BRIEF COMMUNICATION

EFFECTS OF AMMONIUM AND BICARBONATE-CO_2 ON INTRACELLULAR CHLORIDE LEVELS IN *APLYSIA* NEURONS

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ABSTRACT The level of intracellular free chloride in *Aplysia* giant neurons can be made to decline by pretreatment with 50 mM NH_4^+ solution followed by washing with 10 mM HCO_3^-/0.4% CO_2-containing fluids. This effect can be completely blocked by the anion flux inhibitor, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (SITS). The net change of free chloride in the cell cannot be explained by changes in the electrochemical gradient of chloride. These results support the hypothesis that at least one mechanism for intracellular pH regulation involves a Cl^-/HCO_3^- exchange process.

INTRODUCTION

An energy-dependent chloride/bicarbonate exchange process has been postulated to explain, at least partially, intracellular pH (pH_i) regulation in the squid giant axon (Russell and Boron, 1976). Thus when pH_i becomes acidic, HCO_3^- enters the cell and Cl^- leaves. Inasmuch as it is known for a number of excitable cells that pH_i is more alkaline than expected from a Donnan distribution, an active transport process is strongly implied (e.g. Thomas, 1974; Boron and DeWeer, 1976*a*,*b*). An active Cl^-/HCO_3^- exchange process suggests that intracellular free Cl^- (a_{Cl}^i) levels might be lower than would be the case for a Donnan distribution and, furthermore, it predicts that stimulating the process by rendering the cytoplasm acidic would lower a_{Cl}^i even further.

This report represents the results of testing the foregoing hypothesis with the giant neurons of *Aplysia californica*, cells known to maintain a_{Cl}^i lower than equilibrium conditions would predict (Russell and Brown, 1972). Treatment of the neurons in ways known to cause intracellular acidosis resulted in only a slight fall of a_{Cl}^i unless the recovery fluid contained HCO_3^-/CO_2 containing fluids. In the latter case, a_{Cl}^i fell dramatically. The decline of intracellular free chloride levels could be completely inhibited by pretreatment with SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid), a known inhibitor of passive anion fluxes in red blood cells (Cabant-

chik and Rothstein, 1972). Because the fall of a^i_{Cl} could not be explained by changes of membrane potential and no changes in cell volume could be observed, it is concluded that the *Aplysia* giant neurons actively extrude Cl^- in exchange for HCO_3^- when pH_i becomes acidic.

METHODS

Giant neurons (about 500 μm in diameter) of either the abdominal or pleural ganglia of *A. californica* were exposed by removing the connective tissue capsule overlaying the ganglion. A giant neuron was impaled with three electrodes: a 3-M KCl-filled microelectrode for measuring membrane potential (V_m), a current-passing electrode filled with 0.3 M K_2SO_4, and a chloride-selective liquid ion-exchanger microelectrode (Walker, 1971) for measuring the intracellular chloride ion activity (a^i_{Cl}). These electrodes were made fresh daily and had linear slopes of 54-56 mV per 10-fold change in chloride ion activity over the activity range of 7–604 mM. The selectivity of these electrodes for Cl^- over HCO_3^- was tested several times and found to be about 10 to 1 (range 7–12 to 1). A more complete description of the fabrication and testing of these electrodes may be found in an earlier publication (Russell and Brown, 1972).

RESULTS

Two different approaches were used to induce an intracellular acidosis. One was to expose the neuron to CO_2-containing solutions. The other was to pretreat with NH_4^+-containing fluids (Thomas 1974; Boron and DeWeer, 1976*a*,*b*). Such treatment causes a rapid alkalinization of pH_i due to the rapid entry of NH_3 and its subsequent intracellular protonation. Then pH_i slowly declines toward acidic values, presumably as a result of the passive influx of NH_4^+ (Boron and DeWeer, 1976*a*,*b*). Thus, when external NH_4^+ is removed, pH_i falls to values more acidic than those measured before NH_4^+ treatment. This technique has been used by Boron and DeWeer (1976*b*) to study intracellular pH regulation in squid axons.

