Ionic Selectivity of \( I_h \) Channels of Rod Photoreceptors in Tiger Salamanders

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ABSTRACT Ionic selectivity of \( I_h \) channels of tiger salamander rod photoreceptors was investigated using whole-cell voltage clamp. Measured reversal potentials and the Goldman-Hodgkin-Katz voltage equation were used to calculate permeability ratios with 20 mM K\(^+\) as a reference. In the absence of external K\(^+\), \( I_h \) is small and hard to discern. Hence, we defined \( I_h \) as the current blocked by 2 mM external Cs\(^+\). Some small amines permeate \( I_h \) channels, with the following permeability ratios \((P_x/P_K)\): NH\(_3\), 0.17; methylammonium, 0.06; and hydrazine, 0.04. Other amines are tially impermeant: dimethylammonium (<0.02), ethylammonium (<0.01), and tetramethylammonium (<0.01). When K\(^+\) is the only external permeant ion and its concentration is varied, the reversal potential of \( I_h \) follows the Nernst potential for a K\(^+\) electrode. \( I_h \) channels are also permeable to other alkali metal cations \((P_x/P_K)\): Tl\(^+\), >1.55; K\(^+\), 1; Rb\(^+\), >0.55; Na\(^+\), 0.33; Li\(^+\), 0.02. Except for Na\(^+\), the relative slope conductance had a similar sequence \((G_x/G_K)\): Tl\(^+\), 1.07; K\(^+\), 1; Rb\(^+\), 0.37; NH\(_3\), 0.07; Na\(^+\), 0.02. Based on permeabilities to organic cations, the narrowest part of the pore has a diameter between 4.0 and 4.6 Å. Some permeant cations have large effects on the gating kinetics of \( I_h \) channels; however, permeant cations appear to have little effect on the steady-state activation curve of \( I_h \) channels. Lowering K\(^+\) or replacing K\(^+\) with Na\(^+\) reduces the maximal conductance of \( I_h \) but does not shift or change the steepness of its voltage dependence. With ammonium or methylammonium replacing K\(^+\) a similar pattern is seen, except that there is a small positive shift of ~10 mV in the voltage dependence.

INTRODUCTION

\( I_h \) (also called \( I_f \) or \( I_Q \)) is a steeply voltage-gated current activated by membrane hyperpolarization (Bader, Macleish, and Schwartz, 1979; Attwell and Wilson, 1980; Yanagihara and Irisawa, 1980; DiFrancesco, 1981a; Attwell, Werblin, and Wilson, 1982; Bader, Bertrand, and Schwartz, 1982; Spain, Schwindt, and Crill, 1987; Barnes and Hille, 1989). Channels underlying \( I_h \) are permeable to both K\(^+\) and Na\(^+\) and appear quite selective for these ions (DiFrancesco, 1981b; Mayer and Westbrook, 1983; Bader and Bertrand, 1984; Edman and Grampp, 1989). This selectivity profile differs from those for other channel types. K and Na channels are highly selective for...
K⁺ or Na⁺, respectively, whereas nonselective cation channels like the nicotinic acetylcholine receptor (nAChR) channel are highly permeable to all alkali metals, as well as to many other inorganic and organic cations (Adams, Dwyer, and Hille, 1980; Dwyer, Adams, and Hille, 1980). A detailed study looking at a large number of potentially permeant ions in Iₖ channels is needed if we are to analyze the permeation mechanism. Here we quantify ion selectivity in Iₖ channels using changes in reversal potentials under bionic conditions.

Some of this work has appeared in abstract form (Wollmuth and Hille, 1991, 1992).

MATERIALS AND METHODS

Isolation of Rod Photoreceptors

Rod photoreceptors were isolated mechanically from the retina of aquatic tiger salamanders (Ambystoma tigrinum; Kons Scientific Co., Inc., Germantown, WI). Salamanders were maintained at room temperature with ambient illumination for at least 24 h before preparation. An animal was decapitated, the head was hemisected, and the eyeballs were removed. The retina was isolated from the eyeball and a cell suspension was made by triturating the retina in Ringer's solution using a cut-off, fire-polished Pasteur pipette. Recordings were made from solitary rods under constant bright light from the microscope illuminator.

Solutions

Internal. The pipette solution consisted of (mM): 100 KCl, 3.5 MgCl₂, 10 HEPES, 1 EGTA, and 1.5 K₂ATP, pH adjusted to 7.4 with KOH. The total K⁺ concentration was 108 mM. EGTA and ATP were obtained from Sigma Chemical Co. (St. Louis, MO) and HEPES was from Calbiochem Corp. (La Jolla, CA).

