# $I_K$ inactivation in squid axons is shifted along the voltage axis by changes in the intracellular pH

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ABSTRACT   The inactivation curve of the delayed rectifier in internally perfused squid giant axons is shifted along the voltage axis by changes in the pH of the internal perfusate. The amplitude of the shift is 9.5 mV per pH unit ($6 \leq pH_i \leq 10$). No saturation of the effect was observed at either end of the pH range. This result suggests that the inactivation gating mechanism has several titratable groups accessible to protons from the intracellular side of the membrane.

## INTRODUCTION

The effects of hydrogen ions on excitable cells is a topic of long standing interest. For example, changes in the pH alter the properties of ionic currents underlying excitability, including the sodium, potassium, and calcium ion current components (Hille, 1968, 1973; Woodhull, 1973; Wanke et al., 1980; Shrager, 1974; Iijima et al., 1986; Prod'hom et al., 1987). Moreover, almost all cellular processes, including excitability, are sensitive to changes in pH, which may explain the diversity of mechanisms that regulate the intracellular pH (Roos and Boron, 1981).

The purpose of this article is to revisit the effects of changes in the intracellular pH ($pH_i$) on the delayed rectifier potassium ion current, $I_K$, from internally perfused squid giant axons. A little over 10 years ago, Wanke et al. (1979) reported that this current was reversibly reduced by a reduction in $pH_i$ in the 5.2–10 pH range without a significant alteration of $I_K$ kinetics. During experiments on an unrelated topic, I noticed that this effect was dependent upon holding potential ($HP$). Specifically, I observed a lack of effect of $pH_i$ on $I_K$ with $HP \leq -80$ mV or $HP \geq -15$ mV, whereas a clear effect was observed with $-70 < HP < -20$ mV. These results are consistent with a $pH_i$-dependent shift of the inactivation curve of the delayed rectifier along the voltage axis, as described below. Surprisingly, a plot of the voltage at the midpoint of the inactivation curve as a function of $pH_i$ ($6 \leq pH_i \leq 10$) did not reveal saturation of the effect at either end of this pH range. This result suggests that the component of the $I_K$ channel which is responsible for inactivation may have several titratable groups (Discussion).

A brief report of these results has been given (Clay, 1990).

## METHODS

Experiments were performed on voltage-clamped, internally perfused squid giant axons using methods that have been previously described (Clay and Shlesinger, 1983). The temperature in these experiments ranged between 8 and 12°C. In any single preparation it was maintained constant to within 0.1°C. The external artificial seawater solution contained either 10, 50, 100, or 150 mM KCl with, respectively, 430, 390, 340, or 290 mM NaCl. It also contained 50 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM Tris-HCl, and 0.5–1 $\mu$M tetrodotoxin (TTX). The internal perfusate contained 300 mM $K^+$ and, in most experiments, $F^-$ as the major anion, with either glutamate (pH 9–10), phosphate (pH 6.8–8), or citrate (pH 6–6.8) as the buffer at a concentration of 50 mM. The pH in these solutions was adjusted downward with, respectively, free glutamic acid, phosphoric acid, or citric acid. Upward adjustments in pH were made with KOH with appropriate modifications in the potassium ion concentration in the initial preparation of these solutions. The specialized buffers such as Cyclohexylaminopropane sulfonic acid were not used, following the lead of Wanke et al. (1979), who found that these agents produced irreversible alterations of $I_K$. In some experiments glutamate, rather than $F^-$, was used as the major anion. Similar results were obtained with either of these conditions, as noted previously (Clay, 1988). The effects of external pH ($pH_o$) on $I_K$ were carried out with $pH_i = 7$ and $pH_o = 5.8$ or 7.5. The external solution in these experiments contained 430 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 1 $\mu$M TTX, and 10 mM Bis-Tris[bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane]. The pH was adjusted with HCl. Liquid junction potentials for all experimental conditions were <3 mV. The results in this study have not been corrected for these relatively small voltage offsets.

