CONTRIBUTION OF THE Na⁺/H⁺ ANTIPORTER TO THE REGULATION OF INTRACELLULAR pH IN A CRAYFISH STRETCH RECEPTOR NEURONE

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Summary

Regulation of intracellular pH (pHi) following acidosis induced by NH₄⁺/NH₃ exposures was re-investigated in a crayfish stretch receptor neurone using H⁺- and Na⁺-selective microelectrodes. All experiments were performed in nominally HCO₃⁻/CO₂⁻ free salines. From studies in Na⁺-free saline and from electrochemical calculations, we concluded that pHi regulation was dependent on extracellular Na⁺ concentration ([Na⁺]₀). The half-maximal rate of pHi recovery had an apparent Michaelis–Menten constant of 21mmol l⁻¹ [Na⁺]₀. The use of this experimental approach and an improved technique enabled us to observe pHi regulation even in Cl⁻-free saline, in contrast to earlier findings. In addition, amiloride (2mmol l⁻¹) inhibited the maximum rate of pHi recovery by about 80%, SITS (1mmol l⁻¹) by about 20%.

The results strongly suggest the operation of two separate pHi-regulating mechanisms, a Na⁺-dependent HCO₃⁻/Cl⁻ antiporter (probably the Na⁺/H⁺/HCO₃⁻/Cl⁻ antiporter described earlier) and a Na⁺/H⁺ antiporter. Both mechanisms have been described in crayfish ganglion cells and muscle fibres, but the individual contribution to pHi regulation varies considerably in these preparations. Functional aspects of the pHi-regulating mechanisms in relation to ionic changes during the moulting cycle are discussed.

Introduction

Several preparations from various crayfish species have been investigated to find the ion transporters responsible for regulating intracellular pH (pHi). In crayfish, Na⁺, HCO₃⁻ and Cl⁻ are the ions involved in pHi regulation, via a Na⁺/H⁺ and/or a Na⁺-dependent HCO₃⁻/Cl⁻ exchange mechanism. Although these mechanisms operate separately in crayfish ganglion cells (Moody, 1981) and muscle cells (Galler and Moser, 1986), the pHi-regulating mechanism in the sensory neurone of the stretch receptor (Moser, 1985) seems to consist of a single combined mechanism, involving Na⁺/H⁺/HCO₃⁻/Cl⁻ exchange or its equivalent.

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Key words: intracellular pH, ion-transport mechanisms, SITS, amiloride, EIPA, sodium, chloride, bicarbonate, ion-selective microelectrode, moult, sensory neurone, Astacus astacus, crayfish, stretch receptor.
During our recent investigations (Moser et al. 1989; Fresser et al. 1991; Mair, 1992) we noticed that pHi was fully regulated in nominally HCO$_3^-$-free salines. Even under such experimental conditions the operation of the HCO$_3^-$/Cl$^-$ antiporter cannot be excluded since HCO$_3^-$ may be formed from CO$_2$ by the carbonic anhydrase located at the outside of this cell membrane (Kaila et al. 1992b). Complete pHi recovery and the small alkaline shift of the resting pHi value with time during experiments indicate that, in the stretch receptor neurone, pHi regulation was hardly impaired by the nominal absence of HCO$_3^-$. In addition, experiments in Cl$^-$-free saline (Mair, 1992), supported by the results from Voipio et al. (1991), provide further evidence for a minor contribution of the HCO$_3^-$/Cl$^-$ antiporter to pHi regulation. All these results contradict earlier findings and this caused us to re-investigate pHi regulation in the sensory neurone using a different approach and an improved technique.

In this work we demonstrate the presence of two independent pHi-regulating mechanisms, a Na$^+/H^+$ antiporter and a Na$^+$-dependent HCO$_3^-$/Cl$^-$ (probably a Na$^+/H^+/HCO_3^-$/Cl$^-$) antiporter, both known in many other animals (Roos and Boron, 1981; Thomas, 1984; Chesler, 1990; Schwiening and Thomas, 1992). Some aspects of both antiporters were studied in further detail.

Preliminary results have been presented at a scientific meeting (Moser et al. 1989).

**Materials and methods**

Experiments and calibration of ion-selective microelectrodes were performed at 16˚C. *Astacus astacus* L., bought from a hatchery (Keller, Augsburg, Germany), were dissected to obtain the slowly adapting sensory neurone of the stretch receptor, which was mounted in an experimental chamber, connected to a flow-through system, as described earlier (Brown et al. 1978; Moser, 1985; Fresser et al. 1991; Mair, 1992). The flow exchanged bath volume within about 6s.

