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CYTOPLASMIC pH RECOVERY IN ACID-LOADED HAEMOCYTES OF SQUID (*SEPIOTEUTHIS LESSONIANA*)

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Summary

Cytoplasmic pH (pHi) of haemocytes of bigfin reef squid (*Sepioteuthis lessoniana* Lesson) was determined with the fluorescent probe, 2',7'-biscarboxyethyl-5,6-carboxyfluorescein (BCECF). The pHi of haemocytes suspended in nominally HCO_3^--free medium (extracellular pH 7.4) averaged (±S.E.) 7.32±0.02. Intracellular pH was independent of external Na^+ concentration and varied only slightly with changes in extracellular pH (pHe) ($\Delta pHi/\Delta pHe=0.16$ over the pHe range 6.8–7.8). Addition of weak acids (sodium propionate, potassium acetate) to haemocyte suspensions resulted in a rapid decrease in pHi. Haemocyte pHi then recovered with an average half-time of 3–4 min. Recovery of pHi was independent of external Na^+ concentration and insensitive to amiloride, but was abolished by *N*-ethylmaleimide (NEM). These results argue against the involvement of plasma membrane Na^+/H^+ exchange or other Na^+-dependent transport mechanisms in the pHi recovery of acid-loaded haemocytes. The results suggest that there is an NEM-sensitive proton extrusion mechanism in the plasma membrane of squid haemocytes.

Introduction

The haemolymph of cephalopod molluscs contains a single type of circulating cell, a granular leucocyte or haemocyte (Cowden and Curtis, 1974, 1981). This cell participates in internal defence against foreign material and in the cellular responses to injury. Cephalopod haemocytes phagocytose 'non-self' material (Stuart, 1968) and form aggregates that encapsulate foreign material (Jullien *et al.* 1956) and provide dermal plugs to lesions (Polglase *et al.* 1983). The origin, structure and cytochemistry of cephalopod haemocytes have been examined by a number of authors (Bolognari, 1951; Cowden, 1972; Cowden and Curtis, 1973, 1974, 1981). The biophysical properties and membrane transport processes of these cells remain largely unexplored.

Cytoplasmic pH (pHi) is linked to cellular metabolism and a number of membrane transport systems have been delineated for pHi regulation (reviewed

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by Boron, 1985; Hoffmann and Simonsen, 1989). Coupled Na^+/HCO_3^-–Cl^-/H^+ exchange has been demonstrated in invertebrate muscle and nerve cells, including the squid giant axon. Na^+/H^+ exchange has been identified in a variety of vertebrate and invertebrate cells. Additionally, recent studies have found evidence for the existence of proton pumps in the plasma membrane of vertebrate macrophages (Swallow *et al.* 1988, 1989; Bidani *et al.* 1989). This latter finding is of particular interest because invertebrate haemocytes share many characteristics with the vertebrate monocyte–macrophage series (Anderson, 1981). The pHi of cephalopod haemocytes has not been determined previously nor have the ion transport systems that regulate haemocyte pHi been examined.

In this study, we determined the pHi of squid haemocytes, using the pH-sensitive fluorescent probe 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF). BCECF is appropriate for monitoring pHi within the physiological range: it has a pK of approximately 7.0; its peak fluorescence intensity varies markedly with pH over the pH range of approximately 6 to 8; it leaks very slowly across the cell membrane; it becomes localized in the cytoplasm and does not become incorporated into intracellular organelles (Rink *et al.* 1982). Using this pH probe, we monitored transient changes in haemocyte pHi, induced by cellular acidification with weak acids (sodium propionate, potassium acetate), and subsequent pHi recovery. The pHi transients were studied in the presence or absence of external Na^+, amiloride (an inhibitor of Na^+/H^+ exchange) and *N*-ethylmaleimide (NEM, a nonspecific inhibitor of H^+-ATPase), to define the membrane transport processes involved in pHi recovery. A preliminary account of this work has appeared (Heming *et al.* 1988).

