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EXTRACELLULAR Ca^{2+} AND ITS EFFECT ON ACID EXTRUSION IN THE CRAYFISH STRETCH RECEPTOR NEURONE

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Summary

1. In the stretch receptor neurones of the crayfish *Astacus astacus*, the intracellular pH (pHi), the intracellular Na^+ concentration ($[Na^+]_i$) and the membrane potential (E_m) were measured simultaneously using ion-selective and conventional microelectrodes. Normal *Astacus* saline (NAS), and salines containing varying amounts of Ca^{2+} (Ca^{2+}-NAS) but of constant ionic strength, with Na^+, Mg^{2+} or Ba^{2+} as substituting ions, were used to investigate the effects of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on pHi and pHi regulation, on $[Na^+]_i$ and on E_m. The maximum rate of pHi recovery was used as a measure of pHi regulation. Acid loads were imposed using the NH_4^+/NH_3 rebound technique.

2. $[Ca^{2+}]_o$ affected pHi, pHi regulation, $[Na^+]_i$ and E_m. The magnitudes of the effects were inversely related to $[Ca^{2+}]_o$ and were specific to the ion used for $[Ca^{2+}]_o$ substitution.

3. Compared with controls, increasing $[Ca^{2+}]_o$ threefold (in exchange for Na^+) elicited some alkalization, a 7 % faster maximum rate of pHi recovery and generally lower values of $[Na^+]_i$.

4. In low-Ca^{2+} or Ca^{2+}-free NAS (substitutions by Na^+ or Mg^{2+}), pHi became more acid, the maximum rate of pHi recovery was reduced by up to 50 % and $[Na^+]_i$ was generally higher. The effects were faster and larger at lower $[Ca^{2+}]_o$, and stronger with Na^+ than with Mg^{2+} as the substituting ion.

5. In Ca^{2+}-free NAS (Ca^{2+} substituted for by Ba^{2+}), the effects on pHi, on the maximum rate of pHi recovery and on $[Na^+]_i$ were generally small. In this respect, Ba^{2+} had similar physiological properties to Ca^{2+} and was almost equally effective.

6. Changes in E_m, including rapid depolarizations and occasional burst activity in Ca^{2+}-free NAS, indicate that alterations in the properties of the membrane, such as a change in its permeability or selectivity, are occurring. Measurements of $[Na^+]_i$ support this view. In addition, Ba^{2+} *per se* induced a (small) depolarization, as shown when Ba^{2+} was present in NAS or in low-Ca^{2+} NAS.

7. Changes in $[Ca^{2+}]_o$ affected $[Na^+]_i$. *$[Na^+]_i$ is defined as $[Na^+]_i$ determined at the onset of the maximum rate of pHi recovery, and the ratio *$[Na^+]_i/[Na^+]_o$ at that instant was calculated. A linear relationship between the maximum rate of pHi recovery and the *$[Na^+]_i/[Na^+]_o$ ratio was found, irrespective of the amount and of the ion species used for $[Ca^{2+}]_o$ substitution. This is strong evidence that pHi and pHi regulation were indirectly affected by $[Ca^{2+}]_o$, which altered membrane properties and thus caused a change in $[Na^+]_i$. We could find no evidence for a direct contribution of $[Ca^{2+}]_o$ to acid extrusion or to a direct modulatory action on the transport protein of the Na^+/H^+ antiporter.

Key words: intracellular pH, pHi regulation, intracellular Na^+, Ca^{2+} substitutes, Ba^{2+}, Mg^{2+}, EGTA, ion-selective microelectrode, sensory neurone, stretch receptor, crayfish, *Astacus astacus*.

Introduction

Among a variety of other effects, extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is known to affect the properties of membrane proteins such as ion channels (e.g. Frankenhaeuser and Hodgkin, 1957; Armstrong and Cota, 1991; Schirrmacher and Deitmer, 1991; Hille, 1992) and ion carriers (Vaughan-Jones *et al.* 1983). In this work, we are mainly concerned with the effects of $[Ca^{2+}]_o$ on the ion carriers involved in the regulation of intracellular pH (pHi).

Both Na^+/H^+ and $Na^+/H^+/HCO_3^-/Cl^-$ antiporters contribute to acid extrusion in different crayfish cells (sensory neurones, Moser, 1985; Mair *et al.* 1993; ganglion cells, Moody, 1981; muscle cells, Galler and Moser, 1986) as well as in many other cells (e.g. Roos and Boron, 1981; Moody, 1984; Thomas, 1984; Chesler, 1990). Mair *et al.* (1993) investigated the relationship between acid extrusion and $[Na^+]_o$ in sensory neurones. They found that almost 80 % of pHi recovery was due to the activity of the Na^+/H^+ antiporter, with the Na^+ gradient serving as its main source of energy. The experiments described below were performed in order to examine whether, and in what way, $[Ca^{2+}]_o$ affects the operation of the Na^+/H^+

(and the $Na^+/H^+/HCO_3^-/Cl^-$) antiporter. Every $[Ca^{2+}]_o$-induced effect on the Na^+/H^+ antiporter should result in a change in the rate of pHi recovery, from which the maximum rate of recovery and related parameters (see Materials and methods) could be determined using conventional and Na^+- and H^+-selective microelectrodes to measure membrane potential (E_m), $[Na^+]_i$ and pHi.

Materials and methods

Slowly adapting stretch receptor neurones from abdominal segments 2–5 of *Astacus astacus* L., bought from a hatchery in Augsburg (Germany), were dissected as described previously (Moser *et al.* 1979). Calibration of ion-selective microelectrodes (ISMs) and experiments were carried out in an experimental chamber connected to a flow-through system, which exchanged the bath volume of 0.7 ml within 5–6 s, at a temperature of 16 °C (Fresser *et al.* 1991). From 50 cells tested, the results from only 20 could be evaluated quantitatively, mainly because of difficulties with the electrodes.

Chemicals and solutions

Chemicals of highest purity were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany). Normal *Astacus* saline (NAS), modified from that devised by van Harreveld (1936), consisted of (in mmol l^{-1}): 207 NaCl, 5.4 KCl, 2.4 $MgCl_2$, 13.5 $CaCl_2$, 10 Hepes, adjusted to pH 7.4 with NaOH. The pH of all solutions was controlled using an Orion 8162 (Ross-type) glass electrode. For pH 6.4 salines, Pipes was used instead of Hepes.

