Bimodal agonism in heteromeric cyclic nucleotide-gated channels

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Abbreviations: CNG, cyclic nucleotide-gated; BD, binding domain; P₀, open probability

Introduction

Cyclic nucleotide-gated (CNG) channels are non-selective cation channels activated by direct binding of cGMP or cAMP, with important roles in visual1 and olfactory2 sensory transduction. These channels are also a biophysical model for ligand-dependent receptor switching, the structural basis of all signal transduction.3 Functional CNG channels are believed to be tetramers of four homologous subunits4,5 arranged in a ring around a central pore, as in the related potassium channels.6 Conventional subunits (CNGA1, A2, A3) will form homomeric channels when heterologously expressed alone, but in vivo will form heteromers with modulatory subunits (CNGA4, B1, B3).7,8 All conventional and modulatory CNG subunits have a conserved modular architecture (Fig. 1A). The transmembrane region is homologous to voltage-gated potassium channels, with six helices (S1-S6) and a re-entrant “pore-loop” between S5 and S6 that contains the cation selectivity filter. Fused to this is a cytosolic domain containing a “C-linker” region plus a cyclic nucleotide-binding domain (BD) homologous to those of other cyclic nucleotide-dependent proteins.9,10 Agonist-dependent switching of the CNG channel between the open (conductive) and closed (non-conductive) conformation is understood by the principle of allosteric modulation.11,12 Low basal open probability (P₀) is observed without agonist,13,14 and binding of agonist molecules increases P₀ by stabilizing the open state preferentially over the closed state. The essential structural features that couple channel opening to multiple agonist-binding events are still poorly understood.

For homomers of the catfish CNGA2 subtype,15 or of certain derivatives of this subunit,16 the dose-response relation of steady-state channel current vs. cGMP concentration has an unusual biphasic shape: increases in cGMP concentration up to 3 mM will increase channel activity, but further increases in cGMP concentration will decrease channel activity. Single-channel recordings16 showed that the falling phase of the dose-response curve reflects a cGMP-dependent reduction of steady-state P₀ (suggesting a gating effect) rather than a reduction of unitary conductance of the open channel (suggesting agonist pore-block).17,18 This contradicts the natural presumption that steady-state P₀ should increase monotonically with the number of agonist molecules bound. Instead, initial binding events preferentially stabilize the open state to increase P₀ (pro action) whereas subsequent binding...
with CNGA2-specific interactions between BDs of adjacent subunits (as with desensitization of glutamate-gated channels).19 Pro action in normal CNG channels shows a complex form of cooperativity without fully concerted action of the four subunits.20-24 This means the consequences of ligand binding in one subunit might change depending on structural features of neighboring subunits, including both occupancy (liganded vs. unliganded) and primary sequence (e.g., in a heteromer vs. homomer). At the extreme end of the cooperativity spectrum, a prokaryotic homologue of CNG channels is believed to perform pro action with wholly independent action of four subunits.25 To begin understanding how action in the context of competing “concerted” vs. “independent” models, we asked: Can subunits identified as bimodal in a homomeric channel still produce con action in a heteromer, incorporating normal subunits without the catfish CNGA2 BD? Or does bimodal agonism require that all events (to a partially liganded channel) preferentially destabilize the open state to decrease Po (con action). We term this phenomenon bimodal agonism because one agonist has two modes of action caused by distinct binding events on one channel. It was found that the catfish CNGA2 BD contains specific sequence elements essential for bimodal agonism. Two BD-substitution chimeras were constructed with different BD sequences but with identical sequence outside the BD. Whereas homomers of the chimera with the rat CNGA4 BD were “normal” with only conventional (pro) cGMP responses, homomers of the chimera with the catfish CNGA2 BD were bimodal.

Since reports of bimodal agonism have been limited to homomeric CNGA2 derivatives, one could entertain a simple model for con action as a conformational conversion with obligatory participation of all four subunits. This “concerted homomer” model would entail fully cooperative subunit action, perhaps

