A State-independent Interaction between Ligand and a Conserved Arginine Residue in Cyclic Nucleotide-gated Channels Reveals a Functional Polarity of the Cyclic Nucleotide Binding Site*

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Activation of cyclic nucleotide-gated channels is thought to involve two distinct steps: a recognition event in which a ligand binds to the channel and a conformational change that both opens the channel and increases the affinity of the channel for an agonist. Sequence similarity with the cyclic nucleotide-binding sites of cAMP- and cGMP-dependent protein kinases and the bacterial catabolite activating protein (CAP) suggests that the channel ligand binding site consists of a β-roll and three α-helices. Recent evidence has demonstrated that the third (or C) α-helix moves relative to the agonist upon channel activation, forming additional favorable contacts with the purine ring. Here we ask if channel activation also involves structural changes in the β-roll by investigating the contribution of a conserved arginine residue that, in CAP and the kinases, forms an important ionic interaction with the cyclized phosphate of the bound ligand. Mutations that conserve, neutralize, or reverse the charge on this arginine decreased the apparent affinity for ligand over four orders of magnitude but had little effect on the ability of bound ligand to open the channel. These data indicate that the cyclized phosphate of the nucleotide approaches to within 2–4 Å of the arginine, forming a favorable ionic bond that is largely unaltered upon activation. Thus, the binding site appears to be polarized into two distinct structural and functional domains: the β-roll stabilizes the ligand in a state-independent manner, whereas the C-helix selectively stabilizes the ligand in the open state of the channel. It is likely that these distinct contributions of the nucleotide/C-helix and nucleotide/β-roll interactions may also be a general feature of the mechanism of activation of other cyclic nucleotide-binding proteins.

Cyclic nucleotides regulate the activity of a diverse family of proteins involved in cellular signaling. These include a transcription factor (the bacterial catabolite activating protein, CAP), the cAMP- (PKA) and cGMP-dependent protein kinases (PKG) and the cyclic nucleotide-gated (CNG) ion channels involved in visual and olfactory signal transduction (1, 2). Despite obvious divergence among the effector domains of these proteins, the cyclic nucleotide binding (CNB) sites appear to share a common architecture. Solution of the crystal structures of CAP (3) and a recombinant bovine PKA R1α subunit (4) has demonstrated that their CNB sites are formed from an α-helix (A helix), an 8-stranded β-roll, and two more α-helices (B and C), with the C-helix forming the back of the binding pocket. Six residues are invariant among all members of the CAP and kinase families: three glycines involved in turns between strands of the β-roll, an arginine and a glutamate, each of which contact the cyclic nucleotide, and an alanine whose function is uncertain (1) (see also Fig. 1. Strikingly, these six residues are conserved in the CNG channels. Thus, it has been suggested that the invariant residues play important and conserved roles in the folding/function of the CNB sites of these diverse proteins (1–6). Interestingly, only three of these residues (two glycines and the arginine) appear to be conserved among the more distantly related voltage-gated channels that bear the CNB site motif and whose gating may be modulated by direct binding of cyclic nucleotide (KAT1 (7, 8), AKT1 (9, 10), and dEAG (11, 12) see Fig. 1).

Surprisingly, this structural similarity of the CNB site does not appear to be reflected in the conformation of the bound agonist. Thus, the crystal structures reveal cAMP binds in an anti conformation to CAP (3) but in a syn conformation to PKA R1α (4), although this may not reflect the conformation of the ligand bound to the proteins in solution (1, 2, 19). While experiments with cyclic nucleotide analogs and modeling, based upon the CAP and PKA R1α structures, have been used to investigate the conformation adopted by agonists in other CNB sites, this issue is unresolved (1–6). This uncertainty, coupled with the lack of a crystal structure for any of the CNB proteins, in either the absence of bound agonist or presence of antagonist, leaves an important question unresolved: what are the structural changes that take place within these binding sites that result in the activation of each of the CNB proteins?

By employing site-directed mutagenesis and patch clamp recording of CNG ion channels, it is possible to separate the coupled processes of ligand binding from activation, permitting a dissection of the molecular contributions of protein-ligand interactions to each of these events. Such studies have demonstrated that residues within the C-helix selectively contribute to channel activation (20, 21). Indeed, an aspartic acid residue (Asp604) in the bovine rod subunit 1 (bRET1 (16)) C-helix appears to interact with the purine ring of cGMP selectively when the channel is open (21). That is, the binding energy of this interaction predominantly serves to stabilize ligand binding in the active conformation of the binding site, thereby leading to stabilization of the active (open) state of the channel. However, the state dependence of interactions between the cyclic nucleo-
FIG. 1. Schematic representations of the double chimera ROON-S2, the cyclic nucleotide binding site, and the activation gating model of cyclic nucleotide-gated channels. A, diagram of the double chimera ROON-S2 showing the replacement of the bRET1 N-S2 domain (Asn\(^{91}\)-Ser\(^{240}\)) and P-region (Ala\(^{344}\)-Ala\(^{378}\)) by the homologous sequences from fOLF1 (Glu\(^{89}\)-Arg\(^{215}\) and Ser\(^{314}\)-Phe\(^{348}\), respectively). bRET1 sequences are portrayed as light lines and hollow boxes, and fOLF1 sequences are dark lines and filled boxes. The first six boxes correspond to the Cyclic Nucleotide-gated Channel Activation model of cyclic nucleotide-gated channels.
otide and other residues in the binding site are well defined.

