Mechanisms of modulation by internal protons of cyclic nucleotide-gated channels cloned from sensory receptor cells

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SUMMARY

We have examined the modulation by internal protons of cyclic nucleotide-gated (CNG) channels cloned from bovine olfactory receptor cells and retinal rods. CNG channels were studied in excised inside-out membrane patches from *Xenopus laevis* oocytes previously injected with the mRNA encoding for the subunit 1 of olfactory or rod channels. Channels were activated by cGMP or cAMP, and currents as a function of cyclic nucleotide concentrations were measured as pH_i varied between 7.6 and 5.0. Increasing internal proton concentrations caused a partial blockage of the single-channel current, consistent with protonation of a single acidic site with a $\text{p}K_1$ of 4.5–4.7, both in rod and in olfactory CNG channels. Channel gating properties were also affected by internal protons. The open probability at low cyclic nucleotide concentrations was greatly increased by lowering pH_i , and the increase was larger when channels were activated by cAMP than by cGMP. Therefore, internal protons affected both channel permeation and gating properties, causing a reduction in single-channel current and an increase in open probability. These effects are likely to be caused by different titratable groups on the channel.

1. INTRODUCTION

Ionic channels directly gated by the binding of cyclic nucleotides (CNG channels) are responsible for the first step in the generation of the electrical response to external stimuli in both vision and olfaction. Photoreceptors and olfactory receptor cells respond, respectively, to photons or to odorant molecules through the activation of a chain of enzymatic events that produce a change in the intracellular concentration of cyclic nucleotides and the subsequent opening or closing of CNG channels (reviewed by Menini 1995; Torre *et al.* 1995). Channels directly activated by cyclic nucleotides are also present in many other sensory and non-sensory cells, but in many cases their physiological role is not known yet. The bovine retinal rod channel was first cloned (Kaupp *et al.* 1989) and since then many other CNG channels, including the bovine olfactory channel (Ludwig *et al.* 1990), have been cloned, sequenced and functionally expressed. Moreover, it has been shown that native CNG channels are heteromultimers composed of at least two types of subunits, called subunit 1 and subunit 2 (or α and β) (reviewed by Kaupp 1995; Zimmerman 1995; Zagotta & Siegelbaum 1996).

Experiments on native CNG channels have shown

that an increase in internal proton concentration caused a reduction of the maximal current activated by cGMP in retinal rods (Menini & Nunn 1990; Tanaka 1993; Picco *et al.* 1996) or by cAMP in olfactory receptor cells (Frings *et al.* 1992). Maximal currents decreased as a function of pH_i following a curve for titration of a single acidic site with a $\text{p}K_1$ of 5.0–5.2 in both rod and olfactory CNG channels. Moreover, activation of both native (Picco *et al.* 1996) and cloned (Gavazzo *et al.* 1994; Gordon *et al.* 1996) rod channels by cAMP was greatly enhanced by lowering internal pH_i . Site-directed mutagenesis studies have identified at least two amino acids involved in the proton-induced potentiation of activation of rod CNG channels (Gordon *et al.* 1996).

The effect of extracellular pH in cloned CNG channels from catfish olfactory receptor cells has been investigated by Root & MacKinnon (1994). With site-directed mutagenesis studies, they showed that external protons could alter the ion permeation properties by binding to titratable sites formed by glutamate residues with an anomalous $\text{p}K$ of 7.6 located near the extracellular side of the channel.

This study describes for the first time the modulation by internal protons of the properties of cloned olfactory CNG channels, both on macroscopic currents and at the single-channel level. Moreover, the

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mechanisms of action of internal protons on olfactory CNG channels were compared with those on rod CNG channels. Preliminary results have been presented in abstract form (Gavazzo *et al.* 1994, 1996b).

