The Effects of Protons on 3′,5′-cGMP-Activated Currents in Photoreceptor Patches

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ABSTRACT Macroscopic 3′,5′-guanine cyclic monophosphate (cGMP)-activated currents from photoreceptor outer segment membranes were examined as the pH on the cytoplasmic face of inside/out patches was reduced. In the absence of divalent cations, protons reduced the current in both directions without affecting the shape of the current-voltage relation consistent with a voltage-independent block. When Ca^{2+} was added to the bath, increasing the $[H^+]$ relieved the Ca^{2+} block and eliminated the Ca^{2+}-induced reversal potential shifts seen at pH 7.4. These results suggest that protons alter Na^+/Ca^{2+} permeability of the channel and relieve Ca^{2+} block of the sodium transport.

INTRODUCTION

Protons interact directly with many ion channels to reduce the flow of current through the channel. Proton block was first described by Hille (1968) and Hille et al.(1975) in frog nodes of Ranvier where decreasing the extracellular pH leads to a reduction in both the peak sodium and potassium conductances. Exactly how protonation reduces macroscopic currents has been difficult to resolve, in part, because proton effects on channel gating, kinetics, and conductance are not easily separated in macroscopic recordings. It is also not clear to what extent protons mediate physiological responses through direct interactions with ion channels.

In this study, the effect of protons on 3′,5′-guanine cyclic monophosphate (cGMP)-activated channels was examined by recording macroscopic currents from inside/out photoreceptor patches as the bath (cytoplasmic) pH was lowered. In the absence of divalent cations, lowering the pH from 7.4 to 4.7 decreased the current flow in both directions in a voltage-independent manner. The effect of protons on the cGMP-activated current was quite different when Ca^{2+}, a permeant blocker of Na^+ transport through the channel, was added to the cytoplasmic face of the channel. With 1.5 mM Ca^{2+} in the bath, decreasing the pH from 7.4 to 6.5 relieved the Ca^{2+}-induced block of the Na^+ current and eliminated the Ca^{2+}-induced shift in the reversal potential. These changes suggest that protons affect the monovalent/divalent selectivity of the channel. The direct effect of protons on channel selectivity raises the possibility that protons might modulate the Na^+/Ca^{2+} conductance ratio thereby regulating Ca^{2+} levels in the outer segment (OS).

MATERIALS AND METHODS

Materials were obtained as previously reported (Furman and Tanaka, 1990; Tanaka and Furman, 1993). Tetramethyl ammonium hydroxide was obtained from Eastman Kodak Co. (Rochester, NY); Corning 0010 electrode glass was from Garner Glass Co. (Claremont, CA).

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Preparation and solutions

Full details of the preparation have been presented (Furman and Tanaka, 1990). *Rana pipiens* retinas were maintained in cold Ringer's solution containing: 112 mM NaCl, 1.9 mM KCl, 1.1 mM $CaCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1.6 mM $MgCl_2$, and 5 mM glucose. Small aliquots of dissociated photoreceptors were layered onto the bottom of a chamber constructed from a glass coverslip. After the cells settled to the chamber floor, solution was continuously flowed into the chamber through a small S-shaped delivery tube. All experiments were done at room temperature, 22 ± 2°C, and in room light.

The electrode was filled with divalent-free solution, solution A, which contained: 120 mM NaCl, 2 mM EGTA, and 5 mM HEPES adjusted to pH 7.4 with tetramethyl ammonium hydroxide. The divalent-free solutions at pH 6.2 and 4.7 contained the same concentrations as solution A but the pH values were adjusted with HCl. The Ca^{2+} solution contained: 120 mM NaCl, 5 mM HEPES, and 1.5 mM $CaCl_2$. The pH was adjusted to 7.4 with tetramethyl ammonium hydroxide and to 6.2 or 4.7 with HCl. The concentration of cGMP in all test solutions was 200 μM.

Current recording

Details of the patch electrodes and electronics have been reported (Furman and Tanaka, 1990). The tip resistance of the electrodes was 10–20 Mohm. Electrodes were filled with solution A, containing no divalent cations. A patch was excised in the inside/out configuration once a tight seal of 1–10 Gohm was obtained. The electrode was then positioned in the inflow stream of the solution feed which provided sharp solution boundaries as the solutions were changed. This design minimized the equilibration time of each solution switch and was necessary to permit 12 solution changes with little change in the seal resistance. Deterioration of patch integrity was often seen in the low pH solutions, and some patches broke during exposure to these solutions.