Materials and methods

Bigfin reef squid (*Sepioteuthis lessoniana*) were obtained from the Marine Biomedical Institute, University of Texas Medical Branch, Galveston, Texas. Adults of either sex (0.8–2.2 kg live mass) were netted from a rearing tank (water temperature 21–27°C), the branchial hearts visualized *via* a ventral–medial incision of the mantle, and haemolymph collected into plastic syringes by serial puncture of the two branchial hearts. The haemolymph contained $2.8\pm1.0\times10^6$ haemocytes ml^{-1} (mean±S.E., $N=18$), as determined by cell counts with a haemocytometer. Immediately following sample collection, the haemolymph was diluted to 5–6 times its original volume with ice-cold solution *A* (composition in mmol l^{-1}: NaCl, 425; KCl, 10; $MgCl_2$, 63; Hepes, 0.6; dextrose, 0.9; pH 7.4 at 25°C), pelleted (2500 *g*, 10 min, 4°C), washed in 50 ml of ice-cold solution *A*, and resuspended in 10 ml of ice-cold solution *A*.

BCECF was used to determine the cytoplasmic pH of haemocytes. The cells were loaded with the pH probe by incubation in solution *A* with 5 μmol l^{-1} BCECF-AM (acetoxymethyl ester form of BCECF, Molecular Probes Incorporated, Eugene, OR) for 40 min at 25°C. The BCECF-loaded cells were pelleted (1000 *g*, 10 min, 4°C) and resuspended in fresh solution *A* at approximately

10^6 cells ml^{-1} to produce our stock cell suspension. Overall recovery of haemocytes in our hands was 48±5 %, of which 97±1 % of cells were judged to be viable based on Trypan Blue dye exclusion.

To initiate an experimental run, a 100 μl sample of the stock cell suspension was centrifuged (9000 *g*, 2 s, 25 °C) and the pelleted cells resuspended in 100 μl of the appropriate test solution. This suspension was added to 2.5 ml of the same test solution in a cuvette (i.e. final cell density of 4×10^4 cells ml^{-1}). Fluorescence of BCECF in the final cell suspension was measured at 25 °C with a spectrofluorometer (Perkin-Elmer, model LS-5, Norwalk, CT). Fluorescence was monitored at excitation wavelengths of 504 nm (peak fluorescence) and 435 nm (isosbestic point) and an emission wavelength of 530 nm, with excitation and emission slit widths of 3 nm. Leakage of BCECF across the haemocyte membrane during pHi studies was negligible, as assessed by centrifugation of several cell suspensions after pHi determinations had been completed and measurement of BCECF fluorescence of the resultant supernatant. No extracellular BCECF was detected.

The intracellular pH probe was calibrated using the K^+–nigericin technique (Thomas *et al.* 1979). This technique assumes that in the presence of nigericin, a K^+/H^+ ionophore, K^+ and H^+ distribute across the cell membrane such that, at equilibrium, $[K^+]_i/[K^+]_e=[H^+]_i/[H^+]_e$, where i and e indicate intracellular and extracellular, respectively. Thus, in the presence of nigericin, when the extracellular K^+ concentration is approximately equal to the intracellular K^+ concentration, pHi and pHe are approximately equal. The intracellular probe was calibrated from the ratio of fluorescence intensities at the two excitation wavelengths (504 nm/435 nm), using haemocytes suspended in solution *B* (composition in mmol l^{-1}: NaCl, 332; KCl, 103; $MgCl_2$, 63; Hepes, 0.6; dextrose, 0.9) with various values of pHe (6.9–7.7 at 25 °C) to which 1 μg ml^{-1} nigericin was added. A K^+ concentration of 103 mmol l^{-1} was selected for the calibration solution (solution *B*), based on the average potassium concentration of the tissues of *Octopus vulgaris* (D'Aniello *et al.* 1986).

Haemocytes were acidified with the weak acids sodium propionate or potassium acetate (40 mmol l^{-1}), to measure and characterize changes in pHi in response to an intracellular acid load. Subsequent alkalinization (pHi recovery) of the cells was followed in the presence or absence of (1) external Na^+, (2) amiloride hydrochloride, an inhibitor of Na^+/H^+ exchange, and (3) *N*-ethylmaleimide (NEM), a nonspecific inhibitor of H^+-ATPase. In Na^+-replete studies, the cells were tested in solution *C* containing (in mmol l^{-1}) NaCl, 135–425 (NaCl replaced isosmotically with choline chloride); KCl, 10; $MgCl_2$, 63; Hepes, 0.6; and dextrose, 0.9; pHe 6.8–7.8 at 25 °C. Haemocytes in Na^+-replete studies were acidified with sodium propionate. In Na^+-free studies, haemocytes were tested in solution *D* (composition in mmol l^{-1}: choline chloride, 425; KCl, 10; $MgCl_2$, 63; Hepes, 0.6; dextrose, 0.9; pHe 7.4 at 25 °C) and acidified with potassium acetate. All experimental solutions were nominally HCO_3^--free, to minimize the potentially confounding effects of anion exchange. To reduce haemocyte aggregation, calcium was omitted from test solutions.

