregions act independently, and the complete response profiles show further that the subregions participate in different control mechanisms. Thus, hinge replacement and A-helix+β-roll replacement have different effects on efficacy vs. binding. Moreover, hinge replacement and loop-β8 replacement have different effects on cAMP vs. cGMP activation. This finding of agonist selectivity determinants distinct from the C-helix ligand contact reinforces earlier results in X-chimeras, showing that the fCNGA4 roll subdomain imparts K_{1/2} selectivity for cGMP (Fig. 2), and that the fCNGA2 roll subdomain causes cGMP-dependent desensitization(12). The existence of agonist-selective effects, and more generally the finding of distinct mechanisms of action, are important because they show that the individual subregions cannot exert their influence solely
by open state stabilization in an agonist-independent manner, like the N-S2 region(21). Thus models for CNG channel gating must identify plausible agonist-dependent mechanisms by which non-C-helix regions can contribute to efficient activation.

Proposed mechanisms.

Fig. 6C uses the BD of HCN2 (an ion channel from a family closely related to CNG channels(9)) to show putative locations of the BD subregions that we have identified as control elements. Various regions of the roll (including the loop-β8) are clearly implicated in binding the cyclic phosphate and ribose moieties of the ligand. However, formation of specific stable interactions between the ligand and its BD is by itself insufficient to promote channel opening. Rather, ligand-gating requires that interactions of the protein and agonist increase their stability during channel opening, so that more stable protein-agonist interactions form in the open channel than in the closed channel. In this regard, the hinge region is intriguing, because it lies at a remove from the binding pocket. An important contribution from the hinge is plausible in one mechanism proposed for CNG channels(35,37), which posits that docking of the C-helix ligand-contact is the key structural event stabilizing the open channel. In this "C-helix fit" model, the C-helix is a rigid but mobile element whose docking entails rearrangement of the hinge, so the stability of a particular hinge sequence in the open state vs. the closed state would be an efficacy determinant structurally independent of the ligand-contact. To rationalize the observation that the fCNGA4 hinge replacement simultaneously enhances cAMP efficacy and weakens cAMP binding, we speculate that in the closed channel, the fCNGA4 hinge structure is unusually unstable, yet places the C-helix in a position that blocks access of the ligand to the PB cassette. Thus the hinge region could control efficacy by an agonist-independent mechanism (consistent with a previous finding(31) for mutation of Tyr586 to alanine) but also indirectly affect the initial binding step in an agonist-dependent mechanism.

Unfortunately, the C-helix fit model does not readily explain the prominent effects of mutating the roll subdomain. On the contrary, in its minimal form this model implies a "functional polarity" in the BD, in which the β-roll controls only the initial binding of the agonist via the cyclic phosphate and ribose moieties of the ligand.
moieties(22,35,37). One way to address this inadequacy is to posit a conformational change in the β-roll. Indeed, deformation of the PB cassette to fit the agonist molecule better is a defining feature of a model proposed for PKA and EPAC(8). In this “PB fit” model, the PB cassette, B-helix, and C-helix form a network of bonds whose geometry rearranges during activation, so sequence differences in all these regions should determine the energetics of the activating transition. Another way to explain efficacy control by the β-roll is a "purine rotamer" concept, which was implicit in the original proposal of the C-helix fit model(11) but not explicitly stated: namely, that the cyclic nucleotide itself changes conformation during activation. The highly stable interaction between the purine ring and the C-helix in the open channel requires an anti conformation for the cyclic nucleotide(11,38); the rotamer model simply posits that the cyclic nucleotide has a syn conformation in the closed channel that is stabilized (to a lesser extent) by one or more interactions between the purine ring and the β-roll, so that the activation transition requires rotation of the purine ring around its N-glycosidic bond. The β-roll itself need not change conformation, and the particular residues interacting with the purine might even be conserved in many CNG channels, but a small modification to roll geometry from introducing fCNGA4 sequence could destabilize its purine interaction, hence favoring purine rotation towards the C-helix and increasing efficacy.