Here we ask whether regions other than the C-helix of the CNB site are likely to be altered upon channel activation and thereby contribute to the increased affinity of the open channel for agonist. Studies of cyclic nucleotide analogs bearing sulfur substitutions on one or another of the exocyclic oxygens of the cyclized phosphate raise the possibility that residues in the β-roll may also contribute to activation gating. These data show that, in the kinases, the equatorial sulfur-substituted derivative (Rp-cAMPS) is an antagonist, whereas the axial sulfur-substituted compound (Sp-cAMPS) is an agonist (22–24), a profile that appears to be reversed in CAP (4, 25). CNG channels formed from the catfish olfactory neuron subunit 1 (fOLF1 (17)) show an identical pharmacological profile to that of the kinases (26). By contrast, in CNG channels formed from bRET1, both cGMP derivatives are agonists and both cAMP derivatives are antagonists (26). Since the exocyclic oxygen atoms interact with residues in the β-roll, these data raise the possibility that large and possibly divergent structural changes may take place in the β-roll of each of the CNB proteins upon activation (1–6).

We have focused upon the conserved arginine residue in the β-roll (Arg559 in bRET1, Arg529 in fOLF1, see Fig. 1). The homologous residue forms an ionic bond with the cyclized phosphate of the nucleotide in both CAP and the RI subunit of PKA (1–4), which suggests that this residue is well placed to bind ligand to activate the ROON-S2 channel, as determined from the maximum open probability (P_{\text{max}}) of the channel in the presence of a saturating concentration of ligand (27). These data suggest that either the conserved arginine contacts the bound agonist in a state-independent manner (that is, it interacts equally well with ligand in the open and closed states of the channel) or that the polar glutamine residue is able to substitute effectively for the arginine to maintain any state-dependent contacts. Here we explore further the role of Arg559 by studying a wide range of mutations that conserve, neutralize, or reverse the charge of this residue. Such mutations are tolerated and cause a progressive decrease in the affinity of the channel for the agonist with little or no detectable change in the ability of bound ligand to activate the ROON-S2 channel, as determined from the maximum open probability (P_{\text{max}}) of the channel in the presence of a saturating concentration of ligand (27). These data suggest that either the conserved arginine contacts the bound agonist in a state-independent manner (that is, it interacts equally well with ligand in the open and closed states of the channel) or that the polar glutamine residue is able to substitute effectively for the arginine to maintain any state-dependent contacts. Here we explore further the role of Arg559 by studying a wide range of mutations that conserve, neutralize, or reverse the charge of this residue. Such mutations are tolerated and cause a progressive decrease in the affinity of the channel for the agonist with little or no detectable change in the ability of bound ligand to activate the channel. These data are consistent with the formation of a state-independent, electrostatic interaction between this arginine and the cyclized phosphate of the ligand, although they also reveal an unexpected steric influence of chain length.
Cyclic Nucleotide-gated Channel Activation

Fig. 2. Single channel properties of ROON-S2, ROON-S2/Q, and ROON-S2/E. A, representative records of the three channels at the indicated high (top sweep) and low (bottom sweep) concentrations of cGMP. The dashed lines indicate the mean current flow through the patch during the closed (C) and open (O) states of the channels. B, all points amplitude histograms constructed from 20–40 s of data. To enable the closed channel baseline to be clearly resolved in the Gaussian fitting routine, an arbitrary duration of base line was accumulated into the histograms along with each opening. Accordingly, the area of the Gaussian representing the closed component is also arbitrary and does not reflect the closed probability. The relative areas of the three Gaussian components fit to the open channel current does, however, determine the fractional occupancy of the open channel in each of its three conducting states.

(given in parentheses): ROON-S2 (0.3 mM), ROON-S2/K (3 mM), ROON-S2/Q (3 mM), ROON-S2/N (30 mM), RO133 (3 mM), fOLF1 (3 mM), and fOLF1/Q (30 mM). For each patch, 20–40 s of continuous recording was accumulated into an all points amplitude histogram, such as those shown in Fig. 2. As these histograms included all open and closed events, the area of the closed peak represents the closed probability ($P_{\text{closed}}$) and $P_{\text{open}}$ is equal to $1 - P_{\text{closed}}$. However, for ROON-S2/L, ROON-S2/E, ROON-S2/D, and RO133/Q, 30 mM cGMP was not saturating. Higher concentrations of cGMP caused the maximal current to decrease, possibly due to desensitization. Accordingly for these four constructs, we first normalized the dose-response data by the open probability directly measured with 30 mM cGMP. $P_{\text{max}}$ was then obtained by fitting the Hill equation to the normalized data. This introduced only a minor correction for ROON-S2/L, ROON-S2/E, and RO133/Q. The correction was larger for ROON-S2/D, which had the most displaced dose-response curve. For ROON-S2/L and ROON-S2/D, this procedure can lead to $P_{\text{max}}$ values that are slightly larger than 1, reflecting the error inherent in this procedure given that the observed open probabilities are so close to 1 originally. Where appropriate, the values for $P_{\text{open}}$ (with 30 mM cGMP) are reported in the legends to Figs. 4 and 6, in addition to the estimated value of $P_{\text{max}}$. This small error will not significantly affect our estimates of $K_d$. Throughout the manuscript, data are given as mean ± S.E. or mean ± range for those cases in which $n = 2$. Fits are weighted to the reciprocal of the standard deviation of the mean data.

