Time-dependent current decline in cyclic GMP-gated bovine channels caused by point mutations in the pore region expressed in *Xenopus* oocytes

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1. Amino acids with a charged or a polar residue in the putative pore region, between lysine 346 and glutamate 372 of the α-subunit of the cGMP-gated channel from bovine rods were mutated to a different amino acid. The mRNA encoding for the wild-type, i.e. the α-subunit, or mutant channels was injected in *Xenopus laevis* oocytes.

2. When glutamate 363 was mutated to asparagine, serine or alanine, the current activated by a steady cGMP concentration declined in mutant channels. No current decline was observed when glutamate 363 was mutated to aspartate, glutamine or glycine, when threonine 359, 360 and 364 were mutated to alanine or when other charged residues in the pore region were neutralized.

3. The amount of current decline and its time course were significantly voltage dependent. In mutant E363A the current decline developed within about 1·5 s at −100 mV, but in about 6 s at +100 mV. In the same mutant, the current declined to about 55% of its initial level at +100 mV and to about 10% at −100 mV.

4. The current decline in mutants E363A, E363S and E363N was only moderately dependent on the cGMP concentration (from 10 to 1000 μM) and was not caused by a reduced affinity of the mutant channels for cGMP. Analysis of current fluctuations at a single-channel level indicated that current decline was primarily caused by a decrease of the open probability.

5. The wild-type channel was not permeable to dimethylammonium. When glutamate 363 was replaced by a smaller residue such as serine, mutant channels became permeable to dimethylammonium.

6. The current decline observed in mutant channels is reminiscent of desensitization of ligand-gated channels and of inactivation of voltage-gated channels. These results suggest also that gating and permeation through the cGMP-gated channel from bovine rods are intrinsically coupled and that glutamate 363 is part of the molecular structure controlling both the gating and the narrowest region of the pore.

Cyclic nucleotide-gated (CNG) ionic channels functionally belong to the class of ligand-gated channels because they are activated by intracellular ligands (Fesenko, Kolesnikov & Lyubarsky, 1985; and for review see Eismann, Bönigk & Kaupp, 1993; Torre & Menini, 1994; Kaupp, 1995) but share several structural features with voltage-gated channels. In particular, the pore region, the voltage-sensor motif and the transmembrane topology are well conserved in both channel families (Jan & Jan, 1990; Guy, Durell, Warmke, Drysdale & Ganetzky, 1991; Heginbotham, Abramson & MacKinnon, 1992; Goulding *et al.* 1992; Bönigk *et al.* 1993; Henn, Baumann & Kaupp, 1995). Unlike most voltage-gated K^+, Na^+ or Ca^{2+} channels, CNG channels do not inactivate after opening (Karpen, Zimmerman, Stryer & Baylor, 1988). They also do not desensitize in the presence of a steady ligand concentration as do ligand-gated channels like acetylcholine or glutamate receptors (Hille, 1992). Recently, it has been demonstrated that

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glutamate residue 363 in the pore region of the CNG channel from rod photoreceptors is critical for blockage by divalent cations (Root & MacKinnon, 1993; Eismann, Müller, Heinemann & Kaupp, 1994) and for ionic permeation (Root & MacKinnon, 1995; Sesti, Eismann, Kaupp, Nizzari & Torre, 1995*a*, *b*). In this paper we report that, when this glutamate 363 is mutated to some neutral amino acids, such as asparagine, serine or alanine, the channel inactivates or desensitizes in the presence of a steady ligand concentration. Mutant channels become permeable to large organic cations, such as diethylammonium, suggesting that glutamate 363 is located in the narrowest part of the pore. These results indicate that ionic permeation and gating of CNG channels are intrinsically linked and that glutamate 363 is part of a gate structure that controls the flow of ions through the pore.

METHODS

Dissection

Mature *Xenopus laevis* were anaesthetized with 0·2% tricaine methanesulphonate (Sigma) and ovarian lobes were removed surgically. Oocytes from *Xenopus laevis* were prepared as described in Nizzari, Sesti, Giraudo, Virginio, Cattaneo & Torre (1993).

Mutagenesis and oocyte preparation

The point mutations in the α-subunit of the CNG channel from bovine rods (Kaupp *et al.* 1989) were introduced by polymerase chain reaction (Herlitze & Koenen, 1990). All mutations were verified by sequencing of the inserted fragment (Sanger, Nicklen & Coulson, 1977). mRNAs specific for wild-type and mutant channels were synthesized *in vitro* (Melton, Krieg, Rebagliati, Maniatis, Zinn & Green, 1984). Synthesis was primed with m7G(5′)ppp(5′)G. The mRNA was injected into *Xenopus laevis* oocytes prepared and maintained as described in Nizzari *et al.* 1993. The amino acid sequence of the putative pore region of the wild type of the cGMP-gated channel, from aspartate 339 to glutamate 372, is:

DFGRLAR**K**YVYSLYWSTLTL**TT**IG**ET**PPPV**RD**S**E**

The emboldened amino acids were mutated.

