HINDERED DIFFUSION IN EXCISED MEMBRANE PATCHES FROM RETINAL ROD OUTER SEGMENTS

ANITA L. ZIMMERMAN,* JEFFREY W. KARPEN,‡ AND DENIS A. BAYLOR*
Departments of Neurobiology and‡ Cell Biology, Sherman Fairchild Science Building, Stanford University School of Medicine, Stanford, California 94305*

ABSTRACT Excised inside-out membrane patches are useful for studying the cGMP-activated ion channels that generate the electrical response to light in retinal rod cells. We show that strong ionic current across a patch changes the driving force on the current by altering the ionic concentration near the surface membrane, an effect somewhat like that first described by Frankenhaeuser and Hodgkin (1956) in squid axons. The dominant concentration change occurs in the solution adjacent to the cytoplasmic (*inner*) surface of the membrane, where diffusion is impaired by intracellular material that adheres to the patch during excision. The magnitude and time course of the ionic changes are consistent with the expected volume of this material and with an effective diffusion coefficient about an order of magnitude less than that in free solution. Methods are described for correcting current transients observed in voltage clamp experiments, so that channel gating kinetics can be obtained without contamination by changes in driving force. We suggest that restricted diffusion may occur in patches excised from other types of cells and influence rapid kinetic measurements.

INTRODUCTION

The excised inside-out membrane patch (Hamill et al., 1981) has proved useful in studies of the ionic conductance in the surface of retinal rod outer segments (e.g., Fesenko et al., 1985; Stern et al., 1986; Haynes et al., 1986; Zimmerman and Baylor, 1986; Matthews and Watanabe, 1987; Karpen et al., 1988). In this preparation the channels that generate the electrical response to light are decoupled from the intracellular enzyme cascade that regulates channel gating by changing the level of 3′, 5′-cyclic guanosine monophosphate (cGMP, reviewed in Stryer, 1986). When cGMP is applied to the inner surface of an excised patch the channels open, and the current through them can be obtained from the cGMP-dependent increase in patch current.

This paper presents evidence that strong currents change the ionic concentration near the surface membrane, generating a back EMF that opposes current flow. Effects of this kind were discovered by Frankenhaeuser and Hodgkin (1956) in experiments on squid axons. We show that in rod patches the effect results from restricted diffusion of ions in a small region located on the cytoplasmic side of the membrane. The restriction on diffusion is attributed to intracellular material that adheres to the surface membrane during excision. A simple model of the ionic changes is presented, together with a method for correcting raw currents so that reliable kinetic information can be obtained.

METHODS

Inside-out membrane patches were excised from isolated rod outer segments of the tiger salamander, *Ambystoma tigrinum*. The patch pipettes were made from borosilicate glass and had orifices of 0.5–1.5 μm diameter. Isolated outer segments were obtained by teasing small pieces of retina in the experimental chamber. During excision the outer segment was held with a suction electrode. All manipulations were performed under visible light. Preparations were made in salamander Ringer (111 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM glucose, 3 mM Hepes (pH 7.6), 0.02 mM EDTA). The patch pipette and experimental chamber were filled with a low divalent Na solution (130 mM NaCl, 10 mM Tris [pH 6.8], 0.02 mM EDTA). cGMP was applied to the cytoplasmic side of the patch by perfusion of the chamber. The current through the cGMP-activated channels was found from the difference between the currents measured with and without cGMP.

Patch currents were processed by an 8-pole Bessel low-pass filter with selectable cutoff frequency (Frequency Devices, Inc., Haverhill, MA) and digitized at a sampling rate sufficient to prevent aliasing. Currents were not corrected for the series resistance of the pipette, which was usually 3–5 MΩ as measured before a seal was made. Currents were acquired, stored, and analyzed with a PDP 11/73 computer (Indec Systems, Inc., Sunnyvale, CA).

