pH-dependent proton secretion in cultured swim bladder gas gland cells

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Pelster, B., and H. Niederstätter. pH-dependent proton secretion in cultured swim bladder gas gland cells. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1719–R1725, 1997.—The pH dependence of acid production and of acid release has been analyzed in cultured gas gland cells of the European eel using a cytosensor microphysiometer. Total acid release of gas gland cells showed an optimum at pH 7.4–7.6, with only a minor reduction at acidic (pH 7.0) as well as at alkaline pH (pH 8.0). The acid production was largely dependent on the availability of extracellular glucose and was almost completely abolished if glucose was replaced by succinate, alanine, or even pyruvate. Phloretin, an inhibitor of glucose uptake, significantly reduced acid release of gas gland cells with a Ki of ~1 × 10−3 M, irrespective of pH. Although the glucose dependence of acid production was not modified by pH, acid release became increasingly sodium dependent with decreasing pH, but at low pH significantly higher sodium concentrations were necessary to achieve maximal rate of proton secretion. This sodium-dependent proton secretion could only in part be inhibited by application of 5-(N-methyl-N-isobutyl)-amiloride. Removal of extracellular potassium caused a slow reduction in the rate of acid secretion. A similar reduction was observed in the presence of ouabain, a specific inhibitor of Na+/K+-adenosinetriphosphatase, and both effects significantly increased with decreasing pH. The results demonstrate a significant pH dependence of the mechanisms of acid release in swim bladder gas gland cells and indicate that sodium-dependent pathways become especially important at low pH.

The cultured cells retain high activities of enzymes of the glycolytic pathway and the pentose phosphate shunt. This study also demonstrated that, via adenosine 3’,5’-cyclic monophosphate as a second messenger, a significant reduction of acid release can be achieved in gas gland cells. It remains to be shown whether this reduction is due to a decrease in the rate of acid production or to a modification of the mechanisms that contribute to acid secretion.

Although membranes typically are not easily penetrated by protons, gas gland cells apparently are equipped with a number of mechanisms that contribute to the secretion of acid, including sodium-dependent pathways, vacuolar-adenosinetriphosphatase (ATPase), and the diffusion of CO2, combined with carbonic anhydrase activity (15). Depending on the functional state of the swim bladder, gas gland cells in vivo are exposed to a wide range of pH values. Although in inactive, nonsecreting swim bladders blood pH near gas gland cells may be ~7.4–7.6, during periods of gas deposition the pH may decrease down to pH 6.6–6.8 (7, 22). The present study therefore set out to test the idea that either proton production or proton-secreting pathways may be dependent on extracellular pH. A pH dependence of proton secretion would explain why so many different pathways of acid secretion are established in gas gland cells.

MATERIAL AND METHODS

Specimens of the European eel Anguilla anguilla (body mass 350–500 g) were obtained from a local supplier and kept in a freshwater aquarium at 12–16°C until used for the experiments. The fish were not fed and were kept under natural light conditions. All experiments were performed at room temperature of 20–22°C.

To establish primary cell cultures of gas gland cells, the following chemicals were used: Dulbecco’s modified Eagle’s medium nutrient mixture F-12 (DMEM F-12; cat. no. 21331-020) and fetal calf serum (FCS) obtained from Gibco BRL [medium 199 (M 199); Eggenstein, Germany] and deoxyribonuclease (DNase), protease, albumin, putrescine, progesterone, epidermal growth factor (EGF), bovine pituitary extract, gentamicin, and kanamycin obtained from Sigma (Deisenhofen, Germany). Elastase was obtained from Serva (Heidelberg, Germany). Insulin-transferrin-sodium-selenite supplement (ITS), collagen type S, and collagenase P were obtained from Boehringer Mannheim (Mannheim, Germany). Additional salts, enzymes, and drugs were obtained from Sigma and Boehringer Mannheim.

