Modulation by Internal Protons of Native Cyclic Nucleotide-gated Channels from Retinal Rods

CRISTIANA PICCO, CARLO SANFILIPPO, PAOLA GAVAZZO, and ANNA MENINI

From the Istituto di Cibernetica e Biofisica, C.N.R. and Istituto Nazionale per la Fisica della Materia, 16149 Genova, Italy

ABSTRACT Ion channels directly activated by cyclic nucleotides are present in the plasma membrane of retinal rod outer segments. These channels can be modulated by several factors including internal pH (pH_i). Native cyclic nucleotide-gated channels were studied in excised membrane patches from the outer segment of retinal rods of the salamander. Channels were activated by cGMP or cAMP and currents as a function of voltage and cyclic nucleotide concentrations were measured as pH_i was varied between 7.6 and 5.0. Increasing internal proton concentrations reduced the current activated by cGMP without modifying the concentration ($K_{1/2}$) of cGMP necessary for half-activation of the maximal current. This effect could be well described as a reduction of single-channel current by protonation of a single acidic residue with a pK_1 of 5.1. When channels were activated by cAMP a more complex phenomenon was observed. $K_{1/2}$ for cAMP decreased by increasing internal proton concentration whereas maximal currents activated by cAMP increased by lowering pH_i from 7.6 to 5.7–5.5 and then decreased from pH_i 5.5 to 5.0. This behavior was attributed both to a reduction in single-channel current as measured with cGMP and to an increase in channel open probability induced by the binding of three protons to sites with a pK_2 of 6.

KEY WORDS: cyclic nucleotides • retinal rods • ion channels • pH

INTRODUCTION

Ion channels directly gated by cyclic nucleotides (CNG channels)[1] are present in some sensory receptor cells (Fesenko et al., 1985; Haynes and Yau, 1985; Nakamura and Gold, 1987; for review, see Menini, 1995) and in a variety of other cells from kidney, aorta, testis, and heart (for reviews, see Yau, 1994; Kaupp, 1995). These channels have been cloned, and some of them have been shown to be hetero-oligomers composed of several subunits of at least two types, often called α and β subunits (for reviews, see Kaupp, 1995; Zimmerman, 1995).

The physiological role of CNG channels in photoreceptors and olfactory receptor cells is well known, whereas the function of these channels in other cells is not known. In photoreceptors and olfactory receptor cells external stimuli, photons, or odorant molecules cause, through activation of receptors and enzymatic cascades, a change in the internal concentration of cyclic nucleotides, that in turn leads to a change in the current flowing through CNG channels (for review, see Torre et al., 1995).

Channel activation can be modulated by many factors. Some of these could be physiologically important, others probably are not, but, nonetheless, study of their effect could be useful in understanding molecular mechanisms of functioning of channels. It is known that native CNG channels can be modulated by phosphatases (Gordon et al., 1992), divalent cations (Ildefonse et al., 1992; Karpen et al., 1993; Lynch and Lindemann, 1994), Ca^{2+}-calmodulin (Hsu and Molday, 1993; Chen and Yau, 1994; Gordon et al., 1995*b*), and diacylglycerol analogues (Gordon et al., 1995*a*). pH is another modulating agent, since a variation in proton concentration could cause a change in the charge of ionizable amino-acids that can be involved in the gating and/or permeation processes. It has already been shown that intact rods are sensitive to extracellular protons since the dark current could be reversibly reduced by increasing extracellular proton concentration (Liebman et al., 1984). It is also known that cytosolic acidification reduces the current activated by saturating concentrations of cGMP in retinal rods (Menini and Nunn, 1990; Tanaka, 1993) and the current activated by saturating concentrations of cAMP in olfactory receptor cells (Frings et al., 1992).

The aim of this paper is to determine how internal protons modulate the properties of activation by cGMP and cAMP of native channels from retinal rods. It is shown that currents are modulated by protons in different ways depending on which cyclic nucleotide is responsible for channel opening. cGMP-gated currents were reduced by increasing internal proton concentra-

Preliminary results have been previously presented in abstract form (Sanfilippo, C., and A. Menini. 1993. *Biophys. J.* 64:A17; Menini, A., C. Sanfilippo, and C. Picco. *XXXII Congress of the International Union of Physiological Sciences.* Glasgow, 1–7 August 1993. 278:32/P.

Address correspondence to Dr. Anna Menini, Istituto di Cibernetica e Biofisica, C.N.R., Via De Marini 6,16149 Genova, Italy. Fax: 39-10-6475-500; E-mail: menini@icb.ge.cnr.it

[1]*Abbreviation used in this paper:* CNG channels, cyclic nucleotide-gated channels.

tion. cAMP-gated currents were enhanced by lowering pH_i from 7.6 to 5.7–5.5 and then were decreased as pH_i was further lowered to 5.0. These effects could be explained by a reduction of single-channel current, due to protonation of a site with a pK_1 of 5.1 and by an additional increase of open probability for cAMP, caused by binding of three protons to sites with a pK_2 of 6.

METHODS

Cell Preparation

Rods were mechanically isolated from the retina of the larval tiger salamander *Ambystoma tigrinum* as described by Menini et al. (1988). In short, the animal was decapitated and pithed. The retina was kept in Ringer solution and chopped with a small piece of razor blade. An aliquot (0.1 ml) containing many rod outer segments was then transferred to the perfusion chamber. The dissection and experiments were performed in room light at room temperature (20–24°C).

Recording Procedure

Channels activated by cyclic nucleotides were studied in excised inside-out patches from the plasma membrane of isolated retinal rod outer segments as previously described in detail (Menini, 1990; Picco and Menini, 1993).

Channels were activated by the addition of various concentrations of cGMP sodium salt (G6129; Sigma Chemical Co., St. Louis, MO) or cAMP sodium salt (A6885; Sigma Chemical Co.) to the bathing solution. Voltage steps were given from a holding potential of 0 mV, increasing and decreasing in steps of 20 mV. Voltage step duration was usually 100 ms except for instantaneous I-V experiments where it was 16 ms. CNG currents were obtained from the difference of recordings in the presence and in the absence of cyclic nucleotides.

Currents were recorded with a List EPC-7 (Darmstadt, Germany) or with an Axopatch 1D (Axon Instruments, Foster City, CA) patch-clamp amplifier. Currents were filtered through an 8-pole Bessel low-pass filter (Kemo VBF8, Beckenham, UK) usually set at 1 kHz cut-off frequency and digitized on-line at a sampling interval of 0.5 ms. For the instantaneous I-V experiments shown in Fig. 3, currents were filtered at 20 kHz and digitized at a sampling interval of 10 μs. For these experiments the patch pipette was coated with Sylgard to minimize the capacitance transients. Each trace was obtained as subtraction of the average from 16 trials measured in the presence and in the absence of cyclic nucleotides. The rise time of the voltage step was ~10 μs, and the delay and rise time introduced by the 20 kHz low-pass filter were ~40 μs. Instantaneous current values were measured 100 μs after switching to the test voltage from the holding potential of 0 mV (see also paragraph on *Data Analysis*). Acquisition and analysis of data were usually performed by a PDP 11/23 computer and laboratory interface (Indec Systems, Sunnyvale, CA) with a software program written in BASIC 23. For instantaneous I-V experiments a TL-1 DMA interface board (Axon Instruments) was used with a PC-type computer and pCLAMP software (Axon Instruments).

Solutions and Perfusing System

The ion composition of the solution filling the patch pipette was 110 mM NaCl, no added divalent cation salts, 1 mM EDTA and 10 mM HEPES buffered to pH 7.6 with tetramethylammonium hydroxide (TMAOH). The ion composition of the bathing solutions was identical in the pH range from 6.8 to 7.6 whereas HEPES was substituted by MES for pH lower than 6.8. pH was adjusted to the desired value by adding TMAOH or HCl.

The cytoplasmic side of the membrane patch was exposed to the different solutions by using the perfusing system described in Menini and Nunn (1990). Four different solutions flowed simultaneously side-by-side so as to allow several rapid solution changes to be made. In all the experiments complete recovery of the current was checked by comparing, before and after each test solution, the current at pH_i 7.6 in 1 mM cGMP.

Data Analysis

Currents were measured and plotted as a function of membrane potential with the usual sign convention: currents flowing from the previously intracellular side of the membrane patch (bath solution) to the extracellular side (pipette solution) are positive and were plotted upward.

In some experiments a drop in the activated current was observed. This effect was studied by Zimmerman et al. (1988) and is caused by restricted diffusion of ions, probably due to intracellular material that adheres to the surface membrane. The experiments considered in this paper are only those in which this effect was absent.

