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INTRACELLULAR pH OF GIANT SALIVARY GLAND CELLS OF THE LEECH *HAEMENTERIA GHILIANII*: REGULATION AND EFFECTS ON SECRETION

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Summary

1. Intracellular pH (pHi) and membrane potential (E_m) of giant salivary gland cells of the leech, *Haementeria ghilianii*, were measured with double-barrelled, neutral-carrier, pH-sensitive microelectrodes.

2. E_m was -51 ± 11.2 mV and pHi was 6.98 ± 0.1 (mean ± S.D., N=41) in Hepes-buffered saline (nominally HCO_3^--free; extracellular pH, pHe=7.4). pHi was independent of E_m.

3. Amiloride (2 mmol l^{-1}) had no effect on resting pHi or on pHi recovery from an acid load (induced by the NH_4^+ pre-pulse technique). Removal of external Na^+ produced a progressive acidification which was blocked by amiloride, and the drug also slowed the recovery of pHi on reintroduction of Na^+. The results indicate the presence of an electroneutral Na^+/H^+ exchanger whose access to amiloride is competitively blocked by Na^+.

4. In certain smaller cells of the gland, which probably form a separate population, removal of external Na^+ did not affect pHi, and recovery from an acid load was blocked by amiloride. There may, therefore, be two types of Na^+/H^+ exchanger, differing in reversibility and sensitivity to amiloride.

5. Recovery of pHi from NH_4^+-induced acid loading was not affected by bicarbonate-buffered saline (2 % CO_2; 11 mmol l^{-1} HCO_3^-) or by addition of the anion-exchange blocker SITS (10^{-4} mol l^{-1}). This suggests that there is no significant contribution of a HCO_3^--dependent transport mechanism to pHi regulation in the gland cells.

6. Removal of external Cl^- slowly reduced pHi and there was a transient increase (overshoot) in pHi when Cl^- was reintroduced. These effects of Cl^- are probably explained by changes in the Na^+ gradient. Intracellular Na^+ and Cl^- activities were measured with ion-selective microelectrodes.

7. Acidification with NH_4^+ was difficult, probably because of the cells' poor permeability to this ion. Attempts to introduce NH_4^+ *via* the Na^+ pump or Na^+/Cl^-

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transporter were not successful. The H^+/K^+ ionophore nigericin (1 μg ml^{-1}), however, produced a rapid and reversible acidification.

8. *N*-methylmaleimide (0.5–1 mmol l^{-1}), which blocks proton-pumping ATPase, produced a prolonged acidification of almost 1 pH unit, well beyond the level expected for simple equilibration with pHe. The results are consistent with the presence of a vesicular proton pump, acidifying the secretory vesicles which pack the cell body.

9. NH_4^+ (50 mmol l^{-1}) or trimethylamine (50 mmol l^{-1}) increased pHi and stimulated salivary secretion, while propionate (50 mmol l^{-1}) decreased pHi and stopped secretion. The secretory effects of NH_4^+ were associated with little change in intracellular free Ca^{2+} concentration (measured with Fura-2) compared with the change produced by high extracellular K^+ concentration ($[K^+]_e$, 100 mmol l^{-1}) which weakly stimulated secretion. Stimulation of secretion by Ca^{2+}-free saline or high $[K^+]_e$ had no effect on pHi. We conclude that changes in intracellular pH probably do not represent a causal step in excitation–secretion coupling in the gland cells, but have a potential modulatory role.

Introduction

The electrically excitable salivary gland cells of *Haementeria* secrete a variety of peptides by an unknown mechanism: the secretory products are packaged in vesicles, but their release is difficult to explain by exocytosis (Wuttke *et al.* 1989; Cooper and Berry, 1990). Salivary secretion in isolated preparations can be elicited by a variety of means; for example by stimulation of the stomatogastric nerve or removal of external Ca^{2+} (both of which result in gland-cell action potentials), by application of 5-hydroxytryptamine (which has many electrophysiological effects but does not excite the cells), by removal of external Cl^-, or by elevation of external $[K^+]$. Secretion may be prevented by removal of external Na^+ (Wuttke and Berry, 1988, 1990, 1991, 1992; Wuttke *et al.* 1989). In this study, we found that secretion is also influenced by intracellular pH (pHi), and experiments were designed to investigate the regulation of pHi as a prerequisite for detailed analysis of the electrical and secretory effects of changes in intracellular $[H^+]$ (see Moody, 1984). In recent years, the search for additional (complementary) second messengers to Ca^{2+}, which may be involved in secretion, has revealed a possible modulatory role for pH (Lorentzen *et al.* 1983; Cook *et al.* 1984; Cannon *et al.* 1985; Friedman *et al.* 1986; Kao *et al.* 1991). We show that intracellular pH of the salivary cells is regulated by a Na^+-dependent (Cl^-- and HCO_3^--independent) mechanism and that intracellular alkalization produces a powerful stimulation of secretion with little change in cytoplasmic free Ca^{2+} concentration ($[Ca]_i$).

Materials and methods

Preparation

Electrophysiological experiments were performed on isolated anterior salivary glands of the giant Amazon leech *Haementeria ghilianii* (de Filippi) obtained from our breeding colony. The glands were pinned to the Sylgard base of a Perspex experimental bath (volume 0.25 ml) and bathed in a continuous flow of physiological saline at room temperature (18–22 °C). The largest cells (500–1000 μm in diameter) with a fine-grained

appearance were chosen for examination unless specified otherwise (in larger animals these cells may reach 1200 μm). A few experiments on a population of smaller cells are also reported because an important qualitative difference in pHi regulation between the cell types was found.

Studies on secretion were made with isolated preparations in which the proboscis/salivary gland complex was left intact. Secretion was not accurately quantified but was assessed with the help of videotape recordings of the proboscis. Each salivary gland cell extends a single process, or ductule, anteriorly into the proboscis, and secretory products are released at the ductule ending. Some salivary cells secrete into the lumen of the proboscis and others onto its outer surface near the tip (see Wuttke *et al.* 1989). In normal saline, little or no secretory product was seen at the proboscis tip, so that any stimulation of secretion by different solutions could easily be observed and the amounts compared. The secretions are water-immiscible and tend to stick to the surface of the proboscis (Fig. 1). At 15-min intervals they were sucked into a syringe in order to determine more clearly whether secretion had stopped. Although we made no quantitative measurements, the ease of visual observation and the clarity and reproducibility of the results enabled us to distinguish clearly between strong and weak secretagogues.

Solutions and drugs

Physiological saline (nominally HCO_3^--free) contained (in mmol l^{-1}): NaCl, 115; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11; Hepes, 10 (pH 7.4). In Na^+-free solutions, *N*-methyl-D-glucamine was used as a substitute. Cl^--free saline was made with the appropriate salts of D-gluconic acid and contained 9 mmol l^{-1} calcium gluconate to compensate for the possible binding of Ca^{2+} by gluconate. $CaCl_2$ was omitted from Ca^{2+}-free solutions and 1 mmol l^{-1} EGTA was added. Alterations in K^+ concentration were compensated by changes in Na^+ concentration to maintain osmolarity. Solutions containing weak base or acid were made by equimolar substitution of NaCl with NH_4Cl, trimethylamine–HCl or sodium propionate. Solutions were buffered with Hepes (10 mmol l^{-1}) and the pH was adjusted to 7.4 with either NaOH or HCl. Solutions buffered with CO_2/HCO_3^- instead of Hepes were equilibrated with 2 % CO_2 in O_2 and contained 11 mmol l^{-1} $NaHCO_3$.