**Determination of Electrostatic Distance**—Our goal in these experiments is to dissect out the contribution of the conserved arginine in β7 to ligand binding and channel activation. Although $K_d$ values depend, in general, on both ligand affinity and the coupled gating reaction, for those mutations that do not alter channel gating ($P_{\text{max}}$), changes in $K_d$ must reflect a selective change in ligand affinity. Since the $Arg^{559}$ mutations studied here do not alter $P_{\text{max}}$, we have used the observed changes in $K_d$ with the various $Arg^{559}$ point mutants to calculate the change in free energy of the actual binding reactions. Thus, the change in free energy of binding, $\Delta G$, upon changing charge at $Arg^{559}$ is given by,

$$\Delta G = -RT \ln(K_d/K_0)$$

(Eq. 1)

where $K_d$ and $K_0$ are the $K_d$ values for the wild-type and mutant channels, respectively. Assuming that the change in free energy reflects a simple coulombic interaction between the residue at position 559 and the cyclized phosphate of cGMP, it follows that,

$$\Delta G = N\Delta_q q_f - 4RT\epsilon K_d \ln(K_d/K_0)$$

(Eq. 2)

and from Equations 1 and 2, we obtain the electrical distance $r$,

$$r = N\Delta_q q_f - 4RT\epsilon K_d \ln(K_d/K_0)$$

(Eq. 3)

where $R$ is the gas constant, $T$ is the temperature, $\Delta q$ is the change in charge at position 559 between wild-type and mutant channels, $q_f$ is the charge on the cyclized phosphate of the ligand, $N$ is Avogadro’s number, and $\epsilon$ and $\epsilon_0$ are the dielectric constant of the binding site environment and the vacuum permittivity, respectively. As this is a solvent-accessible part of the binding site, we assume that the charged groups are fully ionized and that the dielectric constant equals that of water.

**Fits of the Monod-Wyman-Changeux Gating Model**—We have previously shown (20, 27) that the simplest kinetic scheme that describes the equilibrium gating properties of CNG channels is the cyclical allosteric model of Monod, Wyman, and Changeux (18) (Fig. 1D). According to the model, the channel undergoes an allosteric transition between the closed (C) and open (O) state in the absence of ligand, with an equilibrium constant $L_0$ (equal to $[C]/[O]$). Agonists activate the channel by binding more tightly to the open state than to the closed state (dissociation constants $K_{dO}$ and $K_{dC}$, respectively), thereby shifting the equilibrium from the closed state to the open state by the term $(K_d/K_{dO})^n$ (where $n$ is the number of agonists bound to the channel). To partition the effects of mutating the conserved arginine between ligand-binding reactions and the channel-opening reaction, the increase in open probability as a function of ligand concentration was fit to the MWC model.

$$P_{\text{open}} = (1 + [A]/K_{dO}nL_0)[1 + ([A]/K_{dC}nL_0)]^{n-1}$$

(Eq. 4)

In these fits, the only free parameter was $K_{dO}$. $P_{\text{open}}$ was constrained to the value determined from single channel recording (see above), and the number of ligand-binding sites was assumed to be four (29). $K_{dO}$ was determined from the relation: $K_{dO} = K_{d}/(1 - P_{\text{max}}/[P_{\text{max}}L_0])^{1/n}$. For RO133 (7999) and fOLF1 (443) were constrained to the values previously determined from the unliganded open probability, $P_{\text{max}}$, measured for each of these channels (27). The values of $L_0$ for RO133/Q and fOLF1/Q were assumed to be equal to those of the parent channel. This assumption seems reasonable since we have previously shown that mutation of $Arg^{559}$ to a glutamine had no effect on the value of $L_0$ in the ROON-S2 background (27). For fits of the MWC equation, open probabilities were corrected for subtraction of the unliganded open probability ($P_{\text{open}}$) according to $P_{\text{open}} = (\int_{cGMP}^\infty)P_{\text{max}} - P_{\text{sp}} + P_{\text{sp}}$.

**RESULTS**

The fundamental effects on single channel function of replacing $Arg^{559}$ of the chimeric CNG channel, ROON-S2, with either a neutral (ROON-S2/Q) or acidic (ROON-S2/E) amino acid residue are illustrated in Fig. 2. ROON-S2, ROON-S2/Q, and
ROON-S2/E require progressively greater concentrations of cGMP to open. Whereas 0.003 mM cGMP is sufficient to cause ROON-S2 to be open for more than half the time, even a 10-fold higher concentration of cGMP (0.03 mM) activates ROON-S2/Q to only a relatively low extent. ROON-S2/E exhibits almost no openings, even at 0.3 mM cGMP. Despite this >1000-fold reduction in sensitivity to ligand, the open probability obtained at saturating concentrations of cGMP ($P_{\text{max}}$) for all three constructs is very close to 1 (top traces). These data thus suggest that neutralization and reversal of the charge at position 559 leads to a progressive decrease in the sensitivity of the channel to cGMP. A concern in all mutagenesis experiments is that the observed effects are due to a global disruption of the structure and function of the protein. As the ion conducting pore of the CNG channels is largely formed from the loop between the 5th and 6th transmembrane domains (28), with no detectable contribution from the carboxyl terminus, we determined the single channel conductance properties of each of these mutants. Despite the large change in ligand sensitivity, the representative single channel traces (Fig. 2) reveal that the current flow through the open channel for the two point mutants is indistinguishable from that of ROON-S2. The open states of all three channels are characterized by pronounced open channel noise, which is readily seen by comparison with the base-line noise when the channels are closed (lower traces of each pair). This excess noise is due to the rapid, partial block and unblock of the open channel by external protons (28, 30).

