Permeation Properties of a Ca\textsuperscript{2+}-blockable Monovalent Cation Channel in the Ectoderm of the Chick Embryo: Pore Size and Multioccupancy Probed with Organic Cations and Ca\textsuperscript{2+}

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ABSTRACT A Ca\textsuperscript{2+}-blockable monovalent cation channel is present in the apical membrane of the ectoderm of the gastrulating chick embryo. We used the patch clamp technique to study several single-channel permeation properties of this channel. In symmetrical conditions without Ca\textsuperscript{2+}, the Na\textsuperscript{+} current carried by the channel rectifies inwardly. The channel has an apparent dissociation constant for extracellular Na\textsuperscript{+} of 115 mM at 0 mV and a low density of negative surface charge (−0.03 e/\text{nm}^2) at its extracellular entrance. The minimal pore diameter is \(~\sim 5.8 \text{ Å}\), as calculated from the relative permeabilities of 10 small organic cations. Extracellular application of six large organic cations decreased the inward Na\textsuperscript{+} current in a voltage-dependent manner, which strongly suggests an intrachannel block. The presence of at least two ion binding sites inside the pore is inferred from the Na\textsuperscript{+} dependence of the block by the organic cations. This hypothesis is strengthened by the fact that the extracellular Ca\textsuperscript{2+} block is also modified by the Na\textsuperscript{+} concentration. In particular, the rise of the unblocking rate with increased Na\textsuperscript{+} concentrations clearly suggests the presence of an interaction between Ca\textsuperscript{2+} and Na\textsuperscript{+} inside the channel. A low probability of double occupancy at physiological ionic conditions is implied from the absence of an anomalous mole fraction effect with mixtures of extracellular Li\textsuperscript{+} and K\textsuperscript{+}. Finally, the absence of inward current at very strong hyperpolarizations and in the presence of 10 mM extracellular Ca\textsuperscript{2+} demonstrates the absence of significant Ca\textsuperscript{2+} current through this channel. It is argued that this embryonic epithelial Ca\textsuperscript{2+}-blockable monovalent cation channel is related to both L-type Ca\textsuperscript{2+} channel and cyclic nucleotide-gated channels.

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INTRODUCTION

Poorly selective cation channels blocked by extracellular Ca$^{2+}$ exist in neuronal and epithelial cells. Prominent among them are the cyclic nucleotide-gated (CNG) cation channels of photoreceptors and olfactory neurons, which participate in signal transduction mechanisms (for example, see Yau and Baylor, 1989; Kaupp, 1991). Ca$^{2+}$-blockable monovalent cation currents have also been described in several epithelial preparations (Aelvoet, Erlij, and van Driessche, 1988; Heinz, Krat tenmacher, Hoffmann, and Clauss, 1991; van Driessche, Desmedt, and Simaels, 1991). The physiological functions of the transepithelial Ca$^{2+}$-blockable cation currents have not yet been completely elucidated. Recent work on toad urinary bladder (Das and Palmer, 1989; Erlij, Kaufman, and Gersten, 1992) suggests that the Ca$^{2+}$-blockable cation channel activated by oxytocin could mediate K$^+$ secretion. The oxytocin-stimulated transfer of Na$^+$ from the apical to the basal side creates a large transepithelial potential, negative at the apical side. Under these conditions, the apical membrane potential could be positive and allow an outward K$^+$ flow through the nonselective cation channel. It has also been proposed that the Ca$^{2+}$-blockable channel could be involved in the development of epithelial cells (van Driessche, de Smet, and de Smet, 1994) and in the control of cell volume (van Driessche and Erlij, 1994). We recently have demonstrated the presence of a Ca$^{2+}$-blockable monovalent cation channel in the apical membrane of ectodermal cells of the gastrulating chick embryo and have characterized some of its main properties, such as its apparent Ca$^{2+}$ affinity and its poor selectivity among alkali cations (Li, Prod’hom, and Kucera, 1994). In this report, we extend the description of this channel’s permeation properties and compare them with those of the CNG channels and the L-type Ca$^{2+}$ channel.

METHODS

Embryo Extraction

Fertilized eggs from Warren hens were incubated for 20 h at 37°C and 80% humidity to reach the late gastrula stage (stage 5 from Hamburger and Hamilton, 1951). The yolk, with its vitelline membrane, was separated from the albumen and transferred into a saline solution. A piece of vitelline membrane, 4-cm diam, with the attached embryo, was fixed between two concentric metallic rings to allow further manipulations. After cleaning the ventral side with a gentle flow of saline solution, the embryo was detached from the vitelline membrane and mounted into the recording chamber. Gigaohm seals could be obtained on the dorsal side of the blastoderm without further treatment.

Electrophysiological Recordings

Standard single-channel patch clamp recordings were done following the method of Hamill, Marty, Neher, Sackman, and Sigworth (1981). The electrical signals were amplified by an amplifier (Axopatch model 200, Axon Instruments, Inc., Foster City, CA), digitized, and saved on the disk of an IBM-compatible computer. The composition of the borosilicate capillaries (Clark Electromedical Instruments, Reading, UK) used to make pipettes is as follows (according to the manufacturer): 80.9% SiO$_2$, 12.9% B$_2$O$_3$, 4.4% Na$_2$O, 1.8% Al$_2$O$_3$. The pipette resistance with 140 mM
NaCl varied between 3 and 7 MΩ. PCLAMP (Axon Instruments, Inc.) was used for data acquisition and analysis. The signals usually were sampled at between 10 and 50 KHz and filtered at 2–10 KHz, according to the type of experiment. All of the experiments were done at room temperature. The recordings were performed between 1 and 3 h after the egg was taken out of the incubator. During this time, the embryos showed no signs of development.

Liquid Junction Potential Correction

Liquid junction potentials were measured and corrected for as described by Neher (1992) and Barry and Lynch (1991). Briefly, the voltage reading of the amplifier in current-clamp mode was set to zero in the presence of the test solution in the pipette and in the bath. The bath solution then was exchanged for our standard bath solution. The liquid junction potential was taken as the value of opposite sign from the voltage reading.

Duration of the Channel Activity in Outside-Out and Inside-Out Patches

Most of this work was done in cell-attached patches because the channel activity rapidly fades away in both inside-out and outside-out patch configurations. It was nevertheless necessary to carry out some tests in excised patch modes. The average time of disappearance of the last channel in multichannel (4–10) outside-out patches is 98 s after outside-out patch formation (n = 15). Similarly, in the excised inside-out patch configuration, the average time of last channel disappearance was 94 s (n = 6). Although short, these durations were long enough for the pipette solution to equilibrate close to the membrane.