## RESULTS

The $I_K$ component was significantly increased by an increase in the pH of the internal perfusate. This change occurred rapidly, reaching steady state usually within 1–2

min, and it was readily reversible, also within 1–2 min. An example of the effect with a change in $pH_i$ from 6 to 9 is illustrated in Fig. 1, $A$ and $B$, respectively, with 20 ms duration voltage clamp steps to $-20$, 0, 20, and 40 mV from a holding potential of $-50$ mV. The records for $pH_i$ 6 were scaled upward by a factor of 2.3 (same factor for all four records) and superimposed upon the $pH_i$ 9 results in Fig. 1 $C$. This comparison shows very little effect of the pH change upon the activation kinetics. (The slight discrepancy at $+40$ mV could be due to a relatively greater potassium ion accumulation in the pH 9 record, as compared with the pH 6 record.) These results are similar to those of Wanke et al. (1979). The holding potential dependence of the effect, which is original to this report, is illustrated in Fig. 2. All four panels in Fig. 2 contain $I_K$ records for 20 ms duration steps to $-40$, $-20$, ..., $+40$ mV with a holding potential of $-90$ mV for Fig. 2, $A$ and $C$, and $-50$ mV for Fig. 2, $B$ and $D$. The term holding potential in this study implies a steady-state condition. That is, the holding potential in all of these results was maintained for 3 min, or longer, after a change in this level, before voltage clamp steps were made. (3 min was sufficiently long for the inactivation process to reach steady state, based on the results in Fig. 5 of Clay [1989].) The results in Fig. 2, $A$ and $C$, illustrate a lack of effect of a change in $pH_i$ from 7 to 7.7 with $HP = -90$ mV, whereas a significant effect occurred with $HP = -50$ mV. Specifically, the current was reduced after the change in $pH_i$ from 7.7 to 7 with $HP = -50$ mV. Moreover, both sets of records in Fig. 2 with $HP = -50$ mV show a reduction of $I_K$ relative to $HP = -90$ mV, which is attributable to $I_K$ inactivation (Ehrenstein and
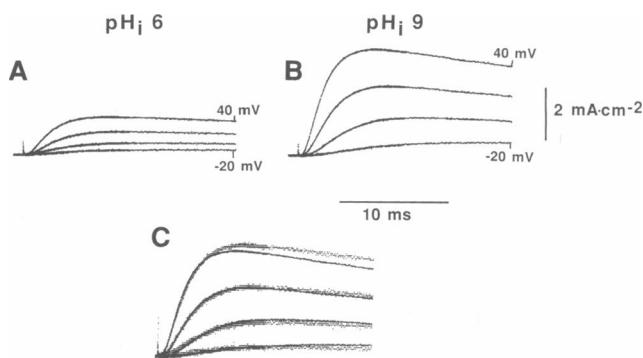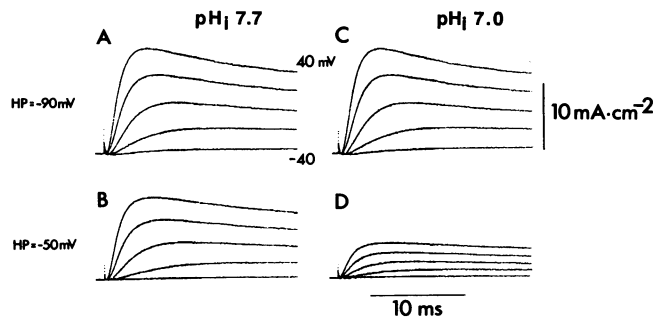


FIGURE 2  Holding potential dependence of the $pH_i$ effect. The membrane potential was initially held in this experiment at $-90$ mV with $pH_i = 7.7$. Voltage clamp steps 20 ms in duration were made to $-40$, $-20$, 0, 20, and 40 mV with a 3 s rest interval between each step (panel $A$). The holding potential was then changed to $-50$ mV. 3 min later voltage clamp steps 20 ms in duration were applied to $-40$, ..., $+40$ mV, as in $A$ (panel $B$). The pH of the internal perfusate was then changed to 7.0. 5 min later the same clamp protocol as in $A$ and $B$ was applied (panel $D$). The holding potential was then returned to $-90$ mV. 3 min later the same sequence of steps as in $A$, $B$, and $D$ was once again applied (panel $C$). All records corrected for leakage current. $K_o = 10$ mM.

