The Journal of Experimental Biology 205, 1069–1075 (2002)
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JEB3907

Determinants of intracellular pH in gas gland cells of the swimbladder of the European eel *Anguilla anguilla*

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Accepted 30 January 2002

Summary

Gas gland cells of the European eel (*Anguilla anguilla*) were cultured on collagen-coated coverslips, and intracellular pH was measured using the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxypropyl)-5-(6)-carboxyfluorescein (BCPCF). The contributions of various proton-translocating mechanisms to homeostasis of intracellular pH (pHi) were assessed by adding specific inhibitors of the various proton-translocating mechanisms at a constant extracellular pH (pHe) of 7.4 and after artificial acidification of the cells using the ammonium pulse technique. The greatest decrease in pHi was observed after addition of 5-(*N*-ethyl-*N*-isobutyl)-amiloride (MIA), an inhibitor of Na^+/H^+ exchange. Na^+/H^+ exchange was active under steady-state conditions at an extracellular pH of 7.4, and activity increased after intracellular acidification. Incubation of gas gland cells with 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), an inhibitor of anion exchange, also caused a decrease in pHi, but this decrease was not as pronounced as in the presence of MIA. Furthermore, at low pHi, the effect of DIDS was further reduced, suggesting that bicarbonate-exchanging mechanisms are involved in maintaining a steady-state pHi but that their importance is reduced at low pH. Bafilomycin A_1, a specific inhibitor of the V-ATPase, had no effect on steady-state pHi. However, recovery of intracellular pH after an artificial acid load was significantly impaired in the presence of bafilomycin. Our results suggest that Na^+/H^+ exchange and anion exchange are important for the regulation of pHi at alkaline values of pHe. When pHi is low, a situation probably often encountered by gas gland cells during gas secretion, Na^+/H^+ exchange continues to play an important role in acid secretion and a V-ATPase appears to contribute to proton secretion.

Key words: swimbladder, gas gland cell, V-ATPase, Na^+/H^+ exchange, anion exchange, European eel, *Anguilla anguilla*, pHi.

Introduction

Many fish possess a gas-filled swimbladder as a hydrostatic organ. To compensate for the compression of the swimbladder in response to an increase in hydrostatic pressure or a volume decrease caused by diffusional loss of gases, gas must be secreted into the swimbladder. Although the commonly used term ‘secretion’ suggests that there is active transport of gas molecules, the swimbladder is actually filled only by diffusion of gas molecules from the blood into the swimbladder lumen. Diffusional gas transport requires gas partial pressure gradients, which are established by reducing the effective gas-transport capacity of the blood during passage through the swimbladder (Kuhn et al., 1963) (for reviews, see Pelster, 1997; Pelster and Randall, 1998).

The reduction in the effective gas-transport capacity of the blood is achieved by the production and secretion of acidic metabolites by swimbladder gas gland cells. Gas gland cells are highly specialized for the production of lactic acid and CO_2 *via* anaerobic metabolic pathways, such as glycolysis or the pentose phosphate shunt (Pelster, 1995b). This acid is released into the bloodstream to reduce the physical solubility of gases (salting out effect) and the haemoglobin oxygen-carrying capacity (Root effect) and, thus, to increase gas partial pressures in the blood. Although the acid secretion of gas gland cells has been characterized in cultured cells (Pelster 1995a; Pelster and Niederstätter, 1997), nothing is known about the intracellular pH of these cells or of how it is regulated.

Three major mechanisms are known to regulate intracellular pH: the Na^+/H^+ exchanger, which uses the Na^+ concentration gradient as a driving force to remove protons from the cell (Roos and Boron, 1981; Doppler et al., 1986; Harvey and Ehrenfeld, 1988; Kramhoft et al., 1988); anion exchangers which, in conjunction with the carbonic-anhydrase-catalyzed equilibrium of the CO_2/HCO_3^- reaction, transfer protons from one side of the membrane to the other; and H^+-ATPases, such as V-ATPase (Deitmer and Rose, 1996). Different types of bicarbonate exchange have been described, the most important here being the $Na^+ + HCO_3^-/H^+ + Cl^-$ exchanger, also called the Na^+-dependent bicarbonate exchanger, and the Na^+-independent HCO_3^-/Cl^- exchanger, which normally promotes an efflux of HCO_3^- to decrease pHi after alkalization

(Reinertsen et al., 1988; Kramhoft et al., 1994). All these mechanisms have been shown to play a role in proton release by gas gland cells in primary cell culture (Pelster, 1995a; Pelster and Niederstätter, 1997). The question unanswered so far is how intracellular pH (pHi) is influenced by these mechanisms, or even by extracellular pH (pHe), which can be as low as pH 6.6–6.8 during periods of gas deposition (Kobayashi et al., 1990). The aim of the present study was therefore to analyze how the various mechanisms of proton secretion contribute to the control of pHi in swimbladder gas gland cells.

