

Extracellular proton alters the divalent cation binding affinity in a cyclic nucleotide-gated channel pore

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Abstract Extracellular protons in the range of 10^{-9} to 10^{-5} M effectively suppressed Na^+ current ($K_{1/2} = 10^{-6.1}$) through the bovine retinal guanosine 3',5'-cyclic mononucleotide-gated ion channel expressed in *Xenopus* oocytes. The reduction of channel current was mediated by a single glutamate residue (Glu363) within the pore-forming region of the channel, also involved in extracellular divalent cation binding. Increasing the concentration of extracellular proton decreased the binding affinity of the extracellular divalent cation (e.g. Sr^{2+}) and the large difference of binding affinity previously observed between the wild-type and E363D mutant channel disappeared. These results indicate that the permeation characteristics of cyclic nucleotide-gated ion channel can be altered by extracellular pH through a single acidic residue in the channel conduction pathway.

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Key words: Extracellular proton; Cyclic nucleotide-gated channel; Binding affinity; Glutamate residue

1. Introduction

The conduction properties of cyclic nucleotide-gated (CNG) channels are determined by a stretch of amino acid residues, the pore-forming regions (P-region), connecting the fifth (S5) and sixth trans-membrane domain (S6) of these membrane proteins [2,3]. Moreover, a conserved glutamate residue within this P-region has been identified as a key player in ion permeation and blockade of the channel. Neutralization of this residue (e.g. Glu363 in the bovine retinal CNG channel α -subunit) dramatically reduced the affinity for divalent cations on the extracellular side without much effect on the internal side [1,7]. A conservative mutation of the glutamate to aspartate (E363D) altered not only the affinity but also the size selectivity of the binding site, as if these acidic residues coordinate divalent cations directly [6]. In a previous study using catfish olfactory CNG channel, Root and MacKinnon [7] also showed that Glu residues (Glu333 in catfish channel) from each subunit may form two identical but independent proton-binding sites within the ion conduction pore of the channel.

In this study, the effects of extracellular protons on the permeation characteristics and the divalent cation blockade of bovine retinal CNG channel were investigated. The reductions in macroscopic current were highly correlated with ex-

ternal proton concentrations without much effect on open probabilities. We also found that the extracellular protons strongly influenced the binding of extracellular divalent cations to the channel pore. The affinity of Sr^{2+} to the channel decreased as the proton concentration increased as if the proton and the divalent cation share the same binding site and the neutralization of the site reduces the electrostatic interaction between ions and the binding site. The results of the present study indicate that the extracellular proton binds to the acidic residue at position 363 in bovine retinal CNG channel and interferes with both the permeation of Na^+ and the coordination of divalent cations.

2. Materials and methods

2.1. Materials

The cDNA of the cGMP-gated channel α -subunit from bovine retina (Goulding et al., 1992) was subcloned into the pGH vector containing 5'- and 3'-untranslated regions of the *Xenopus* β -globin gene for high-level expression [4]. The construction of the E363N, E363G and E363D mutations was previously described [7].

2.2. Expression of CNG channels

The wild-type and the mutant channels were expressed in *Xenopus* oocytes for electrophysiological studies as described previously [4]. Complementary RNAs for the wild-type and mutant CNG channels were synthesized in vitro according to published methods. Oocytes were injected into collagenase treated *Xenopus laevis* oocytes (stage V–VI) in 50 nl of water. Injected oocytes were incubated at 18°C for 1–5 days in ND96 solution containing (in mM) 5 HEPES, 96 NaCl, 2 KCl, 1.8 CaCl_2 , 1 MgCl_2 , and 50 $\mu\text{g/ml}$ gentamicin, pH 7.6 (NaOH).

2.3. Electrophysiological recording

Ionic currents carried by CNG channels were recorded from the devitellinized patches of oocyte membrane in the outside-out configuration. Glass electrodes (Drummond) were firepolished to a resistance of 1–3 M Ω and coated with beeswax, and connected to Axopatch 200A using a CV201 headstage or Axopatch 200B using a CV203BU. Signals were filtered at 1 or 2 kHz using a four-pole low pass Bessel filter and digitized at the rate of 10 points/ms using a Microstar 3200e analog to digital converter (Microstar, Sioux City, SD, USA) or Digidata 1200A (Axon Instruments, Foster City, CA, USA).