$CaCl_2$ was substituted for either by equimolar amounts of the Cl^- salt of a divalent cation (e.g. $BaCl_2$, $MgCl_2$) or by a 1.5-fold higher concentration of NaCl in order to keep the ionic strength constant. 1 mmol l^{-1} EGTA was added to some Ca^{2+}-free salines. In low-Na^+ salines, reductions in [NaCl] were compensated for by the addition of equimolar amounts of *N*-methyl-D-glucamine (NMDG), and HCl was used to adjust pH to 7.4. In NH_4^+-NAS, 20 mmol l^{-1} NH_4Cl was substituted for 20 mmol l^{-1} NaCl. KCl was compensated for by equimolar substitution of NaCl.

All experiments were performed in nominally HCO_3^-/CO_2-free salines. Cells were acidified according to the NH_4^+/NH_3 rebound technique (Boron and de Weer, 1976). The terminology used by these authors will be adopted here.

Special terminology

The maximum pHi recovery rate is the fastest rate of pHi recovery after acid loading under various experimental conditions and usually occurs within 1 min after maximum pHi acidification. It was calculated from the steepest slope of pHi recovery (see Figs 1, 6). Maximum pHi recovery rates of test exposures were compared with those of controls (=100 %).

$*[Na^+]_i$ and $*[H^+]_i$

Concentrations marked with an asterisk (*) were measured at the moment when the maximum pHi recovery rate was achieved (see Fig. 1, arrows). The ratios $*[Na^+]_i/[Na^+]_o$ and $[H^+]_o/*[H^+]_i$ refer to measurements at that instant. Ion concentrations without asterisks are steady-state values.

Evaluation

The values given represent the means ± the standard deviation (S.D.) from the mean from a certain number (N) of experiments. Student's unpaired t-tests were applied and significance was accepted at $P<0.05$. Least-squares regression was used to fit lines to the data.

Microelectrodes, electrical arrangement, recording and display

The tips of single-barrelled Na^+ microelectrodes were filled with Fluka no. 71176 membrane cocktail and the H^+ barrels of double-barrelled pH/E_m microelectrodes with Fluka no. 95293. Electrode fabrication, silanization and calibration are described in detail elsewhere (Mair *et al.* 1993). Pen recordings were digitized, using SummaSketch II hardware, and processed using Sigma-Scan V 3.90 software, converting mV to mmol l^{-1} or pH values. For data presentation, SigmaPlot 4.1 and 5.0 were used, giving rise to the small steps in Figs 1 and 6.

Limitations of Na^+ used as a Ca^{2+} substitute

Alterations to $[Ca^{2+}]_o$ between 0 and 40.5 mmol l^{-1} cause respective changes in $[Na^+]_o$ between 171.5 and 232.25 mmol l^{-1}. Taking into account an additional 20 mmol l^{-1} reduction of $[Na^+]_o$ from exposure to NH_4^+/NH_3, the lowest value of $[Na^+]_o$ should thus be close to 150 mmol l^{-1}.

Four cells were exposed for 10 min each to salines with $[Na^+]_o$ reduced from 212 mmol l^{-1} to as low as 50 mmol l^{-1}, compensating for NaCl with NMDG. Despite this huge $[Na^+]_o$ change, E_m hyperpolarized by only 2.9±0.8 mV, $[Na^+]_i$ decreased by 6.3±3.7 mmol l^{-1} and pHi acidified by 0.078±0.03 units, each parameter obtaining a quasi-stable value within about 3 min. As shown by previous results (Mair *et al.* 1993), the normalized maximum pHi recovery rate is still about 75 % in NAS containing only 50 mmol l^{-1} Na^+.

There is only a limited operational range over which $[Ca^{2+}]_o$ can be increased, otherwise the concomitant changes in $[Na^+]_o$ *per se* will affect pHi recovery. Increasing $[Ca^{2+}]_o$ 10-fold would reduce $[Na^+]_o$ by 182 mmol l^{-1}, and the resulting $[Na^+]_o$ would be close to the K_m value at which the half-maximal pHi recovery rate occurs (see Mair *et al.* 1993). Thus, detailed experiments with $[Ca^{2+}]_o$ increased by 10-fold were excluded.

On the basis of these results, we conclude that E_m, pHi and pHi regulation are minimally, if at all, affected by the much smaller $[Na^+]_o$ changes imposed during the experiments reported below.

Protocol

The sequence of exposures started with controls (NAS), followed by salines with high $[Ca^{2+}]$ (e.g. 40.5 mmol l^{-1}) before testing, in decreasing order, salines with low $[Ca^{2+}]$. Each test was followed by a control exposure. Test exposures at various $[Ca^{2+}]_o$ levels usually lasted for a total of 11 or

12 min, consisting of 3 min of pre-incubation, 3 min of NH_4^+/NH_3 exposure and an additional 5 or 6 min in saline of the same $[Ca^{2+}]_o$ in order to observe effects on pHi recovery, before washout in NAS.

Results

Reciprocal substitution of Ca^{2+} and Na^+

The effects of varying $[Ca^{2+}]_o$ on pHi, $[Na^+]_i$ and E_m were investigated. The superimposed recordings in Fig. 1 are from a representative single cell in which control and test exposures followed alternately, but for clarity of presentation only one control recording is shown. The recordings indicate that pHi, $[Na^+]_i$ and E_m recovered completely between the experiments.