A 700-ms linear voltage ramp from −100 to 100 mV was applied to an excised membrane patch (Tanaka et al., 1989). The current was low-pass filtered at 1 kHz. Currents were displayed on a digital oscilloscope and stored to hard disc at 5 kHz. Leak currents were measured in solutions of the same composition as the test currents except that no cGMP was added. The leak was digitally subtracted during analysis from the current recorded in the presence of cGMP.

Reversal potentials were determined as described previously (Furman and Tanaka, 1990). Ag/AgCl electrodes were maintained in 120 mM KCl/agar bridges to increase the stability of the recording electrodes. Similar agar bridges were used for both the ground electrode and a bath reference amplifier that compensated for the effects of liquid-junction potentials. When measured with respect to a 3 M KCl Ag/AgCl electrode, residual junction potentials were <1 mV. Electrode tip potentials, measured by breaking the pipette tip, were also <1 mV. With each change in pH or Ca^{2+}, currents

were recorded in the presence and absence of cGMP to subtract any changes in resistance due solely to changes in the ions.

RESULTS

Macroscopic currents from an inside/out excised patch activated by 200 μM cGMP are shown in Fig. 1 as the bath pH is lowered. Leak currents in the absence of cGMP have been subtracted from all traces. Fig. 1 *A* shows the effect of decreasing the pH from 7.4 to 4.7 in the absence of divalent cations on both sides of the membrane. The current-voltage relations (IVs) are displayed in the physiological sense where inward currents, carried by Na^+, move from the electrode to