Materials and methods

Eels of the species *Anguilla anguilla* L. (body mass 300–500 g) were obtained from local fishermen and kept in aerated fresh water at 10 °C under a natural photoperiod. Up to 30 eels were kept in a tank of approximately 500 l. In captivity, eels caught in the wild usually do not accept food.

DMEM F12 (Dulbecco's modified Eagle's medium mixture F-12) and foetal calf serum (FCS) were obtained from Gibco-BRL; Ala-Gln (alanine-glutamine), albumin, bafilomycin A_1, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), desoxyribonuclease I (DNAse I), epidermal growth factor (EGF), gentamycin, Hepes, kanamycin, 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA), NH_4Cl, pituitary extract, progesterone, protease, putrescine and valinomycin were obtained from Sigma (Deisenhofen, Germany); 2′,7′-bis-(2-carboxypropyl)-5-(6-)-carboxyfluorescein (BCPCF) and nigericin were obtained from Molecular Probes Europe (Leiden, Netherlands); collagen S (Type I, from calf skin), collagenase and insulin-tranferrin-sodium-selenite supplement (ITS) were obtained from Roche M.B. (formerly Boehringer, Mannheim, Germany).

Cell preparation and cell culture

The eels were quickly killed by decerebration and spinal pithing. The body wall was opened ventrally, and the swimbladder was carefully exposed. A catheter was inserted into the swimbladder artery to perfuse the organ with saline solution (in mmol l^{-1}: 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 Hepes, pH 7.4). The swimbladder epithelium was then removed, cut into small pieces and incubated in saline solution containing (in g l^{-1}) 0.50 albumin, 0.22 collagenase, 0.165 protease, 0.15 DNAse and 3–4 ml l^{-1} elastase for 10 min. The solution was filtered through a 70 μm cell strainer into a stop solution (DMEM F12 with 10 % FCS). For the strained tissue, the enzyme incubation and filtration procedure was repeated twice. Cells were separated from the solution by centrifugation (10 min at 700 revs min^{-1}, 4 °C) and resuspension (see Pelster, 1995a; Pelster and Niederstätter, 1997). Sedimentation and resuspension of the cells were repeated twice to remove all traces of the solution used for the digestion of the tissue. Gas gland cells were cultured on collagen-S-coated coverslips in DMEM F12 (cell culture medium enriched with, in mg l^{-1}, 5 ITS, 0.001 EGF, 0.00629 progesterone, 0.5 pituitary extract, 1000 albumin and 2172 Ala-Glu, and, in ml l^{-1}, 5 FCS, 5 gentamycin (1 mol l^{-1}) and 10 kanamycin (0.5 mol l^{-1}). Cells were incubated at 18–20 °C and 0.6 % CO_2 for 2–5 days until they formed a broad monolayer of cell patches, but not yet a confluent layer. Cell culture medium was renewed as soon as a change in colour indicated low pH.

BCPCF incubation and pHi measurement.

Cell patches were incubated for 30 min with 1 μmol l^{-1} BCPCF, a derivative of the pH-sensitive fluorescent dye bis-carboxyethyl-carboxyfluorescein (BCECF) (Graber et al., 1986; Wood and Pärt, 2000). Excess dye was removed by washing the cells twice with phosphate-buffered saline (PBS; containing, in mmol l^{-1}, 137 NaCl, 2.68 KCl, 1.47 KH_2PO_4, 8.06 $Na_2HPO_4.7H_2O$, 10 glucose, 1 $CaCl_2$ and 1 $MgSO_4$; pH 7.4). Measurements were started approximately 10 min later so that pHi was not influenced by BCPCF incubation itself, which could lower pHi (Negulescu and Machen, 1990). Both cell patches and media were tested before incubation with BCPCF to exclude other disturbing influences such as autofluorescence of the cells or of the chemicals used for measurements (Buckler and Vaughan-Jones, 1990; Kramhoft et al., 1988; Wong and Huang, 1989; Pocock and Richards, 1992).

A common phenomenon was leakage of BCPCF out of the cell (Allen et al., 1990; Muallem et al., 1992), which could not be avoided despite different loading methods; data from experiments with a significant rate of leakage were rejected.

