BRIEF COMMUNICATION

K^+ CONDUCTANCE MODIFIED BY A TITRATABLE GROUP ACCESSIBLE TO PROTONS FROM THE INTRACELLULAR SIDE OF THE SQUID AXON MEMBRANE

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ABSTRACT In the range of pH examined (5.2–10), variations of internal pH from high to low values result in a reversible decrease of the conductance of the open K channels, without significantly affecting the kinetics parameters. A linear plot of the conductance versus internal pH suggests the existence of a titratable group that has an apparent pK_a of about 6.9, and that is accessible to protons only from the intracellular side of the membrane.

INTRODUCTION

Although extracellular pH effects on the ionic currents of voltage-clamped nerve fibers have been widely studied in the past (1–5), relatively few attempts on the results of intracellular pH changes in nerve cells have been reported. To our knowledge, the only published data concern specifically the reversible effects of low pH values on the Na^+ activation (6) and of high pH values on the Na^+ inactivation (7) in squid axons. At present, nothing is known about the action of internal hydrogen ions on the mechanisms of ion transport through potassium channels. We report here that in perfused squid giant axons, internal pH affects the maximum conductance of the potassium channel in a rather unique manner. Our results complement the studies on the effects of internal monovalent (8) and divalent cations (9), which have been shown to influence differently the potassium conductance and the open-closed kinetics of K channels. Part of this work has appeared in a preliminary version (10).

MATERIALS AND METHODS

Isolated giant axons of the squid, *Loligo vulgaris,* were used in this study. The cleaning procedure, the internal electrodes, and the voltage-clamp apparatus were similar to those used in previous works (5, 10). Two major improvements were made here. (*A*) The axon chamber was replaced with one similar to that described by Armstrong et al. (11), except that the central and guard electrodes were made 8 and 5 mm long, respectively, and two air gaps of 3-mm length were used on each side of the space-clamped region. (*B*) Acquisition and storage

of data were obtained by using a PDP11 computer (Digital Equipment Corp., Maynard, Mass.) connected on-line with the voltage clamp apparatus. A computer-programmed digital to analog converter generated a series of stimulating pulses (1–2/s) alternated with a complementary pattern of pulses of attenuated amplitude for leakage and capacitive components subtraction. The membrane current was sampled at intervals of 35 μs with a 12 bit analog to digital converter, and stored after direct memory access.

All the experiments were performed at a constant temperature of 2°C with axons bathed in artificial sea water (ASW) containing 3×10^{-7}M tetrodotoxin, (5) and were perfused according to the procedure of Bezanilla and Armstrong (8). The standard internal solution (SIS) contained (mM): 400 K^+, 317 F^-, 45 $H_2PO_4^-$, and 310 sucrose, with the pH adjusted to 7.2 ± 0.1. Internal solutions of different pH values had the same buffer capacity, ionic strength, and total K^+ content, within 4%. The following buffers were used: K-glutamate (pH range: 9–10.8), K-glutamine (8.5–10.2), K-glycylglycine (7.3–9.2), K-phosphate (6–7.8), K-citrate (2.5–6), and K-succinate (3.5–6). The amount of buffer (45 mM) was determined after a series of preliminary experiments in which the effects of the internal pH were studied as a function of the increasing buffer concentration. Above and below pH 7.2, higher buffer concentrations were proved to be no more effective than 45 mM. It was also observed that several buffer systems like *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, Cyclohexylaminopropane sulfonic acid, and 2-(*N*-morpholino) ethane sulfonic acid, (Sigma Chemical Co., St. Louis, Mo.) produced irreversible modifications of the potassium currents proportional to the amounts of buffer used.

To avoid the effects on membrane currents of K^+-accumulation in the periaxonal space (12), the amplitude of the potassium conductance (g_K) was estimated following the method of Adelman et al. (13). In our case, the time-course of $g_K(t)$ was obtained, without affecting appreciably the shape of the voltage-clamp records, from the tail currents associated to a train of 20-mV brief hyperpolarizations (100 μs) superimposed on the test pulse at a rate of 1 ms^{-1}. The duration of the test pulses varied from a minimum of 20 ms at $E = 110$ mV to a maximum of 60 ms at $E = -40$ mV (E, absolute membrane potential).

The time-course of $g_K(t)$ was analyzed in terms of the Hodgkin and Huxley model (14) by assuming $n_\infty(E_h) = 0$, for the n parameter, (E_h, holding potential), and a constant exponent equal to 4.

RESULTS

The effects of internal pH on the time-course and steady-state value of g_K are illustrated in Fig. 1. High internal pH increase the maximum potassium conductance, $\bar{g}_K$, without much affecting the kinetics of conducting channels. Low internal pH decrease $\bar{g}_K$ and, at pH lower than 6, slightly prolong the $g_K(t)$ curves. In both cases, the action of protons was found to be fast, reaching a steady state within 30–40 s. The recovery of 90% of initial conditions usually required a washing period of 2–3 min, except for pH values lower than 5.2 and higher than 10, for which the reversibility was hampered by a large increase of membrane leakage. Over the entire range of pH examined, g_K changes were found to be independent of the type and amount of buffer used, provided that the internal solutions contained more than 45 mM buffer. This was taken as a good indication that the observed $\bar{g}_K$ changes were due to pH and not to interferences of the buffer system with the membrane.