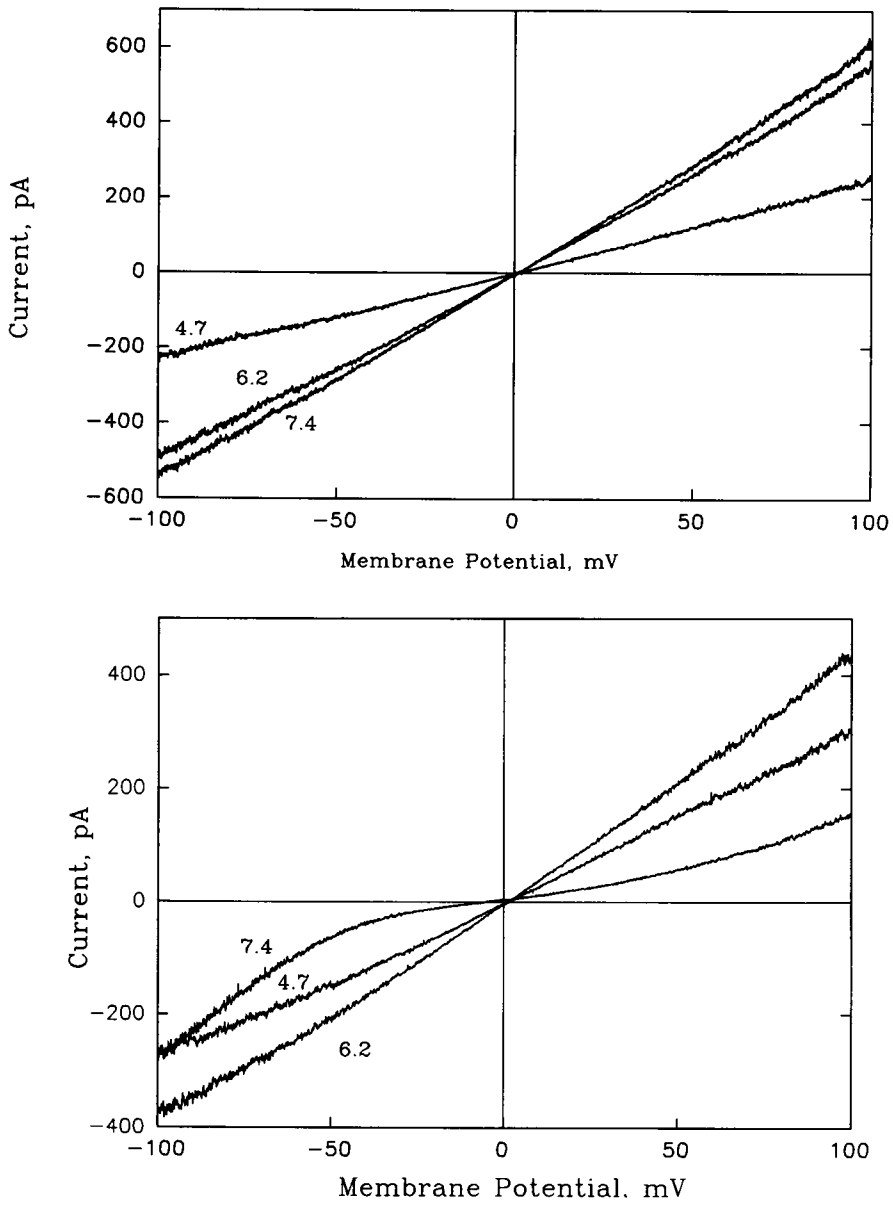

FIGURE 1 The effect of protons on cGMP-activated currents in the presence and absence of divalent cations on the cytoplasmic face of the channel. *A*, IVs in the absence of divalent cations. Net cGMP-activated currents were recorded from excised inside/out patches from *R. pipiens* photoreceptors with symmetrical divalent-free solutions. The membrane potential was ramped from −100 to 100 mV. The trace labeled 7.4 was the control IV measured under the standard conditions at pH 7.4. Smaller currents were recorded at pH 6.2 and 4.7. The nearly linear shape of the IV is characteristic of the nucleotide-activated channel in the absence of divalent cations. The effect of protons is to attenuate the current in both directions without appreciably altering the shape of the IV. At −90 mV, 90% and 42% of the current remains at pH values of 6.2 and 4.7, respectively, and at 90 mV, the fractions are 91% and 42%, respectively. *B*, IVs in the presence of 1.5 mM Ca^{2+} in the bath. Net cGMP-activated currents were measured in the same patch as *A*, but 1.5 mM Ca^{2+} was present in the bath. At pH 7.4, the IV shows the characteristic voltage-relieved Ca^{2+} block. Note that currents in both directions are attenuated, whereas Ca^{2+} is present only at the internal face of the channel. At pH 6.2, the current is increased at all potentials, and the characteristic block is no longer apparent. The current at pH 4.7 is further attenuated but there is still no evidence of Ca^{2+} block. The ratio of current in these traces compared with the traces in *A* are 47% (pH 7.4), 78% (pH 6.2), and 120% (pH 4.7) at −90 mV and 24%, 79%, and 120% at 90 mV. The zero current crossings or E_{Rev} measurements for the traces were: 0.77 mV (pH 7.4), 0.78 mV (pH 6.2), and 0.91 mV (pH 4.7) in the absence of divalents and −4.99 mV (pH 7.4), 1.09 mV (pH 6.2), and 1.3 mV (pH 4.7) in 1.5 mM Ca^{2+}. These values were computed from a linear regression of the averaged current values from −20 mV to 20 mV. (The non-zero E_{Rev} for the control trace reflects some unbalance in the amplifier gain. These traces were recorded at a lower gain than the initial zero adjust.)

the bath at negative potentials. A small decrease in the current is seen at pH 6.2 without any obvious change in the shape of the IV. At pH 4.7, the current is reduced to ~40% of the initial amount, and no apparent change in the shape of the IV is seen. The nearly linear IV in the absence of divalent cations suggests that the cGMP binding reaction and the subsequent channel opening are relatively independent of voltage (Tanaka et al., 1989).

Fig. 1 *B* shows the IV relations in the same patch when 1.5 mM Ca^{2+} is added to the bath. At pH 7.4, the current is attenuated from the divalent-free IV shown in Fig. 1 *A*. (Note the change in the ordinate scale from Fig. 1 *A* to Fig. 1 *B*.) The nonlinear IV in the presence of Ca^{2+} is characteristic of divalent block through the nucleotide-gated channel and suggests that Ca^{2+} blocks at low potentials between ± 40 mV and that, as the magnitude of the electrical driving force is increased, the block is partially relieved (Colamartino et al., 1991; Zimmerman and Baylor, 1992; and Tanaka and Furman, 1993). When the pH is reduced to 6.2 or less in the presence of 1.5 mM Ca^{2+}, the IVs no longer show the characteristic Ca^{2+} block and, in fact, the shape resembles the divalent-free IVs. At pH 4.7, the current in the presence of Ca^{2+} was larger than the divalent-free current at that pH. The effect of lowering the bath pH was rapidly reversible and did not result in any noticeable long-term changes in the magnitude of the cGMP-activated current or the seal resistance.