The similarity of the open channel current properties among the constructs is confirmed from the all-points current amplitude histograms (Fig. 2, right panels). These data are well fitted by four Gaussian functions. The Gaussian function near 0 pA reflects the closed state of the channel. The other three Gaussian components reflect current flow through the open channel, with the largest current component corresponding to the fully open channel (no proton block), the intermediate component corresponding to channels occupied by a single proton, and the small component corresponding to channels occupied by two protons (28, 30). Neither the amplitude (the current value at the peak of each of the fitted Gaussian functions) nor the occupancy (the relative areas under the three open state Gaussian functions) of these open channel current states were significantly affected by the mutations at Arg559 (see also Fig. 5). The small variations in the shapes of the amplitude histograms probably reflect small variations in pH or temperature, and hence the proton block, among the recordings. The fact that the open channel characteristics are unchanged by the point mutations suggests that they do not cause a generalized disruption in channel structure.

To interpret the effect of these mutations quantitatively, we measured $P_{\text{open}}$ over a broad range of cGMP concentrations and fit the dose-response relationships by the Hill equation. As is seen in Fig. 3, the effect of these mutations was to cause essentially parallel shifts in the dose-response curves toward greater concentrations of ligand. Thus, the slope of the relationships and the $P_{\text{max}}$ values were largely unaltered while the $K_{n}$ for activation of ROON-S2, ROON-S2/Q, and ROON-S2/E by cGMP increased from 1.8 $\pm$ 0.3 $\mu$M ($n = 10$) to 50 $\pm$ 8 $\mu$M ($n = 8$) and 3379 $\pm$ 1005 $\mu$M ($n = 5$), respectively. That is, neutralization of Arg559 resulted in a 28-fold increase in the $K_{n}$ value, whereas charge reversal increased further the $K_{n}$ value by 68-fold.

We next asked if the chemical identity of the residue at position 559 was important or if the altered activation of the mutant channels was simply a consequence of the charge in the side chain. To investigate this, we constructed a more extensive series of mutations in the ROON-S2 background, generating channels with basic (arginine or lysine), neutral (glutamine, asparagine, or leucine), or acidic (glutamate or aspartate) residues at position 559. The gating properties of each construct were then determined.

Fig. 4A shows that $P_{\text{max}}$ for all of the constructs was $\geq 0.98$, indistinguishable from the parent chimera ROON-S2. Together, the data in Figs. 2, 3, and 4A show that neither the charge nor chemical identity of the side chain of residue 559 has a detectable influence upon the ability of bound ligand to open the channel.

In contrast, a plot of $K_{n}$ versus charge on the side chain of residue 559 reveals that there are both electrostatic and steric effects of side chain substituents upon the apparent affinity for ligand (Fig. 4B). Thus, introducing the charge-conserving lysine (ROON-S2/K) residue resulted in a decrease in apparent affinity. Surprisingly, the 70-fold increase in $K_{n}$ was larger than the 28-fold increase seen upon neutralization with glutamine. Lysine has two important differences when compared with arginine. First, it is the equivalent of one methylene bridge shorter, and second, it has a point charge on a primary amine, whereas arginine has the charge delocalized over the guanidinium group. As there are no other amino acids with basic side chains, it is not possible to distinguish between the steric effect of shortening the side chain from an effect of alteration in local field strength.

Mutation of Arg559 to neutral and acidic amino acids does permit us to address this question further. Replacement of Arg559 with an asparagine (which is one methylene bridge shorter than glutamine, but otherwise identical), to generate the ROON-S2/N mutant, gives rise to a far more pronounced increase in $K_{n}$ (392-fold) than does replacement with glutamine (ROON-S2/Q). This result suggests that chain length or the exact location of the polar groups, in addition to charge, is an important determinant of ligand affinity. The importance of side chain polarity is demonstrated upon introduction of the non-polar residue leucine, which increased the $K_{n}$ by 882-fold, a more pronounced modification than that seen with either of the polar substitutions or with lysine. Leucine is effectively an asparagine in which the carbonyl oxygen and amino group of the side chain have been replaced by methyl groups and which has a volume intermediate between that of glutamine and arginine.

The importance of charge at position 559 was further ex-
However, here again we see that amino acid residue generation of a repulsive interaction between the acidic side chain of residue 559 either glutamic or aspartic acid. The plied conductance level next to the two dominant conductance states. Taken together, the lack of an effect of the mutations explored by reversing the sign of the charge by introduction of either glutamic or aspartic acid. The $K_{1/2}$ values of these two mutants was increased by 1877 and 5489 fold, respectively. The magnitudes of these increases in $K_{1/2}$ are consistent with the generation of a repulsive interaction between the acidic side chain of the amino acid and the cyclized phosphate of the cyclic nucleotide. However, here again we see that amino acid residue with shorter side chain produced a more pronounced increase in $K_{1/2}$.