The PB fit and the purine rotamer model both account for roll effects on efficacy and are not mutually exclusive, but the rotamer model has two notable advantages. First, it naturally separates mechanistic contributions of the C-helix ligand-contact, hinge, and β-roll regions, consistent with the finding that at least some sequence changes in these regions have independent effects on gating. Second, purine-roll interactions can explain easily how the β-roll can discriminate between cyclic nucleotides, as found in the loop-β8 replacement. For instance, in the HCN BD structure(9), the PB-loop interacts with the N2 atom of cGMP (absent from cAMP). It is not known whether that structure corresponds to an open or closed channel, and more importantly it remains to be seen whether roll residues interacting with the purine of either cAMP or cGMP can be identified in CNG channels. To this end, the BD-substitution
approach should be valuable as a generally applicable experimental context for performing structural modifications to the BD (by genetic or chemical methods) and quantifying the resultant energetic effects on channel gating.

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REFERENCES


**FOOTNOTES**

Abbreviations used are: CNG, cyclic nucleotide-gated; BD, ligand-binding domain; bCNGA1, bovine CNGA1; fCNGA2, catfish CNGA2; rCNGA4, rat CNGA4; fCNGA4, catfish CNGA4; PB, cyclic-phosphate-binding; EPAC, exchange protein activated by cAMP, PKA, cAMP-dependent protein kinase.
Fig. 1: **Chimeras for comparing BDs from diverse CNG channel isoforms.** A, Topology schematic of CNG channel subunit sequence shows transmembrane segments (vertical rectangles) and a cytoplasmic BD with a roll subdomain (omega-shaped loop) and C-helix (horizontal rectangle) containing a ligand-contact (star). Examples of X-chimeras with different BD sequences and ligand-contacts are shown, using line styles to indicate sequence derivation: bCNGA1, thin black solid; fCNGA2, thick gray solid; rCNGA4, gray-and-white striped; fCNGA4, black-and-white striped. B, Additive-branch phylogenetic tree (PHYLIP) of BD sequences from rat CNG genes, and additional BD sequences used in this study (boldface). C, Alignment of BD sequences (numbers refer to bCNGA1; dots indicate identity with bCNGA1) with dashes indicating gaps. Helices (marked "α") and strands ("β") are predicted by homology with the HCN2 channel BD(9); star marks the ligand-contact. Dashed lines delimit sequences exchanged in construction of BD-substitution chimeras; brackets delimit fCNGA4 residues exchanged in subsequent construction of subregion chimeras (Fig. 5.)

Fig. 2: **The fCNGA4 BD mediates efficient activation with cGMP selectivity.** A, Excerpted recordings of a single homomeric X-fA4 channel in an inside-out patch held at -80 mV, with steady-state cAMP concentrations indicated. Dashes mark closed ("C") and open ("O") channel currents. B, Macroscopic currents of X-fA4 or X-rA4/fA4 homomers (line styles as in Fig. 1A) in inside-out patches at -80 mV, in response to perfused agonists (open bars; dotted outlines indicate agonist washout). Gaps in traces are arbitrary time intervals. C. Relative currents of X-fA4 (circles) and X-rA4/fA4 (triangles) activated by cAMP (solid) and cGMP (open), in the patches shown in B at -100 mV. Lines are fits of the Hill equation. D, Each connected pair of points plots $K_{1/2,cAMP}$ (solid) and $K_{1/2,cGMP}$ (open) obtained from an individual patch from a distinct oocyte expressing either X-fA4 (circles), X-rA4/fA4 (up-point triangles), or X-rA4 (down-point triangles). Point pairs are horizontally displaced for visual clarity. Data for X-rA4 from ref.(12).
Fig. 3: Chimeras with disfavored opening reveal efficacy differences. A, Design and single-channel recordings of RO-chimera homomers containing fCNGA4 or rCNGA4 BDs. Note that except for the P-loop and BD, sequence is identical to that of intact bCNGA1 (thin black lines). Recording excerpts (collected as in Fig. 2A) have $P_{\text{open}}$ values of 0.964 (RO-fA4) and 0.436 (RO-rA4). B, Points plot cAMP (solid) and cGMP (open) activation data from representative patches of RO-fA4 (circles) and RO-rA4 (triangles), normalized using respective $P_{\text{max,cAMP}}$ values of 0.974 and 0.468 (means from single-channel measurements); lines show Hill fits.