Electrical recordings and solutions

The pipette solution contained 2 mM EDTA, 10 mM Hepes, 110 mM NaCl buffered to pH 7·6 with tetramethylammonium (TMAOH) or with NaOH. A similar solution was used to superfuse the intracellular side of the patch which could contain specified amounts of cGMP (between 5 and 1000 μM). In some control experiments solutions were buffered with Tris. The technique for measuring macroscopic current was described previously (Sesti *et al.* 1995*a*). Data were stored on a videotape and were analysed off-line. Data shown in Figs 1, 2 and 3 were filtered at 200 Hz.

Determination of the macroscopic *I–V* relations

Macroscopic *I–V* relations were determined with voltage ramps as described in Sesti *et al.* 1995*a*. The *I–V* relations reported in this paper were obtained as the difference between the currents measured with voltage ramps in the presence and in the absence of 500 μM cGMP. Each *I–V* relation was the average of at least five individual trials.

RESULTS

The mRNA of wild-type, i.e. the α-subunit of the bovine rod CNG channel, and mutant channels was injected into *Xenopus* oocytes and cGMP-activated currents were studied in excised patches in the inside-out configuration. CNG channels were activated by adding cGMP to the medium bathing the cytoplasmic side of the membrane patch.

Time-dependent current decline in mutant channels

Similar to the native CNG channel (Fesenko *et al.* 1985; Karpen *et al.* 1988), the wild-type channel does not desensitize or inactivate in the presence of a steady cGMP concentration (Fig. 1*A*). In contrast, a large decline of the current activated by a steady concentration of cGMP was observed in mutants E363N, E363S and E363A (Fig. 1*D*, *E* and *F*). At +50 mV the current decreased to approximately 60 and 45% of its initial level within 30 s in mutants E363S and E363A, respectively. The decline of the current was larger at −50 mV and the activated current decreased to about 35 and 10% of its original level in the same two mutants. The current decline was not observed or was very small in mutants E363D, E363Q (Fig. 1*B* and *C*) and E363G (data not shown). The decline of the current in mutants E363N, E363A and E363S was observed when Hepes was neutralized either with TMAOH or NaOH or when bathing solutions were neutralized with another buffer, such as Tris. This current decline could be caused by an open-channel blockage produced by an unknown cationic contaminant moving slowly into the channel pore. In this case the current decline is expected to depend on the voltage applied to the membrane patch. However, a current decline was also observed with pulses alternating between positive and negative voltages (Fig. 2*A*) and when cGMP was added in the absence of any driving force (Fig. 2*A*). If the current decline is produced by a cationic contaminant entering the channel by moving down its electrochemical gradient, the time course of current decline is expected to be different in the presence of a steady applied voltage and in the presence of alternating voltage pulses. The current decline observed in the presence of a steady voltage (dashed line) and with alternating voltage pulses (continuous line) is compared in Fig. 2*B*. It is evident that the time course of current decline observed with the two experimental protocols is almost identical. The results of these experiments indicate that the current decline was not caused by ionic accumulation or depletion near the membrane patch or by the slow blockage of the channel by an unknown cationic contaminant.

This current decrease can be analysed with different experimental protocols. For instance, when the mutant E363A was activated by 100 μM cGMP a drop was observed (Fig. 2*C*), but the addition of 1000 μM cGMP during the exposure to 100 μM cGMP produced only a

moderate (Fig. 2C; upper trace at +50 mV) or a small (Fig. 2C; lower trace at −50 mV) additional increase in current amplitude. However, the addition of 1000 μM cGMP long before and after the exposure to 100 μM cGMP activated a much larger current. Other experiments show that exposure to 100 μM cGMP, immediately following a prolonged exposure to 500 μM cGMP, activated a current which was significantly smaller than that activated by the same cGMP concentration before the exposure to 500 μM cGMP (Fig. 2D).

Similar results were obtained with mutants E363S and E363N. These results indicate that, once opened by a steady cGMP concentration, mutants E363N, E363S and E363A enter a different microscopic state reminiscent of desensitization in ligand-gated channels or inactivation in voltage-gated channels.

Voltage dependence of current decline

In order to understand the mechanisms underlying the current decline, it is useful to study its voltage dependence.