Data calculated from N different patches are shown as mean±SD. Curve fitting was done with Kaleidagraph (Abelbeck Software) and figures were prepared with Canvas software (Deneba Systems).

Plotted values of macroscopic currents, due to the opening of many CNG channels, were usually measured when currents reached a steady-state value. In the simplest hypothesis that channels have only one conductance state, and that gating and permeation are two independent processes, the steady-state macroscopic current, I, can be described by the following relation (see Hille, 1992):

$$I(V, cNMP, pH) = N_c \cdot p(V, cNMP, pH) \cdot i(V, cNMP, pH), \quad (1)$$

where N_c is the number of channels present in a patch, V is the applied voltage, $cNMP$ is the type and concentration of cyclic nucleotide, p is the open probability, and i is the single-channel current. In the experiments shown in Fig. 3, instantaneous currents were also measured (see previous paragraph on *Recording Procedures* and Fig. 3). Instantaneous currents are defined as currents measured at a time after switching the voltage, V, so short that the equilibrium between open and closed channels is not yet affected by V, and therefore that the open probability is still the same as that at the previous voltage, V_0, while ion permeation is already affected by V. The instantaneous current, I_i, is therefore given by:

$$I_i(V, cNMP, pH) = N_c \cdot p(V_0, cNMP, pH) \cdot i(V, cNMP, pH), \quad (2)$$

and instantaneous current-voltage relations are expected to describe the I-V curve of a single channel.

RESULTS

pH_i-induced Changes in CNG Currents

The modulation by internal protons of CNG channels was studied in excised inside-out patches from retinal rods of the salamander. Solutions at different pH_i were changed at the cytoplasmic side of the patch and currents were activated by various concentrations of cGMP or cAMP and voltage pulses between −100 and +100 mV. Both inward and outward currents were affected

by changing pH_i, and the effect was completely and rapidly reversible in the pH_i range from 7.6 to 5.0.

Fig. 1 shows the effects caused on the same patch by the reduction of pH_i from 7.6 to 5.5. When channels were activated by cGMP, a pH_i reduction caused a similar decrease in current at every cGMP concentration and voltage (Fig. 1 *A*). On the contrary, when channels were activated by cAMP, a pH_i reduction produced an increase in the current. The relative current increase was higher at negative voltages and when the cAMP concentration was lowered (Fig. 1 *B*).

Voltage Dependence of pH_i Effect

The dependence on voltage of the changes in current caused by cytosolic acidification is illustrated in Fig. 2. Current-voltage (I-V) relations from the data shown in Fig. 1 for cGMP or cAMP were plotted in Fig. 2, *A* and *B*, respectively. When channels were activated by cGMP, the shape of the I-V relations at each cGMP concentration was not altered by reducing pH_i from 7.6 to 5.5 (Fig. 2 *A*). The shape of the I-V relations was instead altered by decreasing pH_i when currents were activated by cAMP (Fig. 2 *B*). At pH_i 7.6 I-V curves activated by cAMP showed a strong outward rectification while at pH_i 5.5, they became more linear at every cAMP concentration. The voltage dependence of the effect of pH_i is further illustrated in Fig. 2 *C*. Currents at pH_i 5.5 were normalized to the current at pH_i 7.6 at the same voltage and cyclic nucleotide concentration, and the ratio was plotted as a function of voltage from −100 to +100 mV. The current decrease produced by pH_i 5.5

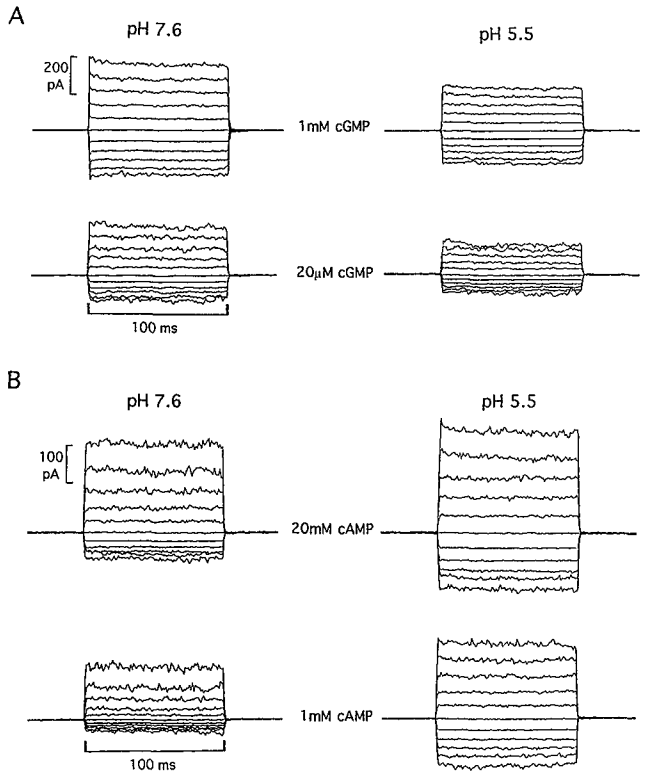

FIGURE 1. Effects of cytosolic acidification on currents activated by cGMP or cAMP. Traces are CNG currents from the same excised inside-out patch. Voltage steps of 100-ms duration from a holding potential of 0 mV were given from −100 to +100 mV in steps of 20 mV. Currents were measured as the difference between currents in the presence and in the absence of cyclic nucleotides. pH_i was reduced from 7.6 to 5.5 as indicated in the figure, and currents were activated by 1 mM or 20 μM cGMP (*A*) and by 20 mM or 1 mM cAMP (*B*).

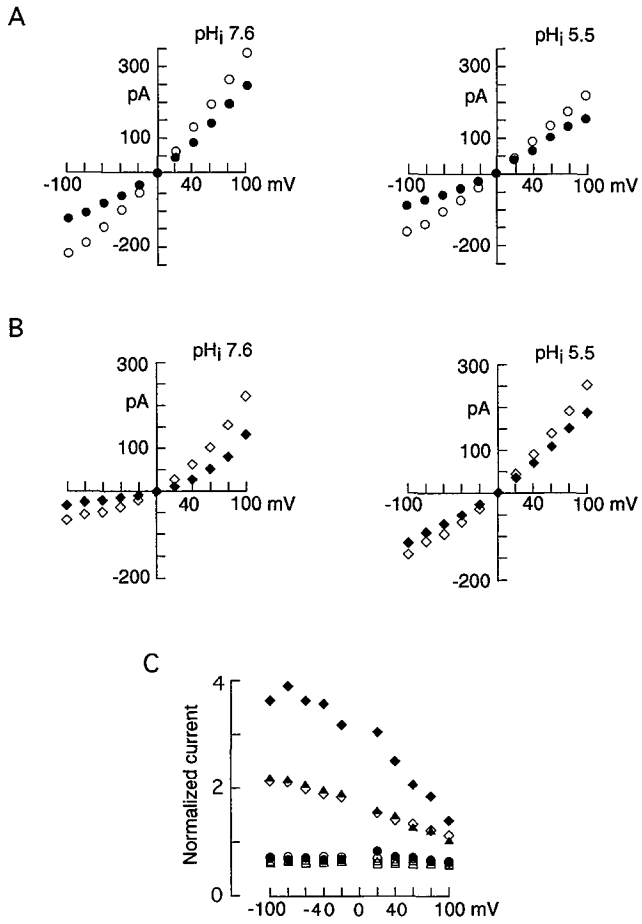

FIGURE 2. Voltage dependence of proton modulation of CNG currents. (*A*) Current-voltage relations from the experiments shown in Fig. 1 *A*. Currents were activated by 1 mM cGMP (*open circles*) or by 20 μM cGMP (*filled circles*) at pH_i 7.6 or 5.5 as indicated in the figure. (*B*) Current-voltage relations from the experiments shown in Fig. 1 *B*. Currents were activated by 20 mM cAMP (*open diamonds*) or by 1 mM cAMP (*filled diamonds*) at pH_i 7.6 or 5.5 as indicated in the figure. (*C*) Currents measured at pH_i 5.5 at various concentrations of cGMP or cAMP were normalized to the current measured at pH_i 7.6 at the same voltage and cyclic nucleotide concentration and plotted as a function of the applied voltage. Data from the same patch shown in Fig. 1. Concentrations for cGMP were: 1 mM (*filled circles*), 50 μM (*open squares*), 20 μM (*open circles*), 10 μM (*open triangles*). Concentrations for cAMP were: 20 mM (*open diamonds*), 5 mM (*filled triangles*), 1 mM (*filled diamonds*).

on cGMP-gated currents did not depend on voltage or concentration and, for the patch shown in Figs. 1 and 2, was ~0.7 (see Fig. 7 for mean values).