The data are presented as arithmetic means±S.E. Statistical analyses of the data included linear regression using the least-squares method and Student's *t*-tests. A probability of 5% was adopted as the level of significance.

Results

Measurement of haemocyte pHi with BCECF

The spectral properties of cytoplasmic BCECF in a representative sample of haemocytes are shown in Fig. 1A. Peak fluorescence intensity increased as a function of pHi. The relative fluorescence intensity (RFI, fluorescence ratio 504 nm/435 nm) of BCECF in haemocytes was linearly related to pHi over the calibration pH range 6.9–7.7 (r=0.961) (Fig. 1B).

To examine the relationship between pHi and external Na^+ concentration, haemocytes were incubated in solution *C* containing various Na^+ concentrations (0–425 mmol l^{-1} Na^+) at pHe 7.4. Intracellular pH was not significantly correlated with external Na^+ concentration (r=0.058) (Fig. 2A).

To investigate the relationship between pHi and pHe, haemocytes were incubated in solution *C* (425 mmol l^{-1} Na^+, 10 mmol l^{-1} K^+) at various pHe values. The observed pHi/pHe relationship is given in Fig. 2B. Haemocyte pHi varied only slightly over the experimental pHe range of 6.8–7.8 (ΔpHi/ΔpHe=0.16, r=0.560). The relative constancy of pHi in the face of changes in pHe suggests that protons were not in electrochemical equilibrium across the haemocyte membrane. At pHe 7.4, the pHi of squid haemocytes averaged 7.32±0.02 (N=18).

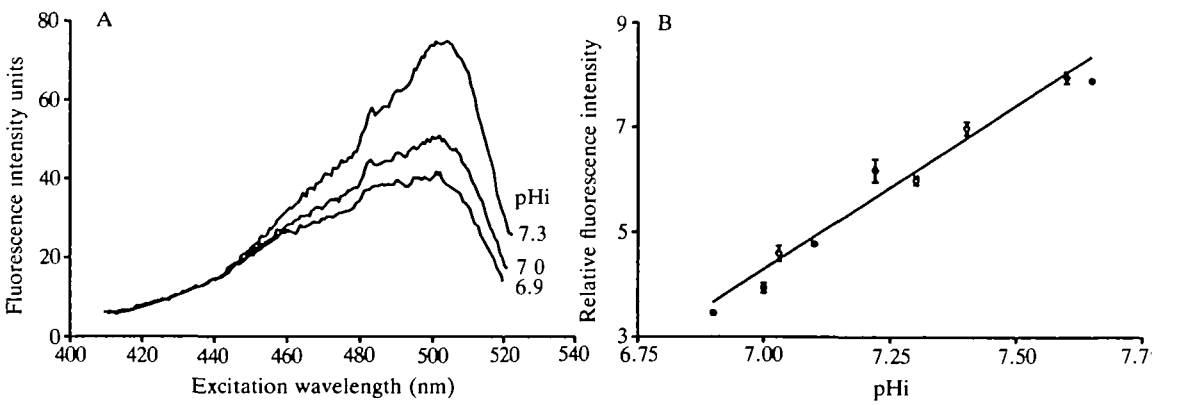

Fig. 1. Measurement of cytoplasmic pH (pHi) of squid haemocytes using the fluorescent probe BCECF. (A) Representative calibration scans of the fluorescence intensity of BCECF in squid haemocytes as a function of excitation wavelength and pHi. The emission wavelength was 530 nm. Cells were suspended in a solution containing 103 mmol l^{-1} K^+ and 1 μg ml^{-1} nigericin, such that when $[K^+]_e=[K^+]_i$ pHi=pHe. (B) Relationship between the relative fluorescence intensity (RFI, fluorescence ratio 504 nm/435 nm) of BCECF in squid haemocytes and haemocyte pHi. Data are mean values±S.E.

