Light-induced proton permeability changes in retinal rod photoreceptor disk membranes

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ABSTRACT We have used the membrane-permeant charged fluorescent dye, 3,3'-dipropylthiadicarbocyanine iodide (diS-C3[5]), to monitor electrical potentials across the membranes of isolated bovine disks. Calibration curves obtained from experiments where a potential was created across the disk membrane by a potassium concentration gradient and valinomycin showed an approximately linear relation between dye fluorescence and calculated membrane potential from 0 to −120 mV. Light exposure in the presence of the permeant buffer, imidazole, caused a rapid decay of the membrane potential to a new stable level. Addition of CCCP, a proton ionophore, in the dark produced the same effect as illumination. When the permeant buffer, imidazole, was replaced by the impermeant buffer, Hepes, neither light nor CCCP discharged the gradient. We interpret the changes in membrane potential measured upon illumination to be the result of a light-induced increase in the permeability of the disk membrane to protons. A permeant buffer is required to prevent the build-up of a pH gradient which would inhibit the sustained proton flow needed for an observable change in membrane potential.

INTRODUCTION

Photons absorbed by rhodopsin in the disk membranes of vertebrate rod photoreceptors initiate processes which lead to a hyperpolarization of the rod cell plasma membrane and in turn to vision. An extensive literature exists on the physiological response of photoreceptor cells to illumination and on the light-regulated biochemical processes within the photoreceptor outer segment (see Dahlem Workshop report edited by H. Stieve, 1986, and reviews by Korenbrot, 1985, Schwartz, 1985, and Pugh, 1987). Less information is available on the specific role which the disk membrane itself plays in these processes. We have monitored electrical potentials across the membranes of isolated bovine disks using the membrane-permeant, cationic, fluorescent dye, 3,3'-dipropylthiadicarbocyanine iodide (diS-C3[5]) (reviews by Waggoner, 1979, Cohen and Salzberg, 1978, and Grinvald et al., 1988). We interpret the changes in membrane potential observed upon illumination in the presence of permeant buffers to be the result of a light-induced increase in the permeability of the disk membrane to protons.

Other workers have investigated the role of protons in visual transduction. Proton uptake by disks accompanies the metarhodopsin I to metarhodopsin II transition (Bennett, 1980; McConnell et al., 1968; Maloney et al., 1980); however, experiments with membrane-permeant buffers indicate little effect of cytoplasmic pH changes on the transduction process (Pinto and Ostroy, 1978; Yoshikami and Hagins, 1984). Buffer microinjection experiments in invertebrates yielded similar conclusions (Coles and Brown, 1976). Calcium-proton exchange sites on disks have been examined in detail, but only in the presence of ionophore (Schnetkamp and Kaupp, 1985). One specific model suggests that protons produced by the light-activated phosphodiesterase activity may drive calcium release from disks (Mueller and Pugh, 1983; Liebman et al., 1984). Light scattering and other measurements suggest that there is a light-regulated proton pump in the disk membrane (Borys et al., 1985). Fluorescent and radioactive membrane-permeant cations have been used to monitor disk membrane potentials and their light-induced changes (Bennett et al., 1980; Hughes and Brand, 1985); however, without the presence of a permeant proton acceptor these measurements could not clearly identify the light-induced proton permeability.

MATERIALS AND METHODS

A potassium concentration gradient was established across isolated bovine photoreceptor disk membranes by adding aliquots of disk suspensions prepared in 100 mM K^+ buffer to "external" solutions containing lower K^+ concentrations. The latter also contained the potential-sensitive, fluorescent dye, diS-C3(5). Disks (Smith et al., 1975; Smith and Litman, 1982) were suspended at 2 mg/ml rhodopsin in a solution containing 100 mM K-glutamate, 50 mM imidazole-glutamate (pH 7.4) and 0.5 mM dithiothreitol, and allowed to equilibrate at 4°C overnight. For experiments requiring a membrane-impermeant buffer, the imidazole-glutamate was replaced with a solution containing 50 mM *N*-methylglucamine (NMG) and 50 mM Hepes with the pH adjusted to 7.4.

