The Single-Channel Dose−Response Relation Is Consistently Steep for Rod Cyclic Nucleotide-Gated Channels: Implications for the Interpretation of Macroscopic Dose−Response Relations†

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Received March 8, 1999; Revised Manuscript Received June 10, 1999

ABSTRACT: Cyclic nucleotide-gated channels contain four subunits, each with a C-terminal binding site for cGMP or cAMP. The dose−response relation for activation is usually fit with the Hill equation, $I/I_{max} = [cGMP]^n/([cGMP]^n + K_{1/2}{}^n)$, where I/I_{max} is the fraction of maximal current, $K_{1/2}$ is the concentration of cGMP that gives a half-maximal current, and n is the Hill coefficient, taken as the minimum number of ligands required for significant activation. The dose−response relations in multichannel patches are often fit with Hill coefficients of ≤2.0, even though other lines of evidence indicate that these channels contain four binding sites and that the binding of three or four ligands is required for significant opening. We have measured dose−response relations for a large number of single cyclic nucleotide-gated channels composed of the bovine rod α subunit. We find that the single-channel Hill coefficient is consistently higher than 2.5, with an average of 3.0 ± 0.37 over 16 patches. In both multichannel and single-channel patches, large variations in $K_{1/2}$ have been observed, and are thought to arise from modifications such as phosphorylation. Here we show that mixtures of single channels with high Hill coefficients and variable $K_{1/2}$ values will give rise to shallow macroscopic dose−response relations with anomalously low Hill coefficients. This is because activation occurs over a broad range of cGMP concentrations. Thus, dose−response relations from multichannel patches should be interpreted with caution, particularly when detailed mechanistic issues such as cooperativity are being investigated.

Cyclic nucleotide-gated (CNG)[1] channels generate the electrical response to light in retinal photoreceptors and to odorants in olfactory receptors (*1*−*5*). Ca^{2+} entering through CNG channels is an important feedback signal in transduction and adaptation (*6*-*13*), and has also been implicated in the regulation of transmitter release at photoreceptor synaptic terminals (*14*, *15*). CNG channels are known to be present in a variety of other cell types, where their functions are, as yet, unclear (*4*, *16*, *17*).

CNG channels can assemble as homomultimers of α subunits or as heteromultimers comprised of α and β subunits (*18*−*25*). Each subunit contains a cytoplasmic cyclic nucleotide-binding domain near the C-terminus (*18*, *26*). There is evidence that homomultimeric channels form tetramers (*27*, *28*), like voltage-gated potassium channels (*29*, *30*). CNG channels have become a popular system for investigating mechanisms of gating and allosterism (*31*, *32*), in part because they share structural similarities with voltage-gated channels and functional similarities with other ligand-gated channels, but lack the experimental disadvantages of inactivation or desensitization (*1*, *33*).

To assess the degree to which CNG channels open with different numbers of ligands bound, a technique was developed recently that allowed single channels to be locked in every possible liganded state (*34*). In these studies, a photoaffinity analogue of cGMP, 8-*p*-azidophenacylthio-cGMP (APT-cGMP; *35*), was used to covalently tether cGMP moieties to the binding sites of single homomultimeric rod channels expressed in *Xenopus* oocytes. This work indicated that maximal opening of the channel required the binding of four cGMPs and that significant opening required the binding of three cGMPs (33% of maximal). The binding of two cGMPs caused a low level of opening (1%), and the binding of one cGMP did not measurably increase the level of opening above spontaneous levels ($\sim 10^{-5}$).

A common experiment that is done to study the activation of a CNG channel is to measure the dose−response relation for cGMP, cAMP, or other ligands. Steady-state currents are measured in excised, inside-out membrane patches in response to different concentrations of activating ligand. The dose−response relations are often fit with the Hill equation, which describes a model in which there are only two states, unliganded and fully liganded (*36*). When this model is applied to channel activation, the unliganded channel is

† This work was supported by National Eye Institute Grants EY09275 (to J.W.K.), EY11397 (to R.L.B.), and EY06713 (individual NRSA to ML.R.).

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[1] Abbreviations: CNG, cyclic nucleotide-gated; APT-cGMP, 8-*p*-azidophenacylthio-cGMP.