**Figure 1.** Bimodal agonism in chimera X-bimP containing 87% normal BD sequence. (A) Topology of the CNG channel subunit, showing BD sequences substituted in X-chimeras (top to bottom) from rat CNGA4 (grey), catfish CNGA2, and the chimeric BD of X-bimP. Black ticks mark 17 amino acids unique to CNGA2. (B) Macroscopic current traces from homomers of X-chimeras in inside-out patches held at -40 mV and perfused with cyclic nucleotide. Two patches of X-bimP are shown, one on each row. Solid and dotted bars mark wash-in and wash-out respectively, with “spikes” of maximal current marked by asterisks. Arrows mark steady-state region used for quantitation; for this patch, g_{10 mM cGMP}/g_{3 mM cGMP} = 0.63. (C) Macroscopic steady-state conductances of X-bimP homomers activated with cGMP (down-triangles) or cAMP (up-triangles), after normalization to the steady-state conductance measured at 3 mM cGMP (solid down-triangle) in the same patch. Points show means (n = 2 for 6 mM cGMP, n = 4 for other points); error bars show SD. Black curve shows fits (excluding cGMP data at >3 mM) to the Hill equation, g/g_{3 mM cGMP} = (g_{max}/g_{3 mM cGMP})/(1 + K_{1/2}/[cNMp])^h. Fitted parameters (±SE) are: for cAMP, K_{1/2} = 3.6 ± 0.3 mM, h = 0.72 ± 0.03, 1/g_{3 mM cGMP} = 4.1 ± 0.1; for cGMP, K_{1/2} = 402 ± 4 μM, h = 1.23 ± 0.01; 1/g_{3 mM cGMP} = 1.085 ± 0.005. Dashed grey curve shows cGMP dose-response curve for X-rA4 homomers.16 (D) General allosteric reaction scheme for bimodal agonism based on ref. 24. Favoured directions of C-O equilibria are indicated by arrow sizes.
four subunits contribute BD sequence elements unique to catfish CNGA2? We find that con action can indeed be produced with fewer than four CNGA2 BD's, but that con action also depends on the arrangement of the different subunits and therefore is a cooperative phenomenon.

Results

A 70-residue portion of catfish CNGA2 BD sequence can support bimodal agonism. It was previously shown that bimodal agonism relies on sequence elements of the BD from catfish CNGA2;16 that work made use of BD-substitutions in chimeric proteins, in a series of “X-chimera” channel subunits which all shared identical sequence outside the BD region. Each X-chimera subunit incorporates a different BD sequence (Fig. 1A), and each can be classed as either bimodal or normal according to its cGMP dose-response curve when expressed alone in Xenopus oocytes to form functional homomeric CNG channels. In this study, to test whether contributions from all four subunits might be required for bimodal agonism, we sought to characterize cGMP responses of heterotetrameric channels in which the bimodal subunits (B) were combined with normal subunits (N).

The normal (N) subunit selected for this test was X-rA4 (reviewed in ref. 16) in which the entire BD was derived from rat CNGA4. This chimera expresses robustly as homomers in oocytes and exhibits monotonic dose-response curves (i.e., with no con phase) for cAMP and cGMP. The CNGA4 BD shows agonist selectivity for cAMP over cGMP,16,26 due to a methionine residue on the final “C-helix” of the BD (M475 in CNGA4 numbering) that makes a discriminator-like contact.25

For the bimodal (B) subunit, we constructed and tested a new X-chimera, named X-bimP, which was identical to X-rA4 except that a limited portion of its rat CNGA4 BD was replaced with corresponding sequence from the catfish CNGA2 BD. The region replaced was 70 residues long at the N-terminal end of the BD, spanning from the A-helix through the phosphate-binding “PB” loop. This means that 113 out of 130 BD residues (87%) are conserved between X-bimP and the normal X-rA4 subunit. The conserved BD sequence elements include the methionine ligand-discriminator, as well as the “hinge” region and β8 strand previously implicated as additional determinants of agonist selectivity and gating efficacy.26 We found that X-bimP showed the distinctive features of bimodal agonism in its cGMP response (outlined below), as well as more reliable expression levels in oocytes in comparison to other bimodal constructs studied.

Figure 1B shows macroscopic current responses of X-rA4 homomers (normal channels) and X-bimP homomers assayed in excised inside-out patch membranes, where cyclic nucleotide solutions could be perfused to access the channel’s cytoplasmic BDs. The X-bimP current trace shows a distinctive two-spiked appearance in response to wash-in and wash-out of 10 mM cGMP, typical of previous reports of bimodal agonism (reviewed in ref. 16); these spikes are not seen with the normal X-rA4, or with X-bimP tested with cGMP at 3 mM or less. In between the wash-in and wash-out spike, the steady state current amplitude (white arrow) at 10 mM cGMP is notably lower than that recorded in 3 mM cGMP, a concentration that approximates the maximum of the cGMP dose-response curve (Fig. 1C, down triangles).

