Molecular determinants of a Ca^{2+} -binding site in the pore of cyclic nucleotide-gated channels: S5/S6 segments control affinity of intrapore glutamates

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Cyclic nucleotide-gated (CNG) channels play an important role in Ca^{2+} signaling in many cells. CNG channels from various tissues differ profoundly in their Ca^{2+} permeation properties. Using the voltage-dependent Ca^{2+} blockage of monovalent current in wild-type channels, chimeric constructs and point mutants, we have identified structural elements that determine the distinctively different interaction of Ca^{2+} with CNG channels from rod and cone photoreceptors and olfactory neurons. Segments S5 and S6 and the extracellular linkers flanking the pore region are the only structural elements that account for the differences between channels. Ca^{2+} blockage is strongly modulated by external pH. The different pH dependence of blockage suggests that the pK_a of intrapore glutamates and their protonation pattern differ among channels. The results support the hypothesis that the S5–pore–S6 module, by providing a characteristic electrostatic environment, determines the protonation state of pore glutamates and thereby controls Ca^{2+} affinity and permeation in each channel type.

Keywords: cAMP/cGMP/olfactory neurons/
photoreceptors/signal transduction

Introduction

Cyclic nucleotide-gated (CNG) channels are non-selective cation channels that conduct both mono- and divalent cations. Ca^{2+} entry through CNG channels in rod and cone photoreceptors and olfactory neurons provides an intracellular Ca^{2+} signal that is important for both excitation and adaptation (for a review, see Finn *et al.*, 1996). In order to permeate, Ca^{2+} must bind to a site inside the pore and thereby blocks the current of more permeant Na^+ and K^+ ions in a voltage-dependent fashion (for reviews, see McNaughton, 1990; Zufall *et al.*, 1994; Kaupp, 1995; Finn *et al.*, 1996). A glutamate (Glu) residue in the pore loop (P) of the CNG channel α -subunit from rod photoreceptors has been recognized as an important structural element of Ca^{2+} binding. When this Glu is replaced by a neutral amino acid, Ca^{2+} blockage is almost

entirely abolished (Root and MacKinnon, 1993; Eismann *et al.*, 1994). Because a Glu residue is provided by each α -subunit in homo-tetrameric channels (Gordon and Zagotta, 1995; Liu *et al.*, 1996), a set of four glutamate residues probably forms the high-affinity intrapore Ca^{2+} -binding site. The four Glu carboxylates form two identical non-interacting acidic sites in channels composed of olfactory α -subunits (Root and MacKinnon, 1994). Protonation of these sites produces two substates of smaller conductance. Various homomeric CNG channels distinctively differ in their sensitivity to blockage by extracellular Ca^{2+} (Frings *et al.*, 1995), implying that the sites inside the pore bind Ca^{2+} with different affinity. The key glutamate is conserved in the pore loops of all known CNG channel α -subunits, suggesting that different affinities arise from other residues that either participate directly in the binding of Ca^{2+} ions or modify the geometric arrangement of glutamates.

We used the voltage-dependent blockage of CNG channels by extracellular Ca^{2+} as a sensitive monitor to map sites in CNG channels that control Ca^{2+} affinity. To our surprise, sequence variations in the pore loop itself are not responsible for the differences between CNG channel types. Instead, we identify transmembrane segments S5 and S6 and the cytoplasmic linkers that connect these segments with the pore loop as important determinants of Ca^{2+} affinity. The efficacy of Ca^{2+} blockage is strongly reduced at low pH, consistent with the idea that protonation of intrapore glutamates is an important control mechanism of Ca^{2+} binding. The characteristic differences in Ca^{2+} blockage itself and its dependence on pH can be transferred between channels by transplanting the S5–P–S6 region. These results provide evidence that the S5–P–S6 region defines a pore module that determines the affinity of the intrapore Ca^{2+} -binding site by producing a characteristic dielectric environment that sets the state of protonation of intrapore glutamate residues. In the accompanying paper (Dzeja *et al.*, 1999), we demonstrate that changes in Ca^{2+} blockage are associated with changes in Ca^{2+} permeation in a simple and predictable way. These findings are likely to hold for other CNG channels and, therefore, provide a quantitative framework to assess Ca^{2+} permeation in CNG channels of different subunit composition and stoichiometry.

Results

Intrapore Ca^{2+} -binding sites differ among channels

Interaction of Ca^{2+} with CNG channels was studied by measuring the blockage of monovalent current by extracellular Ca^{2+} ions as illustrated in Figure 1 for the α -subunit of the rod CNG channel and described in Materials and methods. The voltage dependence of Ca^{2+}

Fig. 1. Blockage of the CNG channel α -subunit from rod photoreceptors by extracellular Ca^{2+} . (A) Current–voltage (I – V_m) recordings obtained from outside-out patches of *Xenopus* oocytes at different concentrations of Ca^{2+} in the bath and 1 mM cGMP in the pipette. $[\text{Ca}^{2+}]_o$ is in μM as indicated on the left and right of current traces. (B) Voltage dependence of the ratio of unblocked current I/I_{max} derived from the I – V_m recordings of (A). (C) Dose dependence of Ca^{2+} blockage (I/I_{max}) at -70 mV. The solid line represents a fit of the Hill equation to the data from (B). The constant K_i of half-maximal inhibition was $5.6 \mu\text{M}$ and the Hill coefficient n was 1.0. (D) Dependence of inhibition constant K_i on voltage. Average values and SD from nine experiments. Inset: voltage dependence of K_i on an expanded scale to illustrate the position of V_c . (E) Dependence of the Hill coefficient n on voltage. Average values and SD from nine experiments.

blockage is characteristic of a positively charged, permeable channel blocker: starting from $+80$ mV, blockage efficacy increased, when V_m was made less positive, because access of Ca^{2+} to the blocking site in the channel lumen was facilitated (Figure 1B). At a characteristic voltage, V_c , Ca^{2+} blockage became maximal (Figure 1D, inset arrow) and was relieved again at values more negative than V_c (Figure 1B and D). The relief of blockage has been interpreted as facilitation of Ca^{2+} permeation (Colamartino *et al.*, 1991; Frings *et al.*, 1995). The dependence of blockage on $[\text{Ca}^{2+}]_o$ was consistent with binding of Ca^{2+} to a single site, because dose–response relationships were well described by a simple binding isotherm (Figure 1C). The concentration of half-maximal blockage (K_i) progressively decreased from $220 \mu\text{M}$ at $+80$ mV to $3.3 \mu\text{M}$ at $V_c = -36.5$ mV, which reflects the increase of the apparent Ca^{2+} affinity of the intrapore binding site (Figure 1D). Hill coefficients were unity over the entire voltage range (± 80 mV) (Figure 1E), suggesting that a single Ca^{2+} ion is sufficient to occlude the pore. In utilizing this binding formalism, we treat the blocking process as if it were in equilibrium; however, Ca^{2+} ions escape to the cytoplasm and, therefore, the measured K_i constants represent only approximations of the true equilibrium constants.

