Monovalent Selectivity of the Cyclic Guanosine Monophosphate–activated Ion Channel

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ABSTRACT Monovalent cation selectivity has been characterized for the 3',5'-cyclic guanosine monophosphate (cGMP)-activated channel in vertebrate photoreceptor outer segment plasma membranes without divalent cations. Macroscopic currents in excised, inside-out patches were activated with saturating concentrations of cGMP (200 μM). Using a bi-ionic protocol with symmetrical 120 mM ion concentrations across the membrane, alkali metal ions and certain organic cations were substituted for sodium on the cytoplasmic face. The relative permeabilities, determined from shifts in the reversal potential (E_r<sub>rev</sub>), were NH<sub>4</sub> ≫ Na > guanidinium > K > Li > Rb > Cs (3.34 : 1.0 : 0.97 : 0.93 : 0.92 : 0.74 : 0.50, respectively). E_r<sub>rev</sub>'s were also measured as a function of [Na], [NH<sub>4</sub>], and [Cs], and the slope of the relation was -59.8, -52.1, and -49.1 mV/decade, respectively. The slopes for NH<sub>4</sub> and Cs differ significantly from the Nernst-Planck prediction of -58.2 mV/decade expected for a single ion channel. Relative permeabilities were also determined for the alkali metal series of ions with 20 mM ionic concentrations on both sides of the membrane. The permeability sequence at 20 mM was unchanged, but the relative permeability for NH<sub>4</sub> and Cs deviated significantly from the measurements at 120 mM with 1.46 and 0.75 ratios, respectively. The dependence of E_r<sub>rev</sub> on absolute concentrations and the deviation from Nernst-Planck predictions are best explained by multi-ion occupancy of the cGMP-activated channel. Selectivity was also examined by comparing the conductance ratios as a function of potential. The conductance sequence relative to sodium at +80 mV was NH<sub>4</sub> > K > Rb > Cs > Li with values of 0.93 : 0.79 : 0.43 : 0.33 : 0.27, respectively. We conclude that the photoreceptor cGMP-activated channel shows little discrimination among monovalent cations without divalent cations, and that the relative permeability follows an Eisenman series IX for a high field strength site. The channel appears to process more than one ion at a time and to have asymmetric energy barriers with respect to the membrane plane for several ions.

INTRODUCTION

The rod outer segment of vertebrate photoreceptors is a unique neural structure designed for the transduction of environmental light signals into electrical messages.

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for transmission into the brain. From extensive biochemical and physiological study, we know the details of how the photoisomerization of rhodopsin initiates a multistep enzymatic cascade that results in the hydrolysis of 3',5'-cyclic GMP (cGMP). This cytosolic messenger couples the photochemical machinery of the disk membranes to the ionic conductance of the plasmalemma that is responsible for the first step in the electrical signaling of photon capture (for reviews, see Pugh and Cobbs, 1986; Stryer, 1986; and Yau and Baylor, 1989). In the dark, cGMP directly and cooperatively activates the ion channels of the outer segment membrane generating a tonic, dark current carried by the influx of both sodium and calcium ions. In the light, the hydrolysis of the ~5 μM free, cytosolic cGMP (Yau and Nakatani, 1985) decreases the dark current producing a hyperpolarization of the outer segment, which, in turn, activates voltage-dependent channels of the inner segment as the next step in the electrical transmission of information into the nervous system.

The vertebrate rod outer segment is a structure highly specialized for light transduction and contains primarily the photochemical machinery and a single class of ion channels, the cGMP-activated channel (Baylor et al., 1984), which is present in high copy numbers with 100–400 channels/μm². Such membrane specialization is in contrast to many central nervous system (CNS) nerve and muscle membranes that have arrays of different channel types each specialized for the conduction of a particular ion. The outer segment channel conducts two ions, sodium and calcium, under physiological conditions in ~4:6:1 ratio (Yau and Nakatani, 1984). The sodium currents function as an electrical signal that contains short-term information on changes in light intensity. The role of the calcium currents, however, is only beginning to be understood. One role for Ca is improving the photoreceptor’s signal-to-noise ratio by blocking Na current through a single channel by a factor of 1,000 and, thereby, attenuating false signals produced by random, spontaneous channel closings. Another role hypothesized for Ca involves setting the photoreceptor’s light sensitivity which can reliably respond to signals ranging from single photons to bombardments with 10⁷ photons (Matthews et al., 1988; Nakatani and Yau, 1988).

This unique, important, and prototype channel of a class of sensory transduction channels has been reconstituted into planar bilayers from both native membranes (Tanaka et al., 1987; Bennett et al., 1989) and biochemically isolated and purified bovine preparations (Hanke et al., 1988), but comparison of results between the two preparations and with in situ data shows differences in details. Unlike the experiences reconstituting the acetylcholine receptor (see review in Montal et al., 1986) and the voltage-dependent sodium channel (Rosenberg et al., 1984; Hartshorne et al., 1985; Furman et al., 1986), which has undergone 40+ years of extensive biophysical investigations of the in situ channels upon which to compare native with reconstituted properties, comparable biophysical study of the cGMP-activated channel is in its infancy.

The general biophysical question to be addressed in this study is what is the ion selectivity of the cGMP-activated channel in rod vertebrate photoreceptors? Ion selectivity, the ability to conduct one ion from a pool of ions having similar size, valence, and coordination chemistry, is both a fingerprint for a channel protein and a functional probe of the architectural features of the walls of the channel's
conducting pore through the membrane. To define these properties in situ and to
develop insight about the permeation process, membrane currents are measured as a
function of voltage, ion species, and ion concentration. Using these approaches, we
want to eventually develop an understanding of how the cGMP-activated channel
passes both sodium and calcium.

Selectivity studies of the photoreceptor channel are complicated by the fact that
calcium blocks the sodium current through the channel. For these particular studies,
we have chosen conditions that eliminate divalent block of the channels and permit
us to compare the selectivity of the open channel for a series of monovalent metal
and organic cations. The following questions have been addressed: (a) How selective
is the channel for monovalent cations? (b) Can the channel process more than one
ion concurrently? (c) What are the relative rates of ion transport? (d) How does the
concentration and species of the ion influence conduction? (e) What are the
molecular dimensions of the pore?

Preliminary reports of this work have been published previously (Furman and

METHODS

Materials

EGTA, KCl, CsCl, HEPES, and tetraethylammonium (TEA) chloride were obtained from
Sigma Chemical Co., St. Louis, MO; tetramethylammonium (TMA) hydroxide was obtained
from Eastman Kodak Co., Rochester, NY. N-methyl-D-glucamine and RbOH were from
Aldrich Chemical Co., Milwaukee, WI. Mannitol, KOH, NaCl, and LiCl were from J. T. Baker
Chemical Co., Phillipsburg, N J., and LiOH and NaOH were from Fisher Scientific Co.,
Pittsburg, PA. cGMP was obtained from Chemical Dynamics Corp., S. Plainfield, NJ;
Calbiochem-Behring Corp., San Diego, CA; and Sigma Chemical Co.

Preparation

*Rana pipiens* were used for most experiments although *Rana catesbiana*, *Rana temporaria*, and
*Bufo marinus* were also used during the course of these experiments because, at various times,
the *Rana pipiens* cells would not permit stable seals. Systematic variation in ionic selectivity or
cGMP activation was not observed among these species (Tanaka et al., 1989).