**Chemicals and solutions**

Chemicals of highest purity were obtained from Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) and Fluka (Buchs, Switzerland). The amiloride analogue EIPA (ethylisopropylamiloride) was a present from Ciba-Geigy (Basel, Switzerland). The pH of all solutions was measured with an Orion 8162 (Ross-type) glass electrode. Normal *Astacus* saline (NAS) was modified from that devised by van Harreveld (1936) and had the following composition (mmol l$^{-1}$): 207 NaCl; 5.4 KCl; 2.4 MgCl$_2$; 13.5 CaCl$_2$; 10 Hepes, adjusted to pH7.4 with NaOH. In NH$_4^+$-NAS, NH$_4^+$ was substituted for 20mmol l$^{-1}$ Na$^+$.

N-Methyl-D-glucamine (NMDG) was used as an equimolar substitute for Na$^+$, when reduced-Na$^+$ (e.g. 30Na$^+$-NAS), or Na$^+$-free (0-Na$^+$-NAS) salines were applied. In all NMDG-containing solutions, HCl was used to adjust pH to 7.4.

Cl$^-$-free salines (0-Cl$^-$-NAS) had the following composition (mmol l$^{-1}$): 207 sodium isethionate; 5.4 potassium gluconate; 9.6 D-gluconic acid hemimagnesium salt; 54 D-gluconic acid hemicalcium salt; 10 Hepes, adjusted to pH7.4 with NaOH. For 0-Cl$^-$-NH$_4^+$-NAS, 20mmol l$^{-1}$ sodium isethionate was substituted by NH$_4$NO$_3$. 
Measurements with Ca\(^{2+}\) macroelectrodes showed that calcium gluconate concentration had to be increased fourfold to obtain the free Ca\(^{2+}\) concentration found in NAS, owing to the substantial chelating action of the Cl\(^{-}\) substitutes used. A similar action was assumed for Mg\(^{2+}\), although the amount could have been overestimated. In view of our recent (N. Mair, H. Moser and F. Fresser, unpublished observations) results with varying amounts of Ca\(^{2+}\), an overestimation of divalent cation concentration seems less critical than an underestimation.

SITS (1mmol l\(^{-1}\)), a blocker of the HCO\(_3\)–/Cl\(^{-}\) antiport, was added to NAS, and exposed to ultrasound just before being used. Amiloride and EIPA, blockers of the Na\(^{+}/\)H\(^{+}\) antiport, were dissolved in distilled water by ultrasound and kept light-protected and cooled as a 20 or 10mmol l\(^{-1}\) stock solution. Before use, the amounts for the final amiloride or EIPA concentration were taken and diluted to half the final volume with distilled water. This was made up to the final volume with double-concentrated NAS to give the correct concentration after mixing.

**Electrodes**

Glass for both single- (GC 150) and double-barrelled (TGC 150) microelectrodes was obtained from Clark (Reading, UK) and pulled vertically (model 700C, David Kopf Instruments, USA). Simultaneous intracellular recordings were performed with a double-barrelled microelectrode, monitoring membrane potential (\(E_m\); and serving as an internal reference) and Na\(^{+}\) concentration ([Na\(^{+}\]); thus assuming an equal activity coefficient in the neurone and the saline), and a single-barrelled microelectrode, monitoring H\(^{+}\) concentration ([H\(^{+}\)] or pH. Signals from the \(E_m\) barrel (filled with a mixture of K\(_2\)SO\(_4\) and KCl; Deisz and Lux, 1982; Moser, 1985) and from an additional bath microelectrode (filled with 3mol l\(^{-1}\) KCl) were measured with an electrometer (WPI 750) against a grounded calomel electrode (Amagruss, Halmstadt-Bargen, Germany) connected to the sink of the bath through a 3mol l\(^{-1}\) KCl/agar bridge. The signal of the bath electrode was subtracted from \(E_m\), which is only important during exposures in Cl\(^{-}\)-free salines, when the (external) reference potential changes.

Ion-selective microelectrodes (ISMs) were made as described by Fresser et al. (1991) and Grafe et al. (1985), using Fluka no. 71176 and no. 95293 (TDDA) as Na\(^{+}\) and H\(^{+}\) membrane cocktails, respectively. In a few cases, Fluka no. 95297 (H\(^{+}\) cocktail) was used, but this proved to interfere with organic blocker substances.

Na\(^{+}\)- and H\(^{+}\)-ISMs were calibrated in parallel, using five calibrating solutions (S1–S5) of constant ionic strength (215mmol l\(^{-1}\)). In the sequence S1–S5 the concentrations (mmol l\(^{-1}\)) for NaCl (+NaOH) were: 205 (+5), 50, 21, 10, 5; and for KCl (+KOH): 5, 157 (+8), 189 (+5), 191.5 (+13.5), 205 (+5). The buffers used were 10mmol l\(^{-1}\); Hepes, Hepes, Pipes and Hepes, respectively, which, in combination with the above-mentioned base, resulted in alkaline salines which were adjusted to pH7.4, 8.0, 7.4, 6.4 and 7.4, respectively, by the addition of HCl.