Fig. 1 A illustrates the effects of a 30-min exposure to NH_4-artificial sea water, (ASW). The compositions of the external solutions are given in the legend. After a 5- to 10-min latency a^i_{Cl} began to increase slightly. In 23 neurons, it increased an average of 2.3 mM after 30 min of exposure. Russell and Brown (1972) previously reported that treatment with NH_4^+ was without effect on a^i_{Cl}, however, in those experiments the exposure was terminated after no more than 15–20 min. Removal of the external NH_4^+ by washing with Tris-ASW should cause an intracellular acidosis, and resulted in a slight decline of a^i_{Cl} (Fig. 1 A). In six neurons, the average a^i_{Cl} declined to a level 1.5 mM less than that measured in the same neurons immediately before NH_4^+ treatment.

This result suggests that intracellular acidosis may promote a net loss of cellular chloride. If the mechanism involved is similar to that proposed by Russell and Boron (1976) for the squid axon, then supplying HCO_3^- might promote an even greater Cl^- loss. This was tested by washing the NH_4^+-treated neurons with an ASW buffered with 10 mM HCO_3^-/0.4% CO_2. Fig. 1 B shows that washing with 10 mM HCO_3-ASW

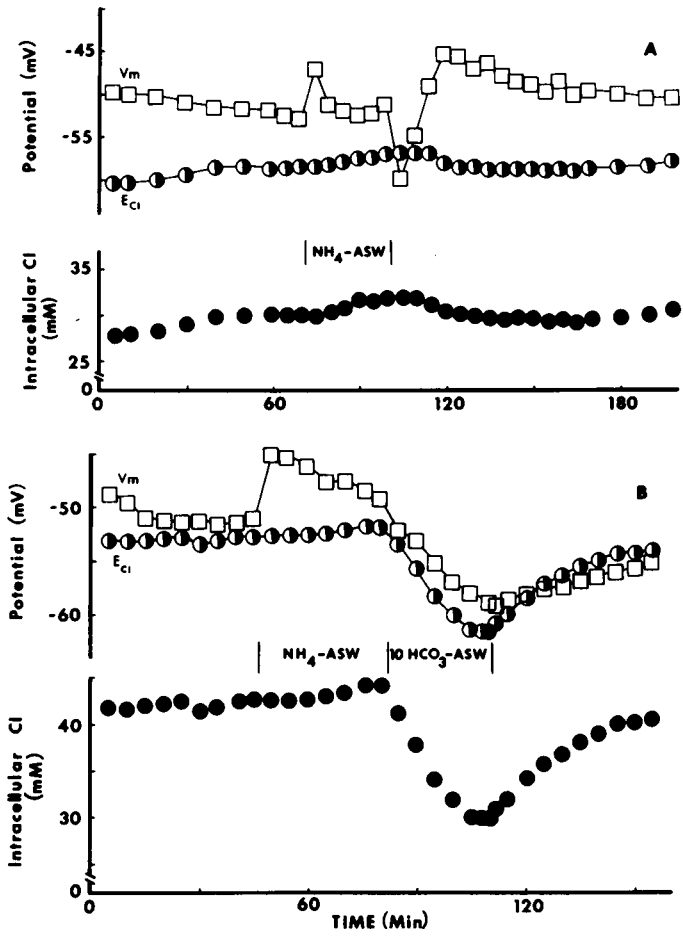

FIGURE 1 Effects of 50 mM NH_4^+ on intracellular chloride ion activity. Unless otherwise indicated in this and subsequent figures, the neurons were bathed in Tris-ASW whose composition in millimoles per liter was: NaCl, 494; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 20; $MgSO_4$, 30; Tris-(hydroxymethyl)-aminomethane buffer, 10; pH 7.9. NH_4-ASW was prepared by substituting 50 mM NH_4Cl for an equimolar amount of NaCl. The HCO_3^-/CO_2 solutions were prepared by substituting $NaHCO_3$ for NaCl and buffering with the HCO_3^-/CO_2 mixture. These latter solutions were continuously bubbled with the appropriate CO_2/O_2 mixtures in a reservoir. Glass tubing was used to connect the reservoir to the chamber containing the ganglion to minimize CO_2 loss. The chloride equilibrium potential (E_{Cl}) was calculated from direct measurements of intra- and extracellular ion activities with the Nernst equation. V_m, membrane resting potential. (A) Effect of pretreating with NH_4-ASW for 30 min and then returning to Tris-ASW. No change in cell diameter could be detected in this cell during the experiment. Accuracy of diameter measurement, ±5%. (B) Effect of pretreating with NH_4-ASW for 40 min and then washing with 10 mM HCO_3^-/0.4% CO_2 (pH 7.9) for 30 min. No change in cell diameter was noted (temperature, 20°C).