Gilbert, 1966; Chabala, 1984; Clay, 1989). Consequently, these results suggest that $I_K$ inactivation is the process which is modified by $pH_i$.

The results given in Fig. 1 and 2 were obtained with holding potentials $\leq -50$ mV. An increasingly significant degree of activation of $I_K$ in the steady state occurs as the holding potential is depolarized relative to $-50$ mV. Consequently, the external potassium ion concentration was elevated ($K_o = 50$–$150$ mM) to minimize the current during the relatively long times for which the potential was held at these levels in these experiments. I have previously shown that this modification in the external solution does not alter either activation or inactivation kinetics (Clay, 1984, 1989). Three examples of experiments with elevated $K_o$ are shown in Fig. 3. In Fig. 3 $A$ the holding potential was $-80$ mV with $pH_i = 6$ or 9 ($K_o = 150$ mM). The potential was stepped for 20 ms to $-10$ mV followed by steps to $+40$, 10, $-20$, $-50$, and $-80$ mV. Relatively little net current was present either at the holding potential, $-80$ mV, which is below the range of activation of $I_K$, or during the prepulse to $-10$ mV. Activation of $I_K$ channels does occur at $-10$ mV, but the driving force is virtually nil, because this potential is close to $E_K$ ($E_K \sim -15$ mV with $K_o = 150$ mM and $K_i = 300$ mM). The degree of $I_K$ activation during the prepulse was determined by stepping to $-80$, $-50$, ..., and $+40$ mV and measuring the current 100 $\mu s$ after this second step of the voltage clamp sequence. (A rest interval of 3 s was used between each two step protocol.) These results are plotted in the right hand panel of Fig. 3 $A$. They further show a lack of effect of a change in $pH_i$
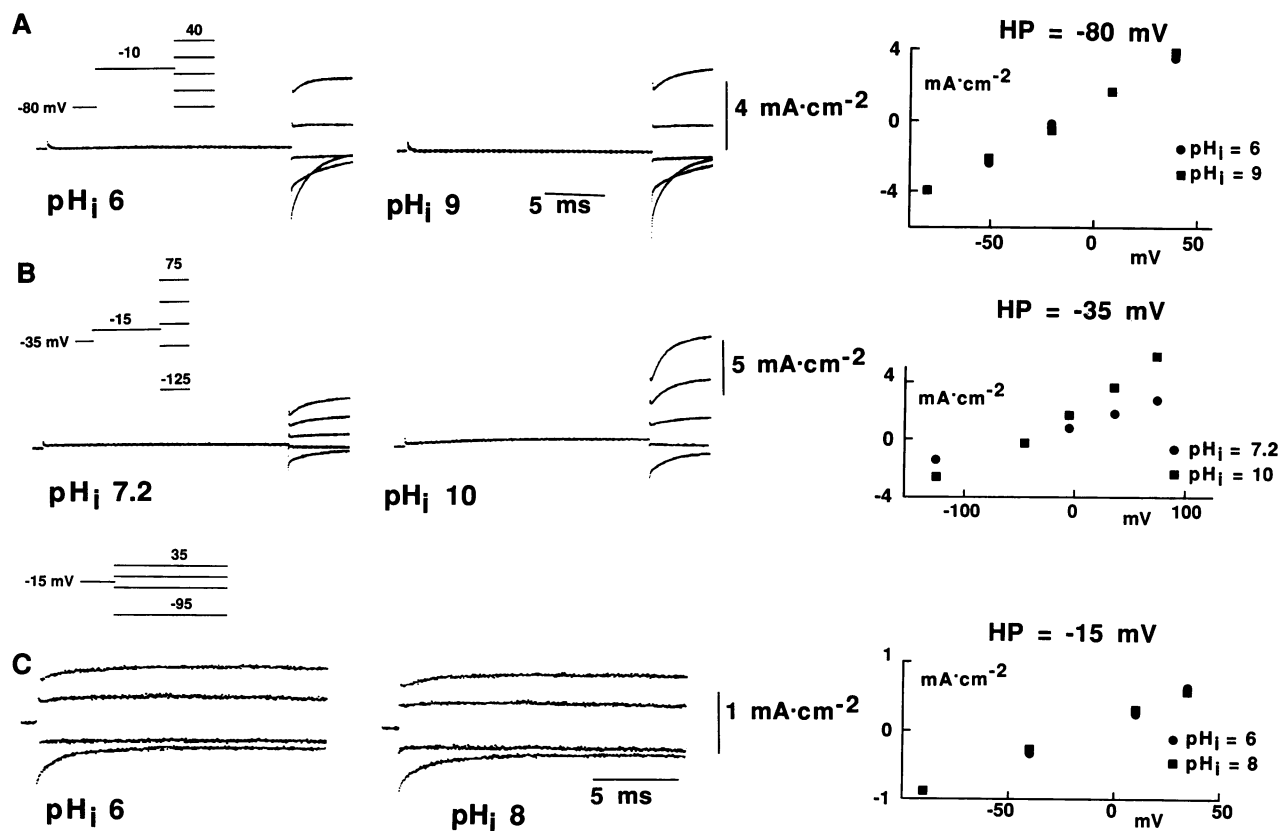