Pre- and post-calibration curves of Na\(^{+}\)-ISMs \((N=50)\) were not significantly different and showed slopes of 54mV/pNa in the 210–21mmol l\(^{-1}\) Na\(^{+}\) range, and 40mV/pNa in the 50–5mmol l\(^{-1}\) Na\(^{+}\) range. A difference of 16.1mV between NAS and S1 saline indicated a selectivity value of +1.8 for Ca\(^{2+}\), which was higher than values in the
literature (Ammann, 1986). The mean post-calibration potential value of (Fluka no. 95293) H⁺-ISMs was 30.9mV between pH7.4 and 8.0, and 56.1mV between pH7.4 and 6.4, these being smaller by 1.8mV and by 5.7mV, respectively, than during pre-calibration. Pre- and post-calibrations with Fluka no. 95297 H⁺-ISMs differed by only about 1mV in the ranges given above.

*Electrical arrangement, recording and display*

Electrical arrangement and recording were as reported in Fresser *et al.* (1991). In addition, original pen recordings were digitized, using SummaSketch II hardware, and processed with Sigma-Scan V 3.90 software, converting mV to mmol l⁻¹ or pH. To present the data, the graphics program Sigma Plot 4.1 (Jandel Scientific) was used. Most of the fine steps in the illustrations are due to limitations of resolution.

*Measurement of the rate of acid extrusion*

**Maximum pHᵢ recovery rate**

In single cells and in most of our results this rate remained constant for about 1.5–4min, during which time pHᵢ recovered by 0.3–0.5 units. For example, the same maximum recovery rate could be obtained if the measurement was taken at a pHᵢ of 6.4 or 6.7. The main criterion for evaluation was that the ranges of maximum pHᵢ recovery rate to be compared should overlap. Under these conditions, the error due to pHᵢ-dependent effects on intracellular buffering capacity and on allosteric modulation of the pHᵢ-regulating transporters should be small.

**Normalized maximum rate of pHᵢ recovery**

The maximum rate of pHᵢ recovery from the control is taken as 100%. This type of evaluation had to be used when there was no overlap in the pHᵢ range of maximum recovery (e.g. ‘low-Na⁺’-experiments). This measure is much more subject to experimental error, because of the reasons given above, which may now become important.

The values given are means ± standard deviation (S.D.) from a certain number (N) of experiments, unless stated otherwise.

**Results**

*Controls*

All experiments were performed in nominally HCO₃⁻/CO₂-free solutions. Cells were acidified according to the NH₄⁺/NH₃ rebound technique (Boron and De Weer, 1976), and the terminology introduced by these authors will be used here.

Controls were usually performed prior to a test. A control run started with exposure to normal *Astacus* saline (NAS) until the variables of interest (pHᵢ, [Na⁺], Eᵣ₉) had stabilized to a reasonable extent. The cells were then exposed to 20mmol l⁻¹ NH₄⁺-NAS for 3min, followed by a washout in NAS. A test run consisted of a 3-min NH₄⁺-NAS
exposure with a subsequent 6-min exposure to the test saline (e.g. amiloride-NAS) and a final washout in NAS. Modifications are noted in the text.

Measurements in 231 control exposures gave a mean pH of 7.36±0.10. During NH4+-NAS exposures the initial alkalization reached a pH maximum of 7.70±0.10 at a rate of 0.91±0.28 pH units min\(^{-1}\). The maximum rate of slow acidification was 0.24±0.05 pH units min\(^{-1}\), resulting in a final pH of 7.27±0.10 at the end of the NH4+-NAS exposure. During washout in NAS, the cells acidified at a maximum rate of 0.96±0.21 pH units min\(^{-1}\), resulting in a maximum acidification to a pH of 6.50±0.31, from which pH finally recovered at a maximum rate of 0.19±0.06 pH units min\(^{-1}\).

Intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) was measured in 178 control experiments. At rest, [Na\(^+\)]\(_i\) was 18.8±9.4 mmol l\(^{-1}\). During fast acidification, [Na\(^+\)]\(_i\) increased at a maximum rate of 8.2±3.1 mmol l\(^{-1}\) min\(^{-1}\), to reach a peak value of 31.1±11.0 mmol l\(^{-1}\) during the early period of pH recovery. The maximum rate of [Na\(^+\)]\(_i\) decrease during pH recovery was 2.9±1.3 mmol l\(^{-1}\) min\(^{-1}\).

At rest the mean membrane potential (\(E_m\)) from 231 control experiments was −65.7±5.0 mV. At the end of the NH4+-NAS exposure the cells had depolarized to −55.3±6.1 mV.