Amiloride hydrochloride, bovine serum albumin, *N*-methylmaleimide, and SITS (4-acetamido-4′-isothio-cyanato-stilbene-2,2′-disulphonic acid) were directly added to the solutions. Nigericin was dissolved in ethanol (1 mg ml^{-1}) and then added to the solutions to give a final nigericin concentration of 1 μg ml^{-1}. All chemicals were obtained from Sigma.

Measurement of intracellular pH and Na^+ and Cl^- activities with ion-selective microelectrodes

Double-barrelled, ion-selective microelectrodes (ISMEs) were made from borosilicate capillaries with internal filaments (Clark Medical Instruments). One barrel was silanized with a solution of 3–5 % tributylchlorosilane (Fluka) in reagent grade carbon tetrachloride (BDH) by the method of Borrelli *et al.* (1985).

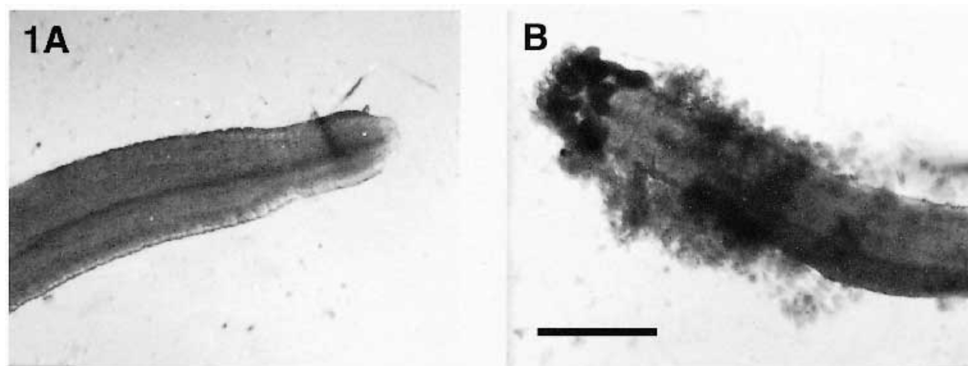

Fig. 1. Measurement of salivary secretion. (A) The ending of the proboscis in a semi-intact preparation bathed in normal saline; no secretion is apparent. In (B), the same proboscis shows the copious secretion that is typically produced by 15 min of exposure to 20–50 $\text{mmol}\,\text{l}^{-1}$ NH_4^+ or trimethylamine, or to Cl^--free or Ca^{2+}-free saline. Two different types of secretory product are apparent: dense globular masses at the tip of the proboscis and strings of thread-like material which mix together over a wider area. Secretions were removed periodically by sucking them into a syringe. A third type of secretion did not stick to the proboscis but fell to the bottom of the bath as oil-like droplets. This cannot be seen in the photograph and was not measured in these experiments. Scale bar, 1 mm.

H^+-ISMEs were made using a proton cocktail (Fluka 95297) with a backfill solution containing 100 $\text{mmol}\,\text{l}^{-1}$ NaCl and 20 $\text{mmol}\,\text{l}^{-1}$ Hepes (pH 7.0). The reference barrel was filled with 3 $\text{mol}\,\text{l}^{-1}$ lithium acetate (pH 6.8), containing 10 $\text{mmol}\,\text{l}^{-1}$ LiCl. Electrodes were calibrated in normal saline (pH 7.4) and in normal saline at pH 6.4 in which Hepes was substituted by Pipes. They had an average slope of 51.2±3.7 $\text{mV}\,\text{pH}\,\text{unit}^{-1}$ (mean ± S.D., N=32).

Na^+-ISMEs were prepared using the neutral ligand ETH 227 with tetraphenylborate (Fluka 71176; see Steiner *et al.* 1979) and a backfill solution of 150 $\text{mmol}\,\text{l}^{-1}$ NaCl. The reference barrel was filled with 3 $\text{mol}\,\text{l}^{-1}$ KCl instead of lithium acetate because of the considerable sensitivity of the ligand to Li^+ (Steiner *et al.* 1979).

Cl^--ISMEs were made using the Corning exchanger 477913. The backfill solution was 0.5 $\text{mol}\,\text{l}^{-1}$ KCl and the reference barrel was filled with 3 $\text{mol}\,\text{l}^{-1}$ lithium acetate (pH 6.8) containing 10 $\text{mmol}\,\text{l}^{-1}$ LiCl. For further details on the manufacture and calibration of Na^+- and Cl^--selective microelectrodes, and on the correction for interference with Cl^--ISMEs, see Wuttke and Berry (1990).

Double-barrelled ISMEs were mounted on a high-speed stepper (Digitimer SCAT-02) and the active and the reference barrels were connected by chlorided silver wires to the inputs of a varactor bridge amplifier (311J, Analogue Devices; input resistance $10^{14}\,\Omega$) and the headstage of a Digitimer NL 102 amplifier, respectively. The potential of the reference barrel was subtracted electronically from the potential of the active barrel by a home-made differential amplifier. The differential signal (i.e. the ion signal) and the potential of the reference barrel were displayed on an oscilloscope and recorded by a two-channel pen recorder (Gould 2200) and stored on tape (Thorn EMI 3000 FM tape-recorder).

Measurement of intracellular [Ca^{2+}] with Fura-2

Changes in $[Ca^{2+}]_i$ were determined by intracellular injection of the calcium indicator dye Fura-2. Single gland cells were penetrated with a microelectrode containing 10 mmol l^{-1} Fura-2 (dissolved in 100 mmol l^{-1} KCl) and a steady hyperpolarising current of 40 nA was passed for 10–30 min. After injection, the electrode was withdrawn and the preparation was transferred to an upright microscope (Axioskop, Zeiss). The intracellular Fura-2 was excited alternately at 350 nm and 380 nm (band width 4 nm) with a Deltascan dual-wavelength spectrofluorimeter (PTI, Wedel, Germany) using a 75 W xenon arc lamp. The light was directed onto the preparation through a water immersion 40× objective (Achroplan 40*/w, Zeiss). Fluorescence intensity was measured at 510 nm using a photon-counting photomultiplier tube. Measurements were limited to a rectangular field of view from a peripheral region of the gland-cell soma by a diaphragm. Shutters, monochromators and data acquisition were controlled by computer software and by interfaces from PTI. Fluorescence imaging of the gland cells was begun 5–10 min after Fura-2 injection, and changes in $[Ca^{2+}]_i$ were determined from the ratio of fluorescence at 350 nm and 380 nm.

The experiments with Fura-2 and with ion-selective microelectrodes were all performed on the cell body, whereas secretion was monitored at the ductule ending, which is inaccessible to these techniques. In the following discussion, it is assumed that the two sites of the cell have similar properties, but this has not been demonstrated.

Results

Intracellular pH and secretion

Effects of NH_4Cl, trimethylamine and sodium propionate on E_m, *pHi and secretion*

Impalement of a gland cell with a double-barrelled, pH-sensitive microelectrode showed a resting membrane potential of −51.5±11.2 mV (*N*=41) and an intracellular pH (pHi) of 6.98±0.1 (*N*=41) when the salivary gland was bathed in physiological saline buffered to pH 7.4 with Hepes. These values remained constant for several hours, though it was difficult to maintain the impalement without making occasional readjustments of the microelectrode position. This was probably caused by the extremely convoluted nature of the cell membrane (Walz *et al.* 1988), but usually resulted in no apparent damage. The cells have the unusual advantage of lacking electrical coupling (Wuttke and Berry, 1988) so that the activity of any cell was not influenced by that of its neighbours.