The fluorescence intensity of BCPCF is independent of proton concentration at an excitation wavelength of 440 nm,

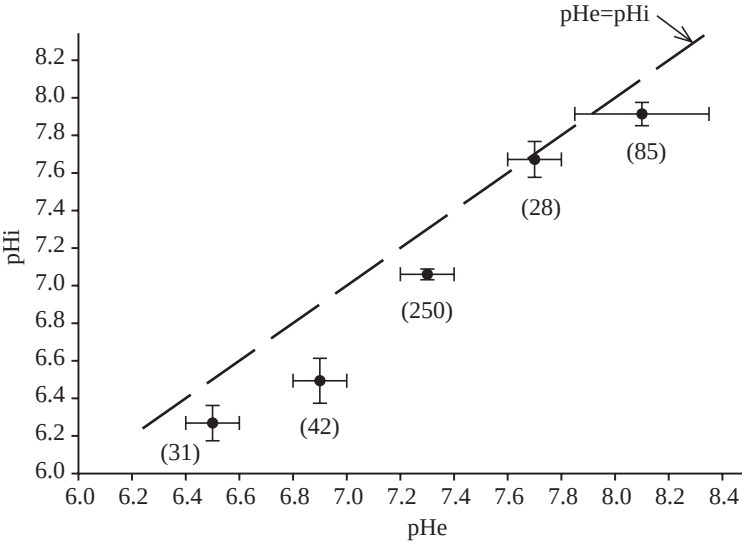

Fig. 1. Changes in intracellular pH (pHi) at varied extracellular pH (pHe) in swimbladder gas gland cells. The values in parentheses are the number of cell cultures measured. Values are means ± S.E.M.

but changes according to pH when excited at 490 nm. Intracellular pH was therefore quantified by calculating the ratio of fluorescence recorded at these two excitation wavelengths (Graber et al., 1986; Negulescu and Machen, 1990). Measurements were supported by Tillvision 3.3 software from T.I.L.L. Photonics (Martinsried, Germany). Measurements were acquired once every minute, or once every 15 s, immediately after the incubation medium had been changed.

The excitation ratio was calibrated for pH by constructing a calibration curve. This was achieved by equilibrating pHi and pHe. Cells were permeabilized by treatment with the cation ionophores nigericin (10^{-5} mol l^{-1}) and valinomycin (5×10^{-6} mol l^{-1}) and exposed to a modified PBS medium containing a high concentration of K^+ (NaCl replaced by KCl) (Pocock and Richards, 1992; Seo et al., 1994). A time-dependent bleaching of the BCPCF (Borzak et al., 1990; Weinlich et al., 1993) was occasionally observed.

Experimental protocol

In the first series of experiments, cells were exposed to PBS titrated to different pH values, and the fluorescence ratio was recorded without any further treatment to analyze the relationship between pHe and pHi. In the second set of experiments, the effects of specific inhibitors of the various proton-translocating mechanisms (MIA, DIDS and bafilomycin A_1) were analyzed at a constant pHe (7.4). In the third series of experiments, the influence of these inhibitors on pHi was examined during artificial lowering of pHi using the NH_4^+ pulse technique, i.e. by incubating the cells with 10 mmol l^{-1} NH_4Cl in PBS (pH 7.4) for 10 min (Negulescu and Machen, 1990). Addition of NH_4Cl solution, in which an equilibrium is established between gaseous NH_3 and NH_4^+, results in the diffusion of NH_3 into the cell. Protonation of NH_3 results in alkalization of pHi. On removal of NH_4Cl, the opposite takes place: NH_3 rapidly leaves the cell by diffusion and the proton stays behind, causing an acidification of the cell, which is then compensated by active or secondary active removal of protons out of the cell.

Statistical analyses

Values are presented as means ± S.E.M. (*N* is the number of cell patches examined). For each set of experiments, cells from at least four different cell preparations were used. Differences from control values were tested by analysis of variance (ANOVA). Significance was accepted at $P<0.05$. Values of $P<0.01$ are reported as highly significant.

Results

Changes in pHi in relation to changes in pHe

For our experiments, the pH of the extracellular fluid was routinely set to 7.4, and mean pHi under these conditions was 7.20±0.03 (N=19). The response of pHi to changes in pHe was not instantaneous; it usually took approximately 2–5 min to establish a new steady state. A summary of all measurements is shown in Fig. 1. On average, pHi is slightly more acidic than pHe over the whole range of pHe values tested (6.4–8.4). At low pHe, the effect of changes in pHe appeared to be more pronounced than at high pHe.

