brief communication

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

John R. Clay

Laboratory of Biophysics, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543 USA

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).

Dr. Clay's mailing address is National Institutes of Health, Building 9, Room 1E131, Bethesda, MD 20892.

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 μM 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 μM 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

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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

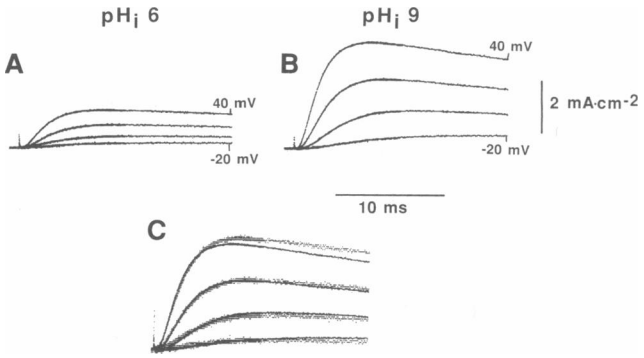

FIGURE 1 Effect of a change in pH from 6 to 9 in the internal perfusate on I_K. (*A*) Membrane current records in response to 20 ms duration voltage clamp steps to −20, 0, 20, and 40 mV applied sequentially with a 3 s rest interval between each step. Holding potential = −50 mV; $pH_i = 6$; $K_o = 10$ 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.

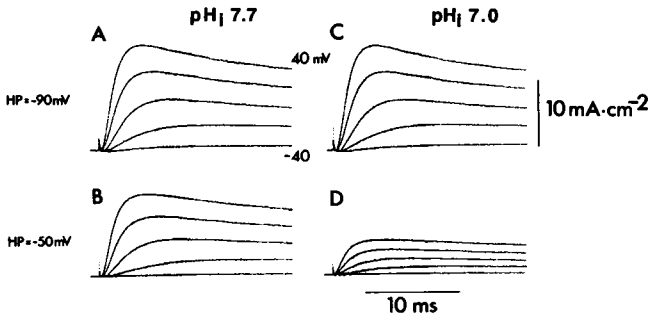

FIGURE 2 Holding potential dependence of the pH_i effect. The membrane potential was initially held in this experiment at −90 mV with $pH_i = 7.7$. Voltage clamp steps 20 ms in duration were made to −40, −20, 0, 20, and 40 mV with a 3 s rest interval between each step (*panel A*). The holding potential 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 ≤ −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 μs 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