Measurement of the Permeability Ratios

To calculate the relative permeability of an extracellular cation in a cell-attached patch, it is necessary to estimate the intracellular K⁺ concentration as well as the membrane potential. The membrane potential was measured in current-clamp mode after breaking the patch. The pipette solution contained 140 mM KCl, 10 mM HEPES, 5 mM EDTA, and 4.55 mM CaCl₂. The average value taken immediately after the break was −24.2 ± 1.5 mV (n = 28) after liquid junction potential correction. Because the reversal potential in the presence of 140 mM K⁺ in the pipette in a cell-attached patch is 20.2 mV (Li et al., 1994), the intracellular K⁺ concentration estimated from the Nernst equation is 155 mM. The permeability ratios \( \frac{P_{X^+}}{P_{K^+}} \) were calculated from the reversal potential of the current with 140 mM of the tested cation in the pipette in cell-attached patches. The following version of the Goldman-Hodgkin-Katz equation was used:

\[
P_{X^+} / P_{K^+} = \frac{\text{[K⁺]}_\text{i} \exp(VF/RT) - \text{[K⁺]}_\text{o}}{\text{[X⁺]}_\text{o}}
\]

where \([K⁺]_\text{i}\) is the intracellular K⁺ concentration, \([K⁺]_\text{o}\) is the pipette concentration of KOH used to titrate the solution to pH 7.4 when the tested cation was available as chloride salt only, \([X⁺]_\text{o}\) is the pipette concentration of the tested cation, \(V\) is the measured reversal potential corrected for the resting and liquid junction potentials, and \(F, R,\) and \(T\) have their usual thermodynamic meaning. The permeability ratio for extracellular ammonium vs intracellular K⁺ was also measured in the outside-out patch configuration under bionic conditions and compared with the value obtained in cell-attached patch. The average reversal potential in outside-out patches was 21.7 ± 0.2 mV (n = 8), which gives a permeability ratio \(\frac{P_{\text{NH}_4^+}}{P_{K^+}}\) of 2.37. This value compares reasonably well with the permeability ratio measured in the cell-attached mode, 2.59, and validates our estimate of the intracellular K⁺ concentration. Organic cation radii were taken from Cohen, Labarca, Davidson, and Lester (1992) and Tinkler and Williams (1993).
Correction of the Kinetics Data

The 50% threshold method was used to determine the opening and closing durations. The histograms were well fitted with monoexponential functions. The dwell times were corrected as described by Kuo and Hess (1992):

\[ \tau_o(\text{corrected}) = \tau_o(\text{crude}) \exp \left[ -0.179/f \tau_o(\text{crude}) \right] \]  
\[ \tau_c(\text{corrected}) = \tau_c(\text{crude}) \exp \left[ -0.179/f \tau_o(\text{crude}) \right] \]

where \( f \) is the filter cutoff frequency and \( \tau_o(\text{crude}) \) and \( \tau_c(\text{crude}) \) are the fitted mean closed and open times.

Test with Different Glass Pipettes

Current-voltage curves and kinetics at -30 mV in the presence of 140 mM NaCl, 10 mM HEPES, and 1 mM EDTA in cell-attached patches were measured with four types of glass pipettes to determine whether leachable components from the glass could affect these parameters (see, for example, Furman and Tanaka, 1988; Rae and Levis, 1992). In addition to the Clark pipette, we tested glasses Nos. 0010, and 8161 (World Precision Instruments, Inc., Sarasota, FL), and No. 7052 (AM Systems, Everett, WA). The current-voltage curves were identical with all glasses (three experiments with Nos. 0010 and 7052, and six with No. 8161). The open time at -30 mV was 1.74 ± 0.25 ms (\( n = 3 \)) with No. 0010, 1.41 ± 0.20 ms (\( n = 3 \)) with No. 7052, 2.02 ± 0.90 ms (\( n = 3 \)) with No. 8161, and 4.8 ± 0.9 ms with Clark (\( n = 14 \)) (Li et al., 1994). The closed time was 1.87 ± 0.30 ms with No. 0010, 1.73 ± 0.21 ms with No. 7052, 1.08 ± 0.08 ms with No. 8161, and 1.77 ± 0.17 ms with Clark. The longer open time with the Clark pipette suggests that this glass leached the smallest amount of contaminants into the pipette solution. In the presence of 1 mM EDTA at -50 mV, the open time was ~30 times longer than with 1 mM EGTA (Li et al., 1994). This led us to use 5 mM EDTA to control the free Ca\(^{2+}\) concentration.

Solutions

The embryo usually was bathed in a solution containing 140 mM NaCl, 1 mM CaCl\(_2\), and 10 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethane-sulphonic acid]. The pH of the solutions was adjusted to 7.4 with NaOH, KOH, or HCl. The pipette solutions included usually 5 mM EDTA and the appropriate amount of cation chloride. The Ca\(^{2+}\) concentrations were calculated as described in Li et al., 1994. The osmolarity of the low-Na\(^+\) solutions (5–70 mM) used to estimate the channel affinity for Na\(^+\) and the surface potential of the Ca\(^{2+}\)-blockable monovalent cation (CMC) channel was maintained at ~300 mOsm with the appropriate amount of sucrose. The solutions of the organic permeant cations were prepared the day of the experiments to avoid contamination by possible hydrolysis products (Seoh and Busath, 1993). All chemicals came from Fluka Chemie AG, (Buchs, Switzerland), Aldrich Chemical Co. (Steinheim, Germany), or Merck (Darmstadt, Germany).

RESULTS

The CMC Channel Has Slow Voltage-independent Kinetics

Single-channel recordings of the CMC channel showed consecutive active and silent periods (Fig. 1 A). The current of the active periods was interrupted by short-closures of unknown origin (Li et al., 1994). Histograms of both active and silent times collected from several cell-attached patch experiments have monoexponential distributions (Fig. 1 B). The closing \( (k_{\text{off}} = 1/\tau_o = 0.143 \text{ s}^{-1}) \) and opening rates \( (k_{\text{on}} = 1/\tau_o = 0.077 \text{ s}^{-1}) \) did not vary significantly with membrane potential (Fig. 1
Permeation Properties of a Cation Channel

Figure 1. Slow kinetics of the CMC channel. (A) Traces at +80 mV and −30 mV holding potential in cell-attached patch configuration. The solid lines represent the closed level. The pipette solution contains 140 mM NaCl, 10 mM HEPES, 5 mM EDTA, and 4.55 mM CaCl₂, at pH 7.41; the bath solution contains 140 mM NaCl, 10 mM HEPES, and 1 mM CaCl₂, at pH 7.4. Traces filtered at 1 KHz and sampled at 5 KHz. Only one sampled value of 50 was printed. (B) Distribution histograms of the active and silent periods collected at −30 mV (n = 10) and at +80 mV (n = 20). The solid lines superimposed on the experimental data represent an expected average distribution of 7 s for the active period and 13 s for the silent period.

B), which indicates that the CMC channel does not belong to the functional family of voltage-activated channels.

Inward Rectification of the CMC Single-Channel Current

In cell-attached patches, millimolar concentrations of Ca²⁺ in the extracellular solution blocked the inward current completely. In these almost physiological conditions, the CMC current was strongly outwardly rectifying (Li et al., 1994). In the absence of extracellular Ca²⁺, the single-channel inward current was larger than the outward current (Fig. 2 B). This inward rectification can originate, in principle, either from the channel itself or from the presence of an intracellular blocker or a low intracellular cation concentration. To distinguish between these possibilities, single-channel currents were recorded in both excised inside-out and outside-out patch configurations with symmetrical Na⁺ concentrations. Fig. 2 A shows a continuous single-channel recording before and after inside-out excision. The current reached a new value very quickly (~200 ms) after excision, and this value did not change as long as the channel was active, which suggests that both change in membrane potential and equilibration of the solution at the intracellular side of the inside-out patch occurred rapidly. This rapid change was in accord with Cannell and Nichols's (1991) estimate of equilibration in inside-out patches. They showed that 90% of the solution at the tip of patch pipettes (of resistance similar to ours) is changed within 400 ms. Clearly, the channel activity lasted long enough in both inside-out and outside-out patches (~1 1/2 min) to allow the solution at the intracellular side of the patch to equilibrate fully.
The single-channel current rectifies inwardly in both inside-out and outside-out patches. (A) Continuous recording of a CMC channel before and after inside-out excision. The single-channel current rapidly reaches (within 200 ms) a new stable value after excision (arrow). The trace was recorded at −30 mV and filtered at 1 kHz. Only one point of 50 was printed. (B) Current-voltage curves from a cell-attached patch (open circle), an outside-out patch (closed circle; n = 10), and an inside-out patch (open triangle; n = 6), with 140 mM NaCl, 10 mM HEPES, and 15 mM EGTA at pH 7.4 in the pipette and 140 mM NaCl, 10 mM HEPES, and 1 mM CaCl₂ (cell-attached patch) or 1 mM EGTA (inside-out and outside-out patch) at pH 7.4 in the bath. Note that because the positive current is defined as going from the bath into the pipette, the polarity of the outside-out patch current must be inverted to be compared with the other patch configurations.