Influence of MIA

Incubation of cells with 10^{-5} mol l^{-1} MIA caused a more-or-less continuous decrease in pHi during the time of MIA

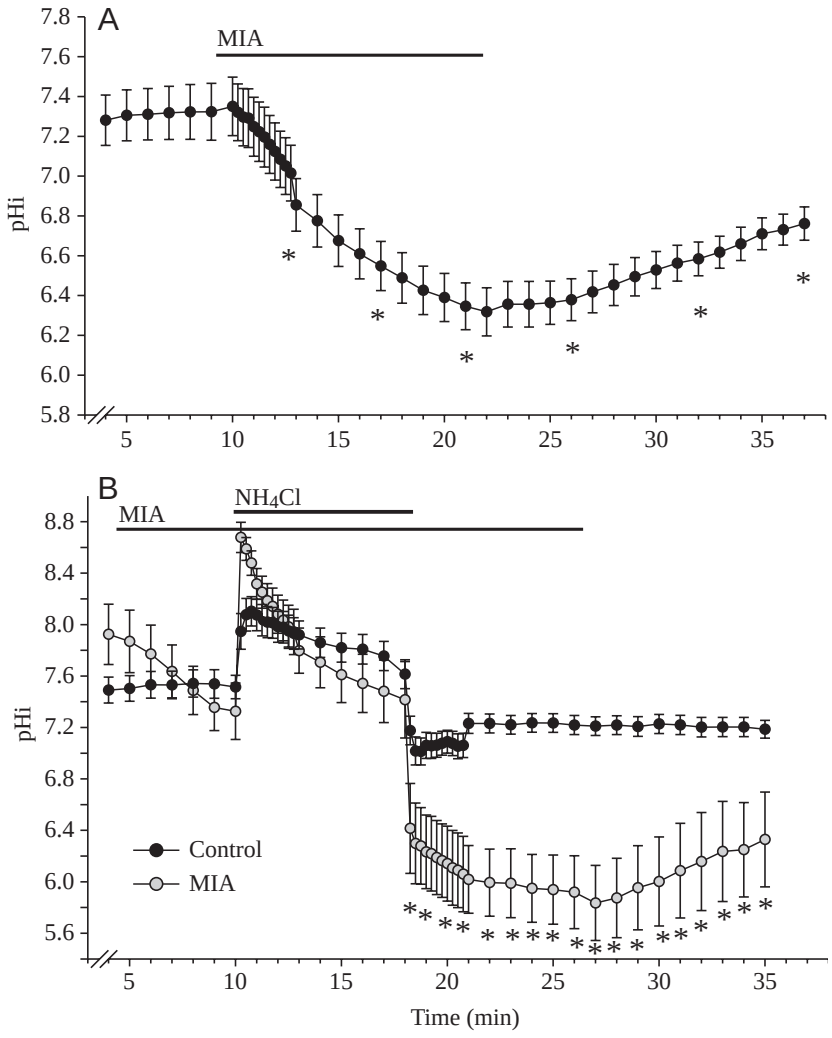

Fig. 2. The influence of 5-(*N*-methyl-*N*-isobutyl)-amiloride (MIA; 10^{-5} mol l^{-1}) on intracellular pH (pHi) of gas gland cells at a constant extracellular pH (pHe) of 7.4 (A) and following an artificial intracellular acidification induced using the ammonium pulse technique (10 mmol l^{-1} NH_4Cl in phosphate-buffered saline for 8 min) (B). *Highly significantly different from the control ($P<0.01$). Values are means ± S.E.M.