The CNG channels from rod (bR) and cone (bC) photoreceptors, olfactory neurons (bO) and *Drosophila melanogaster* (Dm) are set apart from each other by characteristic values for V_c and K_i (-80 mV). The large

differences among CNG channels are illustrated by the channels from retinal rods (bR) and *Drosophila* (Dm) (Figure 2A). High-affinity Ca^{2+} sites, i.e. low K_i values, are correlated with more negative values of V_c (bR; $K_i = 7.4 \mu\text{M}$, $V_c = -36.5$ mV), whereas low-affinity Ca^{2+} sites, i.e. high K_i values, are associated with less negative values for V_c (Dm; $K_i = 352 \mu\text{M}$, $V_c = \sim 0$ mV). The K_i and V_c values from cone and olfactory CNG channels are intermediate between those of the channels from rod photoreceptors and *Drosophila* (Figure 2B). The interdependence of K_i and V_c is consistent with the idea that changes in the free energy necessary to leave the pore through the cytoplasmic exit are larger for high-affinity compared with low-affinity sites and, consequently, facilitation of Ca^{2+} permeation requires a higher driving force, i.e. more negative V_m values. The K_i and V_c values are characteristic of each subclass of CNG channels, because members from other species display the same properties as their respective bovine orthologs (Figure 2B). We will show below that not all changes in K_i necessarily result in changes in V_c ; this result will advance our insight into the molecular interaction of Ca^{2+} with the pore of CNG channels.

Woodhull model of blockage

In order to illustrate the differences and similarities among CNG channels in more quantitative terms, we developed a theoretical framework to describe the voltage dependence of Ca^{2+} blockage. The model is based on a similar analysis

Fig. 2. Differences in Ca^{2+} blockage between distinct CNG channels. (A) Comparison of the voltage dependence of the inhibitory constant K_i from α -subunits of rod photoreceptors (bR) and *Drosophila* (Dm). (B) Comparison of average values and SD (number of experiments in parentheses) for K_i (at -80 mV) (left panel) and V_c (right panel) from α -subunits of rod and cone photoreceptors, olfactory neurons and *Drosophila*. Dm, *Drosophila* (Baumann *et al.*, 1994); rO, rabbit OSN (Biel *et al.*, 1993); bO, bovine OSN (Ludwig *et al.*, 1990); cP, chicken pineal (Bönigk *et al.*, 1996); cC, chicken cone (Bönigk *et al.*, 1993); bC, bovine cone (Weyand *et al.*, 1994); cR, chicken rod (Bönigk *et al.*, 1993); bR, bovine rod (Kaupp *et al.*, 1989).

that originally has been used to describe the proton block of Na^+ channels (Woodhull, 1973). The model assumes a simple two-barrier model with a single binding site. The energy minimum and the energy maxima are localized at fixed positions part way across the electric field (Figure 3A). In order to reach the binding site from either side of the membrane, Ca^{2+} ions need to cross one of these energy barriers. The rate constants of binding (k_1 , k_{-2}) and unbinding (k_{-1} , k_2) determine the kinetics of the blocking process and are assumed to be voltage dependent (as in the Eyring rate theory).

A number of conditions need to be fulfilled in order to apply this model. (i) When Ca^{2+} occupies the binding site, monovalent cations cannot pass through the channel. This condition is fulfilled: in fact, in the accompanying paper (Dzeja *et al.*, 1999), it is shown that all CNG channels investigated here carry pure Ca^{2+} currents at millimolar concentrations of external Ca^{2+} in the presence of physiological Na^+ concentrations. (ii) The channels must not change their open probability, P_o , upon transition from the unblocked to the Ca^{2+} -blocked state. Information on the P_o of CNG channels in the presence of external divalent cations is sparse due to the small single-channel conductance under these conditions. For native olfactory CNG channels, Kleene and co-workers using noise analysis showed that, at saturating cAMP concentrations, P_o is not significantly different with and without millimolar external divalents (Kleene, 1997; Larsson *et al.*, 1997). Since all our experiments were carried out at saturating concentrations of cGMP (1 mM), we are confident that changes in P_o do not compromise the applicability of the model. (iii) Currents carried by Ca^{2+} must be negligibly small compared with the current carried by monovalent cations. From our blocking data and from measurements of Ca^{2+} currents in high Ca^{2+} (Menini, 1990; Frings and Lindemann, 1991; Baumann *et al.*, 1994; Dzeja *et al.*, 1999), we can safely conclude that $<5\%$ of monovalent current can be carried by Ca^{2+} under our conditions.

In Materials and methods, a detailed description of the parameters and the equations is given. In brief, the model uses six independent parameters: three parameters characterize the fraction of the electric field that is effective at the respective energy minima and maxima (δ , ξ , ζ , see Figure 3A). The 'effective valence' z represents the number of blocking charges and indicates whether a single Ca^{2+}

ion blocks the channel ($z = 2$) or more than one ion is needed for blockage ($z > 2$). The combined parameters b_{-1}/b_1 and b_2/b_1 (see Materials and methods) represent the ratios of the voltage-independent terms of the rate constants and are determined by the relative heights of the corresponding barrier energy U_i (Figure 3A). We fit Equation 4 (see Materials and methods) to the voltage dependence of unblocked currents I/I_{max} for bR (Figure 3C), bC (Figure 3D) and bO (Figure 3E). To test how sensitively the model responds to changes in parameter values, we varied z , δ , ξ and ζ independently and plotted the resulting norm value (square root of the sum of squares of the residuals) for each parameter (Figure 3B). In all channel types, the most sensitive parameters were δ and z , whereas perturbation of ξ and ζ resulted in rather small changes in the norm value. This analysis indicates that the values of δ and z are quite reliably determined by the model, while the other parameters should be regarded as good approximations.

