HIGH INTRACELLULAR pH REVERSIBLY PREVENTS GATING-CHARGE IMMOBILIZATION IN SQUID AXONS

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ABSTRACT Squid giant axons were used to study the reversible effects of high intracellular pH (pH_i) on gating currents. Under depolarization, when Na channels are activated, internal solutions buffered at high pH_i (10.2) affect considerably the time course of gating charge associated with channel closing, Q_{OFF}, with almost no alteration of Q_{ON} records. In particular, at pH_i 10.2 the charge corresponding to the fast phase of I_gOFF, measured after long depolarizing pulses (7.7 ms), was consistently larger than that recorded at physiological pH_i (7.2). This suggests that high pH prevents immobilization of gating charges induced by Na inactivation. In this respect, the present data agree reasonably well with previous observations, which show that $pH_i > 7.2$ reversibly removes the fast Na inactivation with little effects on activation kinetics (Carbone, E., P. L. Testa, and E. Wanke, 1981, *Biophys. J.*, 35:393–413; Brodwick, M. S., and D. C. Eaton, 1978, *Science [Wash. DC]*, 200:1494–1496). Unexpectedly, high pH increases the amount of charge associated with the slow phase of I_gOFF. In our opinion, this might be the result of either an increment of the net charge produced by the exposure to high pH_i or that gating charges that return to the closed state might experience a larger fraction of the potential drop across the membrane (Neumcke, B., W. Schwarz, and R. Stampfli, 1980, *Biophys. J.*, 31:325–332).

INTRODUCTION

Gating currents in nerve membranes are widely believed to originate from the molecular rearrangements of ionic channels as they open and close (1–3). As previously observed, the steep dependence of membrane permeability on voltage (4) implies that each channel bears dipolar or charged molecular groups that move transiently in response to changes in the membrane electric field. From theoretical considerations (4), the charged groups associated with each channel gating are expected to be more than one, so that electrostatic interactions are likely to arise either among them or between charges belonging to different gating processes. The best-known example of charge interaction is that postulated to occur between activation- and inactivation-gating charges of Na channels (5, 6). Briefly, the development of Na conductance inactivation reduces the total charge associated with the closing of channels, as if a fraction of the charge displaced during channel opening were immobilized by the inactivation process (5).

Here we describe the effects of high pH_i on the properties of gating currents, with particular attention to the phenomenon of gating-charge immobilization. High pH_i is known to remove reversibly fast Na inactivation, possibly by neutralizing the net positive charge associated with the normally functioning inactivation gate (7, 8). Because of the correlation between Na inactivation and gating-charge immobilization (9), we thought it interesting to use H^+ ions as a tool for studying in more detail the mechanism of gating-charge immobilization as well as its dependence on Na inactivation. The present data show that high pH_i increases the amount of total charge moved during channel closing, with little effects on the charge movement associated with channel opening. In several aspects, this action resembles that of pronase (10) and scorpion venoms (11), and leads us to conclude that high pH_i is an effective agent that reversibly removes the immobilization of ON gating charges.

MATERIALS AND METHODS

All experiments were performed on giant axons of the squid, *Loligo Vulgaris*, internally perfused and voltage clamped as described in previous works (7, 12). Major improvements were made on the geometry of the external current electrodes (13), and on the dynamic and stationary responses of the voltage-clamp apparatus (14). This latter was accomplished by using fast (model, 9862; Optical Electronics Inc., Tuscon, AZ) and slow operational amplifiers (model OP-16; Precision Monolithics, Santa Clara, CA). Before analog-to-digital conversion (sampling interval variable between 8 and 512 μs) the signal bandwidth was corrected for sampling effects by a programmable 8-pole Bessel filter (model 745-OL4; Frequency Devices, Inc., Haverhill, MA). Fast and accurate (12 bit, 2 μs) analog-to-digital and digital-to-analog (12 bit, 0.2 μs) converters (model

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DAS-250 and 681E; Datel-Intersil, Mansfield, MA) connected the voltage-clamp unit to a slave microprocessor (model F8; Fairchild Optoelectronic Div., Mountain View, CA) under the control of a desktop microcomputer (AIM-65; Rockwell International Corporation, Pittsburgh, PA). Digital data were transferred to a DECLAB minicomputer (Digital Equipment, Corp., Marlboro, MA) for OFF-line data analysis.