All animals were housed in the lab at room temperature with a natural light-dark cycle,
except *Rana temporaria* which were stored in the refrigerator. Before dissection, the animals
were dark-adapted for several hours. The retinas of dark-adapted animals were dissected
under red light in cold frog Ringer’s solution (112 mM NaCl, 5 mM HEPES, pH 7.4, 1.1 mM
CaCl2, 1.6 mM MgCl2, and 1.9 mM KCl). One of the dissected retinas was finely minced in a
small volume of Ringer’s solution containing 0.5% Percoll (Schneitkamp and Bounds, 1987).
When the retinas were healthy, this procedure resulted in a large fraction of intact
photoreceptors. The other retina was placed in Ringer’s solution and cells were harvested by
gently tapping the tube. This latter procedure was more likely to produce outer segments only.
Both preps were stored in the dark on ice and used for as long as 8 h after the dissection. A
10-μl aliquot of cells was drawn from a gently swirled container and layered onto the floor of
the experimental chamber. A fraction of cells remained at the elution site, and the remaining
cells were flushed from the chamber before attempting to patch. All experiments were
performed in room light at 20–21°C.
Current Recording

Patch electrodes were fabricated from capillary tubes of potash soda lead glass (Corning no. 0010, 1.60 mm o.d., 1.15 i.d.), or, where indicated, of lead potash glass (Corning no. 8161, 1.65 mm o.d., 1.20 mm i.d.). Both glasses were obtained from Garner Glass Co., Claremont, CA. The electrodes were pulled in two stages, but not firepolished, to bubble numbers in methanol of 3.0–4.0 and to resistances of 8–20 MΩ when filled with standard bath solution A (see below).

Electrodes were connected to the headstage of a patch clamp amplifier (model 8900, Dagan Corp., Minneapolis, MN) by a 120 mM KCl/agar bridge formed from fine polyethylene tubing slipped over the end of a Ag/AgCl wire held in a polystyrene electrode holder (E. H. Wright Co., Guilford, CT). Similar agar bridges were used for both the ground electrode and a bath reference amplifier that compensated for the effects of liquid-junction potentials. When measured with respect to a 3 M KCl Ag/AgCl electrode, residual junction potentials were <1 mV. Electrode tip potentials, measured by breaking the pipette tip, were also <1 mV. Amplifier output was low pass filtered at 1 kHz (eight-pole Bessel, model 902LPF, Frequency Devices, Haverhill, MA) before display on an oscilloscope and acquisition by an IBM AT (5 kHz, 12 bit A/D, Labmaster, Scientific Solutions, Solon, OH).

A 4 × 12-mm chamber with a 150-μl volume was milled from Plexiglass. The bottom of the chamber was a glass coverslip attached by heating Kronig’s cement. Solution was introduced into the experimental chamber through small bore polyethylene tubing attached to a selectable reservoir of twelve solutions. Two banks of six solutions each were manually selected by a Teflon rotary flow valve (type 5011, Rheodyne Inc., Cotati, CA), and the solution from either bank was electronically selected by microvolume, two-way Teflon switches at the chamber (P/N LFAA 1201618H, Lee Co., Westbrook, CT). Outflow was via a fine suction capillary covered with a 0.2-μm nylon mesh screen to reduce noise and fluid level fluctuations. After forming an inside-out patch, the micromanipulator was adjusted to position the pipette tip in the stream of the inflow tube. Perfusion solutions contacted only plastic syringe barrels, polyethylene, or Teflon.

Solutions

The standard solution, solution A, for the patch electrode and the bath consisted of 120 mM NaCl, 5 mM HEPES-NaOH, pH 7.3, and 2 mM EGTA-NaOH. The standard monovalent cation test solutions contained 120 mM of the cation chloride salt, 5 mM HEPES and 2 mM EGTA. The EGTA stock and the pH of the final solution were adjusted to 7.3 with the corresponding hydroxide base, LiOH, KOH, and RbOH. In all solutions containing CsCl, however, RbOH was used because it was most similar to Cs with respect to relative Na permeability. The pH of all organic cation solutions was adjusted with TMA, except for NH₄Cl which was adjusted with NH₄OH.

Reversal potential (Eᵅ) measurements were made both in solution A and a low salt, iso-osmotic solution, B, containing 20 mM total cation with 2 mM EGTA/cation OH and the remainder cation chloride, 80 mM mannitol, and 5 mM HEPES, pH 7.3. Solution B pH was adjusted with base as described for solution A.

The Nemst potential was measured with Na, NH₄, and Cs at concentrations from 10 to 500 mM. For these experiments, solution C contained the indicated concentration of the cation chloride, 2 mM EGTA and 5 mM HEPES adjusted as described above. Sucrose was added to maintain the osmotic strength of solutions that were below 120 mM cation concentration.

The solutions used to test organic cation block of the sodium current, solution D, contained solution A with 120 mM NaCl besides 120 mM of the indicated organic cation chloride. In
these experiments, therefore, the ionic concentration of the test solution was 240 mM while the patch electrode contained the standard 120 mM NaCl, solution A.

**Correction of Offset Currents**

The voltage protocol used in most experiments to drive current across a patch was a 700-ms linear voltage ramp from -90 to +90 mV followed by a decreasing, symmetrical ramp that was generated by a custom microprocessor-controlled 14-bit D/A converter. Correction for linear capacitative offset currents produced by stray capacitance between the preamplifier input and ground was made by several techniques: (a) maintaining a constant, low solution level with flow, (b) electronically compensating residual capacitance with feedback, (c) digitally combining the up and down ramps, (d) employing slow ramps (260 μV/ms), and (e) digitally computing any residual capacitative current from the leak resistance in symmetrical solutions by comparing the mean holding current at 0 mV before the ramp with the mean current around 0 mV during the ramp (the current–voltage [IV] relation is linear in sufficiently small neighborhoods). The corrected currents stimulated by a voltage ramp were identical to the currents produced by a series of steady-state, constant-voltage steps (see below).

**cGMP-activated Currents**

The sodium current recorded from an inside-out excised patch in response to a 700-ms linear voltage ramp is shown in Fig. 1 A. Currents, potentials, and membrane surfaces are reported in physiological conventions. The small linear current, representing typical seal resistances of 1-5 GΩ, was recorded with symmetrical sodium solutions without cGMP. At saturating cGMP (200 μM), the current displays a characteristic slight outward rectification. The deviation from linearity with a slight decrease in current with hyperpolarization and increase with depolarization may arise from weak voltage dependent gating of the channel between a fully liganded closed state and a single open state (Karpen et al., 1988; Tanaka et al., 1989).

The glass–membrane interface demonstrated weak cation selectivity as expected for fixed anionic sites on the glass, although the shifts in $E_{\text{rev}}$ were small, <2 mV. To eliminate this contribution to the selectivity measurements of the cGMP-activated currents, each test ion was recorded in the absence and presence of 200 μM cGMP, and the leak current was digitally subtracted from the total current to give the net, cGMP-activated current. Activated currents were rapidly reversible, but we waited 1 min between solution changes to insure complete exchange and a new steady-state condition.

To insure that the voltage ramp produced steady-state cGMP-activated currents and that stray capacitances were compensated properly, we compared the ramp current to currents produced by constant voltage steps. First, the standard linear voltage ramp was applied to an excised outer segment patch as described. Then, the same patch was stimulated with a series of 10-mV voltage steps applied for 100 ms each. The current from the last 50 ms of each step was digitally averaged and compared to the ramp-stimulated current (Fig. 1 B). No difference is seen between the ramp and step currents leading us to conclude that the slow voltage ramp produces steady-state currents. Voltage ramps were preferred for most experiments because the full IV relation was quickly obtainable, and the lifetime of the patch was enhanced compared with voltage step protocols.

