Photoreceptor Channel Activation: Interaction between cAMP and cGMP†

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ABSTRACT: cAMP activates a current in excised patches from rod outer segments. The current at saturating concentrations of cAMP is ~25% of the current activated with 200 μM cGMP, the terminal cytoplasmic messenger in phototransduction. The $K_{0.5}$ for cAMP is >1.5 mM, and the index of cooperativity is ~1.4. cAMP activates the same population of channels as activated by cGMP since currents in the presence of both nucleotides are less than the sum of the individual responses. When increasing concentrations of cAMP, less than its $K_{0.5}$, are added to a fixed, subsaturating concentration of cGMP, cAMP significantly enhances the total current compared with the current produced by cGMP alone. These results are predicted by a three-site, linear, sequential binding scheme where either cAMP or cGMP may bind to the same site on the channel. At ~5 μM cGMP, which is estimated to be the steady-state dark level in vertebrate photoreceptors, cAMP between 1 and 100 μM produces a large increase in the photoreceptor current. A possible physiological role for cAMP–cGMP interaction in phototransduction is discussed.

Cyclic GMP has been established as the terminal cytosolic messenger in phototransduction [Fesenko et al., 1985; Matthews & Watanabe, 1987; also see a review by Pugh and Cobbs (1986)], and many details of the light-activated cascade, which results in the hydrolysis of cGMP, are known. The less well understood features of phototransduction involve the reopening of cGMP-gated channels following a light pulse and the establishment of current in steady light (light adaptation).

In the first of two papers presented here (Tanaka et al., 1989), we investigated the ability of a series of cGMP derivatives to activate photoreceptor currents in the absence of the photochemical machinery. In contrast to previous reports (Fesenko et al., 1985; Cobbs et al., 1985; Haynes & Yau, 1985), we found that cAMP is a partial agonist of photoreceptor channels activating only a fraction of the maximal cGMP current. The experiments reported here were designed to ask whether cAMP and cGMP interact with the same population of channels. Such a competition would offer the possibility that cAMP has a role in either phototransduction or light adaptation.

The notion that cGMP and cAMP act in concert is not new. Goldberg et al. (1975) proposed the "Yin-Yang" hypothesis in which cGMP opposes the effects of cAMP. While the original Yin-Yang concept has not been well substantiated, several recent experiments demonstrate physiological interaction between cyclic purine nucleotides. In pinealocytes, norepinephrine elevates the accumulation of both cAMP and cGMP through a dual synergistic interaction of α_1- and β-adrenoceptor regulation. β_1 receptor activation modestly stimulates both adenylyl cyclase and guanylyl cyclase. Activation of α_1 receptors alone has no effect on either cyclase; however, concurrent α_1- and β-adrenergic activation increases cAMP and cGMP levels >100-fold. The elegant details of nucleotide regulation and interaction in these cells has been resolved by Klein and collaborators [Ho et al. (1988) and references cited therein].

Fischmeister and Hartzell (1987) studied a calcium current in cardiac cells which is increased by application of isoprenaline or by intracellular perfusion of cAMP. cGMP perfusion, which had no effect on the basal calcium current, reduced the cAMP-stimulated current by 67%. The authors proposed that cGMP increases hydrolysis of cAMP by stimulating a phosphodiesterase.

Both nucleotides may play a role in olfaction. Nakamura and Gold (1987) found that either cAMP or cGMP directly activates a current in membrane patches excised from olfactory cilia with $K_{0.5}$[1] values <10 μM for both although cGMP has an ~2-fold lower $K_{0.5}$ than cAMP. Additional biochemical and physiological studies are necessary to determine the conditions under which each nucleotide acts as a transmitter for olfactory transduction.

These recent experiments extend the domain of cellular functions mediated by purine cyclic nucleotides. Beside the well-recognized role of stimulating nucleotide-specific protein kinases and phosphodiesterases, cGMP and possibly cAMP are internal ligands for cooperative activation of membrane ion channels involved in sensory transduction.

Materials and Methods

Materials. cGMP was obtained from Sigma, Chemical Dynamics Corp., and Calbiochem. cAMP was obtained from Sigma and Boehringer Mannheim. *Rana pipiens* were used for all experiments.

