Online Supplemental Material for

Cherney et al. http://www.jgp.org/cgi/doi/10.1085/jgp.200308813

Effect of Proton Depletion/Accumulation on Estimates of γ_H and N Obtained from y-g$_H$ Plots

It is self-evident that H^+ current changes pH. In the whole-cell configuration, these pH changes can be large and slowly reversible, even with 100 mM buffer in the pipette solution. The present experiments were done on excised, inside-out patches in order to minimize pH changes. Here we evaluate the magnitude of any pH changes under the conditions of this study, and their effects on estimates of γ_H and N. Two types of measurements are affected. First, any pH change during the noise measurement itself will directly alter the result. Second, pH changes during the tail current measurements used to estimate V_{rev} will also affect the outcome. Here we show that systematic errors in γ_H and N are relatively small under the conditions of this study.

Proton Depletion and Accumulation in the Steady-state

During gigaseal formation, part of the cell membrane is drawn into the pipette, so that after excision the patch lies some distance from the tip. Outward proton current under voltage clamp leads to a depletion of protons at the intracellular side of the patch and an accumulation at the extracellular side. First, consider the depletion of protons at the intracellular side of the patch under steady-state conditions. Because the proton concentration, $[H^+]$, is miniscule compared to the concentration of protonated buffer, $[BH]$, we assume that I_H is sustained almost entirely by the diffusion of BH from the bath to the membrane patch. And since in the steady state the diffusive flux of BH is balanced by an equal and opposite electrodiffusive flux of deprotonated buffer, B^-, we have:

$$I_H = -\pi r^2 F D_{BH} \frac{d[BH]}{dx} = \pi r^2 F D_{B^-} \left(\frac{d[B^-]}{dx} - \frac{F[B^-]}{RT}\frac{dV}{dx} \right),$$
(A1)

where r is the internal pipette radius at some distance x from the tip, D_{BH} and D_{B^-} are diffusion coefficients, and F, R, and T have their usual meanings. We assume that activity coefficients are concentration independent (Schultz, 1980). Solving Eq. A1 for $[BH]$ and $[B^-]$ gives:

$$[\mathrm{BH}] = [\mathrm{BH}]_0 - \frac{I_\mathrm{H}}{\pi F D_\mathrm{BH}} \int_0^x \frac{x \, \mathrm{d}x}{r^2} \; , \tag{A2}$$

$$[\mathrm{B}^-] = [\mathrm{B}^-]_0 + \frac{I_\mathrm{H}}{\pi F D_{\mathrm{B}^-}} \int_0^x \frac{x \, \mathrm{d}x}{r^2} + \frac{F}{RT} \int_0^x [\mathrm{B}^-] \left(\frac{\mathrm{d}V}{\mathrm{d}x} \right) \mathrm{d}x \; , \tag{A3}$$

where $[\mathrm{BH}]_0$ and $[\mathrm{B}^-]_0$ are the bath concentrations of protonated and deprotonated buffer, respectively. $[\mathrm{BH}]$ and $[\mathrm{B}^-]$ are related by the buffer dissociation constant:

$$K_a = \frac{[\mathrm{H}^+][\mathrm{B}^-]}{[\mathrm{BH}]} \tag{A4}$$

whence the degree of dissociation (α) is given by

$$\alpha = \frac{[\mathrm{B}^-]}{[\mathrm{B}_{\mathrm{total}}]} = \frac{K_a}{K_a + [\mathrm{H}^+]} \; , \tag{A5}$$

where $[\mathrm{B}_{\mathrm{total}}] = [\mathrm{BH}] + [\mathrm{B}^-]$ is the total buffer concentration.

To simplify matters, suppose that $D_{\mathrm{BH}} = D_{\mathrm{B}^-} = D_\mathrm{B}$ and assume the pipette can be approximated as the frustum of a cone, with r_0 the internal pipette-tip radius, r_p the patch radius, and b the internal shank taper ($= \mathrm{d}r/\mathrm{d}x$). Then on defining a parameter

$$\beta_i = \frac{1}{\pi F D_\mathrm{B} [\mathrm{B}_{\mathrm{total}}]_0} \int_0^p \frac{\mathrm{d}x}{r^2} = \frac{1}{\pi b F D_\mathrm{B} [\mathrm{B}_{\mathrm{total}}]_0} \left(\frac{1}{r_0} - \frac{1}{r_p} \right) \; , \tag{A6}$$

where p is the distance of the patch from the pipette tip, Eq. A2 can be written

$$[\mathrm{BH}]_i = [\mathrm{B}_{\mathrm{total}}]_0 \left(1 - \alpha_0 - \beta_i I_\mathrm{H} \right) \tag{A7}$$