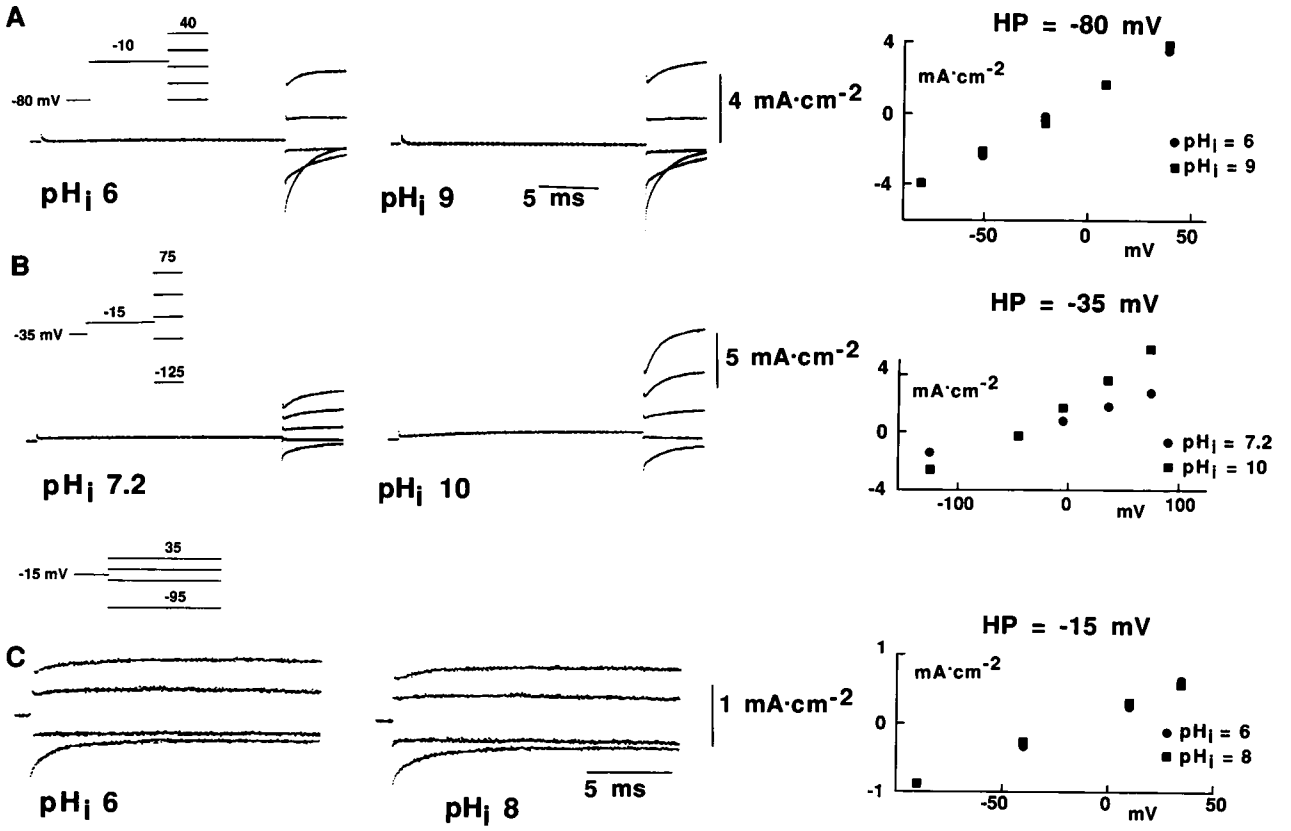

FIGURE 3 Holding potential dependence of the pH_i effect in elevated K_o. (*A*) Membrane currents from a holding potential of −80 mV with a prepulse to −10 mV followed by steps to 40, 10, −20, −50, and −80 mV with 3 s rest interval between each of these two pulse sequences. K_o = 150 mM. The same clamp sequence was applied with pH_i = 6 and pH_i = 9. The current-voltage plots on the right were taken 100 μs 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 μs 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,

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

where NI is the noninactivated state (either the open state

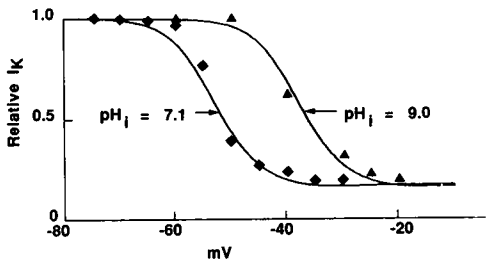

FIGURE 4 Steady-state inactivation curves from two different axons measured using the protocol described in Fig. 3. K_o = 100 mM for both experiments. The pH_i in one experiment was 7.1; it was 9.0 in the other. The curves correspond to $[1 + a/b + ac/(bd) + ace/(bdf)]^{-1}$, where $a = \{1 + \exp[-(V + V_o + 50)/5]\}^{-1}$ 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 ~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

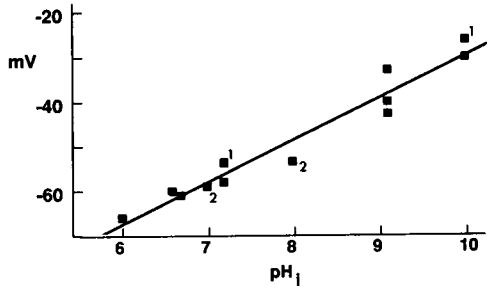

FIGURE 5 Dependence of the midpoint of the I_K inactivation curve on pH_i determined from the protocol illustrated in Fig. 3. Each data point is taken from a different axon, except for the results labeled 1 and 2 in which the inactivation curve was measured in one axon at two different values of pH_i. 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 ~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.

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