**Effect of 0-Na\(^+\)-NAS on pH recovery**

In the control exposure shown in Fig. 1, pH recovered at a maximum rate of 0.23 pH units min\(^{-1}\) and reached its original value after about 20 min. In a subsequent experiment, exposure to 0-Na\(^+\)-NAS, immediately after NH4+-NAS, resulted in a block of pH recovery. Twenty-three similar experiments were performed in 16 different cells, the exposures to 0-Na\(^+\)-NAS lasting 6 or 12 min. Five different types of pH recovery were found, as shown in Fig. 2 and these were studied in greater detail. By calculating the (individual) electrochemical equilibrium pH (\(p\text{Hi} = p\text{He} + 0.0174E_m\), where pHe is extracellular pH), we were able to decide whether the responses were below or above equilibrium. The majority of cells (\(N=7\)) showed a type 1 response, i.e. a slow increase in pH even in 0-Na\(^+\)-NAS. This pH change generally slowed down with time, revealing a passive movement of H\(^+\), until electrochemical equilibrium was achieved. In other cells (\(N=4\)) we found type 2 and type 3 responses where a stable pH value was finally obtained. In these cells the measured pH was close to, or identical with, the calculated electrochemical equilibrium pH. A type 4 response, also seen in Fig. 1, was found in three additional cells. The measured pH values were clearly above their electrochemical equilibrium so that the H\(^+\) gradient remained inwardly directed. It was characteristic of the type 4 response that acidification in 0-Na\(^+\)-NAS proceeded with time, but became slower. Only 2 of 23 exposures exhibited a type 5 response, in which some pH regulation may have taken place in 0-Na\(^+\)-NAS.

Electrochemical considerations of H\(^+\) gradients with respect to the calculated H\(^+\) equilibrium are sufficient to explain 21 (out of 23) pH responses observed during 0-Na\(^+\)-NAS exposures. The results strongly suggest a dominant role of Na\(^+\) in the pH-regulating mechanism in the sensory neurone. In addition, the type 1 response shown in Fig. 2 suggests either a permeability of the plasma membrane to H\(^+\) or OH\(^−\) or indicates that some of the pH-regulating mechanism is driven by the H\(^+\) gradient.
Approximately 30% more acidification occurred in 0-Na\(^+\)-NAS than in controls (Fig. 1). Releasing the block on pH\(i\) regulation by addition of NAS resulted in an accelerated rate of pH\(i\) recovery compared with controls.

The slower pH\(i\) recovery rate in controls could arise from the fact that they still contain intracellular NH\(_4^+\) ([NH\(_4^+\)]\(_i\)), which exerts a prolonged acidifying action. However, we exclude this argument since, at the moment of maximum acidification, all [NH\(_4^+\)] has probably left the cell as NH\(_3\) (see Fig. 5 in Fresser et al. 1991), which is in line with the Boron and de Weer (1976) model. Instead, the acceleration of the pH\(i\) recovery rate on exposure to 0-Na\(^+\)-NAS might be due partly to a steeper (outwardly directed) H\(^+\) gradient resulting from the stronger acidification and partly to a steeper (inwardly directed) Na\(^+\)

![Fig. 1. Simultaneous measurements of pH\(i\) (A), intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\), B) and membrane potential (\(E_m\), C). After exposure to NH\(_4^+\)-NAS (black bar), the cell was washed either in NAS (control) or, for 6min, in 0-Na\(^+\)-NAS (test). In 0-Na\(^+\)-NAS, pH\(i\) recovery stopped and [Na\(^+\)]\(_i\) remained at a low level.](image-url)
gradient caused by a considerable loss of $[\text{Na}^+]_i$ in 0-Na$^+$-NAS. Such factors would indicate that a combined Na$^+$ plus H$^+$ gradient could act as the driving force for the operation of the (subsequently identified) Na$^+/\text{H}^+$ exchanger. Further results (Moser et al. 1989) support this view, since pH$^+$ recovered in cells heavily loaded with Na$^+$, in which the transmembrane ratio $[\text{Na}^+]_o/[\text{Na}^+]_i$ was close to 1, rendering the Na$^+$ gradient negligible. From an electrochemical point of view, the H$^+$ diffusion gradient could have served to drive H$^+$ transport, mimicking some kind of pH$^+$ regulation in these cells.

**Effect of 0-Cl$^-$-NAS on pH$^+$ recovery**

There are two major differences in experimental method between this and previous work: (1) while Moser (1985) used CO$_2$/HCO$_3^-$, we have applied the NH$_4^+$/NH$_3$ rebound technique (using NH$_4$NO$_3$ instead of NH$_4$Cl) for intracellular acidification in nominally CO$_2$/HCO$_3^-$-free salines, thus obtaining larger intracellular acidification; (2) we have compensated for the chelating action of the Cl$^-$ substitutes. As we will show, this approach brought new insight into the mechanisms of pH$^+$ regulation.