The coexistence of pro and con action in X-bimP channels can be described with the general scheme of Biskup et al.24 in Figure 1D: For a channel with x bound molecules of agonist (A), the pore-gate undergoes a closed-open (C-O) transition with a characteristic equilibrium constant, \( Q = \frac{[OA]}{[CA]} \). The consequence of an additional (x + 1)th binding event is either pro action where \( Q_{x+1} > Q_x \) or con action where \( Q_{x+1} < Q_x \). A normal channel shows pro action for all binding events up to the binding-site occupancy limit. For a bimodal channel, there must be some form of the channel where \( P_\text{c} \) has a maximum value and where the occupancy \( m \) is below the occupancy limit, so that additional binding gives con action with \( Q_{m+1} < Q_m \). At steady-state (conformational equilibrium) for a given cGMP concentration, a mixture of occupancies will exist in the channel population, and con action may or may not also occur for some binding events involving channels with occupancy less than \( m \). At concentrations near the maximum of the steady-state dose-response curve, however, the m-ligated channel is most relevant to the con action observed. (Channels with a given occupancy below \( m \) may exist as isomers with different arrangements of occupied binding sites, but are not explicitly distinguished in the model).

In the terms of this model, we interpret the wash-in spike (marked * in Fig. 1C) as a rapidly equilibrating channel population with an average occupancy that slowly increases in conjunction with the increase in cGMP concentration within the gravity-perfusion chamber. The concentration briefly reaches 3 mM where the channel population is dominated by m-ligated channels with maximal \( P_\text{c} \), but the cGMP concentration continues rising up to 10 mM cGMP where most channels have occupancy of \( m + 1 \) or more. Likewise, the wash-out spike (marked ** in Fig. 1C) reflects the average occupancy decreasing to \( m \) and then below \( m \).

As an objective measure of the con effect in X-bimP for comparison to other channels, we measured the steady-state conductance elicited by 10 mM cGMP and normalized it to the value of \( g_{10 \text{mM cGMP}} \) in the same patch; this ratio was \( g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}} = 0.711 \pm 0.096 (n = 18) \), significantly below unity in every patch. In contrast, homomers of the normal subunit X-rA4 reach a \( P_\text{c} \) of 0.94 at 3 mM cGMP,16 and produced macroscopic currents with \( g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}} \) close to unity (1.01 ± 0.03, \( n = 4 \)). Cyclic AMP elicited only pro action in X-bimP, in keeping with previous observations of bimodal subunits.16 Notably, there was strong cAMP selectivity as expected because of the methionine discriminator, with high (30 mM) cAMP concentrations eliciting conductances that were several-fold higher than those elicited by 3 mM cGMP (at the cGMP dose-response curve maximum). We can quantify an “efficacy selectivity ratio” of \( g_{30 \text{mM cAMP}} / g_{3 \text{mM cGMP}} \) which is 3.38 ± 0.59 (\( n = 10 \)) for X-bimP homomers. This is significantly higher than the value 1.38 for the previously studied X-fA2 homomers containing the full catfish CNGA2 BD with a glutamate at the discriminator position.16

B. N2 Long channels exhibit bimodal agonism. To constrain the stoichiometry of heteromeric CNG channels, we ligated different subunit sequences together (via a 6-residue linker) to form...
a tandem tetramer of the form B-N-N-B, where B represents the bimodal X-bimP sequence and N represents the normal X-rA4 sequence which is identical except in the N-terminal portion of the BD. Assembly of the pseudosubunits around the central pore axis is expected to produce functional channels with two adjacent bimodal pseudo-subunits and two adjacent normal pseudo-subunits, the cis configuration (Fig. 2A).

In contrast with B₄ channels (X-bimP homomers), the B₂N₂ cis channel conductances elicited by 3 mM cGMP and 10 mM cGMP were very similar to each other, suggesting a dose-response saturation plateau. The ratio $g_{(10 \text{ mM cGMP})}/g_{(3 \text{ mM cGMP})}$ for cis channels was 0.98 ± 0.02 (n = 14), very close to unity though lower by a statistically significant amount (t-test $p < 0.005$). The wash-in and wash-out current spikes indicative of bimodal agonism during application of 10 mM cGMP were no more than a few percent of total channel current amplitude and were indeed not discernable in some patches. However, evaluation of small spikes is subjective and may depend on vagaries of perfusion rates. When 30 mM cGMP was applied, the presence of con action was more clearly in evidence with a low ratio $g_{(30 \text{ mM cGMP})}/g_{(3 \text{ mM cGMP})} = 0.87 ± 0.05$ (n = 12) and substantial wash-in and wash-out current spikes seen in every experiment (Fig. 2B). (Note the higher viscosity of the 30 mM cyclic nucleotide solution led in some cases to a prolongation of the time required for wash-out). This strongly suggests that two X-bimP subunits can indeed produce cGMP-specific bimodal agonism when incorporated with two normal X-rA4 subunits. In comparison with B₄ channels, higher cGMP concentrations are required and the con action is apparent to a lesser degree.