RESULTS

Current Changes Ionic Concentration Near Patch Membrane

Fig. 1 *A* presents evidence that a strong current changed the ionic concentration near an excised patch. The trace is

Dr. Zimmerman's present address is Section of Physiology and Biophysics, Box G, Brown University, Providence, RI, 02912

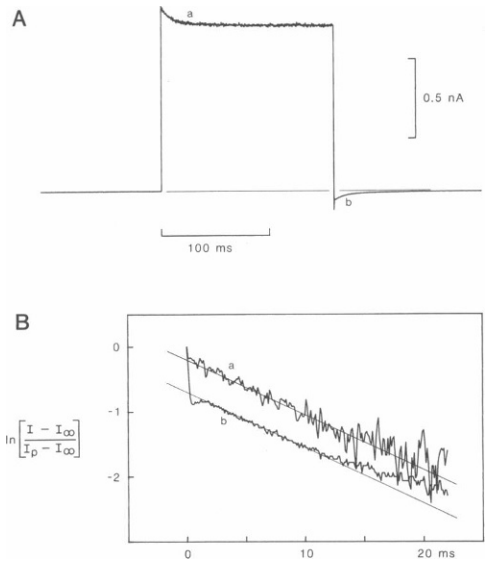

FIGURE 1. Relaxations in patch current due to change in reversal potential. (*A*) cGMP-induced current as a function of time during a voltage pulse to +50 mV from a holding voltage of 0 mV. The outward current at the make of the pulse drooped (*a*). At the break of the pulse there was a brief inward current transient followed by a slower tail of inward current (*b*). Identical solutions (Methods) bathed the inner and outer surfaces of the patch. Current is the difference between currents measured in the presence of 100 μM cGMP (average of four sweeps) and in the absence of cGMP (average of seven sweeps). Bandwidth 0–3 kHz, sampling rate 10 kHz, patch resistance, 4.4 GΩ without cGMP. (*B*) Semilog plots of current transients *a* and *b* shown above; I_p is the peak current and I_∞ the final value. Straight lines have time constant 12.7 ms.

the cGMP-activated current (current with saturating cGMP minus current without cGMP) evoked by a +50 mV pulse from a holding voltage of 0 mV. There was a droop in the outward current and a transient inward current at the end of the pulse. Since the bulk solutions on both sides of the patch had identical ionic compositions, the inward current at 0 mV cannot result from a conductance change. The charge movement during the transients seems much too large to be consistent with displacement currents within the membrane. Instead, outward current caused a time-dependent reduction in the driving force (difference between patch voltage and reversal potential). During the depolarizing pulse the reversal potential became more positive; this effect persisted at the end of the pulse, causing the transient inward current. The transient at the end of the outward current showed a brief spike which was not apparent at the start of the outward current; as explained on p. 5, the absence of the spike is due to a compensating change in the number of open channels. The semilog plots in Fig. 1 *B* show that the final decline of both transients was exponential, with the time constant 12.7 ms. The different amplitudes of the exponential components are explained by the outward rectification in the current-voltage relation of the conductance under these conditions (Fig. 2). At +50 mV the slope conductance is about twice that at 0 mV, and accordingly the same change in reversal potential perturbs the current more at +50 mV. Inward currents during hyperpolarizing pulses sagged with a similar final time course and were followed by rebound outward currents. Changes in reversal potential occurred consistently in many patches when the current exceeded roughly 500 pA.

Ionic Concentration Change Occurs at Inner Surface of Patch

The shift in reversal potential illustrated in Fig. 1 resulted from changes in Na ion concentration, because the conductance is strongly cation-selective (Fesenko et al., 1985) and Na was the current carrier. A positive shift in reversal potential, which is required to explain the direction of the effect, might result if Na efflux (*outward current*) increased the Na concentration at the external patch surface, lowered the concentration at the internal surface, or both. The results in Fig. 2 indicate that the change occurred mainly at the inner surface. Here a patch's cGMP-activated currents were observed at different concentrations of internal Na. For outward currents of similar size, the droop was much larger at lower internal Na concentrations. Even large outward currents showed virtually no droop when the internal Na was high (500 mM). These results are readily explained if the droop results from depletion of internal Na: the fractional concentration change, which determines the shift in the Nernst potential, is larger at a lower starting concentration. The results would not be expected if outward current increased the Na concentration in the external solution. Apparently, ionic diffusion is restricted near the inner surface of the excised patch.