The increase in current measured with cAMP was instead both voltage and concentration-dependent. The current ratio at +60 mV in 20 mM cAMP was ~1.2 and increased to 2 at −60 mV. Lowering the cAMP concentration to 1 mM gave a current ratio of ~2 at +60 mV that became 3.6 at −60 mV. These results indicate that cytosolic acidification strongly affects the activation of the channels by cAMP.

Comparison of Steady-state and Instantaneous Current-Voltage Relations Activated by cAMP

To investigate whether pH_i modifies the voltage dependence of macroscopic currents activated by cAMP, by acting on the channel open probability or on the ionic permeation process, steady-state and instantaneous I-V curves were measured and compared (see Eqs. 1 and 2 in METHODS).

Fig. 3 *A* shows currents from the same patch activated by 200 or 500 μM cAMP at pH_i 7.6 or by 100 μM cAMP at pH_i 5.7. Voltage pulses of 16-ms duration were given from a holding potential of 0 mV in steps of 20 mV between −60 and +60 mV, and each trace was obtained from the average of 16 trials. 100 μM cAMP at pH_i 7.6 activated a current of only a few pA (data not shown), whereas reducing the pH_i to 5.7 caused an increase in the activated current to ~400 pA at +60 mV.

A time-dependent current relaxation was observed in all recordings after switching the voltage to a new value. Currents as a function of time increased at positive voltages and decreased at negative voltages until a steady-state value was reached. Lower panels in Fig. 3 *A* illustrate on an expanded time scale the time course of the onset of the currents shown in *A*. These current relaxations are similar to those previously recorded in channels activated by cGMP at physiological pH (Karpen et al., 1988; Zimmerman and Baylor, 1992).

Instantaneous currents were measured 100 μs after switching the voltage (see METHODS), and steady-state currents were calculated as the average current between 10 and 15 ms. Fig. 3 *B* shows both normalized steady-state (*filled symbols*) and instantaneous (*open symbols*) currents measured from the experiments shown in Fig. 3 *A* and plotted versus voltage. Currents were normalized to the value measured at +60 mV for each pH_i and cAMP concentration.

All the normalized instantaneous I-V curves at various cAMP concentrations and pH_i superimposed (*open symbols*) suggesting that the voltage dependence of the ionic permeation process is not influenced by pH_i or by cAMP concentration. Instantaneous I-V relations of Fig. 3 are very similar to those previously measured in channels activated by cGMP at pH 7.6 (Zimmerman and Baylor, 1992; Taylor and Baylor, 1995). Steady-state I-V curves showed instead rectification properties dependent both on pH_i and cAMP concentration. The outward rectification at pH_i 7.6 was more pronounced as the cAMP concentration was reduced from 500 to 200 μM. When the cAMP concentration was further re-

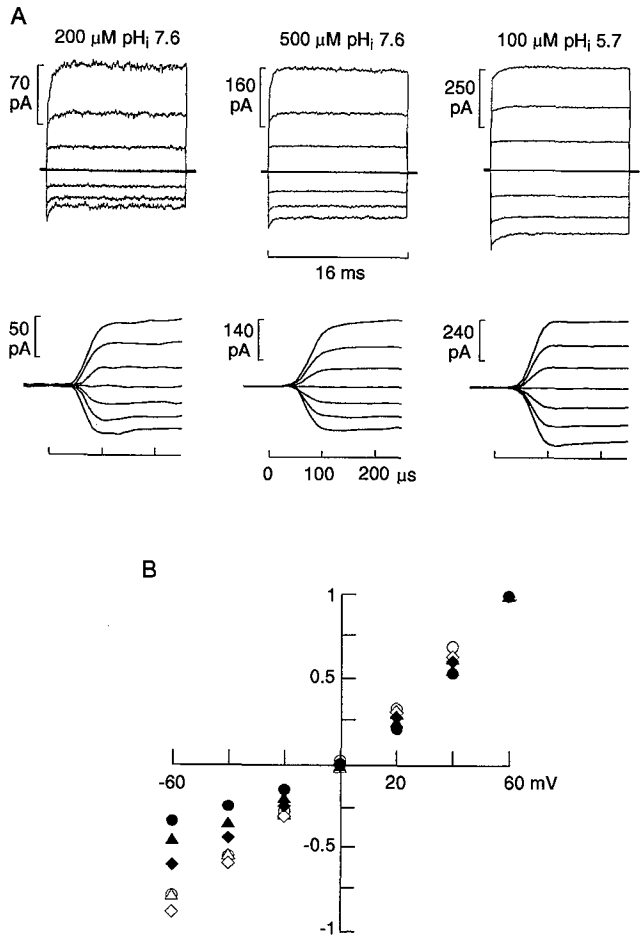

FIGURE 3. Voltage dependence of gating for cAMP-activated currents. (*A*) Currents from the same patch were activated by 200 or 500 μM cAMP at pH_i 7.6 or by 100 μM cAMP at pH_i 5.7 as indicated in the figure. *Lower panels* in *A* show currents from the corresponding *upper panels* on an expanded time scale. Voltage pulses of 16-ms duration were given from a holding potential of 0 mV in steps of 20 mV between −60 and +60 mV. The voltage was switched at time 0. Each trace was obtained from the difference between the averages from 16 trials in the presence and in the absence of cAMP. (*B*) Current-voltage relations from the experiments shown in *A* for 200 μM (*circles*) or 500 μM (*triangles*) cAMP at pH_i 7.6 or for 100 μM cAMP at pH_i 5.7 (*diamonds*). Instantaneous currents (*open symbols*) were measured at 100 μs after switching the voltage and normalized to the value measured at +60 mV. Steady-state currents (*filled symbols*) were calculated as the average value between 10 and 15 ms at each voltage and normalized to the average current at +60 mV. Steady-state (or instantaneous) currents at +60 mV had the following values in pA: 114 (79) for 200 μM cAMP at pH_i 7.6; 280 (214) for 500 μM cAMP at pH_i 7.6; 412 (380) for 100 μM cAMP at pH_i 5.7.

duced to 100 μM and pH_i was decreased to 5.7, the opposite trend was observed: the steady-state I-V relation became more linear. Similar results were obtained in two other patches.

A comparison between steady-state and instantaneous I-V relations indicates that both pH_i and cAMP concentration influence the channel open probability. Also the decrease in rectification observed by lowering pH_i to 5.7 when the channels are activated by cAMP seems to be mainly due to a pH_i-induced change in the open probability.

It has been recently shown that open-closed transitions in CNG channels from retinal rods are very fast and therefore that instantaneous current could be affected by these transitions at early times and that the real single channel I-V could be linear (Taylor and Baylor, 1995). However these observations do not modify the interpretation of our data since the voltage dependence of open probability would become even more pronounced.

In conclusion our results indicate that a pH_i reduction does not modify the voltage dependence of ion permeation and that the voltage dependence measured in the presence of cAMP could be attributed to a pH_i-induced modification of open probability.

Dose–Response Relations at pH_i 7.6 and 5.7

Fig. 4 *A* shows the dose–response curves for cGMP and cAMP at pH_i 7.6 and 5.7 at +60 mV. Currents from the same patch were plotted versus the concentration of cGMP and cAMP. Dose–response data were fitted by the Hill equation:

$$I = I_{max}\frac{c^n}{c^n + K_{1/2}^n}, \qquad (3)$$

where I is the current activated by the concentration c of cyclic nucleotide, I_{max} is the maximal current, $K_{1/2}$ is the cyclic nucleotide concentration necessary to activate half of the maximal current, and n is the Hill coefficient.

At pH_i 7.6 and at +60 mV, the maximal current activated by cAMP in the patch shown in Fig. 4 was 152 pA, which was ~40% of the maximal current activated by cGMP, 345 pA. Moreover $K_{1/2}$ for cAMP, 846 μM, was almost 90-fold larger than $K_{1/2}$ for cGMP, 9.8 μM. The Hill coefficient for cAMP was 1.8, slightly lower than the value (2.2) obtained with cGMP.