The external, dye solution was prepared by adding 4 μl of 1 mM diS-C3(5) in *N*-methylformamide to 2 ml of a pH 7.4 buffer containing

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100 mM *N*-methylglucamine-glutamate (NMG-glut), 50 mM imidazole-glutamate (or 50 mM NMG-Hepes) and 1.0 mg/ml bovine serum albumin to give a final dye concentration of 2 μM. External potassium concentrations from 1 to 100 mM were obtained by replacing some of the NMG-glutamate in the dye buffer with equimolar amounts of K-glutamate.

Additions were made directly into a fluorescence cuvette containing the 2 ml dye solution and are indicated in the figures as follows: "disks"—15 μl of disk suspension; "Val"—5 μl of 1 mM valinomycin in ethanol; "Hv"—illumination with yellow light (Kodak Wratten 8 filter; Eastman Kodak Co., Rochester, NY) for the times indicated in the figure legends; "KCl"—50 μl of 4 M KCl; "CCCP"—1 μl of 0.5 mM carbonyl cyanide m-chlorophenylhydrazone (a protonophore) in dimethylsulfoxide (Kasianowicz et al., 1984).

The fluorescence of the dye solution was monitored using an SLM model 8000C fluorescence spectrophotometer (Aminco, Urbana, IL). The excitation wavelength was 650 nm with a bandwidth of 4 nm; the emission wavelength, 670 nm with a bandwidth of 2 nm. The fluorescence of the initial dye solution was taken as 100%. All additions, except for light, were done under dim red illumination. The shutter was closed during each addition as indicated by the breaks in the traces. The fluorescence cuvette was thermostated at 25°C.

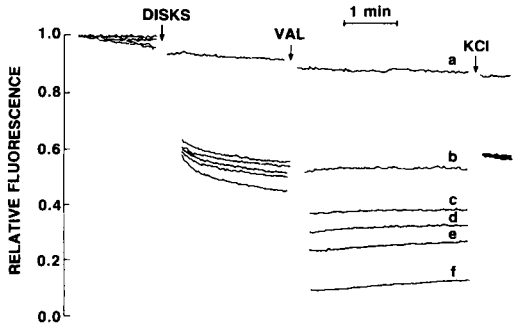

FIGURE 1 The response of diS-C3(5) fluorescence to membrane potentials created by varying external [K^+] in the presence of valinomycin. Traces *b–f* are experiments with disks conducted as described in Materials and Methods. The external [K^+] and calculated membrane potentials were as follows: trace *b*, 100 mM (0 mV); trace *c*, 20.75 mM (−41 mV); trace *d*, 10.75 mM (−58 mV); trace *e*, 5.75 mM (−74 mV); trace *f*, 1 mM (−120 mV). The initial internal [K^+] was 100 mM. These experiments were buffered with Hepes, but similar results were obtained with imidazole. Trace *a* shows a control experiment in which buffer containing 1 mM KCl was added instead of the usual aliquot of disks. After the final addition of KCl, traces *b–f* were contiguous with the individual traces overlapping.

RESULTS

These experiments investigated light-induced changes in the membrane potential of disk membranes that had been hyperpolarized by the presence of a potassium gradient and valinomycin. Valinomycin is assumed to clamp the membrane potential to the potassium equilibrium potential, which, in these experiments, is monitored by the diS-C3(5) fluorescence (Figs. 1 and 2). The observed decrease in membrane potential thus reflects the dissipation of the potassium gradient. In the dark, the potential is relatively stable because the membrane has no significant permeabilities to charged species other than potassium (Uhl et al., 1980; Brierley et al., 1968; Bauer and Mavrommati, 1986).