Whereas it has been shown that concatenation will preserve pro activity in CNG subunits, this is the first use of tandem oligomers to study bimodal agonism. To test for artifactual effects of concatenation, we linked identical subunits in tandem homodimers, B-B (two X-bimP subunits) and N-N (two X-rA4 subunits). For each homodimer, expressed alone in oocytes, the cGMP response closely matched that of channels formed from unlinked monomers (Fig. 2C, grey data points). The homodimers also matched monomers in their cAMP pro action, with $g_{(30 \text{ mM cAMP})}/g_{(3 \text{ mM cGMP})} = 3.00 ± 0.58$ (n = 4) for B-B and $1.018 ± 0.007$ (n = 3) for N-N. This shows notably that incorporating an N subunit into a tandem oligomer without B subunits present will not be sufficient to produce bimodal agonism. Likewise, the dose-response behaviour of the B-subunit will not be distorted simply by its concatenation if N subunits are absent. Thus the distinctive dose-response behaviour of B-N-N-B reflects the simultaneous presence of B and N subunits.

Increasing cGMP concentration from 3 mM to 10 mM cGMP produces very little conductance change of B₂N₂ cis channels, which suggests unchanged occupancy or perhaps binding events with little effect on $P_o$. But as cGMP concentration is increased from 10 mM to 30 mM, average occupancy of B₂N₂ cis channels certainly increases to $m + 1$ or higher. We considered that between 3 and 10 mM cGMP, average occupancy might indeed increase over $m$, but the difference between $Q_m$ and $Q_{m+1}$ might go undetected because both values are numerically high enough that $P_o$ approaches unity. For instance, if $Q > 50$ then for a 2-fold change in $Q$ the associated open probability $P_o = Q/(Q + 1)$ will change by <1%. We can argue against this high-$P_o$ scenario for B₂N₂ cis channels because 30 mM cAMP (which showed no con action) could elicit significantly higher conductance than did 3 mM cGMP. Agonist selectivity for cAMP over cGMP was apparent (Fig. 2C) with an efficacious selectivity ratio $g_{(30 \text{ mM cAMP})}/g_{(3 \text{ mM cGMP})} = 1.18 ± 0.07$ (n = 7) significantly above unity ($p < 0.0004$). Assuming a fixed unitary conductance as previously demonstrated, the reciprocal of the efficacy selectivity ratio ($1/1.18 = 0.85$) gives an upper limit for $P_o$ at 3 mM cGMP. This corresponds to an equilibrium constant no higher than $0.85/(1 - 0.85) = 6$, not high enough to obscure the observation of, for instance, a 2-fold difference between $Q_m$ and $Q_{m+1}$. 

**Figure 2.** Bimodal agonism in heteromeric B₂N₂ cis channel. (A) Schematic shows fusion of B subunits (X-bimP) and N subunits (X-rA4) in a B-N-N-B tandem tetramer with expected cis arrangement. (B) Macroscopic current trace from example patch of B-N-N-B with $g_{(3 \text{ mM cGMP})} = 0.85$. (C) Steady-state conductances of tandem oligomers activated with cGMP (down triangles) and cAMP (up triangles), normalized as in Figure 1C (n = 3 for each point). Data is shown with black open points for B-N-N-B, grey solid points for B-B tandem dimers, and grey open points for N-N tandem dimers. Black line shows Hill fit for B-N-N-B cGMP dataset excluding 30 mM, with parameters as follows: $K_i = 95 ± 5 \mu\text{M, } h = 1.0 ± 0.5; g_{(3 \text{ mM cGMP})}/g_{(30 \text{ mM cGMP})} = 1.00 ± 0.04$. Grey lines show cGMP dose-response curves from Figure 1C for homomers of X-bimP (solid) and X-rA4 (dashed).
Thus we must conclude that either there is little change in site occupancy between 3 and 10 mM cGMP (saturation of m sites), or else the new sites that are being filled in this concentration regime produce a small net effect on Q.

He et al. previously suggested that modulatory subunits fused into tandem oligomers could misassemble, so that the tetrameric channel incorporated pseudosubunits from two different polypeptide chains. Could the bimodal agonism observed in our B-N-N-B recordings arise only from channels with four B pseudosubunits, formed by misassembly? The misassembly of modulatory subunits observed by He et al. should not apply in our case, since our X-chimeras contain sequence from a conventional subunit (bovine CNGA1) in the assembly-control region, C-terminal of the BD. Moreover, we can show that a mixture of bimodal B4 and normal N4 channels (whose dose-response relations are known from experiments on pure populations), could not have produced the observed dose-response relation of our B-N-N-B recordings. We took the assumption of a B4 + N4 mixture, in 10 out of 10 measurements at 10 mM cGMP and in 6 out of 7 measurements at 30 mM cAMP.