In order to decrease intracellular [Cl$^-$], the cells were incubated in 0-Cl$^-$-NAS for a minimum of 6min, but usually for longer. After a 10-min incubation, intracellular [Cl$^-$] should be very low (Deisz and Lux, 1982; Voipio et al. 1991) and be unable to play a role in pH$^+$ regulation, if this involves a HCO$_3^-$/Cl$^-$ exchange mechanism.
Incubation in 0-Cl⁻-NAS had minor effects on the steady-state variables, generally causing short transient changes in pHi and [Na⁺], as can be seen in Figs 3 and 4. These might indicate a short reversal in the operation of a Cl⁻-dependent pH-regulating mechanism or changes in cell volume. Compared with controls, the maximum intracellular acidification after NH₄⁺-0-Cl⁻-NAS exposure in 0-Cl⁻-NAS was markedly reduced (by about 50%; N=15). This phenomenon will be the subject of a separate paper and is not relevant to the pH regulation process. The main conclusion from Fig. 3 is that pHi recovered even in the absence of Cl⁻, supporting the findings of Voipio et al. (1991). Fig. 4, showing [Na⁺]ᵢ measurements, suggests that Na⁺ was directly involved in pH regulation even in 0-Cl⁻-NAS. The operation of a Na⁺/H⁺ exchanger, already assumed by Moser et al. (1989), could explain both results.

Similar experiments, performed in salines with uncompensated levels of divalent cations, help to explain results obtained previously (Moser, 1985). Cells placed in ‘uncompensated’ 0-Cl⁻-NAS showed a several-fold increase in [Na⁺]ᵢ within 3 min, which considerably reduced the transmembrane Na⁺ gradient. This, together with a minor

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**Fig. 3.** Simultaneous measurements of pHi (A) and membrane potential (Eₘ, B), under control conditions or in 0-Cl⁻-NAS, in a cell acid-loaded with 20 mmol l⁻¹ NH₄NO₃-NAS. pHi recovered in the absence of extracellular Cl⁻, when levels of divalent cations were corrected for the chelating action of the Cl⁻ substitutes.
Acid load caused by CO₂/HCO₃⁻-exposure, tends to reduce the activity of the Na⁺/H⁺ antiporter and, thus, electrochemical equilibrium conditions with no net flux of these ions could have been achieved.

**Effects of SITS on pHi regulation**

SITS, a specific blocker of both Na⁺-dependent and Na⁺-independent Cl⁻/HCO₃⁻ exchange (Cabantchik *et al.* 1978; Roos and Boron, 1981), was used at a concentration of 1 mmol l⁻¹. When SITS was applied immediately after NH₄⁺-NAS for 6min, the maximum rate of pHi recovery was decreased by 21±9% (N=3) compared with controls without SITS. This result can be explained only by assuming the operation of two separate pHi-regulating mechanisms, both of which depend on extracellular Na⁺, as shown before. We suggest that these two mechanisms are (1) a Na⁺-dependent Cl⁻/HCO₃⁻ exchange, which is responsible for approximately 20% of total pHi regulation (in nominally HCO₃⁻-free medium; see Discussion), and (2) a Na⁺/H⁺ exchange, dominating pHi regulation under such conditions. The experiments below were performed in order to obtain further information on the Na⁺/H⁺ exchange mechanism.

**Effects of amiloride or EIPA on pHi regulation**

Amiloride, a specific blocker of the Na⁺/H⁺ antiport (Gaillard and Rodeau, 1978; Grinstein *et al.* 1989), exerts its blocking action by competing with Na⁺ (Roos and Boron, 1981). The effects of various amiloride concentrations on pHi, [Na⁺]ᵢ and Eₘ in a single cell are illustrated in Fig. 5. Control and amiloride exposure were alternated but, for clarity, only one control trace is shown.

The dose-dependent effect of amiloride and the amiloride analogue EIPA (Kleyman and Cragoe, 1988) on inhibition of the maximum rate of pHi regulation is shown in Fig. 6. Amiloride had an instant effect, even at low concentrations. In the range 10⁻⁶ to 10⁻⁴ mol l⁻¹ both drugs showed a similar potency, with a calculated half-maximal
inhibition at $3 \times 10^{-5}$ mol$^{-1}$ amiloride. At the same concentrations, there was no significant difference (at the 0.05% level) between the mean values for amiloride and EIPA (Student’s $t$-test).

Compared with amiloride, EIPA showed pronounced side-effects: within 6 min, EIPA ($10^{-4}$ mol$^{-1}$) depolarized the membrane potential by about 30 mV, an effect never seen even with higher concentrations of amiloride. During washout in NAS, complete recovery of pH$i$ after EIPA exposures took much longer and was accompanied by a slower recovery of [Na$^+$].

Compared with controls, amiloride inhibited the maximum rate of pH$i$ regulation by $15.0 \pm 3.6\%$ ($N=3$) at $10^{-6}$ mol$^{-1}$, the lowest concentration used. Maximum inhibition of

![Graph showing pH$i$, intracellular Na$^+$ concentration, and membrane potential changes over time with different amiloride concentrations.](image)

Fig. 5. Simultaneous measurements of pH$i$ (A), intracellular Na$^+$ concentration ([Na$^+$], B) and membrane potential ($E_m$, C). The effects on a single cell of various amiloride concentrations, given after exposure to NH$_4^+$-NAS, are shown.
about 80±5% (N=7) was obtained at an amiloride concentration of 2mmoll⁻¹. This value did not differ significantly from that obtained at 1mmoll⁻¹ (0.05%, unpaired Student’s t-test).