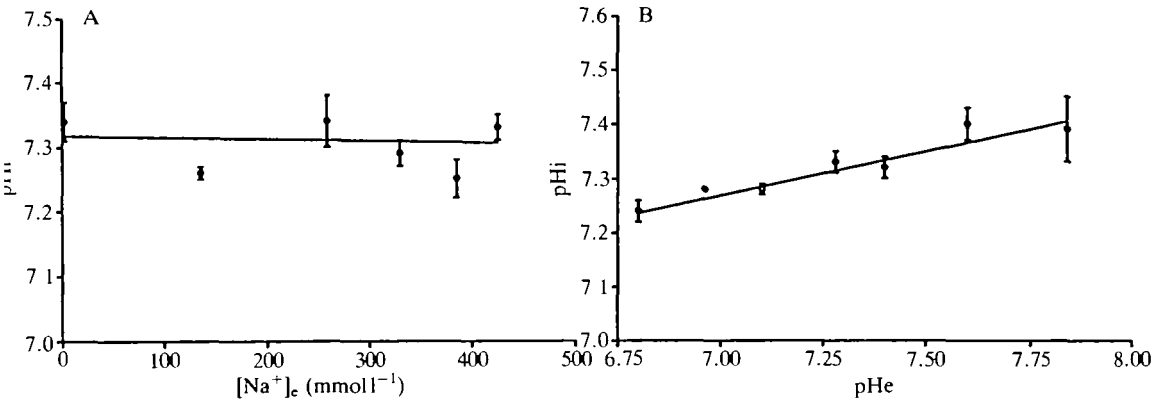

Fig. 2. Intracellular pH (pHi) of squid haemocytes at 25°C. Data are mean values±S.E. (A) pHi as a function of the external Na^+ concentration at a pHe of 7.4. (B) pHi as a function of extracellular pH (pHe), in the presence of 425 mmol l^{-1} external Na^+.

Effects of weak acids on haemocyte pHi

In the presence of 425 mmol l^{-1} Na^+ and at pHe 7.4, addition of weak acids (sodium propionate, potassium acetate) to haemocyte suspensions caused a rapid decrease in RFI, corresponding to an average fall in pHi of 0.25±0.02 (N=6) units in sodium propionate studies and 0.20±0.02 (N=5) units in potassium acetate studies (Fig. 3A, Table 1). The half-time of the acidification process was approximately 6–12 s. Following the acidification phase, pHi slowly recovered with an average half-time of 2.9±0.4 min in studies with sodium propionate and 3.5±0.9 min in studies with potassium acetate (Table 1).

Effects of external Na^+ on pHi recovery

In the absence of external Na^+, addition of potassium acetate to haemocyte suspensions resulted in a rapid decrease in pHi of 0.17±0.01 (N=6) units (Fig. 3B, Table 1). Cytoplasmic pH then recovered with a half-time of 3.1±1.2 min. Haemocyte acidification and subsequent alkalinization were independent of external $[Na^+]$. The ΔpHi observed with potassium acetate in the absence of external Na^+ was not statistically different from values observed with sodium propionate or potassium acetate in the presence of external Na^+ (Table 1). In addition, the half-time of pHi recovery did not differ significantly between Na^+-replete and Na^+-free studies (Table 1).

Effects of amiloride on pHi recovery

Acidification of haemocytes with weak acids and the subsequent alkalinization process were unaffected by 0.1–1.0 mmol l^{-1} amiloride (Table 1, Fig. 3C). Because competition between Na^+ and amiloride for any potential Na^+/H^+ exchanger on the haemocytes would be enhanced at the relatively high Na^+ concentrations of squid saline (425 mmol l^{-1}), 1 mmol l^{-1} amiloride was tested in