At pH_i 5.7 and +60 mV data obtained with cGMP were fitted with values for $K_{1/2}$ (10.6 μM) and n (2.2) very similar to those found at pH_i 7.6, while I_{max} (273 pA) was 80% of that at pH_i 7.6. From the dose–response with cAMP at pH_i 5.7 very different results from those at pH_i 7.6 were found: $K_{1/2}$ for cAMP (280 μM) decreased threefold and the maximal current (246 pA) increased to ~160% of that at pH_i 7.6. The Hill coefficient (1.8) was similar to that at pH_i 7.6.

By comparing the maximal currents activated by cGMP and cAMP at +60 mV at the same pH_i, it was found that I_{max} activated by cAMP was almost 90% of that activated by cGMP at pH_i 5.7, whereas it was only 40% at pH_i 7.6.

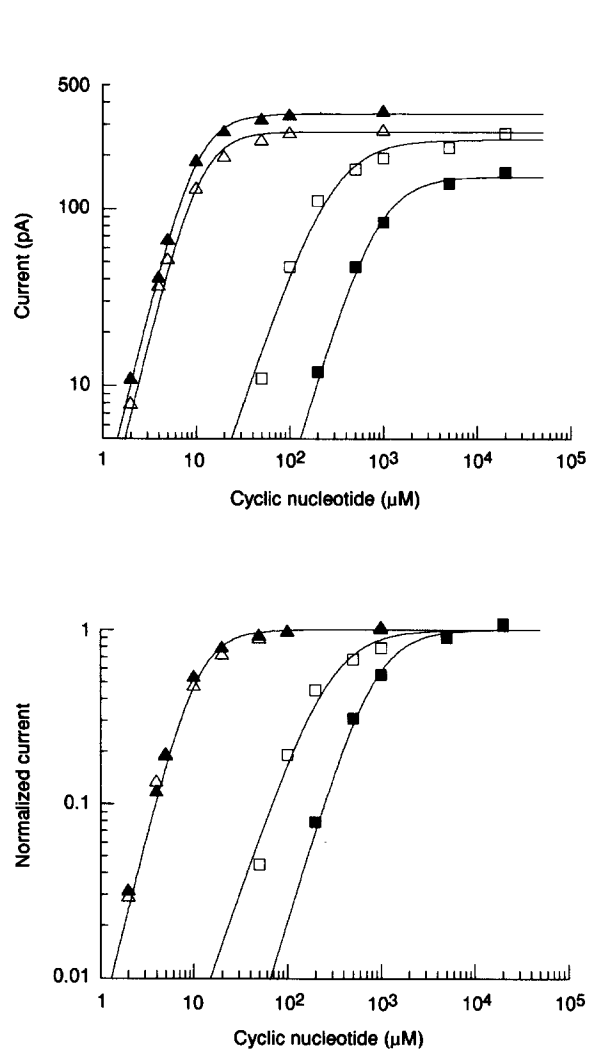

FIGURE 4. Dose-response relations at pH_i 7.6 or 5.7 for cGMP and cAMP in the same patch. (*A*) Steady-state currents activated by cGMP at pH_i 7.6 (*filled triangles*) and 5.7 (*open triangles*), or by cAMP at pH_i 7.6 (*filled squares*) and 5.7 (*open squares*) were measured at +60 mV and plotted versus cyclic nucleotide concentrations. Continuous lines were the best fit of the Hill equation (Eq. 3) to the data with the following values: I_{max} = 345 pA, $K_{1/2}$ = 9.8 μM, n = 2.2 for cGMP at pH_i 7.6; I_{max} = 273 pA, $K_{1/2}$ = 10.6 μM, n = 2.2 for cGMP at pH_i 5.7; I_{max} = 152 pA, $K_{1/2}$ = 846 μM, n = 1.8 for cAMP at pH_i 7.6; I_{max} = 246 pA, $K_{1/2}$ = 280 μM, n = 1.8 for cAMP at pH_i 5.7. (*B*) Currents from *A* were normalized to the maximal currents calculated from the Hill equation and listed above and plotted versus cyclic nucleotide concentration. Symbols as in *A*.

At $V = -60$ mV the effect of reducing pH_i to 5.7 on the currents activated by cGMP was similar to that observed at +60 mV. When currents were activated by cAMP, the relative increase in current at −60 mV was higher than that at +60 mV: I_{max} at pH_i 5.7 was ~250% of that at pH_i 7.6 (data not shown). For mean values of $K_{1/2}$, n, and normalized I_{max}, see Figs. 5–8.

These results show that pH_i 5.7 did not affect $K_{1/2}$ for activation by cGMP whereas it decreased $K_{1/2}$ for activation by cAMP; moreover it decreased the maximal current activated by cGMP and greatly increased that activated by cAMP.

Normalizing Eq. 1 to the maximal current ($I_{max,cN}$) measured in the presence of saturating concentrations of cyclic nucleotide, indicated by *cN*, and assuming that the single-channel current does not depend on cyclic nucleotide concentration, the following relation is obtained:

$$\frac{I(V, cNMP, pH)}{I_{max,cN}(V, pH)} = \frac{p(V, cNMP, pH)}{p_{max,cN}(V, pH)}. \quad (4)$$

Currents from Fig. 4 *A* were normalized to the maximal value obtained for each cyclic nucleotide and pH_i and plotted in Fig. 4 *B* as a function of cyclic nucleotide concentrations. Decreasing pH_i to 5.7 did not modify the normalized dose–response curve for cGMP whereas it shifted to the left that for cAMP. A comparison of Eq. 4 and Fig. 4 *B* indicates that the relative open probability for cAMP is affected by pH_i whereas that for cGMP does not depend on pH_i.

$K_{1/2}$ and n as a Function of pHi at Different Voltages

Dose–response curves were measured at different values of pH_i ranging from 7.6 to 5.0 and at various voltages both for cGMP and cAMP and fitted by the Hill equation (Eq. 3) as shown in the previous section. Fig. 5 shows mean values (±SD) of $K_{1/2}$ obtained from several patches as a function of pH_i at +60 (*filled symbols*) and −60 mV (*open symbols*).

$K_{1/2}$ for cGMP (Fig. 5 *A*) was not significantly affected by pH_i and had a consistently higher value at −60 mV (12 μM at pH_i 7.6) compared to that at +60 mV (8 μM at pH_i 7.6). $K_{1/2}$ for cAMP (Fig. 5 *B*) decreased about tenfold as the pH_i was reduced from 7.6 to 5.0. These results suggest that gating of the channel by cGMP was not modified by pH_i in the range from 7.6 to 5.0 whereas gating by cAMP was progressively enhanced by protonation. The dependence of $K_{1/2}$ for cAMP on pH_i could be well described by Eq. 5, which is consistent with the model used to explain the effects of pH_i on the maximal open probability (see Eq. 8 and Fig. 8):

$$K_{1/2\,cA}(V) = K_{min}(V) + K_{\Delta}(V)\frac{[K_0]^n}{[H^+]^n + [K_0]^n}, \quad (5)$$

where $[H^+]$ is the proton concentration, $K_{min}(V)$ is the value of $K_{1/2}$ at very high proton concentrations, $K_{\Delta}(V)$ is the difference between the value of $K_{1/2}$ at very low proton concentrations and $K_{min}(V)$, $[K_0]$ is the proton concentration giving 50% of $K_{\Delta}(V)$ and n is the Hill coefficient. Continuous lines in Fig. 5 B were obtained from a fit of Eq. 5 to the data with $pK_0 = 6.1$ and $n = 1.5$ at both +60 and −60 mV. K_{min} and K_{Δ} were respectively: 80 and 830 μM at −60 mV, 68 and 588 μM at +60 mV. Eq. 5 can be derived by a model in which $K_{1/2}$ is decreased by the cooperative binding of at least n protons. Therefore the shift of dose-responses to lower cAMP concentrations when internal proton concentration is increased can be described by the cooperative action of at least two protons ($n = 1.5$) binding to sites with a pK_0 of 6.1.