Significantly different forms of the cGMP-activated channel’s macroscopic IV relation can be obtained with different compositions of the patch electrode glass (Furman and Tanaka, 1988) secondary to leaching of multivalent ions from the glass. The IV relation obtained with Corning no. 0010 glass, e.g., Fig. 1, is believed to represent transmembrane sodium current free of divalent contamination (Furman and Tanaka, 1988).

The patches used in this report produced cGMP currents from several hundred picoamperes to 4 nA at +90 mV. The variation in current amplitude, however, did not appear to vary
**FIGURE 1.** cGMP-activated currents from *Rana pipiens* retinal rod outer segments. (A) Macroscopic current–voltage (IV) relations were recorded by applying a 700-ms linear voltage ramp from −90 to 90 mV across an excised, inside-out patch. The low, linear trace is the current driven across an unactivated patch bathed in symmetrical, 120 mM NaCl solutions (solution A, Methods). The larger, concave-upward response is the total current activated by 200 μM cGMP applied to the cytoplasmic surface, and it represents the sum of leak currents (low trace) and specific Na currents through the cGMP-activated photoreceptor channel. In all other figures in this paper, net cGMP-activated traces are shown in which the leak current has been subtracted digitally from the total current shown here. The slight outward rectification of the cGMP-activated current is seen without divalent cations and reflects the voltage dependence of the closed–open channel transition at saturating ligand concentrations (Karpen et al., 1988; Tanaka et al., 1989). Currents were filtered at 1 kHz and digitized at 5 kHz. The electrode resistance was 10 MΩ and the seal resistance was 6 GΩ. Linear, capacitative currents produced by the voltage ramp were compensated by several techniques (see Methods). Patch 7914. (B) The continuous trace is the net cGMP-activated current collected under voltage clamp while applying a linear voltage ramp as in A. The filled circles are time-averages of the last 50 ms of a 100-ms constant voltage step applied at 10-mV increments from a 0-mV holding potential over the interval −90 to 90 mV. No difference in net current response was seen using these two pulse protocols in this or any other patch (n > 20). To forestall seal breakdown, data was collected routinely with the voltage ramp. The shape of the IV relation in this patch is typical of ~80% of our patches; the other 20% showed less outward rectification and were more linear (see Fig. 9 A and Tanaka et al., 1989). The factors responsible for the variation in the shape of the response are not currently known. The current magnitudes shown in A and B are typical for patches using no. 0010 glass electrodes. Solutions and conditions are described in A. Patch 9410.

in a systematic fashion with the average size of the rod or the location patched along the rod nor did the selectivity properties vary with current amplitude.

**Determination of Reversal Potentials**

Reversal potentials were measured graphically from the amplified zero current crossings displayed by the computer. Permeabilities relative to sodium (P_{on}/P_{Na}) were calculated from
differences in $E_{rev}$'s using the Goldman-Hodgkin-Katz (GHK) equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$
\Delta E_{rev} = \frac{RT}{F} \ln \frac{a_{ion} \cdot P_{ion}}{a_{Na} \cdot P_{Na}},
$$

where $a_{ion}$ is the ionic activity of the species and $R$, $T$, and $F$ have their usual meanings. Activity coefficients for the alkali metal cations were taken from Robinson and Stokes (1965) and were given for 0.1 molal solutions at 25°C. Concentration values were used to calculate the relative organic permeabilities.

**Determination of Conductance-Voltage Relations**

Macroscopic conductance-voltage (GV) curves were calculated from values of steady-state currents sampled every 10 mV from −90 to +90 mV by averaging the currents in 1-nW neighborhoods and then by dividing the averaged current by the thermodynamic driving force, $E - E_{rev}$, where $E$ is the externally applied, transmembrane clamp potential and $E_{rev}$ is the zero current crossing of the IV relation. Undefined values (0/0) were replaced by linear interpolation between adjoining, defined conductance values.

**RESULTS**

**Selectivity Measures**

The selectivity of an ion channel for permeant species is commonly characterized by two measures: reversal potentials and conductances for each of the species (Hille, 1985). The $E_{rev}$ is an equilibrium measurement of the transmembrane voltage at which the unidirectional ion influx equals the efflux, i.e., an ion has an equal probability of moving in either direction across the membrane. Although the original equilibrium theories of Nernst and Goldman-Hodgkin-Katz posit several assumptions not commonly observed experimentally, nevertheless, a difference in $E_{rev}$'s between two permeant species can operationally define their relative permeability, $P_{ion}/P_{Na}$, by using Eq. 1. This ratio, in the general case, may depend on the concentration of the ions, the transmembrane potential, and the orientation of permeant species across the membrane. Permeability is invariant only for a restricted, but important, class of channels, i.e., those containing both a single ion and a symmetrical electrical architecture normal to the plane of the membrane. The second measure, conductance, is commonly performed under quasi-steady-state conditions and measures the net rate of ion transport across the membrane through the open channel. Like all transport phenomena, conductance displays competition, block, and saturation effects with changes in the ionic milieu. Only at infinite dilutions of the permeant species does the conductance ratio equal the permeability ratio for the class of ion channels that process only one ion at a time (Lauger, 1973; Coronado et al., 1980; Eisenman and Horn, 1983).

**Permeability: Bi-ionic Alkali Metal Cations**

Macroscopic IV relations of the cGMP-activated channel were measured in excised inside-out patches of outer segment plasma membranes at saturating concentrations of cGMP with equimolar substitution of single permeant ions for Na at the cytoplasmic face. Intracellular and extracellular solutions were otherwise identical.
and contained 2 mM EGTA to eliminate channel block by divalent cations. These conditions were chosen to minimize interaction among ion species within the channel and to maintain the channel in a fully ligand-bound state favoring channel opening. Bi-ionic IV relations from −90 to +90 mV for a typical patch are shown in Fig. 2 A. The most notable features are the small shifts in $E_{rev}$ with all alkali metal cations, the attenuation of both the inward sodium current (negative currents) and the outward currents carried by the test ions, and the similar shapes of the IV curves.

To evaluate the permeability of the test ions relative to Na, the differences in $E_{rev}$'s were measured, (Fig. 2 B) and the relative permeabilities were calculated using Eq. 1. From the similarity of the zero current crossings, one can see that the channel poorly discriminates among the smaller cations, Na, Li, and K. The increasingly more positive $E_{rev}$'s of Rb and Cs indicate the channel prefers sodium to ions with larger...
ionic crystal radii. Averaged data from a series of patches produced the permeability sequence Na > K > Li > Rb > Cs with permeability ratios of 1 : 0.93 : 0.92 : 0.74 : 0.50, respectively (Table I).

The relative conductance of these and other ions will be discussed in a later section.