Addition of 50 mmol l^{-1} NH_4Cl (in exchange for 50 mmol l^{-1} NaCl) produced a maintained alkalization of 0.82±0.22 pH units (*N*=19; Fig. 2A), and 50 mmol l^{-1} sodium propionate (in exchange for NaCl) produced an acidification of 0.31±0.05 pH units (*N*=5; Fig. 2B). NH_4Cl (20–50 mmol l^{-1}) elicited copious secretion at the tip of the proboscis which started within 4 min of application (*N*=12), while sodium propionate (20–50 mmol l^{-1}) abolished or reduced secretion in preparations which were secreting spontaneously or had been stimulated to secrete with NH_4Cl (*N*=6). The effects on secretion did not appear to be related to changes in membrane potential since there was little change in response to propionate, and there was usually a small, transient

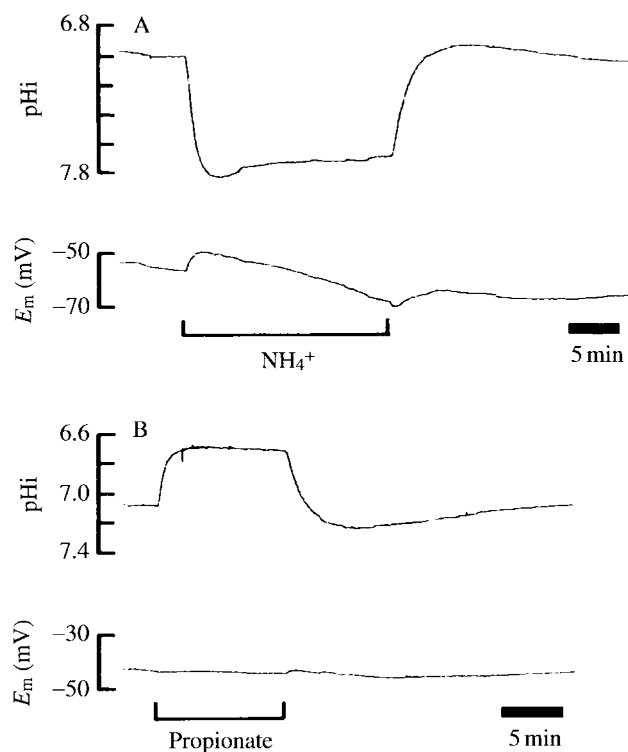

Fig. 2. (A) Effect of 50 mmol l^{-1} NH_4^+ (substituted for Na^+) on intracellular pH (pHi) (upper trace) and membrane potential (E_m) (lower trace) of a salivary gland cell. (B) Effect of 50 mmol l^{-1} propionate (substituted for Cl^-) on pHi and E_m. NH_4^+ is a potent secretagogue whereas propionate tends to inhibit secretion. Both solutions were buffered to pH 7.4 with Hepes.

depolarizing response to NH_4^+. Depolarization to about −10 mV with 100 mmol l^{-1} K^+ (substituted for Na^+) had a relatively small stimulatory influence on secretion and no effect on pHi (*N*=9; Fig. 3A). Furthermore, the weak base trimethylamine (50 mmol l^{-1}) was similar to NH_4^+ in both its effect on pHi (Fig. 3B) and its effectiveness as a secretagogue (*N*=8), whereas its depolarizing action on the membrane potential was almost identical to that of acetylcholine, which does not elicit secretion (see Wuttke *et al.* 1989). These results indicate that increase in pHi is the likely stimulus for secretion by trimethylamine and NH_4^+.

Effect of NH_4Cl on $[Ca^{2+}]_i$

To determine whether an increase in pHi caused an increase in $[Ca^{2+}]_i$, we monitored cytosolic $[Ca^{2+}]$ with Fura-2. Application of 50 mmol l^{-1} NH_4Cl produced a reversible increase in cytoplasmic free calcium concentration which reached a steady peak level after 4–8 min. This increase, however, was only 11±3.6 % of that induced by 90 mmol l^{-1} external K^+ (*N*=3; Fig. 4). This suggests that the effects of NH_4Cl on secretion are not solely mediated by internal Ca^{2+} because NH_4Cl is much more effective as a

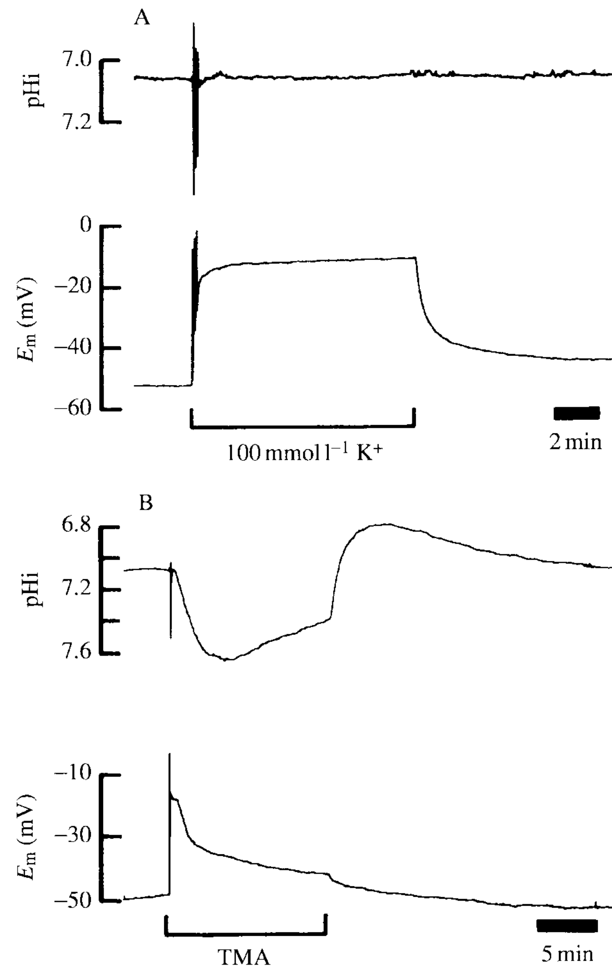

Fig. 3. (A) Depolarization in response to addition of 100 mmol l^{-1} K^+ (substituted for Na^+) is not associated with a change in pHi. Action potentials were elicited during the development of the depolarization (producing artefacts in the pHi signal as a result of high-frequency filtering by the active barrel of the microelectrode). (B) The powerful secretagogue trimethylamine (TMA, 50 mmol l^{-1}) increases pHi. It also produces a depolarizing response which is similar to that produced by acetylcholine, which does not stimulate secretion (see Wuttke and Berry, 1991; Wuttke *et al.* 1989). High [K^+] weakly stimulates secretion.

secretagogue than is high $[K^+]_e$. The elevation of $[Ca^{2+}]_i$ by high $[K^+]_e$ was transient, lasting 6–9 min, but this is unlikely to account for the lower potency of K^+ as a secretagogue because the secretory effect of NH_4Cl is much greater within this time scale.