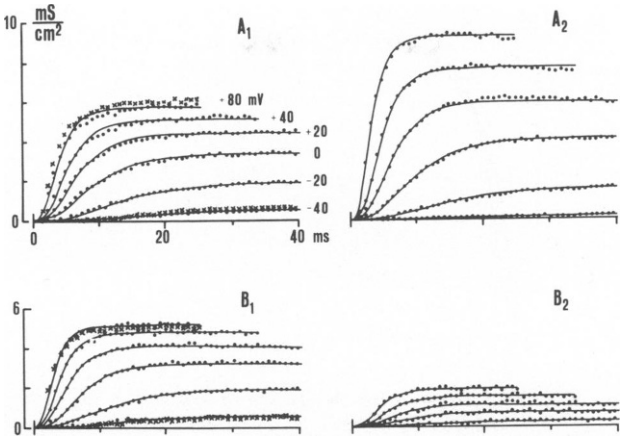

FIGURE 1 g_K records taken from two squid axons perfused with high (A) and low (B) internal pH values. In A_1 and B_1 dots refer to control experiments, while the crosses on the upper and lower traces of each figure are relative to the recovery from the test pH measurements. Depolarizations to levels are indicated in millivolts on the right of each record of A_1. In B_2, the curve at $E = -40$ mV is missing. All data points are evaluated from the tail currents of the voltage-clamp records associated to a 20 mV-repolarization lasting 100 μs. Solid lines are the result of a curve-fitting program based on a Hodgkin and Huxley type of analysis (see text). Internal solutions were SIS containing 45 mM of the following buffers: (A_1) and (B_1)—K-phosphate, pH 7.2; (A_2)—K-glutamate, pH 9.2, and (B_2)—K-citrate, pH 5.5. The temperature was 2°C.

The influence of internal pH on $\bar{g}_K$ becomes more evident if the steady-state values of each g_K curve, $g_{K\infty}$, are plotted against membrane potential (Fig. 2). As shown in the figure, the effects of internal pH can be separated into an amplitude variation of the $\bar{g}_K$ and a shift of the $g_K(E)$ curve along the voltage axis. The observed $\bar{g}_K$ variation suggests the existence of a blocking mechanism of K channels by hydrogen ions, similar in many respects to those reported in other preparations (1, 4, 15, 16), while the lack of any clear effect on the shape of the g_K-E characteristic indicates that protons interfere with the channels differently than Na^+ and Cs^+ ions (10). Na^+ and Cs^+ are known to reduce the outward K^+ currents, providing g_K-E characteristics with negative slope at high depolarizing voltages ("negative conductance"). No such effects are visible in Fig. 2.

The pH dependency of the $\bar{g}_K$ is shown in Fig. 3. The $\bar{g}_K$ values normalized to the conductance observed at pH 7.2 are plotted as a function of internal pH. Data are pooled from 19 axons with an average of about 2 test pH measurements for each fiber. The two lines represent the results of a best-fitting analysis according to a theoretical model based on the assumption that: (*a*) each single conducting channel binds with one hydrogen ion through a first-order chemical reaction with an apparent dissociation costant, K_a; (*b*) K channels have two discrete conductance states in the protonated and uprotonated form: g_{Kmin} and g_{Kmax}, respectively. Under these conditions, the normalized $\bar{g}_K(pH)$ is found to be related to the pH values of the bulk solution by the expression:

$$\frac{\bar{g}_K(pH)}{\bar{g}_K(7.2)} = \frac{\bar{g}_{Kmax}}{\bar{g}_K(7.2)}\left[1 + 10^{(pK_a - pH)}\right]^{-1} + \frac{\bar{g}_{Kmin}}{\bar{g}_K(7.2)}\left[1 + 10^{(pH - pK_a)}\right]^{-1},$$

where $\bar{g}_{Kmin}$ and $\bar{g}_{Kmax}$ indicate the $\bar{g}_K$ values that would be reached if all the K channels were

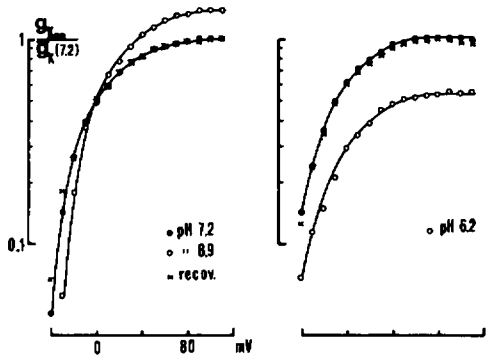

FIGURE 2 g_K-E characteristics of two axons perfused with solutions of high (left) and low (right) internal pH. The steady-state conductance values estimated from the $g_K(t)$ records of Fig. 1, $g_{K\infty}$, are normalized with respect to the $\bar{g}_K$ measured at the reference pH, $\bar{g}_K$ (7.2), and plotted against membrane potential. Solid lines through the control data (filled circles) were drawn by eye. Those at low and high pH were the same curve shifted along the abscissa and the ordinate axes to best fit the circles. The voltage shifts toward more depolarizing voltages were, respectively, 14 mV at pH 8.9, and 4 mV at pH 6.2. Internal solutions were SIS containing K-glycylglycine (pH 8.9) and K-phosphate (pH 6.2). The temperature was 2°C.

either protonated or unprotonated. The solid line shown in the figure is obtained for $\bar{g}_{Kmin} \neq 0$, the dashed curve for $\bar{g}_{Kmin} = 0$.