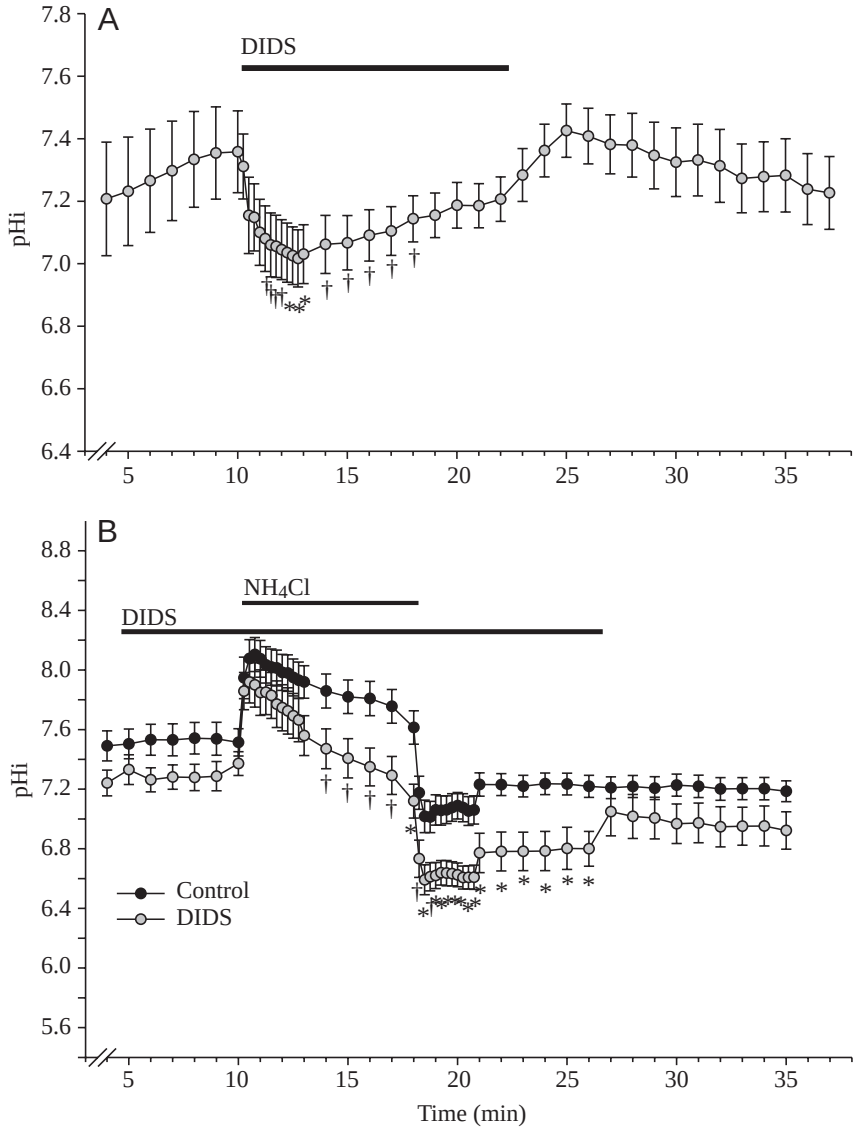

Fig. 3. The influence of 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS; 10^{-4} mol l^{-1}) on intracellular pH (pHi) of gas gland cells at a constant extracellular pH (pHe) of 7.4 (A) and following an artificial intracellular acidification induced using the ammonium pulse technique (10 mmol l^{-1} NH_4Cl in phosphate-buffered saline for 8 min) (B). †Significantly different from the control (P<0.05); *highly significantly different from the control (P<0.01). Values are means ± S.E.M.

exposure (Fig. 2A). After 10 min of exposure, pHi had decreased by approximately 1 unit. After removal of MIA, recovery of pHi was very slow. pHi still was significantly lower than control values 15 min after a return to control medium. Even after 1 h, some cells had not returned to control pHi (results not shown).

A similar result was observed after artificial acidification of the cells using the NH_4^+ pulse technique. In the presence of MIA, the decrease in pHi following the removal of ammonium was significantly more pronounced than in the absence of MIA. After removal of ammonia, the pHi of control cells remained only slightly below the prepulse value, but in the presence of MIA the cells were acidified by more than 1 pH unit, and recovery from this severe acidification was very slow (Fig. 2B).

It is also noteworthy that, during the ammonium pulse, pHi in MIA-incubated cells became more alkaline than in control cells. At pH values higher than 7.8, however, the relationship between fluorescence intensity and pHi changes (Graber et al., 1986), so pHi values calculated at this point may represent an overestimate.

Influence of DIDS

Addition of DIDS (10^{-4} mol l^{-1}) to the incubation medium resulted in a rapid decrease in pHi, but this decrease was not as pronounced as in the presence of MIA. Intracellular pH decreased by approximately 0.3 unit, and after 5 min started to return towards control levels, even in the presence of DIDS (Fig. 3A).

Compared with controls, acidification of cells after the NH_4^+ pulse was more pronounced in the presence of DIDS (Fig. 3B). While pHi stabilised at 7.2 in control cells after the NH_4^+ pulse, in the presence of DIDS it stabilised at approximately 6.8. After removal of DIDS, pHi returned to control values. Towards the end of the NH_4^+ pulse, pHi was less alkaline in DIDS-incubated cells.