TABLE I

EFFECT OF SITS ON THE RESPONSE TO POST-NH_4^+ WASHING WITH 10 HCO_3-ASW

	Control				0.5 mM SITS			
	No.	a^i_{Cl}	E_{Cl}	V_m	No.	a^i_{Cl}	E_{Cl}	V_m
Tris-ASW	8	36.3 ± 2.4	−55.9 ± 1.3	−54.7 ± 4.2	8	34.3 ± 2.2	−56.6 ± 1.1	−52.3 ± 3.4
NH_4-ASW	8	38.5 ± 2.4	−54.6 ± 1.3	−50.2 ± 3.4	8	36.8 ± 3.2	−54.9 ± 2.1	−47.6 ± 3.7
10 HCO_3-ASW	8	26.4 ± 1.4	−61.7 ± 1.1	−60.8 ± 5.2	8	36.3 ± 3.7	−54.1 ± 2.2	−56.8 ± 4.8

All eight control neurons and eight SITS-treated neurons (16 different neurons) were followed through the entire three-step sequence of solution changes from Tris-ASW to HCO_3-ASW. E_{Cl} was calculated from the directly measured values for intracellular and extracellular chloride ion activities by the Nernst equation. Values in the NH_4-ASW row are the minimum ones attained, usually within 30 min of its application. SITS was applied in Tris-ASW about 30 min before treatment with NH_4-ASW.

caused a^i_{Cl} to fall markedly, reaching a minimum within 20–40 min. In eight neurons, a^i_{Cl} declined by about 10 mM below control, pre-NH_4^+-treatment, values (Table I).

Fig. 2 A shows that treatment with 10 mM HCO_3-ASW without prior exposure to NH_4-ASW resulted in very little decline of a^i_{Cl}. Inasmuch as superfusion with such low CO_2 tensions results in only slight intracellular acidification (Boron and DeWeer, 1976*b*), it must be the combination of an acidotic pH_i with the presence of external HCO_3^- that represents the stimulus for a net Cl^- loss. Therefore, exposure to a solution containing a higher CO_2 content should also cause a fall in a^i_{Cl}, because it is well known that higher CO_2 levels cause intracellular acidosis (Caldwell, 1958; Thomas, 1974; Boron and DeWeer, 1976*a*,*b*). As seen in Fig. 2 B, treatment with an artificial seawater buffered with 50 mM HCO_3^-/5.4% CO_2 (pH 7.6) resulted in a fall of a^i_{Cl}.

The amino group reactive agent, SITS, is known to block chloride movements in several cell types (Cabantchik and Rothstein, 1972; Russell and Brodwick, 1976; Ehrenspeck and Brodsky, 1976). Furthermore, it inhibits pH_i readjustment after intracellular acidosis as well as the extra ^{36}Cl efflux associated with intracellular acidosis in squid giant axons (Russell and Boron, 1976). When *Aplysia* neurons were treated with 0.5 mM SITS in Tris-ASW for 30 min before the NH_4-ASW:HCO_3-ASW treatment sequence, no post-NH_4^+ decline in a^i_{Cl} was observed (Table I). Although not shown in Table I, SITS also abolished the effect of 50 mM HCO_3/5.4% CO_2 to decrease a^i_{Cl}.