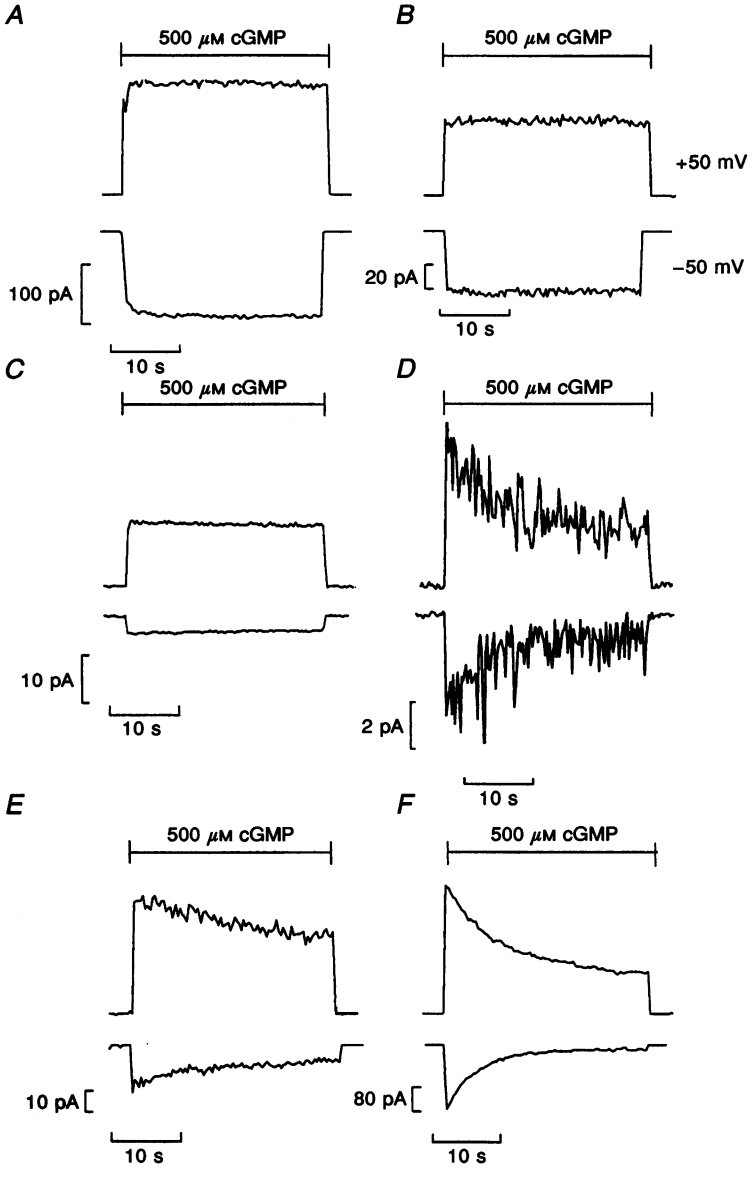

Figure 1. cGMP-activated current of wild-type and mutant channels in the presence of a steady cGMP concentration

Current recordings during an exposure of about 30 s to 500 μM cGMP at +50 (top traces) and −50 mV (bottom traces) from the wild-type channels (*A*) and mutants E363D (*B*), E363Q (*C*), E363N (*D*), E363S (*E*) and E363A (*F*). The duration of exposure to cGMP was manually operated, so that the effective duration varied between 28 and 32 s.

At negative membrane voltage, the time course of current decline was faster and the fractional current decrease was larger than at positive membrane voltages (Fig. 3*A* and *C*). The *I–V* relations of the mutant E363A measured immediately after the exposure to 1000 μM cGMP (Fig. 3*B*, ○) and after development of current decline (Fig. 3*B*, ●) had a different degree of rectification. The ratio of the current flowing at +100 and −100 mV was about 3 before current decline and became greater than 12 after completion of current decline (Fig. 3*C*). The time constant of development of current decline was between 6 and 7 s at +100 mV and decreased to about 2·5 s at −100 mV. The fraction of current decline and its time constant change by about e-fold at 200 mV, indicate an equivalent amount of charge movement associated with the current decline of about 0·1 elementary charge. The dependence of current