2. METHODS

(a) *In vitro* transcription and functional expression

The cDNAs encoding for subunit 1 of the bovine rod and olfactory CNG channels were kindly provided by Dr E. Eismann and Professor U. B. Kaupp (Jülich, Germany). RNA was synthesized (Melton *et al.* 1984) for the olfactory channel from XhoI-linearized pCHOLF102b (Altenhofen *et al.* 1991) using T3 RNA polymerase (Stratagene) and, for the rod channel, from NheI-linearized pGRodawt (Sesti *et al.* 1995) using T7 RNA polymerase. 50 ng of mRNA at a concentration of $0.5 \mu\text{g } \mu\text{l}^{-1}$ was then injected in *Xenopus laevis* oocytes with a Drummond 'Nanoject' automatic injector (Drummond Scientific Company, Broomall, PA, USA). Oocytes were obtained from large females of *Xenopus laevis* (Centre National de la Recherche Scientifique, Montpellier, France). Animals were anaesthetized with 3-amino benzoic acid ethyl ester methanesulfonate salt (tricain) 1 g l^{-1} (Sigma A5040) for about 30 min. The ovarian lobes were surgically removed and the oocytes separated and treated for 1 h in a Ca-free solution with Collagenase type IA (1 mg ml^{-1}) (Sigma C9891) to remove the follicle layers. Oocytes were incubated at 19°C in Barth's solution (88 mM of NaCl, 1 mM of KCl, 0.82 mM of MgSO_4 , 0.33 mM of $\text{Ca}(\text{NO}_3)_2$, 0.41 mM of CaCl_2 , 2.4 mM of NaHCO_3 , 5 mM of Tris-HCl, pH 7.4) containing $50 \mu\text{g ml}^{-1}$ of Gentamycin (Sigma G3632). Channel expression was usually obtained 1–3 days after mRNA injection. Before electrical recordings, the vitelline membrane was removed after incubation of the oocyte for a few minutes in a hyper osmotic medium (200 mM K-gluconate, 20 mM of KCl, 1 mM of MgCl_2 , 10 mM of EGTA, 10 mM of Hepes, pH 7.4).

(b) Current recordings and solutions

Currents were recorded under voltage-clamp conditions from patches excised in the inside-out configuration from the plasma membrane of oocytes. Channels were activated by perfusing the cytoplasmic side of the oocyte membrane patch with various concentrations of cGMP or cAMP, using the perfusion system described by Menini & Nunn (1990). For macroscopic current recordings, voltage steps of 150 ms duration were given from a holding potential of 0 mV. CNG currents were obtained from the difference of recordings in the presence and in the absence of cyclic nucleotides.

The patch-clamp pipette was filled with 110 mM of NaCl, no added divalent salts, 1 mM of EDTA and 10 mM of HEPES, buffered to pH 7.6 with tetramethylammonium hydroxide (TMAOH). The ionic composition of the bath solutions was similar except that HEPES was substituted by MES for pH < 6.8. pH_i effect was tested only in the range 7.6–5.0, since lower pH_i values irreversibly damaged the patch.

Currents were measured with the Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), filtered at 1 kHz and sampled at 2 or 2.5 kHz using a TL-1 DMA interface board (Axon Instruments) with a PC-type computer and pCLAMP software (Axon Instru-

ments).

All experiments were performed at room temperature (20 – 24°C).

(c) Single-channel recordings

Single-channel recordings were obtained 1–3 days after mRNA injection in oocytes. A patch was assumed to contain one CNG channel when only one channel was opened in the presence of saturating cGMP concentrations, i.e. at 1 mM of cGMP. All-points amplitude histograms were constructed from 30 s of data recordings. The single-channel current and the open probability were determined by analysis of amplitude histograms normalized to a total integral area of 1. Histograms were fitted by the sum of two or more Gaussian functions.

In cases when two or three channels were present in the patch, the probability that an individual channel was open, p_o , was estimated by taking into account that in the presence of M independent and identical channels, the probability that r channels are open simultaneously is given by the binomial distribution

$$P(r) = \frac{M!}{r!(M-r)!} p_o^r (1-p_o)^{M-r}.$$

(d) Data analysis

Data calculated from N different patches were shown as mean $\pm$ s.d.

In patches containing many channels, the macroscopic current, I , can be described as $I = N_c p_o i$, where N_c is the number of channels in the patch, p_o is the open probability and i is the single-channel current. Macroscopic currents as a function of cyclic nucleotide concentration were fitted by the Hill equation

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

where I is the activated current, $I_{\max}$ is the maximal current, c is the cyclic nucleotide concentration, $K_{1/2}$ is the cyclic nucleotide concentration activating half of the maximal current and n is the Hill coefficient. If channels have only one conductance state at a given pH_i , and the single-channel current does not depend on cyclic nucleotide concentration, the following equation can be derived from equation (1) to describe the open probability, p_o , as a function of cyclic nucleotide concentration:

$$p_o = p_{o,\max} \frac{c^n}{c^n + K_{1/2}^n}, \quad (2)$$

where $p_{o,\max}$ is the maximal open probability, $K_{1/2}$ is the cyclic nucleotide concentration giving the half-maximal open probability and n is the Hill coefficient (see also Picco *et al.* 1996).

Curve fitting was generally done using the Kaleidagraph software package (Synergy Software, Reading, PA, USA). Fitting of amplitude histograms with Gaussian functions was done with PCLAMP software (Axon Instruments). With both software packages, the Levenberg–Marquardt least squares algorithm was used.