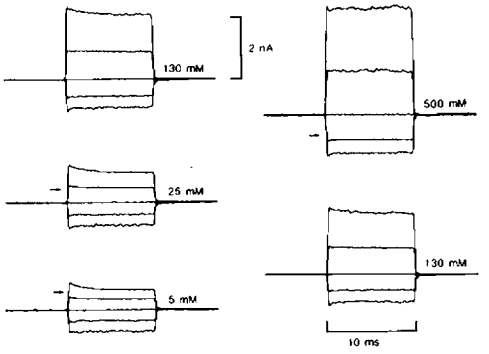

FIGURE 2. Effect of varying internal Na concentration on droop in cGMP-activated currents. Patch voltage was held at 0 mV (not corrected for junction potential changes) and switched between −100 and +100 mV in 50 mV steps. Internal Na concentrations as indicated. Sequence of runs, 130, 25, 5, 500, and 130 mM. Arrows indicate zero membrane current (when different from baseline). Pipette was filled with 130 mM Na, and cGMP concentration was 50 μM. Sucrose was added to keep the osmolarity constant in the internal solution when the Na concentration was lowered; at 500 mM NaCl there was an uncompensated increase in osmolarity. Bandwidth 0–2 kHz, sampling rate 10 kHz. Patch resistance without CGMP was 11 GΩ initially, 6 GΩ at end of the experiment. Temperature 22.5°C.

Dependence of Droop Amplitude on Current Intensity for Fixed Voltage Step

There was a square law relation between the size of the ionic transient and the current that evoked it, when the number of open channels in the patch varied. This was first noted when a patch, subjected to a constant voltage pulse protocol, was exposed to different concentrations of cGMP. In several such experiments the size of the zero millivolt transient at the end of a pulse was found to vary with the square of the preceeding steady current. Square law behavior also occurred in collected results from different patches, activated by saturating cGMP (100 μM), in which the number of channels differed because of variations in patch area or channel density (see fig. 3). In this figure, the size of the droop in the outward current at +50 mV is plotted against the amplitude of the steady current intensity on double log scales. The straight line, determined by linear regression analysis, has a slope of 2.24. The square law seems to have the following basis. The macroscopic current I flowing across the N open channels in a patch is given by

$$I = G_V V' N, \tag{1}$$

where G_V is the chord conductance of a single open channel at voltage V and $V' = V - V_r$ is the voltage driving force, V_r being the reversal potential. The change in current $\Delta_i I$ resulting from a change in reversal potential is

$$\Delta_i I = g_V \Delta V' N, \tag{2}$$

where g_v is the slope conductance of an open channel at V. Eqs. 1 and 2 assume that a change in reversal potential simply translates the current-voltage relation on the voltage axis. If the ionic concentration change varies linearly with the current intensity and is small enough to give a linear change in the reversal potential, then the change in driving force is

$$\Delta V' = R I. \tag{3}$$

The constant R in this expression has dimensions of resistance and depends on the properties of the patch. From relations 1–3,

$$\Delta_i I = \frac{g_V R I^2}{G_V V'}. \tag{4}$$

For a constant voltage pulse this implies a square law relation between the size of the ionic transient and the steady current that produces it. The fact that the results in Fig. 3 conform to this prediction implies that the generalized resistance R in Eq. 3 was relatively constant across patches even though N varied considerably. In general, ionic transients will be most pronounced when the patch contains a large number of channels.

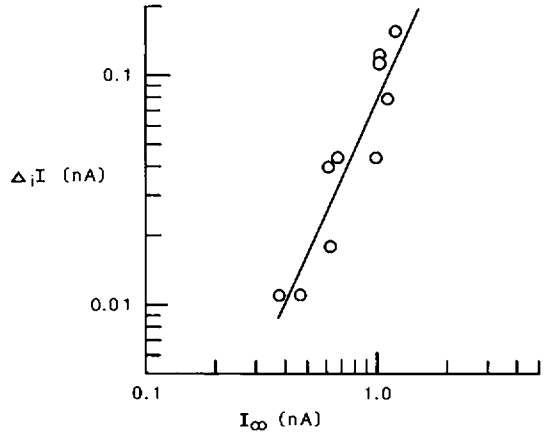

FIGURE 3. Collected results from 10 patches demonstrating a square law relation between droop in cGMP-activated patch current ($\Delta_i I$) and final current amplitude (I_∞). Double log scales. Currents in 100 μM cGMP were measured after switching voltage to +50 mV from a holding voltage of 0 mV. Straight line, obtained by linear regression on log values, drawn according to the equation $\Delta_i I = 0.08\, I_\infty^{2.24}$ in which I_∞ and $\Delta_i I$ are expressed as dimensionless multiples of 1 nA. Correlation coefficient was 0.92.

Quantitative Treatment; Slab Volume and Diffusion Coefficient

Assume that next to the inner surface of the membrane is a uniform slab of finite thickness in which ionic diffusion is restricted (Frankenhaeuser and Hodgkin, 1956, "hypothesis 2"; see also Carslaw and Jaeger, 1986, pp. 93–97 for the analogous problem of heat diffusion in a solid bounded by two parallel planes). For the one dimensional model, an inward current step causes the concentration of ions in the slab just adjacent to the membrane to rise along a biphasic time course. Initially, the rise is rapid as ions accumulate near the membrane, while later there is an exponential rise as the ionic concentration changes over the entire slab. Symmetrical changes occur for outward current steps and at the end of inward and outward currents.