We found that the actual observed value of $g_{30 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ (3 mM cGMP) in 10 mM cGMP and 30 mM cAMP in the same patch (Fig. 3 dashed lines, confidence intervals defined by gray stripes). We found that the actual observed value of $g_{30 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ lay outside the predicted confidence intervals for this concentration in a B4 + N4 mixture, in 10 out of 10 measurements at 10 mM cGMP and in 6 out of 7 measurements at 30 mM cAMP. Thus the majority of B-N-N-B patches show a characteristic dose-response relation that is inconsistent with mixtures of homomers but is consistent with reproducibly assembled heteromers.

B2N2 trans channels show normal agonism but are sensitive to variations in biogenesis. Since interactions between adjacent subunits have been proposed to influence CNG channel gating, we hypothesized that the capability of two B subunits to confer con action might be different in a B-N-B-N tetramer where the bimodal subunits are diagonally opposite, in the trans configuration (Fig. 4A).

The steady-state currents from the B2N2 trans tetramer showed evidence for a saturation plateau from 3 to 10 mM cGMP, with a mean value for the ratio $g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ of 1.008 ± 0.034 (n = 21), not significantly different from unity (p > 0.15). Wash-in and wash-out current spikes were observed in one single “outlier” recording tested with 10 mM cGMP; this recording had an unusually low $g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}} = 0.92$, even lower than any value observed for the consistently bimodal B2N2 cis channel recordings. No spikes were observed in the remaining 20 of 21 recordings, with $g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ ranging from 0.95 to 1.05 and a mean of 1.012 ± 0.029 (n = 20), which is in fact higher than unity by a small but statistically significant amount (p < 0.04).

For 30 mM cGMP, the ratio $g_{30 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ had a mean of 1.02, indicating normal agonism, but had a relatively large standard deviation of 0.11 (n = 15), deriving from a non-normal distribution of values. This arose because of a minority of patch recordings (4 of 15 where 30 mM cGMP was tested) with qualitatively different behaviour. In the “majority-type” recordings (11 patches, from oocytes derived from nine frogs) there were no wash-in and wash-out spikes (Fig. 4Bi) and $g_{10 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ ratios were in the range 0.97 to 1.18 with a mean 1.071 ± 0.064 (n = 11). This value is significantly higher than unity (p < 0.003), so majority-type recordings can be classed objectively as normal, not bimodal. In the “outlier-type” recordings (4 of 15 patches, from oocytes from two of the nine frogs, including the outlier patch with spikes in 10 mM cGMP), wash spikes in 30 mM cGMP were observed (Fig. 4Bii), and $g_{30 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ ratios were in the range 0.83 to 0.92, with a mean 0.877 ± 0.046 (n = 4). Although majority- and outlier-type groups were categorized by a subjective visual inspection, the non-overlapping ranges of the $g_{30 \text{mM cGMP}} / g_{3 \text{mM cGMP}}$ ratio values in the two groups (Fig. 4C) objectively confirms their clear divergence.

For each of the two batches of oocytes giving rise to an outlier-type recording, at least one majority-type B-N-B-N recording was also collected from an oocyte taken from the same ovary and injected with RNA from the same microneedle at one sitting. Therefore the outlier-type recordings did not arise from misidentified RNA samples, but from cell-to-cell variations in biogenesis of the B-N-B-N polypeptide; biogenesis is defined as translation, folding, trafficking, assembly, and other post-translational modifications. Such variation might be due to subtle differences in each oocyte’s developmental stage.
The change in $P_o$ at high cGMP concentrations was too small to be detected.

We can never prove that a $B_{2N_2}$ trans channel is absolutely incapable of bimodal agonism under all conditions, because a negative result can never be definitively proven. Our conclusion, taking into account all of our observations, is that the majority-type recordings represent the most typical cGMP response of $B_{2N_2}$ trans channels in our experimental system, with only pro action up to 30 mM agonist concentrations. In contrast, the cis channels consistently showed con action between 10 and 30 mM cGMP and a maximum $P_o$ below unity for cGMP-activation. The simplest explanation is that the arrangement of B subunits within the $B_{2N_2}$ tetramer affects the open probability of the channel and specifically the ability to support con action at concentrations near 30 mM.

**Discussion**

The most important conclusions of this work are as follows: First, we showed that the peculiar phenomenon of bimodal agonism is not confined to homomeric assemblies of specific CNG channel subunits (such as intact catfish CNGA2 subtype or its derivative X-bimP). Rather, at least one form of heteromer containing normal (N, X-rA4) subunits mixed with bimodal subunits (B, X-bimP) can support bimodal agonism. Second, we examined cis and trans forms of the $B_{2N_2}$ channel and found that having adjacent B subunits is important for con action in the range 3 through 30 mM. Quite apart from the specific issue of a mechanism for con action, the finding of any difference between cGMP dose-response curves of cis vs. trans channels is important evidence that Neighbor-interactions influence coupling of ligand-binding to channel opening in CNG channels. A final additional advance of our work is the more narrow delineation of the catfish CNGA2 BD sequence elements that are sufficient to support bimodal agonism in our X-chimeras.