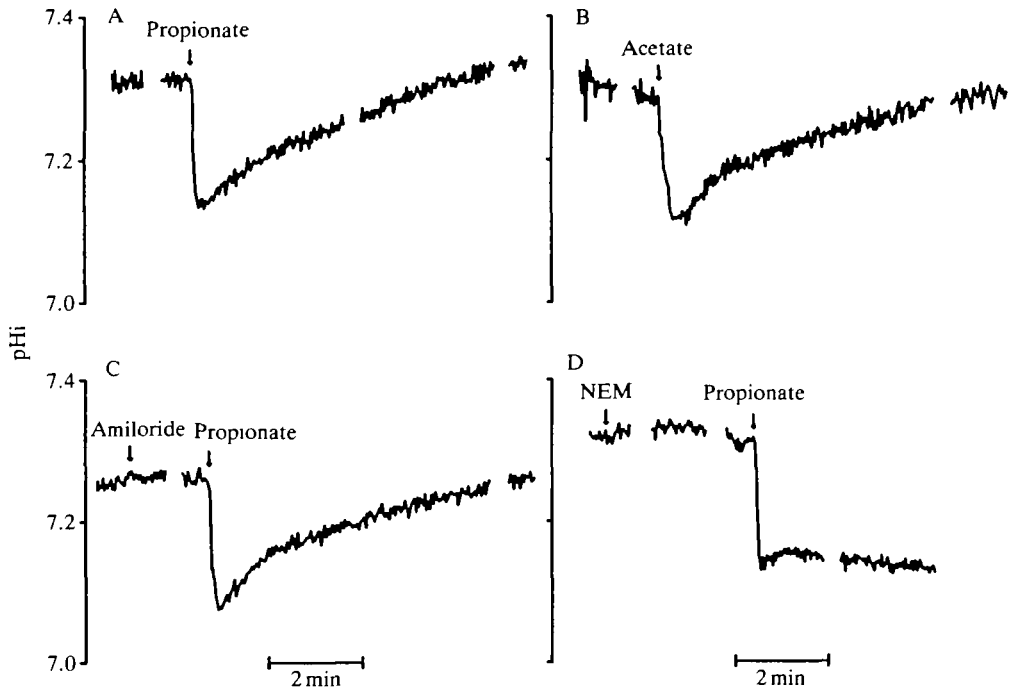

Fig. 3. Representative tracings of the intracellular pH (pHi) of squid haemocytes acidified with weak acids. Breaks in the traces occur when the isosbestic fluorescence (excitation wavelength 435 nm) was determined. (A) Cells acidified with sodium propionate in the presence of 425 mmol l^{-1} Na^+. (B) Cells acidified with potassium acetate in the absence of external Na^+. (C) Cells pretreated with 1 mmol l^{-1} amiloride and then acidified with sodium propionate, in the presence of 425 mmol l^{-1} external Na^+. (D) Cells pretreated with 1 mmol l^{-1} *N*-ethylmaleimide (NEM) and then acidified with sodium propionate, in the presence of 425 mmol l^{-1} external Na^+.

Na^+-replete studies. As a further precaution against bias due to competition between Na^+ and amiloride, pHi recovery of haemocytes was followed in Na^+-free medium in the presence of 0.1 mmol l^{-1} amiloride. Neither the observed ΔpHi nor the half-time of pHi recovery differed significantly among studies with and without amiloride, regardless of the external Na^+ concentration (Table 1).

Effects of N-*ethylmaleimide on pHi recovery*

The ΔpHi observed during acidification of squid haemocytes was unaffected by 1 mmol l^{-1} NEM (Table 1, Fig. 3D). Recovery of pHi, however, was abolished by NEM, regardless of the external Na^+ concentration. When haemocytes were acidified in the presence of NEM, no recovery of pHi towards baseline was evident (observation period ≤10 min).

Discussion

The pHi of squid haemocytes under the present conditions (pHe 7.4, nominall

Table 1. *Effects of extracellular sodium, amiloride and* N-*ethylmaleimide (NEM) on the recovery of intracellular pH (pHi) of squid haemocytes, following a 40 mmol l^{-1} acid load at extracellular pH 7.4*

Pretreatment	Weak acid	$[Na^+]_e$ (mmol l^{-1})	*N*	Baseline pHi	Acidified pHi	ΔpHi	$t_{1/2}$ recovery (min)
None	Sodium propionate	425	6	7.29±0.03	7.05±0.02	0.25±0.02	2.9±0.4
	Potassium acetate	425	5	7.30±0.02	7.12±0.06	0.20±0.02	3.5±0.9
	Potassium acetate	0	6	7.35±0.07	7.18±0.09	0.17±0.01	3.1±1.2
Amiloride	Sodium propionate	425	4	7.35±0.04	7.12±0.05	0.23±0.02	3.5±0.4
	Potassium acetate	0	2	7.28±0.02	7.08±0.01	0.20±0.02	2.8±1.4
NEM	Sodium propionate	425	5	7.37±0.06	7.15±0.02	0.20±0.05	NR
	Potassium acetate	425	3	7.29±0.03	7.14±0.03	0.15±0.01	NR
	Potassium acetate	0	5	7.38±0.07	7.21±0.07	0.16±0.01	NR

Values are means±S.E.
NR, no recovery when observed for ≤10 min.