As shown in Fig. 2 B, the current-voltage curves retained their typical rectifying shape in both inside-out and outside-out patch configurations. This finding argues against unequal concentrations of permeant ion or presence of a cytoplasmic blocker as the origin of the rectification. The inward rectification is therefore an intrinsic property of the channel. Note that the shift of the reversal potential between the cell-attached and the excised configurations is ∼21 mV, close to the measured membrane potential (−24.2 mV; see Methods), as expected for a nonselective cation channel.

An Estimate of the Affinity of the CMC Channel for Extracellular Na⁺

The simplest way to study the affinity of a channel for a permeant ion is to measure its current at different permeant ion concentrations while keeping constant the ionic strength with an impermeant one. Unfortunately, even large cations such as N-methyl-d-glucamine (NMDG) (which is often used as a cation substitute), blocked the current of the CMC channel in a voltage-dependent manner (see later). The Na⁺ current has therefore been measured in the presence of decreasing concentrations of Na⁺ (from 280 to 5 mM in the pipette) without substitution by an impermeant ion (Fig. 3 A). The current-concentration curve of a singly occupied channel follows a rectangular hyperbola. The CMC channel current deviates from a rectangular hyperbola at low concentrations. This usually is interpreted as being attributable to the presence of a negative surface potential, which raises the cation-concentration at the channel entrance (McLaughlin, 1977; Green and Andersen, 1991).
The surface potential at the entrance of the channel depends on the geometry of the channel and the presence of charges and dipoles. Because no geometrical data is available, we used as a first approximation the Gouy-Chapman model of surface charge density:

\[ s = \frac{(2\varepsilon_\text{w}e_o kT)}{\varepsilon} \sum G_i [\exp(-z_i e_\Psi/kT) - 1]^{1/2} \]  

(4)

where \( s \) is the charge density, \( \varepsilon_\text{w} \) is the relative dielectric constant for water, \( e_\text{o} \) the permittivity of free space, \( G_i \) the extracellular ionic concentrations, \( z_i \) the ionic charges, \( \Psi \) the surface potential, and \( k, T, \) and \( e \) have their usual meaning. The cation concentration at the entrance of the channel was assumed to follow a Boltzmann distribution. The hyperbolic curves, corrected for a surface charge density of \(-0.03 \text{ e/nm}^2\), fit the experimental current-concentration curves (Fig. 3 B). The ex-

**Figure 3.** Apparent \( K_d \) for extracellular Na\(^+\). (A) Current-voltage curves obtained from cell-attached patches with 287 mM Na\(^+\) (closed circle; \( n = 7 \)), 147 mM Na\(^+\) (open circle; \( n = 11 \)), 77 mM Na\(^+\) (closed square; \( n = 9 \)), 42 mM Na\(^+\) (open square; \( n = 9 \)), 24.5 mM Na\(^+\) (closed triangle; \( n = 7 \)), 17.8 mM Na\(^+\) (open triangle; \( n = 8 \)), and 5 mM Na\(^+\) (closed diamond; \( n = 5 \)), with 10 mM HEPES, 5 mM EDTA, and 4.55 mM CaCl\(_2\) at pH 7.4 in the pipette, and 140 mM NaCl, 10 mM HEPES, and 1 mM CaCl\(_2\) at pH 7.4 in the bath. (B) Single-channel currents as a function of the pipette Na\(^+\) concentrations at -180, -150, -120, -80, -50, -30, and 0 mV holding potentials. The dotted lines are the expected currents calculated from \( i = i_{\text{max}}([\text{Na}^+] / K_\text{d}) / [1 + ([\text{Na}^+] / K_\text{d})] \) after correction for a surface charge density of \(-0.05 \text{ e/nm}^2\). (C) Reversal potentials shifted by 24.2 mV vs the natural logarithm of the corrected Na\(^+\) concentrations. The dotted line represents the expected reversal potentials from the Goldman-Hodgkin-Katz equation, with 155 mM K\(^+\) in the cell with surface potential correction. (D) Apparent \( K_d \) for extracellular Na\(^+\) as a function of the holding potential obtained from the fit of current vs concentration curves (B).
tracted $K_M$ for the extracellular Na$^+$ varies between 28 mM at -200 mV and 115 mM at 0 mV holding potential (Fig. 3 D). These $K_M$ values estimate the probability of occupancy of the channel by extracellular Na$^+$ in the presence of intracellular permeant K$^+$ and therefore include the competition between Na$^+$ and K$^+$. However, at negative potentials, the contribution from intracellular K$^+$ becomes negligible, and the measured $K_M$ simply reflects channel occupancy. The dependence of the reversal potential on the Na$^+$ concentration is shown in Fig. 3 C. It follows approximately the value expected from the Goldman-Hodgkin-Katz equation (Hille, 1984), with an average difference of 3.7 mV between the theoretical values and the experimental data.

**Probing the Pore with Small Organic Cations**

Fig. 4, A–C, presents current-voltage curves from cell-attached patches with small organic cations in the pipette. The reversal potentials can be used to determine the permeabilities of the organic cations relative to intracellular K$^+$ (see Methods). As

![Figure 4](image_url)

**Figure 4.** A measure of the minimal pore radius. (A–C) Current-voltage curves in cell-attached patches, with 140 mM organic cation in the pipette: (A) ammonium (closed circle), methylammonium (open circle), dimethylammonium (closed square), trimethylammonium (open square); (B) ethylammonium (closed triangle), diethylammonium (inverted closed triangle), ethanolammonium (open triangle); (C) hydrazinium (inverted open triangle), guanidinium (closed diamond), and piperazinium (open diamond). (D) Relative permeability of the organic cations as a function of the ionic radius (closed circle). The almost identical permeability for Li$^+$, Na$^+$, K$^+$, and Ca$^+$ (open triangles) (Li et al., 1994) are shown for comparison. The dotted line represents the theoretical curve expected for a pore radius of 2.9 and a K$^+$ radius of 2.0. These values were obtained by least square fit of the linearization of the excluded-area equation as described by Cohen et al. (1992), with $r = 0.82$. 


shown in Fig. 4D, the relative permeability of an organic cation decreased when its radius increased. This trend was used to estimate the minimal radius of the pore. We used the simplest excluded-area theory that relates the permeability to the surface left empty when the ion occupies the pore:

\[ \frac{P_{\text{X}^+}}{P_{\text{K}^+}} = \frac{(R_c - R_{\text{X}^+})^2}{(R_c - R_{\text{K}^+})^2} \]  

where \( R_c, R_{\text{X}^+}, \) and \( R_{\text{K}^+} \) are the radius of the pore, the tested cation, and the \( \text{K}^+ \) ion, respectively. The minimal diameter of the channel was calculated to be 5.8 Å, with a \( \text{K}^+ \) ion radius of 2.0. Because the crystal radius of \( \text{K}^+ \) clearly is smaller (1.3–1.4) (Hille, 1984), the estimated value of 2.0 could indicate that the \( \text{K}^+ \) ion keeps part of its hydration shell in the channel. This is compatible with the fact that the CMC channel discriminates poorly between the alkali cations (Li et al., 1994). The conductance sequence is roughly in the same order as the relative permeability sequence (Table I), consistent with a permeation mechanism controlled by the energy barrier created by the narrowest section of the pore. Piperazine was an exception because it blocked the outward \( \text{K}^+ \) current whereas the other organic cations did not (Fig. 4C). Presumably, it binds more tightly to the pore than do the other organic cations. The outward current block varied as a function of the potential, with an apparent \( K_m \) equal to ~150 mM at 150 mV. The apparent electrical distance of the piperazine block of the outward current, estimated from a one-site Woodhull model, was 0.53 (not shown).