Fig. 6 shows mean values of the Hill coefficient at +60 mV as a function of pH_i. Values at −60 mV were almost identical to those at +60 mV and were not shown. The Hill coefficient was not affected by pH_i for either

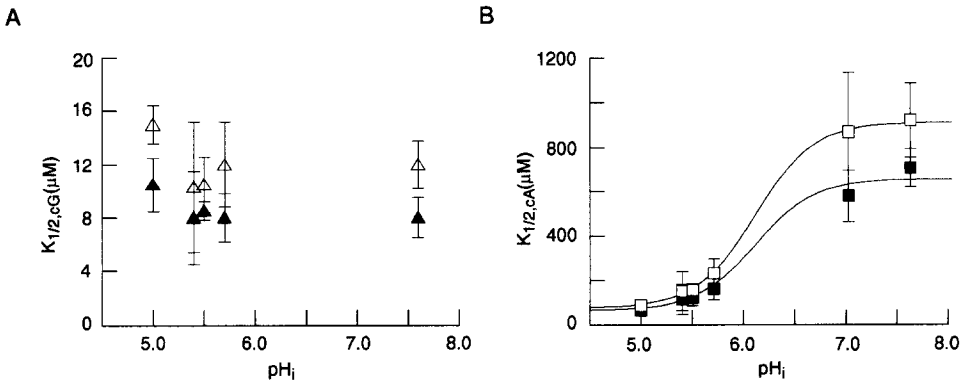

FIGURE 5. $K_{1/2}$ for cGMP and cAMP as a function of pH_i. Mean values of $K_{1/2}$ (±SD) from several patches were calculated from values obtained from the best fit of the Hill equation (Eq. 3) to dose–response curves at various pH_i as shown in Fig. 4. (*A*) $K_{1/2}$ for cGMP at +60 (*filled triangles*) and −60 mV (*open triangles*) and (*B*) $K_{1/2}$ for cAMP at +60 (*filled squares*) and −60 mV (*open squares*) were plotted versus pH_i. Continuous lines were the fit of Eq. 5 to the data with the following values: $pK_0 = 6.1$ and $n = 1.5$ at both +60 and −60 mV; K_{min} and K_{Δ} were, respectively, 80 and 830 μM at −60 mV, 68 and 588 μM at +60 mV. Number of experiments in the presence of cGMP (cAMP) at the indicated pH_i was: 8 (5) at pH_i 7.6, 4 (2) at 5.7, 5 (5) at 5.5, 3 (3) at 5.4, and 2 (2) at 5.0.

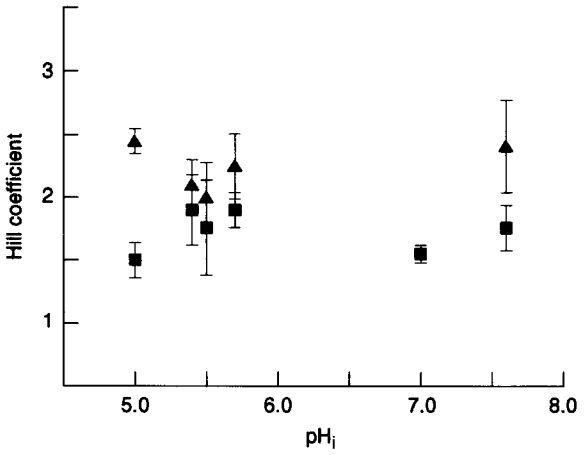

FIGURE 6. Hill coefficients for channel activation by cGMP and cAMP as a function of pH_i. Mean values (±SD) of the Hill coefficients at +60 mV for cGMP (*filled triangles*) or for cAMP (*filled squares*) were plotted versus pH_i. Number of experiments as listed in the legend of Fig. 5.

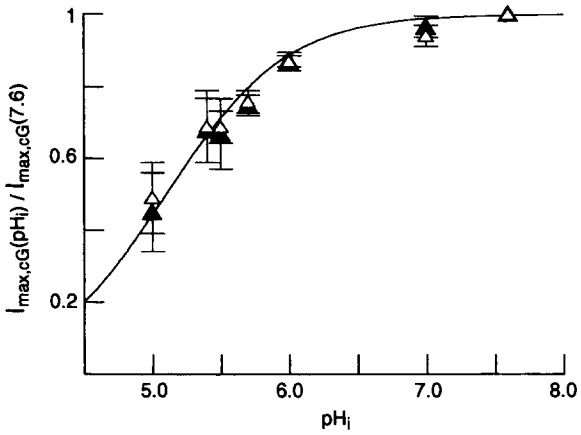

FIGURE 7. Normalized maximal currents activated by cGMP as a function of pH_i. Mean values (±SD) from several patches of $I_{max,cG}$ calculated at a given pH_i and normalized to $I_{max,cG}$ at pH_i 7.6 in the same patch were plotted versus pH_i. Currents were measured at +60 (*filled triangles*) or −60 mV (*open triangles*). Continuous line was the best fit of Eq. 6 to the data with $pK_I = 5.1$. Number of experiments was: 4 at pH_i 7.0, 3 at 6.0, 6 at 5.7, 7 at 5.5, 10 at 5.4, and 9 at 5.0.

cyclic nucleotides: for cGMP n was between 2 and 3, while for cAMP it was consistently between 1 and 2. A lower value of the Hill coefficient for cAMP with respect to cGMP-gated channels is in agreement with previous observations at physiological pH_i (Tanaka et al. 1989, Ildefonse et al., 1992). The independence of n on pH_i indicates that the number of subunits involved in the gating of the channels was not modified by pH_i.

Dependence of I_{max} on pHi and Voltage

Protonation effects were further investigated by studying the relation between maximal currents and pH_i. Maximal currents activated by cGMP or by cAMP were normalized as described below and plotted in Figs. 7–9. Values from several patches were averaged and plotted (±SD) as a function of pH_i.

Fig. 7 shows the plot of maximal currents activated by cGMP at various pH_i at +60 or −60 mV and normalized to the maximal current activated by cGMP at pH_i 7.6 at the corresponding voltage. Current had almost the same value between pH_i 7.6 and 7 and then decreased when pH_i was further lowered reaching 50% of its maximum value at about pH_i 5. The current reduction was not voltage dependent, and data were well fitted by the following equation:

$$\frac{I_{max,cG}(V, pH)}{I_{max,cG}(V, 7.6)} = \frac{[K_I]}{[K_I] + [H^+]}, \qquad (6)$$

where $[H^+]$ is the proton concentration, and $[K_I]$ is the proton concentration necessary to half-inhibit the normalized current. The continuous line in Fig. 7 was the best fit to the data obtained with pK_I of 5.1 for both +60 and −60 mV. Eq. 6 is equivalent to the relation for titration of a single acidic residue. Therefore the effect of pH_i on the current activated by cGMP could easily be explained as partial blockage of the current due to protonation of an ionic group with a pK of 5.1. The absence of a voltage-dependent effect of pH_i on the current activated by cGMP suggests that this ionic group is located near the inner mouth of the channel outside the transmembrane electrical field in agreement with previous observations (Menini and Nunn, 1990; Tanaka, 1993).

It has been suggested in previous sections (see Figs. 4 *B* and 5 *A*) that gating of channels by cGMP is not affected by pH_i. If we therefore assume that the maximal open probability for cGMP, $p_{max,cG}$, is not affected by pH_i it is straightforward to obtain the following relation from Eq. 1:

$$\frac{I_{max,cG}(V, pH)}{I_{max,cG}(V, 7.6)} = \frac{i(V, pH)}{i(V, 7.6)}, \qquad (7)$$

The effect of pH_i on cGMP-gated currents could therefore be described as partial reduction of the single-channel current.

Currents activated by cAMP had a more complex dependence on pH_i. Maximal currents activated by 20 mM cAMP at a given pH_i were normalized to the maximal currents activated in the same patch by 1 mM cGMP at the same pH_i and mean values were plotted in Fig. 8 at +60 (*filled circles*) and −60 mV (*open circles*). Current ratios of cAMP over cGMP increased with proton concentration and could be well described by the following equation:

$$\frac{I_{max,cA}(V,pH)}{I_{max,cG}(V,pH)} = P_{min}(V) + P_{\Delta}(V)\frac{[H^+]^n}{[H^+]^n + [K_2]^n}, \quad (8)$$

where $P_{min}(V)$ is the ratio of maximal currents at low proton concentrations, $P_{\Delta}(V)$ is the difference between the ratio of maximal currents at high and low proton concentrations and $P_{min}(V)$, $[H^+]$ is the proton concentration, $[K_2]$ is the proton concentration giving 50% of $P_{\Delta}(V)$, and n is the Hill coefficient. Continuous lines in Fig. 8 were obtained from a fit of Eq. 8 to the data with $pK_2 = 6$ and $n = 2.8$ at both +60 and −60 mV. P_{min} and P_{Δ} were respectively 0.3 and 0.6 at −60 mV, and both had the same value of 0.5 at +60 mV. Eq. 8 can be derived by a model in which the normalized current is enhanced by the cooperative binding of at least n protons. A fit to the data was also done with a relation describing current enhancement by proton binding to n independent sites, but this relation gave a poorer fit.