Permeability: Organic Cations

We also examined bi-ionic IV relations in solutions with NH₄, choline, TMA, TEA, and guanidinium as current carriers at the cytoplasmic face. Of the five species, only

<table>
<thead>
<tr>
<th>Ion</th>
<th>E_{rev}</th>
<th>P/P_{Na}</th>
<th>n</th>
<th>G_{in}/G_{Na}</th>
<th>G_{out}/G_{Na}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li</td>
<td>2.3 ± 1.2</td>
<td>0.92</td>
<td>11</td>
<td>0.53 ± 0.1</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>K</td>
<td>1.5 ± 1.2</td>
<td>0.93</td>
<td>11</td>
<td>0.73 ± 0.15</td>
<td>0.79 ± 0.15</td>
</tr>
<tr>
<td>Rb</td>
<td>7.3 ± 1.8</td>
<td>0.74</td>
<td>12</td>
<td>0.45 ± 0.16</td>
<td>0.43 ± 0.11</td>
</tr>
<tr>
<td>Cs</td>
<td>16.6 ± 2.7</td>
<td>0.50</td>
<td>12</td>
<td>0.49 ± 0.16</td>
<td>0.35 ± 0.14</td>
</tr>
<tr>
<td>NH₄</td>
<td>−30.4 ± 5.8</td>
<td>3.34</td>
<td>4</td>
<td>0.93 ± 0.42</td>
<td>0.08 ± 0.064</td>
</tr>
<tr>
<td>Guanidinium</td>
<td>1.0 ± 1.8</td>
<td>0.97</td>
<td>3</td>
<td>0.16 ± 0.09</td>
<td>0.08 ± 0.004</td>
</tr>
</tbody>
</table>

Reversal potentials for no. 0010 glass were measured under bi-ionic conditions with 120 mM solution A in the pipette and the test cation in the bath (see Fig. 2). The relative permeabilities were calculated using the GHK relation, Eq. 1. \( G_{out}/G_{Na} \) is the ratio of the outward test conductance to the original conductance of Na in symmetrical solutions; \( G_{in}/G_{Na} \) is the ratio of the inward sodium conductance in the presence/absence of the test ion on the cytoplasmic face (see Fig. 11 A). Conductance values were measured at ±80 mV. The same protocol was used for no. 8161 electrodes except all solutions contained 120 mM NaCl or test cation, 10 mM HEPES/Na, pH 7.3, and 200 μM EGTA. Under these conditions, multivalent cations leach from the electrode glass and block the channel (Furman and Tanaka, 1988).

*Insufficient measurements were available for statistics at these potentials.

The large \( E_{rev} \) for NH₄ shifted the GV curves to potentials <80 mV.

NH₄ and guanidinium supported sufficient current to demonstrate reversal potentials (Fig. 3, A and B). The average \( \Delta E_{rev} \) for NH₄ and guanidinium were −30.4 mV and 1.0 mV corresponding to permeabilities relative to Na of 3.34 and 0.97, respectively (Table I).

Symmetry of the Free Energy Profile

Intuitively, permeability is a measure of the ease, or probability, that a permeant ion can enter into an ion channel's aqueous pathway through a membrane.
probability is a function of the potential energy barriers presented to the permeating species by the amino acids lining the channel's pore. In the simplest case of ion channels that process a single ion at a time, the absolute, equilibrium ionic permeability is solely a function of the tallest barrier height with respect to the bath that the ion experiences. In more complex cases, permeability depends on barrier peaks, well depths, and channel occupancy (Hille and Schwartz, 1978).

Permeability ratios, then, may depend on which side of the membrane the permeant ion species are placed. The only case in which reversal potentials are invariant to the side upon which the test species are substituted is a one-ion channel with a free energy profile that is symmetrical with respect to the plane of the membrane. In a channel with asymmetrical energy barriers, the change in the sign of the transmembrane electrical gradient created by switching the orientation of the permeant species will have an unequal effect on the tallest energy barrier, and, therefore, the magnitude of the thermodynamic equilibrium potential will vary with orientation. Channels with multi-ion occupancy will also show orientation-specificity of the reversal potentials.

Figure 3. Bi-ionic IV relations for organic cations. (A) The permeability of the cGMP-activated channel for NH₄ is greater than for Na as indicated by the shift of $E_{rev}$ to hyperpolarizing potentials when 120 mM Na is replaced by 120 mM NH₄ at the cytoplasmic surface. $E_{rev}$ for NH₄ in this patch is $-23$ mV giving a permeability ratio of 2.6:1 for NH₄/Na. Solutions are described in the Methods and experimental protocol in Fig. 2. Patch 7924. (B) Both inward and outward currents are attenuated when guanidinium is the charge carrier at the cytoplasmic surface, but $E_{rev}$, 3 mV, is slightly greater than for Na, indicating that guanidinium is slightly less permeable than Na. The relative permeability, calculated from Eq. 1, is 0.89. The cytoplasmic solution for the smaller trace, labeled guanidinium, contained 120 mM guanidinium, 1 mM EGTA/TMA, and 10 mM HEPES/TMA, pH 7.3, while the extracellular and intracellular solution for the curve labeled, Na, was solution A. Patch 7723.
To assess the symmetry of the channel, permeability ratios for each orientation of the ion pairs, Li vs. Na, NH$_4$ vs. Na, and Cs vs. Na, were determined from changes in reversal potentials measured under symmetrical 120 mM bi-ionic conditions (Fig. 4). As might be expected from the small value of the $E_{rev}$ for Li in Fig. 2 B, $E_{rev}$ for Li/Na was $\sim 0$ mV. The larger $E_{rev}$ with NH$_4$ at the cytoplasmic face ($\sim -30.4$ mV) was not symmetric when measured in the opposite orientation (14.4 mV). These $E_{rev}$'s reflect a decrease in the relative permeability of NH$_4$/Na from 3.34 to 1.82. With Cs oriented first intracellularly, then extracellularly, an increase in relative permeability from 0.50 to 0.67 was seen corresponding to changes in $E_{rev}$ from 16.5 to $-10.2$ mV. These ratios show the direction of relative asymmetry is ion species-specific: Li enters the channel with equal ease from either side, whereas NH$_4$ enters the channel more easily from the cytoplasmic side, and Cs is more permeant from the extracellular side.

**Figure 4.** Symmetry of $E_{rev}$'s. $E_{rev}$'s were determined for the ion pairs, NH$_4$/Na, Li/Na, and Cs/Na, with both orientations across the membrane of the ions in each pair. NH$_4$ was more permeable from the extracellular side, Cs was more permeable from the intracellular side, and Li was equally permeant from either membrane surface. Error bars represent 1 SD. Cs/Na$_o = -10.2$ mV $\pm 0.84$ (n = 4), Na$_o$/Cs$_i = 16.5$ mV $\pm 0.84$ (n = 12); NH$_4$/Na$_o$ = 14.4 mV $\pm 1.1$ (n = 3), Na$_o$/NH$_4$$_i = -29.3$ mV $\pm 5.7$ (n = 6), Na$_o$/Li$_o$ = 2.3 $\pm 1.2$ (n = 12), Li$_i$/Na$_o$ = 2.0 (n = 1).

**Concentration Dependence of the Reversal Potential**

The classical treatment of ion permeation developed by Nernst (1888, 1889) and Planck (1890a, b) is based on the assumption of independent ion movement through a channel. Under such restrictions, the concentration dependence of the reversal potential for a single, permeant monovalent cation should vary linearly with a slope of $-58$ mV/decade at 20°C. Although most ion channels show numerous deviations from the independence principle, many channels still follow the Nernst-Planck predictions for $E_{rev}$ over a wide range of concentrations.

Initially, we examined the concentration dependence of the reversal potential as the concentration of NaCl in the bath was varied from 10 to 500 mM while the extracellular Na concentration was fixed at 120 mM, (Fig. 5 A). The slope of the relation averaged $-59.8$ mV/decade, in good agreement with the value predicted for a cation selective channel. Although this experiment is consistent with the hypothesis of previous studies that the photoreceptor channel is a single ion channel (see Discussion), we had difficulty reconciling many experimental observations in this
study with such a simple model. Therefore, we also measured the concentration dependence of \( E_{\text{rev}} \) for the most permeable cation, \( \text{NH}_4 \) (Fig. 5 B), and the least permeable cation, \( \text{Cs} \) (Fig. 5 C), with the same protocol as used for Na. For both \( \text{NH}_4 \) and \( \text{Cs} \) the response deviates appreciably from the expected slope of \(-58\) mV/decade.