**Probing the Pore with Large Organic Cations**

Cations slightly larger than the minimal pore diameter should behave as blockers of this channel. Six large organic cations were tested for this purpose. When

<table>
<thead>
<tr>
<th>Ion</th>
<th>Ion radius</th>
<th>Relative permeability</th>
<th>Conductance</th>
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<tbody>
<tr>
<td>Ammonium</td>
<td>1.7</td>
<td>1.93 ± 0.28 (6)</td>
<td>39.3 ± 0.9 (6)</td>
</tr>
<tr>
<td>Hydrazinium</td>
<td>1.8</td>
<td>0.86 ± 0.09 (6)</td>
<td>26.9 ± 1.1 (6)</td>
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<tr>
<td>Methylammonium</td>
<td>1.85</td>
<td>0.45 ± 0.06 (6)</td>
<td>24.7 ± 2.1 (6)</td>
</tr>
<tr>
<td>Ethylammonium</td>
<td>2.25</td>
<td>0.42 ± 0.03 (5)</td>
<td>9.9 ± 0.5 (7)</td>
</tr>
<tr>
<td>Dimethylammonium</td>
<td>2.3</td>
<td>0.29 ± 0.02 (7)</td>
<td>15.7 ± 0.6 (5)</td>
</tr>
<tr>
<td>Ethanolammonium</td>
<td>2.35</td>
<td>0.57 ± 0.09 (5)</td>
<td>9.0 ± 0.2 (5)</td>
</tr>
<tr>
<td>Guanidinium</td>
<td>2.4</td>
<td>0.55 ± 0.09 (7)</td>
<td>19.2 ± 2.6 (5)</td>
</tr>
<tr>
<td>Piperazine</td>
<td>2.5</td>
<td>0.03 (6)</td>
<td>7.2 ± 2.1 (5)</td>
</tr>
<tr>
<td>Diethylammonium</td>
<td>2.55</td>
<td>0.03 (5)</td>
<td>3.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>Trimethylammonium</td>
<td>2.7</td>
<td>0.04 ± 0.01 (4)</td>
<td>8.5 ± 0.2 (4)</td>
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</tbody>
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*From Cohen et al., 1992, Tinker and Williams, 1993; †calculated from reversal potentials, ‡between reversal potential and −1 pA; §number of experiments.
FIGURE 5. Large organic cations behave as weak blockers. The current-voltage curve as well as the fraction of unblocked current vs the membrane potential are shown for six cations in cell-attached mode. The pipette solution contains 140 mM NaCl, 10 mM HEPES, 5 mM EDTA, and 4.55 mM CaCl₂ at pH 7.4, and 50 mM of a blocker. (Upper left) Tetrapropylammonium (TPA; n = 10); (upper middle) tetraethylammonium (TEA; n = 9); (upper right) phenyltrimethylammonium (pTriMA; n = 33); (lower right) choline (n = 6); (lower middle) N-methyl-D-glucamine (NMDG; n = 7); (lower right) tris (hydroxymethyl) aminomethane (TRIS, n = 9). The solid line without symbols of the current-voltage curves is the control current, with 140 mM NaCl, 10 mM HEPES, 5 mM EDTA, and 4.55 mM CaCl₂ at pH 7.4. The solid lines of the plot of the fraction of unblocked current are Woodhull theoretical curves with the following parameters: TPA, $K_m = 66.6$ mM, $\delta$ (electrical distance from the outside) = 0.22; TEA, $K_m = 86.7$ mM, $\delta = 0.27$; pTriMA, $K_m = 200$ mM, $\delta = 0.28$; choline, $K_m = 475$ mM, $\delta = 0.25$; NMDG, $K_m = 149$ mM, $\delta = 0.56$, $k_0 = 0.0278 \times k_1$, electrical distance of $k_0 = 0.36$; TRIS, $K_m = 62.5$ mM, $\delta = 0.56$, $k_0 = 0.0714 \times k_1$, electrical distance of $k_0 = 0.36$. 
present in the extracellular solution, they all reduced the inward Na\(^+\) current (Fig. 5). This effect was more pronounced at more negative membrane potentials, which is an indication that these blockers bind inside the pore (Fig. 5). It was possible to estimate the position of the blocker’s binding site inside the channel with the help of the Woodhull model (Hille, 1984). In this model, the fraction of unblocked current is:

\[
\frac{i_b}{i_c} = \frac{k_{-1} + k_2}{k_{-1} + k_2 + k_k[\text{blocker}]},
\]

where \(i_b\) and \(i_c\) are the blocked and control currents, \(k_1\) is the on rate from the extracellular side, \(k_{-1}\) is the off rate toward the extracellular side, and \(k_2\) is the off rate toward the intracellular side. The extracellular barrier is assumed to be symmetrical. In the presence of tetrapropylammonium (TPA, MW = 186 g), tetraethylammonium (TEA, MW = 130 g), phenyltrimethylammonium (pTriMA, MW = 136 g), and choline (MW = 104 g), fits of the fractions of unblocked current were obtained assuming that these organic cations cannot reach the intracellular side (\(k_2 = 0\)). The average apparent electrical distance of the binding site was 0.26 ± 0.03.

In the presence of NMDG (MW = 196 g) and Tris (MW = 122 g), the fraction of unblocked current was composed of a voltage-dependent part at potentials more positive than -100 mV, followed by a voltage-independent portion at potentials more negative than -100 mV. The simplest interpretation is that these blockers can exit the channel toward the extracellular side (as do the blockers of the first group) but also toward the intracellular side (\(k_2 > 0\)). Fits of the fraction of unblocked current (Fig. 5) were obtained with an electrical distance from the extracellular side of 0.56 and an off rate toward the intracellular side (\(k_2\)) that depended on 0.3626 of the total electrical field. A partial occlusion of the channel could also, in principle, explain the deviation of the block by NMDG and Tris at potentials < -100 mV. In this case, the electrical distance obtained with \(k_2 = 0\) after substracting 17% of the fraction of unblocked current (the current level due to the partial occlusion) was 0.92 for Tris and 0.85 for NMDG. This would indicate that these blockers can cross 90% of the pore and produce inside the narrow pore a partial occlusion. Although not impossible, such a mechanism seems unlikely. The main point is that the steepness of the unblocked current as a function of the membrane potential

![Figure 6](image-url)