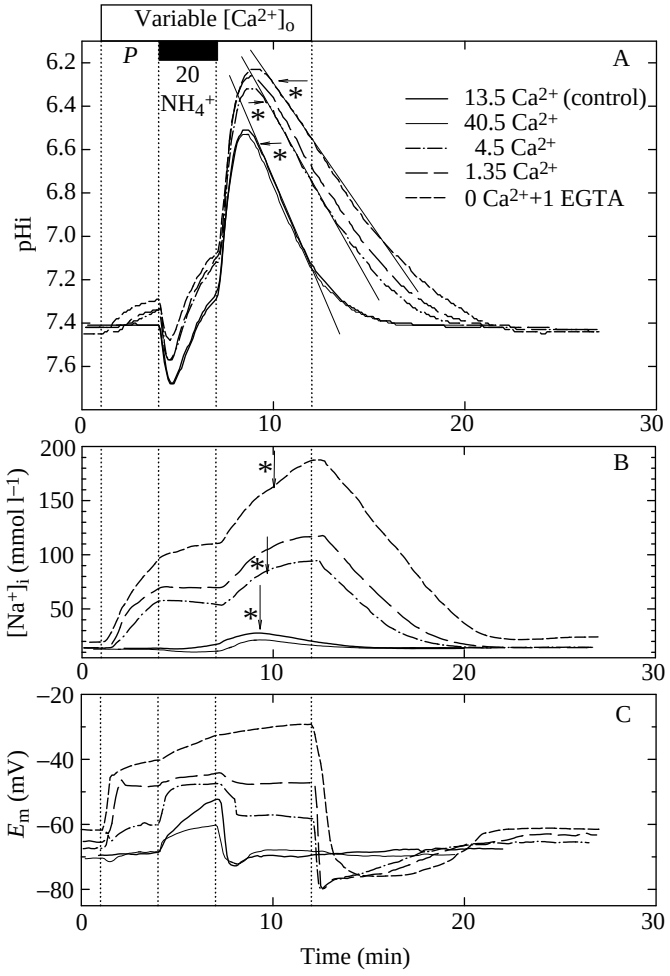

Fig. 1. Superimposed computer tracings from a single cell. Simultaneous measurements of intracellular pH (pHi, A), intracellular Na^+ concentration ($[Na^+]_i$, B) and membrane potential (E_m, C) at various concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$). Ca^{2+} and Na^+ were substituted reciprocally, keeping ionic strength constant. Tangents were drawn in order to calculate maximum pHi recovery rates, and the time at which maximum pHi recovery began is indicated by arrows. Measurements of $[H^+]$ and $[Na^+]$ referring to that instant are denoted by an asterisk ($*[Na^+]_i$, $*([H^+]_i)$ (also see Materials and methods). Concentrations are given in mmol l^{-1}. *P*, pre-incubation period.

Thus, after-effects from prior exposures are unlikely, although dramatic changes in the values of all the parameters of interest could occur during a single test, as will be shown below.

A 3 min exposure to NH_4^+/NH_3 produces essentially the same features of a pHi response in controls and in salines containing different Ca^{2+} concentrations. We found that the pHi recovery curve had a constant portion (see Fig. 1), from which the maximum pHi recovery rate was calculated, and a subsequent declining portion. Usually, the decline started at a value (called the 'set point' by Grinstein *et al.* 1989) which was 0.3–0.4 pH units below steady-state pHi. The onset of maximum pHi recovery was determined. At that instant (arrows in Fig. 1A), the voltages from the Na^+- and H^+-selective microelectrodes were measured and the corresponding values of $*[Na^+]_i$ and pHi or $*[H^+]_i$ were calculated (assuming the same intra- and extracellular activity coefficients).

The main goal of this study was to investigate the effects of $[Ca^{2+}]_o$ on pHi recovery. In Fig. 2, the maximum rate of pHi recovery is plotted *versus* $[Ca^{2+}]_o$. The results obtained during an increase as well as a decrease in $[Ca^{2+}]_o$ will be described in more detail below.

Increase of extracellular $[Ca^{2+}]$

A comparison of controls (=NAS) and cells exposed to Ca^{2+}-NAS containing three times the control $[Ca^{2+}]$ (40.5 mmol l^{-1} Ca^{2+}) showed that there was hardly any effect on the steady-state values of pHi, $[Na^+]_i$ and E_m during the pre-incubation period (Fig. 1). In the presence of NH_4^+/NH_3, the induced depolarization was smaller in 40.5 mmol l^{-1} Ca^{2+} than in controls. The mean values of maximum acidification and the set point for the decline of pHi regulation were hardly affected when compared with controls. $[Na^+]_i$ decreased (Fig. 1B) and the maximum pHi recovery rate was significantly faster (107±6 %;

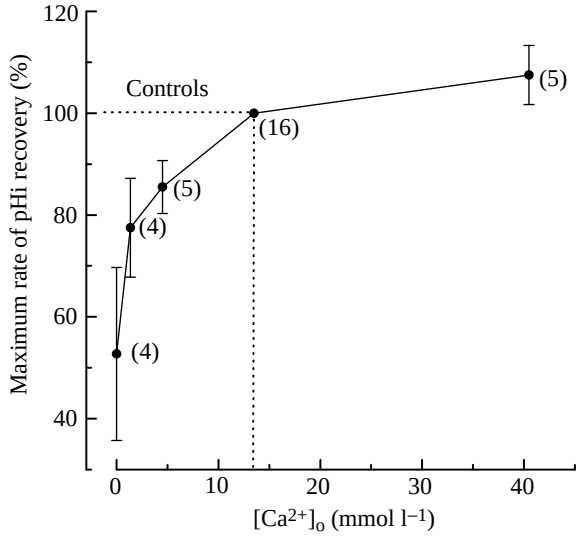

Fig. 2. Plot of normalized maximum pHi recovery rates *versus* extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from different numbers (*N*, given in parentheses) of cells. Ca^{2+} and Na^+ were substituted reciprocally, keeping ionic strength constant. Values are means ± S.D. The mean values are significantly different from each other ($P<0.05$).

N=5) in 40.5 mmol l^{-1} $[Ca^{2+}]_o$ (Fig. 2). At the onset of maximum pHi recovery, $*[Na^+]_i$ decreased from 32.6±15 mmol l^{-1} (controls) to 22.2±3.9 mmol l^{-1} in 40.5 mmol l^{-1} $[Ca^{2+}]_o$. These $*[Na^+]_i$ control values agree well with those reported previously (31.1±11 mmol l^{-1}; Mair *et al.* 1993).

In a pilot study, we substituted all $[Na^+]_o$ iso-osmotically with $[Ca^{2+}]_o$ (the saline contained 0.67 times the control $[Ca^{2+}]$; not shown) and observed a complete inhibition of pHi recovery. If any $Ca^{2+}/2H^+$ exchange were present, some pHi regulation should have been detectable; however, this was not the case.

Decrease of extracellular [Ca^{2+}]

$[Ca^{2+}]_o$ was reduced from its normal concentration of 13.5 mmol l^{-1} in NAS to low-Ca^{2+} NAS containing 4.5 mmol l^{-1} (=one-third normal $[Ca^{2+}]$) or 1.35 mmol l^{-1} (=one-tenth normal $[Ca^{2+}]$) and to nominally Ca^{2+}-free NAS (Fig. 1). The small differences in the steady-state values of pHi, $[Na^+]_i$ and E_m at the beginning of each experiment, and the remarkable consistency in control values measured between test exposures (not shown), indicate that after-effects from prior exposures are negligible.