Preparation of gas gland cells. The eels were quickly killed by decerebration and spinal pithing. The animals were placed into an eel holder, and the gills were irrigated with well-aerated tap water (20–22°C) at a flow rate of ~1.5–2 l/min (13). Because of the autonomic activity of the ventricle, this procedure assures the supply of oxygenated blood to the tissues and prevents hypoxemia. The body wall was opened ventrally, and the swim bladder was carefully exposed. Occlu-
sive catheters were inserted into the swim bladder artery (PE-20) and into the swim bladder vein (PE-50). To remove blood cells from the tissue, the swim bladder was perfused at a rate of ~0.5 ml/min with saline solution, pH 7.4, consisting of (in mM) 140 NaCl, 5.4 KCl, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine-N'2'-ethanesulfonic acid, and 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid. Disintegration of the swim bladder epithelium was initiated by infusing saline solution containing elastase (3.9 U/ml), DNase (70 U/ml), protease (0.3 U/ml), collagenase (0.3 U/ml), and albumin (0.5 mg/ml). About 5–10 min after infusion of the enzyme solution, the outer layers of swim bladder tissue were removed from the swim bladder epithelium. Finally, the epithelium was removed, chopped into small pieces, and incubated in the enzyme solution for 10 min. The solution was filtered through a 70-µm nylon mesh, and cells and cell clusters were dissolved from the tissue transferred to a centrifuge at 600 revolutions/min (100 g) for 10 min (Heraeus Labofuge 400R). The cell pellet was washed twice. After the second wash, cells were suspended in DMEM supplemented with alanine-glutamine (10 µmol/ml), ITS (5 µg/ml), putrescine (16 µg/ml), and albumin (0.1 mg/ml). About 70 µl of this mixture were pipetted onto a capsule cup, and the ethanol was evaporated almost completely polycarbonate cell capsules (Molecular Devices, Munich, Germany) at ~100–200 µl of cell suspension per well. These capsule caps were previously coated with collagen I. The collagen was prepared by mixing one part collagen I with three parts ethanol (60%). About 70 µl of this mixture were pipetted onto a capsule cup, and the ethanol was evaporated overnight. Typical estimates of cell density of the suspension seeded into the cell capsules range from 1.00 to 1.25 × 10⁶ cells. Rate of acid release in the presence of 5 mM glucose was used as a reference value (set to 100%). Normalized data are presented as percent of basal rate (control rate). The amount of protons (dH⁺) actually excreted by the cells in the cytosensor microphysiometer can be calculated from the change in pH observed in a given time period (dpH), the chamber of ~3 s at a flow rate of 100 µl/min. The basic composition of this medium was (in mM) 138 NaCl, 5.1 KCl, 1.1 CaCl₂, 0.93 MgSO₄, 0.81 K₂HPO₄, 0.11 KH₂PO₄, and 5.0 glucose (pH 7.4; control conditions). For preparation of test solutions, various antagonists were added to the basic medium, with the pH of the solution carefully kept constant. In case dimethyl sulfoxide (DMSO) or ethanol (EtOH) was necessary to solubilize a specific component, the same concentration of DMSO or EtOH was also added to the control channel. Experimental temperature was 22 ± 1°C. The pumps, valve switching, and data collection were controlled by a personal computer (Macintosh) and a dedicated software package (Molecular Devices).

In the series of experiments testing the importance of extracellular sodium concentration for acid secretion, sodium chloride was replaced by tetramethylammonium chloride (TMA) or by choline chloride. Replacement of extracellular potassium was achieved by using the appropriate sodium salts. Stock solutions of phloretin were prepared in ethanol, and stock solutions of 5-(N-methyl-N-isobutyl)-amiloride (MIA) were prepared in DMSO. Ouabain was prepared in phosphate buffer.

To calculate the amount of protons secreted by the cells, the buffer values of the phosphate buffer were determined by titration with HCl in the pH range used for the measurements (n = 3), and the appropriate buffer values were obtained from this curve. For pH 7.0, 7.4, and 7.8, the titration curves revealed the following buffer values (P₅₀): 0.413 mM/pH (pH 7.0), 0.272 mM/pH (pH 7.4), and 0.136 mM/pH (pH 7.8).

Data analysis. The rate of acid release was quantified by fitting the cytosensor data collected during periods of interrupted flow to a straight line with the least-squares procedure. The acidification rate is given in microvolts per second. To eliminate the scatter of the data induced by the variable flow rate at which the cells acidify their environment (10, 12), the measuring chambers of the microphysiometer were intermittently perfused by peristaltic pumps with a medium of low buffer concentration (0.92 mM phosphate) to increase the sensitivity of the system. A typical pumping cycle of 60 s consisted of a flow period of 40 s followed by a flow-off period of 20 s. During flow-off periods, protons released from the gas gland cells accumulated in the measuring chamber, and the rate of proton release was quantified by fitting the sensor data to a straight line with the least-squares procedure, the slope of this line representing the acidification rate. Numerically, a slope of 1 µV/s is close to 0.001 pH/min. At the end of a measuring period, flow was resumed and the next pumping cycle began, washing out the protons that had accumulated in the previous measuring cycle. The pH change produced by the cells during a measuring cycle typically was <0.1 pH units.