Patch Recordings. Methods were identical with those previously described (Tanaka et al., 1989; Furman & Tanaka, 1988). Briefly, the retinas of dark-adapted animals were dissected under red light, and harvested photoreceptors were layered into a solution containing 120 mM NaCl, 5 mM HEPES, pH 7.4, and 2 mM EGTA (Na).

Inside-out patches were excised with Corning 0010 glass microelectrodes filled with the bath solution. Voltage-clamped

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[1] Abbreviations: I, transmembrane current; I_{max}, current produced by saturating ligand concentrations; N_h, Hill index of cooperativity; $K_{0.5}$, ligand concentration for half-maximal current activation; IV, plot of membrane current versus membrane voltage.

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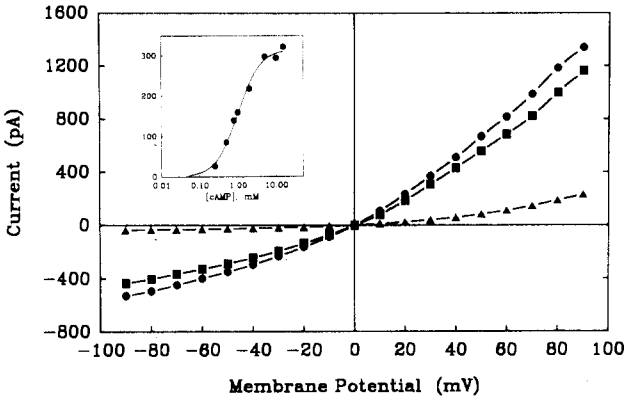

FIGURE 1: cAMP and cGMP current activation. Current vs voltage plot with 200 μM cGMP alone (●), 1 mM cAMP alone (▲), and both cGMP and cAMP present (■). Nucleotides were applied sequentially to the cytoplasmic face of a single patch and currents stimulated by a linear voltage ramp from −90 to +90 mV. Currents were digitally averaged over 0.5 mV and plotted as points; smooth curves connect the symbols. (Inset) The current recorded at +90 mV as a function of cAMP concentrations (●) was fit by the Hill equation (eq 1; solid line). For this patch $K_{0.5}$ = 1.01 mM, N_h = 1.59, and I_{max} = 322 pA. 200 μM cGMP on this patch produced a saturating current response of 670 pA (patch 86152).

membrane currents were collected on-line by digitization at 5 kHz and stored on disk for later analysis. Currents, potentials, and membrane surfaces are reported in physiological conventions.

Nucleotide Solutions. Cyclic nucleotide stock solutions were prepared in water and diluted to the desired final concentration with the bath solution. Adding base to enhance the poor aqueous solubility of cAMP produced slightly higher final concentrations of NaCl in 10 mM cAMP solutions than in the cGMP and bath solutions, but this slight increase was not detected by changes in the reversal potential.

Data Analysis. The *IV* relations at each ligand concentration were converted by computer to current–ligand concentration curves as a function of voltage and analyzed by fitting the data directly to the Hill equation

$$I = I_{max}/[1 + (K_{0.5}/L)^{N_h}] \quad (1)$$

using a Levenberg–Marquardt algorithm for nonlinear weighted least-squares fitting (Press et al., 1986), where I is the measured current and L is the concentration of the applied nucleotide.

RESULTS

cAMP Activation of Membrane Current. In the preceding paper (Tanaka et al., 1989) we reported the activation of sodium currents in excised membrane patches from photoreceptor outer segments using both cGMP and structural analogues of cGMP. cAMP is a partial agonist activating about 25% of the maximal cGMP-activated current at saturating concentrations. The concentration required for half-maximal activation is ~1.5 mM compared with 20 μM for cGMP, but the cooperativity of binding and voltage dependence of channel gating are similar for both ligands, Figure 1, inset. In 12 patches studied, only 3 patches clearly demonstrated current saturation at 10 or 15 mM cAMP. In these three patches, the mean concentration for half-maximal saturation, $K_{0.5}$, was 1.5 mM, and the mean Hill index of cooperativity, N_h, was 1.4. In the remaining nine patches, $K_{0.5}$ was >1.5 mM. This biological variability of $K_{0.5}$ is typical in this preparation (Tanaka et al., 1989).