Effect of impulse activity on pH_i, $[Ca^{2+}]_i$ *and secretion*

The gland cells produce Ca^{2+}-dependent action potentials in response to depolarizing current, but they adapt rapidly and cannot be repetitively activated to determine whether

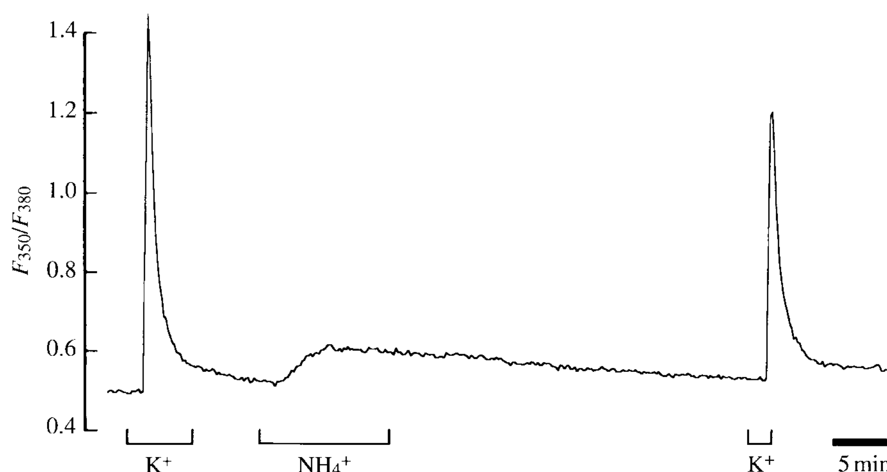

Fig. 4. Fluorescence measurements (fluorescence at 350 nm compared with that at 380 nm) from a salivary gland cell loaded with Fura-2: a small intracellular Ca^{2+} transient is induced by 50 mmol l^{-1} NH_4^+ compared with that elicited by 90 mmol l^{-1} external K^+. The reduced amplitude of the second response to K^+ was probably caused by the hyperpolarization which tends to follow NH_4^+ application: this would reduce the level of K^+-induced depolarization and hence result in the activation of fewer Ca^{2+} channels.

action potentials elicit secretion. In Ca^{2+}-free saline, however, the action potentials become Na^+-dependent and easily stimulated; the gland cells normally fire spontaneously, which results in copious secretion in the proboscis (this does not necessarily mean that secretion is Ca^{2+}-independent: although Ca^{2+} is quickly removed from the salivary glands, it is probably not easily removed from around the site of secretion in the proboscis; see Wuttke and Berry, 1988). Action potentials in Ca^{2+}-free saline did not result in a change in pHi (Fig. 5) or in $[Ca^{2+}]_i$ (not shown).

Intracellular pH regulation

Regulation of pHi was studied by the commonly used method of acidifying the cell and then measuring the subsequent pHi recovery. This was achieved by exposure of the salivary glands to solutions containing NH_4^+, which produces an intracellular alkalization followed by an acidification on removal of the NH_4^+ (Boron and de Weer, 1976; Thomas, 1984). External pH (pHe) was maintained constant at 7.4.

Effect of external Na^+ and amiloride on pHi

The value of normal pHi (6.98) is higher than that expected from passive H^+ distribution (about 6.5), implying the presence of an acid-extrusion mechanism. We tested for Na^+/H^+ exchange by adding the blocking drug amiloride (Bentley, 1968; Bull and Laragh, 1968; Johnson *et al.* 1976) and by removing external Na^+. Amiloride (2 mmol l^{-1}) had no effect on resting pHi (N=5; not shown) or on pHi recovery from an acid load (N=10; Fig. 6A: the average rate of recovery, measured in the absence of amiloride, was 0.0284±0.009 pH units min^{-1}). Removal of external Na^+ produced a

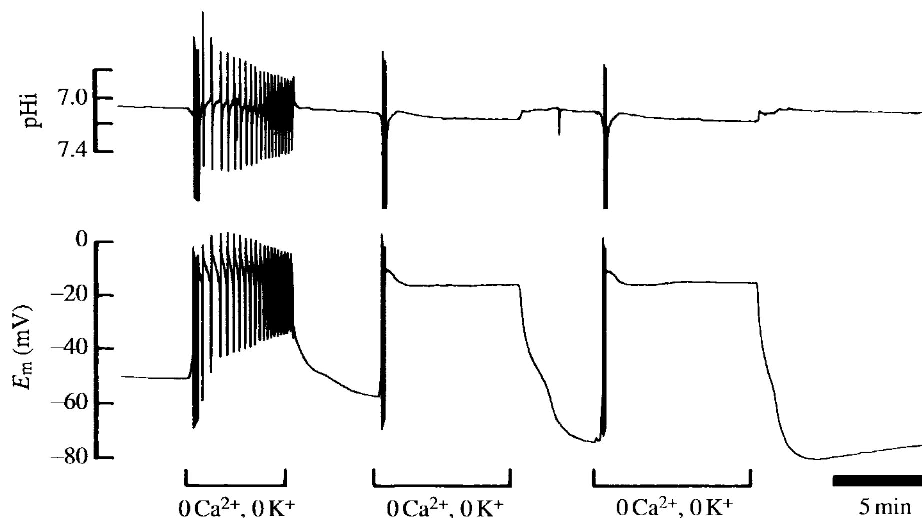

Fig. 5. Activation of a gland cell by Ca^{2+}-free saline (which stimulates secretion) does not markedly change pHi. The solution was added three times and initiated prolonged action potentials and plateau potentials. As part of a different experiment (explained later in the text) K^+ was also omitted from the saline to block the electrogenic sodium pump temporarily, causing Na^+-loading and subsequent pump activation (note the progressive hyperpolarization). The action potentials caused artefacts in the pHi signal.

progressive acidification of 0.015±0.007 pH units min^{-1} which was completely blocked by amiloride (N=4; Fig. 6B). These results can be explained by the presence of a Na^+/H^+ exchanger whose access to amiloride is competitively blocked by Na^+ (see Discussion). The drug also slowed the recovery of pHi on reintroduction of Na^+ to 34±12 % of the control rate (N=4; Fig. 6B). This was unexpected since it suggests that amiloride is slow to come off the Na^+/H^+ exchanger, which is unlikely given the usual affinity of these transporters for amiloride.

The experiments described in this paper were performed on the largest cells in the gland (500–1000 μm in diameter), which are quite distinct in appearance from the others, but generally similar results were found with the smaller cells. Interesting differences in detail, however, were seen with the medium-sized cells (300–400 μm), whose cytoplasm had a more coarse, granular appearance. Amiloride greatly slowed or blocked recovery from an acid load in these cells, whereas removal of external Na^+ had no immediate effect on pHi (N=3; Fig. 7). Although these effects were opposite to those in the largest cells, the results may still be explained by the presence of Na^+/H^+ exchange (see Discussion).

Effect of HCO_3^- and Cl^- on pHi

Another common mechanism of pHi regulation is *via* Cl^-/HCO_3^- exchange, with or without a dependence on external Na^+ (Thomas, 1984). This was of particular interest because it offered a possible explanation for the active accumulation of Cl^- by the salivary cells (Wuttke and Berry, 1990), and also because removal of external Cl^- evokes copious salivary secretion (Wuttke and Berry, 1990).