at $x = p$, where "i" signifies the intracellular side of the patch and "0" signifies the bath. Furthermore, the second integral in Eq. A3 can be written

$$\int_0^x [\mathrm{B}^-] \left(\frac{\mathrm{d}V}{\mathrm{d}x} \right) \mathrm{d}x = \int_{V_0}^{V_x} [\mathrm{B}^-] \, \mathrm{d}V = \overline{[\mathrm{B}^-]}(V_x - V_0) \; , \tag{A8}$$

where V_0 is the electrical potential in the bath, V_x is the electrical potential at x, and $\overline{[\mathrm{B}^-]}$ is a mean concentration in the interval $[0,x]$. Hence, Eq. A3 becomes

$$[\mathrm{B}^-]_i = [\mathrm{B}_{\mathrm{total}}]_0 \left[\alpha_0 + \beta_i I_\mathrm{H} + \frac{F \overline{[\mathrm{B}^-]}}{RT [\mathrm{B}_{\mathrm{total}}]_0} (V_p - V_0) \right] \; , \tag{A9}$$

at $x = p$. Substituting Eqs. A7 and A9 into Eq. A4 and solving for $[H^+]$ gives the proton concentration adjacent to the intracellular side of the patch:

$$[H^+]_i = K_{a,i}\left(\frac{1-\alpha_0-\beta_i I_H}{\alpha_0+\beta_i I_H}\right)\left[1+\frac{F\overline{[B^-]}(V_p-V_0)}{RT[B_{total}]_0(\alpha_0+\beta_i I_H)}\right]^{-1}.$$ (A10)

Removal of protons from the intracellular side ensures that $V_p < V_0$ so that from Eq. A9 $\overline{[B^-]} < [B_{total}]_0(\alpha_0+\beta I_H)$ provided $[B^-]_0 \le [B^-] \le [B^-]_i$. It then follows that,

$$\left|\frac{F\overline{[B^-]}(V_p-V_0)}{RT[B_{total}]_0(\alpha_0+\beta_i I_H)}\right| < \left|\frac{F(V_p-V_0)}{RT}\right| < \left|\frac{FI_H R_{pip}}{RT}\right|,$$ (A11)

where R_{pip} is the pipette resistance. The second inequality in Eq. A11 arises from the fact that $I_{B^-} = I_H$ is driven partly by the gradient of $[B^-]$ (Eq. A1) and so $|V_p-V_0| < |I_H R_{pip}|$. (Also the distributed resistance of the pipette between the tip and the patch membrane is less than R_{pip}.) Since I_H was generally <50 pA and R_{pip} < 15 MΩ, $FI_H R_{pip}/RT < 0.03$. Hence, from Eq. A11, the term in square brackets in Eq. A10 will be close to unity, and so the expression for $[H^+]_i$ reduces to

$$[H^+]_i = K_{a,i}\left(\frac{1-\alpha_0-\beta_i I_H}{\alpha_0+\beta_i I_H}\right).$$ (A12)

Exactly analogous arguments yield the following expression for the proton concentration at the "extracellular" ("o") side of the membrane patch, where protons accumulate:

$$[H^+]_o = K_{a,o}\left(\frac{1-\alpha_0+\beta_o I_H}{\alpha_0-\beta_o I_H}\right),$$ (A13)

where

$$\beta_o = \frac{1}{\pi F D_B[B_{total}]_\bullet}\int_p^\bullet \frac{dx}{r^2} = \frac{1}{\pi b F D_B[B_{total}]_\bullet r_p}$$ (A14)

and "•" signifies the pipette-stem lumen.

In order to evaluate β_i and β_o we need an expression for r_p. This is obtained by considering the membrane in the pipette shank (i.e., the patch membrane plus the membrane adhering to the glass between $x = 0$ and $x = p$), the area of which must be some fraction ($q < 1$) of the original cell membrane area. Thus, since $b = dr/dx = (r_p - r_0)/p$, we have

$$\pi p(r_p+r_0)+\pi r_p^2 = \pi(r_p-r_0)(r_p+r_0)b^{-1}+\pi r_p^2 = 4q\pi r_c^2,$$ (A15)

whence,

$$r_{\text{p}} = \sqrt{\frac{4\,b\,q\,r_{\text{c}}^2 + r_0^2}{1+b}} \quad, \tag{A16}$$

where r_{c} is the radius of the cell from which the patch was excised.