10.1021/bi990532w CCC: $18.00
Published on Web 07/23/1999

assumed to be closed and the fully liganded channel is open. Although this model is too simple, and the complete cooperativity of binding is unrealistic, the fit parameters of the Hill equation have been used as indices of the overall sensitivity of the channel to the ligand ($K_{1/2}$, the concentration which causes half-maximal activation) and the minimum number of ligands required for significant opening (n, the Hill coefficient).

The results with locked channels described above predict a Hill coefficient of about 3.0, since three cGMP molecules are required for significant activation. However, this appears to contradict a common finding for homomultimeric rod channels expressed in oocytes: the fact that Hill coefficients of ≤2.0 are often obtained when macroscopic (multichannel) dose−response relations for cGMP are fit with the Hill equation. Such a result implies that the binding of only two ligands is sufficient to cause significant opening. In fact, investigators observing such dose−response relations have had more success fitting the relations with two-binding site models than with three- or four-site models (*37*−*39*). However, the Hill coefficients reported for the rod CNG channel are quite variable, ranging from 1.5 to 3.4.

In this study, we have investigated the basis for the wide variability in Hill coefficients and the frequent observation of low values. We find that the dose−response relations in single-channel patches containing the homomultimeric rod channel are consistently steep, with a mean Hill coefficient of 3.0, and no values below 2.5 in 16 patches. In contrast, macroscopic dose−response relations measured under the same recording conditions were fit with a mean Hill coefficient of 2.1, and values between 1.5 and 2.0 were observed frequently. In both single-channel and multichannel patches, $K_{1/2}$ varied over a wide range. We suggest, on the basis of these data, that multichannel patches are normally a mixture of channels with steep dose−response relations and variable sensitivities to cGMP. The composite dose−response relation is shallow because the channels in the patch respond to different cGMP concentration ranges.

MATERIALS AND METHODS

All measurements were made on homomultimeric channels formed from the α subunit of the bovine rod cyclic nucleotide-gated channel. Channels were expressed in *Xenopus laevis* oocytes after the injection of in vitro-transcribed RNA, and incubation for 3−14 days. Channel activity was recorded in the excised, inside-out patch configuration with symmetrical recording solutions containing 130 mM NaCl, 2 mM HEPES, 0.02 mM EDTA, and 1 mM EGTA (pH 7.6). Cyclic GMP was added to the control solution at different concentrations for dose−response assays. All dose−response relations were fit with the Hill equation, $I/I_{max} = [cGMP]^n/([cGMP]^n + K_{1/2}{}^n)$. In some cases, dose−response relations were fit assuming two or more channel populations; e.g., $I/I_{max} = f_1[cGMP]^{n_1}/([cGMP]^{n_1} + K1_{1/2}{}^{n_1}) + f_2[cGMP]^{n_2}/([cGMP]^{n_2} + K2_{1/2}{}^{n_2})$, where f_1 and f_2 are the fractional contributions of each population.

For macropatches, oocytes were injected with 25−50 ng of in vitro-transcribed RNA and incubated at 18 °C. Recording electrodes had resistances of 0.8−1.2 MΩ, and currents were measured at +50 mV. Currents were recorded with an Axopatch 200A amplifier (Axon Instruments), filtered at 1 kHz with an eight-pole Bessel filter (Axon Instruments), and sampled at 5 kHz. Large currents (>5 nA) were corrected for the voltage drop across the pipet series resistance. Cyclic GMP-activated currents were determined as the difference between currents measured in the presence and absence of cGMP. The fraction of maximal current, I/I_{max}, was the current at a given concentration divided by the maximal current measured at a saturating cGMP concentration. The maximal currents and leak currents were typically measured two or three times during the dose−response assay to minimize the effect of drifts.