Each measuring chamber was constantly supplied by two media (control and test solution), and a change from one to the other was induced by an electromagnetic valve with a lag time between valve switch and fluid arrival at the measuring

![Fig. 1. Substrate-dependent acid release of cultured eel gas gland cells. Rate of acid release in the presence of 5 mM glucose was used as a reference value (set to 100%). For succinate, alanine, and pyruvate, n = 16; for glucose + acetate and glucose + pyruvate, n = 12.](http://ajpregu.physiology.org/ by 10.220.33.1 on April 8, 2017)
buffer value, and the chamber volume (V) (see Ref. 11): 

$$dH^+ = P_{\text{tot}} \times dpH \times V.$$ 

This gives the amount of protons secreted by all cells in the measuring chamber. To recalculate to the rate of proton secretion by an individual cell, the total number of cells in the measuring chamber at the time of measurement must be known, which, however, can hardly be determined (15, 18). Cells are seeded the day before the experiment, and the cell concentration of this solution can be determined. It is not known, however, how many cells actually attach to the polycarbonate membrane and what the proliferation rate is. Furthermore, only ~25% of the total area of the polycarbonate membrane will be in the measuring chamber; the rest is cut off when the system is assembled. The measuring chamber is constructed as a sandwich with polycarbonate membranes on both sides of the cells, so that at the end of the experiment it cannot be disassembled without breaking cells, which prevents an accurate cell count at the end of the experiment. Nevertheless, to get at least an idea about the possible rate of proton secretion by gas gland cells, we used the original cell count of the cell suspension added to the capsule cap and the geometry of the chamber to get an estimate of cell density. Given the uncertainty in the determination of the cell number, the results clearly are estimates of the actual rate of cellular proton secretion but nevertheless are helpful in the comparison with other cell types. Data are given as means ± SE; n refers to the number of cell preparations. Statistical differences from control or between treatments were tested by analysis of variance, followed by a multiple-comparison procedure (SigmaStat) or, where applicable, the Student’s t-test. Significance of differences was accepted at P < 0.05.

RESULTS

Glucose is a well-known substrate for proton secretion of swim bladder gas gland cells, and therefore the rate of acid secretion measured in the presence of 5 mM glucose was used as a control situation. Substrate specificity of proton production in swim bladder gas gland cells was tested by replacing glucose with succinate, alanine, or pyruvate in the extracellular fluid. Any of these replacements of glucose as a substrate resulted in a decrease in the rate of proton secretion by >80% (Fig. 1), and the final rate of proton secretion was hardly different from values obtained simply by removing glucose from the medium (see below). Furthermore,
proton secretion could not be enhanced by addition of acetate or pyruvate to the glucose-containing medium; addition of pyruvate rather resulted in a slight decrease in the rate of proton secretion (Fig. 1).

The influence of extracellular pH on the rate of proton secretion was tested in 16 preparations. The rate of proton secretion was quite stable and at pH 7.4, which was used as a reference value, decreased only by 20% over the time course of 4 h (Fig. 2A). The estimate of the rate of proton secretion per individual cell obtained from these values showed a broad optimum curve (Fig. 2B), with high values at pH 7.4–7.6 and slightly reduced rates at lower as well as at higher pH values.

Figure 3 shows the rate of acid secretion in relation to the extracellular glucose concentration at pH 7.0, 7.4, and 7.8. In this series of experiments, the data were normalized to the rate of acid secretion measured in the presence of 20 mM glucose (control). Compared with this value, the rate of acid secretion decreased by 90% in the absence of glucose. With increasing glucose concentration, acid secretion rapidly increased and at 1.25 mM glucose already reached 75% of control, and this effect was almost independent of pH. The Line-weaver-Burk plot revealed a Michaelis constant ($K_m$) of 0.20 mM at pH 7.0 and 0.13 mM at pH 7.4 and 7.8 (Fig. 3).

Phloretin, an inhibitor of glucose uptake, effectively blocked acid secretion (Fig. 4), and at a concentration of 10$^{-4}$ M phloretin acid secretion was reduced to $\sim$10–15% of control. Again, this effect was very similar at pH 7.0, 7.4, and 7.8. $K_i$ values for phloretin were between 1.0 and 1.4 $\times$ 10$^{-5}$ M, and they were not significantly different between the different pH values tested.