Fig. 4: fCNGA4 BD with a cGMP-selective ligand-contact mutation maintains efficient cAMP activation. A, Points plot representative patch data for cAMP (solid) and cGMP (open) activation of RO-fA4 Asp (circles) and RO-bA1 (triangles), normalized with respective single-channel mean $P_{\text{max,cGMP}}$ values of 0.99 and 0.94. Lines show Hill fits for cAMP (solid) or cGMP (dashed) activation of RO-fA4 Asp and RO-bA1 (black) and of RO-fA4 (gray, curves from Fig. 3B). B, Recordings from a single RO-fA4 Asp homomer. $P_{\text{open}}$ for excerpts: 0.997 in cGMP and 0.609 in cAMP.

Fig. 5: Identifying subregions of the fCNGA4 BD that produce efficient activation. A, Composition of BD subregion chimeras. Horizontal lines represent the BD sequence of each RO-chimera, showing sequence derivation from RO-bA1 (solid black) or RO-fA4 Asp (black-and-white striped); segment lengths are not strictly to scale. Vertical dashed line marks position of proline between the roll and C-helix subdomains; alphanumeric symbols under RO-bA1{5} schematic indicate positions of secondary structure elements (Fig. 1C). All chimeras shown have aspartate at the C-helix ligand-contact position. B, Free energy of opening in saturating cAMP. Each point in a row plots data for one patch of the indicated chimera, from a distinct oocyte. C, $K_{1/2}$ for cGMP activation, plotted as in B.

Fig. 6: fCNGA4 subregion replacements with differential effects on efficacy, binding, and selectivity. A, Replacements of the A-helix+$\beta$-roll and hinge show different effects on sensitivity. Points
plot representative data for activation by cAMP (solid) and cGMP (open) of RO-bA1{1-3} (circles) and RO-bA1{4} (triangles), normalized with arbitrary $P_{\text{max,cGMP}} = 0.99$. Lines show Hill fits for cAMP (solid) or cGMP (dashed) activation of RO-bA1{1-3} and RO-bA1{4} (black) and of RO-bA1 (gray, curves from Fig. 4A). B, Loop-$\beta_8$ replacement shows cGMP selectivity. Plots of RO-bA1{4} and RO-bA1 data are as in A; down-point triangles plot dose-response data for activation of RO-bA1{3} by cAMP (solid) and cGMP (open), normalized as in A. C, Hinge (red) or A-helix+$\beta$-roll and loop-$\beta_8$ (yellow) in the BD's putative three-dimensional structure, represented by the cAMP-liganded HCN2 BD (RASTER3D diagram); star marks C-helix ligand-contact.
Table I.
*Activation properties of BD-substitution CNG channel chimeras ("X-chimera" series).*
Number of patches is given in parentheses for means ± SD of values from individual patches; values without number of patches are ratios of means.