Effect of Proton Depletion/accumulation on y-g*H plots*

To estimate the effects of proton depletion/accumulation on y-g_{H} plots, the following equations (Eqs. A17 and A18) were solved numerically for I_{H} using a modified linear interpolation algorithm (Gerald and Wheatley, 1984):

$$E_{\text{H}} = \frac{RT}{F} \log \frac{[H^+]_{\text{o}}}{[H^+]_{\text{i}}} = \frac{RT}{F} \log \frac{K_{\text{a,o}}}{K_{\text{a,i}}} \left(\frac{1 - \alpha_0 + \beta_{\text{o}} I_{\text{H}}}{\alpha_0 - \beta_{\text{o}} I_{\text{H}}} \right) \left(\frac{\alpha_0 + \beta_{\text{i}} I_{\text{H}}}{1 - \alpha_0 - \beta_{\text{i}} I_{\text{H}}} \right), \tag{A17}$$

$$I_{\text{H}} = \gamma_{\text{H}} N P_{\text{open}} (V - E_{\text{H}}), \tag{A18}$$

where E_{H} is the Nernst potential for protons. α_0 was calculated from Eq. A5 with $[H^+] = [H^+]_0$, while β_{i} and β_{o} were calculated from Eqs. A6, A14, and A16, given assumed values for D_{B}, $[B_{\text{total}}]$, b, q, r_{c} and r_0 (see below). $P_{\text{open}}(V)$ was approximated by a Boltzmann function

$$P_{\text{open}} = \frac{P_{\text{max}}}{1 + e^{-(V - V_{1/2})/k}} \quad. \tag{A19}$$

Values of P_{max} were taken from Table 1, while values of V_{-} and k were obtained by fitting the function

$$I = \frac{g_{\text{H,max}}(V - V_{\text{rev}})}{1 + e^{-(V - V_{1/2})/k}} \tag{A20}$$

to stationary I-V plots, where V_{rev} is an estimate of E_{H} derived from tail currents (see below). The mean value of k was 6.85 ± 0.68 mV ($n = 11$). Consistent with previous studies (DeCoursey and Cherny, 1997; DeCoursey, 2003), $V_{1/2}$ was a linear function of V_{rev}: $V_{1/2} = (45.5 \pm 6.0) + (0.840 \pm 0.075)V_{\text{rev}}$. The dependence of γ_{H} on $[H^+]_{\text{i}}$ was estimated by interpolating the observed γ_{H} values in Table I. Other relevant data are as follows: The molecular weights of the buffers were in the range 200-300, and so from Hobbie (1978) the Stokes radius should be around 0.45 nm. The Stokes-Einstein relation then gives $D_{\text{B}} = 5 \times 10^{-10}$ m^2 s^{-1}. From measurements on seven pipettes, the external shank taper was around 0.1 and the external tip radius was 1 μm. Then assuming that the nominal internal/external diameter ratio of 0.7 is maintained within the shank, we get $b = 0.07$ and $r_0 = 0.7$ μm. Possibly some pipettes had a smaller tip opening, but given an expected pipette

resistance of 1–5 MΩ when filled with 150 mM KCl (Sakmann and Neher, 1983), it seems unlikely that r_0 was much less than 0.5 µm. For most types of glass the internal/external diameter ratio increases near the tip (Purves, 1981), so if anything r_0 has been underestimated. Symmetrical buffer concentrations were used so that $[B_{total}]_0 = [B_{total}]_{\bullet} = [B_{total}] = 100\text{-}200$ mM. The cell radius (r_c) was about 4 µm and the fraction of the cell membrane (q) drawn into the pipette was varied from 0.25 to 0.75. This range of values is somewhat arbitrary, but the form of predicted y-g_H curves does not depend strongly on q. The reason for this is that as the patch approaches the pipette tip (small q) intracellular H^+ depletion decreases but extracellular accumulation increases. Conversely as the patch moves away from the tip (large q) intracellular H^+ depletion increases but extracellular accumulation decreases. Hencem the ratio $[H^+]_o/[H^+]_i$ (and therefore E_H) does not vary greatly with the distance of the patch membrane from the pipette tip.

Having determined I_H for a particular V, σ_H^2 was calculated from the relation,

$$\sigma_H^2 = \gamma_H (V - E_H) I_H - I_H^2 / N \tag{A21}$$

(Sigworth, 1980). The generation of y-g_H plots also requires an estimate (V_{rev}) of E_H (Eqs. 2 and 3), which is obtained from the analysis of tail currents, where possible (Materials and Methods). V_{rev} was calculated assuming a prepulse to 80 mV above the zero-current E_H (i.e., E_H in the absence of proton depletion/accumulation). I_H and E_H during the prepulse were then obtained by solving Eqs. A17 and A18 as before, except that I_H was assumed to reach 50–80% of its stationary value at the end of the prepulse (varying I_H within this range had little effect on y-g_H plots). The value of E_H at that time was set equal to V_{rev} in Eqs. 4 and 5 in order to obtain values of y and g_H. This approach assumes the establishment of a near-steady state by the end of the prepulse; the validity of this assumption is discussed below.