**Bimodal agonism with fewer than four B subunits.** The skeletal nature of our model in Figure 1D focuses our questioning: what we need to understand are the structural features of the channel with occupancy $m$, which is a receptor structure that treats the incoming cGMP molecule as an inverse agonist ($Q_{m+1} < Q_m$) rather than a normal positive agonist ($Q_{m+1} > Q_m$). A maximum $Q$ value at partial occupancy $m$ contrasts with traditional desensitization in acetylcholine- and glutamate-gated channels where $P_o$ increases with occupancy even when desensitization is taken into account. In this light, finding bimodal agonism in our $B_{2N_2}$ cis channels is important because it implies the existence of an $m$-liganded channel (capable of con action) that includes N subunits. If we assume that the BDs of “normal” N subunits are incompetent to participate in the con action mechanism, then our finding leads us to exclude any of the four BDs must perform a concerted action in some reaction step. The possibility of concerted action during pro action is neither refuted nor supported by this conclusion about con action.

Since N subunits in homomeric form show pro action with a submillimolar EC$_{50}$, the two N subunits in the bimodal $B_{2N_2}$ cis channel are likely to be occupied at the relevant concentrations.
3–30 mM cGMP where the m-ligated form exists. From 3–10 mM cGMP, the observed dose-response plateau with P, no higher than 0.85 suggests saturation, so the m-ligated channel may in fact be the predominant form of the channel population in this regime. However, binding of cGMP to the m-ligated channel is notable because of its qualitative consequence of con action rather than its quantitative consequences. To understand this con action, it is not necessary—and indeed it may not even be helpful—for the model to capture details of the mechanism for the first m binding events, such as precise affinities of the m binding sites or the numerical values of Q. We eliminated the possibility that homomeric B, and N channels could account for the bimodal agonism observed in heteromer experiments, by using the dose-response relation of B, channels as a functional fingerprint. Whether B, N or BN, channels are bimodal remains undetermined, since we were unable to isolate DNA constructs for these tandem tetramers (see Methods). However, if B, N and BN, channels are not bimodal then our current analysis in Figure 3 is valid, and if either B, N or BN, channels are bimodal then this would reinforce our refutation of the concerted-homomer model.

A previous study of heteromers23 sought confirmation of stoichiometry without making tandem tetramers, by “tagging” one pseudo-subunit with a pore-loop sequence variant giving a characteristic unitary conductance in single-channel recordings. However, others have argued that varying the pore-loop is likely to influence gating behavior,24 and indeed the ion pathway of CNG channels is gated by the pore-loop35 rather than the S6 helix as in related channels. Moreover, the structural interpretation of conductance differences arising from pore-tags cannot be independently confirmed. Thus even if we were to repeat our experiments using pore-tags, this would not absolutely exclude the possibility of incorrect subunit assembly. We chose to avoid the controversy associated with pore-tags, opting instead to have every subunit of our channels contain identical sequences in the pore (and everywhere else outside the BD). This is especially important because our B, N trans channels preponderantly showed a lack of con action at up to 30 mM cGMP, and this difference from the cis channels can now be attributed wholly to the geometric arrangement of specific binding domains, rather than the arrangement of pore-loops or transmembrane regions with different sequences.

**Neighbor-contributions influence open probability.** We found bimodal agonism is apparent between 10 and 30 mM cGMP in our B, N trans channels when bimodal subunits are adjacent to each other (cis) but not when they are separated by an intervening normal subunit (trans). Even without considering a particular mechanism for con action, a difference between cis and trans B, N channel gating properties would be impossible in an independent-action model,25 or any similar model where binding domains acted on the channel gate with complete equivalence. This provides strong evidence for the general principle that subunit positioning in CNG channels is important in determining how the interaction of cGMP with a BD is transduced into a P, change. Non-equivalence of binding events has already been observed repeatedly in the context of CNG channel pro action,20–24 but our findings directly point to geometric positioning of BDs as a physical determinant of this non-equivalence.

The crucial hypothesis, that rearranging subunits of a heteromeric channel should change the P, elicited by cyclic nucleotide, has only been tested once previously in CNG channels. This was in the previously mentioned study based on conductance-tagged subunits23 co-expressed as monomers or as tandem dimers. Channels were tested with X,Y stoichiometry, combining subunits with mutated or intact binding pockets. The cis and trans forms had respectively lower or higher P, values at saturating cGMP concentrations. The tandem tetramer strategy was also recently applied to cyclic nucleotide-potentiation of X,Y HCN channels, and also found cyclic nucleotides produced less enhancement of opening for cis channels than trans channels.34 Our data explores beyond these previous studies of the conventional (pro) action of cyclic nucleotides, to show that Neighbor-arrangement is also important for the unusual con action. In line with the previous trend that cis channels have less efficient activation than trans channels, it is the B,N cis channels that show reduced P, due to con action at 30 mM cGMP.