Influence of bafilomycin A_1

Addition of bafilomycin A_1 to the incubation medium at pHe=7.4 had no effect on steady-state pHi (data not shown). After acidification of the cells by the NH_4^+ pulse, however, a severe acidification was observed in the presence of 10^{-5} mol l^{-1} bafilomycin (Fig. 4). In the presence of bafilomycin, pHi decreased to 6.2, compared with 7.1 in control cells. After removal of NH_4^+, only a minor increase in pHi was observed in the presence of bafilomycin. Following the removal of bafilomycin, pHi increased significantly, but it did not return to control levels within the first 10 min.

Discussion

Relationship between external and internal pH

While most acid-secreting cells, such as parietal cells, gain the protons required for acid secretion from the dissociation of water catalyzed by carbonic anhydrase (Muallem et al., 1988), gas gland cells are characterized by a very low rate of aerobic metabolism, and acid is produced mainly *via* the anaerobic glycolytic pathway (lactic acid) and the pentose phosphate shunt (CO_2) (Pelster, 1995b). Nevertheless, the results of our study show clearly that, as in most cells (Frelin et al., 1988), intracellular pH in swimbladder gas gland cells is more

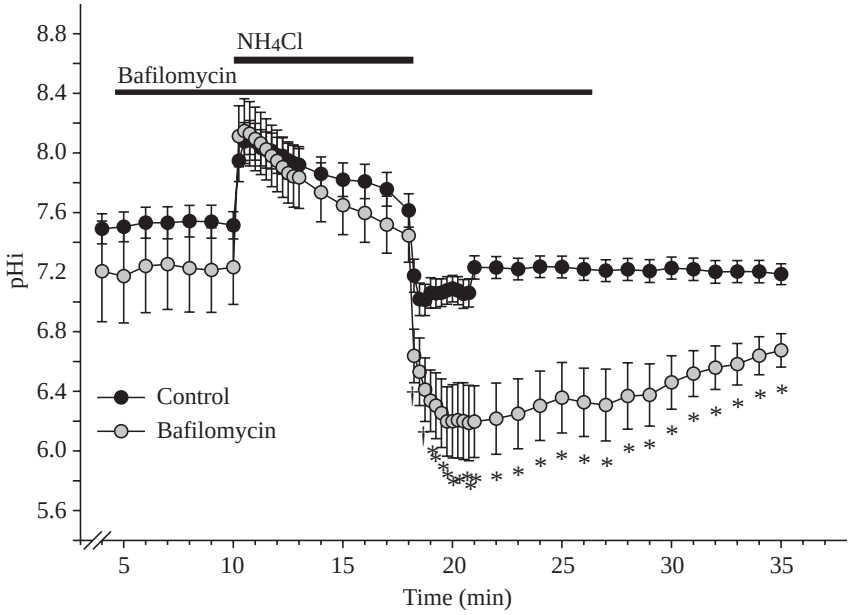

Fig. 4. The influence of bafilomycin A_1 (10^{-5} mol l^{-1}) on intracellular pH (pHi) of gas gland cells following an artificial intracellular acidification induced using the ammonium pulse technique (10 mmol l^{-1} NH_4Cl in phosphate-buffered saline for 8 min, pHe=7.4). †Significantly different from the control (P<0.05); *highly significantly different from the control (P<0.01). Values are means ± S.E.M.

alkaline than expected on the basis of the electrochemical equilibrium. Assuming a membrane potential of approximately –50 mV, pHi at equilibrium would be approximately 1 unit more acidic than pHe. Our results reveal that the pH difference is significantly smaller (approximately 0.2–0.3 unit). If this were an equilibrium state, it would require an unreasonably low membrane potential. We conclude, therefore, that protons are not passively distributed across the plasma membrane of gas gland cells. Active ion transport must contribute to the proton gradient established across the cell membrane in resting gas gland cells. In many cells, changes in cytoplasmic pH are kept small over an extracellular pH range between 7.0 and 7.8, but gas gland cells appear to be most sensitive to changes in extracellular pH in this physiological range.

At low extracellular pH values (near 7.0), pHi in many cells approaches pHe, but in gas gland cells pHi remained significantly lower than pHe. During subsequent alkalization, pHi recovered to alkaline values, which clearly shows that the low pHi does not indicate any loss of control or cell damage. However, the danger of an excessive acidification of the cells is probably alleviated by a reduction in glycolytic activity at low pH (Pelster, 1995b), and the rate of acid secretion indeed decreases with decreasing pHe (Pelster, 1995a).