Simulated y-g_H plots for the 7.5//5.5 and 7.5//6.5 pH regimes are shown in Fig. S1. Values of N were chosen to obtain average and extreme values of g_H (as determined experimentally). For each g_H class the solid line represents no H^+ depletion/accumulation, the dashed curve represents the minimum expected depletion/accumulation ($[B_{total}] = 200$ mM, $r_0 = 0.7$ µm, $q = 0.25$, prepulse I_H to 50% of its stationary value), and the dashed-dotted curve represents the maximum expected depletion/accumulation ($[B_{total}] = 100$ mM, $r_0 = 0.5$ µm, $q = 0.75$, prepulse I_H to 80% of its stationary value). The plots are nearly linear over most of the range of g_H values; certainly the predicted deviations from linearity could not be detected in the experimental data given its scatter.

To quantify the errors in estimates of γ_H and N, regression lines were fitted to the middle one-third of the y-g_H curves. γ_H was then estimated as the y-intercept and N as -1/slope of the fitted lines. For average values of g_H, γ_H was overestimated by 1-4% while N was underestimated by 2-10%. For extreme values of g_H, γ_H was overestimated by 2-8% while N was underestimated by 6-20%. Similar results were obtained for regimes 7.5//5.0 and 6.5//6.5 (not depicted). Hence, the analysis of simulated y-g_H plots suggests that the errors in γ_H and N due to proton depletion/accumulation are relatively small and essentially independent of pH_i and pH_o.

Rate of Establishment of a Steady-state during I_H Activation

Some idea of the time required to establish a steady pH at the patch can be obtained by modeling the pipette-shank lumen for $0 < x < p$ as a well-stirred compartment of volume V, separated from the bath by a diffusive resistance of effective area πr_0^2 and permeability P_s. The time constant (τ_s) for the establishment of a steady state is then:

$$\tau_s = \frac{V}{\pi r_0^2 P_s} \quad . \tag{A22}$$

Substituting Eq. A6 into A7 and solving for I_H/F suggests taking

$$\pi r_0^2 P_s > \pi b D_B \left(\frac{r_0 r_p}{r_p - r_0} \right) . \tag{A23}$$

Furthermore,

$$V < \frac{\pi p}{3} \left(r_0^2 + r_p^2 + r_0 r_p \right) = \frac{\pi \left(r_p - r_0 \right)}{3b} \left(r_0^2 + r_p^2 + r_0 r_p \right) . \tag{A24}$$

Hence given Eq. A16 for r_p, τ_s can be expressed as a function of $D_B = 5 \times 10^{-10}$ m^2 s^{-1}, $b = 0.07$, $r_0 = 0.5$–0.7 μm, $q = 0.25$–0.75 and $r_c = 4$ μm, giving $\tau_s = 0.1$–1.2 s. A small transient "droop" in I_H was sometimes observed during the first few seconds of depolarizing pulses that elicited large currents at $pH_i = 5.0$ (Fig. 2, top panel). Although this calculation is somewhat crude, it seems clear that proton flux–induced pH changes were close to a steady state in inside-out patches during noise measurements (≥ 12 s records). Assumption of a steady state will lead to overestimation of the errors in γ_H and N in simulated y-g_H plots. That this is a small effect can be appreciated from the fact that if proton depletion/accumulation during the prepulse is ignored, the y-g_H curves intercept the y axis at the true value of γ_H (reducing an already small error to zero), but with little change in slope (and hence little change in the estimate of N).

References

Gerald C.F., and P.O. Wheatley. 1984. Applied Numerical Analysis. Third edition. Addison-Wesley, Reading, MA. 579 pp.

Hobbie, R.K. 1978. Intermediate Physics for Medicine and Biology. John Wiley and Sons, New York, NY. 557 pp.

Purves, R.D. 1981. Microelectrode Methods for Intracellular Recording and Ionophoresis. Academic Press, London. 146 pp.

Sakmann, B., and E. Neher. 1983. Geometric parameters of pipettes and membrane patches. *In*: Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 37–51.

Schultz, S.G. 1980. Basic Principles of Membrane Transport. Cambridge University Press, Cambridge. 144 pp.

Sigworth, F.J. 1980. The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol.* 307:97–129.

A

7.5//5.5

Extreme

Average

B

7.5//6.5

Extreme

Average

g_H (pS)

y (fS)