**Neighbor-contributions determine bimodal agonism.** Now we turn specifically to the crucial cGMP binding event producing observable con action, at a subunit of an m-ligated channel. We presume the key binding site to be in a B subunit, but we find that con action is not completely determined by the presence of two B subunits in a B,N tetramer. The majority of trans channel recordings were markedly different from cis channel recordings, implying that con action requires a Neighbor-derived contribution (using the N-terminal region of the BD) whose operation is restricted to B subunits located adjacent to each other. We could not test if the key restriction would also prevent con action in BN, tandem tetramers since we could not isolate that construct. However, functional differences between BN, channels and the bimodal B,N cis channels might arise due to subunit stoichiometry or Neighbor-relationships or both. Our data, though limited to comparing channels with BN, stoichiometry, highlights an effect due to Neighbor-relationships alone.

We found the restriction to be typical for B-N-B-N channels but not absolute, since con action of outlier-type B-N-B-N patches was seen not just in a single channel molecule but in entire populations of channels within individual outlier-type oocytes. If we imagine, for instance, that outlier-type recordings derived from anomalous subunit assembly in a cell, then this anomalous assembly process occurred for a large number of channels in each outlier-type oocyte yet not in majority-type oocytes. A more plausible difference between majority-type and outlier-type cells could be in the average activity of an enzyme such as a kinase, leading to channel populations with different average densities of a covalent modification such as phosphorylation.

Invoking a cell-based explanation for the difference between majority and outlier B-N-B-N patches does not contradict our proposal that the difference between majority B-N-B-N and B-N-N-B patches arises from Neighbor-contributions. In the latter case, the known polypeptide sequence difference (giving trans vs. cis subunit arrangement) provides an explanation
always. This “indirect” model predicts the same phenotypes as our proposed “direct” model. Notably, however, both models force us to postulate that the minimal functional unit for achieving efficient phosphorylation (indirect model) or for directly suppressing $P_o$ (direct model) requires at least two channel subunits, not one subunit alone.

**Rational models for bimodal agonism?** Although we cannot pin down a conclusive picture for the peculiar phenomenon of bimodal agonism, it is useful to try writing a structurally plausible mechanism where (a) B subunits produce both pro and con action, and (b) what a B subunit does somehow depends on the identity of its Neighboring subunits. Figure 5 gives an illustrative example (not intended to exclude other suitable models): binding to a B subunit produces con action only if there is a liganded B subunit as an adjacent Neighbor, and all other binding events produce pro action. This can explain failure of con action in trans channels and coexistence of pro and con action in cis channels and B$_4$ homomers. It does not postulate allosteric binding sites for cGMP outside the canonical binding pocket; that possibility could be incorporated in an alternative model (Suppl. Fig. 1, $m = 4$ canonical sites), where the minimal functional unit for the non-canonical site requires residues from two adjacent B subunits. For any of these models, if BN$_3$ channels (not yet isolated) could be shown to be bimodal, then new rules would be needed to explain the failure of con action in trans B$_N$N$_2$ channels.

Physical interactions between the C-terminal cytoplasmic regions of CNG channel subunits have already been proposed to control longitudinal rotation of the A’ helix and S6, and thereby the position of the pore-loop that gates CNG channels. This type of mechanism could account for either increases or decreases in $P_o$, depending on the A’ helix geometries produced by combinations of adjacent liganded and unliganded subunits. While subunit interactions could operate with the four-fold symmetry seen in the potassium channel pore, electron microscopic ultrastructure analysis of the CNGA1 homomer strikingly shows a two-lobed structure, and a pairwise organization was proposed in a “coupled-dimer” model based on functional analysis of pro action. Our findings suggest that subunit pairing has an important effect on con action in particular, and definitely show that a complete ring of four bimodal BDs is not required for this unorthodox gating phenomenon.