For macroscopic current recordings, the membrane was held at 0 mV and ramped from -100 to 100 mV over 820 ms. Membrane patches with current between 0.5 and 2 nA (at 100 mV) were used for experiments. Otherwise, both intracellular (pipette) and extracellular (bath) solutions contained the following components unless specified otherwise (in mM): 130 NaOH, 3 HEPES (3 MES in low pH buffers), 0.5 $\text{Na}_2\text{-EDTA}$; both solutions were titrated to pH 7.6 using concentrated HCl. Cyclic GMP was added to the intracellular solution at 0.5 mM before the final pH adjustment. External solutions at various pHs (7.0–9.0) were prepared as bath solution but final pH values were adjusted. For the external divalent solutions, SrCl_2 was added to give the desired free concentration calculated using pH and the stability constant of Sr-EDTA , 8.68 [5]. Effects of external divalent cations and external protons were measured by superfusing the extracellular face of the membrane patch with solutions, each containing different con-

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Abbreviations: CNG, cyclic nucleotide-gated; cGMP, guanosine 3',5'-cyclic mononucleotide; P-region, pore-forming region; Asp, aspartate; Glu, glutamate; Gly, glycine

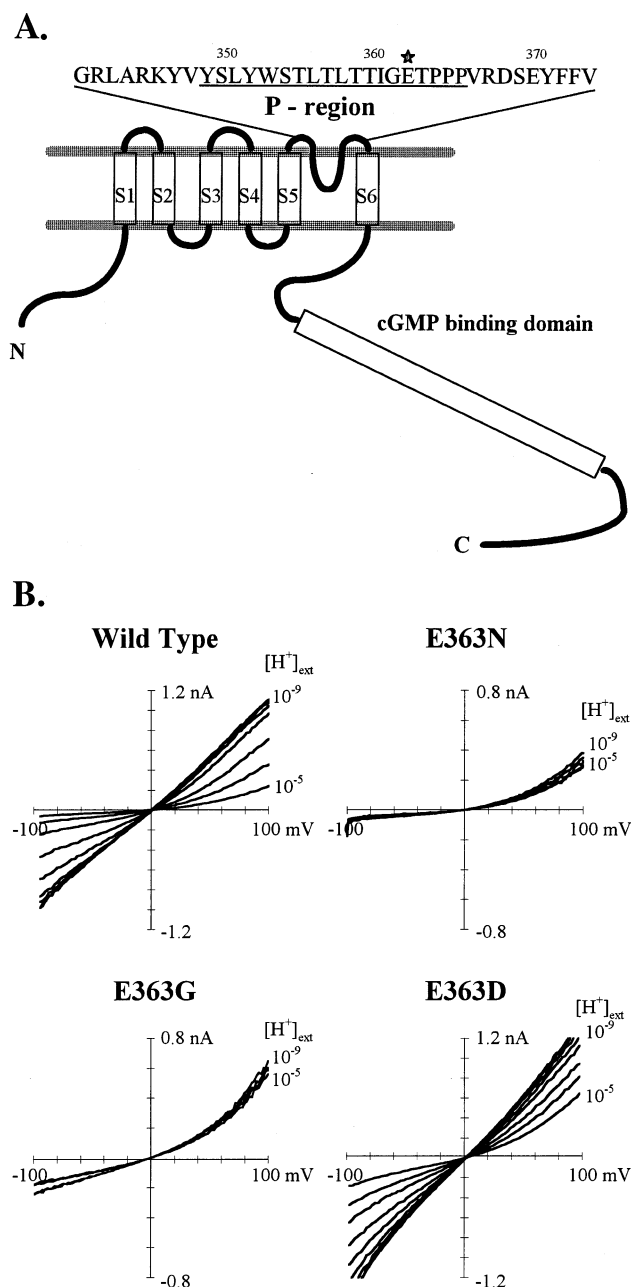


Fig. 1. Membrane topology and effects of extracellular protons on macroscopic currents of CNG channels. A: The proposed membrane topology and amino acid sequence of the S5–S6 linker region in a single α -subunit of the bovine retinal CNG channel are shown. Glu363 in the P-region is marked with a star. B: Macroscopic currents of the wild-type and E363N, E363G, and E363D mutant channels in outside-out membrane patches were blocked as the extracellular proton concentration was raised from 10^{-9} to 10^{-5} . The pipette contained $500 \mu\text{M}$ cGMP to activate the channels. Membrane voltage was held at 0 mV and ramped from -100 to 100 mV over 820 ms . The records shown are averages of five individual ramps.

centrations of Sr^{2+} and/or different pH. All patch-clamp experiments were carried out at room temperature (25°C).