The zero current crossing, E_{Rev}, of the IVs in Fig. 1 provides information about the relative ion permeabilities. For certain simple ion channel profiles, the reversal potential shift in bionic substitution experiments provides an estimate of the relative permeability of the ions (see Hille, 1992). Bionic substitution has not been very useful for studying the Na^+/Ca^{2+} selectivity in the rod channel because the Ca^{2+} current is greatly attenuated, and it is difficult to measure the zero current crossing with precision (Colamartino et al., 1991; Tanaka and Furman, 1993). In the experiments reported here, 1.5 mM Ca^{2+} was added to the bath under conditions of constant, symmetrical Na^+ concentrations. Under these conditions, any shift in the E_{Rev} is an indication that the relative Ca^{2+} permeability is significantly different from that of Na^+ because the [Na^+] is 80-fold higher than [Ca^{2+}]. The E_{Rev} was measured for each of the IVs in Fig. 1 (see details in the Fig. 1 legend) and all traces, except the traces at pH 7.4 in the presence of Ca^{2+}, were within 0.5 mV of the divalent-free control trace. The shift in E_{Rev} for the trace in the presence of 1.5 mM Ca^{2+} at pH 7.4 was −5.77 mV. The negative direction of the E_{Rev} shift suggests that Ca^{2+} is more permeant than Na^+ in the cGMP-activated channel. The loss of the Ca^{2+}-induced shift in E_{Rev} at the lower pH values is another indication that protons relieve Ca^{2+} block of the channel.

In a similar experiment in which accurate zero current crossing could be determined, all traces were within 0.5 mV of the divalent-free control except for the trace with 1.5 mM Ca^{2+} at pH 7.4 (data not shown). The shift in E_{Rev} for this trace was −6.0 mV. In previous experiments examining the effect of divalent additions to the cytoplasmic side of retinal patches, the average shift in E_{Rev} was −2.55 mV (n = 2) with the addition of 1 mM Ca^{2+} and −10.03 mV (± 1.97, n = 7) at 10 mM Ca^{+2} (Tanaka and Furman, 1993).

In Fig. 2, the voltage dependence of the proton block is examined. This parameter has been useful in other channels to determine the relative position of the blocking site within the membrane field (see Hille, 1992). In Fig. 2 *A*, a semilog plot of proton block versus voltage is shown for the currents measured in the absence of divalent cations. Block is independent of voltage at all potentials, suggesting that the site of protonation does not experience the transmembrane field.

To examine the voltage dependence of protons in the presence of Ca^{2+}, the currents at pH 6.2 and 4.7 were normalized to the current at pH 7.4 and this ratio was plotted against the V_m as shown in Fig. 2 *B*. The steep increase in the current ratio from −40 mV to zero reflects relief of voltage-dependent Ca^{2+} block of the inward current at lower pH values. The outward current is much less voltage-dependent, consistent with Ca^{2+} permeation as discussed elsewhere (Tanaka and Furman, 1993).

The fraction of current blocked by Ca^{2+} was then measured as a function of proton concentration. Currents in the presence of Ca^{2+} were divided by the control trace in divalent-free solutions at each pH, and the results are shown in Fig. 3. The characteristic voltage-dependent block by Ca^{2+} is readily seen at pH 7.4. At pH 6.2, the currents with Ca^{2+} are about 80% of the control current and the characteristic voltage dependence of Ca^{2+} block is no longer present. At pH 4.7, the current with Ca^{2+} is larger than the divalent-free current suggesting that protons may block less effectively in the presence of divalent ions.

Because Ca^{2+} is a permeant ion in the cGMP-activated channel, the Woodhull (1973) analysis of block is not applicable; nevertheless, the voltage dependence provides a useful qualitative measure of the effect of the transmembrane field on the Ca^{2+}-binding site. The voltage dependence shown in Fig. 3 was fitted from −90 to −20 mV, and the slope of the inward current ratio was determined for the voltage dependence of Ca^{2+} block at pH 7.4. The slope was 77 mV/decade (plot not shown), consistent with earlier measures of Ca^{2+} voltage-dependent block of 50 mV/decade at 1 mM Ca^{2+} and 38 mV/decade at 10 mM Ca^{2+} (Tanaka and Furman, 1993). The addition of protons completely eliminated this voltage dependence of divalent block as shown in Fig. 3.