For single channels, 50−250 pg of in vitro-transcribed RNA was injected, and incubation was carried out at 16 °C. Recording electrodes were coated with Sylgard and had resistances of 12−20 MΩ. Currents were measured at +50 mV, and single-channel patches were identified when the maximal current amplitude observed at a subsaturating cGMP concentration did not increase at a saturating cGMP concentration. At least 30 s (30 s to 3 min) of channel activity was recorded at each cGMP concentration. Single-channel records were initially filtered at 5 kHz, digitized at 88 kHz with a Neuro-corder DR-484 PCM recording unit (Neuro Data Instruments), and stored on VHS tape. For analysis, the data were played back, filtered at 1 kHz, and sampled at 5 kHz. Analysis was performed with the Fetchan program (Pclamp6, Axon Instruments) using multiple thresholds to resolve four distinct current levels representing a closed state and three different conducting states. For dose−response relations, the mean currents at each concentration of cGMP were calculated via $\Sigma(ip)$, where i is the current amplitude and p is the open probability for each open event. The fractional current, I/I_{max}, was the mean current at a given concentration divided by the mean current measured at a saturating cGMP concentration. Dose−response relations constructed from all-points histograms were indistinguishable from those constructed using the multiple-threshold method. Open probabilities did not change significantly when records were filtered at 5 kHz or when records were corrected for missed events. For example, in one patch, the Hill coefficients were 2.98 when records were filtered at 1 kHz, 2.89 when the same records were corrected for missed events, and 2.90 when records were filtered at 5 kHz. Sometimes the channel exhibited long shut periods on the order of hundreds of milliseconds. We assumed these "quiescent" periods also occurred in macropatches; thus, they were not omitted from the single-channel records. For comparison, in some patches long closed times were removed, and the Hill coefficients remained consistently high.

The single-channel dose−response relations were measured after spontaneous shifts in $K_{1/2}$ were no longer occurring in the patches. These shifts are thought to result from dephosphorylation of the channel; they normally happen during the first 5−10 min in oocyte patches, with a saturating cGMP concentration speeding the process (*40*). To verify that spontaneous shifts were not occurring during the measurement of a dose−response relation, several of the cGMP concentrations were tested at two different times. In these experiments, the solution in the chamber (100 μL) was changed in about 30 s to 1 min with a gravity-driven perfusion system. About half of the macropatch data was obtained in the same way, while the other half was obtained using a rapid solution switcher (Molecular Kinetics) which

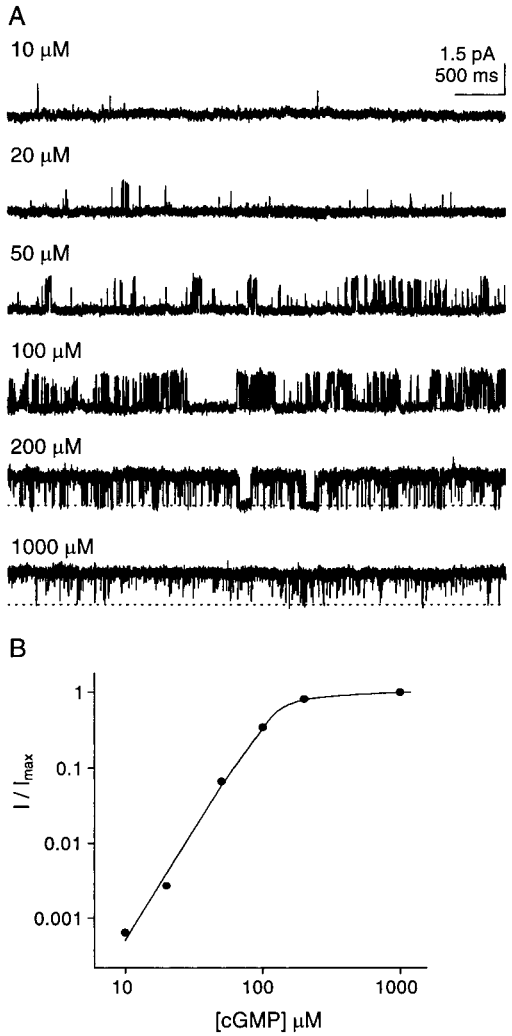

FIGURE 1: (A) Currents through a single CNG channel activated by different cGMP concentrations. The dotted line shows the closed level. The data were from an excised, inside-out patch at a membrane potential of +50 mV. (B) Dose−response relation for the patch shown in panel A. For I/I_{max} values, mean currents at each cGMP concentration were determined from records ranging from 34 to 102 s in length, and expressed relative to the mean current at a saturating cGMP concentration (1 mM). The smooth curve is a fit of the Hill equation to the data, with an n of 3.0 and a $K_{1/2}$ of 127 μM.

allowed an entire dose−response relation to be measured as a snapshot in time (within 1−2 min). The conclusions regarding shallow dose−response relations in macropatches were the same for both perfusion methods.