3. Results and discussion

The effects of extracellular protons on Na^+ current through

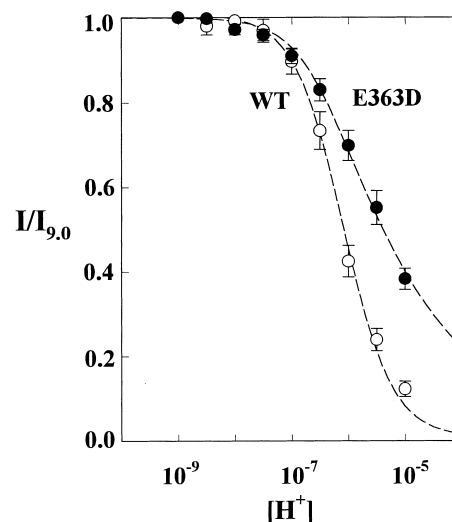


Fig. 2. Proton titration of wild-type, E363G, E363N and E363D mutant channel currents. The fraction of unblocked current (I/I_0) of wild-type (open circle), E363G (open triangle), E363N (filled triangle) and E363D (filled circle) measured at $+30 \text{ mV}$ was plotted as a function of the extracellular proton concentration. The data of wild type and E363D were fit to the equation $I/I_0 = (1 + [H^+]^n/K_{1/2}^n)^{-1}$ with the blocking constant $K_{1/2}$ and the Hill coefficient n . The corresponding data for wild type and E363D were $K_{1/2} = 10^{-6.1}$ ($n = 0.94$) and $K_{1/2} = 10^{-5.4}$ ($n = 0.60$), respectively.

wild-type and two different mutant CNG channels are shown in Fig. 1. As the proton concentration of extracellular solution was increased from 10^{-9} to 10^{-5} M , the macroscopic current of the wild-type channel decreased gradually (Fig. 1B).

To confirm whether the effect of extracellular proton was mediated by a conserved Glu residue within the pore-forming region, several different mutant channels were constructed and the currents were measured at various extracellular pHs. The current expression of mutant channels (E363N and E363G) were lower than that of wild type and the channel currents showed an outwardly rectified I–V relationship, as previously reported [1,7]. When the Glu residues were replaced for neutral Asn or Gly, both the magnitude and the shape of the

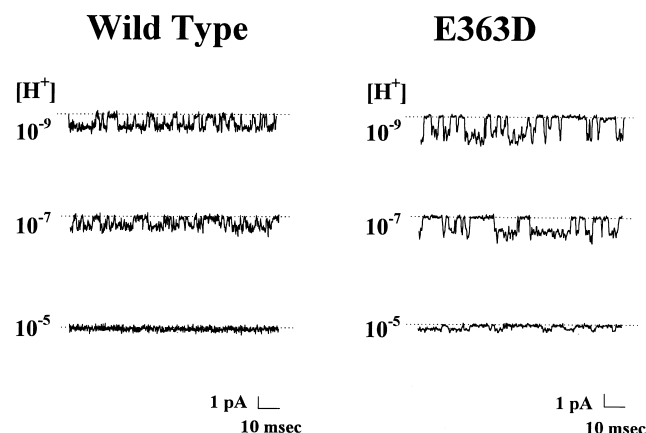


Fig. 3. Single channel current of CNG channels recorded at various extracellular pHs. Single channel currents were recorded for wild-type and E363D mutant channels in the presence of different extracellular proton concentration. The membrane voltage was held at -80 mV . Dotted lines indicate the closed state of channels and individual openings are shown as downward deflections.