FIGURE 1  Effect of a change in pH from 6 to 9 in the internal perfusate on $I_K$. ($A$) Membrane current records in response to 20 ms duration voltage clamp steps to $-20$, 0, 20, and 40 mV applied sequentially with a 3 s rest interval between each step. Holding potential $= -50$ mV; $pH_i = 6$; $K_o = 10$ mM. The leakage current was subtracted from these results. ($B$) Membrane current records using the same protocol as in $A$ with $pH_i = 9$. ($C$) Superposition of the results in $A$ and $B$ in which each record in $A$ was scaled upward by a factor of 2.3.
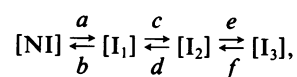
FIGURE 3    Holding potential dependence of the $pH_i$ effect in elevated $K_o$. (*A*) Membrane currents from a holding potential of $-80$ mV with a prepulse to $-10$ mV followed by steps to 40, 10, $-20$, $-50$, and $-80$ mV with 3 s rest interval between each of these two pulse sequences. $K_o = 150$ mM. The same clamp sequence was applied with $pH_i = 6$ and $pH_i = 9$. The current-voltage plots on the right were taken 100 $\mu$s after the second pulse. These results show a lack of effect of $pH_i$ on $I_K$ with $HP = -80$ mV. (*B*) Similar protocol as in *A* with a holding potential of $-35$ mV, a prepulse potential of $-15$ mV followed by steps to 75, 35, $-5$, $-45$, and $-125$ mV with $pH_i = 7.2$ or 10. $K_o = 50$ mM. The current-voltage plots for these results are shown to the right. These results show a significant effect of $pH_i$ on $I_K$ with $HP = -35$ mV. (*C*) Lack of effect of $pH_i$ on the noninactivating $I_K$ component. $K_o = 150$ mM. These results were obtained with $HP = -15$ mV and steps to 35, 5, $-25$, and $-95$ mV with $pH_i = 6$ or 8. The current-voltage plots to the right were measured 100 $\mu$s after the voltage steps from the holding potential.

on $I_K$ with $HP = -80$ mV. (The results in Fig. 3 *A* also show a lack of effect of $pH_i$ on deactivation [tail] current kinetics.) Fig. 3 *B* illustrates a similar experiment from another preparation with $HP = -35$ mV, $K_o = 50$ mM, and $pH_i =$ either 7.2 or 10. The prepulse potential in this experiment was $-15$ mV. These results show a clear effect of $pH_i$ on $I_K$. This observation is not attributable to an effect of $pH_i$ on activation kinetics during the prepulse. The activation process is unaffected by $pH_i$ (Fig. 1). Rather, the results in Fig. 3 *B* are attributable to an effect of $pH_i$ on the relative number of activatable channels in the steady-state at the holding potential ($-35$ mV). The results in Fig. 3 *B* further illustrate a lack of effect of $pH_i$ on tail current kinetics. The third experiment in Fig. 3 was carried out with $HP = -15$ mM, $K_o = 150$ mM, and $pH_i =$ either 6 or 8 (Fig. 3 *C*). This holding potential ($-15$ mV) lies positive to the range of voltage dependence