HCO_3^--free media, 25°C) was approximately 7.3. Howell and Gilbert (1976) suggest that a pHe of 7.4 is normal for the haemolymph of minimally stressed squid at 25°C. It must be pointed out, however, that our estimates of haemocyte pHi were obtained under nominally HCO_3^--free conditions and they are not directly comparable with pHi values *in vivo*.

Cytoplasmic pH was independent of external Na^+ concentration and only marginally affected by pHe in the range 6.8–7.8. Haemocyte pHi was regulated in the face of cellular acidification by a mechanism that was independent of external [Na^+] and insensitive to amiloride. Recovery of pHi following cellular acidification, therefore, did not involve significant plasma membrane Na^+/H^+ exchange. These results also argue against the involvement of other Na^+-dependent transport mechanisms in the observed pHi recovery of acid-loaded haemocytes. Na^+-independent HCO_3^-/Cl^- exchange is an unlikely mechanism for the observed pHi recovery, given the nominal absence of HCO_3^- in haemocyte suspensions. Moreover, in other types of nucleated cells, Na^+-independent HCO_3^-/Cl^- exchange is inactivated under conditions of intracellular acidification (Olsnes *et al.* 1986).

Recovery of haemocyte pHi following cellular acidification was abolished by NEM, a nonspecific inhibitor of H^+-ATPase. These results are consistent with the presence of an NEM-sensitive proton extrusion mechanism in the plasma membrane of squid haemocytes. Similar proton extrusion mechanisms have been described recently for mammalian macrophages, either in parallel with Na^+/H^+ exchange (Swallow *et al.* 1988, 1989) or in the absence of Na^+/H^+ exchange (Bidani *et al.* 1989). The presence of an NEM-sensitive, Na^+-independent mechanism (presumably a proton pump) for pHi recovery in both squid haemocytes and vertebrate macrophages is consistent with Anderson's (1981) proposal that invertebrate haemocytes share many characteristics with the vertebrate monocyte–macrophage series.

Proton pumps have been described previously in a wide variety of intracellular organelles and in the plasma membrane of acid-secreting epithelial cells (reviewed by Stone and Xie, 1988). In acid-secreting epithelial cells, proton pumps are not a constituent element of the plasma membrane but instead are translocated from an intracellular vesicular pool in response to an increase in the intracellular Ca^{2+} concentration (Al-Awqati, 1986). Intracellular Ca^{2+} may play a role in modulating the H^+-ATPase activity of nonepithelial cells (Swallow *et al.* 1988), although it is uncertain whether H^+-ATPase is a constituent element of the plasma membrane of nonepithelial cells or whether an increase in intracellular Ca^{2+} concentration is a necessary signal for insertion of proton pumps into the plasma membrane of such cells. The present study indicates that Na^+-independent alkalinization of acid-loaded haemocytes can occur in the nominal absence of extracellular Ca^{2+}. These results suggest that H^+-ATPase may be a constituent element of the plasma membrane of haemocytes.

NEM is a nonspecific inhibitor of H^+-ATPase. Consequently, alternative interpretations of our NEM data are possible. Assuming BCECF is confined to the

cytoplasm of haemocytes, it is possible that the observed Na^+-independent pHi recovery of acid-loaded haemocytes was attributable to an NEM-sensitive transfer of protons across organelle membranes. A second possibility is that NEM inhibition of pHi recovery resulted from an NEM-induced increase in organic anion (propionate, acetate) efflux across the haemocyte plasma membrane. Nonetheless, the data indicate that Na^+-dependent transport mechanisms, in particular amiloride-sensitive Na^+/H^+ exchange, play an insignificant role in pHi recovery of acid-loaded haemocytes of squid.

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