Which information can be gained from this theoretical model? (i) A single binding site is sufficient to describe Ca^{2+} blockage in CNG channels. (ii) In all channel types, the 'effective valence' z of the blocking process adopts values of ~ 2 (2.4 for bR, 2.1 for bC and 2.3 for bO), demonstrating that a single Ca^{2+} ion is sufficient to occlude the pore. This result supports the interpretation of the analysis of the concentration dependence of Ca^{2+} blockage (Figure 1C–E). (iii) The position of the energy minimum within the electric field, i.e. the fraction of membrane voltage acting at the binding site, is very similar in all channels (0.44 for bR, 0.40 for bC, 0.38 for bO). (iv) Using the values b_{-1}/b_1 and b_2/b_1 , it is possible to derive a quantitative measure of Ca^{2+} permeation: the probability for exit of Ca^{2+} to the cytoplasmic side is given by $P_{\text{in}} = k_2/(k_2 + k_{-1})$. For $V_m = 0$ mV, the voltage-dependent terms of the rate constants disappear, therefore $k_2 = b_2$ and $k_{-1} = b_{-1}$ (see Materials and methods).

$$P_{\text{in}} = \frac{k_2}{k_2 + k_{-1}} = \frac{b_2}{b_2 + b_{-1}} = \frac{\frac{b_2}{b_1}}{\frac{b_2}{b_1} + \frac{b_{-1}}{b_1}}$$

Using values for b_2/b_1 and b_{-1}/b_1 from the fit of the model to the data, we obtain values for P_{in} of 0.03 (bR),

Fig. 3. Woodhull model of Ca^{2+} blockage in three different CNG channels. (A) Parameters of the energy profile (see Materials and methods). (B) Perturbation of z , δ , ζ and ξ for bR (top), bC (middle) and bO (bottom). The norm value $\sqrt{\Sigma(r^2)}$ (r represents the residual, which is the difference between the observed and predicted $I/I_{\max}$ value) is plotted versus the perturbed parameter. (C) Fit of Equation 4 (●, see Materials and methods) to the mean $I/I_{\max} - V_m$ relationship (traces) for the α -subunit from bovine rod photoreceptor (bR). $\delta = 0.44$; $\xi = 0.20$; $\zeta = 0.99$; $z = 2.4$; $b_{-1}/b_1 = 9.8 \times 10^{-6}$ M; $b_2/b_1 = 2.6 \times 10^{-7}$ M. (D) Fit as in (C) for the α -subunit from bovine cone photoreceptors (bC). $\delta = 0.4$; $\xi = 0.5$; $\zeta = 0.87$; $z = 2.1$; $b_{-1}/b_1 = 2.2 \times 10^{-5}$ M; $b_2/b_1 = 9.7 \times 10^{-6}$ M. (E) Fit as in (C) for the α -subunit from bovine olfactory neurons (bO). $\delta = 0.38$; $\xi = 0.58$; $\zeta = 0.99$; $z = 2.3$; $b_{-1}/b_1 = 2.0 \times 10^{-5}$ M; $b_2/b_1 = 8.3 \times 10^{-6}$ M.

0.31 (bC) and 0.29 (bO). The probability that a Ca^{2+} ion leaves to the cytoplasmic side is >10 -fold higher in olfactory or cone CNG channels compared with rod CNG channels. If we assume that the Ca^{2+} entry rates to the binding site from the external medium are similar in all CNG channels, this result predicts 10-fold higher rates of Ca^{2+} permeation in olfactory and cone CNG channels.

This prediction is confirmed in the accompanying paper (Dzeja *et al.*, 1999) by a quantitative analysis of Ca^{2+} permeation.

The S5-P-S6 segment constitutes a pore module

In the following, K_i and V_c values are used as sensitive monitors to identify regions or amino acid residues

responsible for the differences of Ca^{2+} blockage among CNG channels. The amino acid sequences of the pore region of CNG channels are highly conserved; the largest differences exist between CNG channels from rod photoreceptors and *Drosophila* (Figure 4A). In particular, the glutamate residue (E363 in the rod channel) that forms the intrapore Ca^{2+} -binding site (Root and MacKinnon, 1993; Eismann *et al.*, 1994) is conserved in all four CNG channel species (Figure 4A). Replacing four of six non-conserved residues in the pore region of the Dm channel by the respective amino acids found in the rod CNG channel leaves the Ca^{2+} blockage largely unaffected (Figure 4B). These observations strongly argue that the pore loop itself does not account for the differences between channels and that regions outside the pore loop co-determine blockage and permeation.

Chimeras were constructed between the rod and olfactory channels (Figure 4C) and between the rod and cone channels (Figure 4D) to identify these regions. Transplanting the olfactory S5–P–S6 segment (chimera $\text{R}_{\text{S4}}\text{O}_{\text{S6R}}$) to the rod channel also transfers the lower Ca^{2+} sensitivity to the host channel. Similarly, the cone S5–P–S6 segment in an otherwise pure rod channel background ($\text{R}_{\text{S4}}\text{C}_{\text{S6R}}$) and, vice versa, a rod S5–P–S6 segment in a cone channel background ($\text{C}_{\text{S4}}\text{R}_{\text{S6C}}$), yielded chimeras that exactly matched the blockage properties of the donor channel from which the segment has been derived (Figure 4D). A host of modifications outside the S5–P–S6 segment such as deletion of the N-terminal region of the rod channel, the exchange of the entire C-terminal domains that harbor the cyclic nucleotide-binding fold or the exchange of transmembrane segments S1–S4 did not affect Ca^{2+} blockage (Figure 4C and D). In conclusion, the S5–P–S6 segment from either rod, cone or olfactory CNG channels invariably reproduces in various host channels the blockage properties of the donor channel. The results identify the S5–P–S6 segment as the primary, if not only, structural element that determines Ca^{2+} blockage. Because the pore loops themselves cannot account for the different interaction with Ca^{2+} , the regions that set channels apart from each other must reside in S5 or S6 or in the extracellular linkers flanking the pore loop.

Structural determinants in the S5–P–S6 module are delocalized

To map the sites that control Ca^{2+} affinity in the S5–P–S6 segment, a series of chimeras were constructed between channels from rod and cone photoreceptors (Figure 4D). The S5 segment of a cone channel in a rod channel background ($\text{R}_{\text{S4}}\text{C}_{\text{S5R}}$) increased the average K_i from 7.4 μM (rod wild-type) to 17 μM , whereas the mirror construct, the rod channel S5 in a cone channel background ($\text{C}_{\text{S4}}\text{R}_{\text{S5C}}$), decreased the average K_i from 66.3 μM (cone wild-type) to 15 μM . In the same vein, the S6 segment from the rod channel transplanted to a cone channel background ($\text{C}_{\text{P0}}\text{R}_{\text{S6C}}$) enhanced Ca^{2+} blockage ($K_i = 26 \mu\text{M}$) compared with the cone wild-type channel, whereas in the mirror construct (the S6 segment from the cone channel transplanted to a rod channel, $\text{R}_{\text{P0}}\text{C}_{\text{S6R}}$), the efficacy of Ca^{2+} blockage was decreased ($K_i = 36 \mu\text{M}$). These results illustrate that, while the S5 and S6 segments each make a contribution to the Ca^{2+} affinity, either one

alone cannot account entirely for the phenotype of the respective wild-type channel.