3. RESULTS

(a) Modulation of dose-response relations by internal pH_i

The modulation by internal pH_i of the bovine olfactory and rod CNG channels expressed from subunit 1 in *Xenopus oocytes* was studied in excised

Figure 1. Effect of cytosolic acidification on macroscopic CNG currents. Traces of currents activated by cGMP or cAMP were reported for olfactory (a) or rod (c) channels. Currents were activated in excised inside-out patches by various concentrations of cyclic nucleotides as the pH_i was reduced from 7.6 to 5.7, applying voltage steps at +60 and -60 mV from a holding potential of 0 mV. Dose-response relations normalized to the maximal current activated by cGMP at pH_i 7.6 were plotted for olfactory (b) and rod (d) channels. Currents activated at +60 mV by cGMP at pH_i 7.6 (filled triangles) and 5.7 (open triangles) or by cAMP at pH_i 7.6 (filled squares) or 5.7 (open squares) were normalized to the maximal current in 100 μM of cGMP for the olfactory channels or in 1 mM of cGMP at pH_i 7.6 for the rod channels and plotted versus cyclic nucleotide concentrations. For rod channels, the dose responses for cGMP and for cAMP were obtained from two different patches. Continuous lines were the best fit of the Hill equation (equation (1)) to the data with the following values. (b) Olfactory channels: for cGMP at pH_i 7.6, $K_{1/2} = 2 \mu\text{M}$, $n = 2.9$, $I_{\text{max}} = 245 \text{ pA}$; for cGMP at pH_i 5.7, $K_{1/2} = 1.5 \mu\text{M}$, $n = 2.87$, $I_{\text{max}} = 215 \text{ pA}$; for cAMP at pH_i 7.6, $K_{1/2} = 76 \mu\text{M}$, $n = 2.4$, $I_{\text{max}} = 252 \text{ pA}$; for cAMP at pH_i 5.7, $K_{1/2} = 20 \mu\text{M}$, $n = 2.6$, $I_{\text{max}} = 218 \text{ pA}$. (d) Rod channels: for cGMP at pH_i 7.6, $K_{1/2} = 60 \mu\text{M}$, $n = 2.3$, $I_{\text{max}} = 1200 \text{ pA}$; for cGMP at pH_i 5.7, $K_{1/2} = 25 \mu\text{M}$, $n = 2$, $I_{\text{max}} = 1072 \text{ pA}$; for cAMP at pH_i 7.6, $K_{1/2} = 2389 \mu\text{M}$, $n = 1.5$, $I_{\text{max}} = 153 \text{ pA}$; for cAMP at pH_i 5.7, $K_{1/2} = 689 \mu\text{M}$, $n = 1.5$, $I_{\text{max}} = 4912 \text{ pA}$.

inside-out membrane patches. Figure 1 shows macroscopic currents activated by various concentrations of cAMP or cGMP for olfactory (a) and rod (c) channels. Lowering pH_i at the cytoplasmic side of the patch affected both inward and outward currents.

In olfactory channels, cytosolic protonation caused a reduction of maximal currents activated by cAMP or cGMP and an enhancement of currents activated by low cyclic nucleotide concentrations, as shown in figure 1a (see also figure 2f for maximal current reduction). Maximal currents activated by cGMP or by cAMP had very similar values, in agreement with previous results (Altenhofen *et al.* 1991; Gavazzo *et al.* 1996a). At pH_i 5.7, the maximal current activated by 1 mM of cAMP (or 100 μM of cGMP) was $87 \pm 2\%$ ($N = 3$) of the value at pH_i 7.6 and was further decreased by increasing proton concentration, as shown in figure 2f. However, figure 1a also shows that the current activated by 2 μM of cGMP or by 50 μM of cAMP was higher at pH_i 5.7 than at pH_i 7.6, and

therefore protons potentiated channel activation at low cyclic nucleotide concentrations.

Currents were measured at the steady-state and plotted as a function of cAMP or cGMP concentration at various pH_i values. Dose-response data were fitted by the Hill equation (equation 1)). Dose-response curves from the same patch shown in figure 1a were normalized to the current value activated by 100 μM of cGMP at pH_i 7.6 at +60 mV and plotted in figure 1b. Both $K_{1/2}$ for cAMP and for cGMP were reduced by protonation, whereas the Hill coefficient was not affected. $K_{1/2}$ for cAMP was reduced by about threefold from an average value of $60 \pm 12 \mu\text{M}$ ($N = 4$) at pH_i 7.6 to $19 \pm 3 \mu\text{M}$ ($N = 3$) at pH_i 5.7. $K_{1/2}$ for cGMP was reduced by almost twofold from an average value of $2.4 \pm 1.3 \mu\text{M}$ ($N = 9$) at pH_i 7.6 to $1.3 \pm 0.2 \mu\text{M}$ ($N = 4$) at pH_i 5.7. The Hill coefficient was not affected by pH_i and was 2.6 ± 0.3 ($N = 13$) for cGMP and 2.7 ± 0.3 ($N = 7$) for cAMP.