In the simplest hypothesis that the single-channel current at a given voltage and pH_i is the same when channels are activated by cGMP or by cAMP, the following relation is easily obtained from Eq. 1:

$$\frac{I_{max,cA}(V,pH)}{I_{max,cG}(V,pH)} = \frac{p_{max,cA}(V,pH)}{p_{max,cG}(V,pH)}, \quad (9)$$

Eq. 9 is equivalent to Eq. 8, and, if $p_{max,cG}$ is not affected by pH_i, it is suggested that the maximal open probability for cAMP increases as a function of proton concentration due to a cooperative action of at least 3 (n = 2.8) protons with a pK_2 of 6. This model is consistent with that (see Eq. 5 and Fig. 5 *B*) used to explain the shift of dose-responses to lower cAMP concentrations caused by protons.

Another normalization procedure, equivalent to that used for cGMP in Fig. 7, was then applied to maximal currents activated by cAMP. Currents measured at the saturating concentration of 20 mM cAMP at a given pH_i were normalized to the maximal current activated in the same patch by 1 mM cGMP at pH_i 7.6, and mean values were plotted versus pH_i in Fig. 9 at +60 (*filled squares*) and −60 mV (*open squares*). Normalized maximal currents in cAMP increased by increasing proton concentration from pH_i 7.6 to 5.7–5.5 and then decreased as pH_i was lowered to 5.0.

A relation describing normalized currents plotted in Fig. 9 is easily obtained multiplying Eqs. 6 and 8:

$$\frac{I_{max,cA}(V,pH)}{I_{max,cG}(V,7.6)} = \frac{[K_1]}{[K_1] + [H^+]} \left\{ P_{min}(V) + P_{\Delta}(V)\frac{[H^+]^n}{[H^+]^n + [K_2]^n} \right\}. \quad (10)$$

Continuous lines in Fig. 9 were plotted from Eq. 10 with the parameters obtained from fits of Eqs. 6 and 8 to the data in Figs. 7 and 8, respectively. Eq. 10 well describes maximal currents activated by cAMP as a function of pH_i and is very useful to interpret the data. An increase in internal proton concentration produces an enhancement of activation by cAMP increasing the channel open probability through the cooperative binding of at least 3 protons with a pK_2 of 6 (Eqs. 8 and 9) and, at the same time, protons cause a reduction of

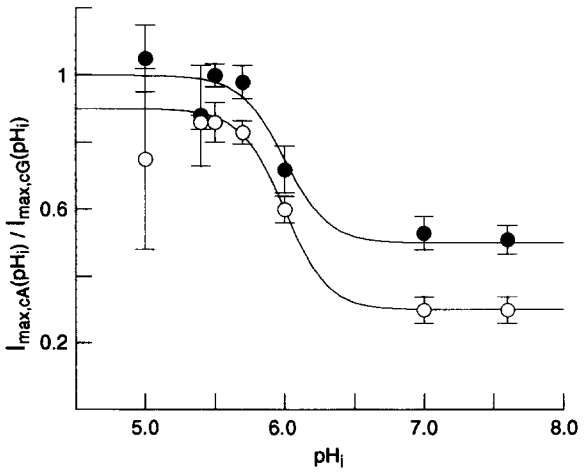

FIGURE 8. Maximal currents activated by 20 mM cAMP at a given pH_i normalized to maximal currents activated in the same patch by 1 mM cGMP at the same pH_i plotted as a function of pH_i at +60 (*filled circles*) or −60 mV (*open circles*). Continuous lines were the fit of Eq. 8 to the data with the following values: $pK_2 = 6$ and $n = 2.8$ at both +60 and −60 mV; $P_{min}(+60) = P_{\Delta}(+60) = 0.5$; $P_{min}(-60) = 0.3$; $P_{\Delta}(-60) = 0.6$. Number of experiments was: 6 at pH_i 7.6, 2 at 7.0, 3 at 6.0, 4 at 5.7, 4 at 5.5, 4 at 5.4, and 2 at 5.0.

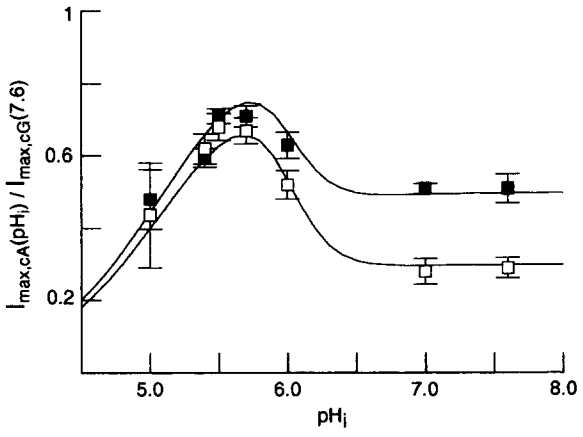

FIGURE 9. Normalized maximal currents activated by cAMP as a function of pH_i. Mean values (±SD) from several patches of the ratio between currents activated by 20 mM cAMP at a given pH_i, and currents activated by 1 mM cGMP at pH_i 7.6 in the same patch were plotted versus pH_i. Currents were measured at +60 (*filled squares*) or −60 mV (*open squares*). Continuous lines were obtained from Eq. 10 with values of parameters listed in the legend of Figs. 7 and 8. Number of experiments was: 9 at pH_i 7.6, 2 at 7.0, 3 at 6.0, 4 at 5.7, 7 at 5.5, 4 at 5.4, and 7 at 5.0.

single-channel current due to the binding of a proton to a site with a pK_1 of 5.1 (Eqs. 6 and 7). Enhancement of gating dominates over single-channel current reduction when pH_i is decreased from 7.6 to 5.7–5.5. A further pH_i decrease to 5.0 does not produce an additional increase in open probability (Fig. 8) while the single-channel current is further reduced (Fig. 7).

DISCUSSION

The experiments described in this paper show that the activation of channels by cyclic nucleotides in retinal rod outer segments could be modulated by cytosolic acidification. Currents activated at every cGMP concentration and applied voltage were reduced by increasing cytoplasmic proton concentration. Currents activated by cAMP were dramatically enhanced by increasing proton concentration in the range of pH_i between 7.6 and 5.7–5.5 and then decreased between pH_i 5.5 and 5.0. Furthermore this effect was both voltage and concentration dependent.

Modulation of cGMP-activated Channels

When native channels were activated by cGMP, $K_{1/2}$ was not modified by pH_i (Fig. 5 *A*) suggesting that the gating of the channel was not altered by pH_i. Currents activated by cGMP were instead reduced by increasing proton concentration. Current reduction as a function of pH_i did not depend on voltage and was well described by a curve for titration of a single acidic residue with a pK of 5.1 (Fig. 7). On the assumption that the gating of the channel is not modified by pH_i, the same relation also describes the reduction of single-channel current by cytosolic acidification (Eqs. 6 and 7). The site of action is voltage independent, and, therefore, must be located at the inner mouth of the channel outside the transmembrane electrical field. These results are consistent with previous studies in which intracellular protons were shown to reduce the current activated by saturating cGMP concentrations in a voltage-independent way (Menini and Nunn, 1990; Tanaka, 1993).

Modulation of cAMP-activated Channels

When channels were activated by cAMP an increase in internal proton concentration caused a decrease in $K_{1/2}$ (Fig. 5 *B*) indicating that pH_i altered the gating process of the channel. Since $K_{1/2}$ is the concentration of cAMP for half-maximal activation of currents, its value reflects both the affinity of cAMP for the binding site and the channel conformational change from the closed to the open state. If the effect of pH_i on currents activated by cAMP is simply due to an increase in affinity for its binding site, dose–response relations are expected to be shifted toward lower cAMP concentrations without modification of the maximal current. Maximal currents activated by cAMP were instead affected by pH_i (Figs. 4 *A* and 9). Therefore cytosolic acidification is acting at some site very important in favoring the open state of the channel. An additional effect on the binding site could also be present.

Both the effect of pH_i on $K_{1/2}$ (Fig. 5 *B*) and on the maximal open probability (Fig. 8) could be well explained (Eqs. 5, 8, and 9) by same mechanism: the cooperative binding of at least 2–3 protons to sites with a pK of ~6. The maximal open probability for cAMP relative to that for cGMP could be obtained from the ratio of maximal currents (see Eq. 9 and Fig. 8). At pH_i 7.6 and +60 mV, $p_{max,cA}$ was ~50% of $p_{max,cG}$ but became ~100% when pH_i was reduced to 5.7–5.5. An even larger increase in open probability was measured at −60 mV: at pH_i 7.6 $p_{max,cA}$ was ~30% of $p_{max,cG}$ and became ~90% at pH_i 5.7–5.5. Therefore when proton concentration is increased, gating of the channels by cAMP becomes more similar to gating by cGMP. This could also explain the change in the shape of I-V curves from the strong outward rectification observed in cAMP at pH_i 7.6, to the more linear form at pH_i 5.5 (compare *left* and *right* panels in Fig. 2 *B*). Indeed I-V curves in cAMP at pH_i 5.5 have a shape similar to I-V curves in cGMP (Fig. 2, *A* and *B*), and this change in voltage dependence could be due, as shown above, to a pH_i-induced change in the gating process by cAMP.