RESULTS

Figure 1A shows currents through a single CNG channel composed of the bovine rod α subunit. The membrane patch was excised from a *Xenopus* oocyte, and the cytoplasmic face of the patch was exposed to the indicated cGMP concentrations. The presence of only one channel was verified initially by a maximal outward current of about 1.5 pA at a holding potential of +50 mV, measured for several minutes at a saturating cGMP concentration (1 mM). Clearly, activation of the channel was strongly dependent on cGMP concentration, with 10 and 20 μM inducing brief openings to the main conducting state and subconducting states (*34*, *41*−*44*). Intermediate concentrations (50 and 100 μM) caused

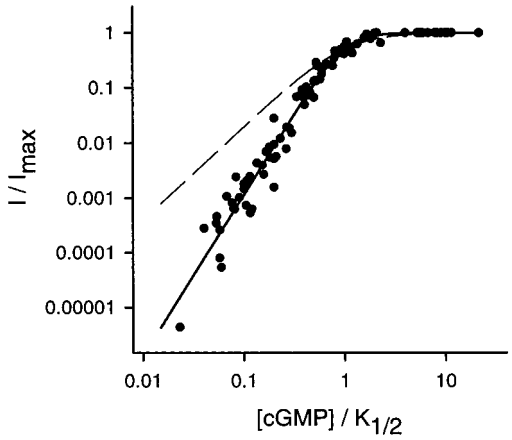

FIGURE 2: Combined dose−response relation from 16 single-channel patches. Cyclic GMP concentrations were expressed relative to the $K_{1/2}$ value for each individual patch. Fractional currents are defined in the legend of Figure 1. The smooth curve is a fit of the Hill equation to the data, with an n of 2.9. For comparison, the dashed curve is generated from the Hill equation with an n of 1.7.

frequent openings that often occurred in bursts. High concentrations caused the channel to spend most of its time in the main conducting state, interrupted by brief sojourns to subconducting and closed states. All of these effects were reversible, and the order of concentrations did not matter. Figure 1B shows the dose−response relation for this patch, plotted as the fraction of maximal current versus cGMP concentration on double log coordinates. At each concentration, the mean current through the channel (see Materials and Methods) was divided by the mean current at a saturating cGMP concentration. The smooth curve is the best fit of the Hill equation to the data, with an n of 3.0 and a $K_{1/2}$ of 127 μM.

Figure 2 shows combined data from 16 single-channel patches. Because the values of $K_{1/2}$ varied among patches, cGMP concentrations were expressed relative to each channel's $K_{1/2}$. This aligns the dose−response relations along the concentration axis and allows the slopes to be compared. The combined dose−response relation was fit with a Hill coefficient of 2.9. When only I/I_{max} values between 0.01 and 1 were considered (a typical range in macropatch experiments), a Hill coefficient of 2.8 was obtained. For comparison, the dashed curve represents a Hill coefficient of 1.7, which is commonly observed in multichannel patches. Clearly, the single-channel relations were quite steep, and they differed markedly from shallower relations often found in macropatches. A quantitative comparison of the two types of patches showed that the mean Hill coefficient in 16 single channel patches was 3.0 ± 0.37 (standard deviation) with a range from 2.5 to 3.9, and the mean Hill coefficient in 112 macropatches was 2.1 ± 0.30 with a range from 1.5 to 2.8. The two data sets barely overlapped ($p < 10^{-10}$, t test).

Figure 3A shows the Hill coefficients and $K_{1/2}$ values for single channels (white symbols) and for multichannel patches (black symbols). As described above, the two sets of Hill coefficients were clearly separate. In contrast, there was considerable overlap in $K_{1/2}$ values between the two data sets. Since the single channels had slightly higher $K_{1/2}$ values on average, we asked if high Hill coefficients were correlated with high $K_{1/2}$ values. The dotted lines in Figure 3A represent linear regressions and show that no such correlation exists,