In rod channels (figure 1c, d), the maximal current

Figure 2. The single-channel current of the olfactory channel as a function of pH_i . Current recordings from a membrane patch containing one channel activated at +80 mV (a) and -80 mV (b) by saturating concentrations of cAMP, 500 μM , or cGMP, 20 μM , at pH_i 7.6 or 5. Current recordings were filtered at 1 kHz and sampled at 2.5 kHz. (c)–(d) Amplitude histograms from the recordings shown in (a) at 500 μM of cAMP and +80 mV at pH_i 7.6 (c) and 5 (d). The continuous lines were obtained from the best fit of the sum of two Gaussians to the amplitude histograms. The single-channel current at +80 mV and the open probability were, respectively, 3.05 pA and 0.985 at pH_i 7.6 and 1.94 pA and 0.995 at pH_i 5. (e) Mean values ($\pm$ s.d.) from three to nine separate patches of normalized single-channel currents activated by cAMP plotted as a function of pH_i . Currents were measured at +80 (filled triangles) or -80 mV (open squares) at various pH_i and normalized to the value at pH_i 7.6 at the corresponding voltage. Continuous lines were the best fit of equation (3) to the data with $\text{p}K_1 = 4.7$ at +80 mV and $\text{p}K_1 = 4.5$ at -80 mV. (f) Mean values ($\pm$ s.d.) from 3 to 13 separate patches of normalized maximal currents activated by cAMP at +60 (filled triangles) or -60 mV (open squares) were plotted versus pH_i . Currents were normalized to the current value in 1 mM of cAMP at pH_i 7.6 in the same patch at the corresponding voltage. Continuous lines were the best fit of equation (3) to the data with $\text{p}K_1 = 4.6$ at +60 mV and $\text{p}K_1 = 4.3$ at -60 mV.

activated by cAMP at pH_i 7.6 was only a small fraction, $2.7 \pm 1\%$ ($N = 2$) of that activated by cGMP,

in agreement with previous results (Altenhofen *et al.* 1991; Gavazzo *et al.* 1996a), and currents activated by cAMP showed a dramatic increase induced by protonation at every concentration, as previously observed (Picco *et al.* 1996; Gordon *et al.* 1996). Potentiation by protons was also observed at a low concentration of cGMP (20 μM in figure 1c), while the maximal current in cGMP was slightly reduced at pH_i 5.7. At pH_i 5.7, I_{max} for cGMP was reduced to $93 \pm 2\%$ ($N = 5$) of the value at pH_i 7.6, while I_{max} for cAMP increased to $77 \pm 5\%$ ($N = 4$) of the corresponding I_{max} for cGMP at pH_i 5.7. Increasing proton concentration produced a further decrease of I_{max} for cGMP and an increase of I_{max} for cAMP, as shown in figure 4d. Figure 1d shows dose-response curves from the patches shown in figure 1c, normalized to the current measured in 1 mM of cGMP at pH_i 7.6. $K_{1/2}$ for cAMP decreased 2.5-fold from $2095 \pm 416 \mu\text{M}$ ($N = 2$) at pH_i 7.6 to $854 \pm 250 \mu\text{M}$ ($N = 6$) at pH_i 5.7. $K_{1/2}$ for cGMP decreased 1.66-fold from $78 \pm 16 \mu\text{M}$ ($N = 15$) at pH_i 7.6 to $47 \pm 19 \mu\text{M}$ ($N = 12$) at pH_i 5.7.

(b) Olfactory single channels

Single-channel properties were therefore measured to distinguish effects of internal protons on channel gating and permeation. Figure 2a, b shows single-channel activity from a patch containing only one olfactory channel. Saturating concentrations of cyclic nucleotides, as determined from dose-response relations measured from macroscopic currents (see figure 1b), were used to activate the channel. The effect of reducing pH_i from 7.6 to 5 was investigated both at positive and negative potentials. Recordings at +80 and -80 mV are illustrated in figures 2a and b, respectively. Amplitude histograms calculated from continuous recordings in cAMP at +80 mV at pH_i 7.6 or 5 are shown in figures 2c and d, respectively. The single-channel current at +80 mV was reduced by cytosolic acidification from 3 ± 0.12 pA ($N = 11$) at pH_i 7.6 to 1.94 ± 0.29 pA ($N = 6$) at pH_i 5.0. The maximal open probability was 0.98 ± 0.01 ($N = 2$) at pH_i 7.6 and 0.99 ± 0.007 ($N = 2$) at pH_i 5.0, and therefore was not appreciably modified by lowering pH_i . At -80 mV, the single-channel current was 3.18 ± 0.16 pA ($N = 16$) at pH_i 7.6 and decreased to 2.48 ± 0.12 pA ($N = 9$) at pH_i 5.0. Similar results were obtained from channels activated by cGMP.