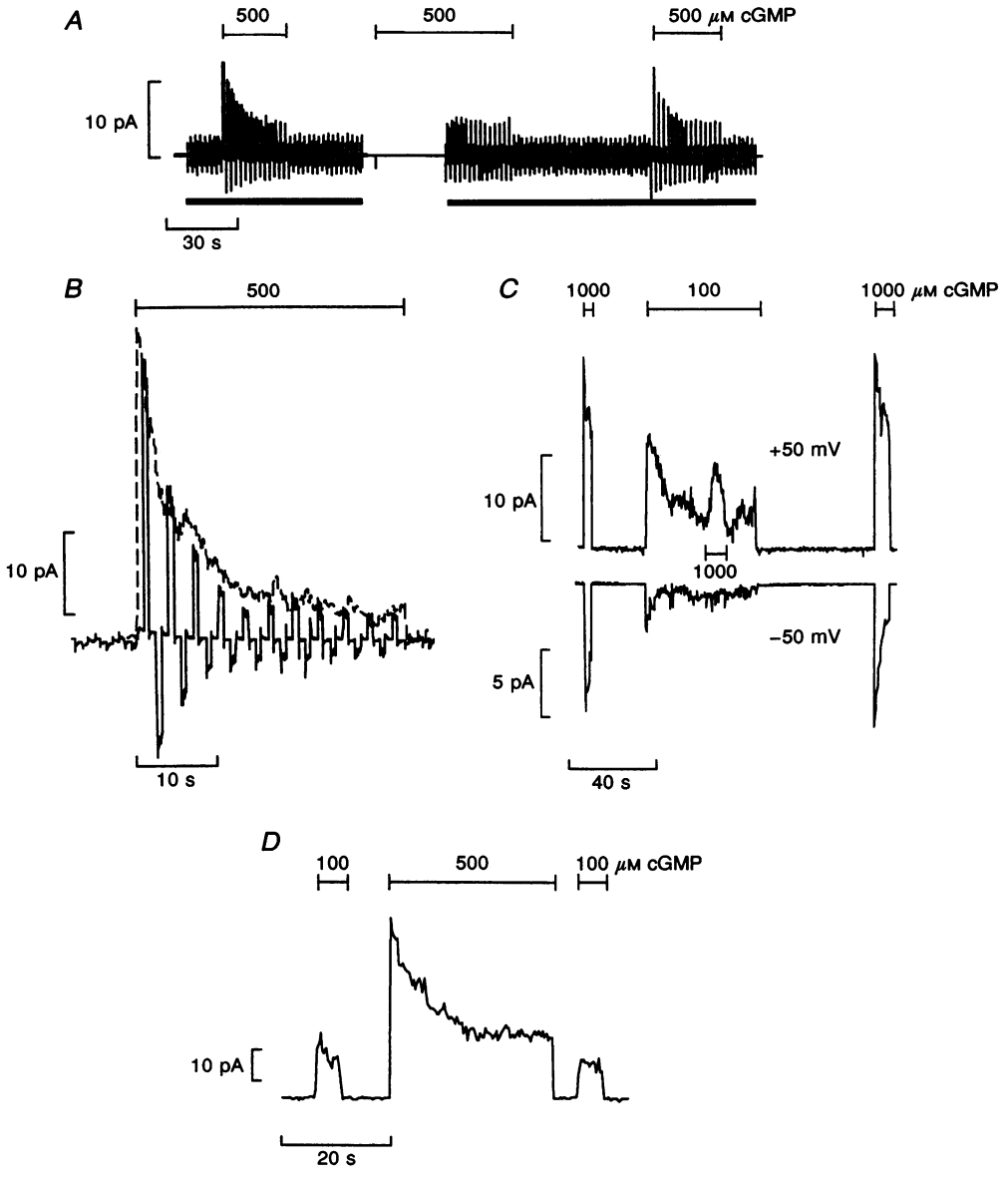

Figure 2. Current decline of mutant E363A

A, current recordings from mutant E363A in the presence of alternating voltage pulses at +50 and −50 mV for the duration indicated by the filled bars under the traces. Pulses had a duration of 200 ms and a frequency of 0·5 Hz. Current decline was also observed when cGMP was added in the absence of any applied voltage. *B*, comparison of current decline in the presence of a steady voltage of +50 mV (dashed line) and in the presence of pulses alternating between +50 and −50 mV (continuous line). *C*, exposure to 1000 μM cGMP during a prolonged exposure to 100 μM cGMP. The upper and lower traces were obtained at +50 and −50 mV, respectively. *D*, addition of 100 μM cGMP before and after a prolonged exposure to 500 μM cGMP. In all panels the addition of cGMP to the superfusing solution is indicated by the bars above the traces.

decline on membrane voltage indicates that the underlying molecular structure does experience the electric field across the membrane and is therefore likely to be located inside the membrane. Indeed the wild-type channel was not permeable to diethylammonium (see Fig. 3*D*) but, when glutamate 363 was replaced with a smaller residue such as serine, the mutant E363S was permeable to diethylammonium (see Fig. 3*E*). The size of the largest permeant organic cations is usually used to probe the pore at its narrowest section (Hille, 1992; Picco & Menini, 1993). As a consequence this result suggests that glutamate 363 is located well within the pore, probably at its narrowest restriction.

Dependence of current decline on the cGMP concentration

The decline of the current observed with mutants E363A, E363S and E363N might be caused by a reduced affinity of cGMP for the channel or by changes in the ionic permeation or gating of the channel. Therefore, the current activated by different amounts of cGMP was analysed (Fig. 4*A*).