of the inactivation process (Clay, 1989). Consequently, the $I_K$ conductance which remains under these conditions is the noninactivating $I_K$ component, which is unaffected by $pH_i$, as shown by the results in Fig. 3 *C*. The holding potential dependence of $I_K$ as a function of pH is further illustrated in Fig. 4, in which the conductance obtained by the method illustrated in Fig. 3 is plotted vs. holding potential for $pH_i$ 7.1 and 9.0. ($K_o$ in both of these experiments was 100 mM.) The curves in Fig. 4 were determined from the model of inactivation given in Clay (1989), which contains three sequential inactivated states. That is,

$$[\text{NI}] \underset{b}{\overset{a}{\rightleftarrows}} [\text{I}_1] \underset{d}{\overset{c}{\rightleftarrows}} [\text{I}_2] \underset{f}{\overset{e}{\rightleftarrows}} [\text{I}_3],$$

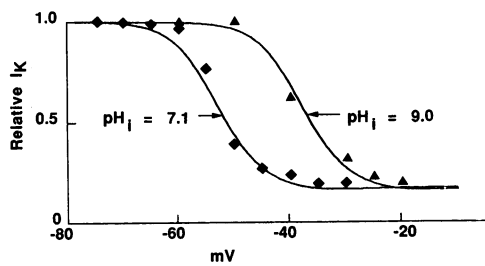where NI is the noninactivated state (either the open state

FIGURE 4 Steady-state inactivation curves from two different axons measured using the protocol described in Fig. 3. $K_o = 100$ mM for both experiments. The $pH_i$ in one experiment was 7.1; it was 9.0 in the other. The curves correspond to $[1 + a/b + ac/(bd) + ace/(bdf)]^{-1}$, where $a = \{1 + \exp[-(V + V_o + 50)/5]\}^{-1}$ $s^{-1}$, $b = 1.75\{1 + \exp[-(V + V_o + 65)/10]\}$ $s^{-1}$, $c = 1 + \exp[-(V + V_o + 57)/10]$ $s^{-1}$, $d = 2\{1 + \exp[-(V + V_o + 50)/15]\}$ $s^{-1}$, $e = 0.2\{1 + \exp[-(V + V_o + 35)/8]\}$ $s^{-1}$, and $f = 0.015\{1 + \exp[-(V + V_o + 40)/5]\}$ $s^{-1}$, with $V_o = 0$ for $pH_i$ 7.2 and $V_o = -16$ mV for $pH_i = 9.0$.

or any one of the closed states of the channel) and the rate constants $a, b, \ldots, f$ are as given in the legend of Fig. 4. The steady-state probability that the conductance is not inactivated is given by $p_{NI} = [1 + a/b + ac/(bd) + ace/(bdf)]^{-1}$. this function is plotted in Fig. 4. The two curves in Fig. 4 are identical except for a voltage shift, which is a reasonable description of the $pH_i$ effect.

The midpoint of the inactivation curve as a function of $pH_i$ is plotted in Fig. 5 for $6 \leq pH_i \leq 10$. The solid line in Fig. 5 is a best fit to these data. The slope of this line is $\sim 9.5$ mV per pH unit. Surprisingly, no saturation of the shift is apparent in these results, which would be expected if the effect were attributable to the binding of protons to a single, titratable group on the $I_K$ channel (Discussion). These results cannot be readily extended to a broader range of $pH_i$ because the leakage current was significantly
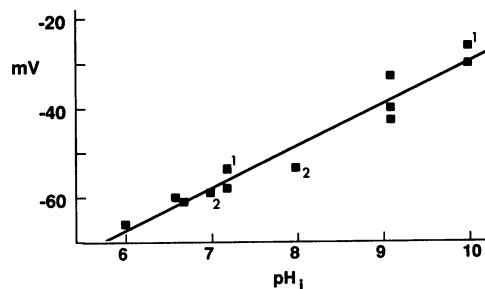