The single-channel current from several patches at various pH_i was normalized to the value at pH_i 7.6 and the average was plotted as a function of pH_i in figure 2e at +80 (filled symbols) and -80 mV (open symbols). Data were well fitted by the following equation:

$$\frac{I(\text{pH}_i)}{I(7.6)} = \frac{[K_1]}{[K_1] + [\text{H}^+]}, \quad (3)$$

where $I(\text{pH}_i)$ is the current measured at pH_i , $[\text{H}^+]$ is the proton concentration, and $[K_1]$ is the proton concentration necessary to half-inhibit the normalized current. Equation (3) is equivalent to the relation for titration of a single acidic site. The contin-

uous lines in figure 2e are the best fit to the data obtained with a pK_1 of 4.7 at +80 mV and 4.5 at -80 mV. These values (and those in figures 2f and 4c-d) are an estimate of the pK_1 since the lowest pH_i value we could test without irreversibly damaging the patch was 5.0. These results demonstrate that the single-channel current was partially blocked by internal protons, and the blockage was slightly higher at positive than at negative potentials.

Figure 2f shows maximal cAMP-activated macroscopic currents, i.e. measured in patches containing many olfactory channels, normalized to the value at pH_i 7.6 and plotted as a function of pH_i . Similar values were measured when channels were activated by saturating cGMP concentrations. The best fit of equation (3) to the data gave a pK_1 value of 4.6 at +60 and 4.3 at -60 mV. These results demonstrate that the dependence on pH_i of maximal macroscopic currents is due to a reduction of the single-channel current (figure 2e). However, recordings from macroscopic currents in figure 1a showed that the effect of pH_i was different when cyclic nucleotide concentrations were lowered: increasing proton concentration potentiated activation of the channels by cyclic nucleotides. The effect of pH_i on single-channel properties was therefore further investigated at lower cyclic nucleotide concentrations.

Figure 3a shows recordings and amplitude histograms from a patch containing two olfactory channels activated at -80 mV by 30 μ M of cAMP at pH_i 7.6, 5.4 or 5. The single-channel current was reduced as previously measured (figure 2e), while the probability of being in the open state was dramatically increased by progressively lowering the pH_i from 7.6 to 5. At pH_i 7.6, the highest peak in the amplitude histogram corresponded to the closed state, while at pH_i 5.0, it corresponded to the open state. The open probability at 30 μ M of cAMP increased from 0.068 ± 0.045 ($N = 3$) to 0.64 ± 0.24 ($N = 3$) to 0.94 ± 0.05 ($N = 2$) at pH_i 7.6, 5.4 and 5, respectively. Therefore, the open probability was greatly increased at sub-saturating concentrations when pH_i was reduced and channels seemed to spend most of their time in the open state at pH_i 5. When channels were activated by cGMP, the open probability also increased by lowering pH_i , but the effect was quantitatively smaller compared to that measured with cAMP. Figure 3b shows the mean open probability from several patches at pH_i 7.6 and 5.4 at -80 mV as a function of cyclic nucleotide concentration for both cAMP and cGMP. Continuous lines in figure 3b are the best fits of equation (2) to the data. $K_{1/2}$ for cAMP was reduced 2.9-fold from 70 μ M at pH_i 7.6 to 24 μ M at pH_i 5.4, while $K_{1/2}$ for cGMP was reduced 1.2-fold from 4 μ M at pH_i 7.6 to 3.4 μ M at pH_i 5.4.

Figure 3c shows $K_{1/2}$ for cAMP as a function of pH_i at +60 and -60 mV. The effect of pH_i on $K_{1/2}$ was not found to be significantly voltage dependent. Data were well fitted by the following equation:

$$K_{1/2} = K_{\min} + K_{\Delta} \frac{[K_2]^n}{[H^+] + [K_2]^n}, \quad (4)$$

Figure 3. The open probability of the olfactory channel as a function of pH_i . (a) Recordings from one patch containing two olfactory channels activated by 30 μ M of cAMP at -80 mV at pH_i 7.6, 5.4 or 5. Current recordings were filtered at 1 kHz and sampled at 2.5 kHz. Amplitude histograms were fitted as the sum of three Gaussians. The single-channel current at -80 mV decreased from 3.16 to 2.75 to 2.48 pA, but the open probability increased from 0.189 to 0.869 to 0.992 at pH_i 7.6, 5.4 and 5, respectively. (b) Mean values ($\pm$ s.d.) from two to six separate patches of open probability for various concentrations of cGMP (filled symbols) and cAMP (open symbols) were plotted for pH_i 7.6 (triangles) and 5.4 (squares). Continuous lines were the best fit of equation (2) to the data with the following values: for cAMP $K_{1/2}$ was 70 and 24 μ M and n was 3 and 2.7 at pH_i 7.6 and 5.4, respectively, while for cGMP $K_{1/2}$ was 4 and 3.4 μ M and n was 2 and 1.9 at pH_i 7.6 and 5.4, respectively. (c) $K_{1/2}$ for cAMP as a function of pH_i at +60 mV (filled circles) and -60 mV (open circles). Mean values of $K_{1/2}$ ($\pm$ s.d.) from two to five separate patches were calculated from values obtained from the best fit of the Hill equation (equation 1) to dose-response curves at various pH_i , as shown in figure 1b. Continuous lines were the best fit of equation (4) to the data with the following values: $pK_2 = 6.2$, $n = 1.8$, $K_{\min} = 14.7 \mu$ M, $K_{\Delta} = 45.3 \mu$ M at +60 mV, and $pK_2 = 6.2$, $n = 1.5$, $K_{\min} = 12.7 \mu$ M, $K_{\Delta} = 46.4 \mu$ M at -60 mV.