**Figure 5.** Concentration dependence of the \( E_{\text{rev}} \). (A) The \( E_{\text{rev}} \) for Na, is plotted as the cytoplasmic concentration of Na is varied from 10 to 500 mM while holding the extracellular Na concentration at 120 mM. The slope of the linear regression (solid line) was \(-59.8\) mV/decade \((r = 0.999)\) in good agreement with an expected Nernst slope of \(-58.2\) mV/decade at 20°C. (○) Averaged data from three patches. (△) Data from a single patch, 6a153. (B) \( \text{NH}_4 \) \( E_{\text{rev}} \)'s were determined as in A with 120 mM \( \text{NH}_4 \) in the electrode, and the data were averaged for three patches except for the 10 mM point which contains two points (○; standard deviation is smaller than the plotting symbols). The solid line is the best linear fit with a slope of \(-52.1\) mV/decade \((r = 0.995)\). For comparison, the dotted line shows the nearly ideal Nernst response observed with Na in A. Although the number of concentration points is limited, \( \text{NH}_4 \) appears to systematically deviate from the Nernst prediction as the cytoplasmic concentration of \( \text{NH}_4 \) is lowered. (C) \( \text{Cs} \) permeability as a function of cytoplasmic concentration also exhibits deviations from the ideal behavior expected for a single-ion channel (dotted line). Data from two patches are shown and fitted by the solid line with a slope of \(-49.1\) mV/decade \((r = 0.994)\). Although there is more scatter in the data than with \( \text{NH}_4 \), \( \text{Cs} \) also appears to systematically deviate from the predicted response at lower concentrations.
mV/decade as the cytoplasmic ion concentration is reduced. Using a linear approximation for the deviation, the average slope for NH₄ was −52.1 and for Cs, −49.1 mV/decade. These deviations from the Nernst predictions are consistent with intrachannel interactions for both ammonium and cesium ions as has been reported in other channels (Begenisich and Cahalan, 1980 and references therein).

Dependence of Bi-ionic Reversal Potentials on Absolute Concentrations

Of the numerous classical tests for multi-ion occupancy, such as Ussing unidirectional flux ratios, non-Langmuir concentration–conductance relations, anomalous mole fraction effects, anomalously high voltage dependence of channel blockers, and dependence of reversal potentials on absolute concentration (Hille and Schwartz, 1978), the latter is most easily determined with our protocols. In channels that can only transport a single ion across the membrane, the bi-ionic reversal potential is independent of the absolute ion concentrations bathing the channel entrances. In channels that process more than one ion at a time, absolute concentration affects the equilibrium occupancy of energy minima (binding sites) in the permeation pathway and, thereby, alters the potential at which the probability of ion movement across the channel is equal for influx and efflux, i.e., the reversal potential.

The simplest experimental approach to determine whether concentration affects ion loading of the channel is to repeat the bi-ionic reversal potential measurements at different, absolute concentrations. By maintaining equal ion concentrations on both sides of the membrane, the thermodynamic equilibrium potential is held constant. Changes in absolute concentration, then, only change the probability that the channel is occupied by an ion; concentration changes do not generate a change in the transmembrane voltage gradient that may alter the “selectivity filter” in the channel’s potential energy profile. At 20 mM ionic strength, the permeability sequence was the same as at 120 mM, but the $E_{rev}$’s were altered (Fig. 6 A). Cs, the least permeant ion, became more permeable relative to sodium increasing from 0.50 at 120 mM to 0.75 at 20 mM (Fig. 6 B). NH₄, the most permeant ion, became less permeant decreasing from 3.34 to 1.46 at 20 mM. Permeability changes for Li and K showed small, but consistent decreases in permeability with low concentrations.

Selectivity Determined by Conductance Ratios

To determine rigorously the selectivity of the cGMP-activated channel based on conductance measurements, either single-channel or instantaneous currents must be measured for each ionic species. In photoreceptors, in situ single-channel measurements are challenging because the high channel density obscures discrete channel events even at low agonist concentrations. Additionally, the individual channel currents suggest at least two conductance levels for the cGMP-activated channel furthering complicating the analysis of single channel currents (Zimmerman and Baylor, 1986; Hanke et al., 1988; Bennett et al., 1989). Measuring instantaneous currents has also proved arduous as the fastest clamping time of our present equipment (30–40 μs) is comparable to the cGMP-activated current relaxation time constant (60 μs), and our patches do not routinely tolerate the voltage jumps required for thorough characterization. Although steady-state macroscopic conductance ratios do not separate gating from conductance effects, they can be used for
Figure 6. Dependence of $E_{rev}$'s on absolute concentration. (A) Bi-ionic IV relations were recorded with NH₄, Na, and Cs in the cytoplasmic medium and Na extracellularly, but the absolute concentration of permeant species on both sides of the membrane was 20 mM compared with the usual 120 mM solutions. In this patch, zero current crossings were 0, 10.5, and -10 mV for Na, Cs, and NH₄, respectively, with relative permeabilities for Cs of 0.66 and for NH₄ of 1.49. Compared with the average values at 120 mM (Table I), Cs is more permeable and NH₄ is less permeable at low, absolute concentrations. Patch 8713. (B) The difference of $E_{rev}$ at 120 and 20 mM absolute concentration on both sides of the membrane is shown for the species NH₄, Cs, K, and Li. All ions, except Cs, show a decline in the relative permeability as the concentration is decreased, but the differences are striking only for the most permeant species, NH₄, and the least permeant species, Cs, tested. Measured $E_{rev}$'s relative permeabilities, and numbers of observations at 120 and 20 mM are given in Tables I and II, respectively. The error bars represent standard errors of the mean. Currents were recorded as described in Fig. 1, but with 20 mM total Na, 80 mM mannitol, 2 mM EGTA/Na and 5 mM HEPES/Na, pH 7.3, inside the patch electrode. Before obtaining a seal, the bath contained the usual 120 mM NaCl solution to maintain the integrity of the outer segments. After obtaining a seal, 20 mM NaCl solution or sodium-free 20 mM test solutions were exchanged into the bath, and currents were recorded with and without 200 µM cGMP.

Table II

<table>
<thead>
<tr>
<th>Ion</th>
<th>$E_{rev}$ (mV)</th>
<th>$P/P_{Na}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄</td>
<td>-9.6 ± 2.1</td>
<td>1.46</td>
<td>5</td>
</tr>
<tr>
<td>Cs</td>
<td>7.1 ± 3.5</td>
<td>0.75</td>
<td>4</td>
</tr>
<tr>
<td>K</td>
<td>3.4</td>
<td>0.87</td>
<td>2</td>
</tr>
<tr>
<td>Li</td>
<td>3.6</td>
<td>0.87</td>
<td>2</td>
</tr>
</tbody>
</table>

Bi-ionic conditions with NaCl in the pipette were used to measure reversal potentials as described in Fig. 5. Solutions are described in Methods.
comparison to permeability estimates of selectivity and for comparison to previous studies of selectivity on intact photoreceptors.