Block of the cGMP-activated currents as a function of pH and Ca^{2+} was examined in multiple patches and the averaged results are shown in Fig. 4. In Fig. 4 *A*, the fraction of divalent-free current blocked is shown as a function of pH. The mean current reduction was about 20% at pH 6.2 and 58% at pH 4.7 at 60 mV. In Fig. 4 *B*, the fraction of divalent-free current blocked by 1.5 mM Ca^{2+} is plotted at each pH. These data show that increasing the proton concentration relieves current block by Ca^{2+} from 65% at pH 7.4 to 30% at pH 4.7.

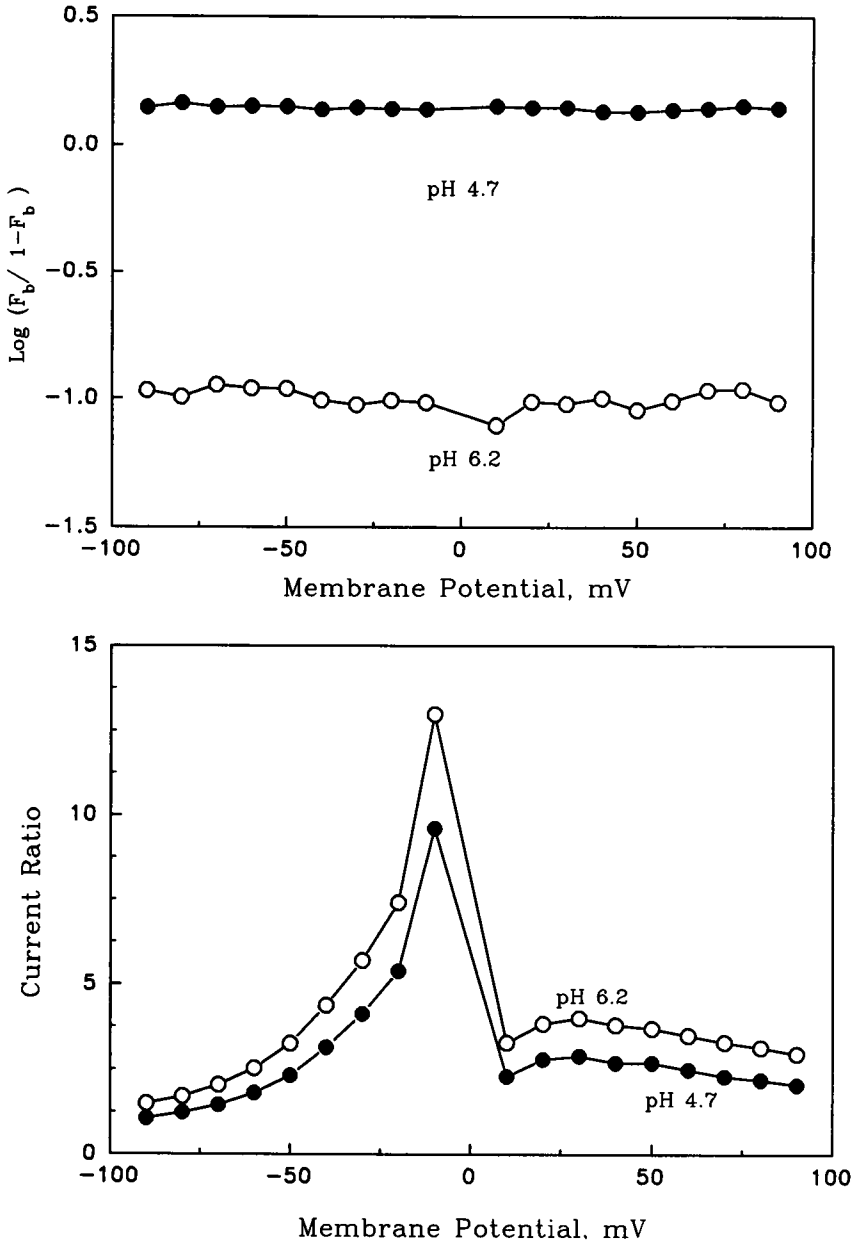

FIGURE 2 Voltage dependence of the proton-induced current changes. *A*, Voltage dependence of the proton block in the absence of divalent cations. Currents were averaged in 2-mV regions every 10 mV and the fraction of block (F_b) calculated at pH 6.2 and 4.7. A plot of the Log (F_b/1−F_b) vs V_m shows no voltage dependence in the absence of divalent cations. *B*, The effect of protons in the presence of Ca^{2+}. Currents in the presence of Ca^{2+} at pH 6.2 and 4.7 were divided by the current at pH 7.4. The curvature seen for the inward currents arises from the relaxation of the control IV to the nearly linear, divalent-free shape as pH is lowered. At all voltages, the ratio is smaller for pH 4.7 than 6.2, suggesting that protons do block the current as in the absence of divalent cations.