Usually, gating-current signals were recorded by repeating 4 to 16 times the P/4 pulse procedure described by Armstrong and Bezanilla (5). The holding potential (E_h) was −64 mV. Test depolarizations (E_t) were applied after a 150-ms hyperpolarizing pulse to −96 mV (E_c) to remove fast Na inactivation. Repolarizations after tests were either to −64 or −96 mV (E_r). Correcting pulses were delivered from a holding level of −144 mV. The standard intracellular solution (350 Cs) contained in millimolars: 350 CsF, 255 sucrose, and 45 tetramethylammonium (TMA)-phosphate buffer (pH_i 7.2 ± 0.1). Solutions of high pH_i had the same ionic composition except that the buffer was 45 mM TMA-glutamate (pH_i 10.2 ± 0.1). The external bath was either TMA-artifical seawater (ASW) or Tris-ASW containing, respectively, in millimolars: 445 TMA-Cl, 10 $CaCl_2$, 40 $MgCl_2$, 20 Tris-Cl (pH_o 8.0 ± 0.1) or 525 Tris-Cl, 10 $CaCl_2$, 40 $MgCl_2$ (pH_o 7.6 ± 0.1). 3×10^{-7} M tetrodotoxin (TTX) was added during current measurements. The bath temperature was kept at 13 ± 0.1°C throughout all the experiments.

RESULTS

Fig. 1 shows typical records of outward and inward gating currents at normal (7.2) and high (10.2) pH_i. Intracellular perfusion with solutions of pH_i 10.2 produces strong alterations to the time course of inward gating currents, I_gOFF, with only small effects on I_gON. In two axons the action of high pH on I_gON was independent of membrane depolarization so that the voltage dependence of the total charge associated with the ON traces, Q_{ON}, was nearly unaffected by pH_i 10.2 (Fig. 2 *a*). Notice that pH 10.2 does not produce any substantial voltage shift of the $Q_{ON}(E)$ data points while under conditions similar to those of Fig. 2 *b*, pH_i 10.2 shifts the sodium conductance, g_{Na}, by nearly 20 mV toward positive voltages (our observation; see also Fig. 7 *a* in reference 7). This peculiarity was not further investigated in the present paper.

The effects of pH_i 10.2 on the I_gOFF were also independent of the level of membrane repolarization. After depolarizations of increasing duration, steps to either −64 or −96 mV produced I_gOFF records that remained of fairly constant amplitude instead of decreasing as normally occurs at physiological pH_i (Fig. 1, upper trace). This suggests that high pH_i might remove reversibly the immobilization of ON gating charges. Further support of this view comes from the results of Fig. 2 *b*, in which the ratio, Q_{OFF} and Q_{ON}, and the time course of Na conductance, g_{Na}, recorded under similar conditions are plotted on the same time scale. At pH_i 7.2 (Fig. 2 *b*, bottom) the ratio Q_{OFF}/Q_{ON} decreases from 1 to 0.3–0.5 with a time course similar to that of Na inactivation, while at high pH_i (Fig. 2 *b*, top) the ratio is nearly constant and deviates considerably from g_{Na} (notice that the ratio is even greater than unity for pulses of 7.7 ms). Indeed, since high pH_i induces an increase of leakage currents (7), it is possible that the increment of Q_{OFF} might be distorted by the presence of a not perfectly compensated leakage-current component. The two following points speak against this possibility. (*a*) Linear leakage current can be fully compensated by the P/4 procedure, as proven by the absence of any slow component in the time course of I_gON; (*b*) there are no appreciable differences in the results of Fig. 2 *b* when repolarizing to −64 (dotted line) or −96 mV (dashed line) (if a nonlinear leakage component was present, the data at −96 mV had to be consistently higher).