where $[H^+]$ is the proton concentration, $K_{\min}$ is the value of $K_{1/2}$ at very high proton concentrations, K_{Δ} is the difference between the value of $K_{1/2}$ at very low proton concentrations and $K_{\min}$, $[K_2]$ is the proton concentration giving 50% of K_{Δ} and n is the Hill coefficient. Continuous lines in figure 3c were obtained from the best fit of equation (4) to the data. The dependence of $K_{1/2}$ for cGMP on pH_i could also be fitted by equation (4) (data not shown). Values for pK_2 were about 6.2 for both cAMP and cGMP, and n was always greater than 1, while K_{Δ} was $0.7 \mu M$ for cGMP and $45.3 \mu M$ for cAMP at +60 mV. Equation (4) can be derived by a model in which $K_{1/2}$ is decreased by the cooperative binding of at least n protons (see also equations (5) and (8) in Picco *et al.* (1996)). Therefore, the increase in channel open probability at low cyclic nucleotide concentrations caused by an increase in internal proton concentration can be described by the cooperative action of at least two protons ($n = 1.8$) binding to sites with a pK_2 of about 6.2.

(c) Rod single channels

Figure 4a shows recordings from a patch containing only one rod channel. The channel was activated by a sub-saturating concentration of cGMP, $100 \mu M$, at +80 mV and pH_i 7.6, 5.7 or 5. A comparison between the amplitude histograms at different pH_i shows that the single-channel current was reduced by cytosolic acidification similarly to the olfactory channel, and that the open probability was increased. The single-channel current was reduced from 2.22 ± 0.31 pA ($N = 15$) to 1.8 ± 0.2 pA ($N = 14$) to 1.52 ± 0.1 pA ($N = 5$) at pH_i 7.6, 5.7 or 5, respectively, while the open probability at $100 \mu M$ of cGMP increased from 0.41 ± 0.09 ($N = 2$) to 0.58 ± 0.2 ($N = 2$) to 0.87 ± 0.1 ($N = 2$). Figure 4b shows recordings from another patch containing only one rod channel activated by a saturating concentration of cAMP, 20 mM. The open probability at pH_i 7.6 in cAMP was extremely small (no openings were detected during 30 s recordings in this patch) but dramatically increased to 0.78 ± 0.2 ($N = 2$) when pH_i was decreased to 5.

The single-channel current as a function of pH_i could be very well described by equation (3) with a pK_1 of 4.8 at +80 mV and 4.7 at -80 mV (figure 4c). Maximal macroscopic currents activated by cGMP were plotted in figure 4d and were also well fitted by equation (3) with a pK_1 of 4.7 at +60 mV and 4.5 at -60 mV. Maximal currents activated by cAMP behaved instead very differently, dramatically increasing when pH_i was lowered. This effect can be explained by the large increase in open probability measured also at saturating cAMP concentrations, as illustrated in figure 4b.

Therefore, the dependence on pH_i of the maximal current activated by cGMP could be explained as partial blockage of the current due to protonation of a single site, with a pK_1 of 4.5–4.8, located close to the intracellular mouth of the channel. The increase of the cAMP current as pH_i was decreased was instead