External. To measure permeability ratios, we used a 20 mM KCl solution as a reference (mM): 20 KCl, 90 tetraethylammonium CI (TEACI), 8 or 16 glucose, and 5 histidine, pH adjusted to 7.4 with HCl. These solutions minimize other currents that may contaminate Iₖ, including a delayed rectifier K⁺ current (blocked by TEA) and Ca²⁺-activated K⁺ and Cl⁻ currents (0 external Ca²⁺, EGTA in pipette, and TEA outside) (Bader et al., 1982). TEA also removes much but not all of a K⁺ current called I₉ (Beech and Barnes, 1989). In a few experiments, 2 mM BaCl₂ and 0.1 mM CdCl₂ were added. In test solutions, the 20 mM KCl was replaced by an equivalent amount of various test cations as the chloride salt. For hydrazine, in both the reference and test solutions, the buffer was 10 mM 2-[N-morpholino] ethanesulfonic acid (MES) instead of 5 mM histidine and the pH was adjusted to 5.75 with TMAOH. In solutions used to measure permeability to Ti⁴⁺, the anion was NO₃⁻ and 20 mM TiNO₃ replaced 20 mM KNO₃ in the test solution. At times we used a control solution that consisted of (mM): 20 KCl, 30 N-methyl-D-glucamine (NMDG), 60 TEACI, 16 glucose, and 5 histidine, pH adjusted to 7.4 with HCl. The NMDG was replaced by an equivalent amount of a test cation. In testing different K⁺ concentrations, we used this control solution and kept the sum of the NMDG and K⁺ concentrations constant at 50 mM.

Whole-Cell Recording

The whole-cell version of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to voltage clamp and dialyze cells at room temperature (21–25°C). Electrodes were pulled from glass hematocrit tubes (VWR Scientific Corp., Seattle, WA) and had resistances of 2–5 MΩ when filled with the pipette solution and measured in the 20-mM K⁺
reference solution. The pipette was sealed to the inner segment of the photoreceptor, the patch was broken by suction, and whole-cell membrane current was measured using an Axopatch 1-C patch clamp (Axon Instruments, Inc., Foster City, CA). Currents were recorded with partial pipette and membrane capacitance compensation, low-pass filtered at 200 Hz, digitized at 1 kHz, and stored and analyzed on an IBM-compatible computer using the BASIC-FASTLAB software and hardware package (INDEC systems, Inc., Capitola, CA). Boltzmann and Hill equations were fitted using nonlinear least squares. Results are reported as mean ± SEM.

The recording chamber consisted of three connected wells cut out from a layer of Sylgard at the bottom of a Petri dish. Cell suspensions were pipetted into the center and largest well (100–200 μl). The two end chambers were the inflow and outflow for superfusion. Tests with dyes indicated that bath solutions were mostly exchanged within 30 s. For all experiments, solution flow was continuous.

Liquid-junction potentials were measured using a Beckman ceramic-junction, saturated KCl electrode and were corrected during data analysis. The junction potential between the 20 mM K⁺ reference solution and the pipette solution was −3 mV (pipette negative). Superfusion of some test cation solutions generated junction potentials between the ground electrode and bath of 1 mV (NaCl, amines), 2 mV (LiCl), or −1 mV (KNO₃, TINOs) (ground electrode 0 mV).

Evaluation of Ih steady-state activation curves. We used tail current amplitudes measured 10 ms after a test step to construct Ih activation curves. Tail amplitudes were converted to conductance by dividing the current by the driving force (E − Eᵣ), where E is the tail potential and Eᵣ the reversal potential for Ih. Conductance−voltage plots were fitted with the Boltzmann equation:

\[
g(E) = \frac{g_{\max}}{1 + \exp\left(\frac{E - E_{1/2}}{S}\right)}
\]

where g is conductance, \(g_{\max}\) is the maximal conductance, \(E_{1/2}\) is the voltage for half-maximal activation, and \(S\) is the slope factor. The slope factor was converted into equivalent gating charge (Q) by dividing RT/F by the measured slope factor. R, T, and F have their normal thermodynamic meanings and the quantity RT/F was 25.5 mV (22°C). Q is an index of the number of charges needed to move all the way across the membrane for channel gating to occur (Hodgkin and Huxley, 1952).

Ionic selectivity. Permeability ratios were determined with the Goldman-Hodgkin-Katz (GHK) voltage equation (Goldman, 1943; Hodgkin and Katz, 1949). If one assumes that K⁺ and Na⁺ are the only permeant ions, it has the form:

\[
E_r = \frac{RT}{F} \ln \left( \frac{P_{K[K]o} + P_{Na[Na]o}}{P_{K[K]i} + P_{Na[Na]i}} \right)
\]

where \(E_r\) is the zero current or reversal potential.

Ionic selectivity of Ih channels was determined by measuring the change of reversal potential for Ih on replacing the 20 mM K⁺ reference solution, which contains no Na⁺ or other permeant ion, with an identical solution except with the K⁺ replaced by a test cation, X⁺. The permeability ratios, \(P_X/P_K\), were calculated according to the relation:

\[
E_{r,X} - E_{r,K} = \Delta E_r = \left(\frac{RT}{F}\right) \ln \left( \frac{P_{X[X]o}}{P_{K[K]o}} \right)
\]

This equation is valid only if the internal concentrations of permeant ions remain constant. To avoid large ionic fluxes, we used short conditioning voltage steps (0.2 s).