**Figure 6.** Single-channel recordings in cell-attached patch configuration with varying Na\(^+\) and NMDG extracellular concentrations. The pipette contained 70, 140, or 280 mM Na\(^+\) and 0, 12.5, 25, or 50 mM NMDG, 1 mM EDTA, and 10 HEPES at pH 7.4. The holding potential is -80 mV. The solid lines represent the zero level. Cut off frequency 2 KHz, refiltered at 1 KHz for the display, with 70 mM Na\(^+\) and 50 mM NMDG.
Figure 7. The NMDG block is modulated by the extracellular Na⁺ concentration. (A) current-voltage curves in cell-attached patches with corresponding block ratio vs NMDG concentration with 70, 140, 280, and 500 mM Na⁺, and 12.5 mM (closed circle), 25 mM (open circle), and 50 mM (closed square) NMDG. Number of experiments: n = 6 (70 mM Na⁺, 12.5 mM NMDG), 7 (70, 25), 6 (70, 50); 8 (140 mM Na⁺, 12.5 mM NMDG), 9 (140, 25), 7 (140, 50); 9 (280 mM Na⁺, 12.5 mM NMDG),
potential between $-100$ and $+100$ mV is clearly larger in the presence of NMDG and Tris than in the presence of the other blockers. This, in turn, can be interpreted as evidence that NMDG and Tris do not bind at the same location as do the other blockers. The conclusion to be drawn from this simple analysis is that the CMC channel does not behave as a one-binding site channel and should contain at least two binding sites. We also tentatively conclude that the TEA-type blockers bind mainly at the external binding site ($0.26$), whereas NMDG and Tris can reach a site deeper inside the channel also. Three remarks should be made. First, the electrical distances were calculated from the ratio of two sets of current-voltage curves. A shift of the membrane potential of one current-voltage curve relative to the other should change the current ratio and the electrical distance. Calculations to test whether the voltage dependence of the electrical distance of the NMDG-type block could originate from a membrane potential shift indicated that the deviation at holding potentials more negative than $-50$ mV is negligible. The voltage-dependent change of the electrical distance cannot be explained by such artifact. Second, the addition of $50$ mM blocker increases the surface charge screening and should reduce the Na$^+$ concentration close to the pore entrance. If one assumes a charge density of $-0.03$ e/nm$^2$ as determined earlier, the Na$^+$ concentration decreases by $<3\%$, and the surface potential changes by $<1$ mV. Because the surface charge density is low, it is possible to compare directly the Na$^+$ current in the presence and in the absence of $50$ mM blocker. Third, the seemingly different block mechanisms between TEA- and NMDG-like blockers are not related to the size of the molecules. Tris is the second smallest blocker we used, whereas NMDG is the largest. In fact, the data presented in the next section suggest that the mechanisms of block of all these organic cations basically do not differ.

Affinity of the Channel for NMDG and TEA

To test further the possibility that multiple binding sites are present inside the pore, the apparent $K_M$ for NMDG (Figs. 6 and 7) and TEA (Fig. 8) were measured.
with increasing extracellular Na\(^+\) concentrations. The rationale for this experiment is the following: if the channel has two binding sites and the blockers have access to both sites, then increasing the extracellular Na\(^+\) concentration should increase the probability that the channel is occupied by the blocker at the deeper binding site and by the Na\(^+\) ion at the more external binding site. In other words, the apparent affinity should increase at high Na\(^+\) concentration, because the blocker will be locked in by the Na\(^+\) ion present at the first binding site and will therefore leave the channel more slowly toward the extracellular side. If the blocker and the Na\(^+\) ion were competing for only one binding site, the apparent \(K_m\) for the blocker should increase linearly with the Na\(^+\) concentration as

\[
K_{mB} = K_{mB}^0(0\ Na^+)\frac{[Na^+]}{K_{mNa}^+},
\]

where \(K_{mB}\) is the apparent dissociation constant for the blocker, \(K_{mB}^0(0\ Na^+)\) the dissociation constant in the absence of Na\(^+\), and \(K_{mNa}^+\) the dissociation constant for Na\(^+\). Clearly, the apparent \(K_m\) for NMDG and TEA did not increase linearly with the Na\(^+\) concentration as would be expected for a one-binding site curve (Figs. 7B and 8B). The fact that the affinity for the blockers increased with increasing Na\(^+\) concentrations relative to the theoretical expectation supports the idea that the CMC channel possesses at least two binding sites that can be occupied by these blockers. As a first approximation, the ratio between the measured \(K_m\) and the expected one-binding site, \(K_m\) reflects the degree of occupancy of the first binding site by Na\(^+\). This ratio is equal to the ratio of the off rates, \(k_{-1}\) (experimental) \(k_{-1}\) (theoretical) if one assumes that the on rates are governed only by the competition between Na\(^+\) and the blocker (i.e., theoretical and measured on rates are equal). The value \((1-\text{ratio})\) varies from 0, when the first binding site is empty, to 1, when it is completely occupied by Na\(^+\). The Na\(^+\) concentration for half occupancy is on the order of 500 mM (Figs. 7C and 8C), which is an order of magnitude higher than the apparent \(K_m\) of the channel for Na\(^+\). This value is an estimate of the reduced affinity of the first binding site for Na\(^+\) when the second one is occupied by a blocker. Note that this estimate does not take into account the fact that the blocker could leave the channel toward the cytoplasmic side. This ratio is not dependent on holding potential, as shown in Figs. 7D and 8D, which indicates that the first binding site is apparently very close to the extracellular entrance. Although we could satisfactorily fit the TEA block of Fig. 5, assuming just one binding site located at 0.2630 from the extracellular side, these supplemental data suggest that TEA, like NMDG, can reach a second binding site. The weak voltage dependency of the apparent electrical distance of the TEA block (Fig. 8E) strengthens this view and might even suggest that TEA can cross the entire channel at strong hyperpolarization.

\(K^+ / Li^+\) Mole Fraction Experiment

A classical way to find evidence for multiple binding sites inside a channel is to look for an anomalous mole fraction effect. The current in the presence of mixtures of varying proportions of K\(^+\) and Li\(^+\), but with a constant total cation concentration, was measured at \(-50\) and \(-30\-mV\) holding potentials. To increase the chance of seeing anomalous effect, the current was measured in the presence of 500 mM cation.
Figure 8. The Na⁺ dependence of the TEA block also shows the presence of more than one binding site inside the pore. (A) Same as Fig. 7 A but with n = 5 for 70 mM Na⁺ and 12.5 mM TEA, 6 (70, 25), 7 (70, 50); 9 (140 mM Na⁺, 12.5 mM TEA), 14 (140, 25), 9 (140, 50); 6 (280 mM Na⁺, 12.5 mM TEA), 8 (280, 25), 8 (280, 50); 10 (500 mM Na⁺, 12.5 mM TEA), 9 (500, 25), 9 (500, 50). (B-E) Same as Fig. 7. Note the weak but clear voltage dependency of the TEA block in E.
ion in the pipette. Even under these conditions, the current diminished monotonically when the Li⁺ fraction was increased from 0 to 1 (Fig. 9), whereas an anomalous effect would appear if the current passed through a minimum with a mixture of Li⁺ and K⁺. These data suggest that the probability of the channel being doubly occupied by these alkali cations is low, even in the presence of 500 mM permeant ion. This agrees well with our estimate of the affinity of Na⁺ for the first binding site when the second one is occupied by an organic blocker, which was ~500 mM.

**The Ca²⁺ Block Depends on the Extracellular Na⁺ Concentration**

The Ca²⁺ ion is known to cross the CMC channel very slowly. Its sojourn time inside the channel varies between 2 ms at -30 mV and 0.2 ms at -120 mV (Li et al., 1994). If our hypothesis that the CMC channel has at least two binding sites is correct, then it should be possible to modulate the sojourn time of Ca²⁺ by varying the

![Figure 9](image_url)

**Figure 9.** Currents with Li⁺–K⁺ mixtures show no signs of the anomalous mole fraction effect. Single-channel current as a function of the Li⁺ fraction with 500 mM total cation concentration, at -30 mV (open triangle) and -50 mV (closed triangle). The dotted lines are the theoretical currents for a one-binding site channel fit from $I_{\text{mixture}} = (k_{\text{in}} [\text{Li}^+] / K_{\text{Li}^+} + k_{\text{out}} [\text{K}^+] / K_{\text{K}^+})/([1 + (k_{\text{in}} [\text{Li}^+] / K_{\text{Li}^+} + k_{\text{out}} [\text{K}^+] / K_{\text{K}^+})])$, with $k_{\text{in}} = -6.5, -4.6 \text{ pA}, k_{\text{out}} = -1.55, -1.15 \text{ pA}, K_{\text{Li}^+} = 51, 46 \text{ mM}, K_{\text{K}^+} = 20, 21 \text{ mM at } -50 \text{ mV and } -30 \text{ mV, respectively. Note that the affinity for Li⁺ is twice as high as for K⁺, consistent with a higher relative permeability and a lower conductance (Li et al., 1994). Solutions: 5 mM HEPES, 1 mM EGTA, with 500 mM KCl (n = 4), 500 mM LiCl (n = 15), 125 mM LiCl + 375 mM KCl (n = 4), 375 mM LiCl + 125 mM KCl (n = 7), and 437.5 mM LiCl + 62.5 mM KCl (n = 4) in the pipettes; bath solution, 140 mM NaCl, 10 mM HEPES, and 1 mM CaCl₂ at a pH adjusted at 7.4.