CNG channels carry between the P loop and S6 a very short linker that consists of a few amino acid residues only, whereas the linker between S5 and the pore loop is much longer (~25 amino acid residues) (Figure 4A). Sequence variation in the S5–P–S6 module is particularly obvious in the linker regions. The influence of the S5/P-linker (amino acids 320–347 in the rod channel) was investigated with the chimeras $\text{C}_{\text{S5}}\text{R}_{\text{S6C}}$, $\text{C}_{\text{S5}}\text{R}_{\text{P0C}}$ and $\text{C}_{\text{P0}}\text{R}_{\text{S6C}}$. Chimera $\text{C}_{\text{S5}}\text{R}_{\text{S6C}}$ carries the S5/P- and P/S6-linker regions and S6 from the rod channel, whereas $\text{C}_{\text{P0}}\text{R}_{\text{S6C}}$ carries the P/S6-linker and S6 from the rod channel in a cone channel background. The linker-P-linker-S6 segment sufficed to reproduce almost completely the rod phenotype in a cone channel background ($K_i = 7 \mu\text{M}$, $\text{C}_{\text{S5}}\text{R}_{\text{S6C}}$), whereas chimera $\text{C}_{\text{P0}}\text{R}_{\text{S6C}}$, which is lacking the long S5/P-linker from the rod channel, displayed an average K_i value intermediate between rod and cone wild-type channels ($K_i = 27 \mu\text{M}$). Similarly, chimera $\text{C}_{\text{S5}}\text{R}_{\text{P0C}}$ which carries the S5/P-linker from rod in a cone channel background, or the mirror construct, $\text{R}_{\text{S5}}\text{C}_{\text{P0R}}$, which carries the respective S5/P-linker from the cone channel in a rod channel background, both displayed average K_i values, intermediate between those from the two wild-type channels (23.3 and 14.7 μM , respectively). Thus, the respective segments from the rod channel enhance Ca^{2+} blockage in a cone channel background, whereas the cone channel counterparts in a rod channel background lower the efficacy of Ca^{2+} blockage. Taken together, these results identify the S5 and S6 segments and the S5/P-linker as important determinants of blockage efficacy. Each of the three segments alone cannot fully account for the difference between rod and cone channels; instead the contributions from segments are additive to yield the respective phenotype of wild-type channels. The P/S6-linker is very short and its boundaries are ill defined; therefore, its contribution to pore properties has been examined by substitution of single amino acid residues.

Single amino acid substitutions

We exchanged several amino acids at positions in the S5–P–S6 segment where rod and cone CNG channels differ (see Figure 4A). In particular, we tested whether amino acid residues at the extracellular ends of S5 and S6, i.e. the interface between membrane and aqueous phase, affect Ca^{2+} blockage. The largest effects were observed by substituting bR(F374) or bC(L398) in the short P/S6-linker for the respective amino acid found in the donor channel (Figure 4E). Mutations at other positions of the P/S6-linker that differ among channels do not cause significant changes in K_i [bC(E395S), bR(R369Q)]. Notably, a quadruple mutant of the *Drosophila* CNG channel Dm (T318P/E320V/N321R/V323S), in which four positions from that linker region have been changed to the respective rod sequence, was indistinguishable from the *Drosophila* wild-type channel (Figure 4B).

Remarkably, mutations of S318 in bR, or the corresponding position A342 in bC, produced significant shifts in V_c and K_i (Figure 4E). Mutations at this position in the human rod CNG channel are associated with a rare form of retinitis pigmentosa (Dryja *et al.*, 1995). Because these mutants, when expressed in a cell line, rarely produced

Fig. 4. Identification of structural determinants of the Ca^{2+} blocking site in chimeric constructs and mutant channels. **(A)** Sequence alignment of the S5–P–S6 segment from α -subunits of rod (bR) and cone (bC) photoreceptors, olfactory neurons (bO) and *Drosophila* (Dm). Black and gray boxes indicate identical or conserved residues, respectively. **(B)** Average values of K_i (–80 mV) and V_c for single and quadruple mutations in the *Drosophila* CNG channel. The number of experiments is in parentheses on the right of the respective V_c error bar. **(C)** Average values of K_i (–80 mV) and V_c for chimeric constructs between bR and bO α -subunits. The closed and open pictograms refer to bR and bO sequences, respectively. **(D)** Average values of K_i (–80 mV) and V_c for chimeric constructs between bR and bC α -subunits. The closed and open pictograms refer to bR and bC sequences, respectively. **(E)** Average values of K_i (–80 mV) and V_c for mutant channels with single amino acid substitutions in bR and bC α -subunits.

Fig. 5. Dependence of Ca^{2+} blockage on extracellular pH. **(A)** Voltage dependence of Ca^{2+} blockage of the bO α -subunit, at different pH values. $[\text{Ca}^{2+}]_o$ was 50 μM . The traces represent the mean normalized current of four (pH 5.5), six (pH 6.5), four (pH 7.4), six (pH 8.2) and two (pH 9) experiments. **(B)** Voltage dependence of Ca^{2+} blockage of the bR α -subunit at different pH values. $[\text{Ca}^{2+}]_o$ was 50 μM . The traces represent the mean normalized current of two (pH 5.75), three (pH 6.25), three (pH 6.5) and four (pH 7) experiments. **(C)** Dependence of Ca^{2+} blockage on extracellular pH for bR (∇), bC ($\circ$), bO ($\bullet$) and chimera $\text{R}_{54}\text{C}_{56}\text{R}$ ($\blacksquare$). Data for bC represent mean normalized currents ($\pm$ SD) of seven (pH 5.5), six (pH 6.5), five (pH 7.1) and five (pH 7.8) experiments. Data for $\text{R}_{54}\text{C}_{56}\text{R}$ represent mean normalized currents ($\pm$ SD) of five (pH 5.5), one (pH 6), seven (pH 6.5), five (pH 7.1), six (pH 7.8) and two (pH 8.2) experiments. Solid lines represent a fit of the equation $I = (1 - I_{\min}) [H^+]^n / ([H^+]^n + K^n) + I_{\min}$ to the data. Parameter values were: $pK_a = 6.3$, $n = 1.96$, $I_{\min} = 0.01$ for bR; $pK_a = 6.5$, $n = 1.31$, $I_{\min} = 0.17$ for bC; $pK_a = 6.7$, $n = 1.02$, $I_{\min} = 0.26$ for bO; and $pK_a = 6.5$, $n = 1.22$, $I_{\min} = 0.08$ for $\text{R}_{54}\text{C}_{56}\text{R}$. $[\text{Ca}^{2+}]_o$ was 50 μM ; $V_m = -30$ mV.