The importance of extracellular sodium for acid secretion was tested by replacing sodium by TMA or choline. Reducing the extracellular sodium concentration caused an immediate marked decrease in the rate of acid secretion, and this reduction was significantly modified by extracellular proton concentration (Fig. 5). Whereas in the absence of sodium acid secretion was reduced to $\sim$35% of control at pH 7.8, at pH 7.0 it was reduced to $\sim$15%. The $K_m$ values calculated from these curves were 7.4 $\pm$ 1.2 mM at pH 7.8, 10.6 $\pm$ 0.6 mM at pH 7.4, and 32.1 $\pm$ 8.1 mM at pH 7.0 and thus showed a clear pH dependence. The sodium-dependent acid secretion was in part inhibited by MIA, and this inhibition was also pH dependent. At 10$^{-5}$ M MIA acid secretion was reduced by $\sim$20% at pH 7.8, whereas at pH 7.0 a reduction of $\sim$40% was observed (Fig. 6). At 10$^{-4}$ M MIA the effect was even more pronounced, but this effect most likely was not specific anymore. At this high concentration, the difference between the different pH values was lost, and the effect was no longer reversible.

In contrast to sodium removal, the removal of extracellular potassium caused a slow decrease in the rate of proton secretion, finally leveling off at $\sim$80% of control at pH 7.8 (Fig. 7). This effect markedly increased with decreasing pH, and at pH 7.0 proton secretion was finally reduced by 65% compared with control values. Quite similar inhibition values were obtained after inhibition of Na$^+$-K$^+$-ATPase using 10$^{-3}$ M ouabain; even the time course of these experiments was quite similar (Fig. 8). Whereas the decrease in proton secretion seen after removal of potassium rapidly recovered to near control rates after returning to the potassium-
containing medium, the effect of ouabain was not easily abolished by removal of the inhibitor.

**DISCUSSION**

The results of the present study demonstrate that extracellular glucose is the by far most important substrate for proton production in swim bladder gas gland cells. Furthermore, with respect to proton production glucose cannot be replaced by other substrates commonly metabolized by other cells. The $K_m$ value of 0.2 mM or even lower indicates, however, that plasma glucose levels in the eel (19) are high enough to assure constant glucose supply to the gas gland cells. This confirms previous studies, which demonstrated that inhibition of aerobic metabolism with cyanid has almost no effect on the rate of acid secretion by gas gland cells (15). Thus protons are almost exclusively produced in the glycolytic pathway, and substrates entering energy metabolism at the level of pyruvate or even below cannot be used for proton production.

Our attempt to obtain a quantitative estimate of the rate of proton secretion revealed that the average rate of proton secretion per cell probably is $\sim 1 - 2 \times 10^{-17}$ mol H⁺/s. Owicki and Parce (11) reported a value of $\sim 1.6 \times 10^{-16}$ mol/s for mammalian cells at 37°C, and the rate of proton secretion of rat parietal cells is $\sim 1 \times 10^{-15}$ mol/s (5). If we account for the difference in temperature, the rate of acid secretion of swim bladder gas gland cells appears to be only slightly lower than values reported for mammalian cells, which underlines the acid-producing capacity of the cells.

Glycolysis usually is inhibited at low pH, and the rate of acid production in gas gland cells was slightly reduced at pH 7.0. If glycolytic ATP production and ATP consumption remain tightly coupled, the rate of proton production per mole glucose does not change with pH (6), so that the decrease in proton secretion at low pH indicates a decrease in the glycolytic activity. A reduction in glycolytic flux at low pH is a common phenomenon that appears to apply to gas gland cells as well, although they are specialized for the production of acid and at least during periods of gas secretion are exposed to low extracellular pH values down to pH 6.6. Unfortunately, no data are as yet available on the intracellular pH of gas gland cells, but the decrease in the rate of acid secretion observed in our study at low extracellular pH suggests that gas gland cells are no exception in this respect.

Glucose uptake is effectively inhibited by phloretin, which binds to the outer glucose carrier site (8). This inhibitory effect of phloretin was not dependent on pH, and our $K_i$ value with 10–14 µM is well in the range of $K_i$ values reported in the literature. Tiihonen and co-workers found a value of 6 µM in carp erythrocytes (23); 48 µM have been reported for erythrocytes of the common eel (24), and 0.24 µM have been reported for human erythrocytes (8).