Chord conductance of the alkali metal cations as a function of voltage is illustrated in Fig. 7. Outward conductances are measured with current carried by the test ion, while inward conductances at hyperpolarizing potentials are attributable to Na ions. Both inward Na and outward alkali metal conductance is decreased at all potentials with equimolar substitution of the test ion for cytoplasmic Na. The selectivity sequence of the alkali metal series and two organic cations (not shown) determined from conductance ratios is Na > NH₄ > K > Rb > Cs > Li >> guanidinium. This sequence differs from the selectivity sequence determined from permeability measurements in the positions of Li and the organic cations, NH₄ and guanidinium, which are significantly more permeable than conductive.

**Figure 7.** Macroscopic conductance–voltage (GV) relations. (A) GV relations were calculated from the IV relations and the differences between the applied potential and the zero crossings for each of the indicated alkali metal cations substituted at the cytoplasmic surface. The inward Na conductance (V < 0 mV) varies significantly depending on the ion in the bath. For a single-ion channel, the Gₙᵣ should not be influenced by the ion species on the cytoplasmic face.

**Concentration Dependence of Conductance**

Because permeability experiments suggested that the cGMP-activated channel is multi-ion in character, we sought evidence of non-Langmuir conductance–concentration relationships. Over the concentration range 10–500 mM, we were unable to observe deviations from Langmuir isotherms (data not shown). Extrapolation of the data to zero concentrations indicated that half-saturation of the conductance for the alkali metal cations was <10 mM. The organic cation, NH₄, however, had a higher apparent half-saturation concentration of ~25 mM. Conductance/concentration data as a function of the thermodynamic driving force is illustrated in Fig. 8 for the NH₄ data from Fig. 5 B. Both inward and outward conductance increased with 10 to 500 mM NH₄ in the bath and appeared to saturate at 120 mM. The magnitude of the inward conductances carried by 120 mM NH₄ showed a dependence on concentration that paralleled the outward conductances at concentrations well below saturation suggesting that inward and outward ion fluxes through the channel were not independent.
Channel Block by Organic Cations

To study selectivity at low concentrations of the permeant species, we wanted to identify a physiologically inert cation which could be used to maintain a fixed ionic strength. As a screening procedure, potentially inert ions, selected either for their large size or for their use as inert substituents in previous studies, were added at the cytoplasmic face in equal concentration to solution A, which contained 120 mM NaCl. If the cation was inert, only modest changes to the sodium currents were anticipated due to the doubling of ionic strength. As shown in Fig. 9, all substituents except NH$_4$ (TMA, guanidinium, choline, and N-methylglucamine) produced significant attenuation of the inward and outward Na currents indicating they block sodium in the channel. In solutions containing only these ions at the cytoplasmic face, no appreciable currents were seen except with NH$_4$ and guanidinium. However, except for N-methylglucamine, the absence of current saturation suggests that TMA and choline may be slowly conducting across the channel, and, therefore, would also be physiologically active from the extracellular side of the channel.

Ion Selectivity using No. 8161 Glass Electrodes

Our initial selectivity studies (Furman and Tanaka, 1987) used electrodes fabricated from a soft lead glass, Corning no. 8161, with filling and bathing solutions containing 0.1 mM EGTA, 0.1 mM EDTA, and no added divalent cations. Realizing that the IV relation of symmetrical sodium solutions was strikingly different with 8161 glass than with electrodes pulled from other glass, we investigated the reasons for these differences and concluded that multivalent cations, most likely Ca or Mg, leach from no. 8161 glass (Furman and Tanaka, 1988). Since calcium and magnesium are known blockers of the cGMP-activated channel (see review, Yau and Baylor, 1989), it seems quite possible that their surreptitious presence accounted for the IV relations. Besides channel block, however, several other interesting features were observed in monovalent, bi-ionic experiments that bear on the topic of channel selectivity.

With no. 8161 pipettes the experimental procedures of ionic substitution at the cytoplasmic surface used in this study (cf. Fig. 2 A) produced significantly different
IV relations (Fig. 10). First, the IV has a shallow slope with slight outward rectification between $-40$ and $40$ mV, but beyond ±$40$ mV the currents increase rapidly. Secondly, the inward sodium currents are not significantly affected by the ion at the cytoplasmic face as were currents recorded with no. 0010 glass (Figs. 2 and 7 and Table I). Thirdly, outward Li current is equal or slightly greater than the Na current, while the K current is appreciably reduced. Fourthly, from Fig. 10 B, we see that the inward Na current is significantly increased when NH$_4$ is present on the cytoplasmic face, and, fifthly, the outward NH$_4$ currents are much larger than the Na currents compared with the response with 0010 pipettes (Fig. 6 A). These latter two observations suggest that NH$_4$ more effectively competes with divalents than does Na for transport through the channel.

The relative permeabilities of the alkali metal cations and NH$_4$ measured with no. 8161 electrodes have been calculated from the GHK Eq. 1 without consideration of the presence of divalent cations and are shown in Table I. Li and Cs are unchanged.

![Figure 9](https://via.placeholder.com/150)

**Figure 9.** Organic cation block of the sodium current. The four panels show the effect of adding high concentrations of an organic cation to the cytoplasmic face of a single patch. The current in A labeled Na was obtained in the usual fashion with 120 mM NaCl solutions bathing both membrane surfaces (solution A). The other responses labeled with the indicated organic cation were recorded after adding 120 mM cation intracellularly to the 120 mM Na already present. Except for N-methylglucamine and NH$_4$, $E_{m}$'s were minimally affected indicating either an impermeant species or permeability comparable to Na. With all organic cations except NH$_4$, both the inward and the outward currents were dramatically attenuated providing evidence of channel block. The absence of current saturation at depolarizing potentials with organics other than N-methylglucamine suggests these ions are slowly conducted through the ion channel. Patch 87202.
with respect to Na whereas K and Rb are slightly more permeable than Na. This change is probably not significant since the $E_{rev}$ measurements with no. 8161 electrodes were more variable than with no. 0010 electrodes.

Conductance ratios for these ions were calculated at $+80$ mV and show $NH_4 > Na \sim Li > K > Rb$ compared with $Na > NH_4 > K \sim Rb > Cs > Li$ with no. 0010 electrodes. The $E_{rev}$'s in this patch were $1$ mV for Na, $5.5$ mV for Li, and $2$ mV for K corresponding to relative permeabilities of 0.80 and 0.92, respectively, assuming true bi-ionic conditions. Averaged values are presented in Table I. Patch 6a085. (A) An experiment similar to A was performed with NH$_4$ as the permeant species substituting for cytoplasmic Na with 8161 electrodes. Compared to NH$_4$ with no. 0010 electrodes in Fig. 3 A, the permeability relative to Na is similar, 2.7 ($E_{rev} = -25$ mV), but the inward Na current is increased when NH$_4$ replaces Na on the cytoplasmic face (larger current at $-90$ mV). The outward NH$_4$ current is much greater than the outward sodium current. This result is a consistent difference between patches with no. 8161 electrodes and no. 0010 electrodes (e.g., Fig. 4 A). Patch 6c022.

In the presence of divalent cations the conductance of NH$_4$ and Li increase about threefold over Na and the relative conductance of K decreases by ~50% over that with 0010 electrodes. Although it is tempting to speculate that divalent cations may alter ion selectivity of the channel, the effects of divalent cations on gating, single-channel currents and block have not yet been analyzed.
The average magnitude of the Na currents in no. 8161 was significantly smaller than with no. 0010, and currents with no. 8161 electrodes also showed significantly more noise. Although we have not examined these parameters quantitatively, the observations are consistent with flickery block from divalent cations solubilized from the no. 8161 glass matrix.