A reduction in $[Ca^{2+}]_o$ affected steady-state pHi, $[Na^+]_i$ and E_m, with the strongest effects observed in Ca^{2+}-free NAS. Generally, we found a change to a more acidic pHi, a rapid increase in $[Na^+]_i$ and a rapid depolarization of E_m; these tendencies may also be seen during the 3 min of pre-incubation in Fig. 1. In this cell, the rate of $[Na^+]_i$ increase was 19 mmol l^{-1} min^{-1} in 4.5 mmol l^{-1} Ca^{2+}-NAS and 40 mmol l^{-1} min^{-1} in 0-Ca^{2+}-EGTA-NAS. In another four cells, exposed to 1.35 mmol l^{-1} Ca^{2+}-NAS for 10 min (without an acid load), we found that pHi acidified by 0.133±0.075 units, $[Na^+]_i$ increased by 36.4±28.6 mmol l^{-1} and E_m depolarized by 7.1±7.4 mV. These effects were most marked immediately following the solution change.

As shown in Fig. 2, the mean values of maximum rate of pHi recovery decreased in 4.5 mmol l^{-1} Ca^{2+} to 85±5 % (N=5), in 1.35 mmol l^{-1} Ca^{2+} to 77±9 % (N=4) and in 0-Ca^{2+} to 53±17 % (N=4), compared with controls (100 %; N=16). Thus, the maximum pHi recovery rate in Ca^{2+}-free saline was only about half that in controls. Upon return to NAS (with normal Ca^{2+}), the maximum pHi recovery rate was not usually enhanced. This can be seen in all the recordings with Ca^{2+}-reduced or Ca^{2+}-free salines (Fig. 1), particularly in the latter, where the maximum pHi recovery rate hardly changed for an additional 3 min after Ca^{2+} had been restored. In this cell, the rate of pHi recovery began to decline at a 'set point' of approximately pHi 7.0, which remained unchanged by the prior low-Ca^{2+} exposures.

Upon Ca^{2+}-free (Na^+) exposure, a small acceleration of acid extrusion was observed in just one cell, which also showed a short, transient retardation of pHi recovery immediately after the solution was changed.

On average (N=4–5), $*[Na^+]_i$ increased several-fold, from 33±15 mmol l^{-1} (control) to 68±24 mmol l^{-1} in 4.5 mmol l^{-1} Ca^{2+}, to 92±36 mmol l^{-1} in 1.35 mmol l^{-1} Ca^{2+} and to 161±23 mmol l^{-1} in 0-Ca^{2+} measured at the onset of maximum pHi recovery. Using these values, $*[Na^+]_i/[Na^+]_o$ ratios of 0.154 (control), 0.30, 0.40 and 0.69 were calculated and plotted *versus* $[Ca^{2+}]_o$ (Fig. 3A). The fourfold higher $*[Na^+]_i/[Na^+]_o$ ratio elicited in Ca^{2+}-free (Na^+) NAS was mainly due to an increase in $[Na^+]_i$. It appears to be unimportant whether a background level of 1 or 10 µmol l^{-1} Ca^{2+} was assumed in 'Ca^{2+}-free' NAS, as the 95 % confidence interval of the regression included both values.

In a similar graph (Fig. 3B), the relationship between the ratio $[H^+]_o/*[H^+]_i$ and $[Ca^{2+}]_o$ is drawn. As $[H^+]_o$ remained constant in all experiments, alterations of the $[H^+]_o/*[H^+]_i$ ratio reflect changes in $*[H^+]_i$. Measured at the onset of maximum

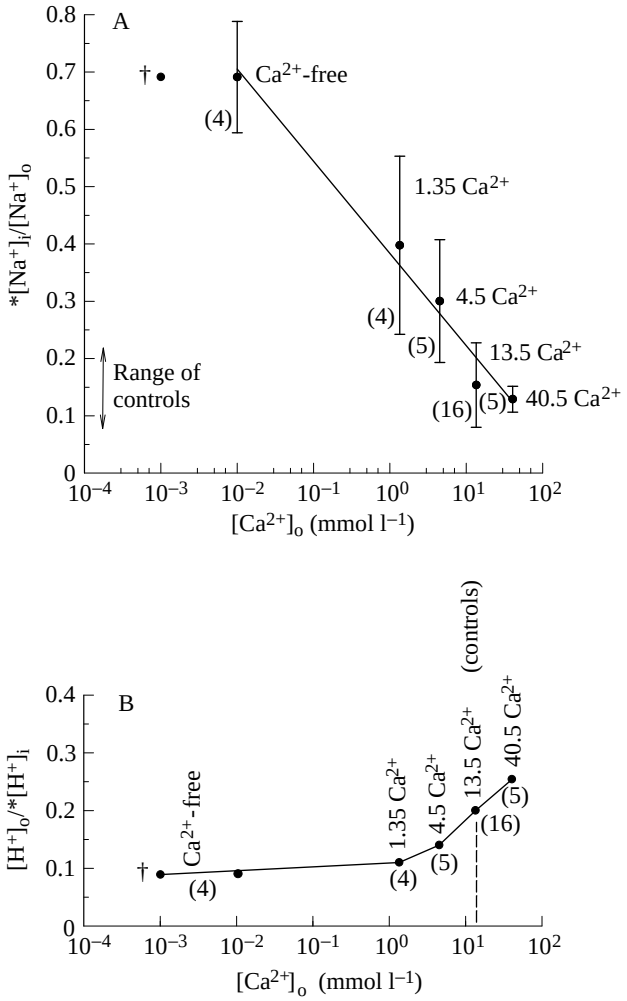

Fig. 3. Semilogarithmic plots of transmembrane ratios of Na^+ (A) and H^+ (B) *versus* extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from the number (N) of cells given in parentheses. Ca^{2+} and Na^+ were substituted reciprocally. Both $[Na^+]_i$ and $[H^+]_i$ were measured at the onset of maximum pHi recovery, as denoted by an asterisk ($*[Na^+]_i$, $*[H^+]_i$). The regression equation in A is y=–0.161x+0.383 (correlation coefficient 0.990). It seems unimportant whether a 1 or 10 µmol l^{-1} Ca^{2+} background (value marked †) was assumed in 'Ca^{2+}-free' saline, as the calculated 95 % confidence interval (not shown) includes both values. Values are means ± S.D. Ca^{2+} concentrations are given in mmol l^{-1}.

pHi recovery, Ca^{2+}-free saline caused a twofold increase of $*[H^+]_i$ when compared with controls. While $[Ca^{2+}]_o$ reduction below 1 mmol l^{-1} hardly affected the $[H^+]_o/*[H^+]_i$ ratio, this range was very important for the $*[Na^+]_i/[Na^+]_o$ ratio. This is shown in Fig. 4, where the maximum rate of pHi recovery is plotted *versus* the $*[Na^+]_i/[Na^+]_o$ ratio.