Na⁺ concentration. Fig. 10 shows the change in Ca²⁺ block kinetics when the extracellular Na⁺ concentration was increased from 35 to 280 mM. The on rates were three times slower, whereas the off rates increased three times when the Na⁺ concentration was raised from 35 to 280 mM. After the analysis of Kuo and Hess (1993), the change in off rates was used to estimate the occupancy by Na⁺ of the first binding site. Fits of the data with a rectangular hyperbola (Fig. 10 B) gave a $K_M$ of 1,200 mM. This value was larger than the estimated $K_M$ of the first binding site in the presence of an organic blocker (~500 mM) and could mean that it is more difficult for Na⁺ to occupy the first binding site when Ca²⁺, a doubly charged cation, occupies the second binding site. The on rates were also affected by the Na⁺ concentration, but the origin of these variations was less clear. The factors involved in these changes are the ionic strength, the competition between Na⁺ and Ca²⁺ for
Figure 10. The extracellular Ca\(^{2+}\) block is modulated by the extracellular Na\(^{+}\) concentration. (A) Single-channel recordings with the dwell time distributions of the same experiments in the presence of 70, 140, and 280 mM Na\(^{+}\), 0.16 mM free Ca\(^{2+}\), at -30 mV holding potential. Cut off frequencies 4 KHz (140 mM and 280 mM Na\(^{+}\)) and 2 KHz (70 mM Na\(^{+}\)). The solid lines represent the zero current level. (B, top) Voltage dependency of the dwell times with 280 mM Na\(^{+}\) (closed circle; n = 5), 140 mM Na\(^{+}\) (open circle; n = 7), 70 mM Na\(^{+}\) (closed square; n = 4), and 35 mM Na\(^{+}\) (open square; n = 4). (Bottom) On (1/\(\tau_{on}\)) and off (1/\(\tau_{off}\)) rates are function of the Na\(^{+}\) concentration at -120 mV (closed triangle), -80 mV (open triangle), -50 mV (closed diamond), and -30 mV (open diamond) holding potentials. The dotted lines of lower left panel are theoretical fits corrected for the cell membrane potential, \(k_{on} = k_{on}(0 \text{ mV})/[1 + ([\text{Na}^{+}]/K_{M^{Na^{+}}})]\) with \(k_{on}(0 \text{ mV}) = 540 \text{ s}^{-1}\), and an electrical distance of \(k_{on} = 0.093\), and \(K_{M^{Na^{+}}}^{Na^{+}}\) taken from Fig. 3D. No surface potential correction for the Ca\(^{2+}\) concentrations. The dotted lines of lower right panel are theoretical fits corrected for the cell membrane potential, \(k_{off} = k_{off}(0 \text{ mV})/[1 + ([\text{Na}^{+}]/K_{M^{Na^{+}}}^{Na^{+}})]\) with \(k_{off}(0 \text{ mV}) = 3,700 \text{ s}^{-1}\), an electrical distance of \(k_{off} = 0.2\), and a \(K_{M}(0 \text{ mV})\) for Na\(^{+}\) = 1,200 mM, with an electrical distance of 0.23.
EDTA and for the channel, and the surface potential. If one assumes that surface potential and ionic strength effects compensate exactly, then the voltage and concentration dependencies of the on rate point to an electrical distance of 0.093 and an association rate constant of $3.41 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (Fig. 10 B). This high value can be compared with the theoretical expectation for the diffusion-limited interaction of Ca$^{2+}$ with the channel (Lansman, Hess, and Tsien, 1986):

$$k_{\text{theory}} = 2\pi \Delta r DN 10^{-3}$$

where $D$ is the diffusion coefficient for Ca$^{2+}$, $N$ the Avogadro number, and $\Delta r$ the difference between the ion and channel radius. If the surface charge density is $-0.03 \text{e/nm}^2$, the Ca$^{2+}$ concentration at the entrance will be, on average, one and a half times the bulk-free concentration. We know that large organic cations can reach the first binding site of the channel. The radius of TPA extrapolated from NH$_4$ (1.7), TMA (2.75) (Lansman et al., 1986), and TEA (4.5) (Armstrong, 1971) is $\sim 6 \text{Å}$. If this value is taken as the entrance radius, then the expected value matches the experiment alone. This rough estimate implies that the high value of the on rate is an indicator of a broad first portion of the pore.

**No Measurable Ca$^{2+}$ Current Is Carried by the CMC Channel**

The CMC channel has several properties reminiscent of the L-type Ca$^{2+}$ channel (see Table II), in particular its high affinity for Ca$^{2+}$ and its multiple binding sites. These two characteristics are required to generate a Ca$^{2+}$ current through the highly selective Ca$^{2+}$ channel at physiological Ca$^{2+}$ concentration (Almers, McCleskey, and Palade, 1984; Tsien, Hess, McCleskey, and Rosenberg, 1987). Single-channel Ca$^{2+}$ current through the Ca$^{2+}$ channel is best observed in the presence of a high Ca$^{2+}$ concentration in the pipette, typically 90 mM. Unfortunately, in this condition, neither inward nor outward currents crossing the CMC channel can be detected in cell-attached patches of the apical membrane of the ectoderm (unpublished observations). However, in the presence of 10 mM extracellular Ca$^{2+}$, it was still possible to observe an outward current at $+150 \text{mV}$. The protocol to test whether the CMC channel conducts a measurable Ca$^{2+}$ current with 10 mM Ca$^{2+}$ consisted of a 50-ms depolarizing pulse at $+150 \text{mV}$, followed by a hyperpolarization at $-180 \text{mV}$ during 450 ms repeated every 2 s. Because the open time ($\tau_o = 7 \text{s}$) and the closed time ($\tau_c = 13 \text{s}$) are voltage independent (see earlier), one can assume that the channel is in an open conformation at $-180 \text{mV}$ if the channel is open before and after depolarizing pulses. The probability that the channel appears open during two consecutive depolarizations but closes and reopens between the depolarizing pulses can be calculated as follows:

$$\Sigma([1 - \exp(-k_{\text{on}}t)][1 - \exp(-k_{\text{off}}(T - t))] \Delta t/T$$

where $T = 1.95$, $s$ is the interval between the depolarizing pulses. Because this probability is only 0.6%, it is possible to correlate the open-channel current that is visible at the $+150\text{mV}$ depolarizing pulses with the total current through the patch (leak current) of the subsequent hyperpolarizations at $-180 \text{mV}$, measured as the average current of the last 150 ms (Fig. 11 A). A Ca$^{2+}$ flow through the CMC channel too small to be identified as a single-channel current could still appear as a
TABLE II

Comparison between Cyclic-Nucleotide Gated Channels, L-type Ca$^{2+}$ Channel and CMC Channel