FIGURE 5 Dependence of the midpoint of the $I_K$ inactivation curve on $pH_i$ determined from the protocol illustrated in Fig. 3. Each data point is taken from a different axon, except for the results labeled 1 and 2 in which the inactivation curve was measured in one axon at two different values of $pH_i$. The straight line is a least squares best fit to these results with a slope of 9.5 mV per pH unit. ($K_o = 50$–$150$ mM).

and irreversibly increased when $pH_i$ was increased beyond 10.0 or decreased below 5.8.

Results similar to those in Figs. 2 and 3 were obtained in a total of 22 axons in which a holding potential dependence of $pH_i$ on $I_K$ was systematically investigated.

In two other experiments the effect of a change in the external pH ($pH_o$) on $I_K$ was studied ($pH_i = 7$). Shrager (1974) has shown that a change of $pH_o$ from 7.5 to 5.8 reversibly slows the activation of $I_K$ in crayfish axons ($HP = -75$ mV) without significantly altering the steady-state $I_K$ conductance. I obtained similar results in squid axons with $HP = -80$ and $-50$ mV. Moreover, the degree of steady-state inactivation with $HP = -50$ mV was not altered by the change in $pH_o$.

## DISCUSSION

The results in this study confirm and extend the earlier work of Wanke et al. (1979) and they also suggest a mechanism for the $pH_i$ effect. They are consistent with the binding of protons to the $I_K$ channel, perhaps the inactivation gate of the channel, in such a way that the voltage dependence of inactivation is shifted along the voltage axis. A major conclusion from these results is that the $pH_i$ effect does not saturate in the 6–10 pH range. The apparent saturation of the effect at both low and high pH reported by Wanke et al. (1979) was attributable, I believe, to their use of a single holding potential (probably in the $-60$ to $-50$ mV range). The $pH_i$ effect would appear to saturate under these conditions, because the voltage dependence of the inactivation curve is a relatively steep function of membrane potential (Fig. 12, Clay 1989 and Fig. 4 of this paper). The maximal $I_K$ conductance (upper left limb of the inactivation vs. voltage relation) is shifted into the $-60$ to $-50$ mV range with $pH_i \sim 8$. Any further increase in pH would not result in an increase of $I_K$ with a holding potential of $-50$ mV. Similarly, the noninactivating $I_K$ component (lower right limb of the inactivation vs. voltage relation) is shifted into the $-60$ to $-50$ mV holding potential range at $pH_i \sim 6.5$. Any further decrease of $pH_i$ below $\sim 6.5$ will not produce a further decrease in $I_K$ with $HP = -50$ mV. Consequently, use of a single holding potential can give the appearance of titration of the $pH_i$ effect, whereas none, in fact, exists, at least not in the 6–10 pH range. This result is surprising, because other reports of pH effects on ion channels do show clear saturation with either increasing or decreasing-pH level, or both. For example, the effect of $pH_o$ on $I_K$ kinetics reported by Shrager (1974) saturated above $pH_o$ 7 and (apparently) below $pH_o$ 5, which is consistent with a single titratable group having a $pK_a \sim 6.3$. Similarly, external protons block the $I_K$ channel with

$pH_o < 6$ with a single titratable group having a $pK_a \sim 4.4$ (Hille, 1973). (The $pH_o$ effect on kinetics [$pK_a = 6.3$] and the $pH_o$ effect on $I_K$ amplitude [$pK_a = 4.4$] appear to represent two different actions of external protons on the $I_K$ channel.) The lack of saturation of the $pH_i$ effect I have observed can be most reasonably explained by the existence of several titratable groups in the inactivation gate having a range of $pK_a$'s. For example, five different groups having hypothetical $pK_a$'s of 6, 7, 8, 9, and 10 would give an approximately linear relationship between $pH_i$ and the voltage at half maximum of the inactivation curve.