A linear regression through the plot of log($K_{1/2}$) values versus charge at position 559 (solid line in Fig. 4B) yields a slope corresponding to a 19.5-fold increase in $K_{1/2}$ for an elementary charge change in $P_{max}$ (mean value of $K_{1/2}$, Equations 1 and 3 under “Experimental Procedures”). Assuming a coulombic interaction between the residue at position 559 and a single negative charge on cGMP, this relationship yields an approximate distance of 2.4 Å between the ligand and the charge at position 559 (determined from Equation 3, under “Experimental Procedures”). Approximate upper and lower bounds for this value are obtained from the largest and smallest changes in $K_{1/2}$ observed upon reversal of charge. The 5489-fold increase in $K_{1/2}$ upon replacing arginine by aspartate is equivalent to a distance of 1.7 Å, whereas the 27-fold increase in $K_{1/2}$ upon replacing lysine by glutamate indicates a slightly longer distance of 4.4 Å. The electrostatic nature of this interaction is supported by the roughly similar fold increase in $K_{1/2}$ seen upon changing the residue at position 559 either from a glutamine to a glutamate or from an asparagine to an aspartate. In each case, chain length is held essentially constant while a negative charge is introduced by conversion of the amide to the acid (Fig. 4B).

Taken together, the data in Fig. 4 are consistent with the formation of a state-independent ionic bond between the side chain of residue 559 and the cyclized phosphate of the nucleotide.

Despite the pronounced effect of these point mutations on cGMP sensitivity, the conductance properties of the mutant channels are essentially identical to the parent chimera, ROON-S2. This is evident in Fig. 5, a two-dimensional plot of conductance versus fractional occupancy of the three open channel conductance states (unprotonated, singly and doubly protonated, see Fig. 2). The variability in the amplitude of the largest conductance state among the different mutants is not correlated with ionic charge at position 559. Rather, it is likely to reflect a technical difficulty in fitting this infrequently occupied conductance level next to the two dominant conductance levels, which represent the partially and fully protonated states. Taken together, the lack of an effect of the mutations upon either the single channel conductance or $P_{max}$ indicate that the mutations of Arg559 result in a discrete disruption of the binding site that selectively lowers the apparent affinity for ligand.
the mutation R559Q in the RO133 background increased the $K_o$ of the resulting construct (RO133/Q) 42-fold with no change in $P_{\text{max}}$. A similar decrease in the apparent affinity was observed upon introduction of the R559Q mutation in bRET1 (52 ± 6 μM, n = 9 to 2793 ± 365 μM, n = 2; data not shown). The qualitative and quantitative similarity of the R559Q mutation in bRET1, RO133, and ROON-S2 demonstrate that the selective change in apparent affinity upon mutation of Arg559 is an intrinsic property of the bRET1 CNB site.

What is the basis for this selective increase in $K_o$? To address this, we have utilized the cyclic allosteric model of Monod, Wyman, and Changeux (18) (Fig. 1D), which we previously demonstrated to be the simplest kinetic scheme that adequately describes CNG channel activation (20, 27). Based upon this model, an increase in $K_o$ can be produced either by a reduction in affinity for ligand (increase in $K_o$ or $K_c$) or from an increase in the allosteric equilibrium constant, $L_o$, between the open and closed state of the channel ($L_o = [C]/[O]$). However, we have previously shown, using measurements of agonist-free openings, that the arginine to glutamine mutation does not alter $L_o$ in ROON-S2 (27). Moreover, a change in $L_o$ in any of the mutants would not only increase $K_o$ but would also significantly reduce $P_{\text{max}}$, which was not observed (Figs. 2A, 4A, and 6A). Rather, these data suggest that mutation of Arg559 lowers the apparent affinity by specifically decreasing the absolute affinity of the CNB site for ligand.

To investigate the effect of the arginine to glutamine muta-

**Fig. 6.** The effect of an arginine to glutamine mutation in RO133 and fOLF1. A, open probabilities of RO133 (○) and RO133/Q (■). Squares represent the open probabilities while the circles are the open probabilities corrected for subtraction of ligand-independent activation. This correction is significant only at very low open probabilities. The solid lines represent fits of the Hill equation while the dashed lines are the fits of the MWC model assuming four binding events (see “Experimental Procedures”). From the fits to the Hill equation, we determined values for the $K_o$ and Hill coefficient ($n = 7$) for RO133, 1712 ± 334 μM, 2.2 ± 0.1, $n = 5$) while $P_{\text{max}}$ was determined from single channel recordings (RO133, 0.948 ± 0.015, n = 6; RO133/Q, 0.964 ± 0.043, n = 3, corrected from the $P_{\text{open}}$ value at 30 mM cGMP of 0.953 ± 0.042, see “Experimental Procedures”). The non-zero asymptote for $P_{\text{open}}$ at low concentrations of cGMP reflects the value for the spontaneous open probability of the channel (see “Experimental Procedures”). B, open probabilities of fOLF1 (○, □) and fOLF1/Q (■, ■). Symbols and lines have the same meaning as in panel A. From the fits to the Hill equation, we determined values for the $K_o$ (fOLF1, 45 ± 11 μM, 1.6 ± 0.1, n = 7; fOLF1/Q, 342 ± 60 μM, 1.9 ± 0.1, n = 7) while $P_{\text{max}}$ was determined from single channel recordings (fOLF1, 0.421 ± 0.128, n = 6; fOLF1/Q, 0.877 ± 0.069, n = 7).