Both kinetic phases are apparent in Fig. 1, *Ab*. If v is the volume of the slab, the time course of the exponential component of the concentration change ΔC at the inner surface of the membrane after the onset of a current step of intensity I is given by

$$\Delta C = -\frac{2I\tau}{Fv}(1 - \exp(-t/\tau)), \tag{5}$$

where F is the Faraday constant and τ is the characteristic time constant of the slab. This equation can be applied to the patch of Fig. 1 as follows. Assuming for simplicity that the fractional change in driving force is equal to the fractional change in current, one finds that the reversal potential changed from 0 to +4.8 mV during the outward current. From the Nernst equation, the steady state Na concentration dropped by 23 mM from the bulk concentration of 130 mM. Using these values, as well as the time constant of 12.7 ms and the measured current, a slab

volume v of 12 μm^3 is calculated by eq. 5. This seems reasonable for a structure at the end of a patch pipette.

The diffusion coefficent D' in the slab is given by

$$D' = \frac{4x^2}{\pi^2 \tau}, \quad (6)$$

where x is the slab thickness. Assuming a value for x of $(v)^{1/3}$ = 2.3 μm, a diffusion coefficient D' of 1.67 × 10^{-6} cm^2 s^{-1} is obtained. This may be regarded as a lumped figure; probably the slab is heterogeneous and consists of membranes and watery spaces. D' is an order of magnitude less than the diffusion coefficient of Na in free solution, which is 1.54 × 10^{-5} cm^2 s^{-1} at room temperature (Weast et al., 1987).

Eleven patches analyzed in this way gave a volume v of 7.5 ± 2.6 um^3 (mean ± S.D.) and a value for D' of 2.7 ± 1.7 × 10^{-6} cm^2 s^{-1}.

DISCUSSION

Jumps in the membrane voltage perturb activation of the rod channel by cGMP and thus provide a useful tool for analyzing the kinetics of activation (Karpen et al., 1988). The following method can be used to separate changes in current due to channel gating from changes in the driving force. The macroscopic current I flowing through the N open channels in an excised patch is Ni, where i is the single channel current. The change in current ΔI at voltage V due to small changes in N (gating) and reversal potential V_r will be given by:

$$\Delta I = \Delta(Ni) = i\Delta N - Ng_V \Delta V_r, \quad (7)$$

where g_V is the slope conductance of an open channel at V. In Eq. 7, i is close to zero at 0 mV with identical solutions on both sides of the patch, so that ΔI depends only on the change in reversal potential. The kinetics of ΔV_r after a current step depend only on the (constant) slab properties. Therefore the gating component $i\Delta N$ can be obtained from the change in current ΔI at any voltage V by subtracting the scaled 0 mV transient $Ng_0 \Delta V_r$. The scaling factor is obtained in the following way. Let $\Delta_i I_0$ denote the 0 mV transient at the end of a steady current I' (see Fig. 4). Assuming that the change in reversal potential varies linearly with the change in steady patch current, it follows that the resulting ionic transient $\Delta_i I_2$ at a new voltage V_2 when the steady current switches from I_1 to I_2 will be given by

$$\Delta_i I_2 = \left(\frac{g_v}{g_0}\right)\left(\frac{I_2 - I_1}{-I'}\right)\Delta_i I_0. \quad (8)$$

The transient $\Delta_i I_2$ derived in this way is subtracted from the raw current at V_2 to obtain the gating transient (*dotted line* in Fig. 4). Eq. 8 applies to small perturbations in which the change in reversal potential varies linearly with the change in ionic concentration. A more general method, which gave very similar scaling factors, is to assume only