functional channels, it has been suggested that a defect in channel assembly or targeting is causing the retinal degeneration (Dryja *et al.*, 1995). Three different mutants of the bovine ortholog, including the one that causes retinitis pigmentosa (S318F), showed robust expression. The V_c values of these mutants were shifted to more negative values (Figure 4E). The resting voltage of rod photoreceptors in the dark ($V_m = -35$ mV) coincides well with a $V_c = -36.5$ mV of maximal Ca^{2+} blockage of bR homomeric channels. A shift of V_c by up to -15 mV is expected to disturb Ca^{2+} entry into the rod photoreceptor cell. Consequently, alterations in cellular Ca^{2+} homeostasis might underlie the degeneration of retinal rods rather than assembly into non-functional channels.

In summary, the results from single amino acid substitutions conform with the chimera experiments in that no single locus in the S5–P–S6 module fully accounts for the difference between channels and that the contributions from several regions or positions are additive.

Dependence of Ca^{2+} blockage on pH

The stability of a complex between Ca^{2+} and pore glutamates will be determined by the spatial arrangement of carboxylic groups and their degree of protonation. The pore of olfactory CNG channels contains two titratable sites (pK_a of 7.6) that are formed by the intrapore Glu residues (Root and MacKinnon, 1994). Depending on the dielectric environment, pK_a values of acidic groups buried inside proteins can vary by several units (Fersht, 1985). Within this framework, it is plausible that the S5–P–S6 module may create in each CNG channel type a characteristic electrostatic environment and, thereby, control the pK_a of glutamate residues. As Ca^{2+} binding is weaker to protonated than to unprotonated carboxylic groups (Falke *et al.*, 1994), this hypothesis predicts that (i) Ca^{2+} blockage is weakened at low pH and (ii) the pH dependence differs among CNG channels.

These predictions were tested by examining the Ca^{2+} blockage at various pH values and fixed $[\text{Ca}^{2+}]_o$ (50 μM) (Figure 5). We were concerned that a change in pH on

both sides of the membrane might, in addition to titrating intrapore Glu residues, alter the gating properties of the channel. In fact, for native CNG channel from rod photoreceptor, it has been shown that internal H^+ attenuates channel current up to 30% when cGMP is used as ligand (Picco *et al.*, 1996). Because this effect is voltage independent, it does not affect our conclusions. Ca^{2+} blockage was suppressed at low pH and completely restored at high pH, as shown for the CNG channels of rod photoreceptors and olfactory neurons (Figure 5A and B). For each channel type, the sensitivity of Ca^{2+} blockage to changes in pH was different. In the rod CNG channel, the pH dependence of Ca^{2+} blockage was characterized by a pK_a of 6.3 and a Hill coefficient of 1.96 whereas, in the olfactory channel, the pK_a was 6.7 and the Hill coefficient was 1.0 (Figure 5C). The cone CNG channel displayed a pH dependence that was intermediate between rod and olfactory channels (Figure 5C). Most significantly, the pH dependence of the chimera $\text{R}_{54}\text{C}_{56}\text{R}$, which carries a S5–P–S6 module of cone in a rod CNG channel background, is largely similar to that observed for the cone wild-type channel, except for a smaller residual current at $\text{pH} \geq 7.5$. Both channels have a pK_a of 6.5 and Hill coefficients of 1.3 ($\text{R}_{54}\text{C}_{56}\text{R}$) and 1.2 (bC) (Figure 5C). These findings suggest that, indeed, the pK_a values of pore Glu differ between CNG channels. It is therefore plausible that, at a given pH, different Ca^{2+} affinities result from different protonation states. However, protonation patterns alone cannot fully account for the differences observed between channels. Although the blocking efficacy is strongly dependent on external pH, values for V_c are practically independent of pH (see Figure 5A and B). As a consequence, it is not possible to turn one blocking phenotype into another by simply changing the pH. Probably coordination of Ca^{2+} by negatively charged Glu $^-$ residues varies slightly between channels, and V_c is a measure of this structural difference in the geometric arrangement of the Ca^{2+} –Glu complex in the pore. Since V_c is not changed by protonation, we speculate that, in order to bind to Glu residues, Ca^{2+} ions have to displace

Fig. 6. pH dependence of current through bR and mutant E363D. (A) pH dependence of K^+ currents in bR ($\circ$) and the bR(E363D) mutant ($\blacktriangle$) measured from outside-out patches of *Xenopus* oocytes (pipette solution was kept at pH 7.4). (B) Voltage dependence of K_i of Ca^{2+} blockage for wild-type bR and the mutant E363D. Inset: voltage dependence of K_i for the mutant E363D on an expanded scale to illustrate the position of V_c (arrow).

H^+ from the intrapore site and that the geometric arrangement of the Ca^{2+} –Glu complex, once formed, is very similar at low and high pH and is characteristic for each channel type.

Variation in side chain length at position 363 of bR

To address experimentally alterations in the geometry of the negatively charged residues, we examined the Ca^{2+} blockage of the mutant E363D of bR. This exchange preserved the negative charge but the side chain of Asp compared with Glu is shorter by one CH_2 group. The pH dependence of K^+ currents through wild-type and mutant channels was largely similar (Figure 6A), indicating that the protonation patterns of glutamates in the wild-type and of aspartates in the E363D mutant are also similar; therefore, any differences in blockage might be attributed to the different length of the amino acid side chains. In fact, the E363D mutant displayed an ~ 5 -fold higher Ca^{2+} sensitivity at -80 mV ($K_i = 1.3$ μM compared with 7.4 μM of wild-type) and almost 30-fold at $+80$ mV ($K_i = 8$ μM compared with 220 μM of wild-type) (Figure 6B). In addition, V_c of the E363D mutant shows a small but significant (t -test: $P = 0.028$) shift to more negative potentials (-40 ± 0.7 mV, see Figure 6B, inset), indicating that the change in side chain length and the consequent change in the geometry of the binding site leads to an altered stability of the Ca^{2+} –Asp $^-$ complex.