Results with cAMP and cGMP Mixtures. The small currents activated by cAMP may reflect binding to a separate

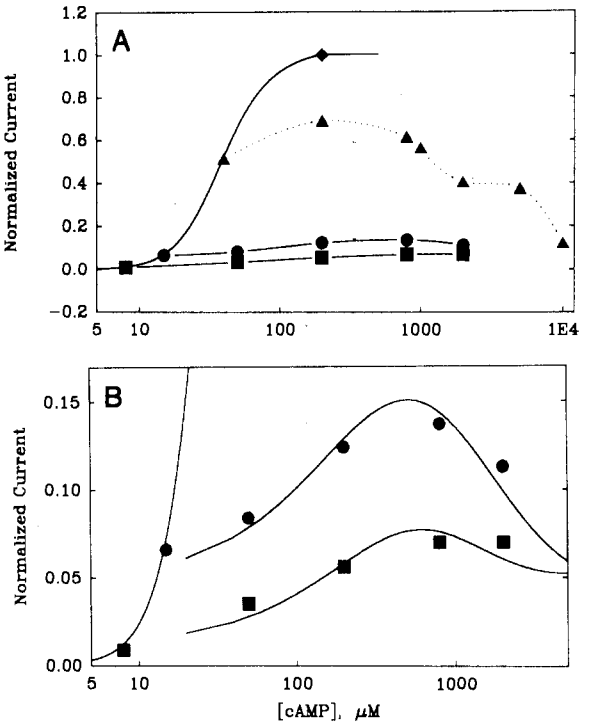

FIGURE 2: Effect of varying cAMP at fixed concentrations of cGMP. (A) Currents from two patches at +80 mV have been combined by scaling the observed current to the maximal cGMP-activated current for that patch. The left-most curve is a cGMP concentration–response curve, while the other curves were activated by exposing the cytoplasmic face to a fixed concentration of cGMP [8 (■), 15 (●), or 40 μM (▲)], and cAMP was added as shown by the abscissa. As cAMP is increased from 50 μM to 10 mM the total current initially increases. The solid curves are the theoretical fit of the mixed nucleotide binding model in Figure 3 with the assumption that two common nucleotides determine the open channel conductance. The dotted line is a spline fit to the data. (B) The above data from a single patch at 8 and 15 μM are shown on an expanded scale. The equilibrium binding constants for cGMP were 99 (k_1), 65 (k_2), and 48 μM (k_3) and for cAMP were 0.56 (j_1), 0.75 (j_2), and 4.0 mM (j_3). The opening constants were 0.146 and 14.6 for cGMP and cAMP, respectively. The Hill parameters for the cGMP curve were $K_{0.5}$ = 39 μM and N_h = 2.6 and for the theoretical cAMP curve were I_{max} = 0.08, $K_{0.5}$ = 4.1 mM, and N_h = 1.3.

class of nucleotide-activated channels, binding to allosteric sites on the channel, or binding to the cGMP sites with only partial activation of the conducting state of the channel. For independent channel populations, currents activated by either nucleotide alone should add when activated by their mixture. Any other current response from dual nucleotide activation argues for interaction. Figure 1 shows that currents activated in the presence of both saturating cGMP and 1 mM cAMP were smaller than those activated by cGMP alone, providing evidence for binding to the same population of channels.

The effect of nucleotides binding to separate, but interacting, classes of receptors on a channel is not predictable a priori, whereas two activating nucleotides competing for the same site might be expected to produce a smaller current when both are present, if one nucleotide is a significantly less potent agonist of channel opening.

To explore the interaction of cGMP and cAMP, increasing concentrations of cAMP were added to fixed, subsaturating concentrations of cGMP. At both low and intermediate concentrations of cGMP, the photoreceptor current was significantly increased by the addition of concentrations of cAMP that alone would not produce appreciable current (Figure 2A). With further titration of cAMP, the current rose to a peak at ~800 μM before tapering to the lower current plateau