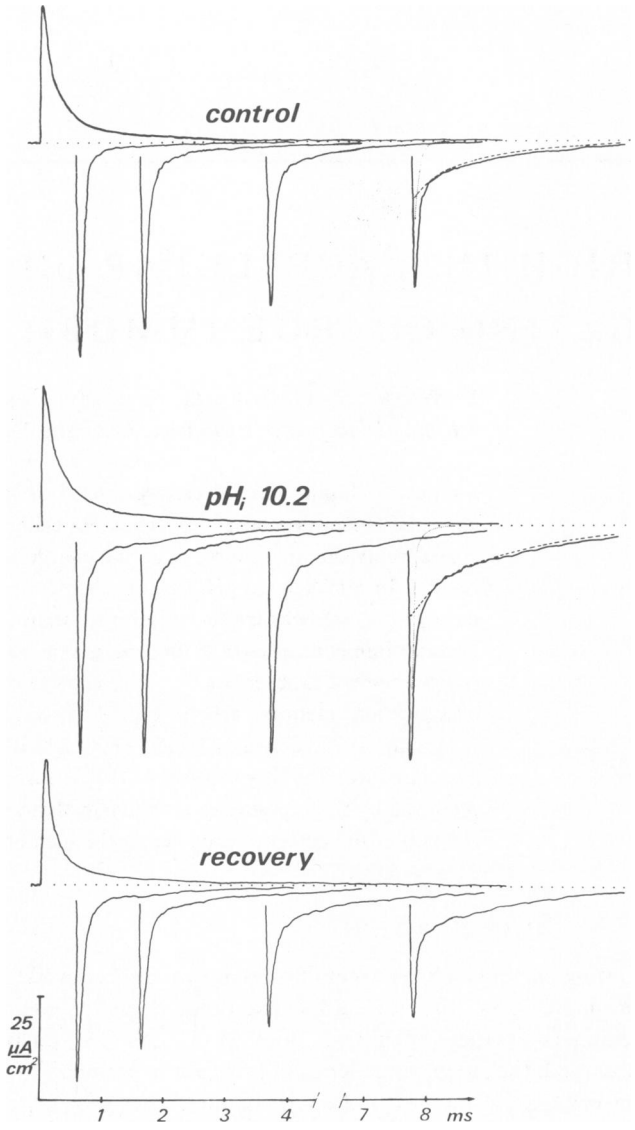

FIGURE 1 Reversible effects of high pH_i on gating currents. The traces are records of outward and inward gating currents before (*upper*), during (*middle*), and after (*lower*) intracellular perfusion with 350 Cs at pH_i 10.2. Inward tails were recorded on return to −96 mV after different times at 0 mV. Traces were low-pass filtered using a digital filter. The dotted and dashed lines are examples of the fast and slow exponential terms used to determine the fast and slow Q_{OFF} components (see text and Fig. 3). The following constants were used: amplitudes ($\mu A/cm^2$), a fast(7.2) = 20; a slow(7.2) = 14.5; a fast(10.2) = 37.5; a slow(10.2) = 21; time constants, τ (ms), τ fast(7.2) = 0.08, τ slow(7.2) = 0.8, τ fast(10.2) = 0.08, τ slow(10.2) = 1.1. *Outside:* Tris ASW.

To quantify the effects of high pH_i on I_gOFF, we fitted (for 5 ms) the time course of the OFF response with a