**DISCUSSION**

**Comparisons with Previous Studies**

Previous selectivity studies of photoreceptor dark currents have used suction electrode recordings and extracellular bath exchanges where the outer segment dark current and light responses are measured without a voltage clamp. In an early study by Yau et al. (1981), selectivity was determined by comparing the levels of dark current in Ringer-type solutions containing mixtures of Ca, Mg, and alkali metal cations. Their selectivity sequence, with 100 μM external Ca, was Na > Li > K > Rb > Cs. The low selectivity ratios among these ions was attributed later to slow solution exchange times and lack of support of Na/Ca exchange by other ions, which is essential for dark current maintenance. In a follow-up study, Hodgkin et al. (1985) used rapid solution switches and measured the currents 350 ms after the switch. From the unclamped current ratios, they determined the selectivity sequence as Li > Na > K > Rb > Cs with relative ratios of 1.4 : 1.0 : 0.8 : 0.6 : 0.15 in 1 μM external calcium. Choline, TMA, and TEA did not carry current.

Yau and Nakatani (1984) also determined the relative selectivity in a similar fashion from suction electrode recordings using rapid solution switches. Their relative selectivity sequence showed Na ~ Li > K > Rb > Cs with current ratios of 1.0 : 1.0 : 0.7 : 0.45 : 0.25. These values were the same in solutions containing either 1 μM or 1 mM Ca externally.

In the first study using excised inside-out patches, Fesenko et al. (1985) compared $E_{rev}$ in bi-ionic solutions containing both Ca and Mg and the test cation. The reversal potential shifts compared to Na were 2.2, 2.7, 5.0, and 6.0 mV, for K, Li, Rb, and Cs, respectively. Permeability ratios under these conditions were not calculated because of the presence of divalent cations.

Excised patches are a better preparation for selectivity measurements because the ionic composition can be controlled on both sides of the membrane, and the potential across the patch is voltage clamped. Nunn (1987) reported permeability ratios in a single patch, calculated from $E_{rev}$ measurements, of 1.13 : 1.0 : 0.91 : 0.74 : 0.55 for Li:Na:K:Rb:Cs. Menini and Torre (1989) reported the same sequence from $E_{rev}$ measurements in patches, but a different sequence of Na > K > Rb > Li > Cs from current ratios. Luhring and Kaupp (1989) determined an identical permeability in bovine rod patches.

In summary, the selectivity profile for the cGMP-activated channel, using several different techniques and variations in the concentration of divalent cations, is in good agreement with a permeability sequence of Na ~ Li > K > Rb > Cs. The qualitative position of Li in the reported sequences varies slightly, but we believe this represents experimental errors as, quantitatively, the channel is nearly equally permeable to Na, Li, and K (Fig. 11 A).
Selectivity determined by conductance ratios, however, is much more sensitive to the levels of calcium in the solution. The suction electrode experiments with intact outer segments have all reported a selectivity (conductance) sequence similar to the above permeability sequence. In the absence of divalent cations, however, the sequence of Nunn (1987) and of Menini and Torre (1989) is Na > K > Rb > Li > Cs. This differs from our sequence and Luhring and Kaupp (1989) only in the position of Cs. We find Cs more conductive than Li, but the Cs ratios are noisy and the difference is probably not statistically significant with 0.33 for Li and 0.27 for Cs, Table I.

**Figure 11.** Relative selectivity of the alkali metal cations determined by conductance and permeability as a function of ionic radius. (A) Average permeability (○) and conductance (▲) values at +80 mV are plotted against the ionic crystal radius. The cGMP-activated channel shows little discrimination in permeability between Li and K, but the larger ions, Rb and Cs, begin to show some decrease. The conductance ratios follow a similar profile, except that the decline with increasing size is a little greater and conductance for Li is reduced compared to its permeability. The permeability ratios follow an Eisenman sequence IX for a high field strength binding site, while the conductance ratios follow Eisenman sequence VII for a less electronegative binding site. (B) The relative permeabilities for alkali metal cations are compared for three monovalent-selective ion channels, the cGMP-activated photoreceptor channel (○), the voltage-dependent sodium channel (▲), and the motor end-plate channel (■). The plot clearly shows the lack of selectivity in the end-plate channel and the high degree of selectivity in the voltage-activated sodium channel. The cGMP-activated channel follows a permeability sequence similar to that of the sodium channel, but the relative permeabilities of the larger cations are much greater than for the sodium channel. Permeability ratios for the frog node sodium channel were taken from Hille (1985, Table II, p. 240) and for the frog muscle end-plate channel from Adams et al. (1970).
Zimmerman and Baylor (1988) and Menini et al. (1988) suggested that the cGMP-activated channel is a single ion pore, although an earlier abstract by Zimmerman and Baylor (1987) showed a concentration-dependent $E_{\text{rev}}$ for Cs. Menini and Torre (1989) saw no concentration dependence of the currents carried by Na, K, or Li, which is consistent with a single-ion channel, but their results are also consistent with our small, but distinct, concentration dependence for these ions (Fig. 6 B). In our experiments, the most permeant ion, NH$_4^+$, and the least permeant ion, Cs, showed clear and convincing deviations from the behavior expected from a single-ion channel that led us not to discount the small changes observed with Li and K.

**Preliminary Views of the cGMP-activated Channel Architecture**

Physical dimensions of ion channels have been estimated by measuring the conductance of a series of similar ions with increasing radii. One of the best examples of this approach was used by Hille and collaborators (Adams et al., 1980; Dwyer et al., 1980) to determine the pore dimensions of the frog muscle end-plate channel. They measured currents with a series of monovalent and divalent cations and >40 polyatomic organic cations and concluded that the endplate channel is a molecular sieve with dimensions of $\sim 6.5 \times 6.5$ Å.

The permeability and conductance ratios for the alkali metal cations as a function of the ionic crystal radius are graphed in Fig. 11 A. Based on permeability measures, the channel discriminates poorly among Li, Na, and K. Permeability gradually declines for Rb and Cs with Pauling radii of 1.48 and 1.69 Å, respectively. This response is typical for an Eisenman sequence IX in the equilibrium theory of ionic selectivity (Eisenman, 1962). In this theory, the observed selectivity sequence is determined by the difference in the energy gained from an electrostatic interaction between the permeant ion and a high field strength binding site within the channel and the energy expended removing the tightly bound waters of hydration from the smaller diameter cations.

The conductance sequence in Fig. 11 A, Eisenman VII, differs from the permeability sequence only in the shift of Li relative to the other ions. In Eisenman’s theory, this is attributable to a decrease in the electrostatic interaction between Li and the channel. In the single-ion Eyring rate theory model of selectivity, the probability that an ion enters a channel (permeability) is determined by heights of energy barriers (relative to the bulk solution) presented by the channel’s structure, and the rate of ion crossing (conductance) is governed by the largest well-to-peak energy barrier in the channel’s free energy profile. The small shift in $E_{\text{rev}}$ for Li, then, suggests that the energy barrier for Li entry into the channel is not significantly higher than for Na, but the small currents carried by Li suggest that binding of Li within the channel is significantly greater than for other ions. Li’s tighter binding to a site inside the pore produces a longer transit time compared with other ions, and, hence, a lower conductance.