In the voltage range negative to −33 mV, rod photoreceptor membranes exhibit two major voltage- and time-dependent conductances, those of Ih and I₅ channels (Beech and Barnes, 1989; Wollmuth, L. P., unpublished observations). The I₅ channels are open at −33 mV and close slowly during hyperpolarization, and Ih channels are shut at −33 mV and open slowly during hyperpolarization. Despite the presence of TEA in our solutions, a residual component of I₅ contaminates the first 50 ms of a hyperpolarizing step, particularly when the bathing
solution contains ions that are quite permeant in K⁺ channels (K⁺, Tl⁺, Rb⁺, NH₄⁺). Since 2 mM Cs⁺ blocks much of Iₜ, without blocking Iₖ, most experiments used a Cs⁺ subtraction procedure described in Results to remove the contaminating Iₖ.

To measure reversal potentials, we used a standard protocol. After break-in to the whole-cell configuration and waiting 3–4 min for intracellular dialysis, we recorded currents in the 20 mM K⁺ reference solution and then in the same solution but with 2 mM CsCl added. Subsequently, we perfused the test solution a minimum of 3 min (~20–40 vol of solution through the chamber). We then recorded currents in the test solution and again in the same solution but with 2 mM CsCl added. In most instances we then retested the 20 K reference solution. In comparison to the pre-20 K, the post-20 K reversal potential never changed by more than 3 mV. When both pre- and post-20 K reversal potentials were measured, they were averaged in calculating changes in reversal potential.

**RESULTS**

The aim of these experiments is to quantify ionic selectivity of Iₜ channels. We first present the basic features of Iₜ and our approach to measuring reversal potentials.

**Features of Iₜ**

Fig. 1A shows current recordings made in a 20 mM K⁺ solution without and with 2 mM Cs⁺ to block Iₜ. The traces on the right are difference currents, representing the Cs⁺-sensitive component. The Iₜ channels are closed at the holding potential of −33 mV. A hyperpolarizing conditioning voltage step to −113 mV activates a slowly developing inward Iₜ current, and test steps to more positive voltages turn the

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**Figure 1.** Reversal of Iₜ. (A) Whole-cell currents from a rod bathed in 20 mM KCl, 90 mM TEACl with 0 (left) or 2 mM (center) added CsCl. The difference between currents in 0 and 2 mM Cs⁺ is shown on the right. Holding potential was −33 mV, indicated at the beginning of the voltage trace, and test steps were in 10-mV increments with 4-s intervals between steps. (B) Instantaneous current amplitude (measured 10 ms after the start of the test step) plotted against test step potential (I-E relation). Points derived from the Cs⁺-difference current (filled circles) or by linear leak subtraction of the 0 Cs⁺ record (open circles). Leak subtraction was performed by scaling a record, produced by stepping from −33 to −43 mV for 0.2 s and returning to −33 mV, and subtracting it from the 0 Cs⁺ records to remove capacity transients and linear leak currents. (Series resistance, 8.5 MΩ; cell capacitance, 16 pF.)
current off again. The reversal potential was determined from “instantaneous” current–voltage (I–E) relations (Fig. 1 B), which for the Cs⁺-sensitive difference current (filled circles) is curved and crosses the voltage axis at $-35 \text{ mV}$, defining the reversal potential. The curvature is an artifact of the Cs⁺ subtraction procedure. The block of $I_h$ channels by Cs⁺ is much less complete at positive potentials than at negative potentials (DiFrancesco, 1982), and therefore the difference current at positive potentials reflects only a fraction of the $I_h$ that is flowing. Nevertheless, this subtraction procedure does not affect the reversal potential for $I_h$. In the absence of Cs⁺ the current is $I_{\text{other}}(E) + I_h(E)$, and the current in the presence of Cs⁺ is $I_{\text{other}}(E) + [1-f(E)]I_h(E)$, where $f(E)$ is the fraction of $I_h$ blocked by Cs⁺ at voltage $E$.

Therefore, the difference current is $f(E)I_h(E)$, which will have the reversal potential of $I_h$. This assumes that $I_h$ is the only Cs⁺-sensitive current and that Cs⁺ ions themselves are not permeant (see Discussion).

Fig. 1 B illustrates that in 20 mM K⁺ the instantaneous I–E relation derived by a conventional linear leak subtraction (open circles) is much more linear and gives the same reversal potential as Cs⁺ subtraction. However, as the contaminating $I_{Kx}$ is voltage and time dependent and, in some solutions, larger than $I_h$, this method often will fail to isolate $I_h$ on its own. The decaying tail of inward current most evident in the hyperpolarization steps in Figs. 4, 9, and 10 (in 2 mM Cs⁺) is $I_{Kx}$.