Internal protons could modulate both gating and permeation of channels activated by cAMP. If single-channel currents in cAMP and cGMP are the same, the relation for titration of a single acidic residue (Eq. 6) will also describe the effect of pH_i on the permeation properties of cAMP-gated channels. Therefore modulation of cAMP-gated channels by internal protons seems to involve at least four protons, one of which combines with a pK_1 of 5.1 and causes a reduction of single-channel current, and the other three protons combine with a pK_2 of 6 and produce an increase in the channel open probability.

Comparison with Modulation by Other Factors

The sensitivity to cyclic nucleotides of native channels from retinal rods can be modulated by various factors. A comparison of modulation properties by transition metal divalent cations and Ca^{2+}-calmodulin shows many differences with modulation by internal protons.

Micromolar concentrations of some transition metal divalent cations applied to the cytoplasmic side of the patch have been shown to modulate the current activated by cGMP (Ildefonse et al., 1992; Karpen et al., 1993) by shifting the dose–response relation to lower concentrations without affecting the maximal current activated by saturating cGMP; moreover, this potentiation effect persisted for many minutes after divalent cations were removed. Currents activated by cAMP

were also potentiated, and maximal currents were increased without modification of their voltage dependence by the addition of Ni^{2+} (Ildefonse et al., 1992). This effect was explained by an increase of the open probability by cAMP.

Ca^{2+}-calmodulin applied to the cytoplasmic side of the patch has been shown to modulate CNG-gated channels from retinal rods by shifting the dose-response for activation by cGMP to higher concentrations (Hsu and Molday, 1993) without changing the maximal current (Gordon et al., 1995*b*). Also this effect persisted for several seconds after calmodulin was removed.

There are many functional differences between modulation by the above described factors and modulation by protons, and, therefore, they are likely to be caused by different molecular mechanisms.

Native and Cloned Channels

CNG channels from retinal rods have been shown to be hetero-oligomers composed of α and β subunits (Kaupp et al., 1989; Chen et al., 1993; Chen et al., 1994; Körschen et al., 1995; and for reviews, see Kaupp, 1995; Zimmerman, 1995). Comparison of properties of native CNG channels with that of cloned CNG channels (Zimmerman, 1995; Gavazzo et al., 1996) composed of one or two types of subunits and site-directed mutagenesis studies are necessary for understanding function and modulation properties at a molecular level.

It has been shown that Ca^{2+}-calmodulin does not have any effect on the α subunit of the rod channel, whereas it seems to interact with the most recently cloned β subunit (Hsu and Molday, 1993; Chen et al. 1994). Modulation by Ni^{2+} ions was instead effective on the α subunit of the bovine rod channel, and site-directed mutagenesis studies have indicated that a histidine residue (H420) is involved in modulation (Gordon and Zagotta, 1995).

Recent studies have shown that modulation by internal protons is effective on channels composed of only α subunits and that there are many similarities and many interesting differences between modulation of native and cloned channels (Gavazzo et al. 1994; Gordon et al. 1996). For example, one difference was found on activation by cGMP: in native channels dose–response curves were not shifted by pH_i (Figs. 4 and 5), whereas in channels composed of α subunits, $K_{1/2}$ for cGMP was decreased as the internal proton concentration was increased. Activation by cAMP of channels composed of α subunits was enhanced by increasing proton concentration, but the effect was quantitatively larger than that observed in native channels. Site-directed mutagenesis studies (Gordon et al. 1996) have shown that aspartic acid (D604) and histidine (H468) residues in the α subunit of the bovine rod channel are involved in modulation by pH_i. In particular, protonation at D604 accounts for the cAMP-specific component of potentiation, and H648 accounts for a nucleotide-nonspecific component of potentiation. Gordon et al. (1996) also found that channels formed by co-expressing the α subunit with the β subunit from Chen et al. (1993) showed a reduced proton potentiation. They suggested that this result could be explained by the presence of asparagines, which are nonprotonatable groups, at the positions corresponding to 468 and 604. However, even their results with channels composed of α and β subunits are still quantitatively different from those on native channels shown in this paper. This discrepancy could at least be partially due, as pointed out by Gordon et al. (1996), to channels composed of different ratios of α and β subunits, since the fraction of channels that incorporated the β subunit in their study is unknown. There could also be a difference between species, since native channels were studied in salamander retinal rods and cloned channels were obtained by co-expressing the α subunit from bovine with the β subunit from human. Moreover, it has been recently shown that the β subunit used by Gordon et al. (1996) and originally cloned by Chen et al. (1993) is a component of a 240-kD protein (Chen et al., 1994; Körschen et al., 1995). It is therefore possible that some other amino acid of this most recently cloned complete β subunit are involved in modulation by internal protons. Further studies on channels composed of α subunit and of the complete β subunit in different ratios will be necessary to identify the reasons for differences between native and cloned channels at a molecular level. Furthermore the amino acid responsible for the reduction in single-channel current and located at the cytoplasmic side of the channel has not been identified yet.

A study of the effect of pH on cloned CNG channels from catfish olfactory receptor cells composed only of α subunit has also been performed (Root and MacKinnon, 1994). Single channels activated by saturating concentrations of cGMP were studied in solutions containing deuterium while pH was changed at both sides of the membrane. Site-directed mutagenesis studies showed that external protons could alter the ion permeation properties by binding to titratable sites formed by glutamate (E333) with an anomalous pK_a of 7.6 and suggested that these sites are located near the extracellular side of the channel.

Comparison of Native Channels from Retinal Rods and Other CNG Channels

The effect of cytosolic acidification on native CNG channels from olfactory receptor cells has been studied activating channels only with saturating concentrations of cAMP (Frings et al., 1992). It has been found that the current decreased as a function of pH_i following a

curve for titration of a single acidic residue with a pK of ~5.0–5.2. These results are similar to those obtained with the rod channel at saturating cGMP concentrations (Fig. 7). The enhancement of cAMP-activated currents by increasing internal proton concentration seems to be a peculiarity of the photoreceptor channel not shared by the olfactory channel and therefore useful to identify their different mechanisms of activation at a molecular level.

In retinal rods cGMP is the internal second messenger while cAMP does not seem to play any role in phototransduction. Moreover pH_i in rods does not seem to vary appreciably during the phototransduction process (Yoshikami and Hagins, 1985). Even if there is a small change in pH_i due to hydrolysis of cGMP, the current activated by the physiological messenger cGMP does not appreciably change when pH_i varies between 7.6 and 7. Also, in olfactory receptor cells, where the physiological internal transmitter is cAMP, the activation of the channels does not seem to depend on pH_i between 7.6 and 7 (Frings et al., 1992). Thus, modulation of CNG channels by internal protons does not seem to play a physiological role in phototransduction or in olfactory transduction.

Other CNG channels not belonging to sensory receptor cells have been recently discovered (for reviews, see Yau, 1994; Kaupp, 1995), but most of their functional properties have not been characterized yet. With respect to the properties of activation by cyclic nucleotides, it was found that, for cloned channels from testis, maximal currents activated by cAMP were <30% of the maximal current activated by cGMP with $K_{1/2}$ of 8 μM for cGMP and 1.7 mM for cAMP (Weyand et al., 1994). These channels seem to be functionally very similar to the rod channel and could have similar modulation properties. Cytosolic acidification is likely to modulate these channels in a similar way to the rod channels, but it is not known whether this could have a physiological relevance. In any case, regardless of the physiological role of pH_i in modulating CNG channels, elucidation of the molecular mechanisms by which it acts will contribute to our understanding of CNG channels. Understanding of molecular mechanisms of channel function and modulation will be accomplished by combining site-directed mutagenesis studies with a continuous comparison of properties of native and cloned channels composed of various subunits.

We thank V. Torre for helpful discussions and comments on the manuscript, A. Sostman for participating in some experiments on instantaneous I-V, and G. Boido, G. Gaggero, D. Magliozzi, and P. Guastavino for technical assistance.