On the basis of our permeability vs. ionic radii data for the alkali metal cations, one could predict that the narrowest region of the channel is somewhat flexible but begins to constrain ions larger than $\sim 3$ Å in diameter. The experiments with a small series of organic cations, however, provide a somewhat different notion of the pore
size. The results in Fig. 3 show that both NH$_4$ and guanidinium are permeant. The radius of NH$_4$, 1.48 Å, falls between that of K and Rb while the dimensions of guanidinium are quite large by comparison, ~3 × 5 Å (Hille, 1971). The results in Fig. 9 demonstrate channel block with other organic ions, TMA, choline, and N-methylglucamine. While these ions may not pass entirely through the channel, the absence of current saturation with TMA and choline suggests that additional studies with larger organic cations should be undertaken.

Is the cGMP-activated channel, then, just a large nonselective pore? Fig. 11 B compares the permeability of the cGMP-activated channel to the highly selective, voltage-dependent sodium channel and the nonspecific acetylcholine end-plate channel. Similar to the cGMP-activated channel, the sodium channel follows an Eisenman sequence X for binding to a “strong” field site and has a relatively narrow selectivity filter that restricts the passage of ions larger than guanidinium, while the end-plate channel is effectively an open hole for ions of this size. The lower conductance for smaller ions in the end-plate channel reflects the greater energy needed to dehydrate the ions before they enter the pore. The lower discrimination among large cations in the cGMP-activated channel compared with the Na channel, despite a similar selectivity sequence and size of the narrowest passage, may reflect a more conformable electrostatic interaction energy for the larger ions or, perhaps, an enhanced exit rate for the larger ions secondary to multi-ion effects within the channel.

If the cGMP-activated channel shows reduced permeation for ions >1.5 Å, how can we account for the high permeability of NH$_4$ and guanidinium? Organic ions can interact with the side chains of the amino acid groups lining the pore of the channel to form highly favorable hydrogen bonds. Such hydrogen bonding contributes to additional channel–ion interaction energy over the purely electrostatic interaction of a metal and also serves to reduce the effective radius of amino groups on the permeant species. Hydrogen bonding may explain the large NH$_4$ currents in cGMP-activated channels compared to similar sized ions, K and Rb (Figs. 2 B and 8 B). In a study by McCleskey and Almers (1985), the highly flexible ion, n-butylammonium, did not carry detectable current in the Ca channel of skeletal muscle nor did it block NH$_4$ currents. Surprisingly, however, the larger ion, 1,4-diaminobutane, did show an inward current through the channel. They attributed these apparently contradictory findings to changes in the bonding interactions within the channel.

**Single- or Multi-ion Channel?**

One of the traditional tests for multi-ion occupancy of a channel is the dependence of the reversal potential on absolute ion concentration as we have found for the cGMP-activated channel (Fig. 6). Although the work of Eisenman and Horn (1983) did show that a single-ion channel with an asymmetrical free energy profile could produce concentration-dependent permeability ratios when characterized by some experimental techniques, they could find no free energy profile for a single-ion channel that falsely produced concentration dependent permeability ratios when studied with bi-ionic conditions. Our experiments were performed with symmetrical concentrations of bi-ionic pairs to eliminate voltage-dependent effects on the
channel's asymmetrical free energy profile (Fig. 4) from being falsely interpreted as concentration effects. Lauger (1980) has shown theoretically that true concentration dependence of the permeability ratio can arise in a single-ion channel if the transitions between conformational states of the protein are coupled to ion translocation, but experimental confirmation of this alternative will require kinetic analysis.

The deviation of the concentration dependence of the reversal potential for NH$_4$ and Cs from the predictions of the Nernst equation are also consistent with the behavior of a multi-ion channel. The equilibrium reversal potential occurs at the voltage where the probability of ion movement across the membrane in either direction is equal. In a single ion channel, the probability of movement is only a function of potential, while, in a multi-ion channel, movement is also a function of the occupancy of adjacent binding sites within the channel. The reversal potential-log (concentration) slopes for NH$_4$ and Cs are both significantly less than the slope for Na (Fig. 5). Deviation from the Nernst predictions might be explained by significant anion permeability in the cGMP-activated channel. The good agreement of the Na curve with the Nernst predictions indicates that anion permeability in this case is small compared to the channel's Na permeability. If anion permeability accounted for the deviation from the Nernst slope, then NH$_4$, which is three times more permeable than Na, should also fit the Nernst prediction, while Cs, which is half as permeable as Na, should deviate from $-58$ mV/decade as it does. However, both NH$_4$ and Cs show similar deviations away from the Nernst prediction suggesting that anion permeability does not account for the reduced slopes.

We were unable to find deviations from the predictions of a single-ion channel in conductance-concentration curves, but we only explored the concentration range 10–500 mM. The classic analysis of multi-ion channels by Hille and Schwartz (1978) showed that concentration effects typically extend over three or more orders of magnitude. In our study most species showed half-saturation at $\leq 10$ mM where the signal-to-noise ratio limited further exploration at lower concentrations, and above 500 mM we had difficulties obtaining stable patches. Multiple ion occupancy would also have been unresolvable in the conductance-concentration relations if binding affinities of the channel differed by less than a factor of 10. NH$_4$, however, supported large currents with a half-saturation of $\sim 25$ mM and would be a useful candidate for future studies of conductance-concentration relations.

By traditional criteria, the concentration dependence of reversal potentials is strong evidence for the multi-ion character of the cGMP-activated channel. The deviations of the reversal potential from the Nernst predictions, while not conclusive in their own right, are also consistent with a multi-ion channel. Future confirmatory evidence for the multi-ion occupancy of the cGMP-activated channel may be sought by extending the concentration range in conductance-concentration experiments, searching for anomalous mole-fraction effects, quantitating the voltage dependence of channel blockers, or measuring unidirectional tracer fluxes through the channel.

**Conclusions**

To begin developing a functional understanding of the photoreceptor channel architecture, we have examined the ability of the channel to discriminate among monovalent cations. Although our experiments have primarily addressed the perme-
ation of monovalent ions, the interaction of monovalent and divalent ions in the channel is an important area for further investigation. The most interesting question about the selectivity of this channel is how does the channel process two ions of similar size, but different valence, under physiological conditions? Presently not much is known about the biophysical details of the interaction between the principal current carriers, Na and Ca, except that calcium permeability appears to be high while the \( K_{\text{d}} \) for calcium block of the Na current is low. At first glance, these features appear similar to those of the voltage-dependent calcium channel, most notably that calcium acts as a permeant blocker (Almers and McCleskey, 1984; Hess & Tsien, 1984). The elegant collective work on the calcium channel, described in a review by Tsien et al. (1987), has provided evidence for a high-affinity Ca-binding site in the channel that is the underlying basis of its high divalent selectivity. The significant difference between these channels, however, is that the voltage-dependent Ca channel does not pass sodium current under physiological conditions while most of the cGMP-activated current is carried by Na. In photoreceptors, electrical signaling is dependent on the sodium current through the cGMP-activated channel and without divalent cations, the selectivity of this channel resembles that of the voltage-dependent Na channel. It appears, then, that the cGMP-activated channel shares functional properties with both the voltage-dependent Na and Ca channels. In the future, combined study of function, using biophysical techniques, and structure, using the powerful tools of molecular biology, may reveal a shared heritage among these channels.

We thank Drs. Paul Mueller and Richard Horn for their insights on channel selectivity.

This work was supported by National Institutes of Health grants NS-00865, University Research Fund, and BRSG S07-RR-05415-26 to Dr. Furman; EY-06640, BRSG 07083-21, and 05415-25 to Dr. Tanaka; and EY-01583 Core support.

*Original version received 3 July 1989 and accepted version received 17 January 1990.*

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