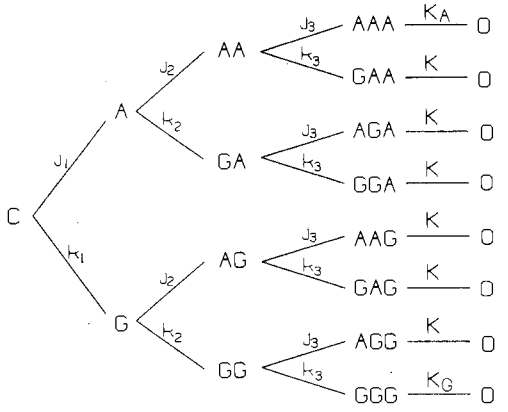

FIGURE 3: Reaction scheme for multiple nucleotide binding. The nucleotide gated channel (C) is assumed to have three nucleotide binding sites, each of which can be occupied by either cGMP (G) or cAMP (A). The binding sites are populated from left to right, and all permutations are allowed. The equilibrium binding constants are determined only by the ligand species (k_n for cGMP and j_n for cAMP). The opening equilibrium constants are balanced such that K_A for cAMP is a fraction of K_G, as reflected by the comparison of the saturating current magnitudes. In mixed-ligand forms, the opening constant, K, was selected equal to K_G or K_A according to one of three hypotheses (see text).

produced by cAMP alone. In four patches the total current with 1 mM cAMP and 8 μM cGMP present averaged >7.3 ± 0.7 mol larger than the current at 8 μM cGMP (Figure 2B). At higher cGMP concentrations, near the $K_{0.5}$, the effect remained prominent, but the relative current increase was less. We attribute the cAMP-induced increase in current to the presence of mixed ligand-bound forms of the channel with a single class of binding sites.

Discussion

Models of cGMP Channel Activation. The nucleotide-activated channel from photoreceptors shows positive cooperativity with at least three cyclic nucleotide molecules required for activation of each channel. In the previous paper (Tanaka et al., 1989), we used two simple schemes to describe nucleotide binding and subsequent channel opening. In both the Hill scheme and the receptor-effector model based on the Hill equation, ligand binding was treated as a lumped equilibrium constant without accounting for individual binding constants. This semiempirical approach is justified since we have no information on individual binding constants. These simple models, however, fail to explain our data with nucleotide mixtures because they do not account for multiply liganded channels and because they predict monotonic competition displacement curves. To interpret the experiments reported here, we have used a linear sequential model with three binding sites, each of which can be occupied by either cGMP or cAMP. This model is based on a scheme originally proposed for hemoglobin (Adair, 1925). The model, however, can be considered as a mechanism-free, thermodynamic analysis of binding that is valid as long as binding does not lead to receptor polymerization (Levitzki, 1978).

The tree of reaction pathways in the presence of cGMP and cAMP is shown in Figure 3. Three sequential binding steps precede isomerization to the open, conducting state of the channel protein. The equilibrium dissociation constant for each binding step is determined by the ligand species bound. Channel opening occurs as an isomerization after the third binding site is occupied. The equilibrium constant for this transition is adjusted to produce a saturating current response, I_{max}, for cAMP that reflects the cAMP/cGMP current ratios at saturating ligand concentrations. Three cases were considered for choosing whether a multiply liganded channel opens to a high or low conductance state: (1) any cGMP bound determines the opening equilibrium constant, (2) two ligands in common determine the equilibrium constant, or (3) the last ligand bound determines the equilibrium constant.