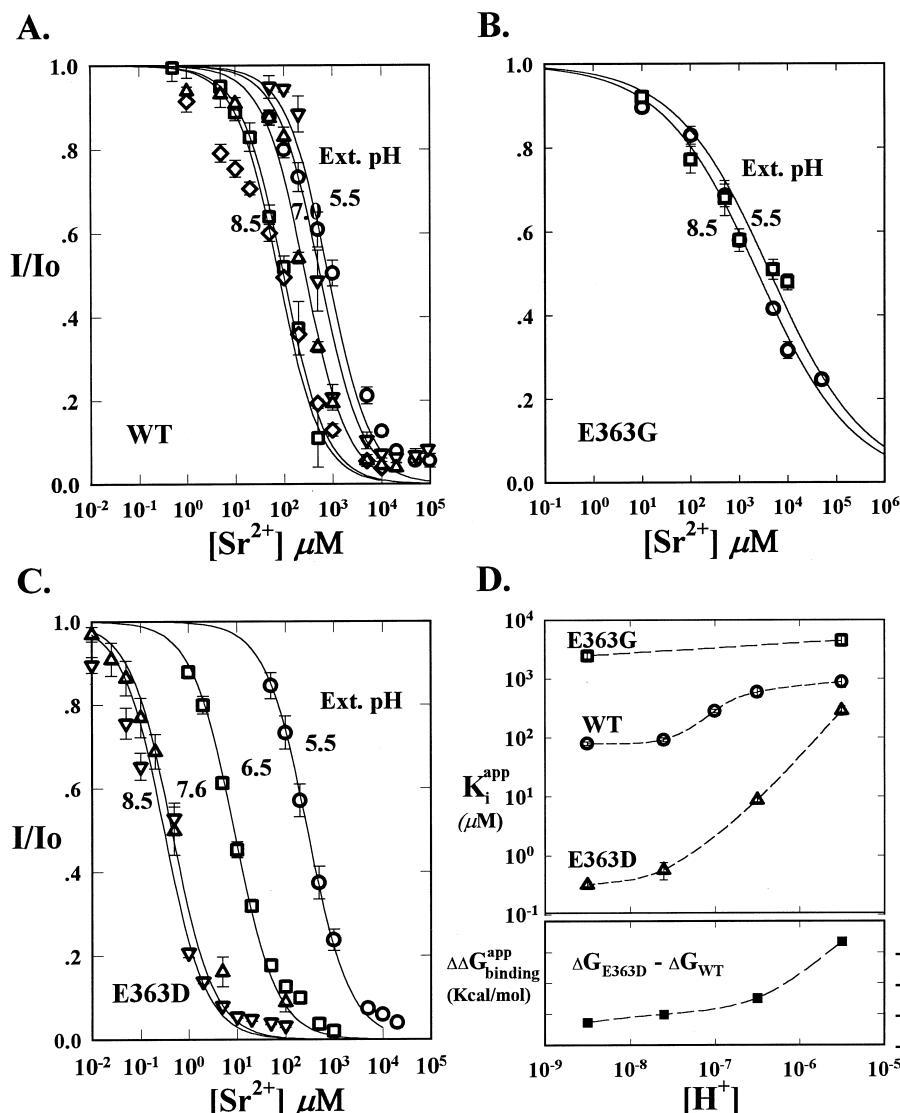


Fig. 4. Divalent cation blockade measured at various extracellular proton concentrations and effects of extracellular protons on the affinity of wild-type and E363D mutant channel to Sr^{2+} . The fraction of unblocked current (I/I_0) at +30 mV was plotted as a function of the divalent cation concentration for wild type (A), E363G (B) and E363D (C). The data were fit to the equation $I/I_0 = (1 + [Sr^{2+}]^n/K_{1/2}^n)^{-1}$ with Hill coefficients (n): wild type and E363D, 1; E363G, 0.44; and half-blocking constants ($K_{1/2}$): wild type 78.9 ± 5.4 (pH 8.5; diamond), 91.3 ± 7.3 (pH 7.6; square), 279.0 ± 20.0 (pH 7.0; triangle), 583 ± 106.89 (pH 6.5; down triangle), 870.7 ± 179 (pH 5.5; circle); E363G, 4447.1 ± 757.19 (pH 5.5; circle), 2444.34 ± 285.66 (pH 8.5; square); E363D, 0.317 ± 0.0420 (pH 8.5; filled down triangle), 0.568 ± 0.185 (pH 7.6; filled triangle), 8.71 ± 0.630 (pH 6.5; filled square), 284.7 ± 53.0 (pH 5.5; filled circle). D: Half-blocking concentration ($K_{1/2}$) values of Sr^{2+} measured at +30 mV were shown for the wild-type (circle), E363G (square) and the E363D mutant (triangle) channel as a function of the extracellular proton concentration. Bottom: The difference in apparent free energy of binding ($\Delta\Delta G_{app} = RT \ln K_{1/2}(E363D)/K_{1/2}(WT)$) of the wild-type vs. the mutant channel is shown for each proton concentration.