This work was supported by EC BRA SSS 6961 and HFSP.

Original version received 2 April 1996 and accepted version received 8 July 1996.

REFERENCES

Chen, T.Y., M. Illing, L.L. Molday, Y.T. Hsu, K.W. Yau, and R.S. Molday. 1994. Subunit 2 (or β) of retinal rod cGMP-gated cation channel is a component of the 240-kDa channel-associated protein and mediates Ca^{2+}-calmodulin modulation. 1994. *Proc. Natl. Acad. Sci. USA.* 91:11757–11761.

Chen, T.Y., Y.W. Peng, R.S. Dhallan, B. Ahamed, R.R. Reed, and K.W. Yau. 1993. A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. *Nature (Lond.).* 362:764–767.

Chen, T.Y., and K.W. Yau. 1994. Direct modulation by Ca^{2+}-calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. *Nature (Lond.).* 368:545–548.

Fesenko, E.E., S.S. Kolesnikov, and A.L. Lyubarsky. 1985. Induction by cyclic cGMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature (Lond.).* 313:310–313.

Frings, S., J.W. Lynch, and B. Lindemann. 1992. Properties of cyclic nucleotide-gated channels mediating olfactory transduction. *J. Gen. Physiol.* 100:1–11.

Gavazzo, P., C. Picco, L. Maxia, and A. Menini. 1996. Properties of native and cloned cyclic nucleotide gated channels from bovine. *In* Neurobiology. V. Torre and F. Conti, editors. Plenum Press, New York. In press.

Gavazzo, P., C. Picco, C. Sanfilippo, and A. Menini. 1994. The effects of cytosolic acidification on native and cloned channels activated by cyclic nucleotide in retinal rods. *Proc. XII Congr. Italian Biophys. Soc., Mondello.* B-P2. (Abstr.)

Gordon, S.H., D.L. Brautigan, and A.L. Zimmerman. 1992. Protein phosphatases modulate the apparent agonist affinity of the light-regulated ion channel in retinal rods. *Neuron.* 9:739–748.

Gordon, S.E., J. Downing-Park, B. Tam, and A.L. Zimmerman. 1995*a*. Diacylglycerol analogs inhibit the rod cGMP-gated channel by a phosphorylation-independent mechanism. *Biophys. J.* 69: 409–417.

Gordon, S.E., J. Downing-Park, and A.L. Zimmerman. 1995*b*. Modulation of the cGMP-gated ion channel in frog rods by calmodulin and an endogenous inhibitor factor. *J. Physiol. (Lond.).* 486: 533–546.

Gordon, S.E., J.C. Oakley, M.D. Varnum, and W.N. Zagotta. 1996. Altered ligand specificity by protonation in the ligand binding domain of cyclic nucleotide-gated channels. *Biochemistry.* 35: 3994–4001.

Gordon, S.E., and W.N. Zagotta. 1995. A histidine residue associated with the gate of the cyclic nucleotide-activated channels in rod photoreceptors. *Neuron.* 14:177–183.

Haynes, L.W., and K.W. Yau. 1985. Cyclic GMP-sensitive conductance in outer segment membrane of catfish cones. *Nature (Lond.).* 317:61–64.

Hille, B. 1992. Ionic Channels in Excitable Membranes. 2nd Ed. Sinauer Associates, Inc., Sunderland, MA. 607 pp.

Hsu, T.Y., and R.S. Molday. 1993. Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature (Lond.).* 361:76–79.

Ildefonse, M., S. Crouzy, and N. Bennet. 1992. Gating of retinal rod

cation channel by different nucleotides: comparative study of unitary currents. *J. Membr. Biol.* 130:91–104.

Karpen, J.W., R.L. Brown, L. Stryer, and D.A. Baylor. 1993. Interactions between divalent cations and the gating machinery of cyclic GMP-activated channels in salamander retinal rods. *J. Gen. Physiol.* 101:1–25.

Karpen, J.W., A.L. Zimmerman, L. Stryer, and D.A. Baylor. 1988. Gating kinetics of the cyclic-cGMP-activated channel of retinal rods: flash photolysis and voltage-jump studies. *Proc. Natl. Acad. Sci. USA.* 85:1287–1291.

Kaupp, U.B. 1995. Family of cyclic nucleotide gated ion channels. *Curr. Opin. Neurobiol.* 5:434–442.

Kaupp, U.B., T. Niidome, T. Tanabe, S. Terada, W. Bönigk, W. Stühmer, N.J. Cook, K. Kangawa, H. Matsuo, T. Hirose, T. Miyata, and S. Numa. 1989. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature (Lond.).* 342:762–766.

Körschen, H.G., M. Illing, R. Seifert, F. Sesti, A. Williams, S. Gotzes, C. Colville, F. Müller, A. Dosé, M. Godde, L. Molday, U.B. Kaupp, and R.S. Molday. 1995. A 240 kDa protein represents the complete β subunit of the cyclic nucleotide-gated channel from rod photoreceptor. *Neuron.* 15:627–636.

Liebman, P.A., P. Müller, and E.N. Pugh. 1984. Protons suppress the dark current of frog retinal rods. *J. Physiol. (Lond.).* 347:85–110.

Lynch, J.W., and B. Lindemann. 1994. Cyclic nucleotide gated channels of rat olfactory receptor cells: divalent cations control the sensitivity to cAMP. *J. Gen. Physiol.* 103:87–106.

Menini, A. 1990. Currents carried by monovalent cations through cyclic GMP-activated channel in excised patches from salamander rods. *J. Physiol. (Lond.).* 424:167–185.

Menini, A. 1995. Cyclic nucleotide-gated channels in visual and olfactory transduction. *Biophys. Chem.* 55:185–196.

Menini, A., and B.J. Nunn. 1990. The effect of pH on the cyclic GMP-activated conductance in retinal rods. *In* Sensory Transduction. A. Borsellino, L. Cervetto, and V. Torre, editors. Plenum Press, New York. 175–181.

Menini, A., G. Rispoli, and V. Torre. 1988. The ionic selectivity of the light-sensitive current in isolated rods of the tiger salamander. *J. Physiol. (Lond.).* 402:279–300.

Nakamura, T., and G.H. Gold. 1987. A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature (Lond.).* 325:442–444.

Picco, C., and A. Menini. 1993. The permeability of the cGMP-activated channel to organic cations in retinal rods of the tiger salamander. *J. Physiol. (Lond.).* 460:741–758.

Root, M.J., and R. MacKinnon. 1994. Two identical noninteracting sites in an ion channel revealed by proton transfer. *Science (Wash. DC).* 265:1852–1856.

Tanaka, J.C. 1993. The effects of protons on 3′,5′-cGMP-activated currents in photoreceptor patches. *Biophys. J.* 65:2517–2523.

Tanaka, J.C., J.F. Eccleston, and R.E. Furman. 1989. Photoreceptor channel activation by nucleotide derivates. *Biochemistry.* 28:2776–2784.

Taylor, W.R., and D.A. Baylor. 1995. Conductance and kinetics of single cGMP-activated channels in salamander rod outer segments. *J. Physiol. (Lond.).* 483:567–582.

Torre, V., J.F. Ashmore, T.D. Lamb, and A. Menini. 1995. Transduction and adaptation in sensory receptor cells. *J. Neurosci.* 15: 7757–7768.

Weyand, I., M. Godde, S. Frings, J. Weiner, F. Müller, W. Altenhofen, H. Hatt, and U.B. Kaupp. 1994. Cloning and functional expression of a cyclic-nucleotide gated channel from mammalian sperm. *Nature (Lond.).* 368:859–863.

Yau, K.W. 1994. Cyclic nucleotide gated channel: an expanding new family of ion channels. *Proc. Natl. Acad. Sci. USA.* 91:3481–3483.

Yoshikami, S., and W. Hagins. 1985. Cytoplasmic pH in rod outer segments and high-energy phosphate metabolism during phototransduction. *Biophys. J.* 47:101*a.* (Abstr.)

Zimmerman, A. 1995. Cyclic nucleotide gated channels. *Curr. Opin. Neurobiol.* 5:296–303.

Zimmerman, A.L., and D.A. Baylor. 1992. Cation interactions within the cyclic GMP-activated channel of retinal rods from the tiger salamander. *J. Physiol. (Lond.).* 449:759–783.

Zimmerman, A.L., J.W. Karpen, and D.A. Baylor. 1988. Hindered diffusion in excised membrane patches from retinal rods outer segments. *Biophys. J.* 54:351–355.