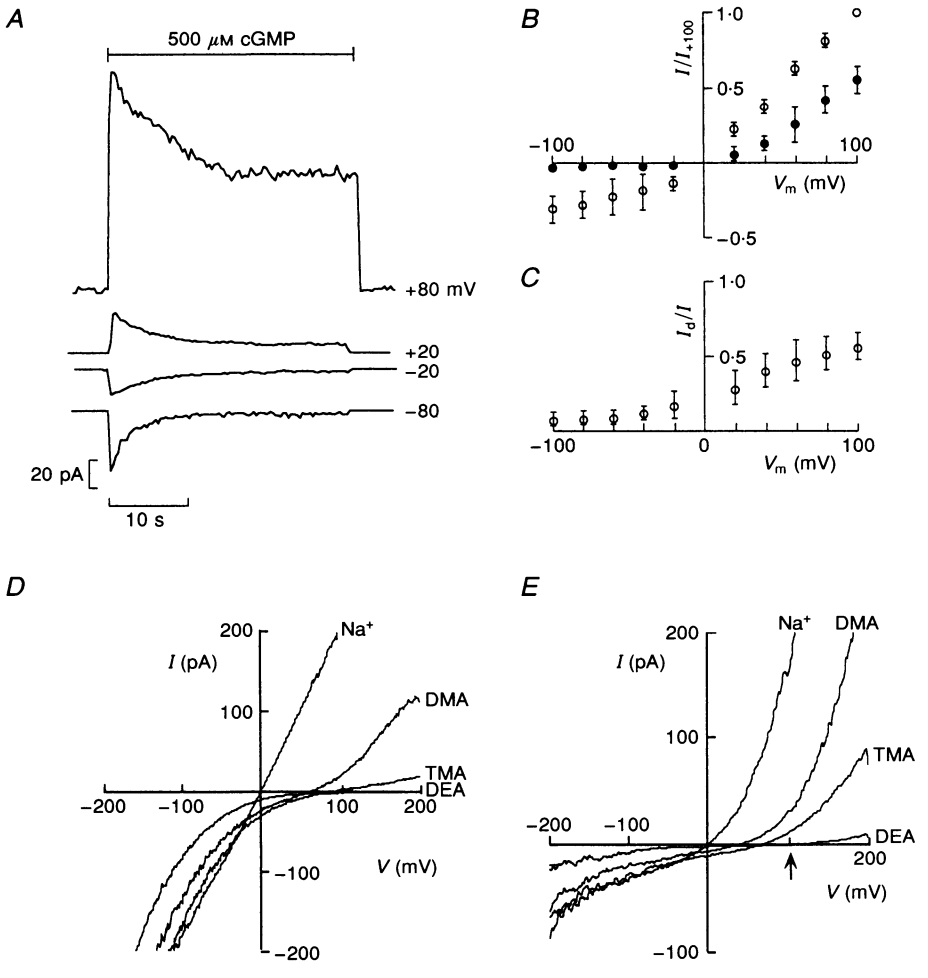

Figure 3. Voltage dependence of current decline of mutant E363A and the ionic permeability of mutant E363S to large organic cations

A, current recordings at +80, +20, −20 and −80 mV during an exposure of about 30 s to 500 μM cGMP. *B*, dependence of the current activated by 500 μM cGMP on membrane voltage. ○, data obtained prior to current decline; ●, data obtained after completion of current decline. Current was normalized to the current flowing at +100 mV (I_{+100}) prior to current decline. *C*, ratio (I_d/I) between the current amplitude after current decline (I_d) and before current decline (I) against membrane voltage (V_m). Data in *B* and *C* were collected from 4 patches; bars indicate the standard deviation. *D* and *E*, *I–V* relations of the wild type and mutant E363S, respectively. The patch pipette was filled with 110 mM Na^+ and the bathing medium contained an equimolar amount of Na^+ or dimethylammonium (DMA), or trimethylammonium (TMA) or diethylammonium (DEA). Channels were activated by 500 μM cGMP and the *I–V* relations were obtained with voltage ramps from −200 to +200 mV. The voltage ramp had a duration of 2 s. Each trace was obtained as the average of at least 5 voltage ramps. In the presence of DEA the reversal potential in mutant E363S was about +100 mV (arrow), while no outward current was observed in the wild type.

The current activated by 500 μM cGMP was usually about 90% of the current activated by 1000 μM cGMP both at the peak and in the steady state, indicating that 1000 μM cGMP elicited a saturating current before and after completion of current decline. At +50 mV, concentrations of 80 and 110 μM cGMP activated half the maximal current in the steady state (◇) and at the peak (◆), respectively (Fig. 4*B*). At −50 mV, half-activation of the current was observed at about 90 and 150 μM cGMP in the steady state (◇) and at the peak (◆), respectively (Fig. 4*C*). These results indicate that the value of $K_{½}$ (the reciprocal of the ion concentration required for half-maximal inhibition) does not change by more than 60% at the peak and at the steady state, thus demonstrating that the current decline is not significantly dependent on the cGMP concentration and is not produced by a reduced affinity of cGMP for the channel in the steady state.

Recovery of current decline

As previously shown, the current initially activated by cGMP in mutant E363A declined within a few seconds. When cGMP was removed from the bathing solution for progressively longer periods of time a further addition of cGMP activated larger currents. Figure 5 reproduces current recordings obtained during experiments in which 500 μM cGMP was added to the bathing medium for the times indicated by the bars. Recordings in *A* and *C* were obtained at +50 and −50 mV, respectively.

The fractional activated current, $I_{\Delta t}/I$, after removal of cGMP for Δt seconds is reproduced in Fig. 5*B* (at +50 mV) and *D* (at −50 mV). Complete recovery of activated current was obtained following removal of cGMP for at least 30 s. The recovery was slightly faster at +50 mV than at −50 mV. Very similar results were obtained with mutant E363S.