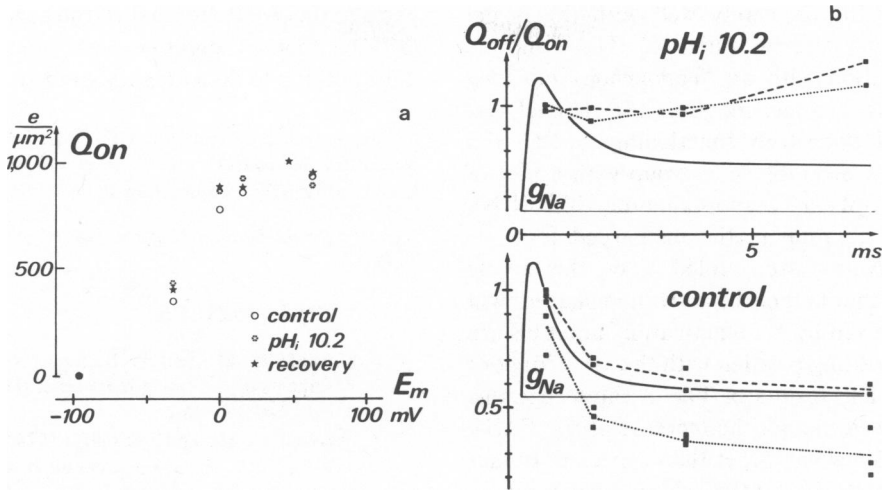

FIGURE 2 (*a*) Distribution of the nonlinear charge movement, Q_{ON}, vs. membrane voltage (E_m) before, during, and after perfusion at pH_i 10.2. Data points were obtained by integrating I_g records over an interval of 7 ms from the onset of the step. This value was chosen because longer time intervals did not increase appreciably the result of the integration. Compared with other data, e.g., Fig. 5 in reference 15, our maximum value of Q is 25% smaller. This might be the result of using less negative holding levels from which we delivered our correcting pulses (−144 mV vs. the usual −170 mV). *Outside:* Tris ASW. *Inside:* 350 Cs. (*b*) Charge immobilization and Na inactivation before (pH_i 7.2, *bottom*) and during perfusion at pH_i 10.2 (*top*). Symbols indicate the ratio Q_{OFF}/Q_{ON} calculated (with the method of Fig. 2 *a*) at various pulse width (vertical scale on the left). Data from different axons. $E_t = 0$ mV. Lines through the symbols represent means at pH 7.2 while they connect data at pH 10.2. Dashed lines refer to $E_r = -96$ mV; dotted lines to $E_r = -64$ mV. The continuous curves are g_{Na} at pH_i 7.2 and 10.2 plotted in arbitrary units and recorded under the following conditions. $E_h = -70$ mV, $E_c = -100$ mV for 160 ms, $E_t = 0$ mV, temperature 12°C. Internal solution (in millimolars): 50 NaF, 267 CsF, 307 sucrose, 45 K-phoshate (pH_i, 7.2) or 45 K-glutamate (pH_i 10.2). *Outside:* ASW (see reference 1).

linear combination of two exponential terms (5, 16). The two exponentials were determined starting ~64 μs after the onset of repolarization (see Fig. 1), and the charge movement associated with each component (FAST OFF, SLOW OFF) was calculated as the product of the initial amplitude by the time constant. The results of the analysis are shown in Fig. 3. In agreement with other authors (5, 16), at pH_i 7.2, the charge movement associated with the FAST component decreases while the SLOW OFF increases with the duration of the depolarizing pulse. At high pH_i the decrease of the FAST OFF is considerably reduced, while the charge movement associated with the SLOW component increases markedly. For the axon of Fig. 3, the time constant of the FAST and SLOW response was 80 μs and 0.8 ms at pH_i 7.2, and 80 μs and 1.1 ms at pH_i 10.2.

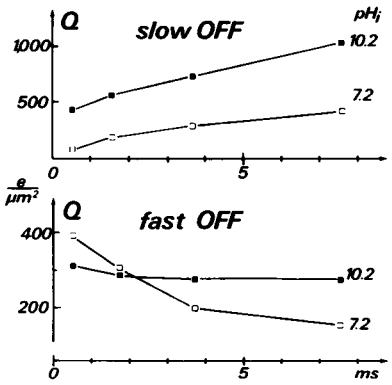

FIGURE 3 Gating charges associated with the FAST and SLOW components of I_gOFF vs. pulse duration as derived from the curve-fitting analysis described in the text at normal (□) and high pH_i (■). Integration of the FAST and SLOW exponential terms started after 64 μs from the onset of step depolarization to −96 mV. *Outside:* Tris ASW. *Inside:* 350 Cs.