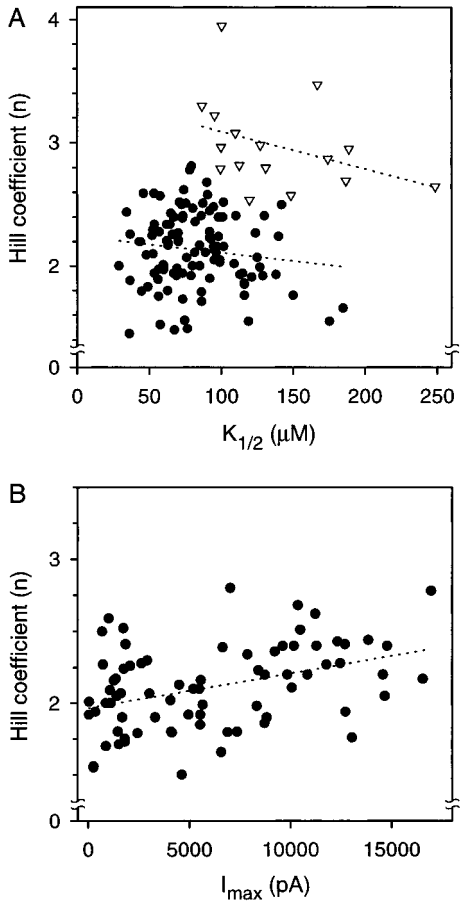

FIGURE 3: Hill coefficient values were not correlated with $K_{1/2}$ values, or with the number of channels in a macropatch. (A) White triangles show the Hill coefficient and $K_{1/2}$ values for each single channel, and black circles show the same parameters for each macropatch. In single-channel patches, the $K_{1/2}$ range was 86−248 μM, with a mean of 137 ± 45 μM; in macropatches, the $K_{1/2}$ range was 27−185 μM, with a mean of 83 ± 29 μM. Hill coefficient ranges are given in the text. The dotted lines are linear regressions for each data set. (B) Hill coefficients for 78 individual macropatches are plotted against maximal currents elicited by 1 mM cGMP (I_{max}). I_{max} values ranged from 40 to 17 000 pA.

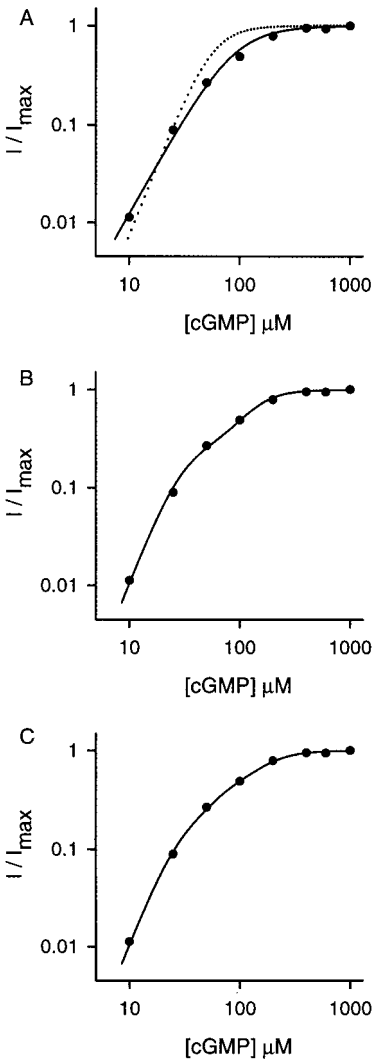

FIGURE 4: Dose−response relation from a macropatch, fit assuming one or more channel populations. Black symbols represent steady-state currents at different cGMP concentrations measured at +50 mV and divided by the maximal current at 1 mM cGMP (892 pA). (A) The smooth curve is a fit of the Hill equation to the data with an n of 2.0 and a $K_{1/2}$ of 86 μM. The dotted curve shows the best fit with the Hill coefficient constrained to be 2.9. (B) The smooth curve is a fit assuming two populations of channels with Hill coefficients of 2.9, the following $K_{1/2}$ values and proportions: 30 μM and 0.26 and 131 μM and 0.74. (C) The smooth curve is a fit assuming four populations of channels with Hill coefficients of 2.9 and the following $K_{1/2}$ values and proportions: 27 μM and 0.17, 64 μM and 0.29, 162 μM and 0.46, and 248 μM and 0.08.

either in single channels or in macropatch data. Thus, the difference in Hill coefficients appears to be unrelated to the slight differences in the distribution of $K_{1/2}$ values. We cannot rule out the possibility that the Hill coefficient may be modulated differently in single channels and macropatches; however, we have found that the number of channels in a macropatch does not influence the value of the Hill coefficient. This is shown in Figure 3B, where the Hill coefficient is plotted against the maximum current for each macropatch. The range of maximum currents indicates the presence of about 30 to about 11 000 channels. If the Hill coefficient were systematically higher in patches with fewer channels, the regression line would slope downward.