Ca^{2+}-free NAS: substitutions with Na^+, Mg^{2+} or Ba^{2+}

In Ca^{2+}-free NAS, with Na^+ as the substituting ion, tests were performed in the absence as well as in the presence of EGTA, which buffers $[Ca^{2+}]_o$ to about 10^{-8} mol l^{-1}. The results were basically similar, except that in the presence of EGTA any kind of recovery was much slower and rather incomplete, as shown in Fig. 5. These recordings were made with a pen recorder, to give a better impression of additional biological actions such as action potentials and late membrane changes.

Fig. 5 shows tracings of pen recordings and Fig. 6 of computer recordings of the superimposed responses from two different cells exposed to Ca^{2+}-free salines in which different substituting ions were used. Tests were separated by control exposures (not shown). Again, the high degree of recovery of pHi, $[Na^+]_i$ and E_m indicates that after-effects from previous exposures are negligible.

During pre-incubation, Ca^{2+}-free NAS usually elicited some depolarization. This was strongest (100 %) with Na^+ as the substituting ion, while values of 77±11 % (N=7) were obtained with Mg^{2+} and 48±13 % (N=12) with Ba^{2+}. In addition, Ca^{2+}-free saline elicited an increase in $[Na^+]_i$, which was greatest (100 %) with Na^+ as the substituting ion, whereas with Mg^{2+} the $[Na^+]_i$ increase was reduced to 53±15 % (N=7) and with

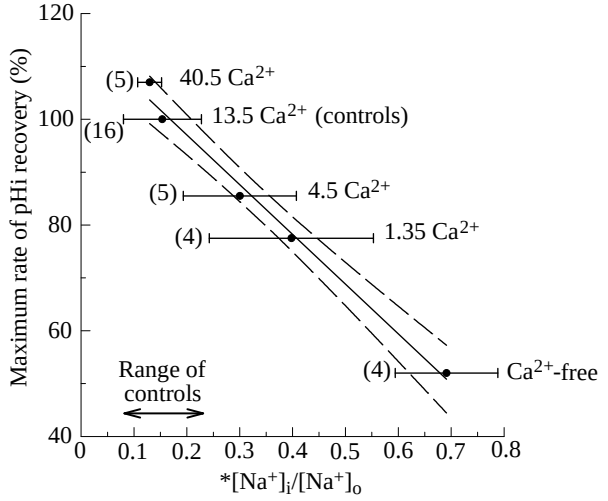

Fig. 4. Plot of normalized maximum pHi recovery rates (%) *versus* transmembrane $[Na^+]$ ratios with $[Na^+]_i$ measured at the onset of maximum pHi recovery as denoted by an asterisk. Ca^{2+} and Na^+ were substituted reciprocally. The regression equation is y=–92.88x+115.87 (correlation coefficient 0.995). The mean values are significantly different from each other. For the sake of clarity, standard deviations for the maximum rate of pHi recovery have been omitted but were as shown in Fig. 2. Values are means ± S.D., values of N are given in parentheses. Ca^{2+} concentrations are given in mmol l^{-1}.

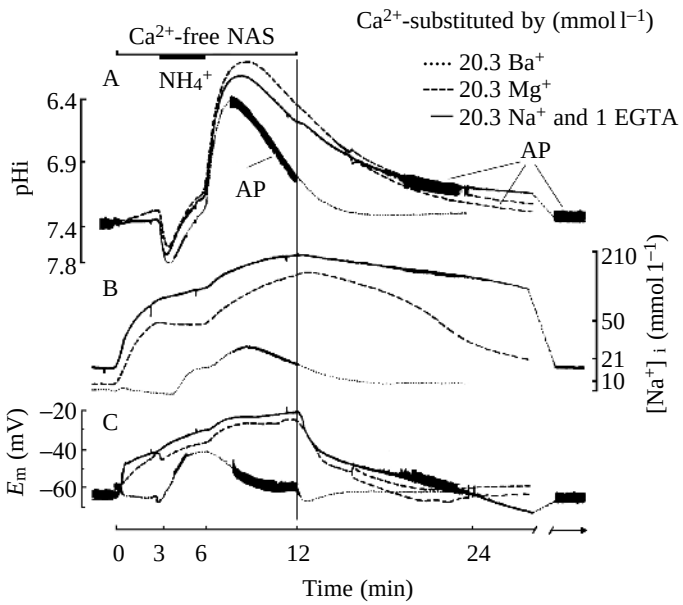

Fig. 5. Superimposed pen recordings (partially redrawn) from a single cell and simultaneous measurements of pHi (A), intracellular Na^+ concentration ($[Na^+]_i$, B) and membrane potential (E_m, C) in Ca^{2+}-free salines. Extracellular Ca^{2+} was substituted by equimolar Ba^{2+} or Mg^{2+} or by 1.5 times $[Na^+]$ to which 1 mmol l^{-1} EGTA had been added. The pHi response in the presence of Ba^{2+} is similar to that seen in controls. The interruption shown at the end of the Na^+-EGTA recording lasted 25 min. AP, action potential; NAS, normal *Astacus* saline.

Ba^{2+} the increase almost disappeared (3±6 %; N=12). Complete substitution of Ca^{2+} by Na^+ or Mg^{2+} resulted in intracellular acidification. Both acidification and the increase in $[Na^+]_i$ were faster and stronger with Na^+ than with Mg^{2+}.

Comparing NH_4^+/NH_3 exposures with controls, the level of maximum acidification was larger, but the maximum rate of pHi recovery slower when Na^+ or Mg^{2+} replaced $[Ca^{2+}]_o$. In three tests each, the maximum rate of pHi recovery was reduced to 55±15 % (Na^+) and 68±4 % (Mg^{2+}) and at the onset of maximum pHi recovery $*[Na^+]_i$ increased to 161±23 mmol l^{-1} (Na^+) and 111±44 mmol l^{-1} (Mg^{2+}), and $[Na^+]_i$ remained high or even increased until the solution was changed to NAS. At the onset of maximum pHi recovery, pHi was 6.36 (Na^+) and 6.39 (Mg^{2+}), resulting in a value for $*[H^+]_i$ of 4.36×10^{-7} mol l^{-1} in Na^+-substituted saline and 4.07×10^{-7} mol l^{-1} in Mg^{2+}-substituted saline.