DISCUSSION

How do protons block macroscopic cGMP-activated currents?

Several features of proton block in divalent-free solutions offer clues about how protons interact with the cGMP-activated channel. First, ion flow is reduced in both directions as the $[H^+]$ is raised and the proton block is independent of the membrane potential. In addition, no change in the E_{Rev} is seen as the $[H^+]$ is increased over a wide range. Interpretation of voltage-independent block depends on whether protons are permeant ions. For an impermeant blocking ion, one would expect to see an effect of the membrane potential on block if the binding site were located within the membrane field. From the absence of a shift in the E_{Rev} at pH 4.7, we can place a limit on the relative proton to Na^+ permeability. Assuming a maximal shift in the E_{Rev} of 1 mV as the pH is decreased from 7.4 to 4.7, the upper limit for the proton to Na^+ permeability ratio can be estimated using the Goldman-Hodgkin-Katz equation (Hille, 1992). Because a 1-mV shift in the reversal potential is well within the experimental limitations of the recording system, the upper limit of the permeability ratio for protons compared with Na^+ is 7.7 in the cGMP-activated channel. Although this limit might seem high compared with the ratio of 3.34 for NH_4^+, the most permeant ion previously measured for this channel (Furman and Tanaka, 1990), voltage-dependent sodium channels have a relative proton to Na^+ permeability ratio of ~250:1 (Mozhayeva and Naumov, 1983), suggesting significant differences between the architecture of the pore regions of these two channels.

If protons are permeable, we still might expect some voltage dependence to the inward current if the binding site is located within the membrane field. The voltage dependence

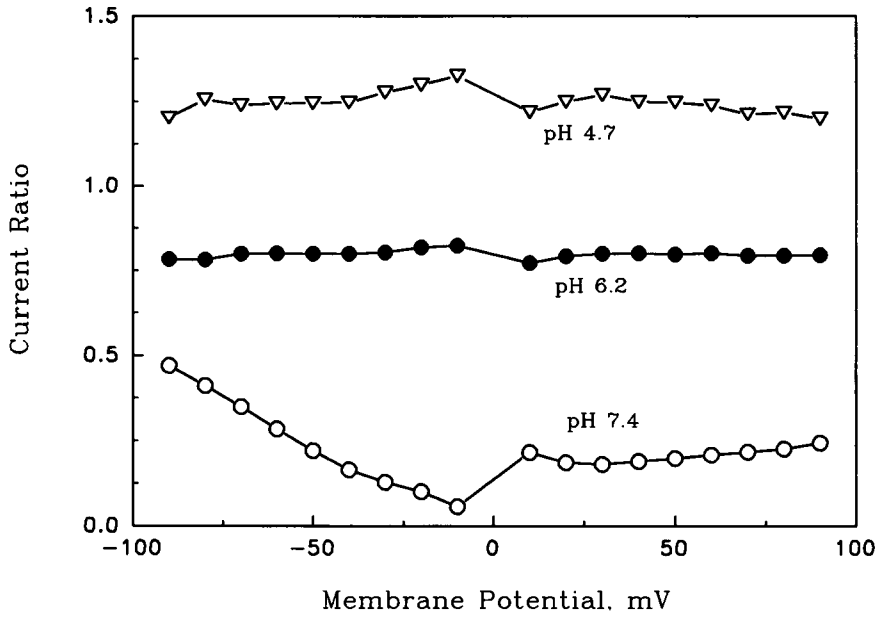

FIGURE 3 Ca^{2+} block as a function of membrane potential. Currents recorded at 1.5 mM Ca^{2+} were divided by the current at the corresponding pH in the absence of divalent cations to give the current ratio as a function of voltage. The only voltage dependence is seen in the inward current ratio at pH 7.4.

would arise from the >1000-fold higher proton concentration in the bath at pH 4.7. When the driving force moves Na^+ inward, protons entering the channel from the bath would increasingly be expelled as the driving force increased. Failure to see any effect of membrane potential on the proton block under these conditions argues that the binding sites are at the surface of the channel pore or acting at a remote site which is outside the transmembrane field. Other studies on nucleotide-activated channels are consistent with the idea that a protonation site is located at the cytoplasmic surface. Menini and Nunn (1990) showed a reduction in cGMP-activated currents in excised salamander patches with an apparent pK_a of 5.4. In cAMP-activated currents recorded from olfactory patches, Frings et al. (1991) showed a reduction in cAMP-activated currents with apparent pK_a values of 5.2 in frog and 5.1 in rat. In both of these studies, proton block was rapid, reversible, and voltage-independent, suggesting a protonation site at the cytosolic surface of the channel.