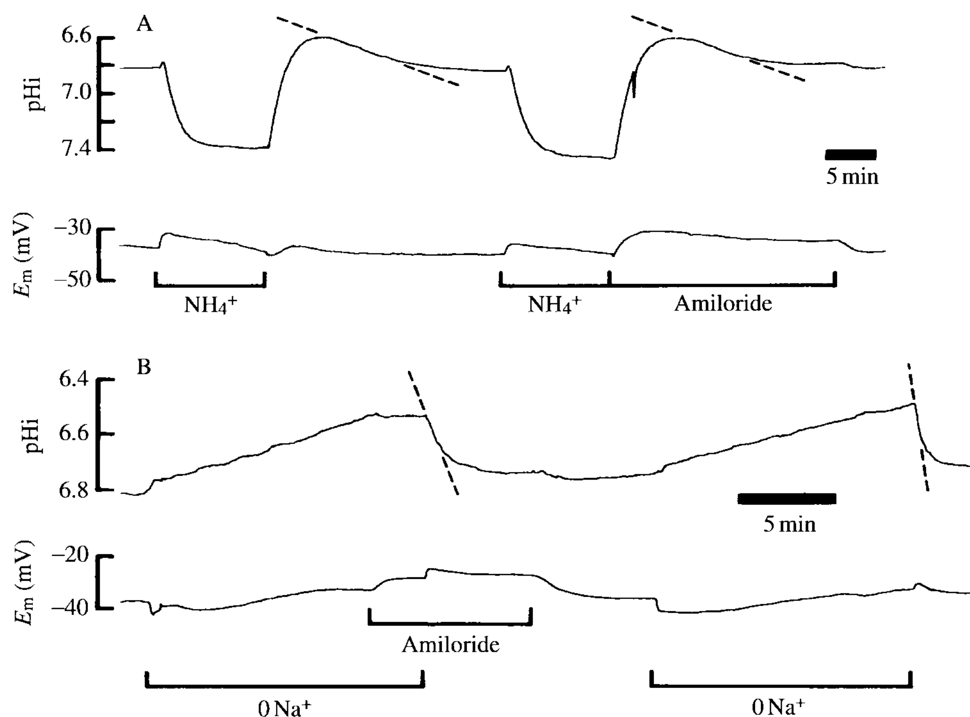

Fig. 6. (A) Amiloride (2 mmol l^{-1}) has little effect on pHi recovery after exposure to 50 mmol l^{-1} NH_4^+. (B) Removal of external Na^+ (substituted by *N*-methyl-D-glucamine) produces an acidification which is blocked by amiloride. Recovery of pHi on reintroducing Na^+ is slowed by amiloride. Dashed lines are drawn to facilitate comparison of recovery rates.

Bicarbonate-buffered saline, equilibrated with 2% CO_2 (pH 7.4), produced a maintained acidification of 0.145±0.03 pH units (*N*=8) with little or no sign of pHi regulation (Fig. 8A). There was a transient overshoot of 0.2±0.05 pH units (*N*=8) on switching back to normal saline (Hepes-buffered) before recovery to the original level. There was little effect of bicarbonate-buffered saline on recovery of pHi from a NH_4^+-induced acid load (Fig. 8B), and addition of the anion-exchange blocker SITS (see Roos and Boron, 1981) also failed to influence pHi recovery (Fig. 8C). These results indicate that Cl^-/HCO_3^- exchange does not play a significant role in pH_i regulation in these cells.

Removal of external Cl^- (replaced by gluconate) does, however, progressively increase pHi of the salivary cells even in the absence of HCO_3^- (0.22±0.16 pH units, *N*=5; Fig. 9A). We do not know the quantitative relationship between pHi and secretion rates, but the effect seems too slow to account for the stimulation of salivary secretion by Cl^--free saline. Reintroduction of external Cl^- causes a transient decrease in pHi (Fig. 9A). These Cl^--induced changes in pHi are probably explained by effects on intracellular [Na^+]. It can be seen from Fig. 9B that removal of external Cl^- lowers intracellular [Na^+] (by reversing the Na^+/Cl^- transport system; see Wuttke and Berry, 1990); this increases the Na^+ electrochemical gradient and presumably potentiates

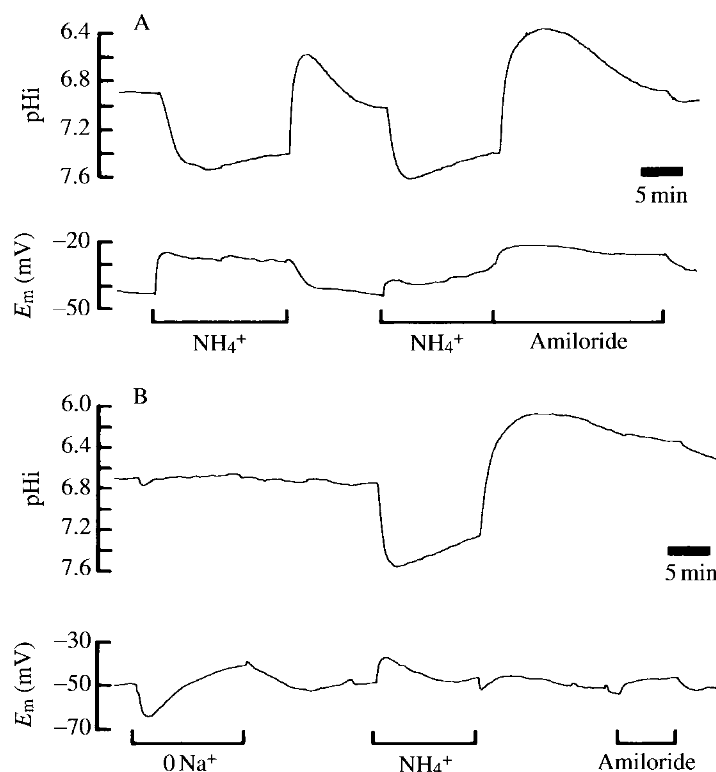

Fig. 7. The effects of amiloride and external Na^+ on pHi of medium-sized cells (300–400 μm) are different from their effects on the largest cells described in the rest of this paper. (A) Amiloride (2 mmol l^{-1}) slows recovery from an acid load. (B) Removal of external Na^+ has no effect on pHi. Amiloride applied during the recovery phase from acid loading, reversibly blocks the recovery.

Na^+/H^+ exchange. Conversely, the Na^+ gradient is reduced (and pHi decreased) on replacement of external Cl^- as Na^+ is accumulated by the Na^+/Cl^- transporter (Fig. 9B).

Intracellular buffering power

The intrinsic (non-bicarbonate) buffering power (β_i) was estimated from the pHi changes induced by 20 mmol l^{-1} NH_4Cl in Hepes-buffered solution (Roos and Boron, 1981; Szatowski and Thomas, 1989). This gave a mean value of 21±15 mmol l^{-1} pH unit^{-1} (N=6). Measurements of β_i are based on a number of assumptions and may vary with the substance used to change pHi. However, a similar result was obtained with 20 mmol l^{-1} propionic acid (β_i=19.2±2.6 mmol l^{-1} pH unit^{-1}; N=3), and is comparable with values reported for leech neurons (12–33 mmol l^{-1} pH unit^{-1}; Deitmer and Schlue, 1987).