The results obtained with Ca^{2+}-free NAS in which Na^+ or Mg^{2+} was substituted for $[Ca^{2+}]_o$ clearly contrast with those obtained when Ba^{2+} was the substituting ion. During pre-incubation, Ba^{2+} caused a small $[Na^+]_i$ decrease and during NH_4^+/NH_3 exposure, the $[Na^+]_i$ response was much like that of a control cell, but at a lower absolute $[Na^+]_i$ level. Upon return from Ca^{2+}-free (Ba^{2+}) NAS to NAS, $[Na^+]_i$ gradually returned to the value attained before $[Ca^{2+}]_o$ was substituted.

In Ca^{2+}-free (Ba^{2+}) NAS, pHi hardly changed during pre-incubation and on exposure to NH_4^+/NH_3 the pHi responses closely resembled those of controls. Small differences in the

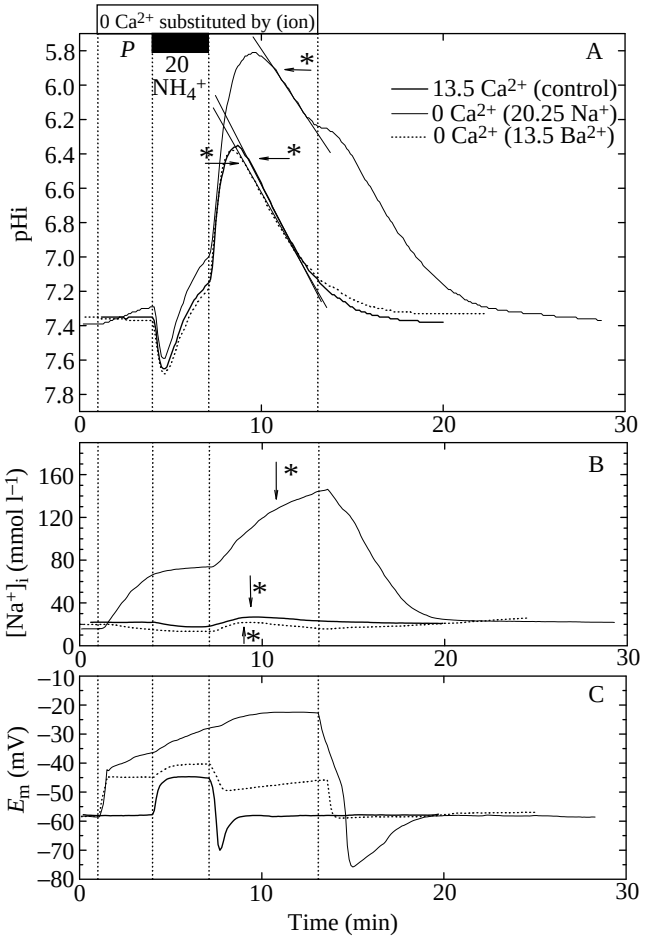

Fig. 6. Superimposed computer recordings from a single cell in Ca^{2+}-free salines. Simultaneous measurements of pHi (A), intracellular Na^+ concentration ($[Na^+]_i$, B) and membrane potential (E_m, C) are shown. $[Ca^{2+}]_o$ was substituted by equimolar Ba^{2+} or by 1.5 times $[Na^+]$ in order to keep ionic strength constant. See Fig. 1 for further details. Concentrations are given in mmol l^{-1}. *P*, pre-incubation period.

maximum amount of acidification as well as in the maximum rate of pHi recovery seem to be within experimental variability. If $[Ca^{2+}]_o$ had any direct or indirect functional role during pHi recovery, it was almost perfectly fulfilled by Ba^{2+} too.

The variable and specific actions of substituting ions in low-Ca^{2+} and in Ca^{2+}-free NAS on the maximum rate of pHi recovery are closely related to the ratio $*[Na^+]_i/[Na^+]_o$, as shown in Fig. 7. For better comparison, the results from Fig. 4 have been redrawn as open symbols in Fig. 7.

Discussion

Effects of $[Ca^{2+}]_o$ on pHi, pHi recovery, $[Na^+]_i$ and E_m

During their lifetime, crustaceans are repeatedly subjected to moulting cycles, accompanied by periodic changes in ion concentrations within their body (e.g. Cameron and Wood, 1985; Roer and Dillaman, 1984; Greenaway, 1985; Wheatly and Ignaszewski, 1990). While levels of ionized Ca^{2+} seem to be fairly well controlled (Greenaway, 1974, 1985; Zanotto and Wheatly, 1993), levels of bound Ca^{2+} show clear changes. However, mechanisms to control pHi are present, with both Na^+/H^+ and $Na^+/H^+/HCO_3^-/Cl^-$ antiporters available for acid extrusion in crayfish stretch receptor neurones (Mair *et al.* 1993; Moser, 1985). The main goal of this study was to investigate whether $[Ca^{2+}]_o$ affected pHi and pHi regulation and, if so, to explain the mechanism.

To a certain extent it seems that $[Ca^{2+}]_o$ affects both pHi and the maximum rate of pHi recovery (Fig. 1) in a way that is similar to $[Na^+]_o$, as demonstrated when the latter was substituted for by NMDG (Mair *et al.* 1993). Both low $[Ca^{2+}]_o$ and low $[Na^+]_o$ elicit some acidification which saturates with time, and both these salines partially inhibit pHi regulation upon exposure to NH_4^+/NH_3. Although in Ca^{2+}-free NAS (Fig. 2), even in the presence of EGTA, pHi recovery is reduced by only 50 %, it is completely stopped in Na^+-free saline. After exposure to low $[Na^+]_o$, washout in NAS always resulted in an instantaneous acceleration of pHi recovery; this was usually not observed after exposure to low $[Ca^{2+}]_o$. Taken together, these results indicate that, at least for pHi recovery, the action and functional roles of Na^+ and Ca^{2+} are quite different. While Na^+ is directly involved in the operation of the Na^+/H^+ antiporter as an essential substrate, Ca^{2+} acts indirectly, exerting its effect *via* the transmembrane Na^+ gradient (see below).