Fig. 2 is an experiment to measure the voltage dependence of the activation of $I_h$ channels using tail currents. The membrane potential is stepped to $-113 \text{ mV}$, as before, to open $I_h$ channels, and then returned to various potentials for 900 ms, both to measure the reversal potential and to allow $I_h$ gating to relax to equilibrium (Fig. 2 A). This gives the steady-state currents plotted as squares in Fig. 2 B. Finally, the potential is stepped to $-63 \text{ mV}$, where the fraction of open channels at each test potential can be measured by the amplitude of the tail current (solid diamonds). The steady-state currents show the inwardly rectifying nature of $I_h$ gating, and the tails show that opening of the channels is steeply voltage dependent between $-74$ and $-94 \text{ mV}$, with a maximal number of $I_h$ channels being activated negative to $-113$
mV. The tail currents are transformed into conductances (open diamonds) to yield an activation curve that is fitted by the following Boltzmann parameters (smooth line): $g_{\text{max}}$, 5.8 nS; $E_{1/2}$, $-85.5$ mV; and $Q$, 4.7. An activation curve derived from the 20 K, 0 Cs record was essentially identical: $g_{\text{max}}$, 5.9 nS; $E_{1/2}$, $-85$ mV; and $Q$, 4.6.

Activation curves measured from tail currents in the same way with 2, 20, and 50 mM K$^+$ are shown in Fig. 3. Increasing the K$^+$ concentration increases the maximum conductance of Ih channels appreciably but has little effect on the midpoint and steepness of the activation curve (summarized in Table I).

**Ammonium (NH$_4^+$) Is Permeant**

We turn now to identify permeant ions other than K$^+$. With some ions the Cs$^+$-difference currents are small and have a different time course from that of Ih in 20 mM K$^+$. In 20 mM NH$_4^+$, without Cs$^+$, there are time-dependent currents (mostly $I_{kx}$) whose time course is not entirely similar to that of Ih (Fig. 4A). However, Cs$^+$

| $X$ | $g_{\text{max}}$ | $E_{1/2}$ | $Q$ | $n$ | $K^*$ |
|-----|-----------------|---------|-----|-----|-----|
| 2 K$^+$ | 1.9 ± 0.1 | $-95 ± 2$ | 5.1 ± 0.3 | 3 | 73 |
| 20 K$^+$ | 5.1 ± 0.3 | $-94 ± 2$ | 5.5 ± 0.2 | 6 | 73 |
| 5 K$^+$ | 2.7 ± 0.2 | $-96 ± 2$ | 5.3 ± 0.4 | 4 | 53 |
| 20 K$^+$ | 4.5 ± 0.2 | $-97 ± 1$ | 5.3 ± 0.2 | 6 | 53 |
| 50 K$^+$ | 8.8 ± 0.3 | $-94 ± 0.5$ | 5.0 ± 0.2 | 3 | 63 |
| 20 K$^+$ | 4.6 ± 0.4 | $-91 ± 2$ | 5.7 ± 0.2 | 4 | 63 |
| 20 NH$_4^+$ | 0.6 ± 0.05 | $-76.5 ± 0.5$ | 4.5 ± 0.3 | 4 | 54 |
| 20 K$^+$ | 4.9 ± 0.2 | $-84 ± 1$ | 5.0 ± 0.3 | 4 | 53 |
| 20 MA | 1.1 ± 0.05 | $-79 ± 3$ | 4.1 ± 0.1 | 3 | 74 |
| 20 K$^+$ | 5.4 ± 0.5 | $-90 ± 2$ | 4.5 ± 0.2 | 4 | 73 |
| 20 Na$^+$ | 0.6 | $-88$ | 4.7 | 2 | 49 |
| 20 K$^+$ | 5.2 ± 0.3 | $-88 ± 3$ | 4.8 ± 0.1 | 3 | 48 |

Activation curves for 20 K$^+$ were measured in the same rod before and/or after that for the test solution. *Tail test potential.
subtraction reveals currents that activate slowly at \(-114\) mV and deactivate at more positive potentials, much like \(I_h\) currents in 20 mM \(K^+\). The activation curve (Fig. 4B) of these currents in \(NH_4^+\), measured as in Fig. 2, shows a somewhat more positive midpoint (\(-76\) instead of \(-85\) mV), a lower slope (\(Q = 3.9\) instead of 5.0), and a much lower maximal conductance (0.54 instead of 3.5 nS), as is summarized in Table I. The reversal potential for currents in \(NH_4^+\) is almost 50 mV more negative than in \(K^+\) (Fig. 4C), showing that \(NH_4^+\) is less permeable, and giving in seven cells a mean permeability ratio \(P_{NH_4^+}/P_{K^+}\) of 0.17 (Table II).