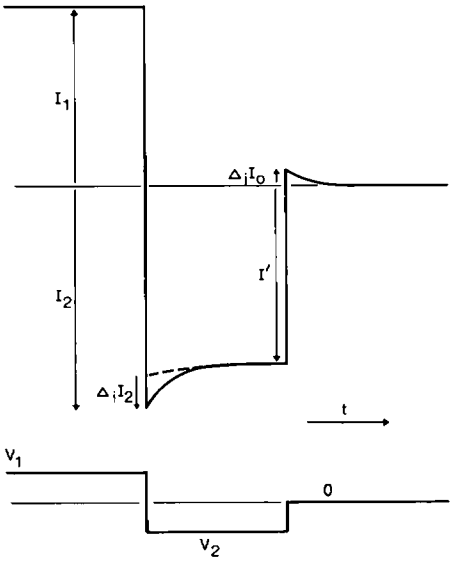

FIGURE 4. Diagram of method for removing ionic transients from cGMP-activated currents in order to obtain channel-gating kinetics. Patch current shown above, voltage below. Zero millivolt ionic transient $\Delta_i I_0$ occurs at the end of current I'. The ionic transient $\Delta_i I_2$ occurring when the current switches from I_1 to I_2 is calculated as a scaled replica of the zero millivolt transient, using Eq. 8 of the text. Subtraction of $\Delta_i I_2$ yields a current proportional to the number of open channels, shown by the dotted line.

that the concentration change varies linearly with current change and to calculate changes in reversal potential from the Nernst relation.

Fig. 5 illustrates the use of Eq. 8 to obtain the time course of channel gating after voltage steps, which perturb activation of the channel by cGMP. Fig. 5 *A* shows difference currents induced by 20 and 50 μM cGMP when the

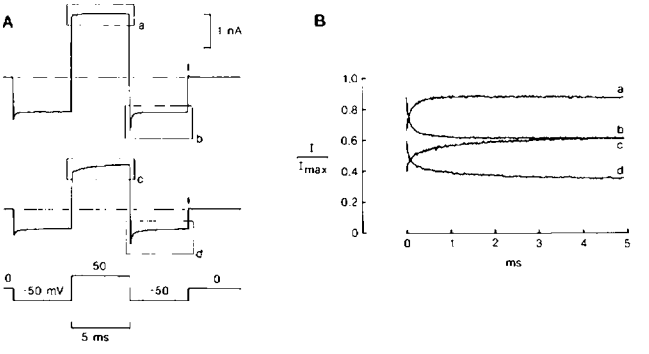

FIGURE 5. Correcting patch currents for changes in reversal potential to obtain time course of channel gating after voltage jumps. (*A*) Raw cGMP-induced currents for the voltage pulse protocol shown at the bottom. Upper record obtained with 50 μM cGMP, lower record with 20 μM cGMP. Boxes show regions that were corrected, using the 0 mV transients indicated by the arrows. (*B*) Ordinate is the cGMP-induced current, corrected by the method described in the text and normalized by the saturating current in 500 μM cGMP. Abscissa is time after the voltage was switched. Letters indicated parts of raw record (*boxed regions* in *A*) from which traces were obtained. Bandwidth 10 kHz, sampling rate 100 kHz, patch resistance without cGMP 1.25 GΩ. Temperature 22.8°C.

voltage was switched in the sequence 0, −50, +50, −50, 0 mV. The currents at the transitions −50 to +50 and +50 to −50 were quite asymmetric for both concentrations of cGMP. Fig. 5 *B* shows the current transients after they were corrected for changes in driving force and normalized by the respective maximal currents at 500 μM cGMP. The corrected transients are nearly symmetrical and begin and end at the same levels, as they should if gating is indeed being measured. Asymmetry was present in the raw records because the ionic transient, which here consisted mainly of a fast spike, added to the gating transient at −50 mV and subtracted from it at +50 mV. The slight differences in the kinetics of the gating transients at a given cGMP concentration reflect the voltage dependence of the open-closed equilibrium of the fully-liganded channel (Karpen et al., 1988).

It seems likely that diffusion is restricted by intracellular material that adheres to the surface membrane during patch excision. Occasionally excision gave a just-discernable blob at the end of the electrode, but usually none was evident; the presence of a blob was not required to observe changes in reversal potential. In rods, the intracellular discs are attached to the surface membrane by filamentous material (Falk and Fatt, 1969; Cohen, 1971; Roof and Heuser, 1982; Molday et al., 1987). Perhaps the slab consists of disc membrane rims that adhere to the surface membrane. Jung et al. (1987) have obtained electron microscopic evidence that cytoplasmic material also clings to patches excised from chick myocytes.

Restricted diffusion, arising from adhering intracellular material, may be a general property of excised patches from a variety of types of cells. The presence of diffusional barriers may be expected to limit the speed of access of ligands to the surface membrane in rapid kinetic experiments and to cause current-dependent changes in ionic concentration at the patch membrane.

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