Simultaneous measurements of $[Na^+]_o$ and pHi enabled us

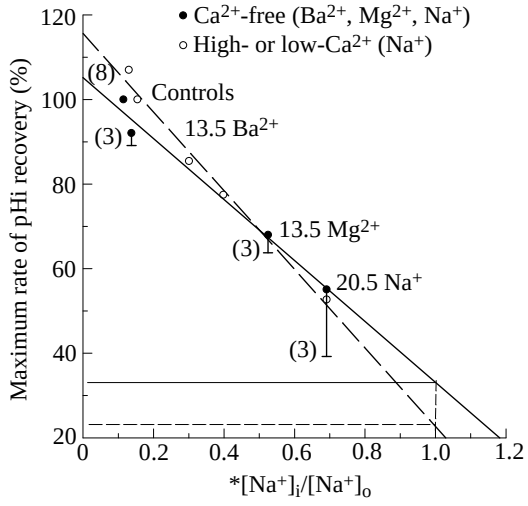

Fig. 7. Plot of normalized maximum pHi recovery rates (%) *versus* the transmembrane ratios of Na^+, with $[Na^+]_i$ measured at the onset of maximum pHi recovery rate, as denoted by an asterisk. Filled circles represent data from cells exposed to Ca^{2+}-free NAS with different substituting ions, while open circles represent data from Fig. 4, for comparison. The regression equation is $y=-72.0x+105.2$ (correlation coefficient 0.992). Using both regressions and extrapolating each for $[Na^+]_i=[Na^+]_o$, as indicated by a solid and a dashed line, the normalized maximum rates of pHi recovery cover a range of about 30 %. Values are means ± S.D., values of *N* are given in parentheses. Concentrations of Ba^{2+}, Mg^{2+} and Na^+ are given in mmol l^{-1}.

to calculate the ratios $*[Na^+]_i/[Na^+]_o$ and $[H^+]_o/*[H^+]_i$ at the onset of maximum pHi recovery. On a semilogarithmic scale, the relationship between mean $*[Na^+]_i/[Na^+]_o$ and $[Ca^{2+}]_o$ was linear, while that between mean $[H^+]_o/*[H^+]_i$ and $[Ca^{2+}]_o$ was nonlinear, as shown in Fig. 3A,B. Since in the sensory cell, the transmembrane Na^+ gradient must be considered to be the main energy source for pHi regulation (Mair *et al.* 1993), the maximum rate of pHi recovery was plotted *versus* the $*[Na^+]_i/[Na^+]_o$ ratio (Fig. 4). Irrespective of $[Ca^{2+}]_o$, all mean values are within the 95% confidence interval of that regression, suggesting a strong correlation between the maximum rate of pHi recovery and the transmembrane $[Na^+]$ gradient. In view of the considerable range of standard deviations for the controls, which has been noticed before (Mair *et al.* 1993), the range of standard deviations in experimental treatments is not surprising.

Fig. 4 also shows a 50% reduction in maximum pHi recovery rate at a $*[Na^+]_i/[Na^+]_o$ of about 0.7; this was due to an increase in $*[Na^+]_i$. Compared with previous results (Mair *et al.* 1993; Fig. 7), the same degree of inhibition of the rate of pHi recovery was observed when $[Na^+]_o$ was reduced to 21 mmol l^{-1} (K_m value). Taking into account the $[Na^+]_i$ decrease that could be elicited in these tests by low-Na^+ saline, it seems probable that a $*[Na^+]_i/[Na^+]_o$ ratio of approximately 0.7 existed. This indicates that, under both experimental conditions, the Na^+/H^+ antiporter was fuelled by the Na^+ gradient.

Relatively rapid depolarizations of E_m as well as burst-like activity were observed when the saline was changed to low-Ca^{2+} or Ca^{2+}-free NAS. In crayfish stretch receptor neurones, similar effects could be elicited by various stimuli (e.g. Wiersma *et al.* 1953; Moser *et al.* 1979; Barrio *et al.* 1991), all of which affected membrane permeability, concomitant with a decrease in membrane resistance. In parallel with depolarizations, we observed rapid increases in $[Na^+]_i$, indicative of an increase in membrane Na^+ permeability (Figs 1, 5, 6).

To determine which type(s) of cation channels are responsible for the increase in $[Na^+]_i$ when $[Ca^{2+}]_o$ is reduced is rather complex. While there is experimental evidence that tetrodotoxin-sensitive Na^+ channels are not involved, the potential role of several types of K^+ channels and stretch-activated channels, which are known to be present in this preparation (Rydqvist, 1992), remains unclear. Using Ba^{2+} to substitute for Ca^{2+}, we observed depolarizations that were probably due to a block of K^+ conductances, but these were accompanied by only small, usually decreasing, changes in $[Na^+]_i$. This contrasts with the results obtained when Na^+ or Mg^{2+} was substituted for Ca^{2+}. This observation, as well as the results from the tetrodotoxin experiments, indicates that voltage-activated cation channels play a minor role in the increase in $[Na^+]_i$. Whether modulation of $[Ca^{2+}]_o$ affects stretch-activated channels directly or indirectly by causing tension changes in the receptor muscle, as is known from other systems (Austin and Wray, 1995), remains open for discussion.

The maximum rate of pHi recovery does not seem to be affected by the rapid 50 mV potential change in E_m (Fig. 1) that accompanies the washout of Ca^{2+}-free NAS, which is in agreement with previous results (Mair *et al.* 1993). Nevertheless, these experiments are further evidence that pHi regulation (especially the Na^+/H^+ exchange) *per se* is not electrogenic in the crayfish stretch receptor neurone. In this respect, the crayfish sensory cell is quite different from leech glial cells, in which an electrogenic Na^+/HCO_3^- cotransporter dominates pHi regulation (Deitmer and Schneider, 1995).

The experiments reported here do not provide conclusive evidence for the effects of $[Ca^{2+}]_o$ on intracellular H^+ buffering for two reasons. (1) $[Ca^{2+}]_o$ generally affects membrane permeability and changes the dynamics of both NH_3 and NH_4^+ influx to an unknown extent. A smaller initial alkalization does not necessarily reflect increased H^+ buffering, but it could result from a faster influx of NH_4^+. (2) In low-Ca^{2+} NAS or in Ca^{2+}-free NAS, the Na^+ gradient changes very quickly. Even on initial exposure to NH_3/NH_4^+, this could result in a lower rate of acid extrusion. In turn, this could affect the degree of initial alkalization, mimicking a direct influence on H^+ buffering. In the light of these arguments, we nevertheless tend to assume that in crayfish sensory neurones the effect of $[Ca^{2+}]_o$ on intracellular H^+ buffering is small.