Whereas glucose uptake and glycolytic proton production were not only to a minor degree dependent on the extracellular pH, the sodium-dependent proton
secretion significantly varied with pH. The relative contribution of sodium-dependent membrane transport processes to total acid release apparently increases with decreasing pH, although sodium affinity decreases, so that higher sodium concentrations are necessary to achieve the maximal rate of acid secretion. Na+/H+ exchange and sodium-dependent Cl-/HCO3- exchange represent acid-secretory pathways (1, 2, 4, 21). The inhibitory effect of MIA clearly demonstrates that Na+/H+ exchange is involved in acid secretion of gas gland cells, although at pH 7.0 only a reduction of ~40% was achieved, and at higher pH the reduction was even less. In fact, while the remaining rate of proton secretion after sodium removal decreased from 35 to ~15% between pH 7.8 and 7.0, the inhibitory effect of MIA (10^-5 M) increased from ~20 to 40% at the same time. This suggests that the increased sodium dependence of proton secretion at low pH is mainly due to an increased contribution of Na+/H+ exchange to total acid secretion. A pH-dependent modification of the Na+/H+ exchange activity has been reported for several cells, including fibroblasts (20) or mammalian kidney cells. In proximal tubular cells of rabbit kidney, the activity of the Na+/H+ antiporter at pH 6.5 was almost three times as high as at pH 8.0 (21). The authors suggest that the increase in activity might be caused by a pH-dependent activation of inactive Na+/H+ antiporters.

Whereas, depending on pH, removal of sodium can inhibit 65–85% of the total acid secretion, inhibition of Na+/H+ exchange accounts only for ~40% of this sodium-dependent acid secretion. Sodium-dependent Cl-/HCO3- transport appears to be present in gas gland cells (15), but its contribution to proton secretion is no more than 10–20%, so that a significant fraction of the sodium-dependent acid release cannot be explained on the basis of acid transfer through membranes using the sodium gradient as the driving force. These considerations as well as the time course of the sodium effect, which is quite similar to the time course of glucose deprivation, lend further support to the notion that sodium-dependent glucose uptake is present in gas gland cells (15).

Sodium-dependent symport and antiport require Na+/K+/ATPase activity to maintain the sodium gradient, and ouabain, a specific inhibitor of Na+/K+/ATPase, also reduced the rate of acid secretion. Na+/K+/ATPase activity is rapidly enhanced following the entry of sodium into the cell. Accordingly, the acidification-induced increase in cytoplasmatic sodium concentration causes a significant stimulation of Na+/K+/ATPase in primary cultures of rat renal proximal tubular cells (9). At pH 7.0, inhibition of Na+/K+/ATPase in gas gland cells finally reduced the rate of acid excretion by ~70%, compared with only 35–40% at pH 7.8, suggesting an increased contribution of sodium-dependent pathways to acid secretion at low pH. As already discussed, the contribution of Na+/H+ exchange to acid secretion indeed increased at low pH.

Inhibition of Na+/K+/ATPase activity not only decreases the sodium and potassium gradients across the cell membrane, but with changing ion gradients it will also induce a change in membrane potential. Removal of extracellular potassium, in turn, causes an immediate hyperpolarization. Nevertheless, in gas gland cells the inhibitory effect of potassium removal and the time course of the inhibition almost exactly mimicked the effect of Na+/K+/ATPase inhibition, whereas sodium removal caused an immediate reduction in acid secretion. This suggests that the sodium gradient is critical for the secretion of protons and that the membrane potential itself is of primary importance. An alternative explanation for the decrease in acid secretion after removal of potassium would be the contribution of K+/H+ exchange to acid secretion. This explanation is not very likely, however, because omeprozole, a specific inhibitor of K+/H+ ATPase, did not reduce acid secretion in gas gland cells (15).

Our results thus indicate that, at alkaline pH, acid-secreting pathways using sodium-dependent mechanisms are not as important for acid release of gas gland cells as at low pH. Alkaline pH values typically are observed in blood of inactive swim bladders (7), and in this situation blood Pco2 typically is low. Stimulation of acid production results in the formation of lactic acid, but also of CO2 via the pentose phosphate shunt (17, 25). These considerations support the idea that, with initial stimulation of acid production in the energy metabolism of gas gland cells, the diffusion of CO2 including carbonic anhydrase activity in the plasma as well as in the cell membrane (15), might be a dominating pathway for acid secretion. With proceeding acidification and raising of Pco2 values, however, additional ion-transporting pathways become more and more important.

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