A further surprise in this analysis concerns the mechanisms of the $pH_i$ effect. A simple voltage shift of a gating parameter, which is suggested by the results in Fig. 4, is a hallmark of a surface charge effect (Hille, 1984). That is, protons bind to the membrane or, perhaps, the $I_K$ channel itself in such a way that the voltage drop across the channel is altered via a change in the surface charge potential. The difficulty with this interpretation is that a change in $pH_i$ does not alter the voltage dependence of activation (Figs. 1–2 and Wanke et al., 1979). That is, the $pH_i$ effect appears to be a specific surface charge effect on inactivation alone, which is, seemingly, a contradiction in terms. The fact that different channels in a membrane might not experience the same voltage shift is not surprising. For example, the shift in the voltage dependence of activation of the sodium channel in squid axons produced by a change in $Ca_o^{+2}$ is not the same as the shift in activation of the potassium channel (Frankenhauser and Hodgkin, 1957). Moreover, Hille (1984) has suggested that the effective surface charge may differ even between separate domains of the same channel. Nevertheless, the specificity of the $pH_i$ effect for inactivation does suggest that alternative interpretations of the effect cannot be excluded.

## REFERENCES

Chabala, L. D. 1984. The kinetics of recovery and development of potassium channel inactivation in perfused squid giant axons. J. Physiol. (Lond.). 356:193–220.

Clay, J. R. 1984. Potassium channel kinetics in squid axons with elevated levels of external potassium concentration. Biophys. J. 45:481–485.

Clay, J. R. 1988. Lack of effect of internal fluoride ions on potassium channels in squid axons. Biophys. J. 53:647–648.

Clay, J. R. 1989. Slow inactivation and reactivation of the K$^+$ channel in squid axons. A tail current analysis. Biophys. J. 55:407–414.

Clay, J. R. 1990. The inactivation curve of the delayed rectifier in squid axons is shifted along the voltage axis by changes in pH$_i$. Biophys. J. 511a. (Abstr.)

Clay, J. R., and M. F. Shlesinger. 1983. Effects of external cesium and rubidium on outward potassium currents in squid axons. Biophys. J. 42:43–53.

Ehrenstein, G., and D. L. Gilbert. 1966. Slow changes of potassium permeability in the squid giant axon. Biophys. J. 6:553–566.

Frankenhauser, B., and A. L. Hodgkin. 1957. The action of calcium on the electrical properties of squid axons. J. Physiol. (Lond.). 137:218–244.

Hille, B. 1968. Charges and potentials at the nerve surface: divalent cations and pH. J. Gen. Physiol. 51:221–236.

Hille, B. 1973. Potassium channels in myelinated nerve. Selective permeability to small cations. J. Gen. Physiol. 61:669–686.

Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associates. Sunderland, MA. 325.

Iijima, T., S. Ciani, and S. Hagiwara. 1986. Effects of the external pH on Ca channels: experimental studies and theoretical considerations using a two-site, two-ion model. Proc. Natl. Acad. Sci. USA. 83:654–658.

Prod'hom, B., D. Pietrobon, and P. Hess. 1987. Direct measurement of proton transfer rates to a group controlling the dihydropyridine-sensitive Ca$^{+2}$ channel. Nature (Lond.). 329:243–246.

Roos, A., and W. F. Boron. 1981. Intracellular pH. Physiol. Rev. 61:296–434.

Shrager, P. 1974. Ionic conductance changes in voltage clamped crayfish axons in low pH. J. Gen. Physiol. 64:666–690.

Wanke, E., E. Carbone, and P. L. Testa. 1979. K$^+$ conductance modified by a titratable group accessible to protons from the intracellular side of the squid axon membrane. Biophys. J. 26:319–324.

Wanke, E., E. Carbone, and P. L. Testa. 1980. The sodium channel and intracellular H$^+$ blockage in squid axons. Nature (Lond.). 287:62–63.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. J. Gen. Physiol. 61:687–708.