<table>
<thead>
<tr>
<th>CNG channels</th>
<th>CMC channel</th>
<th>L-type Ca$^{2+}$ channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selectivity</td>
<td>K$^+$$&gt;$Na$^+$$&gt;$Rb$^+$$&gt;$Li$^+$$&gt;$Ca$^{2+}$</td>
<td>Li$^+$$&gt;$Na$^+$$&gt;=$Ca$^+$</td>
</tr>
<tr>
<td>Sequence type</td>
<td>Eisenman VIII-IX or polarizability</td>
<td>Eisenman XI</td>
</tr>
<tr>
<td></td>
<td>Li$^+$ anomaly</td>
<td></td>
</tr>
<tr>
<td>Pore diameter (Å)</td>
<td>5.9 $^b$, 6.3 $^i$</td>
<td>5.8</td>
</tr>
<tr>
<td>Current rectification</td>
<td>yes $^k$</td>
<td>yes</td>
</tr>
<tr>
<td>Current sublevel</td>
<td>yes (olfactory) $^l$</td>
<td>no</td>
</tr>
<tr>
<td>Multiple binding sites</td>
<td>probable $^o$</td>
<td>yes</td>
</tr>
<tr>
<td>Ca$^{2+}$ affinity</td>
<td>1–2 μM $^p$</td>
<td>&lt;1 μM $^q$</td>
</tr>
<tr>
<td>Electrical distance of extracellular Ca$^{2+}$ block</td>
<td>0.35 $^i$</td>
<td>0.1 to 0.335 $^e$</td>
</tr>
<tr>
<td>Possible role for high Ca$^{2+}$ affinity</td>
<td>low noise membrane</td>
<td>avoid sodium load</td>
</tr>
<tr>
<td>Voltage-dependency</td>
<td>?</td>
<td>no</td>
</tr>
<tr>
<td>Run-down after patch excision</td>
<td>?</td>
<td>yes</td>
</tr>
</tbody>
</table>

*From relative permeability; $^b$rod (Goulding et al., 1993; Eismann et al., 1994); $^i$olfactory (Goulding et al., 1993); $^j$cone (Picones and Korenbrot, 1992); $^k$L (Li et al., 1994); $^l$cardiac muscle (Hess et al., 1986); skeletal muscle (Coronado and Smith, 1987); $^m$Eisenman and Horn, 1983); $^n$rod (Goulding et al., 1993); $^o$olfactory (Goulding et al., 1993); $^p$skeletal muscle (McCleskey and Almers, 1985); $^q$retinal (Root and MacKinnon, 1993); $^r$(Goulding et al., 1992); $^s$(Eis-mann, 1983); $^t$olfactory (Zufall and Firestein, 1993), Na$^+$ as permeant ion; $^u$Na$^+$ as permeant ion, −30 mV; $^v$olfactory (Zufall and Firestein, 1993), Na$^+$ as permeant ion; $^w$Na$^+$ as permeant ion, −30 mV; $^x$(Lansman et al., 1986), Li$^+$ as permeant ion, −40 mV; $^y$rod (Eismann et al., 1994).

change of the leak current at −180 mV. It should coincide with the number of open channels seen during the depolarizing pulses. Fig. 11 B presents the fluctuation of the leak current at −180 mV during an experiment. There was no obvious correlation between the opening of the channels at +150 mV and the following leak current at −180 mV. The standard deviation of the fluctuation of the leak current at −180 mV was 49 ± 18 fA (n = 3). This value can be regarded as an estimate of the resolution of the system and an upper limit for the Ca$^{2+}$ current through the CMC channel at −180 mV. In other words, the largest possible Ca$^{2+}$ current through the CMC channel at −180 mV should be <49 fA.

At −180 mV with 10 mM extracellular Ca$^{2+}$, the on rate will be $6.8 \times 10^7$ s$^{-1}$ and the off rate $4 \times 10^4$ s$^{-1}$ (Li et al., 1994). A one-binding site channel would be almost completely saturated as the on rate is 1,700 times faster than the off rate, and the Ca$^{2+}$ flux would depend only on the off rate (i.e., 13 fA at −180 mV holding potential). This value is not far from the estimated upper limit for a possible Ca$^{2+}$ current (49 fA), which suggests that in the presence of 10 mM extracellular Ca$^{2+}$, no more than one Ca$^{2+}$ ion can bind at one time inside the CMC channel or that the multi-
occupancy does not enhance the Ca$^{2+}$ current through the CMC channel as it does through the L-type Ca$^{2+}$ channel. For comparison, the Ca$^{2+}$ current through the Ca$^{2+}$ channel at $-70$ mV with 10 mM extracellular Ca$^{2+}$ is $-0.5$ pA (Gollasch, Hescheler, Quayle, Patlak, and Nelson, 1992) or $-1.0$ pA if linearly extrapolated at $-180$ mV.

![Graph showing current rectification](image)

**Figure 11.** No inward Ca$^{2+}$ current is measurable at strong hyperpolarization in the presence of 10 mM extracellular Ca$^{2+}$. (A) Three consecutive traces filtered at 1 KHz and sampled at 0.5 KHz. The first two traces show the presence of an open channel at $+150$ mV. The dotted lines represent the zero levels obtained from the average of the traces without openings. Recording from a cell-attached patch with 10 mM CaCl$_2$, 140 mM NaCl, and 10 mM HEPES at pH 7.4. (B) Fluctuation of the average current of the last 150 ms of the $-180$-mV pulse. The average leak current over the whole experiment was subtracted from the leak currents at each pulse. The leak current preceded by a prepulse at $+150$ mV without opening is shown as an empty circle, with one open level as a closed square and with two open levels as closed triangles. The average leak current from three experiments is 26.9 fA when no opening was visible during the prepulse, 6.4 fA with one open level, and $-14.5$ fA with two open levels.

**DISCUSSION**

Some basic permeation properties of the CMC channel are described in this article: (a) The current rectifies in the presence of symmetrical Na$^+$ concentrations and in the absence of Ca$^{2+}$. (b) The extracellular surface potential is $\sim 0.03$ e/\text{nm}^2. (c) Functional measures of the minimal pore size give a diameter of 5.8. (d) Quaternary ammonium derivatives bind inside the channel at an apparent electrical distance of 0.26. Other organic blockers such as NMDG or Tris seem to bind deeper inside the pore. (5) The block of the inward Na$^+$ current by organic block-
ers and Ca$^{2+}$ varies with the permeant ion concentration, which is interpreted as being attributable to the presence of at least two binding sites.

**Rectification**

Cell-attached experiments with K$^+$ in the pipette have shown that the CMC channel rectifies inwardly in the absence of extracellular Ca$^{2+}$ (Li et al., 1994). However, it was not clear whether this rectification was due to the presence of blockers or was an intrinsic property of the channel. In this article, we show that the inward rectification cannot originate from the presence of blockers. An intrinsic rectification can originate from asymmetrical charge or dipole distributions, or from unequal cytoplasmic and extracellular surface potentials. It is interesting to mention the appearance of rectification when a negatively charged glutamate is replaced by a neutral amino acid at the extracellular entrance of the retinal CNG channel (Root and MacKinnon, 1993; Eismann, Müller, Heinmann, and Kaupp, 1994). The presence of a negative charge at the extracellular entrance of the CMC channel could indeed explain both the rectification and the small electrical distance of the on rate of the Ca$^{2+}$ block (Li et al., 1994). It has also been proposed that the macrodipoles of $\alpha$-helices parallel to the pore could create an asymmetrical energy profile and produce a similar rectification in peptide models (Kienker, DeGrado, and Lear, 1994; but see Eisenman and Alvarez, 1991, for a possible impermeable parallel $\alpha$-helices bundle if electrostatically uncompensated). A rectification can also occur in case of unequal intracellular and extracellular surface potentials or partition coefficients (Skinner, Ward, and Bardakjian, 1993).