The fluorescence decreased when an aliquot of disks with a high internal potassium concentration was added to a dye solution with low potassium and no valinomycin (Fig. 1). The magnitude of this decrease was independent of the potassium gradient across the disks and, therefore, appears to be potential insensitive. The fluorescence decrease may be due to dye molecules binding to the disk membranes. The magnitude of the decrease was insensitive to the buffer used but varied somewhat from sample to sample probably reflecting variation in a small background permeability to potassium. The lack of potential in the absence of an ionophore is consistent with other reports of the impermeability of dark adapted disks (Uhl et al., 1980; Brierley et al., 1968).

If the concentration of potassium was the same on both sides of the disk membrane, then addition of valinomycin caused no further change in fluorescence; however, if a potassium concentration gradient had been established across the membranes, then addition of valinomycin caused a further decrease in fluorescence which was graded with the magnitude of the potassium gradient (Fig. 1, *traces b–f*). This decrease is caused by movement of dye into the disks in response to the

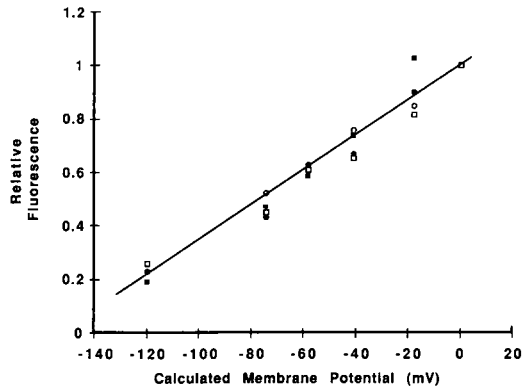

FIGURE 2 Correlation of diS-C3(5) fluorescence with calculated membrane potential. Data were taken from sets of experiments like that shown in Fig. 1. These included two sets buffered with Hepes (●, ■) and two sets buffered with imidazole (○, □). The fluorescence after addition of valinomycin at a given external [K] is plotted vs. the estimated potential calculated from the Nernst relationship, using the known external [K] and an internal [K] estimated to be that of the buffer in which the disks were incubated (100 mM). The line is a linear least squares fit to all the data.

hyperpolarization created by the potassium concentration gradient and valinomycin, and the subsequent quenching of the fluorescence of the concentrated dye within the disks. Subsequent addition of potassium to the external medium decreases the membrane potential and produces a recovery of the fluorescence as the dye is driven back out of the disks. Waggoner (1979) discusses the redistribution and quenching of diS-C3(5) in response to membrane potentials.

Calibration curves obtained from experiments where the external potassium concentration ranged from 1 to 100 mM showed an approximately linear relation between dye fluorescence and calculated membrane potential from 0 to −120 mV (Fig. 2). These results are similar to those found by Simchowitz et al. (1982) in their measurements of potentials across leukocyte membranes. The same linear relationship between calculated membrane potential and fluorescence was observed when the buffer was either imidazole or Hepes. It was thus possible to analyze the data from sets of experiments by estimating the potential using a linear extrapolation between the fluorescence of an experiment with 100 mM external potassium (0 mV) and one with 1 mM external potassium (−120 mV).

The specific dependence of the observed fluorescence upon membrane potential is a function of the concentration of both disks and dye. The conditions used in these experiments were empirically chosen to optimize the relative change in fluorescence observed upon light exposure.

Illumination in the presence of imidazole caused the membrane potential to decay to a new stable level (Fig. 3). In eight experiments with 86% bleaching the plateau was −64 ± 7 mV (mean ± sd). The rate at which the new level was reached increased with increasing light exposure. Addition of CCCP, a proton ionophore, in the dark produced the same effect as illumination (Fig. 4). When the permeant buffer, imidazole, was replaced by the impermeant buffer, Hepes, neither light (Fig. 3, *trace g*) nor CCCP (Fig. 4, *trace d*) discharged the gradient. Neither valinomycin, light, nor CCCP caused a change in fluorescence if there were no potassium gradient across the disk membrane (Figs. 3 and 4, *traces a*). None of the fluorescence changes reported here were observed in the absence of disks.