Discussion

The pore contains a single blocking site

The analysis of inhibition of monovalent currents by Ca^{2+} reveals a single blocking site inside the pore. This conclusion is supported by two independent measures, namely the Ca^{2+} dependence of blockage at constant V_m (Figure 1C–E) and the voltage dependence of blockage at fixed Ca^{2+} (Figure 3). In two previous studies, Hill coefficients of 2.7 (Park and MacKinnon, 1995) and 0.9–2.3 (Eismann *et al.*, 1994) have been reported for the Ca^{2+} blockage of the α -subunit from rod photoreceptor and taken as evidence that the pore is occupied by at least two Ca^{2+} ions. In the present study, whenever Hill coefficients >1 were observed, subsequent control of the

free $[Ca^{2+}]$ in buffer solutions uncovered deviations from calculated values, indicating that large Hill coefficients are artifacts resulting from improper control of $[Ca^{2+}]$. Our results further show that the binding site, at least at low $[Ca^{2+}]_o$, is occupied by only one Ca^{2+} ion at a time. We cannot rule out, however, double occupancy of the Glu site at high $[Ca^{2+}]_o$, as has been suggested for voltage-activated Ca^{2+} channels (see below). In fact, in the accompanying paper (Dzeja *et al.*, 1999), we show that key aspects of Ca^{2+} permeation are determined by this intrapore site for ion binding and that the permeation process may require that more than one Ca^{2+} ion populates the pore.

The S5–P–S6 region represents a basic pore module

A striking finding of this study is that when the S5–P–S6 segment is exchanged between different channels, all aspects of Ca^{2+} blockage are transferred from one channel to another, whereas swapping of domains outside this region has only marginal effects on Ca^{2+} blockage. These results identify the S5–P–S6 region as a basic pore module that governs key properties of ion permeation, consistent with a model in which a ring of four S5–P–S6 modules, one from each subunit, forms the inner core of the channel. The pore loops extend towards the central axis of the channel, much like the blades of an iris-like structure (Sun *et al.*, 1996), whereas the S5/S6 segments shield the pore interior from the surrounding S1–S4 segments. Shaker-type channels, like CNG channels, encompass six transmembrane-spanning regions. For K^+ channels, the concept of a basic pore module is well established. In fact, a number of K^+ channels have been identified, with only two transmembrane segments flanking a central pore loop (TM1–P–TM2). Recently, the first high-resolution structure of such a channel from *Streptomyces lividans* has been solved by X-ray crystallography (Doyle *et al.*, 1998). Moreover, the similarity of pore structures from this bacterial channel and the Shaker K^+ channel is suggested by similar binding of scorpion toxins to the extracellular orifice of the two channel types (MacKinnon *et al.*, 1998). Our results support the notion that CNG channels are organized in a modular way similar to K^+

channels and underscore the structural similarity between CNG channels and the family of voltage-gated K^+ channels (Jan and Jan, 1990; Heginbotham *et al.*, 1992; Sun *et al.*, 1996).

The pore loops of CNG channels themselves are not responsible for the differences among channels. Instead, several amino acid residues at the extracellular end of the S5 and S6 segments, located near the membrane–water interface, as well as the linkers that connect these segments with the pore loop, determine how strongly Ca^{2+} interacts with the pore. The string of residues at the transition between membrane and extracellular space could serve as a flexible ‘suspension’ that fastens the pore loop proper to the transmembrane segments. Such a ‘suspension’ model provides a simple molecular explanation for variations of Ca^{2+} affinity. The pore ‘suspension’ may control the geometric arrangement of glutamates in the pore vestibule and at the same time their degree of protonation. Thus different pK_a values of intrapore glutamates may underlie the large differences in Ca^{2+} binding.

Protonation of the intrapore site controls Ca^{2+} blockage

The negative charge at the intrapore ion-binding site controls ion permeation and blockage of CNG channels. Manipulation of the intrapore charge density has been shown to result in a change in the interaction between binding site and ions: protonation of Glu gives rise to subconductance states in the case of monovalent charge carriers (Root and MacKinnon, 1994) and reduces blocking efficacy of Ca^{2+} (this study). Neutralizing the charge by site-directed mutagenesis almost completely eliminates divalent block (Root and MacKinnon, 1993; Eismann *et al.*, 1994). Partial neutralization of the charge in hetero-oligomeric channels leads to a reduced blocking efficacy (Körschen *et al.*, 1995). Thus, experimental maneuvers expected to reduce or eliminate the negative charge of the Ca^{2+} -binding site by three independent routes also reduce or eliminate Ca^{2+} binding. These results underscore the importance of negative charges in binding of Ca^{2+} inside the pore vestibule.

The pK_a of pore glutamates is modulated

The inhibition of monovalent currents by protons occurs with a pK_a of 6.5 in rod (Figure 6A) and 7.6 in the olfactory channel (Root and MacKinnon, 1994). Similarly, the unblocked current versus pH relationship is shifted by up to +0.4 log units in the olfactory and cone compared with the rod CNG channel. Interestingly, along with the changes in apparent pK_a , we find considerable differences in the steepness of the pH dependence of Ca^{2+} blockage. These observations strongly argue that different protonation patterns of the pore Glu residues cause the variations among channels. As transplantation of the S5–P–S6 region also transfers pH sensitivity of the Ca^{2+} blockage (Figure 5C), it is plausible that the S5–P–S6 module also controls the pK_a of Glu protonation.

How do residues flanking the pore loop affect the pK_a of the glutamate’s carboxylic group? The pK_a of an acidic group located at the interface between two dielectric media depends on the difference of the dielectric constants ϵ_1 and ϵ_2 (Figure 7) (Edmonds, 1989). Furthermore, the protonation and thereby the partial negative charge of two

Fig. 7. Electrostatic model of protonation of intrapore glutamates.

acidic groups in juxtaposition to each other, as illustrated in Figure 7, will depend on their mean distance. As the distance is made shorter, the carboxylates become protonated and thereby reduce electrostatic repulsion by like charges. Thus the S5/S6 segments including the linkers may provide a characteristic dielectric micro-environment in the vicinity of the carboxylates and thereby control their pK_a . Alternatively, these segments may determine the ‘packing’ density of the pore module in a tetrameric channel and, along with it, the mean distance between carboxylic groups. In practice, however, the two effects are difficult to separate from each other.