**Fig. 7.** Free energy plot of cGMP dissociation constants for binding to bRET1 and fOLF1 cyclic nucleotide binding sites and the destabilization of binding by the arginine to glutamine mutation. A, values of the dissociation constants of MWC model for RO133 (■, $K_o = 2.0 ± 0.2$ μM and $K_c = 39$ μM) and RO133/Q ($K_o = 88 ± 11$ μM and $K_c = 1852$ μM) were determined from fits in Fig. 6 and are plotted as free energy terms according to $\Delta G = -RT \ln(1/K)$. The change in free energy, $\Delta G$, upon mutation of the arginine to glutamine is shown as a filled circle (●) in each plot. B, values of the dissociation constants of MWC model for fOLF1 (●, $K_o = 3.0 ± 0.6$ μM and $K_c = 12.7$ μM) and fOLF1/Q (■, $K_o = 43 ± 9$ μM and $K_c = 322$ μM) were determined and plotted as described above. No error is reported for $K_c$ since this was constrained as described under “Experimental Procedures.” The errors in $K_o$ are smaller than the symbols.

The effect of this mutation on the interaction of arginine with cyclic nucleotide analogs by bRET1 and fOLF1 (26), which suggests that the $\beta$-roll portion of the binding site of fOLF1 may differ significantly from that of bRET1. Fig. 6B compares dose-response curves for fOLF1 and fOLF1/Q. Although there is a shift in the fOLF1/Q dose-response curve to higher concentrations, this effect is less marked (7.6-fold) than in the bRET1 CNB site (28–54-fold, depending upon the channel background). Surprisingly, the fOLF1/Q mutant shows a higher $P_{\text{max}}$ compared with wild-type fOLF1, despite the decrease in cGMP sensitivity.

These data raise two questions. First, to what extent do these differences in gating properties between bRET1 and fOLF1 result from a fundamental difference in the mechanistic behavior of their binding sites? Second, how can a mutation destabi-
lize binding but increase efficacy? To investigate these questions, we fit the dose-response data for fOLF1 and fOLF1/Q with the MWC model and determined $K_a$ and $K_C$ for these two channels. This analysis reveals that the impact of the R to Q mutation on ligand binding in fOLF1 is, in fact, very similar to the effect observed in the bRET1 CNB site. Thus, the destabilization of $K_a$ (1.57 kcal mol$^{-1}$) and $K_C$ (1.90 kcal mol$^{-1}$) in fOLF1/Q are similar in sign and magnitude to the changes seen in the bRET1 background. The less marked shift in the dose-response curve and the increase in $P_{\text{max}}$ seen with the arginine to glutamine mutation in fOLF1 arise from small quantitative differences in the magnitude of the effect of the mutation upon binding of agonist to the open and closed states of the channel, not from a qualitatively different utilization of the binding energy.

**DISCUSSION**

Here we have investigated which regions of CNB sites contribute to activation and, in particular, whether there is likely to be a significant change in the interaction between the $\beta$-roll of the CNB site and the ligand upon activation. Our studies focused on an arginine residue in the $\beta$-roll that is conserved among diverse CNB proteins and that makes an ionic interaction with the cyclized phosphate of cyclic nucleotides in both the bacterial CNB protein CAP as well as in the regulatory subunit of cAMP-dependent protein kinase (1–4) (see also Fig. 1).

Mutations of this conserved arginine, in the background of the chimeric CNG channel ROON-S2, to a series of residues that conserve, neutralize, or reverse its charge, caused a progressive decrease in apparent affinity of the channel for ligand. Although an unexplained steric effect of chain length contributed to this decrease, the clear dependence of the $K_a$ values on charge at position 559 strongly supports the formation of an ion pair between Arg$^{559}$ and the cyclized phosphate. This result is consistent with the x-ray crystallographic structural studies of PKA RII$\alpha$ and CAP (1–4). Indeed, the estimate of the electrostatic distance between Arg$^{559}$ and the cyclized phosphate from these experiments (1.7–4.4 Å) is close to that predicted from the crystal structures of CAP (3.1–3.5 Å, (3)) and PKA RII$\alpha$ (<3.3 Å, (4)). Despite the large changes in ligand sensitivity with the Arg$^{559}$ mutants (spanning nearly four orders of magnitude), the ability of the bound ligand to activate the channel (as determined from $P_{\text{max}}$) was virtually unaltered. These data suggest that Arg$^{559}$ plays an important role in stabilizing cyclic nucleotide and that these interactions do not contribute to channel activation. The absence of an effect of the mutations on the single channel conductance or on $P_{\text{max}}$ shows that these mutations are unlikely to cause a global disruption of the protein.

This surprising result, that the Arg$^{559}$ point mutants have large effects on ligand sensitivity but little effect on activation gating, can be readily explained within the context of the MWC allosteric reaction scheme (18). According to this scheme, a concerted allosteric conformational change in the channel both opens the channel pore and alters the binding site, causing the ligand affinity of the open state to be considerably higher than the ligand affinity of the closed state (dissociation constants $K_a$ and $K_C$, respectively). By measuring ligand-independent openings, we previously determined the allosteric equilibrium constant between closed and open channels in the absence of agonist, $L_0$, for both bRET1 and fOLF1 (27). We found that a 20-fold difference in $L_0$ between bRET1 and fOLF1, which contributes to physiologically important differences in ligand gating (20, 27, 31), was localized to the amino-terminal N-S2 domain (27). Since this region of the channel interacts with the carboxyl terminus (32, 33) and is involved in subunit assembly in the homologous voltage-gated K channels (34–38), we have postulated that channel activation involves a change in quaternary structure.