The baseline after addition of valinomycin was more stable in experiments buffered with Hepes than in those buffered with imidazole. This probably represents the permeability of the disk membrane to imidazolium ions and proton permeability caused by background bleaching.

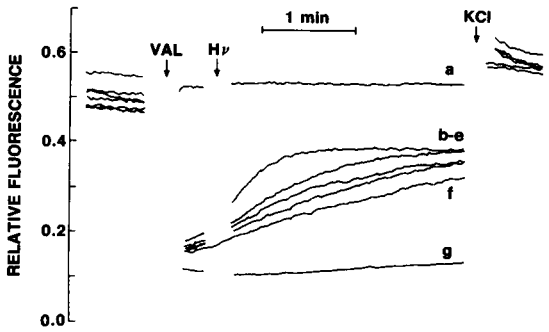

FIGURE 3 Fluorescence signals reflecting changes in disk membrane potential upon illumination in the presence of the permeant buffer, imidazole. The fluorescence signals are plotted beginning after addition of the disks. Light which bleached 86.1% of the rhodopsin in the sample caused no change in the fluorescence signal if there were no potential across the membrane (*trace a*, external $[K^+]$ = 100 mM). The external $[K^+]$ was 1.0 mM for traces *b–g*. Traces *b–e* show the results of light exposures that bleached 86.1%, 21.0%, 11.9%, and 3.7% of the rhodopsin in the sample respectively. The duration of the light exposure was less than 10 s. Trace *f* was taken in the dark. Illumination which bleached 86.1% of the rhodopsin caused no change in potential when the impermeant buffer, Hepes, was used (*trace g*).

DISCUSSION

When a permeability for protons is created, either by the action of light or by a protonophore such as CCCP, the electrical gradient will drive a proton flux. If a permeant buffer like imidazole is present, as in Fig. 3 (*traces b–f*), the uncharged imidazole will cross the membrane and act as an acceptor for the protons entering the disks. These protons are taken up by imidazole to form imidazolium thereby preventing build-up of a proton chemical gradient and enabling a sustained proton flow.

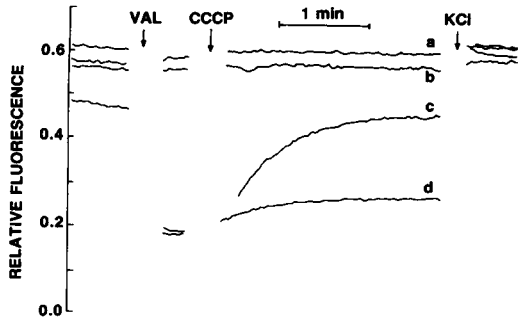

FIGURE 4 The protonophore, CCCP, mimics the effect of light. Experiments are shown with imidazole buffer at 1 mM and 100 mM external $[K^+]$ (*traces c and a* respectively), and with Hepes buffer at 1 mM and 100 mM external $[K^+]$ (*traces d and b* respectively).

Potassium ions will leave the disks to compensate for the charge carried by the protons. The potassium gradient will rapidly dissipate and the membrane potential will decrease. For the conditions of our experiments, the final potential estimated from Eq. 8 in Appendix A is −56 mV compared with the −64 ± 7 mV calculated from the fluorescence data.

With an impermeant buffer such as Hepes, the amount of proton acceptor available is limited by the small internal volume of the disks. The internal buffering capacity can be easily overcome as protons enter the disks and the resulting pH gradient will prevent a sustained proton flux from being maintained (see Fig. 3, *trace g*). Potassium ions, which are present in much higher concentrations than are protons, can easily redistribute to maintain the membrane potential near the original potassium equilibrium potential. Thus, creation of a proton permeability causes no significant change in either the potassium gradient or the fluorescence. The final potential estimated from Eq. 3 in Appendix A is −113 mV for the conditions of our experiments using Hepes.