Because a continuum of $K_{1/2}$ values is observed in different patches, it is likely that $K_{1/2}$ also varies among individual channels in a given macropatch. Strong evidence in support of this idea comes from experiments in which the photoaffinity analogue APT-cGMP was used to covalently tether cGMP moieties to the binding sites of homomultimeric rod channels in macropatches (*45*). A population of channels that contained covalently attached ligands at all but one site was isolated. The dose−response relations for free cGMP usually indicated a significant heterogeneity in the apparent affinities at the last binding sites. At a minimum, there were two types of binding sites with widely disparate dissociation constants. In a homotetrameric channel, this is expected to give rise to at least five distinct classes of channels with different $K_{1/2}$ values (cf. ref *46*). Such a phenomenon could explain the shallow nature of dose−response relations in macropatches. Figures 4 and 5 illustrate this point. In Figure 4A, a macropatch dose−response relation was fit with a Hill coefficient of 2.0 and a $K_{1/2}$ of 86 μM. For comparison, the dotted curve shows a poor fit to the data assuming a single population with a Hill coefficient of 2.9. In Figure 4B, a very good fit to the same relation was obtained assuming two populations of channels with Hill coefficients of 2.9 and $K_{1/2}$ values of 30 and 131 μM (fractional abundances of 0.26

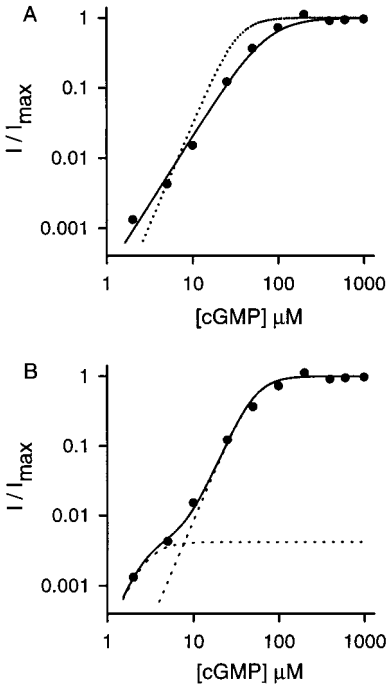

FIGURE 5: Dose−response relation from a macropatch different from that depicted in Figure 4, extending to lower I/I_{max} values, and fit assuming one or two channel populations. Fractional currents are defined in the legend of Figure 4. The maximal current of this patch was 3040 pA. (A) The smooth curve is a fit of the Hill equation to the data with an n of 1.9 and a $K_{1/2}$ of 73 μM. The dotted curve shows the best fit with the Hill coefficient constrained to be 2.9. (B) The smooth curve is a fit assuming two populations of channels with Hill coefficients of 2.9, the following $K_{1/2}$ values and proportions: 2.7 μM and 0.0042, 52 μM and 0.9958. The dotted curves show the two individual components that were summed to produce the smooth curve.

and 0.74, respectively). The smoother fit in Figure 4C was obtained assuming four populations of channels with Hill coefficients of 2.9 and the following $K_{1/2}$ values and fractional abundances: 27 μM and 0.17, 64 μM and 0.29, 162 μM and 0.46, and 248 μM and 0.08. In panels B and C of Figure 4, it is important to note that the $K_{1/2}$ values used in the fits have been observed in either single-channel patches or macropatches (see Figure 3). The fits suggest that shallow dose−response relations in macropatches may arise from a small number of channel populations each with high Hill coefficients but different $K_{1/2}$ values. The number of data points in the dose−response relation has little bearing here; a comparison of the solid line fits in panels A and B of Figure 4 shows that it would be very difficult to discern the presence of the two populations used in the fit, even if a much larger number of concentrations were tested.