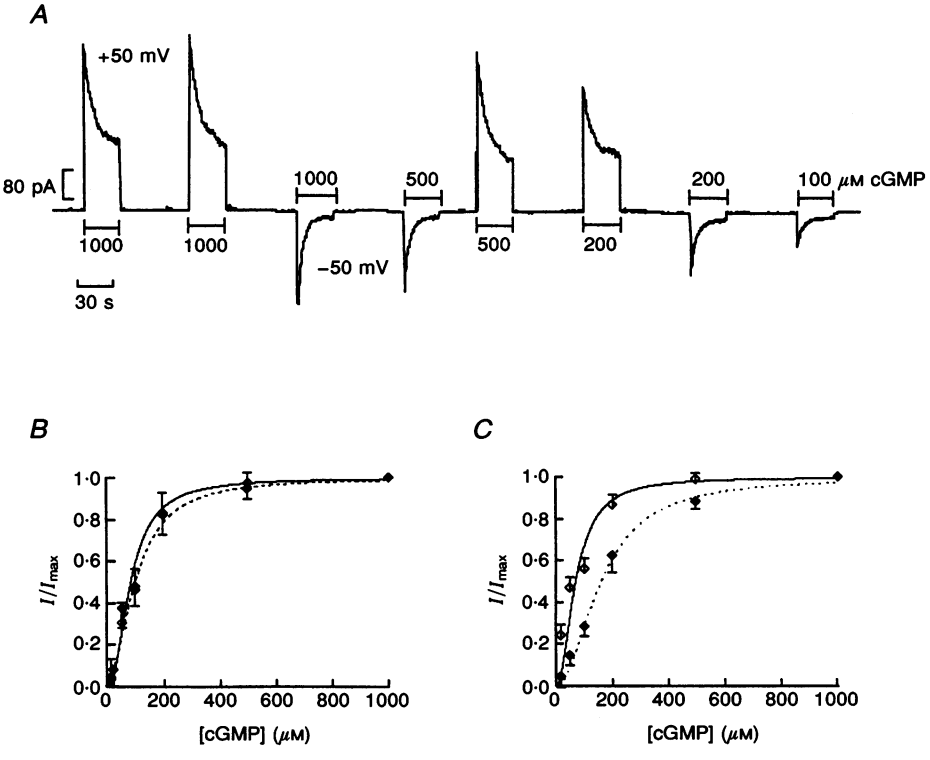

Figure 4. Dependence of current decline of mutant E363A on cGMP concentration

A, current recordings in the presence of different amounts of cGMP at either +50 or −50 mV in mutant E363A. Upwards (downwards) currents were obtained at +50 mV (−50 mV). cGMP concentrations are indicated by the bars. *B* and *C*, relation between ligand concentration and normalized current at the peak (◆) and in the steady state (◇) at +50 mV (*B*) and −50 mV (*C*) in mutant E363A. The current was normalized to the current measured at the peak (◆) or at the steady state (◇) in the presence of 1000 μM cGMP (producing the maximum current, I_{max}). Average values from 4 different patches. Bars indicate standard deviation. In *B*, the continuous and dashed curves were drawn from the equation: $[cGMP]^2/([cGMP]^2 + (K_{½})^2)$, with values of 80 and 110 μM for $K_{½}$, respectively. In *C*, the continuous and dotted curves through the points were drawn from the same equation with values of 90 and 150 μM for $K_{½}$, respectively. The value of $K_{½}$ at the peak of 110 and 150 μM for mutant E363A is significantly larger than the value found in the wild-type channel which is about 80 μM (Kaupp *et al.* 1989; Nizzari *et al.* 1993). The value of $K_{½}$ was obtained by a mean least-squares fit of the data.

The results of the experiments described in Fig. 5 provide additional evidence against the view that the current decline is caused by an open-channel blockage by a cationic contaminant entering the pore. As shown in Fig. 3 the current decline is faster and more pronounced at negative voltages, as if a cationic contaminant entered the pore of the open channel from the extracellular medium within 2–10 s. The experiments illustrated in Fig. 5, however, show that this presumed contaminant is able to leave the closed channel within 5–20 s. Thus, the presumed open-channel blocker is able to leave a closed channel with a time constant similar to the time constant of the open-channel blockage. This last possibility cannot be ruled out completely, but appears highly unlikely.

Current decline at the single-channel level

The results presented in the previous section indicate that the current decline is caused by a decrease in the open probability and/or by a decrease in the single-channel conductance. As a consequence, it is useful to analyse the current decline at the single-channel level. Figure 6*A* (upper trace) shows a current recording from a patch containing very few CNG channels of the mutant E363N. In the absence of cGMP the trace was quiet; upon addition of 500 μM cGMP it became noisy and was characterized by brief current pulses whose frequency decreased after some tens of seconds (see second and third trace in Fig. 6*A*). Analysis of amplitude histograms (Fig. 6*B*) indicates that the single-channel conductance was 15 pS before and after completion of current decline, much smaller than the value of 27 pS observed in the wild type (Kaupp *et al.* 1989; Nizzari *et al.* 1993).

Figure 6*C* and *D* illustrates current recordings from a membrane patch containing very few channels of mutant E363A activated by 500 and 50 μM cGMP. As evident from Fig. 6, the single-channel conductance of mutant E363A is significantly smaller than that of mutant E363N and it is more difficult to measure precisely. However, it is evident that the frequency of channel openings induced by cGMP significantly decreased after some tens of seconds (see second and third trace in Fig. 6*C* and *D*). These data suggest that the current decline is a consequence of a reduction in the open probability and is not due to a decrease in the single-channel conductance.

The open probability at saturating cGMP concentrations of mutant E363G that did not show any current decline within the time resolution of our experiment, was also very low and smaller than that of the wild-type channel. Indeed the open probability, P_o, of mutant E363G at 20 °C in the