One way protons might produce block at the cytoplasmic surface of the channel is by screening the negative surface charges on the local protein and lipid environment as was recently shown for the voltage-dependent sodium channel by Zhang and Siegelbaum (1991). In addition to changes in surface potential, protons might act directly on side chains involved in cGMP binding to increase the $K_{0.5}$ for cGMP activation, thereby decreasing the number of channels opened at 200 μM cGMP. A recent abstract by Sanfilippo and Menini (1993) suggests this is not the explanation for the pH-induced reduction in current in the presence of cGMP. They measured the $K_{0.5}$ for cGMP, as the pH at the cytoplasmic face of inside/out patches in photoreceptors was decreased and showed that the cGMP-activated currents were attenuated at elevated proton levels but the cGMP dose-response relation was not altered. They also examined the effects of a partial agonist, cAMP, on activation of the retinal channel and found that as the bath pH was lowered, the maximal current activated by cAMP was increased and the $K_{0.5}$ was decreased. These results suggest that protons may affect nucleotide activation of the channel by partial agonists in addition to producing changes in the current at constant nucleotide levels.

How do protons interact with Ca^{2+} binding in the channel?

The effect of protons on cGMP-activated currents in the presence of Ca^{2+}, provides additional clues about where protons may act. In the presence of Ca^{2+}, protons affect the relative Na^+/Ca^{2+} permeability as shown by the linearization of the IV relation at pH 6.2 and 4.7 and the Ca^{2+}-induced shift in the E_{Rev} which is eliminated at the lower pH values. Relief of Ca^{2+} block seems complete by pH 6.2, whereas proton block continues to increase from 6.2 to 4.7. This apparent differential effect of protons hints at different sites with different pK_a values. Careful titration of pH to examine the shape change should provide insight about which amino acid residue(s) are involved in altering the IV shape as Ca^{2+} block is relieved. The amino acid side chains usually considered as possible protonation sites have pK_a values of 6.15 for histidine, 3.9 for aspartic acid, and 4.3 for glutamic acid. Although protonation of particular amino acids has been targeted in a few channels, for example, the quisqualate/kainate channel in catfish cone horizontal cells (Christensen and Hida, 1990), the protein environment of an amino acid residue may significantly alter the apparent pK_a of a side chain making such assignments difficult.

Finally, protons might be acting allosterically. Precedent for this mechanism was shown by the work of Hess and collaborators on L-type Ca^{2+} channels (Prod'hom et al., 1987; Pietrobon et al., 1988). Their work suggests that protons act allosterically to induce a conformational change in an ion-binding site within the pore. In another Ca^{2+} channel study using dorsal root ganglion cells (Konnreth et al., 1987), protons altered a Ca^{2+}-selective current by changing the