**Several Larger Amines Are Not Measurably Permeant**

When ethylammonium (EtNH\(_4^+\)) is the test cation, the Cs\(^+\)-difference current is small and outward at all test potentials down to \(-154\) mV. Nevertheless, the time

| X               | \(\Delta E\) ± SEM | \(P_x/P_K\) | n  |
|-----------------|---------------------|-------------|----|
| \(K^+\)         | 0                   | 1.0         | 1  |
| Ammonium        | \(-44 \pm 1.7\)     | 0.17        | 7  |
| Methylammonium  | \(-71 \pm 2.0\)     | 0.06        | 9  |
| Hydrazine (pH 5.75) | \(-81 \pm 3.5\) | 0.04        | 6  |
| Dimethylammonium| \(> -100\)          | <0.02       | 8  |
| Ethylammonium   | \(> -120\)          | <0.01       | 4  |
| Tetramethylammonium | \(> -120\)       | <0.01       | 9  |

Reference and test solution: 20 XCl, 90 TEACL, 18 glucose, 5 histidine, except for hydrazine, where the buffer was 10 MES titrated with TMAOH.
dependence of this outward current seems to correspond to gating of \( I_h \) channels. At \(-114 \) mV there is a slow increase of conductance and at \(-64 \) mV there is a slow decrease (Fig. 5 A). The reversal potential is shifted by at least \(-120 \) mV (Fig. 5 B), indicating that the permeability relative to \( K^+ \) must be <0.01 (Table II). When the \( K^+ \) solution is returned, the normal \( I_h \) returns undiminished and with the expected reversal potential (Fig. 5 B). Two other amines of similar or larger size than \( \text{EtNH}_2^+ \), dimethylammonium and tetramethylammonium (TMA), gave similar results

![Figure 5](image)

**Figure 5.** Ethylammonium is poorly permeant. (A) A rod bathed in \( \text{EtNH}_2^+ \) test solution. The most negative test potential was \(-153 \) mV. (B) Instantaneous \( I-E \) relation for the \( \text{Cs}^+ \)-difference current and for the same rod in 20 K reference solution before (filled circles) and after (open circles) exposure to 20 \( \text{EtNH}_3 \) (filled squares). (C) Instantaneous \( I-E \) relation for \( \text{Cs}^+ \)-difference currents for different rods tested in hydrazine (\( \text{NH}_2\text{NH}_2^+ \), circles) or methylammonium (MA, diamonds). (5.5 MΩ; 12.5 pF.)

(5.5 MΩ; 12.5 pF.)

Two smaller amines, hydrazine and methylamine, had a small but measurable permeability, shifting the reversal potential by \(-81 \) and \(-71 \) mV (Table II). To be sure that the small inward currents in these cations were not due to an incomplete washout of \( K^+ \) ions from the control solution, we switched first to the TMA solution, in which only outward currents were obtained, before going to the hydrazine or methylammonium solutions (Fig. 5 C).
**Na⁺ and K⁺ Ions Are Both Permeant**

The reversal potential for $I_h$ changes when the external K⁺ concentration is changed (Fig. 6A). In the absence of Na⁺, the measured values agree well with the predictions of the Nernst equation for a K⁺ electrode (Fig. 6B), showing that $I_h$ channels are much more permeable to K⁺ ions than to any of the other cations in these internal and external solutions. The Na⁺ ion is also known to be permeant (DiFrancesco, 1981b; Mayer and Westbrook, 1983; Bader and Bertrand, 1984), a result we confirm. When 30 mM Na⁺ is added to the 20 mM K⁺ solution (Fig. 7A), the reversal potential shifts ~6 mV more positive (Fig. 7B), consistent with a permeability ratio $P_{Na}/P_{K}$ of 0.36 ± 0.02 ($n = 3$).

Whole-cell currents change substantially when K⁺ ions are removed from Na⁺-containing solutions. The Cs⁺-difference current is inward at −114 mV, but surprisingly shows little time dependence there (Fig. 8A). Subsequent test steps to more positive potentials turn off an outward current that looks like $I_h$. Although the currents are smaller, the shape of a steady-state activation curve measured in the usual way from tails is the same in 20 mM Na⁺ as that for $I_h$ in 20 mM K⁺ solution (Fig. 8B; Table I). The reversal potential of the instantaneous currents is shifted by −27 mV (Fig. 8C) and the calculated mean permeability ratio $P_{Na}/P_{K}$ is 0.33 (Table III), in good accord with the results in Na-K mixtures.
Metal Ions: Tl⁺ and Rb⁺

We used KNO₃ as a reference solution in measuring the relative permeability of Tl⁺; the reversal potentials for the Cs⁺-difference current in KNO₃ and KCl were identical. Fig. 9 shows whole-cell currents for a rod bathed in a 20 mM TlNO₃, 90 mM TEANO₃ solution. In 0 Cs, whole-cell currents are large and comparable to those in the 20 mM KNO₃ reference solution and in 20 mM KCl. Further, with conditioning steps to −112 mV, there is a time-dependent inward current in the 0 Cs and the Cs⁺-difference current. With test steps returning to more positive potentials, however, difference currents show essentially no time dependence, nor are they outward even at very positive potentials. One possible explanation is that Tl⁺ reduces the block by Cs⁺. However, in the 20 mM Tl⁺, 0 Cs record, there are only small time-dependent currents at positive potentials, possibly reflecting only capacity transient currents. Also, in this example we used a fivefold higher concentration of Tl⁺.