macroscopic channel current were not significantly altered. However, when the glutamate residue was replaced with another acidic residue, aspartate, the macroscopic current of the E363D mutant channel was greatly reduced in the same range of extracellular pH. It was noteworthy that the rectified I–V curve of both the wild-type and E363D channel obtained at low pH (presumably the I–V curve of fully protonated channel) resembled that of the neutral mutants, E363N and E363G.

Although the effects of extracellular protons on E363D mutant channels appeared to be similar to those on the wild-type channel, there were clear quantitative differences (Fig. 2) ($K_{1/2} = 10^{-6.1}$ M for wild type and $10^{-5.4}$ M for E363D, Hill coefficient for E363D: $n = 0.60$ and for wild

type: $n = 0.94$). The current reduction at low extracellular pH was due to the reduction of single channel current amplitude of the mutant channel without significant alteration in channel gating (Fig. 3). The apparent pK_a s of the wild type (6.1) and E363D (5.4) were significantly higher than those of Glu and Asp (~ 4.4). This discrepancy in pK_a values can be understood if the negative charges (possibly four) within the pore significantly increase the effective concentration of protons near the external entryway of CNG channel. Alternatively, in analogy with catfish olfactory CNG channels [8], the Glu or Asp residues of bovine retinal CNG channel may also form pairs of carboxyl-carboxylate which can have an anomalously high pK_a .

To investigate the effects of proton titration on the binding

of extracellular divalent cations to this site, we measured the reduction of macroscopic current of the wild-type and mutant channels by extracellular Sr^{2+} at various proton concentrations. In a previous study, it was shown that a conserved mutation of Glu to Asp at the 363 position greatly increased external divalent cation affinity and altered the selectivity profile of the channel pore [6]. Fig. 4 shows the blocking curve of external Sr^{2+} measured at +30 mV for the wild-type (Fig. 4A) and the mutant channels (Fig. 4B and C). As the extracellular proton concentration was increased from $10^{-8.5}$ to $10^{-5.5}$, the affinities for Sr^{2+} on both channels were decreased but the effects were quantitatively quite different. In Fig. 4D, the affinities of Sr^{2+} (shown as apparent inhibition constants, K_i^{app}) on the wild-type, E363G and E363D channels are plotted against the external proton concentration. In the case of E363D, the binding affinity for divalent cations decreased more than 1000-fold while wild type decreased only a few-fold over three orders of magnitude of proton concentration and E363G channel showed only about two-fold decrease. As a result, all the channels exhibited similar affinities for Sr^{2+} at low pH as if the favorable electrostatic interaction (e.g. negative $\Delta\Delta G$) between a divalent cation and the negatively charged binding site disappeared (Fig. 4D, bottom). Therefore the binding energies of the wild-type and the E363D channel to divalent cations depended on the extracellular pH and thus the extent of protonation of the acidic residues in the channel pore.

In this study, we were able to show that extracellular protons effectively reduced the macroscopic current of bovine retinal CNG channel. An acidic residue at position 363 within the pore-forming region of the channel was responsible for this effect. The reduction of channel current appeared to be the result of a specific blockade rather than an influenced channel gating and the detailed characteristics of proton blockade were sensitive to the exact nature of the acidic res-

idue at position 363. The large difference in the binding affinity of the wild-type and E363D channel for Sr^{2+} disappeared at high concentrations of protons, suggesting that a negative charge(s) is required for the high-affinity binding of Sr^{2+} to the external divalent cation site created in E363D [6]. Thus, when fully protonated, divalent cations no longer interact favorably with a binding site composed of Asp residues over Glu residues.

In conclusion, the protonation state of the acidic residues in the channel pore determines the permeation characteristics of the CNG channel such as ion conduction and blockade. This study underscores the importance of the conserved Glu residue in the channel pore for the conduction properties of CNG channels.

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