Figure 4. The single-channel current of the rod channel as a function of pH_i . Current recordings from membrane patches containing one channel activated at +80 mV by (a) $100 \mu M$ of cGMP at pH_i 7.6, 5.7 and 5, and (b) 20 mM of cAMP at pH_i 7.6 and 5. Recordings in (a) and (b) were from two different patches. Currents were filtered at 1 kHz and sampled at 2 kHz in (a) and at 2.5 kHz in (b). Amplitude histograms were fitted as the sum of two Gaussians. The single-channel current was 2.24, 1.99 and 1.26 pA for cGMP at pH_i 7.6, 5.7 and 5, respectively, and 1.6 pA for cAMP at pH_i 5. The open probability was 0.422, 0.766, 0.968 for cGMP at pH_i 7.6, 5.7 and 5, respectively, and 0.735 for cAMP at pH_i 5. At 20 mM of cAMP and pH_i 7.6, no single-channel openings were observed. (c) Mean values ($\pm$ s.d.) from 4 to 14 separate patches of normalized single-channel currents activated by cGMP plotted as a function of pH_i . Currents were measured at +80 mV (filled squares) or -80 mV (open squares) at various pH_i and normalized to the value at pH_i 7.6 at the corresponding voltage. Continuous lines were the best fit of equation (3) to the data with $pK_1 = 4.8$ at +80 mV and $pK_1 = 4.7$ at -80 mV. (d) Mean values ($\pm$ s.d.) from two to six separate patches of normalized maximal currents activated by cGMP at +60 mV (filled squares) and -60 mV (open squares) or by cAMP at +60 mV (open circles) were plotted versus pH_i . Currents were normalized to the current value in 1 mM of cGMP at pH_i 7.6 in the same patch at the corresponding voltage. Continuous lines were the best fit of equation (3) to the data with $pK_1 = 4.7$ at +60 mV and $pK_1 = 4.5$ at -60 mV.

caused by a dramatic increase in the channel open probability.

4. DISCUSSION

We have shown that increasing internal proton concentration had a similar blocking effect on the single-channel current of both bovine olfactory and rod CNG channels composed of subunit 1 (Kaupp *et al.* 1989; Ludwig *et al.* 1990) and that this blockage can be described by the binding of protons to a site with an apparent pK_1 of about 4.5–4.7. In addition, internal protons modified the open probability of the channels favouring the open state. Potentiation of activation was more pronounced when channels were activated by cAMP than by cGMP.

(a) Proton-induced reduction of single-channel current

We have investigated the blocking effect of internal protons on cloned bovine CNG channels, both on macroscopic and on single-channel currents. Previous studies on the effect of cytosolic acidification on macroscopic currents in native CNG channels have shown that maximal currents decreased as a function of pH_i following a curve for titration of a single acidic site with a pK_1 of 5.0–5.2 in salamander rod (Picco *et al.* 1996) and rat and frog olfactory CNG channels (Frings *et al.* 1992). The pK_1 measured in the present study for cloned channels was about 4.5–4.7, slightly lower than that measured in native channels. Moreover, the effect of internal pH_i in native channels was not voltage dependent (Menini & Nunn 1990; Frings *et al.* 1992; Tanaka 1993; Picco *et al.* 1996), whereas in cloned channels it was found to be slightly voltage dependent. Native CNG channels are heteromultimers formed by at least two types of subunits, while cloned channels in the present study were homomers composed only of subunit 1. Therefore, the differences observed between native and cloned CNG channels could be due to differences in their subunit composition and/or to differences in the amino acid composition of the channels from different species. Since CNG channels are composed of several subunits, a single binding site, to which hydrogen ions could bind and block the channel, could be formed by groups from the side chains of residues at the same position in different subunits. The similar apparent pK values between rod and olfactory channels suggest that the proton binding site could be formed by corresponding residues located at the cytoplasmic side of the pore. A comparison of channel sequences and site-directed mutagenesis studies will help identifying at the molecular level the sites responsible for blockage of the single-channel current by internal protons.

(b) Proton-induced potentiation of channel activation

When internal proton concentration was increased, the open probability at sub-saturating concentra-

tions increased in both rod and olfactory channels, and the effect was more pronounced when channels were activated by cAMP than by cGMP. In addition, the maximal open probability for the rod channels in the presence of cAMP greatly increased by lowering pH_i , almost approaching the value for cGMP. For olfactory channels, the maximal open probability for both cGMP and cAMP was close to 1 and did not change with pH_i .

Previous studies on macroscopic currents both in native (Picco *et al.* 1996) and cloned (Gordon *et al.* 1996) CNG channels from rods have shown that cAMP-activated currents were greatly increased by lowering internal pH_i . However, the proton-induced potentiation of cAMP activation in native channels was quantitatively smaller than that observed in cloned channels and, moreover, activation by cGMP in native channels was not affected by pH_i . A possible molecular explanation of these differences has been suggested by Gordon *et al.* (1996) and will be discussed in the following section.

We have shown in this study also that activation of cloned bovine olfactory CNG channels composed of subunit 1 was potentiated by protons. Moreover, recent experiments have also shown that activation by both cAMP and cGMP of native bovine olfactory channels was potentiated by protons (C. Picco, P. Gavazzo & A. Menini, unpublished results). Decreasing pH_i from 7.6 to 5.7 caused a 2.7-fold decrease of $K_{1/2}$ for cAMP at +60 mV (from about 4 to 1.5 μ M), and a 1.7-fold decrease of $K_{1/2}$ for cGMP (from about 0.7 to 0.4 μ M). The maximal current at pH_i 5.7 for both cAMP and cGMP was 81% of that at pH_i 7.6 (data not shown). Therefore, the decrease in maximal currents and the relative decrease of $K_{1/2}$ for both cGMP and cAMP were similar in native and in cloned olfactory channels, indicating that the difference between native and cloned olfactory channels was not as pronounced as in the corresponding rod channels.