Figure 7. Permeability to Na⁺. (A) A rod bathed in 20 mM K⁺, 30 mM Na⁺ solution. (B) Leak-subtracted instantaneous I-E relations for currents in A (open squares) and for the same rod with 30 mM NaCl replaced by 30 mM NMDG (open circles). Arrow indicates $E_r$ calculated from the GHK voltage equation (see Materials and Methods), assuming that only K⁺ and Na⁺ are permeant, $P_{Na}/P_{K} = 0.36$ and $[Na]_i = 0$. (6 MΩ; 16.5 pF.)

Figure 8. Reversal of $I_h$ in 20 mM Na⁺, 0 K⁺. (A) A rod bathed in 20 mM Na⁺ test solution. (B) Steady-state activation curves for a rod bathed in 20 K (diamonds) or 20 Na (triangles) solutions (tail potential −48 mV). Dashed line, Boltzmann fit to Na⁺ record scaled by 7.6. (C) Instantaneous I-E relation for Cs⁺-difference current in 20 mM K⁺ solution before (filled circles) and after (open circles) exposure to 20 Na (filled squares). (9 MΩ; 21 pF.)
Cs⁺. Because of the lack of outward current, the Cs⁺-sensitive instantaneous $I-E$ relation in Ti⁺ (closed squares) does not have a defined reversal potential (Fig. 9B). In any case, clear inward currents are present at potentials negative to −30 mV and $P_{Ti}/P_K$ can be estimated to be $>1.55$ (Table III).

Like Cs⁺, Rb⁺ blocks $I_h$ channels (DiFrancesco, 1982; Wollmuth, L. P., and B. Hille, unpublished data), but Cs⁺ and Rb⁺ seem to block at different sites and Rb⁺ is much less efficacious. Fig. 10A shows that when Rb⁺ is the test cation there still is a large Cs⁺-sensitive inward current. However, this current shows no time dependence either with the conditioning step or, like the Ti⁺ difference current, with positive test steps. Despite the lack of time dependence, there are properties of this Cs⁺-sensitive current that suggest that it flows through $I_h$ channels. The steady-state currents in Rb⁺ and K⁺ appear at the same potentials (Fig. 10B). In addition, the Cs⁺-sensitive current in Rb⁺ is completely blocked by 500 μM 9-amino-1,2,3,4-tetrahydroacridine ($n=3$), a blocker of the time-dependent component in K⁺ solutions (DiFrancesco, 1982).

### Table III

| Cation  | $\Delta E$ (mV) | $P_x/P_K$ | $n$ |
|---------|-----------------|-----------|-----|
| K⁺      | 0               | 1.0       |     |
| Na⁺     | $-28 \pm 1.4$   | 0.33      | 9   |
| Li⁺     | $-96 \pm 2.3$   | 0.02      | 7   |
| Rb⁺     | $<-16$          | $>0.55$   | 7   |
| Ti⁺     | $>+10$          | $>1.55$   | 7   |

Reference and test solution: 20 XCl, 90 TEACl, 18 glucose, 5 histidine.

*Reference and test solution: 20 XNO₃, 90 TEANO₃, 18 glucose, 5 histidine.
Porciatti, Janigro, Maccaferri, Mangoni, Tritella, Chang, and Cohen, 1991; Wollmuth, L. P., and B. Hille, unpublished data), and it persists even in 2 mM BaCl₂ (n = 2), a blocker of most K⁺ channels, and in 0.1 mM CdCl₂. Fig. 10 C shows the instantaneous I-E relation in Rb⁺. Again, since there is no outward current the reversal potential is not defined, but it must be positive to −60 mV. Hence, $P_{Rb}/P_K$ is > 0.55 (Table III).

**Relative Conductance**

Another measure of selectivity is relative conductance. In measuring relative conductance we used 0 Cs records, since the block of Ih channels by Cs⁺ gets stronger at more negative potentials. Steady-state I-E plots (cf. Fig. 10 B), which were leak-subtracted by fitting a line to positive potentials, were fitted with a line over the very negative region to derive the steady-state limiting slope conductance (G). The conductance was normalized to 20 K. The relative conductance ($G_X/G_R$) was: Tl⁺, 1.07; K⁺, 1; Rb⁺, 0.37; NH₄⁺, 0.07; Na⁺, 0.02. Each value has a minimum of three observations.

**DISCUSSION**

**Identification of the Current**

This paper describes measurements of Cs⁺-sensitive currents in photoreceptors using solutions containing 90 mM TEA. An important and not completely resolved issue is whether the current sensitive to 2 mM Cs⁺ is exclusively Ih or is contaminated by other currents. In K⁺- or amine-containing solutions, the Cs⁺-sensitive current has all the kinetic features of Ih, both with hyperpolarization and with subsequent depolar-
ization (Figs. 1, 4, and 5). However, with K\(^+\)-free Na\(^+\), Ti\(^+\), or Rb\(^+\) solutions, not all of the expected kinetic features are seen; with Na\(^+\) there is no \(I_h\)-like time dependence during the hyperpolarizing pulse (Fig. 8), with Ti\(^+\) there is none during the depolarizing pulse (Fig. 9), and with Rb\(^+\) there is none during either pulse (Fig. 10). Three hypotheses need be considered: (1) The Cs\(^+\)-sensitive currents seen are flowing in other channels. (2) The ionic conditions alter \(I_h\) channels so that they do not conduct in the outward direction and therefore gating cannot be observed. (3) Permeant ions have profound effects on the gating of \(I_h\) channels. Hypotheses 2 and 3 could be closely related.