DISCUSSION

Our present results suggest the existence of a proton-titratable group controlling the conductance of the late channels of the squid axon membrane. The results of Fig. 3 may indicate that the candidate group has a pK_a characteristic of hystidyl residues and that when protonated, the permeability of the channel does not fall necessarily to zero. This, in turn, would imply that a complete block of K channels most likely requires the protonation of a second side-chain group with more acidic pK_a. The alternative case of a single titrating group with a pK_a of 6.3, which completely occludes the channel when protonated, seems unlikely. The dashed curve in Fig. 3 is seen to be too steep to fit the experimental points, while the spreading of data would rather suggest the existence of more than one receptor side for protons having pK_a values restricted to the range 5.5–7.2. Although significantly different from the former, this latter possibility would still be consistent with the idea that a imidazole group may affect the permeability of K channels. A distinction between the two plausible models needs at present further investigations. Measurements of $\bar{g}_K$ below 5.2 could also help to clarify this point, but unfortunately, more acidic solutions increase the leakage conductance to levels that make the decrease of $\bar{g}_K$ poorly reversible.

The location of the titratable residues seems to be restricted to the intracellular side of the K channel. This is suggested by the present results, combined with the observation that the extracellular pH in the range 4.7–10 does not appreciably affect the $\bar{g}_K$ of the squid giant axon (5). In a series of experiments, we have extended those measurements to lower pH values and found that in two axons bathed in ASW at pH 4.36, $\bar{g}_K$ decreases $<10\%$, excluding a direct interaction of external protons with the ion-transport mechanism within a wide range of pH values. Further evidences for the inability of external pH to affect the g_K were obtained from

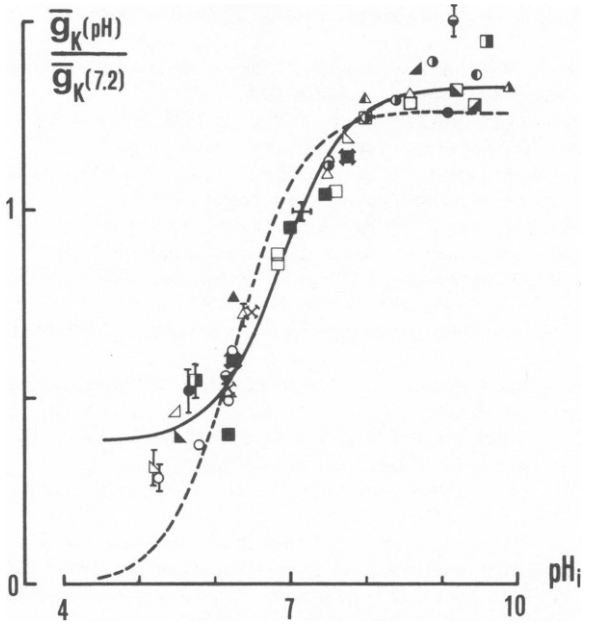

FIGURE 3 Relative g_K as a function of internal solution pH. The $\bar{g}_K$ are normalized to the reference maximum conductance, $\bar{g}_K(7.2)$. Data points represents test pH measurements taken from nineteen axons. Each axon is indicated by a different symbol. Unless marked with bars, errors for all data points are within the size of the symbols. The two lines were computed by a least-squares best-fitting program, according to the theoretical model described in the text. The continuous curve was obtained with $\bar{g}_{Kmax}/\bar{g}_K(7.2) = 1.33$, $\bar{g}_{Kmin}/\bar{g}_K(7.2) = 0.39$, and $pK_a = 6.9$, and the dashed line for $\bar{g}_{Kmax}/\bar{g}_K(7.2) = 1.26$, $\bar{g}_{Kmin}/\bar{g}_K(7.2) = 0$, and $pK_a = 6.3$.

experiments in which the extracellular and intracellular pH were varied simultaneously. The degree of block of the K channel by internal hydrogen ions was found to be independent of whether the external bath was made more acidic or basic.

The voltage shift of the g_K–E characteristics at high internal pH (Fig. 2, left) can be partially described as a result of an interaction of internal protons with fixed surface charges on the axonal membrane. As discussed elsewhere (4, 5), a detailed study of these effects would require a more complex analysis of the action of internal pH on the voltage clamp parameters, which would not add anything more to the present discussion. The two effects (voltage shift and block) have been clearly separated in other preparations (1, 3, 16), and found to provide different information about distinct membrane structural components.

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