(c) Molecular mechanisms of channel gating

It is certainly very important to identify the molecular mechanisms of the effect of intracellular protons on the gating of CNG channels. Olfactory and rod CNG channels have different intrinsic gating properties: the maximal open probability and the apparent affinity of cAMP and cGMP is different in olfactory and rod channels. In addition, channels from different species are activated differently; for example, the catfish olfactory CNG channel is activated almost equally well by cGMP and by cAMP, while the rat and bovine CNG channels have a much higher affinity for cGMP than for cAMP.

Recent work aimed at understanding the molecular mechanisms controlling gating in CNG channels have shown that several domains are involved in the gating process (Goulding *et al.* 1994; Gordon & Zagotta 1995; Bucossi *et al.* 1997; Tibbs *et al.* 1997). One domain is the cyclic nucleotide binding site located in the carboxy terminus that is homologous with the cyclic nucleotide binding domain of other cAMP and

cGMP-activated proteins (Kaupp *et al.* 1989). Two other domains have been shown to influence gating as well. One domain (N-S2) is located in the amino terminus (Goulding *et al.* 1994; Gordon & Zagotta 1995) and, rather surprisingly, the other domain is located in the putative pore region (Bucossi *et al.* 1997). The involvement of these three regions implies that global conformational changes are involved in the nucleotide gating of CNG channels. Consequently, it is expected that changes of internal pH_i that affect gating could have multiple sites of action.

Within the binding domain, site-directed mutagenesis studies (Altenhofen *et al.* 1990; Varnum *et al.* 1995) and molecular models (Scott & Tanaka 1995; Scott *et al.* 1996) have suggested the importance of specific residues in determining the cyclic nucleotide affinity and specificity. In particular, aspartate 604 in the bovine rod channel and the corresponding amino acids in the other channels (see Yau & Chen (1995) for alignments of the complete sequences of many CNG channels), play an important role in channel gating and ligand recognition. Varnum *et al.* (1995) suggested, from the results of mutations of D604, that this residue is the major determinant of agonist efficacy and specificity. However, Scott *et al.* (1996) argued that if D604 is the sole determinant, the D604E bovine rod channel mutant should reflect the nucleotide specificity of the bovine olfactory channel that has a glutamate in the corresponding position. The wild-type bovine olfactory channels display the same level of current for both cGMP and cAMP (figure 1a; see also Altenhofen *et al.* 1990), whereas cAMP activates a small fraction of the cGMP current in the D604E mutant bovine rod channel (Varnum *et al.* 1995). D604Q mutation dramatically altered the dose-response relations and relative currents activated by the two nucleotides. Using molecular models based on the crystal structure of the catabolite gene activator protein (CAP), Scott *et al.* (1996) suggested that the effect of residue D604 of the bovine rod channel and corresponding residues on the other channels depends also on the chemical identity of other residues within the binding domain. Therefore, the cyclic nucleotide affinity and specificity within the CNG channel family depend on several residues within the binding domain working in concert as well as inter-domain or inter-subunit interactions.

Previous mutagenesis work also led to the identification of two residues involved in proton potentiation of the bovine rod CNG channel (Gordon *et al.* 1996). One important residue affected by changes in the internal pH_i is D604. Protonation of this aspartate accounts for the cAMP-specific component of potentiation. Gordon *et al.* (1996) hypothesized that the open conformation of the cAMP-activated rod channel is stabilized by protonation, and our single-channel data provide experimental evidence for this hypothesis. Gordon *et al.* (1996) further identified a second residue, H468, which accounts for a nucleotide non-specific component of potentiation and is located in an intracellular region of the channel between

the sixth transmembrane segment and the cyclic nucleotide binding domain. Further indirect support for the involvement of these residues is that human subunit 2 (Chen *et al.* 1993) has non-protonatable asparagine residues in both the corresponding positions. When subunits 1 and 2 were coexpressed, they showed a reduced proton potentiation (Gordon *et al.* 1996) more similar to our previous work with native channels (Picco *et al.* 1996). We therefore compared the sequences of subunit 1 of the bovine olfactory and rod channels to see whether similar amino acids could also be involved in proton-induced potentiation of the bovine olfactory channel. As already pointed out, a glutamate (E581) residue is present at the position corresponding to D604 in the bovine rod channel, and a histidine (H445) is present at the position corresponding to H468. Both residues could therefore be involved in potentiation. Site-directed mutagenesis will help expand our emerging understanding of the molecular mechanisms of proton-induced potentiation of activation of the family of CNG channels.

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