**Surface Potential**

The surface potential at the extracellular entrance of the CMC channel is estimated to be $-0.03 \, \varepsilon/\text{nm}^2$. This value is much lower than the ones estimated from other channels. For example, the voltage-dependent Na$^+$ channel ($-0.38 \, \varepsilon/\text{nm}^2$) (Green, Weiss, and Andersen, 1987), or the L-type Ca$^{2+}$ channel ($-0.16 \, \varepsilon/\text{nm}^2$) (Kuo and Hess, 1992) have surface charge densities 13 and 5 times larger. In our experiments, the charge density includes the lipid membrane and the channel itself. If the lipid bilayer comprises 20% negatively charged lipid and the average surface occupied by a phospholipid molecule is 0.6 nm$^2$, then the surface charge density of the lipid membrane is $\sim 0.3 \, \varepsilon/\text{nm}^2$ (McLaughlin, 1977). Our low measure of the CMC channel surface charge density could suggest that the pore is actually insulated from the lipid membrane surface potential (see, for example, Coronado and Affolter, 1986).

The estimate of the surface potential is difficult and uncertain on several grounds. The Gouy-Chapman equation is only a rough approximation of the physical situation (McLaughlin, 1977; Green and Andersen, 1991). The calculation also depends markedly on the permeation model used (see, for example, Naranjo and Latorre, 1993). Moreover, it is probably impossible to find a molecule that can efficiently screen the surface charge without blocking the CMC channel. In this respect, the experimental protocol described by Kuo and Hess (1992) could be applied to the CMC channel to improve the evaluation of the surface potential.
**Pore Size**

In a previous article, we showed that the CMC channel selects only weakly between the alkali cations (Li et al., 1994). Reuter and Stevens (1980) have developed a selectivity theory that relates the permeabilities to physical properties of a pore with one rate-limiting barrier. In their theory, the barrier height is dependent on the reciprocal of the ion radius, and this dependence can be expanded as a power series. The first coefficient $a_1$ of the expansion reflects, in part, the "fluidity" of the channel water. In the case of the CMC channel, $a_1 = 0.5 \text{ kJ} \cdot \text{Å}^{-1} \cdot \text{mol}^{-1}$, which is very low and could indicate a large pore (unpublished observation). For comparison, $a_1 = 1.4 \text{ kJ} \cdot \text{Å}^{-1} \cdot \text{mol}^{-1}$ for the acetylcholine receptor (Bormann, Hamill, and Sakmann, 1987).

It is therefore not surprising to find that the CMC channel has a relatively large pore, 5.8 Å diam. The skeletal muscle Ca$^{2+}$ channel (6.0 Å) (McCleskey and Almers, 1985), the retinal CNG channel (5.9 Å), and the olfactory CNG channel (6.3 Å) (Goulding, Tibbs, Liu, and Siegelbaum, 1993) have similar pore sizes. The conductance sequence of the organic cations follow approximately the same sequence as the relative selectivity. This suggests that the selectivity mechanism of the CMC channel for organic cations, unlike alkali cations (Li et al., 1994), rests mainly on the height of the barriers and not on the detail of the wells of the energy profile.

**Block**

All organic cations tested can travel at least 26% of the total length of the ionic path from the extracellular side. The diameter of the first quarter of the pore is therefore quite large. The decrease of the electrical distance with strong hyperpolarizations, as observed with TEA, NMDG, and Tris, could be an indication that these blockers can cross the CMC channel. The minimal pore diameter could therefore be larger than the 5.8 Å estimated from the permeability ratio experiments. The blockers that could most obviously cross the channel are NMDG and Tris. They differ from the others by their many hydroxyl groups, three for Tris and five for NMDG. We propose that the presence of these hydroxyl groups could facilitate the transport of these molecules along the ionic path because they can form hydrogen bonds with the wall residues.

The data presented in this article are analyzed in terms of simple Eyring models, and the conclusions depend heavily on their many simplifying assumptions. The most puzzling one is perhaps the fact that the large NMDG molecule could cross the CMC channel. Indeed, with 140 mM NMDG ($n = 4$) or Tris ($n = 4$) as the only cation in the pipette, no inward current could be observed (not shown). An analysis of the variations of the leak current at high negative potentials ($-150$ to $-200$ mV), as was done to test the Ca$^{2+}$ permeability, was impossible because of the large-leak current fluctuations, which presumably are the result of the low seal resistance in the absence of divalent cation in the pipette. The absence of NMDG and Tris currents in these conditions could be rationalized if one assumes that these blockers can pass through the channel only if they are pushed by an ion present at the external binding site and if two blocker molecules cannot occupy the channel simultaneously.

Other mechanisms could explain the independence of the fraction of unblocked...
current at strong hyperpolarizing potentials (Fig. 5), without the assumption that the blocker can pass through the channel. For example, the control current at very negative potentials could begin to saturate because of the interfacial polarization (Andersen, 1983). The fraction of unblocked current being the ratio between the current in the presence of the blocker and the current in its absence, such effect will decrease the denominator and increase the ratio. Another possible explanation is that the assumption that the rate constants are exponentially dependent on the membrane potential is no longer valid at strong voltages. We could also invoke a conformational change of the pore at strong hyperpolarizations, which could decrease the affinity of the channel for the blockers.

**Multioccupancy**

The ability to modulate the organic cations and Ca\(^{2+}\) blocks by changing the extracellular Na\(^+\) concentration strongly suggests that the CMC channel has more than one binding site inside its pore. The change in \(K_m\) for the organic blockers is a kind of lock-in effect, whereas the increased off rate of the Ca\(^{2+}\) block by the extracellular Na\(^+\) is also called an enhancement effect (Kuo and Hess, 1993). These mechanisms have previously been demonstrated in the Ca\(^{2+}\)-activated K\(^+\) channel (Neyton and Miller, 1988) and in the L-type Ca\(^{2+}\) channel (Kuo and Hess, 1993). Taken together, the lock-in and enhancement experiments with the mole fraction data point to a channel that has at least two binding sites but that is occupied by only one ion at a time under physiological conditions.

**Ca\(^{2+}\) Permeability**

In our experimental conditions, the CMC channel, contrary to the L-type Ca\(^{2+}\) channel, carries no significant Ca\(^{2+}\) current. It is interesting to note that Ag\(^+\) can induce a divalent cation current through the poorly selective channel in toad urinary bladder (van Driessche, 1987), possibly through a direct interaction with the channel. Our data do not exclude the possibility that the permeation properties of the CMC channel can be modulated by some intracellular compound.

**A Possible Structural Model**

The data described in this work suggest that the embryonic CMC channel is closely related to the L-type Ca\(^{2+}\) channels and to the CNG channels (Table II). A possible model of the CMC channel, based on our results and the proposed structures for the CNG channels and the Ca\(^{2+}\) channel, is the following: the first part of the path is large enough to allow a block by large quaternary ammonium derivatives. This portion is mainly hydrophobic but contains one negative charge responsible for both the Ca\(^{2+}\) block and the inward rectification. The rest of the path is narrower and more hydrophilic because NMDG and Tris can reach a second binding site downstream of the pore more easily than can the other blockers. It is this portion of the path that is responsible for organic cation selectivity according to size. The affinity of the second binding site for Ca\(^{2+}\) probably is much lower than the first site because of the presence of an outward K\(^+\) current in the presence of 1 mM extracellular Ca\(^{2+}\).
This paper is dedicated to the memory of Peter Hess.

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