Problems of acid loading

The technique of acid loading of the salivary cells by NH_4^+ exposure proved difficult,

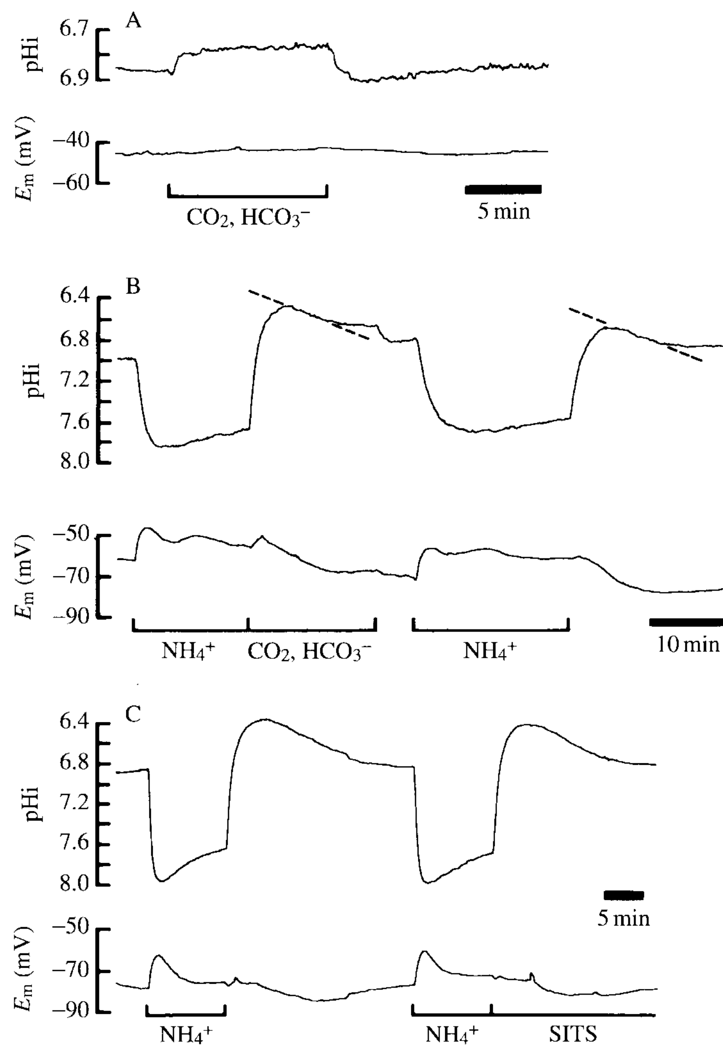

Fig. 8. Effects of 2% CO_2, 11 mmol l^{-1} HCO_3^- (pH 7.4) on pHi regulation. (A) There is a small acidification in response to CO_2/HCO_3^- with little effect on pHi recovery after acid loading by the NH_4Cl method (B). SITS (10^{-4} mol l^{-1}), which blocks Cl^-/HCO_3^- exchange, is also ineffective on recovery (C).

and many attempts were unsuccessful; there was little or no acidification, irrespective of the exposure time (from a few minutes to 30 min). The method appears to depend on an influx of NH_4^+ which acts as a proton carrier, shedding H^+ and leaving the cell as NH_3 (Boron and de Weer, 1976). The failure of pHi to fall possibly reflects a low permeability to NH_4^+, and we therefore tried alternative means to introduce this cation into the cells. For example, NH_4^+ may replace K^+ in the Na^+/K^+ pump (Aickin and Thomas, 1977). The gland cells have an electrogenic sodium pump which may be activated by removal of external Ca^{2+} (which elicits spontaneous Na^+-dependent action potentials; Wuttke and Berry, 1988). The cells were repeatedly exposed to Ca^{2+}-free, K^+-free saline to block the

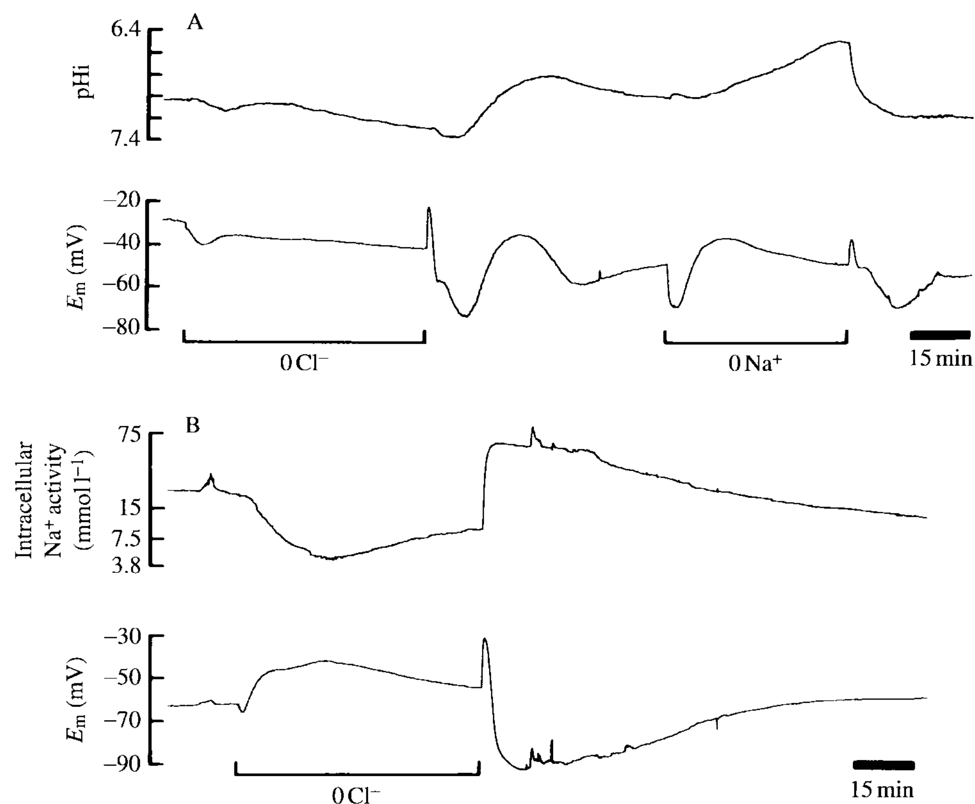

Fig. 9. (A) Removal of external Cl^- increases pHi and its reintroduction transiently lowers pHi. (B) The effects of Cl^- are probably caused by changes in intracellular Na^+ activity (top trace). The Na^+ electrochemical gradient (which energises H^+ extrusion) is increased by removal of external Cl^- and transiently reduced by its reintroduction. Movements of Na^+ and Cl^- are linked by an active cotransport system. In A, Na^+ removal demonstrates that pHi of this cell depends on the Na^+ gradient.

pump and load the cells with Na^+ (see Fig. 5) and NH_4^+ was then added during recovery (NH_4^+ replaced K^+ and Na^+ in normal saline), but this did not enhance its ability to acidify the cells (not shown).

Another possible means of NH_4^+ entry is *via* cation-dependent anion transporters, substituting for Na^+. The salivary gland cells have a powerful Na^+/Cl^- cotransport system whereby Cl^- is accumulated to a level which is three times its passive distribution, energised by the Na^+ gradient. Transport can be maximally activated by washing Cl^- from the cells in Cl^--free solution and then re-adding external Cl^- (Wuttke and Berry, 1990). Unfortunately, NH_4^+ did not replace Na^+ in this transport system (Fig. 10).