Whereas the difference in the allosteric transition between fOLF1 and bRET1 is localized to the amino terminus of the channel, the postulated increase in ligand affinity of the open state of these channels is mediated, at least in part, by interactions of the cyclic nucleotide with the C-helix of the carboxyl terminus CNB domain (20, 21). In particular, an aspartate residue in the C-helix of bRET1, Asp$^{604}$, has been shown to make important contacts with cGMP in the open state, but not closed state, of the channel (21). These results suggested a model of channel gating in which the allosteric transition that opens the channel is coupled to a change in the orientation of the C-helix relative to the $\beta$-roll, leading to an enhancement of C-helix/ligand contacts. According to this model, the $\beta$-roll would provide a relatively stable structure that is involved in the initial binding of ligand, which orients the nucleotide within the binding pocket. The lack of effect of mutation of Arg$^{559}$ on ligand-dependent gating is consistent with this hypothesis.

A quantitative analysis of the effect of mutating arginine 559 to glutamine was performed by fitting the MWC model to the cGMP dose-response data. This was done in the background of a chimeric channel, RO133 (bRET1 with the fOLF1 P region), because the gating properties and large single channel conductance of this construct facilitated accurate determination of $P_{\text{max}}$ and hence, the channel activation parameters (28). This analysis shows that the R559Q mutation decreases the affinity of the open ($K_a$) and closed ($K_C$) state of the channel for ligand by an identical amount. From these data we can conclude that there is no significant structural rearrangements between this deep part of the $\beta$-roll and the ligand upon channel activation. Conversely, we can also conclude that all bonds between the protein and the ligand that are made more favorable when the channel goes from the closed to the open state, and stabilize the latter, are unaffected by the electrostatic and steric effects of substitutions at position 559.

Does this analysis of the interaction between Arg$^5$ in bRET1 and the cyclic phosphate hold true for other cyclic nucleotide binding pockets? Although mutation of the conserved arginine in CAP (to lysine, histidine, glutamine, or leucine) and PKA (to either lysine or tryptophan) has been shown to interfere with ligand-dependent activation, it has been difficult in these molecules to separate out effects of binding from activation (1, 25, 39–43). To address this question, we therefore constructed the homologous mutation in the fOLF1 CNB domain. This is particularly interesting given the different actions of Rp- and Sp-substituted ligands in fOLF1 and bRET1 (26). In the background of the olfactory channel, mutation of the homologous arginine (Arg$^{529}$) actually enhanced $P_{\text{max}}$ despite a decrease in ligand sensitivity. This result suggested that there might be a qualitatively different interaction between cGMP and the $\beta$-roll of the fOLF1 binding site compared with the cGMP/bRET1 $\beta$-roll interaction. However, a fit of the MWC model showed that these differences can be explained by relatively small quantitative changes, amounting to only an $0.3$ kcal mol$^{-1}$ difference between the effects of the R529Q substitution on $K_a$ and $K_C$, in which closed state binding is decreased to a slightly greater extent than open state binding. Such small changes (equivalent to a fraction of a hydrogen bond) may readily be explained by indirect effects of the R529Q mutation on the orientation of the bound ligand rather than a large scale change in the structure of the fOLF1 $\beta$-roll during channel activation.

The state-independent interaction with the conserved argi-
nine in β7 in the CNG channels is in contrast to results suggesting that the neighboring residue, Thr360 in bRET1, may contribute to activation gating (21, 44). Thus, the mutation T560A produces a somewhat greater decrease in binding to the open state compared with the closed state, resulting in a 6–7-fold decrease in \( P_{\text{max}} \). Although this suggests that there might be a state-dependent interaction between Thr360 and ligand, this effect on gating could also be due to an indirect effect of the mutation, either by altering the conformation of the binding pocket or the orientation of the ligand in the binding site. For example, the T560A mutation might slightly decrease the ability of bound ligand to form optimal contacts with the C-helix in the open state. Although there are many possible interpretations for mutations that alter gating, only one interpretation is consistent with the profound state-independent changes in ligand binding seen with the wide range of Arg359 mutations, that this region of the channel does not alter its contacts with ligand during gating.

The data presented here, taken together with previous results, suggest that the CNB site of both FOLF1 and bRET1 CNG channels comprises two distinct structural and functional domains. The β-roll forms state-independent contacts with ligand that are important for stabilizing the ligand in the binding pocket, whereas the C-helix makes state-dependent contacts that increase ligand affinity upon channel activation and stabilize the channel in its open state (1–4, 20, 21). Based on the qualitatively similar effects of the mutation in bRET1 (R559Q) and FOLF1 (R529Q), our data suggest that these proteins undergo a common structural change upon activation despite their different patterns of activation with Rp- and Sp-cyclic nucleotide analogs (26). The distinct pharmacology of these two proteins probably reflects relatively small variations in the conformation of the binding pockets or the bound ligand rather than qualitatively different mechanisms of activation. Given the sequence similarity among CAP, the kinases, and the CNG channels and the similar effects seen in each upon mutation of the conserved arginine residue, we expect that the CNB sites of these diverse proteins share a similar functional organization that underlies the mechanism of ligand activation.