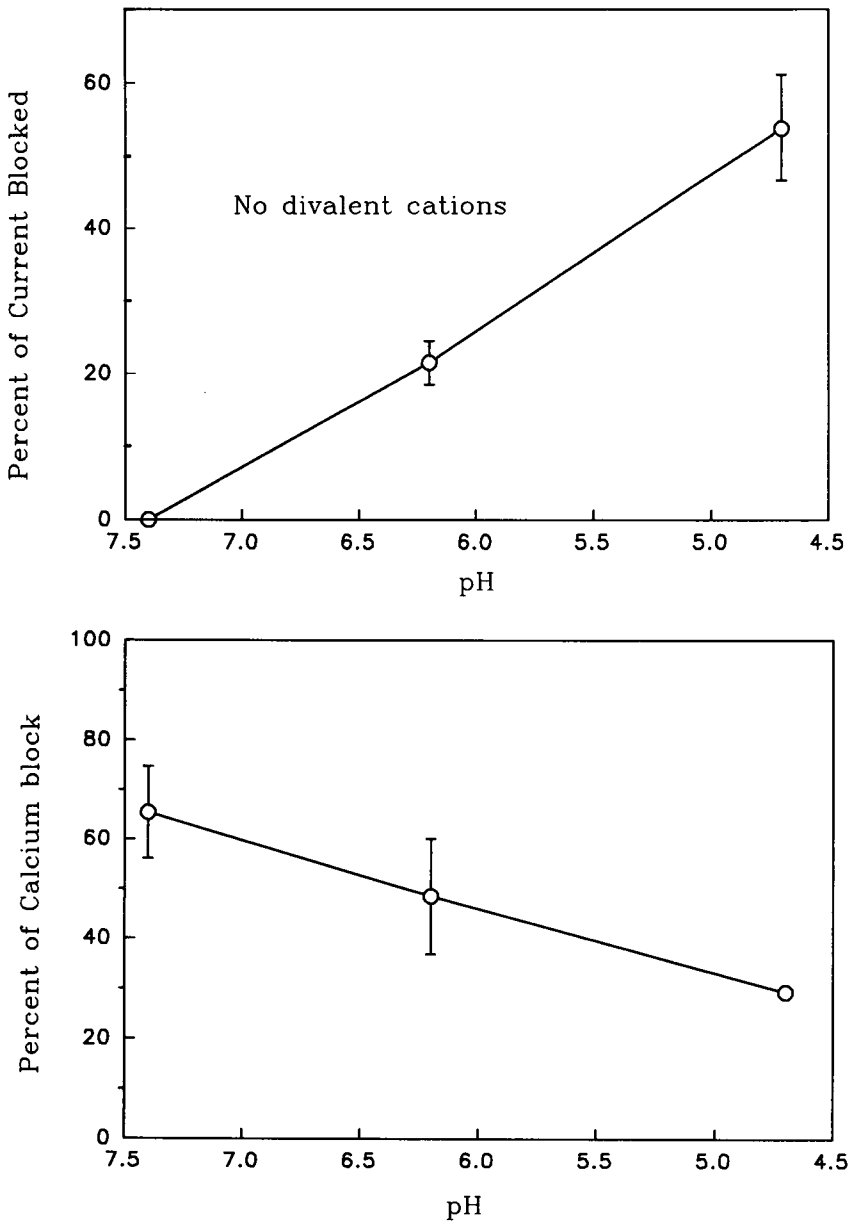

FIGURE 4 Averaged current changes as a function of pH. *A*, The averaged percent of current blocked by protons in the absence of divalent cations. Currents were normalized to the control at pH 7.4 at 60 mV for six cells. The mean fractional currents and standard errors are plotted as pH is varied. The pH values of the solutions varied by 0.15 pH units within the experiments reported here. The plot shows that the current changes over 3 log units in proton concentration. *B*, The fraction of Ca^{2+} block decreases as the proton concentration increases. The fraction of Ca^{2+} block was calculated at 60 mV by normalizing the current at 1.5 mM Ca^{2+} against the control current in the absence of divalents. Plotted are mean values and standard errors for all but pH 4.5 ($n = 2$). Patch instability increased substantially with decreasing pH, and many patches did not withstand the low pH condition in either the divalent-free or the Ca^{2+}-containing solutions.

monovalent/divalent selectivity of the channel. This Ca^{2+} channel is thought to exist in two mutually exclusive states which are regulated by a displacement of Ca^{2+} by protons at the channel binding sites which, in turn, alters the selectivity of the channel.

Single cGMP-activated channel measurements may provide us with the ability to distinguish between the effects of protons on channel gating and conductance. Goulding et al. (1992) have recorded single-channel currents from nucleotide-gated channels in catfish olfactory epithelia. The conductance of the fully open state was 55 pS, with a subconducting state of 32 pS; the subconducting state was both voltage- and pH-dependent.

Recent experiments with both retinal and olfactory nucleotide-gated channels offer evidence for the importance of cytosolic modulation of nucleotide-gated channels especially in feedback mechanisms. The cytosolic components identified as channel modulators to date include $[Ca^{2+}]_i$ (Kramer and Siegelbaum, 1992), a calcium calmodulin complex (Hsu and Molday, 1993), and phosphorylation (Gordon et al., 1992). Although protons are no longer thought to play a direct role in phototransduction, the hydrolysis of 3′,5′-cGMP by phosphodiesterase generates 5′-GMP and protons. The relief of Ca^{2+}-induced block of the cGMP-activated channel by protons suggests an alteration in the Na^+/Ca^{2+} selectivity of the channel which might have important consequences for the gain of phototransduction in the OS by affecting intracellular Ca^{2+} levels.

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