The role of Na^+/H^+ exchange in gas gland cells

Previous studies have demonstrated the existence of a variety of proton-translocating mechanisms in gas gland cells, such as Na^+/H^+ exchange, anion exchange and V-ATPase, but their importance for the homeostasis of pHi remains unclear (Pelster, 1995a). The results of the present study reveal that Na^+/H^+ exchange is essential for the stability of pHi in resting cells, but also for the recovery of pHi from an artificial acid load. The Na^+/H^+ exchanger is present in almost all cells and contributes significantly to the removal of protons from the cytoplasm (Frelin et al., 1988; Fliegel and Dibrov, 1996). Even in parietal cells, which during stimulation secrete protons *via* the K^+/H^+-ATPase, Na^+/H^+ exchange appears to be the main route of proton release after an artificial acid load (Rossmann et al., 2001).

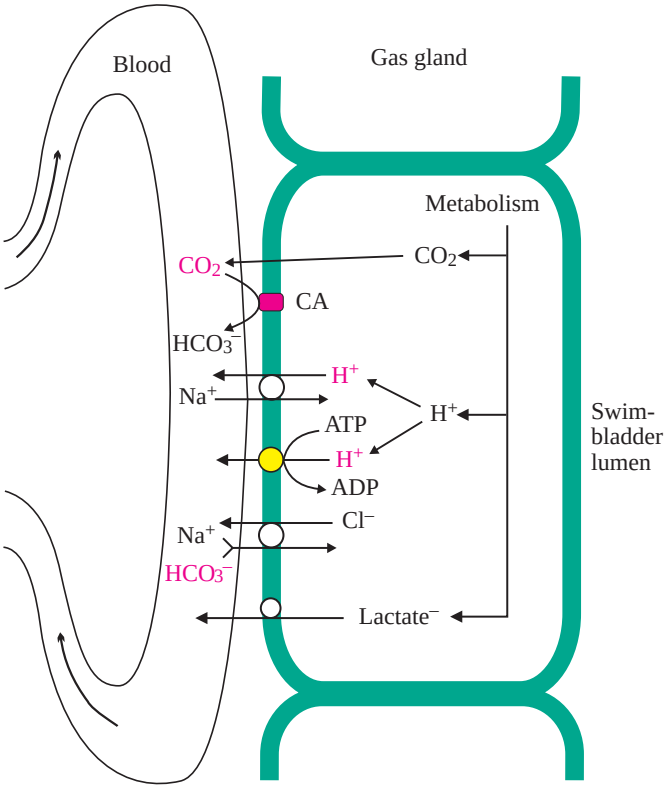

Fig. 5. Hypothetical model showing the various proton-translocating mechanisms identified so far in swimbladder gas gland cells of the European eel. Acidic metabolites (lactic acid and CO_2) are produced in the glycolytic pathway and in the pentose phosphate shunt. This acid is secreted at the basolateral membranes to reduce the effective gas-transport capacity of the blood *via* the Root effect and the salting out effect (see Pelster, 1997; Pelster and Randall, 1998). The decrease in gas-transport capacity induces an increase in gas partial pressures in the blood, which provides the necessary partial pressure gradient towards the swimbladder lumen so that gases can enter the swimbladder by diffusion. The mechanisms of lactate transport have not yet been characterized in detail. CA, membrane-bound carbonic anhydrase.

Na^+/H^+ exchange is typically activated at low pHi and inactived at alkaline pH (Frelin et al., 1988). As shown previously, the relative contribution of Na^+/H^+ exchange to acid secretion by gas gland cells is enhanced at low pHe (Pelster and Niederstätter, 1997), and this is supported by the results of the present study. The use of Na^+-dependent mechanisms for ion transport requires Na^+/K^+-ATPase activity, and the importance of this ATPase for acid secretion by gas gland cells has been demonstrated previously (Pelster and Niederstätter, 1997). Incubation of gas gland cells with 1 mmol l^{-1} ouabain induced a slow decrease in the rate of proton secretion, and this effect was particularly pronounced at low pH, which is in line with our results on the pH-dependence of Na^+/H^+ exchange. The rapid decrease in the rate of acid secretion on removal of extracellular Na^+ in comparison with the slow response to removal of K^+ or application of ouabain suggested that the Na^+ gradient was critical for acid secretion and that the membrane potential was not of primary importance (Pelster and Niederstätter, 1997).