In the presence of valinomycin and a permeant base such as imidazole, the rate at which the potassium gradient and the membrane potential decay will be limited by the proton permeability of the disk membrane. Because the intradiskal volume can be estimated, the change in potassium concentration enables calculation of the total molar efflux of potassium which is equal to the proton influx. The rate of change in fluorescence can thus be used to calculate the light-induced proton permeability as described below and shown in Fig. 5.

From the fluorescence traces one can calculate the initial rate of the potential decay ($d\psi/dt$) after light exposure. In seven sets of experiments like that shown in Fig. 3, the rate of change after addition of valinomycin was 0.35 ± 0.05 mV/s in the dark and 1.3 ± 0.2 mV/s after a light exposure which bleached 86% of the rhodopsin in the sample. One can then estimate the rate of change in the intradiskal potassium concentration, $d[K^+]_i/dt$, from the following derivative of the Nernst equation (assuming the external potassium concentration to be constant):

$$\text{Proton Flux} = -d[K^+]_i/dt = (F/RT)[K^+]_i d\psi/dt.$$

With an initial intradiskal potassium concentration of 100 mM, $-d[K^+]_i/dt$ was 1.3 ± 0.2 mM/s in the dark and 4.7 ± 0.9 mM/s after 86% bleaching.

Because the efflux of potassium is equivalent to the proton flux across the disk membrane, one can calculate the number of protons entering each disk. Fig. 5 provides these calculated proton fluxes normalized to the rhodopsin content as a function of the percentage of bleached rhodopsin using an estimated intradiskal volume of 2.2×10^{-16} l per disk and 50,000 rhodopsin molecules per disk. Subtracting the background flux of 3.5 H^+/Rh/s measured in the dark (0% bleaching), and dividing by the fraction of rhodopsin bleached gives the light-induced proton fluxes per absorbed photon. These ranged from 11 H^+/s/absorbed photon at 86% bleaching to 24 H^+/s/absorbed photon at 3.7% bleaching. In work with planar lipid bilayers containing rhodopsin, Antanavage et al. (1977) reported that a total of 30–1,000 protons crossed the membrane for each absorbed photon.

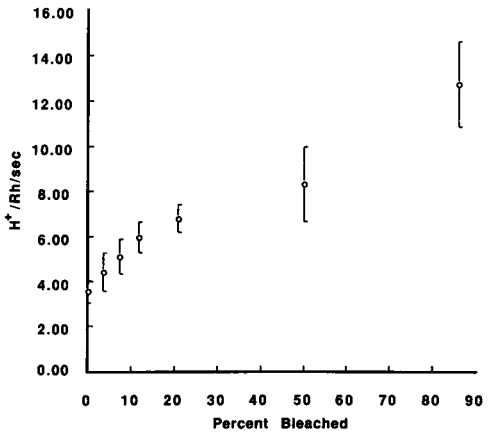

FIGURE 5 Dependence of calculated initial proton flux upon rhodopsin bleaching. The proton flux was calculated from the equation given in the discussion using the initial slopes of the fluorescence change after light exposure taken from experiments such as those shown in Fig. 3 and the relationship between fluorescence and potential shown in Fig. 2. The mean ± standard deviation is plotted for seven sets of experiments except for the data at 50% bleaching which is comprised of four experiments.

These results are all consistent with light increasing the proton permeability of the disk membrane. This suggests that one of the ways that light may modulate rod cell function is by controlling the movement of protons through the disk membrane. Protons generated by the action of the light-activated phosphodiesterase activity within the rod cell cytoplasm might enter the disk lumen via this permeability to trigger a release of calcium. Rhodopsin could thus have a dual role of both initiating the production of protons and of providing a pathway which would allow protons to act within the disks. One could speculate that rhodopsin itself might serve as a light-regulated proton pore. Changes in the proton gradient or electrical potential across the disk membrane which result from this permeability might also modulate the behavior of the disk membrane-linked

biochemical elements of the system as suggested by Bennett et al. (1980).