Consider hypothesis 1, that other channels are involved. Salamander rods have conventional voltage-gated Ca and K channels, \(I_{K_s}\) channels, and Ca\(^{2+}\)-activated Cl and K channels (Bader et al., 1982; Beech and Barnes, 1989). These are, however, not good candidates to carry a Cs\(^+\)-sensitive, inwardly rectifying Na\(^+\), Ti\(^+\), or Rb\(^+\) current at potentials between \(-130\) and \(-60\) mV. As there may be still other channels, such as inward rectifiers, hypothesis 1 remains possible and difficult to exclude. One possibility is that the presence of Na\(^+\), Rb\(^+\), or Ti\(^+\) interferes with the action of Cs\(^+\) on \(I_h\) channels (so that \(I_h\) would no longer be isolated as the difference current). This would seem more likely if the difference currents in Na\(^+\), Rb\(^+\), or Ti\(^+\) could not be blocked by other blocking agents of \(I_h\) channels. However, we find here that, like the normal difference currents in K\(^+\) (DiFrancesco et al., 1991; our results), the unusual currents in Rb\(^+\) are blocked by 500 \(\mu\)M 9-amino-1,2,3,4-tetrahydroacridine but not by 2 mM Ba\(^{2+}\), a blocker of many K channels including inward rectifiers (Rudy, 1988).

Hypothesis 2 might be applied to Ti\(^+\) or Rb\(^+\) solutions, where no outward currents are seen. It would suggest that complete replacement of K\(^+\) with Ti\(^+\) or Rb\(^+\) greatly reduces the ability of the \(I_h\) channel to carry outward currents. Indeed, some such postulate seems necessary since we know that \(I_h\) channels are present in the membrane and that permeant K\(^+\) ions are present inside the cell, yet no outward current is observed. Remotely analogous examples in the literature are the establishment of normal inward rectification in cardiac \(i_{K1}\) (and other) channels by internal Mg\(^{2+}\) (Vandenberg, 1987; Matsuda, Saigusa, and Irisawa, 1987) and the absolute requirement of certain K channels for extracellular K\(^+\) ions to conduct outward current at all (Carmeliet, 1989; Pardo, Heinemann, Terlau, Ludewig, Lorra, Pongs, and Stühmer, 1992). Such properties presumably depend on occupancy of ion-binding sites at or within the pore and indeed we find that \(I_h\) channels have numerous flux properties suggestive of multiple binding sites in the pore (Wollmuth and Hille, 1992).

An alternative but similar explanation for the apparently instantaneous nature of rectification in, for example, Rb\(^+\) is that normal \(I_h\) channel gating has become very fast (hypothesis 3) at all voltages. This would fit well with the finding that the voltage dependence of the steady-state K\(^+\) and Rb\(^+\) conductances are nearly superimposable (Fig. 10 B) and that the currents in Rb\(^+\) are sensitive to the aminoacridine blocker. The literature does give several examples of major changes of channel gating occurring when an ion is added or taken away from the medium. For example, the skeletal muscle inward rectifier is a time-dependent increasing current in K\(^+\) but a time-dependent decreasing current in Ti\(^+\) (Stanfield, Ashcroft, and Plant, 1981;
Ashcroft and Stanfield, 1983). Also, in the delayed rectifier of squid giant axons, activation gating is slowed by intracellular fluoride (Adams and Oxford, 1983) and by extracellular Zn\(^{2+}\) (Gilly and Armstrong, 1982), and closing is lost when extracellular Ca\(^{2+}\) is removed (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990).

Perhaps the most difficult records to identify with \(I_h\) channels are the difference currents in Na\(^+\) (Fig. 8). Here all currents are small and even a small contamination would be significant. With steps to \(-114\text{ mV}\) the difference current is only \(-30\text{ pA}\) and shows little time dependence. Nevertheless, the tails during steps back to more positive potentials do show a time course like the deactivation of \(I_h\). In addition, they have three other properties expected of \(I_h\) currents: Activation curves constructed with these tails indicate that they depend on preceding hyperpolarizing pulses with exactly the same voltage dependence as currents in K\(^+\) (Fig. 8 B). Furthermore, the tails grow with increasing duration of the activation pulse much as they do in K\(^+\) solution (Wollmuth and Hille, 1992). Finally, the reversal potential measured from the tails gives the same \(P_{Na}/P_K\) as that from larger \(I_h\) currents in Na\(^+\)/K\(^+\) mixtures. Therefore we suggest that \(I_h\) channels are activating normally in Na\(^+\) solution during the hyperpolarizing prepulse; however, as no time dependence is seen in the small inward current measured at \(-114\text{ mV}\), this may be contaminated with current in some other channel. Apparently in K\(^+\)-free Na\(^+\) solutions, the ability of the channels to conduct inward current is highly attenuated. In cardiac cells, the ability of the \(I_h\)-like current \(I_f\) to carry inward Na\(^+\) currents also depends on external K\(^+\) (Frace, Maruoka, and Noma, 1992).