Comparison with Ca^{2+} channels

CNG channels and Ca^{2+} channels share several functional key properties: (i) both channels are highly selective for Ca^{2+} ; (ii) monovalent currents are blocked by 1–200 μM of extracellular divalents in a voltage-dependent fashion; and (iii) blockage is relieved at negative V_m . These functional similarities would argue that the mechanisms of Ca^{2+} permeation and the underlying structural elements are similar as well. In fact, the pore regions of Ca^{2+} channels also contain conserved Glu residues that provide the structural basis for the high-affinity intrapore binding site (Kim *et al.*, 1993; Yang *et al.*, 1993). Protonation of Glu residues in Ca^{2+} channels also gives rise to subconductance states and, at the same time, lowers the efficacy of the divalent blockage (Chen *et al.*, 1996, see also Prod’hom *et al.*, 1987, 1989; Pietrobon *et al.*, 1989; Kuo and Hess, 1993). Despite these striking parallels, it has been speculated that the high-affinity Ca^{2+} sites in CNG and Ca^{2+} channels might be organized in different ways (Yellen, 1993). While all four Glu residues in the pore loops of repeats I–IV of Ca^{2+} channels participate in the coordination of Ca^{2+} ions, their contributions are far from equal (Yang *et al.*, 1993; Ellinor *et al.*, 1995). The inherently asymmetric arrangement of carboxylates could be a distinguishing feature of ‘sticky’ pores such as

Table I. Structure of chimeras between rod and olfactory CNG channels

bR		r: M ₁ - D ₆₉₀		
-NR		r: M ₁ , I ₁₅₆ - D ₆₉₀		
R _{S2} O _{S4} R		r: M ₁ - Q ₂₂₄	o: G ₂₀₂ - I ₂₇₅	r: S ₂₉₉ - D ₆₉₀
R _{S6} O		r: M ₁ - I ₃₉₀	o: V ₃₆₈ - P ₆₆₃	
R _{S4} O		r: M ₁ - I ₂₉₈	o: S ₂₇₆ - P ₆₆₃	
R _{S4} O'		r: M ₁ - F ₂₈₃	o: D ₂₆₁ - P ₆₆₃	
R _{S2} O'		r: M ₁ - K ₂₄₅	o: L ₂₂₃ - P ₆₆₃	
R _{S2} O		r: M ₁ - Q ₂₂₄	o: G ₂₀₂ - P ₆₆₃	
O _{S2} R _{S4} O		o: M ₁ - Q ₂₀₁	r: G ₂₂₅ - I ₂₉₈	o: S ₂₇₆ - P ₆₆₃
R _{S4} O _{S6} R		r: M ₁ - I ₂₉₈	o: S ₂₇₆ - I ₃₆₇	r: V ₃₉₁ - D ₆₉₀
bO		o: M ₁ - P ₆₆₃		

r and o refer to bovine sequences of the α_1 subunit from rod photoreceptors and the α_3 subunit from olfactory neurons, respectively.

in Ca^{2+} channels that reconcile a high Ca^{2+} affinity, i.e. selectivity, with large ion fluxes (Yellen, 1993). However, as we will show in the accompanying paper (Dzeja *et al.*, 1999), some homomeric CNG channels, which are expected to display a symmetric arrangement of pore Glu, also support large Ca^{2+} fluxes and carry a pure Ca^{2+} current at physiological $[\text{Ca}^{2+}]_o$. Therefore, the differences in Ca^{2+} selectivity of CNG channels and Ca^{2+} channels may be only quantitative rather than qualitative in nature.

Materials and methods

Construction of channel chimeras

Several chimeras were constructed between rod and olfactory CNG channels and rod and cone CNG channels. The nucleotide sequences of the chimeras have been verified by sequencing of cDNA. The structures of the chimeras are given in Tables I and II.

Blockage of CNG channels by extracellular Ca^{2+} and protons

The blockage of K^+ currents by extracellular Ca^{2+} was studied in outside-out patches from *Xenopus* oocytes as described (Baumann *et al.*, 1994; Frings *et al.*, 1995). The voltage and concentration dependence of the blockage was determined as illustrated in Figure 1 and described in Frings *et al.* (1995). A series of current-voltage relationships were recorded in the presence of various $[\text{Ca}^{2+}]_o$ (Figure 1A). The voltage dependence of Ca^{2+} blockage was analyzed by plotting the ratio of unblocked currents I/I_{max} against V_m (Figure 1B). The constant of half-maximal blockage, K_i , and the Hill coefficient, n , were derived for a given V_m from a plot of I/I_{max} against $[\text{Ca}^{2+}]_o$ (Figure 1C). The voltage dependence of K_i and n was determined by calculating K_i and n for each V_m between +80 and -80 mV (Figure 1D and E).

The pipette solution contained (mM): 125 KCl, 25 KOH, 10 HEPES, 10 EGTA and 1 cGMP (pH 7.4). The bath solution contained (mM): 100 KCl, 50 KOH, 10 HEPES, 10 EGTA and the concentration of CaCl_2 necessary to give the indicated free Ca^{2+} concentration, $[\text{Ca}^{2+}]$. The $[\text{Ca}^{2+}]$ in Ca^{2+} buffers made from EGTA was calculated with the chelator program (Schoenmakers *et al.*, 1992), using the association constants from Martell and Smith (1974). The free $[\text{Ca}^{2+}]$ of the solutions was measured with a Ca^{2+} -sensitive electrode (KwikCal, WPI, Germany). The electrode was calibrated with a set of Ca^{2+} buffers (Tsien and Rink,

Table II. Structure of chimeras between rod and cone CNG channels

bR		r: M ₁ -D ₆₉₀		
C _{S4} R _{S6} C		c: M ₁ -T ₃₁₂	r: R ₂₈₉ - T ₃₈₉	c: I ₄₁₄ - P ₇₀₆
C _{S5} R _{S6} C		c: M ₁ -I ₃₄₃	r: S ₃₂₀ - T ₃₈₉	c: I ₄₁₄ - P ₇₀₆
C _{S4} R _{S5} C		c: M ₁ -T ₃₁₂	r: R ₂₈₉ - I ₃₁₉	c: S ₃₄₄ - P ₇₀₆
C _{S5} R _{P0} C		c: M ₁ -I ₃₄₃	r: S ₃₂₀ - P ₃₆₇	c: V ₃₉₂ - P ₇₀₆
C _{P0} R _{S6} C		c: M ₁ -P ₃₉₁	r: V ₃₆₈ - T ₃₈₉	c: I ₄₁₄ - P ₇₀₆
R _{S5} C _{P0} R		r: M ₁ -I ₃₁₉	c: S ₃₄₄ - P ₃₉₁	r: V ₃₆₈ - D ₆₉₀
R _{S4} C _{S5} R'		r: M ₁ -T ₂₈₈	c: R ₃₁₃ - S ₃₅₃	r: W ₃₃₀ - D ₆₉₀
R _{S4} C _{S5} R		r: M ₁ -T ₂₈₈	c: R ₃₁₃ - I ₃₄₃	r: S ₃₂₀ - D ₆₉₀
R _{P0} C _{S6} R		r: M ₁ -P ₃₆₇	c: V ₃₉₂ - T ₄₁₃	r: I ₃₉₀ - D ₆₉₀
R _{S5} C _{S6} R		r: M ₁ -I ₃₁₉	c: S ₃₄₄ - T ₄₁₃	r: I ₃₉₀ - D ₆₉₀
R _{S4} C _{S6} R		r: M ₁ -T ₂₈₈	c: R ₃₁₃ - T ₄₁₃	r: I ₃₉₀ - D ₆₉₀
bC		c: M ₁ -P ₇₀₆		