An earlier paper showed that light-induced calcium release from disks was more efficient in the presence of imidazole than with other buffers and was also more efficient at basic pH where imidazole would have been in its uncharged, permeant form (Smith et al., 1977). It seems likely that a sustained proton flux by the mechanism described here allowed proton-calcium exchange and thereby enhanced the light-induced calcium releases observed in that earlier work. Tyminski et al. (1982) also noted a requirement for proton permeability for optimal calcium release from rhodopsin-containing membrane vesicles.

APPENDIX A

Calculation of final equilibrium membrane potentials with permeant and impermeant buffers

We assume that initially, before light exposure, both the buffer and pH are equilibrated across the membrane. In the experiments described here the internal volume is <0.1% of the total volume; thus, the internal concentration changes occur without significant changes in the external concentrations. We also assume that the uncharged form of imidazole is always in equilibrium and that the major permeability of the membrane after addition of valinomycin is to potassium. The potassium gradient is thus the major determinant of the membrane potential.

After addition of valinomycin, the membrane potential can be calculated from the Nernst equation using the internal and external potassium concentrations. Under the experimental conditions used, the interior of the disks is at a negative potential with respect to the exterior. When a proton permeability is created, protons will move into the disks. Potassium ions will leave the disks to maintain electroneutrality. These flows will continue until a new electrochemical equilibrium is reached in which both potassium and protons are at equilibrium. The membrane potential will then be

$$\psi_f = (RT/F)\ln\{[K^+]_o/([K^+]_{i\phi} - x)\}, \quad (1)$$

where ψ_f is the final equilibrium membrane potential; $[K^+]_o$, the external potassium concentration; $[K^+]_{i\phi}$, the initial internal potassium concentration; and x, the reduction in internal potassium concentration required to maintain electroneutrality. At equilibrium, all monovalent cations must obey the same Nernst relationship; therefore,

$$[K^+]_o/[K^+]_i = [H^+]_o/[H^+]_i. \quad (2)$$

At this point both potassium and protons will be distributed in the same ratios across the membrane. As potassium is present at a much higher concentration than are protons, this redistribution can take place without significantly depleting the internal potassium concentration so long as no permeant base (such as imidazole) is present to buffer the protons that enter the disks. For example, at pH 7.5 with initial concentrations of $[K^+]_o$ = 1 mM and $[K^+]_i$ = 100 mM, protons will enter the disks to produce an internal pH of ~5.5 (a 100:1 internal/external concentration ratio) or a change in proton concentration within the disks of ~3 μM. The efflux of potassium will reduce the internal $[K^+]$ by this same concentration (x in Eq. 1). This is a negligible change in $[K^+]_i$ and there will be little change in membrane potential when no proton acceptor is present within the disks.

If a proton acceptor is present within the disks, then protons must overcome its buffering capacity before the internal proton concentration will rise to create the gradient necessary for electrochemical equilibrium. If the buffer is impermeant, the buffer capacity within the disks is finite and only a small additional movement of protons and potassium ions is needed to establish the new electrochemical equilibrium. The internal potassium concentration will be depleted by an amount approximately equal to the internal concentration of the basic form of the buffer. Thus, the final potential which would be reached at equilibrium after creation of a proton permeability in the presence of an impermeant buffer can be estimated from Eq. 3 in which x has been replaced by the initial concentration of the basic form of Hepes ($[Hep^-]_\phi$).

$$\psi_f = (RT/F)\ln\{[K^+]_o/([K^+]_{i\phi} - [Hep^-]_\phi)\}. \quad (3)$$

At pH 7.5, which is near the pKa for Hepes (7.55) ~47% of the Hepes will be in the basic form. Under the conditions of our experiments with an initial membrane potential of −120 mV, the creation of a proton permeability in the presence of 50 mM Hepes at pH 7.5 will result in a decrease in potential to ~ −113 mV.