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REFERENCES
1. Shabb, J. B., and Corbin, J. D. (1992) J. Biol. Chem. 267, 5723–5726
2. Zagotta, W. N., and Siegelbaum, S. A. (1996) Annu. Rev. Neurosci. 19, 235–263
3. Weber, I. T., and Steitz, T. A. (1987) J. Mol. Biol. 198, 311–326
4. Su, Y., Dostmann, W. R. G., Herberg, K., Xuong, N.-h., Ten Eyck, L., Taylor, S. S., and Varughese, K. I. (1995) Science 269, 807–813
5. Kurnar, V. D., and Weber, I. T. (1992) Biochemistry 31, 4643–4649
6. Scott, S. P., Harrison, R. W., Weber, I. T., and Tanaka, J. C. (1996) Protein Eng. 9, 333–344
7. Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., and Gabel, R. F. (1993) Proc. Natl. Acad. Sci. U.S.A. 88, 3736–3740
8. Hoshi, T. (1995) J. Gen. Physiol. 105, 309–328
9. Sentenac, H., Bonneau, N., Minet, M., Lacroute, F., Salmon, J.-M., Gaymard, F., and Grigioni, C. (1992) Science 256, 663–665
10. Gaymard, F., Cerutti, M., Horeau, C., Lemailliet, G., Urbach, S., Ravallec, M., Devauchelle, G., Sentenac, H., and Thibaud, J.-B. (1996) J. Biol. Chem. 271, 22863–22870
11. Warmke, J., Drysdale, R., and Gazetzk, B. (1991) Science 252, 1560–1562
12. Bruggemann, A., Pardo, L. A., Stühmer, W., and, Pongs, O. (1993) Nature 365, 445–448
13. Aiba, H., Fujiyama, K., and Oikazaki, N. (1982) Nucleic Acids Res. 10, 1345–1361
14. Cossart, P., and Gicquel-Sanze, B. (1982) Nucleic Acids Res. 10, 1363–1378
15. Titani, K., Saasagawa, T., Ericsson, L. H., Kumar, S., Smith, B. S., Kebes, E. G., and Walsh, K. A. (1994) Biochemistry 33, 4193–4199
16. Kaupp, U. B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N. J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T., and Numa, S. (1989) Nature 342, 762–766
17. Goulding, E. H., Nagi, J., Kramer, R. H., Calicos, A., Axel, R., Siegelbaum, S. A., and Chess, A. (1992) Neuron 8, 45–58
18. Monod, J., Wyman, J., and Changeux, P.-J. (1965) J. Mol. Biol. 12, 88–118
19. Gronenborn, A. M., and Clore, G. M. (1982) Biochemistry 21, 4040–4048
20. Goulding, E. H., Tibbs, G. R., and Siegelbaum, S. A. (1994) Nature 372, 369–374
21. Varnum, M. D., Black, K. D., and Zagotta, W. N. (1995) Neuron 15, 619–625
22. De Wit, R. W., Hekstra, D., Jastrow, B., Stec, W. J., Baraniak, J., Van Driel, R., and Van Haastert, J. M. (1984) Eur. J. Biochem. 142, 255–260
23. Dostmann, W. R. G., and Taylor, S. S. (1991) Biochemistry 30, 8710–8716
24. But, E., Van Berendonk, P., Fischer, L., Walter, U., and Jastrow, B. (1990) FEBS Lett. 263, 47–50
25. Gronenborn, A. M., Sandulache, R., Gartner, S., and Clore, G. M. (1988) Biochem. J. 250, 601–607
26. Kramer, R. H., and Tibbs, G. R. (1996) J. Neurosci. 16, 1285–1293
27. Tibbs, G. R., Goulding, E. H., and Siegelbaum, S. A. (1997) Nature 386, 612–615
28. Goulding, E. H., Tibbs, G. R., Liu, D., and Siegelbaum, S. A. (1993) Nature 364, 61–64
29. Liu, D. T., Tibbs, G. R., and Siegelbaum, S. A. (1996) Neuron 16, 983–990
30. Root, M. J., and MacKinnon, R. (1994) Neuron 12, 857–860
31. Gordon, S. E., and Zagotta, W. N. (1995) Neuron 14, 857–864
32. Gordon, S. E., Varnum, M. D., and Zagotta, W. N. (1997) Neuron 19, 431–441
33. Varnum, M. D., and Zagotta, W. N. (1997) Science 278, 110–115
34. Li, M., Jan, Y. N., and Jan, L. Y. (1992) Neuron 6, 767–767
35. Biale, T. M., Moscucci, A., Wang, H., Weaver, F. E., and Koren, G. (1994) Neuron 12, 615–626
36. Cabo, J. M., and Stühmer, W. (1998) J. Bacteriol. 174, 807–813
37. propionyl-CoA (5). The distinct pharmacology of these two proteins probably reflects relatively small variations in the conformation of the binding pockets or the bound ligand rather than qualitatively different mechanisms of activation. Given the sequence similarity among CAP, the kinases, and the CNG channels and the similar effects seen in each upon mutation of the conserved arginine residue, we expect that the CNB sites of these diverse proteins share a similar functional organization that underlies the mechanism of ligand activation.

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A State-independent Interaction between Ligand and a Conserved Arginine Residue in Cyclic Nucleotide-gated Channels Reveals a Functional Polarity of the Cyclic Nucleotide Binding Site
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