Because post-NH_4^+ washing with 10 HCO_3-ASW always resulted in membrane hyperpolarization, it was important to know whether the hyperpolarization caused the lowering of a^i_{Cl}. This seemed unlikely because, as Fig. 2 A shows, exposure to 10 HCO_3-ASW without NH_4^+ pretreatment resulted in little or no change of a^i_{Cl} whereas V_m hyperpolarized. Fig. 3 shows the results of an experiment in which the Vm was voltage-clamped to −45 mV while the preparation was washed with 10 HCO_3-ASW. Even when V_m was substantially less negative than the chloride equilibrium potential (E_{Cl}), there was a net fall of intracellular Cl^-. Conversely, after treatment

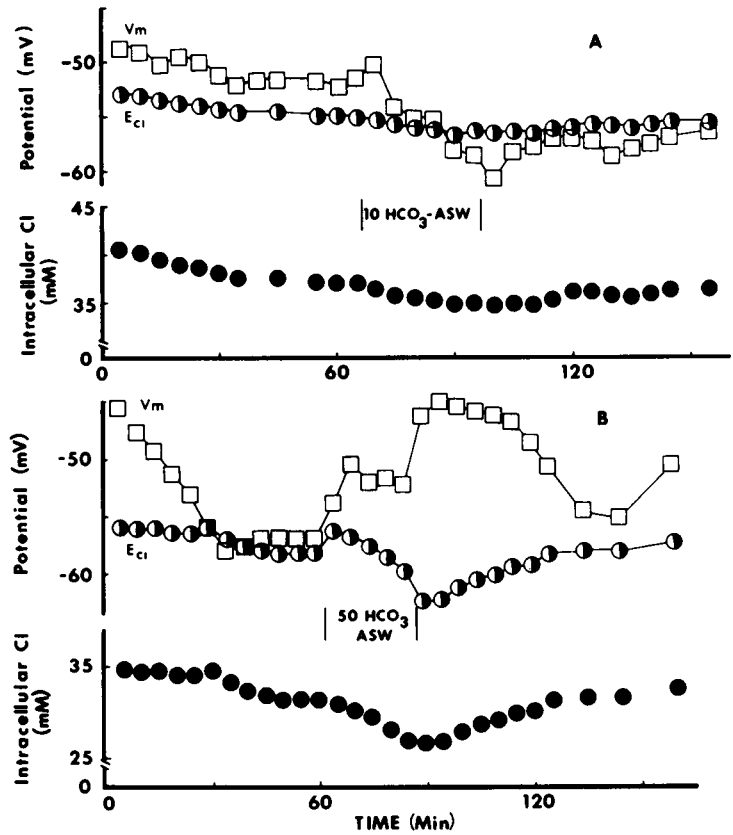

FIGURE 2 Effect of different HCO_3^-/CO_2 buffers on intracellular chloride ion activity. (A) 10 mM HCO_3^-/0.4% CO_2-99.6% O_2 (pH 7.9). The effect on a_{Cl}^i was slight although the effect on V_m was marked. (B) 50 mM HCO_3^-/5.4% CO_2-94.6% O_2 (pH 7.6). The solution was identical to Tris-ASW except 50 mM NaCl was replaced by 50 mM $NaHCO_3$ and Tris was left out. The fall of external chloride ion activity as a result of this replacement accounts for the abrupt change in E_{Cl} going from Tris-ASW to 50 HCO_3-ASW. Notice that although the V_m depolarized, there was a net efflux of Cl^- under these conditions. No correction for HCO_3^- contribution to the apparent a_{Cl}^i was made in either of these experiments. In the case of the higher bicarbonate concentration the actual a_{Cl}^i might be as much as 3 mM less due to the HCO_3^- error of the microelectrode. Temperature, 20°C.

with SITS, V_m still hyperpolarized when 10 HCO_3-ASW was applied, but now a_{Cl}^i was unchanged (Table I). Thus, the net movements of Cl^- described here appear to be unrelated to passive forces represented by changes in the electrochemical gradient of chloride.