If the basic form of the buffer is permeant, as is the case with imidazole, then entering protons will again be bound by the basic form of the buffer, but the proton flux will continue as additional imidazole enters the disk maintaining equilibrium of the permeant form. Here the buffering capacity within the disks is no longer limited by the small internal volume, and the final electrochemical equilibrium includes imidazole/imidazolium equilibria on either side of the membrane as well as the transmembrane gradients of potassium and protons.

Most of the protons entering the disks will be bound by imidazole (Im) to form imidazolium ions (Im^+). There will be an efflux of potassium of the same magnitude as the proton influx in order to maintain electroneutrality. Thus,

$$[K^+]_{i\phi} - [K^+]_{if} = [Im^+]_{if} - [Im^+]_{i\phi},$$
$$\text{or } [K^+]_{i\phi} + [Im^+]_{i\phi} = [K^+]_{if} + [Im^+]_{if}, \quad (4)$$

where the terms all refer to concentrations within the disks (subscript i) and the second subscripts, f and ϕ, refer to final and initial conditions respectively. As the internal volume is small with respect to the external volume and the initial conditions are defined as being at equilibrium in terms of imidazole and pH, $[Im^+]_{i\phi}$ can be replaced with $[Im^+]_o$ the external imidazolium concentration. Using the equation for the imidazole dissociation constant (K_a) to substitute for $[Im^+]_{if}$, one obtains

$$[K^+]_{i\phi} + [Im^+]_o = [K^+]_{if} + [H^+]_{if}[Im]_o/(K_a). \quad (5)$$

As imidazole is assumed to be freely permeant, the internal and external concentrations are the same. From the Nernstian equilibrium condition, Eq. 2, one can now substitute $[H^+]_{of}[K^+]_{if}/[K^+]_{of}$ for $[H^+]_{if}$ which gives

$$[K^+]_{i\phi} + [Im^+]_o = [K^+]_{if} + [H^+]_{of}[K^+]_{if}[Im]_o/[K^+]_{of}(K_a). \quad (6)$$

Again, because the external concentrations do not change significantly, $[H^+]_{of} = [H^+]_o$; therefore, $[H^+]_{of}[Im]_o/(K_a) = [Im^+]_o$. Making

these substitutions and rearranging terms gives

$$[K^+]_{i\phi} + [Im^+]_o = [K^+]_{if} + [K^+]_{if}[Im^+]_o/[K^+]_{of}$$
$$\text{or, } [K^+]_{i\phi} + [Im^+]_o = ([K^+]_{of} + [Im^+]_o)([K^+]_{if}/[K^+]_{of})$$
$$\text{or, } [K^+]_{of}/[K^+]_{if} = ([K^+]_{of} + [Im^+]_o)/([K^+]_{i\phi} + [Im^+]_o). \quad (7)$$

Because the final external potassium concentration is the same as the initial external potassium concentration, one can substitute $[K^+]_o$ for $[K^+]_{of}$, and because $\psi_f = (RT/F)\ln([K^+]_{of}/[K^+]_{if})$, the expression for the final membrane potential is

$$\psi_f = (RT/F)\ln\{([K^+]_o + [Im^+]_o)/([K^+]_{i\phi} + [Im^+]_o)\}, \quad (8)$$

where ψ_f is the final equilibrium membrane potential; $[K^+]_o$, the external potassium concentration; $[K^+]_{i\phi}$, the initial internal potassium concentration; and $[Im^+]_o$, the external imidazolium concentration. Under the conditions of our experiments with an initial membrane potential of −120 mV, the creation of a proton permeability in the presence of 50 mM imidazole at pH 7.5 (~12 mM imidazolium) is calculated to result in a decrease in potential to −56 mV.

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