The problems of acidifying the salivary cells by the ammonia prepulse method prompted consideration of other possible approaches. Propionate, for example, acidified the cells (Fig. 2B), but this has the disadvantage that it must continually be present for the acid load to be maintained (see Roos and Boron, 1981). Injection of acid (e.g. Thomas,

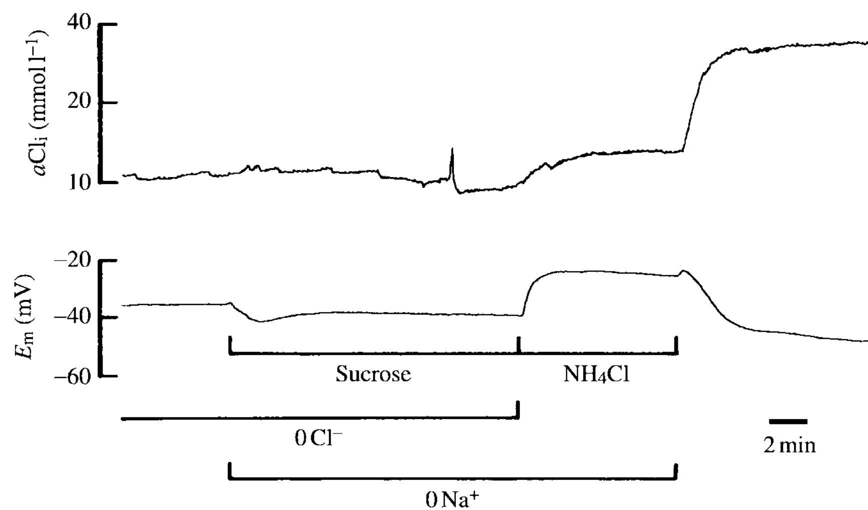

Fig. 10. Attempt to load a salivary gland cell with NH_4^+ *via* the Na^+/Cl^- transporter. Upper trace is a record of intracellular Cl^- activity (aCl$_i$) (uncorrected for interference) after prolonged washing in Cl^--free solution (Cl^- substituted by gluconate). Lower trace shows the membrane potential (E_m). External Na^+ was removed (substitution of sodium gluconate by equimolar sucrose) and then Cl^- was re-added as NH_4Cl (115 mmol l^{-1}). Intracellular Cl^- rose only to its passive level until Na^+ was replaced, showing that NH_4^+ cannot substitute for Na^+ in this transport system.

1984) would be difficult because the cells are greatly invaginated and also they do not easily tolerate multiple impalements. We therefore investigated the ionophore nigericin, which couples K^+ and H^+ gradients across the membrane, inducing an efflux of cellular K^+ in exchange for external H^+ (Grinstein *et al.* 1984). Fig. 11 shows that this worked well, producing a rapid acidification and normal recovery which started almost as soon as the nigericin was washed out of the bath. Decreasing pHe to 6.4 increased the intracellular acidification (not shown). Surprisingly, however, addition of 100 mmol l^{-1} K^+ (replacing Na^+) had little or no effect on pHi in the presence of nigericin, though prolonged exposure was not examined. It was intended to use K^+ to monitor the washout of nigericin (until the effect of K^+ on pHi ceased), but the speed of pHi recovery suggested that this was unnecessary. Similarly, a lack of effect of bovine serum albumin (normally used for rapid termination of nigericin's action; Grinstein *et al.* 1984) indicated an effective removal of the ionophore from the membrane within about 10 min (Fig. 11).

Effect of N*-methylmaleimide on intracellular pH*

The salivary gland cells of *Haementeria* are densely packed with storage vesicles (Walz *et al.* 1988), and we considered whether these may influence pHi. In many types of cell, such organelles are acidic as the result of a proton-pumping ATPase in the membrane which drives H^+ into the vesicle against its electrochemical gradient (Njus *et al.* 1986). In some cases Cl^- acts as a counterion, reducing the potential change across the vesicle membrane and perhaps allowing greater acidification (Al-Awqati, 1986; Nelson, 1991). This H^+ pump of acidic organelles is rapidly inactivated by sulphydryl

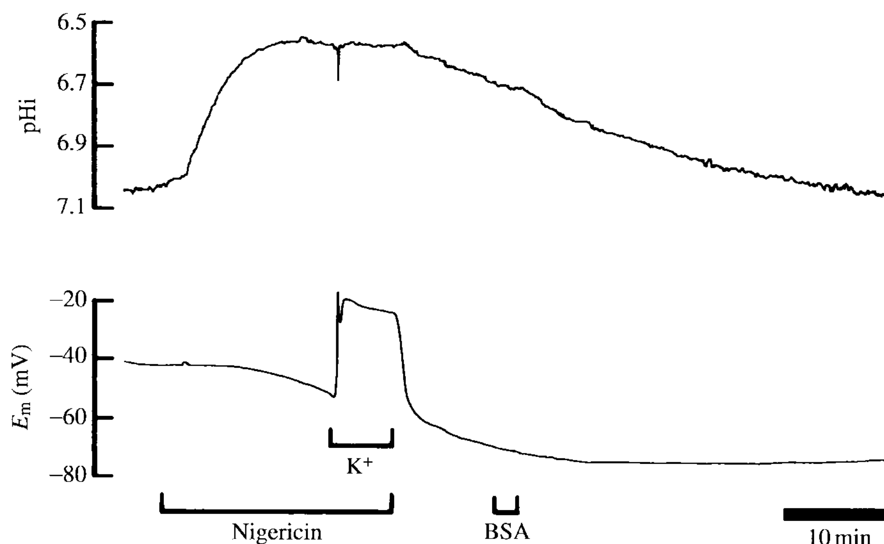

Fig. 11. Effect of nigericin (1 μg ml^{-1}) on pHi and E_m. The ionophore produced a rapid acidification, with normal recovery on washout. The associated hyperpolarization is probably at least partly due to activation of the electrogenic sodium pump as a result of Na^+ influx during regulatory Na^+/H^+ exchange. Application of bovine serum albumin (BSA, 5 mg ml^{-1}), which terminates the action of nigericin, has no effect on pHi, indicating effective removal of nigericin from the membrane. Addition of 100 mmol l^{-1} K^+ (substituted for Na^+) does not influence pHi, even though nigericin usually couples K^+ and H^+ gradients.

reagents (Rudnick, 1986; Mellman *et al.* 1986) and we therefore tested for effects of *N*-methylmaleimide (NMM).

Application of 0.5–1 mmol l^{-1} NMM resulted in an acidification of almost 1 pH unit which continued for up to 30 min after the drug had been washed from the bath. There was also a progressive loss of membrane potential (Fig. 12A). Fig. 12B is a plot of pHi against membrane potential on exposure to NMM, and the dashed line shows the relationship expected for passive distribution of H^+ across the cell membrane. It can be seen that pHi decreased well beyond the level expected for simple equilibration with pHe and is consistent with leakage of H^+ from storage vesicles.

Discussion

Exposure of the salivary gland cells of *Haementeria* to NH_4^+ or TMA increased pHi and stimulated secretion in amounts which exceeded those produced by K^+-depolarization, stimulation of the stomatogastric nerve or by addition of the putative neuroglandular transmitter 5-hydroxytryptamine (see Wuttke *et al.* 1989). This suggested a possible role of pHi in stimulus–secretion coupling, perhaps as a modulator. In order to understand the relationships between pHi and the electrical and secretory properties of the cells, it is necessary to determine the mechanisms of pHi regulation. The principal