<table>
<thead>
<tr>
<th>Chimera</th>
<th>BD source</th>
<th>$K_{1/2}$ (µM)</th>
<th>Selectivity Ratios</th>
<th>Synonym and reference for previous data</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
<td>Sensitivity $K_{1/2,cGMP} / K_{1/2,cAMP}$</td>
</tr>
<tr>
<td>X-fA4</td>
<td>catfish CNGA4</td>
<td>1.01 ± 0.17 (11)</td>
<td>0.73 ± 0.10 (11)</td>
<td>0.721 ± 0.034 (11)</td>
</tr>
<tr>
<td>X-rA4</td>
<td>rat CNGA4</td>
<td>81 ± 85 (9)</td>
<td>120 ± 140 (9)</td>
<td>1.45 ± 0.14 (9)</td>
</tr>
<tr>
<td>X-fA2</td>
<td>catfish CNGA2</td>
<td>643 ± 29 (6)</td>
<td>261 ± 12 (6)</td>
<td>0.407 ± 0.026</td>
</tr>
<tr>
<td>X-bA1</td>
<td>bovine CNGA1</td>
<td>432 ± 99 (4)</td>
<td>1.8 ± 1.0 (10)</td>
<td>0.0042 ± 0.0025</td>
</tr>
<tr>
<td>X-TAX4</td>
<td>C. elegans TAX-4</td>
<td>680 ± 37 (2)</td>
<td>3.70 ± 0.59 (2)</td>
<td>0.00546 ± 0.00057 (2)</td>
</tr>
<tr>
<td>X-TAX2</td>
<td>C. elegans TAX-2</td>
<td>74 ± 33 (6)</td>
<td>2.0 ± 1.3 (6)</td>
<td>0.0286 ± 0.0049 (6)</td>
</tr>
</tbody>
</table>
Table II.

*Activation properties of RO-chimeras.*

Values are mean ± SD of measurements from individual patches (number of patches in parentheses).

<table>
<thead>
<tr>
<th>Chimera</th>
<th>BD source</th>
<th>( \text{K}_{1/2} ) (µM)</th>
<th>Selectivity Ratios</th>
<th>Efficacy Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
<td>( \frac{\text{K}<em>{1/2, \text{cGMP}}}{\text{K}</em>{1/2, \text{cAMP}}} )</td>
</tr>
<tr>
<td>RO-fA4</td>
<td>catfish CNGA4</td>
<td>14.5 ± 0.4.4 (8)</td>
<td>11.9 ± 3.3 (8)</td>
<td>0.829 ± 0.075 (8)</td>
</tr>
<tr>
<td>RO-rA4</td>
<td>rat CNGA4</td>
<td>1800 ± 1300 (3)</td>
<td>5000 ± 1900 (3)</td>
<td>3.7 ± 1.6 (3)</td>
</tr>
<tr>
<td>RO-TAX2</td>
<td>C. elegans TAX-2</td>
<td>460 ± 190 (4)</td>
<td>13.5 ± 6.0 (5)</td>
<td>0.034 ± 0.019 (4)</td>
</tr>
<tr>
<td>RO-bA1</td>
<td>bovine CNGA1</td>
<td>2140 ± 410 (4)</td>
<td>55 ± 27 (18)</td>
<td>0.0196 ± 0.0088 (4)</td>
</tr>
</tbody>
</table>
### Table III.

**Activation properties of subregion replacement RO-chimeras.**

Values are mean ± SD of measurements from individual patches (number of patches in parentheses).