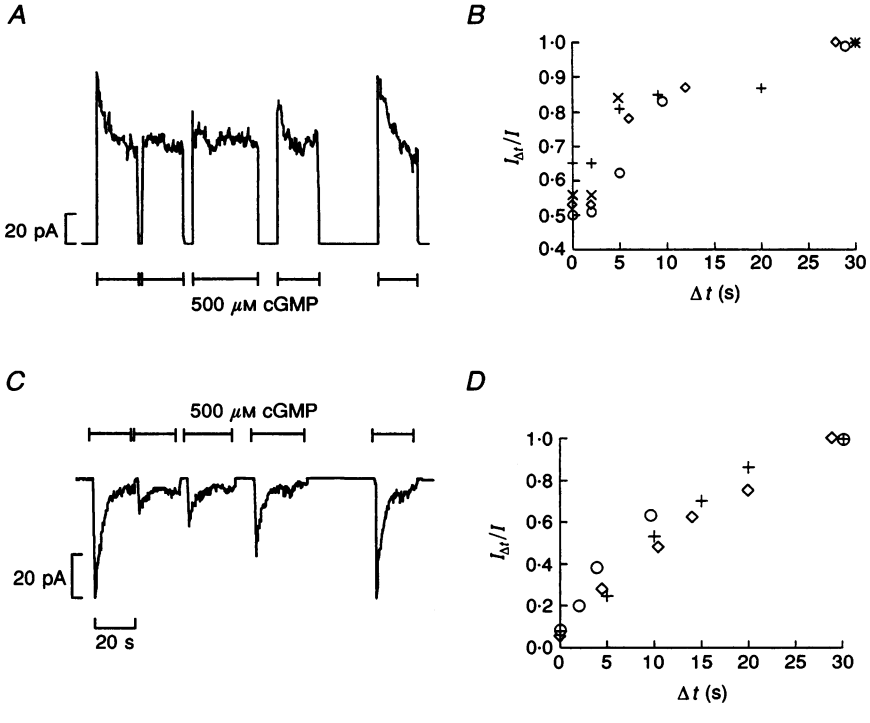

Figure 5. Recovery from current decline in mutant E363A

In *A* (+50 mV) and *C* (−50 mV) current recordings obtained during exposures to 500 μM cGMP for the times indicated by the bars. In *B* (+50 mV) and in *D* (−50 mV) recovery of normalized activated current $I_{\Delta t}/I$ following the removal of cGMP for Δt seconds. *I* is the maximal activated current before the initiation of current decline. In *B* and *D* different symbols refer to different patches.