Figure 5 shows fits to a different patch in the which the dose−response relation extended to lower I/I_{max} values. In Figure 5A, the relation was fit with a Hill coefficient of 1.9 and a $K_{1/2}$ value of 73 μM; again, the dotted curve shows that a single population with a Hill coefficient of 2.9 does not fit the data. In Figure 5B, the relation was fit well assuming two populations of channels with Hill coefficients of 2.9 and $K_{1/2}$ values of 2.7 and 52 μM. Remarkably, the fractional abundances were 0.0042 and 0.9958. Thus, less than one percent of the channels with a low $K_{1/2}$ value were sufficient in this case to generate a shallow relation with an overall Hill coefficient of 1.9. The dotted curves in Figure 5B illustrate the two component populations that were summed to produce the fit. Even though the population with a $K_{1/2}$ value of 2.7 μM is present in very low abundance, it influences the current significantly at low concentrations; at these concentrations, the fractional activation of the more abundant population is very small. Unlike the fits in Figure 4, a $K_{1/2}$ value of 2.7 μM has not been observed experimentally in either single-channel or multichannel patches. Such values are likely to exist, however, since shallow macropatch dose−response relations are observed with overall $K_{1/2}$ values of $\leq$30 μM. It should be noted that if the fit in Figure 5B does reflect reality for the expressed retinal rod CNG channel, the very low fractional abundance of the high-affinity population dictates that the probability of observing this in a single-channel patch would be very small.

There are two reasons for displaying dose−response data on double log coordinates. First, when a wide range of cGMP concentrations are tested, currents can vary over several orders of magnitude. On both linear and semilog scales, the data at low concentrations would be severely compressed. Second, for a single channel or a homogeneous population of channels, the slope of the relation at low concentrations (limiting slope) on a double log plot approaches the minimum number of ligands required for significant activation. This number does not depend on a particular model of channel activation; the Hill model, which assumes complete cooperativity of binding, and an independent model, which assumes no cooperativity of binding, both predict the same limiting slope (*42*). For mixed channel populations, the slope at low concentrations can be shallow as shown in Figures 4 and 5. In theory, it should be possible to test if mixed channel populations exist by measuring the level of activation at extremely low concentrations, where only one channel population is responding. At these concentrations, the slope should ultimately become steeper and reflect the number of ligands required for significant activation. As a practical matter, however, it is often difficult to measure such small currents in macropatches. The leak, background channels, and capacitance all make it hard to resolve very small currents.

The limiting slope of the single-channel dose−response relation in Figure 2 is 2.9. About the same slope was obtained over the entire range of I/I_{max} values below 0.1. This slope is consistent with three ligands being required for significant activation, and is the same as the Hill coefficient of 2.9 given earlier. When data are plotted on double log coordinates, it is natural to fit the Hill equation to the log values of I/I_{max}, rather than the actual values, because one obtains a better looking fit. A disadvantage is that this assumes uncertainties in the data are equal on a log scale, which usually underestimates the uncertainties at low I/I_{max} values. However, because it emphasizes the foot of the curve, the fit to log values has the advantage of yielding a Hill coefficient that is close to the limiting slope. In contrast, the fit of the Hill equation to the actual values of I/I_{max} (on a linear scale) yields a Hill coefficient that usually reflects the cooperativity of binding more than the number of ligands required for activation (*47*). This is because of the physically implausible assumption of complete cooperativity in the Hill mechanism. For example, for a four-site model of activation in which the binding steps occur independently and there is a favorable conformational change to the open state in a fully liganded

channel (equilibrium constant of 19, which confers positive cooperativity), a Hill coefficient of only 2.15 is obtained. (For this same mechanism, the fit to the log values of I/I_{max} yields a Hill coefficient of 3.3, and the limiting slope is 3.7, reflecting the number of ligands required for activation.) For single channels in Figure 2A, a Hill coefficient of 2.6 was obtained when the Hill equation was fit to the actual I/I_{max} values. In comparison to the value of 2.15 obtained for the model above, this suggests that there is significant cooperativity of binding in the mechanism.

DISCUSSION

The main conclusion of this study is that the slope of the dose−response relation for single rod CNG channels expressed in oocytes is much steeper, on average, than the slope of the dose−response relation for multichannel patches, measured under the same recording conditions. Because $K_{1/2}$ values are known to vary as a result of modifications such as phosphorylation (*40*, *48*), and previous evidence suggests that $K_{1/2}$ values of individual channels vary widely within a given macropatch (*45*), we propose that shallow dose−response relations (Hill coefficients of 1.5−2.4) arise in macropatches from mixtures of channels with high Hill coefficients (∼3.0) and variable sensitivities to cGMP. The continuum of $K_{1/2}$ values in both macropatches and single-channel patches argues for many modulated states of the channel with different ligand sensitivities. For example, if phosphorylation of a single amino acid residue on one subunit can affect sensitivity, then at least five different modulated states are predicted. The fits show that two channel populations may be enough in some cases to give rise to the shallow relations. Four channel populations can yield shallow dose−response relations that are also very smooth. We regard this proposal as a minimal explanation for shallowing, but other mechanisms may also be involved. For example, we cannot completely rule out the possibility that the higher channel densities in macropatches somehow change the cooperativity of activation in the individual channels, but such a mechanism seems unlikely because there was no obvious dependence of the Hill coefficient on the number of channels in a patch.