We conclude that, in nominally HCO₃⁻-free saline, about 80% of pH regulation is due to Na⁺/H⁺ antiport and about 20% to Na⁺-dependent Cl⁻/HCO₃⁻ antiport, this value being...
close to that determined in SITS. Summarizing, the mean values of inhibition of the maximum rate of pH regulation obtained either with SITS or with amiloride (2mmol\textsuperscript{-1}) account for complete blockage of pH regulation.

We tried to demonstrate total inhibition of pH regulation by blocking both antiporters simultaneously. However, any attempt to combine the blocker of the Na\textsuperscript{+}-dependent Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} antiport (SITS) with the inhibitor of the Na\textsuperscript{+}/H\textsuperscript{+} antiport (amiloride) resulted in precipitation of amiloride, and it appears that this experiment cannot be performed.

The effects of various concentrations of amiloride on [Na\textsuperscript{+}]\textsubscript{i} and on maximum pH\textsubscript{i} regulation were not as consistent as those found for the maximum rate of pH\textsubscript{i} regulation. We argue that amiloride, and more clearly EIPA, interacted in a concentration-dependent manner not only with the Na\textsuperscript{+}/H\textsuperscript{+} exchanger but also with Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. Compared with controls, slower rates of [Na\textsuperscript{+}]\textsubscript{i} recovery were seen during and after amiloride exposures in previously acid-loaded cells (Fig. 5). Similar amiloride interactions have been reported by Grinstein et al. (1989) for a different preparation (for EIPA interactions, see Kleyman and Cragoe, 1988).

Dependence of the operation of the Na\textsuperscript{+}/H\textsuperscript{+} and the Na\textsuperscript{+}/H\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{-}/Cl\textsuperscript{-} antiporter on extracellular [Na\textsuperscript{+}]

As reported above, pH\textsubscript{i} regulation was completely inhibited in 0-Na\textsuperscript{+}-NAS. As soon as 5 mmol\textsuperscript{-1} Na\textsuperscript{+} was added (5Na\textsuperscript{+}-NAS) acid extrusion started at a normalized maximum rate of pH\textsubscript{i} recovery of 24±3\% (N=5). The normalized maximum pH\textsubscript{i} recovery rate in 10Na\textsuperscript{+}-NAS was 31±2\% (N=6), in 30Na\textsuperscript{+}-NAS it was 68±4\% (N=9), in 50Na\textsuperscript{+}-NAS it was 75±7\% (N=6) and in NAS (equivalent to 210mmol\textsuperscript{-1} Na\textsuperscript{+}) it was 100\%. A plot of these values (Fig. 7) indicates a hyperbolic saturation curve, allowing application of the Michaelis–Menten equation. The calculated apparent Michaelis–Menten constant (\(K_m\)) was 21.1±0.7mmol\textsuperscript{-1} (extracellular) Na\textsuperscript{+}.

Discussion

Steady-state values of pH\textsubscript{i}, [Na\textsuperscript{+}]\textsubscript{i} and \(E_m\)

A comparison of the pH\textsubscript{i}, [Na\textsuperscript{+}]\textsubscript{i} and \(E_m\) values, obtained at physiological rest, with previous data (Moser, 1985) shows minor differences. The 0.15 pH units more alkaline pH\textsubscript{i} found here seems to be caused by several factors, such as a different mode of calibration of the pH electrodes, which accounts for a difference of 0.06 pH units. Additional effects could arise from altered measurements of \(E_m\) (K\textsubscript{2}SO\textsubscript{4}/KCl electrode versus 3mmol\textsuperscript{-1} KCl electrode), from seasonal effects (H. Moser, unpublished observation) and from chemical differences in the water in which the animals were kept for several months. We also noted a slow alkaline shift of pH\textsubscript{i} with time of experimentation. The deviation in mean pH\textsubscript{i} values cannot be explained by the effects of temperature on pH\textsubscript{i} as they are inversely related to the observations (Aickin and Thomas, 1977; Rodeau, 1984).

Such arguments could also serve to explain the 4mmol\textsuperscript{-1} higher [Na\textsuperscript{+}]\textsubscript{i} value previously observed, but the most likely explanation is that we did not wait long enough
until we started the tests. When the cells were penetrated by the double-barrelled electrode we observed a substantial increase in [Na\(^+\)] from which the cells slowly recovered. Comparing ‘early’ with ‘late’ controls in a single cell, we often found that [Na\(^+\)] had decreased in later controls, a result similar to that of Edman et al. (1983, 1986).