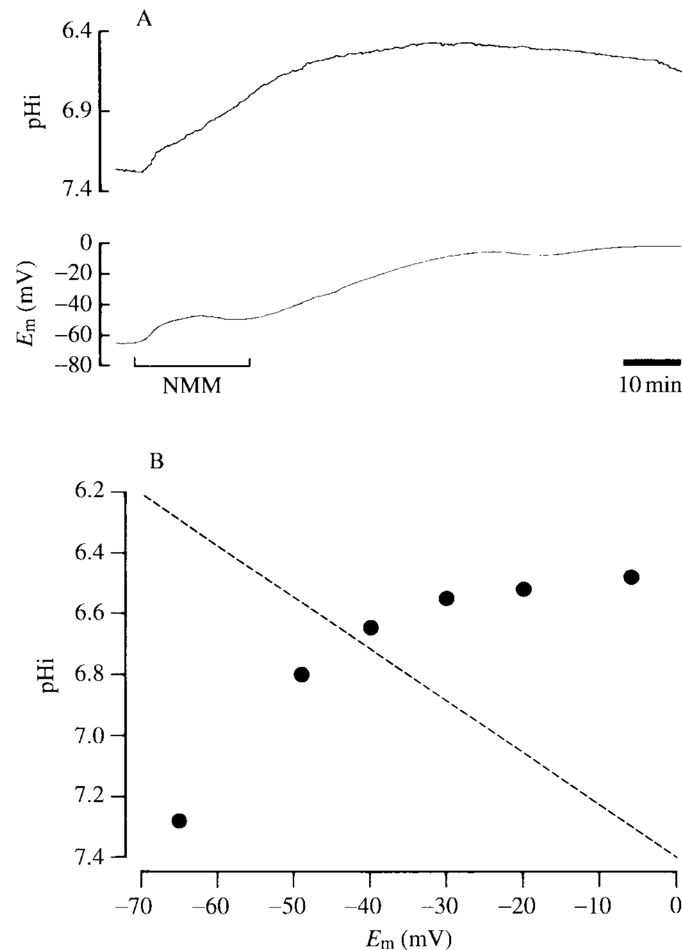

Fig. 12. (A) *N*-methylmaleimide (NMM, 0.5 mmol l^{-1}), which inactivates acidic organelle H^+ pumps, induces a prolonged decrease in pHi. (B) Plot of pHi against E_m (filled circles) from the recording in A for a few selected values. The dashed line shows the expected relationship for passive distribution of H^+ across the cell membrane, and demonstrates that pHi falls well below its equilibrium value. This may be explained by loss of H^+ to the cytoplasm from the secretory vesicles which pack the cell body.

mechanisms in animal cells are Na^+/H^+ exchange and various bicarbonate-transport systems.

Na^+-dependence of pHi regulation

In nominally HCO_3^--free saline, the giant salivary cells have an average pHi of 6.98, which is about 0.5 units above the value expected for passive H^+ distribution and which decreases when external Na^+ is removed. Acid loading by the NH_4^+ prepulse technique is followed by a Na^+-dependent recovery which is unaffected by amiloride, a blocker of Na^+/H^+ exchange. Amiloride is known, however, to compete with Na^+ for the Na^+/H^+

exchanger (Boron and Boulpaep, 1983) and this is consistent with our observation that acidification on removal of external Na^+ is blocked by amiloride, which also slows the subsequent recovery when Na^+ is reintroduced (Fig. 6B). The results indicate a significant contribution of Na^+/H^+ exchange to the control of the resting level of pHi and to recovery from acidosis.

The salivary gland cells do not constitute a homogeneous population; they differ in histochemistry, content of secretory product and in their size and appearance (Sawyer *et al.* 1982; Walz *et al.* 1988; Wuttke *et al.* 1989). The results of the present study refer to the largest cells, which appear quite distinct from their neighbours and seem to represent a separate population. Nevertheless, we have been unable to identify any electrophysiological differences amongst the cells of the gland, except in the nature of their pHi regulation. The smaller cells (300–400 μm) with a coarse-grained cytoplasm showed an amiloride-induced block of pHi recovery from an acid load, whilst removal of external Na^+ had no immediate effect on pHi. These results suggest the presence of two distinct Na^+/H^+ exchangers. In the giant cells, the exchange mechanism is insensitive to amiloride in the presence of external Na^+ and can probably operate in reverse, in view of the progressive decline in pHi when external Na^+ is removed (see Deitmer and Ellis, 1980). In the smaller cells, the exchanger is inhibited by amiloride in the presence of external Na^+ and appears unable to run backwards, since removal of external Na^+ has no immediate effect on pHi.

The lack of effect of E_m on pHi regulation suggests an electroneutral exchange of external Na^+ for internal H^+. Thermodynamic equilibrium of the Na^+/H^+ exchange would be expected with a pHi of about 8.17 (calculated using the Na^+ equilibrium potential of +45 mV; see Wuttke and Berry, 1990). This suggests the presence of a controlling mechanism that slows the exchanger when pHi is returned to its original physiological level after cytoplasmic acidification (Aronson *et al.* 1982).

Absence of HCO_3^--dependent mechanisms of pHi regulation

None of the results indicates the presence of significant Cl^-/HCO_3^- exchange or Na^+/HCO_3^- cotransport: recovery from an acid load is not blocked by the anion-exchange inhibitor SITS and is not affected by substitution of Hepes-buffered saline with a bicarbonate-buffered solution. There remains the possibility that in nominally HCO_3^--free solution the metabolic production of CO_2 could lead to the generation of HCO_3^- in unstirred layers surrounding the cells. In barnacle muscle, however, whose cells have a similar large diameter and highly infolded surface membrane, it was estimated that HCO_3^- in unstirred layers could support the transport system at about 12 % of its maximal rate (Boron *et al.* 1981). Furthermore, in the gland cells a lower steady-state pHi value was found in bicarbonate-buffered solution compared with Hepes-buffered solution; in cells possessing a bicarbonate-dependent acid extrusion mechanism, the steady-state pHi tends to be rather higher in the presence than in the absence of bicarbonate (e.g. Deitmer and Schlue, 1987).

pHi and secretion

Intracellular pH has been implicated as a component in stimulus–secretion coupling in

several cell systems. In rat brain synaptosomes, Drapeau and Nachsen (1988) found a direct Ca^{2+}-independent stimulation of dopamine release by reduced pHi, and an increase in evoked release. Acidic pH also favours the occurrence of exocytosis in bovine adrenal chromaffin cells (Knight and Baker, 1982; Kao *et al.* 1991), whilst cytoplasmic alkalization inhibits secretion (O'Sullivan and Burgoyne, 1988; Kuijpers *et al.* 1989; Rosario *et al.* 1991). Cholinergic agonists or K^+-depolarization, which stimulate secretion in these chromaffin cells, also produce a cytoplasmic acidification, though the changes in pHi do not appear to represent a causal step in stimulus–secretion coupling (Rosario *et al.* 1991). Acidic pH enhances synexin-mediated fusion of vesicular membrane and also the unpolymerised state of actin, which may favour exocytosis (Tilney *et al.* 1978; Stutzin *et al.* 1987). Stimulation of secretion by alkalizing agents appears less common. Shorte *et al.* (1991) found that NH_4^+ stimulates secretion by bovine anterior pituitary cells mainly by releasing Ca^{2+} from secretory vesicles. This effect, however, appeared to be caused by increased pH of the vesicles rather than the cytoplasm, because methylamine increased pHi without stimulating secretion (the concentration of uncharged methylamine was considered to be too low to enter the vesicles to produce sufficient increase in their pH). In contrast, reduction in the H^+ gradient across the chromaffin vesicle membrane caused by NH_4^+ had no effect on catecholamine secretion (Holz *et al.* 1983; Knight and Baker, 1985).

Our results add to this variability of effects of pHi on secretion by showing that cytoplasmic alkalization stimulates secretion independently of changes in E_m or $[Ca^{2+}]_i$ in leech salivary cells. There is no evidence that the Na^+/H^+ exchanger may be a component in excitation–secretion coupling, but factors influencing pHi would have a modulatory role.

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