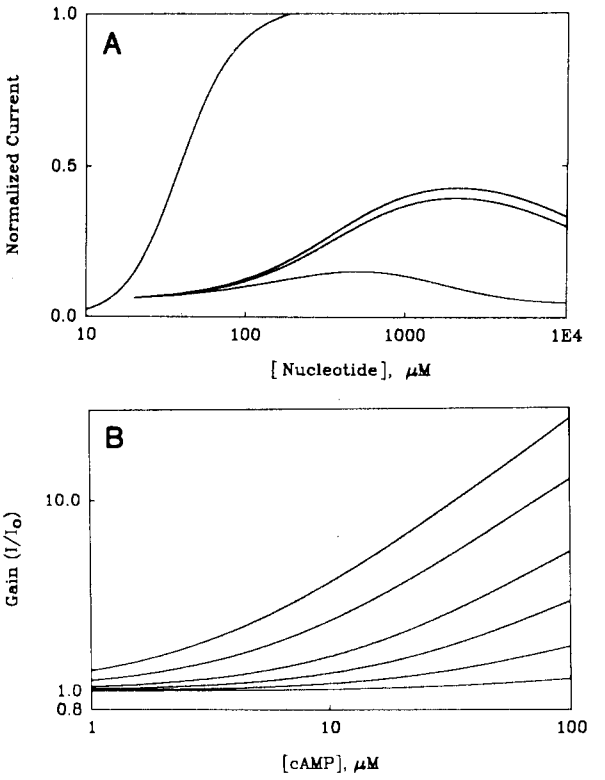

FIGURE 4: Predicted responses of cGMP and cAMP competition models. (A) The curve at the left is the fit of the cGMP concentration–response data shown in Figure 2A. The other curves are theoretical responses for 8 μM cGMP with increasing cAMP predicted by the three assumptions for determining the opening equilibrium constant discussed in the text. The lowest curve was fitted by assuming two common ligands determined the open state; the middle curve assumed the last ligand bound determined the open state, and the top curve assumed any cGMP bound determined the open state. The values chosen for the fit are given in Figure 2B. (B) The gain of nucleotide-activated current at increasing cAMP concentrations was plotted for the following fixed [cGMP], beginning with the uppermost curve: 1, 2, 5, 10, 20, and 40 μM. The $K_{0.5}$ for cGMP used was 40 μM and 1.5 mM for cAMP. The theoretical curves were based on the assumption that two common ligands determine the open state.

The experimental data from the patch in Figure 2B were fitted with this model. The equilibrium binding constants for cGMP were determined from the best fit of the normalized current in the presence of cGMP alone. The binding constants and opening equilibrium constant for cAMP were then manually adjusted to reasonably fit the mixed cGMP–cAMP current response simultaneously at both fixed concentrations of cGMP. The only assumption about the open conducting state that would fit the data was two common ligands determine the open state.

Theoretical current plots using the parameters from Figure 2B are shown in Figure 4A for all three hypotheses for selecting the opening equilibrium constant. In each case, cAMP first increases and then decreases the total current. The relative magnitude of the current increase is greatest when any cGMP bound determines the conductance state and least when two common ligands determine the open state. Only with the latter assumption, however, were we able to chose a parameter set that simultaneously predicted the cGMP curve, the peak of the mixed cGMP–cAMP response at different concentrations, and a cAMP response with a $K_{0.5}$ and I_{max} within the

experimentally observed range.

Fixed concentrations of cAMP decrease the $K_{0.5}$ and cooperativity of a cGMP concentration–response curve. Under the two common ligand assumption for the opening state and with the Hill parameters that fit the cGMP data in Figure 2B, 200 μM cAMP reduces the estimated Hill parameters by $\sim$20% ($K_{0.5}$ from 39 to 31 μM and N_h from 2.6 to 2.0). The greatest effect of cAMP at concentrations between 1 and 100 μM occurs at the foot of the cGMP concentration–response curve. Figure 4B shows the increase in total membrane current (gain) produced by cAMP at low, fixed cGMP concentrations. At the lowest concentrations of cGMP, the current gain is almost directly proportional to the rise in cAMP from 10 to 100 μM even though cAMP alone would not produce detectable current levels. Qualitatively, the addition of low concentrations of cAMP increases the total number of pathways available to the conducting state, and the magnitude and position of the maximal current increase are a function of the number of pathways available to the high conductance state.

Speculation on a Physiological Role for cAMP. Could intracellular cAMP regulate the photoreceptor membrane current in vivo? Increasing cAMP would both decrease the channel's sensitivity to a rise in free cGMP levels and shift the apparent $K_{0.5}$ of the cGMP-response curve to the left. For such a mechanism to be feasible, cAMP must be less sensitive to light-activated phosphodiesterase hydrolysis than cGMP, the free cGMP concentration in the dark must be near the foot of the cGMP-response curve, and free cAMP levels must be in the 10 μM to 1 mM range. The specificity of PDE for cGMP over cAMP is between 6-fold (Sitaramayya Ari, personal communication) and 23-fold (Miki et al., 1973). Yau et al. (1986) have estimated the intracellular free cGMP concentration in dark-adapted rods to be 6 μM, a range where our model predicts the largest relative increase in current by varying cAMP levels. Although we could not locate data on intracellular free cAMP levels in outer segments, the experiments by Farber et al. (1981) estimated cAMP/cGMP ratios in the rod-dominant retinas (mouse, rat, and toad) to be $\sim$0.2 and between 2 and 8 for the cone-dominated retinas (ground squirrel and Western fence lizard). Experiments using intact outer segments may demonstrate a physiologically relevant interplay between cGMP and cAMP.

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