The configuration of the double-barrelled electrode may have caused an extra activation of the stretch-activated channels (Erxleben, 1989) or the electrode seal may have been less than perfect, resulting in a 1mV lower mean value for \(E_m\).

**Ion transport involved in pH regulation in the sensory neurone**

In addition to NH\(_4^+\)-NAS exposures, we carefully studied pH changes in 0-Na\(^+-\)NAS (Figs 1 and 2) and concluded that pH regulation was Na\(^+-\)dependent. From further experiments, we concluded that two separate exchange mechanisms existed in the sensory neurone, a Na\(^+-\)dependent HCO\(_3^-\)/Cl\(^-\) antiporter and a Na\(^+/H^+\) antiporter.

In addition to the observations of Moser (1985), two observations support the existence of a Na\(^+-\)dependent HCO\(_3^-\)/Cl\(^-\) antiporter: (1) high concentrations of amiloride (2mmol l\(^-1\); Figs 5 and 6), a specific blocker of the Na\(^+/H^+\) antiporter, never inhibited pH regulation completely, suggesting that an additional pH-regulating mechanism exists (with Na\(^+\) also being involved); (2) SITS, which blocks the HCO\(_3^-\)/Cl\(^-\) antiporter, reduced the maximum rate of pH regulation by about 20%. This reduction in the rate of recovery was approximately equal to the amount needed to cause complete inhibition of pH regulation in amiloride experiments.

The fact that all salines were nominally HCO\(_3^-\)-free cannot be taken as an argument against the existence of an HCO\(_3^-\)/Cl\(^-\) antiporter, as two sources of HCO\(_3^-\) are always present: the CO\(_2\) of the air and metabolically produced CO\(_2\). CO\(_2\) can easily diffuse through the plasma membrane and will then be converted to HCO\(_3^-\) in a reaction mediated by carbonic anhydrase, known to be located at the outside of plasma membranes even in excitable cells (Chen and Chesler, 1992; Kaila et al. 1990, 1992a).

From the results presented here, there is no clear evidence to indicate whether Na\(^+\) acted indirectly as a modifier of HCO\(_3^-\)/Cl\(^-\) antiport or whether it was directly transported by a Na\(^+/H^+\)/HCO\(_3^-\)/Cl\(^-\) exchanger or an equivalent. As found by several authors (Aickin et al. 1982; Deisz and Lux, 1982; Kaila et al. 1992b), \(E_{Cl}\) is more negative than \(E_m\), resulting in an inwardly directed driving force for Cl\(^-\). If a Na\(^+-\)-modulated 1:1 HCO\(_3^-\)/Cl\(^-\) antiport exists, it should export HCO\(_3^-\), causing an intracellular acid load. However, an inwardly directed driving force for HCO\(_3^-\) is unlikely, since metabolic CO\(_2\) production together with both intra- and extracellular carbonic anhydrase (Chen and Chesler, 1992; Kaila et al. 1992a) will keep CO\(_2\) at about the same level on both sides of the plasma membrane. Thus, electrochemical considerations favour the presence of Na\(^+/H^+\)/HCO\(_3^-\)/Cl\(^-\) antiport, as already indicated by Moser (1985).

The existence of Na\(^+/H^+\) antiport can be deduced (1) from the effect of external Na\(^+\) on pH regulation and the direct involvement of Na\(^+\), as sensed with Na\(^+-\)ISM (Figs 1 and 7), (2) from experiments with selective blockers of Na\(^+/H^+\) antiport, amiloride and EIPA (Figs 5 and 6), which cause a maximum inhibition of pH regulation of about 80% at a concentration of 1 or 2 mmol l\(^-1\), and (3) from the observation that pH was well regulated.
even when Na\(^+\)/H\(^+\)/HCO\(_3^-\)/Cl\(^-\) antiport was blocked by SITS or by prolonged exposure to 0-Cl\(^-\)-NAS in nominally HCO\(_3^-\)-free saline (Fig. 3). During such 0-Cl\(^-\)-NAS exposures, the cells should have lost most of their internal Cl\(^-\) (Deisz and Lux, 1982; Voipio et al. 1991), thus retarding the operation of the HCO\(_3^-\)/Cl\(^-\) exchanger or even stopping it. In 0-Cl\(^-\)-NAS, we observed considerable changes in [Na\(^+\)]\(_i\) (Fig. 4), suggesting direct involvement of Na\(^+\) in an additional pH\(_i\)-regulating mechanism, Na\(^+\)/H\(^+\) antiport.

At physiological pH, the Na\(^+\)/H\(^+\) antiport is half-maximally inhibited at an amiloride concentration of 3\(\times\)10\(^{-5}\)mol\(\cdot\)l\(^{-1}\). In addition, previous results (Moser et al. 1989) indicated that it was the combined Na\(^+\)/H\(^+\) gradient that fuelled Na\(^+\)/H\(^+\) antiport (Kinsella and Aronson, 1980; Grinstein and Rothstein, 1986) and that the Na\(^+\)/H\(^+\) antiport was largely, or totally, unaffected by the transmembrane potential.