r as in Table I. c refers to bovine sequences of the α_2 subunit from cone photoreceptors.

1980) (Calbuf-1, WPI, Germany), each time before the $[\text{Ca}^{2+}]$ in the test solution was measured. Alternatively, $[\text{Ca}^{2+}]$ was determined using the metallochromic indicator dye Arsenazo III. Difference spectra of Arsenazo III (1 μM) were recorded between 400 and 730 nm with an Aminco DW2a spectrophotometer (SLM) in the split-beam mode. Ca^{2+} was added sequentially to the sample cuvette in small volumes that caused $\leq 1\%$ dilution of the dye. The reference cuvette contained Ca^{2+} -free Arsenazo III in an identical solution. A plot of the changes in absorbance ΔA at 652 nm versus total $[\text{Ca}^{2+}]$ yielded the calibration curve. Test and calibration solutions had the same composition, except that test solutions contained various $\text{CaCl}_2/\text{EGTA}$ (10 mM) ratios. The determination of free $[\text{Ca}^{2+}]$ using Arsenazo III is accurate and reliable: (i) the intrinsic calibration procedure does not rely on the knowledge of binding constants of the dye, the binding stoichiometry and the extinction coefficient; (ii) because of the low dye concentration, the free $[\text{Ca}^{2+}]$ almost equalled the added $[\text{Ca}^{2+}]$; therefore, no calibration buffers are needed; and (iii) the dye is most sensitive within the range of $[\text{Ca}^{2+}]$ used for Ca^{2+} blockage of CNG channels (1–500 μM).

The solutions used to measure the pH dependence of Ca^{2+} blockage and of proton blockage of monovalent current contained the following. (i) Ca^{2+} -free bath solution and pipette solution (mM): 125 KCl, 10 EGTA and 10 pH buffer, titrated to the desired pH with KOH (~25 mM). The pipette solution contained in addition 0.5 mM cGMP. (ii) Ca^{2+} -containing bath solutions (mM): 150 KCl, 0.05 CaCl_2 and 10 pH buffer, titrated to the desired pH with *N*-methyl-D-glucamine (NMDG). The pH buffers were: MES (pH 5–6), PIPES (pH 6.25–6.5), HEPES (pH 6.75–7.8), TAPS (pH 8–8.5), CHES (pH >8.5). The pipette solution to measure proton block of K^+ currents always contained HEPES at pH 7.4 and 0.5 mM cGMP.

Woodhull model of Ca^{2+} blockage

Blockage of CNG channels by extracellular Ca^{2+} was described quantitatively using a modification of the model by Woodhull (1973). The rate equation for binding of Ca^{2+} ions to an intrapore binding site is:

wherein BS denotes the Ca^{2+} -binding site, $[\text{Ca}^{2+}]_o$ and $[\text{Ca}^{2+}]_i$ the extracellular and intracellular $[\text{Ca}^{2+}]$, respectively, and CaBS the complex

between the binding site and Ca²⁺. The probability P that the pore is not occupied by Ca²⁺, i.e. the fraction of unblocked current $I/I_{\max}$, is:

$$P = I/I_{\max} = \frac{k_{-1} + k_2}{k_1 [\text{Ca}^{2+}]_o + k_{-1} + k_2 + k_{-2} [\text{Ca}^{2+}]_i} \quad (1)$$

The rate constants k_i are defined as

$$\begin{aligned} k_1 &= a_1 \exp\left(-\frac{U_1}{RT} - \frac{z\delta\zeta FV_m}{RT}\right) \\ k_{-1} &= a_{-1} \exp\left(-\frac{U_{-1}}{RT} + \frac{z\delta(1-\zeta) FV_m}{RT}\right) \\ k_2 &= a_2 \exp\left(-\frac{U_2}{RT} - \frac{z(1-\delta)\zeta FV_m}{RT}\right) \\ k_{-2} &= a_{-2} \exp\left(-\frac{U_{-2}}{RT} + \frac{z(1-\delta)(1-\zeta) FV_m}{RT}\right) \end{aligned} \quad (2a-d)$$

wherein a_i are constants, z is the 'effective' valence of the blocking ion, U_i refer to the height of the energy barrier; δ , ξ and ζ refer to the 'electric' distance of the energy minima and maxima within the electric field (see scheme in Figure 3A). The set of independent variables can be reduced to six by defining:

$$b_i = a_i \exp(-U_i/RT) \quad (3)$$

and using the ratios b_{-1}/b_1 and b_{-2}/b_1 instead of individual rate constants (Woodhull, 1973). If $[\text{Ca}^{2+}]_i = 0$, these simplifications yield Equation 4:

$$\frac{I}{I_{\max}} = \frac{\frac{b_{-1}}{b_1} \exp\left(-\frac{z\delta(1-\xi) FV_m}{RT}\right) + \frac{b_2}{b_1} \exp\left(-\frac{z(1-\delta)\zeta FV_m}{RT}\right)}{[\text{Ca}^{2+}]_o \exp\left(-\frac{z\delta\zeta FV_m}{RT}\right) + \frac{b_{-1}}{b_1} \exp\left(-\frac{z\delta(1-\xi) FV_m}{RT}\right) + \frac{b_2}{b_1} \exp\left(-\frac{z(1-\delta)\zeta FV_m}{RT}\right)}$$

Acknowledgements

We thank Drs J. Weiner and Y. Fujita for construction of several chimeras, Dr S. Frings for careful reading of the manuscript, and A. Eckert for preparing the manuscript. This work was supported by a grant from the Land Nordrhein-Westfalen.

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Received September 14, 1998; revised and accepted November 6, 1998