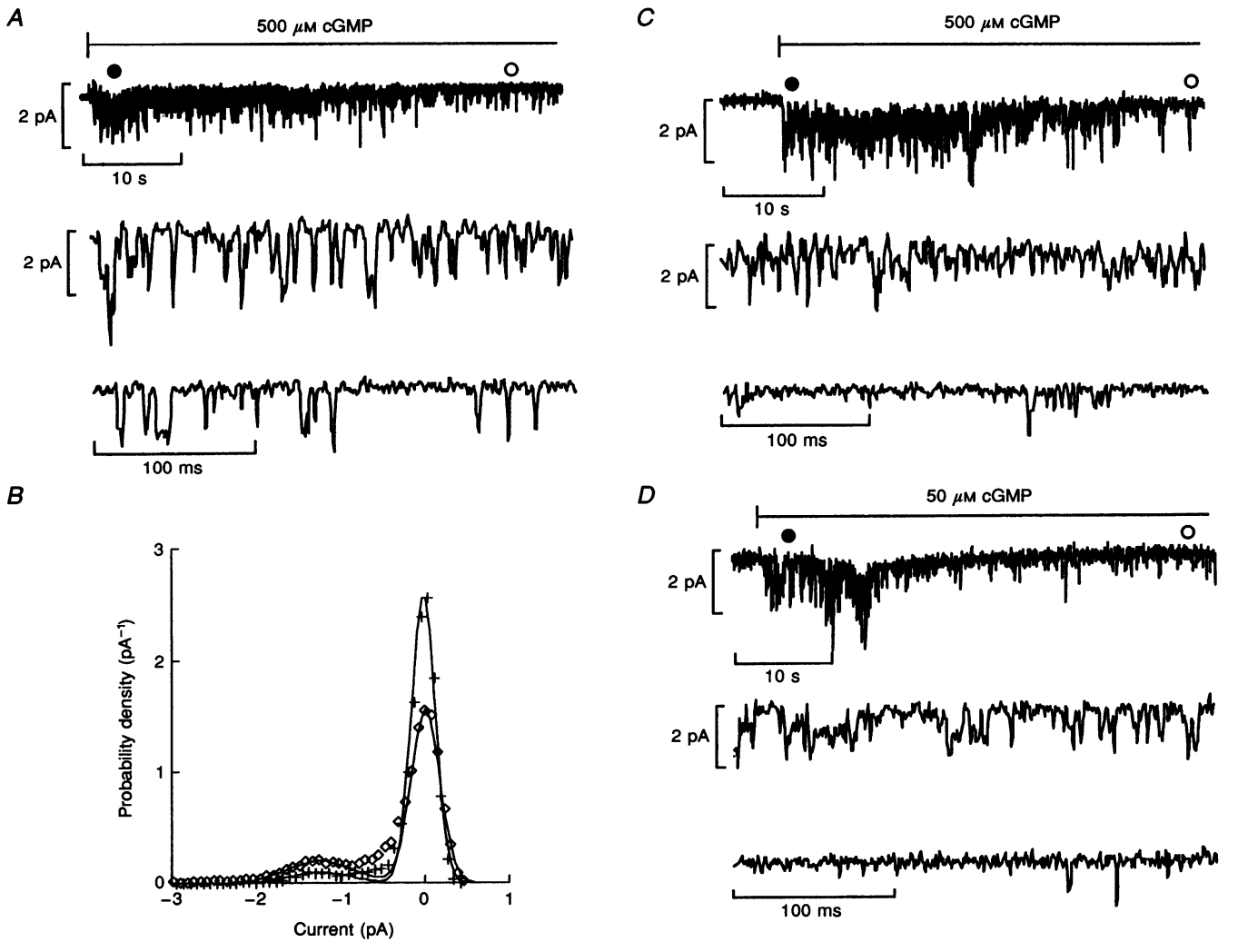

Figure 6. Current decline at the single-channel level in mutants E363N and E363A

Current recordings from mutant E363N at −100 mV in the presence of 500 μM cGMP (*A*), from mutant E363A at −100 mV in the presence of 500 μM (*C*) and 50 μM cGMP (*D*). The upper traces are continuous recordings of about 60 s and were filtered at 200 Hz. The portion of the recordings indicated by ● and ○ are illustrated on an expanded time scale in the second and third trace, respectively, and were filtered at 2 kHz. *B*, amplitude histograms of current fluctuations of the recording shown in *A* between 3 and 13 (◇), and between 30 and 40 s (+) after cGMP application. Histograms were normalized so as to have unitary area. The amplitude histograms were fitted as the sum of two Gaussian distributions; the area of the Gaussian distribution centred around 0 pA was taken as the closed probability and was 0·75 (◇) and 0·88 (+); the single-channel current was 1·5 pA in both histograms. The closed probability of mutant E363N in the presence of a saturating concentration of cGMP was significantly higher than that observed in the wild-type channel, which is about 0·25 (data not shown), because of development of current decline (see also Fig. 1*D*).

presence of 500 μM cGMP at +60 mV was much less than 0·1 (data not shown), i.e. at least ten times less than the value of about 0·8 observed in the wild-type channel (Nizzari *et al.* 1993). This result indicates that, even in the absence of a resolvable time-dependent current decline, mutations of glutamate 363 modify the gating of the channel.

DISCUSSION

The current decline described in this paper is specifically caused by mutations of glutamate 363. Mutations of other charged residues in the pore region, such as lysine 346, arginine 369, aspartate 370 and glutamate 372, and of threonines in position 359, 360 and 364 did not produce any time-dependent current decline (data not shown).

Origin of the current decline

The current decline in mutants E363N, E363A and E363S can in principle be caused by an open-channel blockage by an unknown cationic contaminant slowly moving into the channel mouth. However, several observations argue against this possibility. Firstly, the current decline was observed during a multitude of experiments during which solutions were made with chemicals from different bottles, patch pipettes pulled with different glasses and in the presence of different buffers. The same time-dependent current decline was observed in the presence of 0·2 or 2 mM EDTA, indicating that heavy metals are unlikely to act as blocking contaminants. In addition, when glutamate 363 is neutralized, the blocking effect of divalent cations and protons is drastically reduced (Root & MacKinnon, 1993, 1994; Eismann *et al.* 1994). Secondly, as shown in Fig. 2*B*, the time course of the current decline did not depend on the voltage applied to the membrane patch, as would be expected from a large cation slowly obstructing the channel mouth. Thirdly, as shown by the data in Fig. 5, the presumed open-channel blocker can move out of the closed channel with about the same time constant controlling the open-channel blockage. These observations do not rule out completely the possibility of an open-channel blockage by some unknown contaminant, but indicate that this mechanism is unlikely or rather peculiar.

The current decline described in this paper is similar to inactivation of voltage-gated channels and desensitization of ligand-gated channels. It is important, however, to note that the current decline of mutant CNG channels is not characterized by large changes in ligand sensitivity, i.e. of at least 2-fold, as is often observed in ligand-gated channels (Ochoa, Chattopadhyay & McNamee, 1989). The current decline observed in mutant CNG channels is reminiscent of C-type inactivation of voltage-gated channels (Lopez-Barneo, Hoshi, Heinemann & Aldrich, 1993), which is affected by mutations in the pore region, but differs because it depends both on membrane voltage and ligand concentration. As in the case of desensitization and of inactivation, the current decline described in this paper is likely to be caused by a molecular conformational change of the channel developing with time.

Permeation and gating in CNG channels

Residue 363 from the bovine rod CNG channel which is critically located within the pore region controls several features of ion permeation such as blockage by Ca^{2+}, the multi-ion nature and the single-channel conductance (Root & MacKinnon, 1993, 1995; Eismann *et al.* 1994; Sesti *et al.* 1995*a*, *b*). In this paper, we show that exchange of glutamate 363 by a neutral and smaller amino acid, such as asparagine, alanine or serine, causes the mutant channels to desensitize or to inactivate in the presence of a steady cGMP concentration, indicating that this residue is involved in the gating, i.e. the transitions between the open and closed states of the channel.

Residue 363 controls the size of the largest permeant ions that can flow through the CNG channel (see Fig. 3) and therefore appears to be located at the narrowest section of the pore. The observation that residue 363 controls the size of permeating ions and the open probability shows that gating and ionic permeation are intrinsically linked and suggests that opening and closing of the CNG channels are produced by a global conformational change of the protein (Goulding, Tibbs & Siegelbaum, 1994). It is tempting to propose that this global conformational change leads to movements of residue 363 within the pore region and not to the movement of a different domain of the channel into a fixed pore. With this view, the binding of cGMP to the channel causes a steric rearrangement of the array of glutamates 363, which perhaps is an essential part of both the gate and the selectivity filter.

The results reported in this paper raise the possibility of the existence of native cyclic nucleotide-gated channels exhibiting the kind of desensitization or inactivation of mutants E363A, E363S and E363N. This important issue and the relation with the gating of voltage-gated channels, such as the well-studied K^+ channels, will be elucidated in future work.

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