At an extracellular pH of 7.4, the normalized maximum rate of pH\(_i\) recovery depends on [Na\(^+\)]\(_o\) following a simple Michaelis–Menten relationship, suggesting a single binding site (Fig. 7). We assume that the \(K_m\) value is mainly linked to the Na\(^+\)/H\(^+\) antiport, which carries out about 80% of pH\(_i\) regulation in our experiments. We found an apparent Michaelis–Menten constant \(K_m\) of 21mmol\(\cdot\)l\(^{-1}\) [Na\(^+\)]\(_o\), indicating that the operation of the Na\(^+\)/H\(^+\) antiporter is unlikely to be limited by physiological [Na\(^+\)]\(_o\) changes, since [Na\(^+\)]\(_o\) normally exceeds this value by approximately 10-fold. Even when [Na\(^+\)]\(_o\) is lowered to about half of its normal value, the action of the Na\(^+\)/H\(^+\) antiporter is hardly impaired (Fig. 7) and the pH\(_i\) value will only change a little in the acid direction.

**The mechanisms of pH\(_i\) regulation in crayfish preparations**

All three preparations of crayfish, ganglion cell (Moody, 1981), muscle cell (Galler and Moser, 1986) and the sensory neurone of the stretch receptor, have two separate pH\(_i\)-regulating mechanisms in common, a Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) antiport and a Na\(^+\)/H\(^+\) antiport. Interestingly, the contribution of each mechanism to pH\(_i\) regulation seems to be different. Muscle fibres had the slowest rate of pH\(_i\) recovery, the Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) antiport contributing 75% and the Na\(^+\)/H\(^+\) antiport 25% to pH\(_i\) regulation (Galler and Moser, 1986). The sensory cell showed the fastest pH\(_i\) recovery, mainly due to Na\(^+\)/H\(^+\) antiport (by about 80%) and, to a lesser extent (20%), to Na\(^+\)-dependent HCO\(_3^-\)/Cl\(^-\) antiport. Between these two extremes, the crayfish neurone (Moody, 1981) exhibited an intermediate rate of pH\(_i\) recovery, with both mechanisms contributing substantially to pH\(_i\) regulation under normal conditions. All these observations are in line with the observation that amiloride hardly affects pH\(_i\) regulation in muscle fibres (Galler, 1985) but has a considerable influence on the sensory cell. The opposite was true for exposure to SITS, 0-Cl\(^-\)-saline or nominally HCO\(_3^-\)-free saline, which moderately inhibited pH\(_i\) recovery in the sensory neurone but had a considerable effect in the muscle fibres.

**Moulting and pH\(_i\) regulation**

During the moulting cycle of crustaceans, cuticular CaCO\(_3\) mineralization and demineralization is accompanied by changes in [Ca\(^{2+}\)], [HCO\(_3^-\)] and [H\(^+\)] (e.g. Cameron and Wood, 1985; Greenaway, 1985; Roer and Dillaman, 1984; Wheatly and Ignaszewski, 1990). The increase in body size in freshwater crayfish is accomplished by uptake of environmental water. Both processes affect the concentration of ions involved in pH\(_i\)
regulation. The effects of concentration changes on the mechanisms of pH i regulation are discussed below.

The dependence of the normalized maximum pH i recovery rate on [Na\(^+\)]\(_o\) (Fig. 7) gives \(K_m\) values of 21 mmol l\(^{-1}\) for the crayfish sensory cell and 39 mmol l\(^{-1}\) for the neurone (Gaillard and Rodeau, 1987). Although direct comparisons cannot be drawn, because the experimental situations were somewhat different, we assume that pH i remains regulated to a reasonable extent (and may be fully regulated) during the moulting cycle, thus keeping the nervous system functioning, mainly by the operation of the Na\(^+\)/H\(^+\) antiport.

In crayfish muscle, the functional role of the Na\(^+\)/H\(^+\) antiport, contributing approximately 25% to pH i regulation (Galler and Moser, 1986), is less clear. Assuming a \(K_m\) value in the range 3–50 mmol l\(^{-1}\) [Na\(^+\)]\(_o\), as found in many preparations from vertebrates and invertebrates (Aronson, 1985; Moolenaar, 1986), the Na\(^+\)/H\(^+\) antiport could provide some kind of ‘safety mechanism’ for pH i regulation with variable importance during the moulting cycle. At moulting, ionic conditions could have slowed or stopped the operation of the Na\(^+\)/H\(^+\)/HCO\(_3\)\(^-\)/Cl\(^-\) antiport, but the Na\(^+\)/H\(^+\) antiport, hardly affected by ionic alterations, would remain functional.

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References