**Selectivity of \(I_h\) Channels**

Others have shown that \(I_h\) is carried by both K\(^+\) and Na\(^+\) (DiFrancesco, 1981b; Mayer and Westbrook, 1983; Bader and Bertrand, 1984; Hestrin, 1987). When \(P_{Na}/P_K\) has been explicitly calculated, it was for mixtures of Na\(^+\) and K\(^+\) as in Fig. 7 giving values from 0.2 to 0.3 (Hestrin, 1987; Maricq and Korenbrot, 1990). In mixtures of external K\(^+\) and Na\(^+\), we found \(P_{Na}/P_K\) to be 0.36, and with only Na\(^+\) outside and K\(^+\) inside it was 0.33.

Edman and Grampp (1989) addressed the permeability of ions other than K\(^+\) and Na\(^+\) in \(I_h\) channels. They tested Li\(^+\), hydrazine, NH\(^+\), and Rb\(^+\) and concluded that none permeated \(I_h\) channels. However, permeabilities were tested in the presence of K\(^+\) and calculated from the change in amplitude of the time-dependent current recorded at a constant voltage. A major drawback of this approach is that it assumes independence of permeant ions. This condition is not met in \(I_h\) channels, which are blocked by some permeant ions (NH\(^+\), Tl\(^+\), Rb\(^+\); Wollmuth and Hille, 1992), have permeability ratios that depend on permeant ion concentrations (Hestrin, 1987), and show anomalous mole fraction dependence (Wollmuth and Hille, 1992). Measurements of changes in reversal potentials are insensitive to these deviations from independence (Hille, 1971).

**Comparison to Other Channels**

\(I_h\) channels have been called nonselective cation channels since they are permeable to Na\(^+\) and K\(^+\). Yet, in comparison to other “nonselective” cation channels such as the nicotinic acetylcholine receptor (nAChR) channel or the cGMP-gated channel in rod
outer segments, this one is highly selective. The nAChR channel, for example, is almost equally permeable to all alkali metal ions and is permeated by >41 organic cations and several divalent cations (Dwyer et al., 1980; Adams et al., 1980). Further, ethylammonium, which is essentially impermeant in Ih channels, has a permeability equal to that of K+ in nAChR channels.

The selectivity of Ih channels shows few similarities to that of Na channels. In Na channels, Li+ and hydroxylamine are about equally permeant, whereas hydrazine is half as permeant as Na+ (Hille, 1971, 1972). We were unable to assess the permeability of hydroxylamine since rods became unstable in the presence of this ion, but Ih channels show a very low permeability to Li+ ($P_{Li}/P_{Na} = 0.06$) and hydrazine ($P_{hydrazine}/P_{Na} = 0.12$). Perhaps more revealing, in Na channels $P_{Na}/P_{K}$ is < 0.1, whereas in Ih channels it is almost 3.

The selectivities of Ih and K channels show few similarities and a significant difference. Like K channels (Hille, 1973), Ih channels are permeable to Tl+, Rb+, and NH4+. Nevertheless, they differ in that Ih channels have a relatively high permeability to Na+. For most K channels, the $P_{Na}/P_{K}$ is < 0.03.

Possible Explanations for Selectivity

Our results with organic cations allow us to estimate the diameter of the most restricted part of the pore. Ammonium ions are reasonably permeant ($P_{NH_4}/P_K = 0.17$) and have a diameter of 3.7 Å, assuming that all hydrogens can form hydrogen bonds. Methylammonium (MA) is also permeant ($P_{MA}/P_K = 0.06$). It is a linear molecule with a methyl group at one end and an amine group at the other. The methyl group, which cannot form hydrogen bonds, has a diameter of 4 Å, so the minimum profile diameter for MA is dictated by the methyl group. In contrast, ethyl- and dimethylammonium are essentially impermeant. They are slightly larger than MA with a minimum profile diameter of ~4.6 Å. Hence, the minimum pore diameter of Ih channels seems to be between 4 and 4.6 Å (based on Corey-Pauling space-filling models). These dimensions are larger than those for K and Na channels but smaller than those for nAChR channels (Dwyer et al., 1980).

Ih channels display properties that seem to put them in a class of their own within the superfamily of voltage-gated channels: like Na, Ca, and delayed rectifier K channels, Ih channels have steeply voltage-dependent gating and activate with a sigmoid time course, but unlike the others they also deactivate with a sigmoid time course. Like inward rectifier channels, Ih channels open at negative potentials, close at positive potentials, and are blocked by Cs+ and Rb+ ions. Unlike inward rectifier channels, however, they have a high permeability to Na+, have a nearly linear instantaneous $I-E$ relation, are not strongly blocked by Ba2+ ions, and have a voltage dependence that does not shift with changes of external K+.

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