In Ca^{2+}-free NAS, pHi and the maximum rate of pHi recovery seem to be closely related to the ion species used as a Ca^{2+} substitute (Figs 5–7). With Ba^{2+}, the maximum rate of pHi recovery is similar to that of controls, while the rate is reduced by about one-third with Mg^{2+} and by about half with Na^+. The relationship between the maximum rate of pHi recovery and the ratio $*[Na^+]_i/[Na^+]_o$ was found to be linear, with a slope similar to that obtained in low-Ca^{2+} experiments. This observation is strong evidence that it was actually Ca^{2+}, and not a substituting ion, that caused the effects on pHi and pHi recovery, and that the substituting ions affect, to varying degrees, the same membrane mechanisms as Ca^{2+} does in NAS. Direct involvement of $[Ca^{2+}]_o$ in the activity of the Na^+/H^+ exchanger seems to be rather unlikely. To explain the action of $[Ca^{2+}]_o$ on pHi and pHi regulation, the following model is proposed.

Model of $[Ca^{2+}]_o$ action on pHi regulation

Membrane permeability is controlled in $[Ca^{2+}]_o$. A reduction of $[Ca^{2+}]_o$ causes an increase in permeability, favouring the transmembrane passage of Na^+, while an increase in $[Ca^{2+}]_o$ decreases membrane permeability, impeding Na^+ passage and resulting in a decrease of $[Na^+]_i$. The observed $[Na^+]_i$ changes are probably due to the effects of Ca^{2+} on cation channels (see above), as will be discussed in detail in a separate article.

Any change in $[Na^+]_i$ affects the $[Na^+]_i/[Na^+]_o$ ratio, which represents the main driving force for pHi recovery. Low $*[Na^+]_i/[Na^+]_o$ ratios are consistent with fast pHi recovery rates during exposure to NAS or high-Ca^{2+} NAS, while exposure to low-Ca^{2+} NAS or Ca^{2+}-free NAS causes higher $*[Na^+]_i/[Na^+]_o$ ratios, which are consistent with slower rates of pHi recovery.

The effects on maximum pHi recovery rate of different ions substituting for Ca^{2+} seem to be mainly due to ion-specific

actions on membrane permeability. Consequently, the substituting ions do not act directly on the H^+-transporting proteins. With Na^+ and Mg^{2+} as Ca^{2+} substitutes, membrane depolarizations and the increase in $[Na^+]_i$ are larger, and pHi recovery lower, than with Ba^{2+}. Substitution with Ba^{2+} produces only small changes in E_m, $[Na^+]_i$ and the rate of pHi recovery compared with controls.

Implications of the model

If the Na^+ gradient were the only factor supplying energy for pHi regulation, recovery should stop when the $*[Na^+]_i/[Na^+]_o$ ratio is 1 (see Fig. 7). However, at this value we found a residual maximum pHi recovery rate that was still between 23 and 33 % of that of controls (100 %). This result can be explained in two different ways: (1) the remaining pHi recovery could reflect activity of the $Na^+/H^+/HCO_3^-/Cl^-$ exchanger, which we know (Mair *et al.* 1993) to contribute about 20 % of the pHi regulation; (2) the residual pHi recovery could reflect the contribution of the H^+ gradient to Na^+/H^+ antiport, since it is known that both the Na^+ gradient and the H^+ gradient fuel Na^+/H^+ antiport (Aronson, 1985; Grinstein and Rothstein, 1986; Kinsella and Aronson, 1980; Mair *et al.* 1993). Further investigations are necessary to decide between these possibilities, but the results are of little importance for this article.

At steady state, the $[Na^+]_i/[Na^+]_o$ ratio is close to 0.1. Exposure to NH_4^+/NH_3 increased the $*[Na^+]_i/[Na^+]_o$ ratio ($[Na^+]_i$ measured at the onset of maximum pHi recovery) to a value of about 0.15, thereby changing $[Na^+]_i$ by almost 50 %, but decreasing the maximum rate of pHi recovery by about 7 %. When $*[Na^+]_i$ increased twofold, the maximum rate of pHi recovery was about 15 % lower than in controls. When $[Na^+]_i$ increased to half $[Na^+]_o$, the maximum rate of pHi recovery was reduced by about 30 %. From these examples, we conclude that the maximum rate of pHi recovery is relatively insensitive to changes in $[Na^+]_i$.

The addition of chelating substances to physiological salines could result in considerable changes in $[Ca^{2+}]_o$. Such an effect is known from experiments performed in Cl^--free salines, in which Cl^- salts were substituted for by salts of gluconic acid. In NAS, an equimolar substitution of $CaCl_2$ will result in a reduction of free $[Ca^{2+}]_o$ to one-quarter of the original level. From the experiments reported above, it is evident that such a substitution will result in a considerable increase in $[Na^+]_i$, with implications for pHi and pHi recovery. To avoid all these effects, $[Ca^{2+}]_o$ compensation seems inevitable.

The question of a $Ca^{2+}/2H^+$ exchanger

In crayfish stretch receptor neurones, any significant contribution of a $Ca^{2+}/2H^+$ exchanger or an equivalent Ca^{2+} exchange mechanism to pHi recovery is unlikely for two reasons. (1) Substantial pHi regulation continued to be observed in Ca^{2+}-free NAS (Figs 1, 5), even in the presence of EGTA. A 50 % lower maximum pHi recovery rate can be fully explained by a concomitant change in the $*[Na^+]_i/[Na^+]_o$ ratio induced by Ca^{2+}-free conditions. (2) Exposures to high-Ca^{2+} NAS, substituting all $[Na^+]_o$ by 0.67 times the $[Ca^{2+}]_o$ of NAS (not shown), resulted in complete inhibition of pHi recovery. If any $Ca^{2+}/2H^+$ antiport were present, some pHi regulation should have been detectable. This, however, was not the case.

Since the Na^+/H^+ antiporter seems to be in more or less full operation even during ecdysis (Mair *et al.* 1993), this could have minimized the evolutionary demand of developing an additional $Ca^{2+}/2H^+$ antiporter.

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