DISCUSSION

In our opinion a possible interpretation of the present findings is that high intracellular pH_i reduces the immobilization of ON gating charges, occurring when Na channels inactivate. As shown, our conclusions are based primarily both on the Q_{OFF}/Q_{ON} ratio, and the analysis of the time course of the Q_{OFF} components. Such analysis is sufficient in describing the phenomenon of gating-charge immobilization (17). For this reason, we considered it unnecessary to attempt a full characterization of the voltage dependency of the charge immobilization in the present work (see references 5 and 6 for a detailed description). With this in mind, we feel the following points are reasonably well established. First, despite the fact that the amount of gating charges displaced by Na-channel activation is hardly affected by high pH_i, the total charge moved during OFF responses increases considerably after long depolarizations at pH 10.2 (Fig. 1 and 2 *b*). Second, at high pH_i the Q movement associated with the FAST component of I_gOFF decreases by only 15% compared with the 60% decrement occurring after long depolarizations at pH_i 7.2 (Fig. 3). Finally and unexpectedly a remarkable increase of the SLOW OFF component was clearly detected (Fig. 3).

Our present data fit reasonably well with the model proposed by Armstrong and Bezanilla (5). These authors identify Na inactivation with an "endogenous blocking particle" located near the inner mouth of the channel that is positively charged when fully functioning (8, 18), and supposed to possess a titratable side group with a pK_a of 10.4. (7, 8). Thus, at pH_i 10.2 approximately 50% of Na channels have such a group in the uncharged form. If activation and inactivation are coupled (5, 6), this simple model would predict that half of the activation charges will no longer be immobilized by Na inactivation, and thus are free to return to the resting position with the same kinetics of channel closing. The results of Fig. 3 show that this might be the case, even though the increase of the FAST OFF component is somewhat larger than expected. In fact at pH_i 10.2, the fraction of OFF charges that return quickly to the closed state after a pulse of 7.7 ms is nearly 80% of that available with a 0.5-ms pulse (Fig. 3, bottom), while the fraction of noninactivated channels is only 50% (Fig. 2 *b*).

Supporting evidence of this interpretation comes from the similarity of action that high pH_i and two other inactivation removing agents, such as pronase and scorpion venoms, have on the FAST component of I_gOFF (10, 11). Like high pH, pronase and scorpion venoms remove fast Na inactivation and increase the FAST OFF response following pulses of long duration. This reinforces the idea that whatever the mechanism of action is, removal of Na inactivation is always followed by an increase of the FAST component of I_gOFF recorded after long depolarizations. However, the reverse is not necessarily true. For instance, in *Myxicola* axons internal application of gallamine enhances the FAST OFF response without affecting Na inactivation (18).

Indeed, according to the above interpretation (5), one should also expect that the quantity of charges that return slowly to the closed state decreases at pH_i 10.2 by the same amount that increases the FAST component. This is in contrast with the results of Fig. 3 (upper part) that show a remarkable increase of the SLOW OFF component at high pH_i. An explanation for the above discrepancies could be that the relationship between Na inactivation and charge immobilization is more complicated than that simply hypothesized (6). Along this line, there are at least two ways in which high pH_i might increase the SLOW OFF component. First, high pH_i might create new gating charges that do not necessarily move with the time constant of recovery from inactivation (5). In this respect note that a lowering of the external pH also produces an increase of the ON gating charges. This occurs in frog nodes (19) as well as in squid axons (E. Carbone, G. Prestipino, P. L. Testa, and E. Wanke, unpublished results). Secondly, high pH_i might facilitate a deeper penetration of charges in the membrane phase, so that gating charges experience a larger fraction of the voltage drop across the membrane (19). Indeed, one cannot also exclude the possibility that the large increase of the SLOW OFF component might be contaminated by processes that have nothing to do with gating currents (16).

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