DISCUSSION

The effects of pretreatment with NH_4^+ and HCO_3^-/CO_2 on a_{Cl}^i reported here may be most easily understood in terms of a Cl^-/HCO_3^- exchange mechanism stimulated by intracellular acidosis. Both NH_4^+ pretreatment and exposure to 5% CO_2 resulted in an

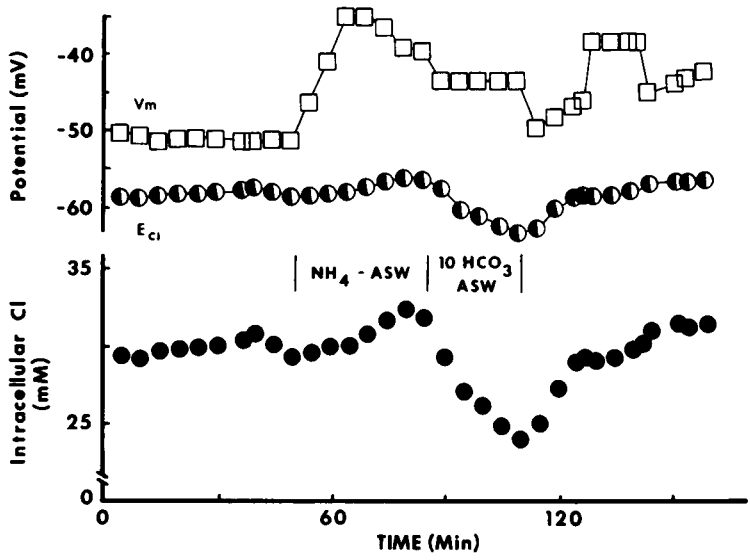

FIGURE 3 Effect of membrane potential on the post-NH_4^+, HCO_3^--stimulated fall of a_{Cl}^i. This neuron was treated exactly like that in Fig. 1 B, except V_m was voltage-clamped to −45 mV during the wash with 10 HCO_3-ASW. For Cl^- to be in equilibrium with the membrane potential, a_{Cl}^i would have to be about 59 mM when V_m = −45 mV; it actually fell to about 21 mM during the HCO_3^- treatment. It was voltage-clamped to −40 mV for a 20-min period in Tris-ASW, during which time some increase of intracellular chloride was noted. Current was injected into the cell from a 0.3-M K_2SO_4-filled microelectrode. Temperature, 20°C.

intracellular acidosis, and it is shown here that both treatments also induce a net efflux of chloride from *Aplysia* neurons. This net fall was particularly enhanced by the presence of extracellular HCO_3^-. In the absence of treatments inducing a fall of pH_i, exposure to the same HCO_3^- concentration has very little effect on a_{Cl}^i. An ATP-dependent Cl^-/HCO_3^- exchange mechanism has been postulated to explain, at least in part, the regulation of pH_i in squid giant axons (Russell and Boron, 1976). Just as in the squid axon, SITS inhibited the movement of chloride from *Aplysia* neurons induced by intracellular acidosis and extracellular HCO_3^-. However, in one important respect the *Aplysia* mechanism may differ from that in the squid axon. In the *Aplysia* neurons, the acidosis-induced chloride movement was clearly against an electrochemical gradient, whereas in squid axons chloride efflux was thermodynamically downhill, even though it required ATP. In both cases, however, an inward movement of HCO_3^- would occur against passive driving forces.

Chloride extrusion mechanisms blocked by ammonium have been described in other neuronal preparations (Lux, 1971; Llinás et al., 1974). In view of the present results, it is tempting to speculate that these extrusion processes are also pH_i sensitive. If so, ammonium could be acting to make pH_i alkaline, thereby inhibiting Cl^-/HCO_3^- exchange. The present results support this idea because a slight increase in a_{Cl}^i was noted during ammonium treatment.

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