<table>
<thead>
<tr>
<th>Chimera</th>
<th>bCNGA1 sequence replaced</th>
<th>$K_{1/2}$ (µM)</th>
<th>Efficacy for cAMP</th>
<th>$\Delta G_{sat,cAMP}$ (kJ/mol)</th>
<th>$\Delta G_{sat}$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
<td>$P_{max,cAMP}$</td>
<td></td>
</tr>
<tr>
<td>RO-bA1</td>
<td></td>
<td>2140 ± 410 (4)</td>
<td>55 ± 27 (18)</td>
<td>0.025 ± 0.12 (17)</td>
<td>+9.4 ± 1.3</td>
</tr>
<tr>
<td>RO-fA4 Asp</td>
<td>V487-N613</td>
<td>264 ± 62 (7)</td>
<td>0.82 ± 0.19 (9)</td>
<td>0.71 ± 0.18 (10)</td>
<td>-2.5 ± 2.1</td>
</tr>
<tr>
<td>RO-bA1[{1}]</td>
<td>V487-R512</td>
<td>2160 ± 730 (2)</td>
<td>23 ± 13 (8)</td>
<td>0.082 ± 0.097 (9)</td>
<td>+6.9 ± 2.4</td>
</tr>
<tr>
<td>RO-bA1[{2}]</td>
<td>I530-Y541</td>
<td>949.5 ± 2.1 (2)</td>
<td>9.1 ± 1.8 (7)</td>
<td>0.058 ± 0.023 (7)</td>
<td>+7.06 ± 0.93</td>
</tr>
<tr>
<td>RO-bA1[{3}]</td>
<td>S553-C573</td>
<td>1910 ± 870 (4)</td>
<td>17.2 ± 4.5 (9)</td>
<td>0.042 ± 0.23 (10)</td>
<td>+8.1 ± 1.6</td>
</tr>
<tr>
<td>RO-bA1[{4}]</td>
<td>D577-M592</td>
<td>3550 ± 760 (6)</td>
<td>13.5 ± 4.8 (10)</td>
<td>0.47 ± 0.14 (12)</td>
<td>+0.4 ± 1.5</td>
</tr>
<tr>
<td>RO-bA1[{5}]</td>
<td>K598-N613</td>
<td>n.d.</td>
<td>185 ± 52 (3)</td>
<td>0.0028 ± 0.0019 (4)</td>
<td>+14.9 ± 1.5</td>
</tr>
<tr>
<td>RO-bA1[{4.1}]</td>
<td>D577-Y586</td>
<td>n.d.</td>
<td>16.5 ± 3.9 (6)</td>
<td>0.154 ± 0.061 (5)</td>
<td>+4.3 ± 1.1</td>
</tr>
<tr>
<td>RO-bA1[{4.2}]</td>
<td>D588-M592</td>
<td>2130 ± 320 (4)</td>
<td>22 ± 17 (10)</td>
<td>0.164 ± 0.074 (9)</td>
<td>+4.4 ± 1.7</td>
</tr>
<tr>
<td>RO-bA1[{1-3}]</td>
<td>V487-C573</td>
<td>586 ± 63 (8)</td>
<td>1.48 ± 0.27 (9)</td>
<td>0.424 ± 0.098 (11)</td>
<td>+0.8 ± 1.0</td>
</tr>
<tr>
<td>RO-bA1R/fA4 Asp</td>
<td>D588-N613</td>
<td>n.d.</td>
<td>72 ± 25 (6)</td>
<td>0.0171 ± 0.0099 (7)</td>
<td>+10.5 ± 1.7</td>
</tr>
<tr>
<td>RO-fA4R/bA1C</td>
<td>V487-Y586</td>
<td>284 ± 71 (6)</td>
<td>0.361 ± 0.069 (6)</td>
<td>0.825 ± 0.054 (9)</td>
<td>-4.0 ± 1.0</td>
</tr>
</tbody>
</table>

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Figure 3

A

RO-fA4, 3 mM cAMP

RO-rA4, 30 mM cAMP

B

$P_{\text{open}}$ vs. Cyclic nucleotide (µM)

cGMP

cAMP

RO-fA4

RO-rA4
Figure 5
Figure 6

A

\[ P_{\text{open}} \]

\[ \text{Cyclic nucleotide} (\mu\text{M}) \]

B

\[ P_{\text{open}} \]

\[ \text{Cyclic nucleotide} (\mu\text{M}) \]

C

A-helix+β-roll

Hinge

Loop β8

cAMP

cGMP

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Distinct structural determinants of efficacy and sensitivity in the ligand-binding domain of cyclic nucleotide-gated channels
Edgar C. Young and Natalia Krougliak

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