**Materials and Methods**

**Recombinant DNA.** Previous reports have described the design and construction of BD-substitution chimeras, and the specific “X-chimera” series, which incorporate 130-residue BD sequences from catfish CNGA2 (ref. 15; residues L455-G584) and rat CNGA4 (refs. 36 and 37; residues L356-A485). All X-chimeras have invariant sequence outside the BD, including bovine CNGA1 sequence in the region C-terminal of the BD that regulates subunit assembly. This enables efficient formation of homomers in the oocyte expression system, even though BD sequence (in X-rA4) derives from a modulatory subunit. The new X-chimera, X-bimP, contains a BD with catfish CNGA2 residues L455-M525 followed by rat CNGA4 residues G427-A485,
and was constructed using standard PCR and restriction/ligation techniques as previously reported. All genes for monomer subunits in this study were deoxyrase sequenced in entirety, and cRNA was generated by in vitro transcription of the linearized plasmids (Ambion mMessage).

For construction of tandem tetramers, two individual M or N genes (where M and N are either X-rA4 or X-bimP subunits) were first fused to form a tandem dimer M-N, and pairs of dimer genes were then fused to produce tandem tetramers. To form an M-N fusion, the M and N gene sequences were ligated at compatible cohesive ends from non-identical restriction sites giving a ligation splice site that was resistant to restriction cleavage. In this case, the PsPOMI cohesive end (found within gene M at its 3’ end) was ligated to a EagI cohesive end (engineered into the plasmid bearing gene N, at a position 11 bp 5’ of the start codon). In the resultant fusion protein, amino acids TDSTQD at the C-terminus of subunit M were replaced with amino acids ST, followed by the initial methionine of subunit N. Incorrect clones of abnormally short length were frequently isolated after transformation of plasmids containing tandem direct repeats even though a recA mutant *E. coli* strain (STBL2, Invitrogen) was used for transformation. The prevalence of false clones was so high that our attempts at isolating tandems with three or more direct repeats (e.g., B-B-B-B, B-B-B-N) were unsuccessful. The successfully isolated clones (B-B dimer, N-N dimer, B-B-N-B tetramer and B-B-N-B-N tetramer) were verified by restriction mapping of a unique EcoRV site in the B subunit genes, in lieu of sequencing. Denaturing gel electrophoresis confirmed the expected differences in molecular weight between cRNA transcripts from monomer, dimer and tetramer genes.

**Electrophysiology.** All constructs were expressed in Xenopus oocytes by injection of cRNA and assayed in the excised inside-out patch-clamp configuration using previously reported procedures. In brief: Oocytes were harvested from mature female *Xenopus laevis* frogs (procedures approved by the Canadian Council on Animal Care), defolliculated by collagenase (Sigma) and injected with 15–40 ng RNA; 1–7 days later, inside-out membrane patches were excised from oocytes, using heat-polished borosilicate glass micropipettes (0.5–5 MΩ). Pipette and bath solutions both contained (in mM): 67 KCl, 30 NaCl, 10 HEPES, 10 EGTA, 1 EDTA, pH 7.2 with KOH. As needed, the sodium salts of cAMP or cGMP (Sigma) were included in the bath solution of cAMP or cGMP (Sigma) were included in the bath solution by iso-osmolar replacement of NaCl, and applied by gravity perfusion. Current data was acquired at room temperature (19–23°C) with either an Axopatch 200B or GeneClamp500 amplifier, filtered at 10 kHz using the amplifier’s low-pass Bessel filter, and recorded at 0.1–1 kHz by a Digidata 1322 and pClamp 9.0 software (Axon Instruments).

Patches were held at -40 mV and steady-state currents in the presence of cyclic nucleotide were corrected by subtraction of leak currents in the absence of cyclic nucleotide. Channel conductance in 3 mM cGMP was used in each patch to normalize conductances in other conditions. Unless otherwise noted, means are reported ± SD with n the sample size, and conductance ratios were compared to unity using t-test. Excised patches nearly always show variation (run-up or run-down) between macroscopic conductances elicited by repeated applications of the same agonist solution, due to unknown spontaneous changes such as dephosphorylation. All data reported were collected after waiting a sufficient time for completion of most runup, as shown by a difference of <10% among multiple measurements of current in 3 mM cGMP, but some residual variation of a few percent could not be avoided.

**Prediction of relative conductances in B4 + N4 mixtures.** (Fig. 3) We considered high cAMP or cGMP concentrations >3 mM where N4 channels have g(3 mM cGMP) closely approximated as unity. Then for a given agonist condition, a mixture of B4 and N4 homomers has g(3 mM cGMP) given by the linear combination:

\[
g(3 \text{mM cGMP}) = fR + (1 - f)
\]

where f is the B4 mole fraction, and R is the value of g(3 mM cGMP) determined experimentally for a pure population of B4 channels in the same condition. For each B-N-N-B recording, the observed value of g(30 mM cGMP)/g(3 mM cGMP) was used in the linear combination equation to predict f, which was then used to find the expected values of g(30 mM cGMP)/g(5 mM cGMP) for the 10 mM cGMP and 30 mM cAMP conditions.

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/ChanCHAN3-6-Sup.pdf

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