Ideally, it should be possible to reproduce a shallow dose−response relation by combining data from single channels in which disparate $K_{1/2}$ values were measured. When this was tried with the two extreme $K_{1/2}$ values (83 and 249 μM), the Hill coefficient was reduced from 2.9 to as low as 2.3. The shallowest macroscopic dose−response relations were not reconstructed because the $K_{1/2}$ values in the single-channel patches were slightly toward the high end of the scale. It is possible that channel sensitivity to ligand is regulated somewhat differently when channels are expressed at very high or very low densities. Nevertheless, to account for macropatch dose−response relations, it is the $K_{1/2}$ values of the individual channels in a macropatch that are relevant. The overall $K_{1/2}$ values in macropatches ranged from 27 to 183 μM, which means that individual channels *must* have $K_{1/2}$ values lower than those observed in the single-channel patches. In fact, the dose−response relation in Figure 4B was fit using $K_{1/2}$ values of 30 and 131 μM, well within the observed range of macropatch values. Moreover, the four $K_{1/2}$ values used to obtain a smooth fit to the same relation in Figure 4C are realistic because they were observed in either single-channel patches or macropatches.

We propose that mixtures of channels with variable $K_{1/2}$ values also explain the wide variability in Hill coefficients and the frequent observation of low values in studies of the native channel in photoreceptor cells. Although some of the early work on excised patches from rods indicated a Hill coefficient of about 3.0 (*41*, *42*), a number of subsequent studies have reported lower values (*45*, *49*). To our knowledge, there are 20 dose−response relations reported for single native photoreceptor channels [$n = 6$ (*43*), $n = 3$ (*44*), $n = 8$ (*50*), and $n = 3$ (*51*)]. In all cases, the Hill coefficients were near 3.0, consistent with the values we report here on single expressed homomultimeric channels. Matthews (*52*) has suggested that a mixture of single native channels with Hill coefficients of 3.0 and variable $K_{1/2}$ values could explain the shallow nature of the upper portion of macroscopic dose−response relations. However, his observations did not account for the wide variation in the overall Hill coefficients (particularly where the foot of the dose−response relation is shallow).

It should be noted that in assessing the physiological behavior of channels in their native settings, it may often be more appropriate to use macropatch data which reflect the presence of channel mixtures with different sensitivities to ligand.

The observation of 36 single channels with high Hill coefficients (16 reported here and 20 reported previously for the native photoreceptor channel) is completely consistent with our measurements on channels locked in different liganded states (*34*), described in the introductory section. In fact, the locked channel data have been used to successfully reconstruct the steep dose−response relation of single-channel patches (*53*). In contrast, the recent data of Liu et al. (*54*) on single channels with mutated binding sites and exogenous pore regions do not appear to reflect the opening of wild-type rod channels. In their study, two ligands were found to significantly open the channel, in a way that was nearly indistinguishable from that with three ligands. Their data reconstructed a dose−response relation that was too shallow to explain single-channel data.

In the sixteen single-channel patches reported here, there was some variability in the Hill coefficient (values ranged from 2.5 to 3.9). We found in our studies of locked channels that the degree of opening in different liganded states varied somewhat among individual channels (*34*). These variations predict a range of Hill coefficients such as those observed in single channels presented here.

In summary, we urge caution in interpreting macroscopic dose−response relations, or other data in which the activities of a large number of protein molecules are being measured in response to a variable stimulus or inhibitor. This is particularly true if the sensitivity to the stimulus is known to be a modulated property, as is the case for CNG channels. This is a common situation for enzymes, transporters, and other ion channels. The measurement of cooperativity and the number of ligands required to produce an effect, in particular, can be subject to large errors. Once again, the power of single-channel recording is apparent. Even something as simple as a steady-state dose−response relation can be misinterpreted in a situation where the average behavior of a group of proteins is being measured.

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BI990532W