The role of anion exchange

DIDS, as an inhibitor of bicarbonate exchangers, also caused a decrease in pHi, but this decrease was not as pronounced as that observed after addition of MIA. It can therefore be concluded that an anion exchanger is involved in maintaining steady-state pHi. The Cl^-/HCO_3^- exchanger typically extrudes base, and an inhibition would therefore result in an accumulation of base inside the cell, which is equivalent to an alkalization. The Na^+-dependent anion exchanger, however, uses the Na^+ gradient and transports HCO_3^- into the cell. In this case, inhibition of the anion exchanger would result in a lower HCO_3^- concentration inside the cell and, thus, in a reduced buffering capacity. In consequence, pHi will become more acidic. The observed decrease in pHi in the presence of DIDS is therefore in line with the conclusion that a Na^+-dependent anion exchanger is present in swimbladder gas gland cells (Pelster, 1995a). The influence of DIDS was reduced after the ammonium pulse, suggesting that the importance of bicarbonate-exchanging mechanisms is reduced at low pHi. Similar results have been reported for muscle and in Ehrlich ascites tumour cells, in which bicarbonate exchange appears to be mainly responsible for the regulation of steady-state pHi, but Na^+/H^+ exchange is activated at low pHi (Kramhoft et al., 1994). This conclusion is also supported by the observation that, during the NH_4Cl incubation phase, the alkalization of control cells was not as pronounced as in the presence of DIDS. HCO_3^- extrusion by the Na^+-independent HCO_3^-/Cl^- exchanger would have resulted in the downregulation of pHi in this situation (Reinertsen et al., 1988; Wood and Pärt, 2000).

The role of V-ATPase

A reduction in the rate of acid secretion in the presence of bafilomycin A_1 suggests that a V-type ATPase is present in gas gland cells (Pelster, 1995a), and two isoforms of the B-subunit of V-ATPase have recently been cloned and sequenced (Niederstätter and Pelster, 2000). The presence of two different isoforms may, of course, be related to different properties and/or functions of the protein. Gas gland cells are responsible not only for the production and secretion of acidic metabolites but also for the production and secretion of surfactant at their luminal surface (Prem et al., 2000). Before exocytosis, surfactant is stored in multilamellar bodies, which can be stained with the fluorescent dye LysoTracker Green (Mair et al., 1999), a weak base that accumulates in acidic organelles. This staining can be prevented by preincubation of the gas gland cells with submicromolar concentrations of bafilomycin A_1 (H. Niederstätter and B. Pelster, unpublished observations), which demonstrates that the low pH established in these vesicles requires the presence of V-ATPase. The present study revealed that inhibition of V-ATPase with bafilomycin A_1 significantly impairs proton extrusion after an artificial acid load. Therefore, at low pHi, V-ATPase is activated and required for the regulation of cell pH. At alkaline pH values, bafilomycin A_1 had no effect on pHi in the present study, but in an earlier study a 15–20 % reduction in the rate of acid secretion was observed (Pelster, 1995a). It appears quite possible that these different results may be related to a possible shuttling of V-ATPase in relation to the activity status of the cell, as observed in kidney cells for example (Gluck et al., 1998). Like parietal cells, in which K^+/H^+-ATPase is mainly involved in acid secretion during periods of stimulation and Na^+/H^+ exchange is mainly responsible for homeostasis of intracellular pH (Muallem et al., 1988; Yanaka et al., 1991; Rossmann et al., 2001), in gas gland cells, V-ATPase would be responsible for the secretion of acid during periods of gas deposition into the swimbladder and pHi would be controlled mainly by Na^+/H^+ exchange and in part by anion exchange at alkaline pHe values. Unfortunately, attempts to stimulate gas gland cells and thus to enhance acid secretion have not been successful (Pelster and Pott, 1996). Verification of this hypothesis must await further experimentation.

Concluding remarks

Our results suggest that Na^+/H^+ exchange and anion exchange are important for the regulation of pHi at alkaline pHe values. Recovery from an intracellular acidification depends mainly on the activity of the Na^+/H^+ exchanger, and a V-ATPase contributes significantly to the removal of protons from the cytoplasm. The activity of bicarbonate-exchanging mechanisms, however, is greatly reduced at low pHi. A model of a gas gland cell and the mechanisms of acid transport developed on the basis of the present study and the results of previous studies (Pelster, 1995a; Pelster and Niederstätter, 1997) is shown in Fig. 5. The presence of various mechanisms for the secretion of protons therefore appears to be attributable to the fact that gas gland cells need to secrete protons at a wide range of pHe values. The mechanisms that control the activity status of gas gland cells are still unknown, but this promises to be a fascinating area of research.

Parts of this study were financially supported by the Fonds zur Förderung der wissenschaftlichen Forschung (FWF, P11837-BIO).

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