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INTRACELLULAR pH REGULATION IN THE EMBRYONIC CHICKEN LENS EPITHELIUM

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SUMMARY

1. The intracellular pH (pH_i) of embryonic lens epithelia was measured by the emission ratio technique using the fluorescent pH probe carboxy-seminaphthorhodafluor-1 (Snarf-1).

2. In artificial aqueous humour solutions (AAH) containing HCO_3^-, pH_i was 7·45, a value more alkaline than that of the bathing medium (pH = 7·3). In HCO_3^--free AAH, pH_i was 7·29.

3. Acetazolamide, an inhibitor of carbonic anhydrase, had no effect on resting pH_i.

4. The pH_i could be manipulated experimentally by changing the external pH (pH_o) of HEPES-buffered AAH, the addition or withdrawal of CO_2–HCO_3^-, or by perfusion with the weak bases NH_4Cl and procaine.

5. The pH_i change induced by withdrawal of 5 mM-procaine was used to calculate a value for the intrinsic cytoplasmic buffering capacity (β_i) of 16·5 mM.

6. The addition of amiloride (1 mM) or treatment with low-Na^+ AAH solutions led to a decrease in pH_i of 0·23 over the 10 min exposure. In addition, these treatments inhibited pH_i recovery from NH_4^+-induced acidosis. These observations are consistent with the presence of amiloride-sensitive Na^+–H^+ antiport.

7. Addition of exogenous antiport activity in the form of 50 μM-monensin caused an increase in pH_i of 0·24.

8. In HCO_3^--containing media, replacing Cl^- by gluconate or isothionate led to an immediate, reversible increase in pH_i which could be completely inhibited by 2 mM-4-acetamido-4′-isothiocyanato-stillbene-2,2′-disulphonic acid (SITS). This indicates the presence of Cl^-–HCO_3^- exchange in this tissue.

9. Under HCO_3^--free conditions, replacement of Cl^- by gluconate or isothionate caused a small transient acidification followed, 5 min later, by a large sustained alkalinization. The delayed increase in pH_i could be completely blocked by 1 mM-amiloride and may reflect volume-sensitive stimulation of the Na^+–H^+ antiporter as cell volume (estimated by cell height measurements) was shown to decrease significantly during this period.

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INTRODUCTION

The vertebrate lens is an avascular structure bathed by the humours of the eye. At the anterior surface, the lens is bounded by an inwardly facing epithelium, the basement membrane of which constitutes the collagenous lens capsule. Near the lens equator, epithelial cells differentiate to form the lens fibre cells that comprise the bulk of the tissue. This process continues throughout life with accretion of newly formed fibres at the periphery and a gradual increase in the size of the lens. The fibre cells are electrically coupled, both to each other and to the overlying epithelium, by an extensive network of communicating junctions (Duncan, 1969; Rae & Kuszak, 1983) which in some species can account for 50% of the membrane area present (Kuszak, Maisel & Harding, 1978). Terminal differentiation in lens cells is accompanied by the loss of membrane-bound organelles. Consequently, the well-being of the older inner fibre cells is thought to depend on metabolic co-operativity with the overlying epithelium (Goodenough, Dick & Lyons, 1980).

Lens metabolism is predominantly anaerobic (Kinoshita, 1965), leading to the production and accumulation of lactic acid within the tissue (Bassnett, Croghan & Duncan, 1987). The permanent anaerobic status of the vertebrate lens has prompted several studies on lens intracellular pH (pH_i) utilizing a variety of techniques including ^{31}P-NMR (Greiner, Kopp, Sanders & Glonek, 1981), pH microelectrodes (Bassnett & Duncan, 1985, 1988) and fluorescent indicators (Wolosin, Alvarez & Candia, 1988, 1989; Bassnett, Reinisch & Beebe, 1990). Each of these studies has shown H^+ to be far from electrochemical equilibrium, indicating the presence of mechanisms capable of extruding H^+ equivalents against an electrochemical gradient. Using double-barrelled pH microelectrodes, Bassnett & Duncan (1986) demonstrated the presence of pH gradients within the rat lens. These intracellular gradients extended across many cell diameters and led to pH_i values in the lens nucleus that were 0·2–0·3 pH units more acidic than in the outer cortical layers. Similar gradients have since been reported within the frog lens (Mathias, Riquelme & Rae, 1988). Bassnett *et al.* (1987) suggested that lenticular pH gradients resulted from the diffusion of lactic acid from the lens and stressed the importance of both CO_2 as a mobile buffer within the bulk of the tissue and putative H^+ extruding mechanisms at the lens surface.

It is known that pH_i influences both intercellular communication (Peracchia & Peracchia, 1980; Mathias *et al.* 1988) and membrane permeability properties (Bassnett & Duncan, 1988) in the lens. In view of the importance of these parameters in the etiology of cataract, an understanding of pH_i regulation in the lens is clearly important. Most of the studies thus far performed on the lens have dealt with the tissue as a whole and have been limited by the slow pH response of this large, well-buffered system. However, by analogy with other transport processes, it is likely that the pH_i-regulating mechanisms in the lens are located primarily in the epithelium and peripheral fibre cells. In the present study, I utilized isolated epithelia, explanted from the embryonic chicken lens, to study the ionic basis of pH_i regulation mechanisms. With a diameter $< 10\ \mu m$, these cells are too small to impale with conventional ion-sensitive microelectrodes. Consequently, I used a newly introduced fluorescent probe (carboxy-seminaphthorhodafluor-1) in conjunction with scanning laser microscopy to investigate the regulation of lens pH_i.

METHODS

Animals. Fertilized chicken eggs were obtained from Truslow Farm (Chestertown, MD, USA) and stored at 12 °C for periods of up to 1 week before incubation at 38 °C in a forced-draft incubator (Humidaire, New Madison, OH, USA). Lenses were removed from 6- to 8-day-old embryos and placed in a 35 mm Petri dish containing warm artificial aqueous humour (AAH, for composition see below) where the adhering ciliary epithelium and vitreous humour were carefully removed. Using fine forceps, the posterior capsule was peeled back to the equator of the lens and the fibre mass lifted clear of the anterior capsule/epithelium. The isolated capsule/epithelium was then placed apical face down and attached to the Petri dish base by pushing the marginal tissue directly into the plastic with the tips of the forceps. The isolated explant was typically 1–2 mm in diameter and contained 10–20000 cells. The tissue was utilized for pH_i measurements immediately following dissection.

Solutions. Explants were maintained at 37 °C in control artificial aqueous humour solution (control AAH) of the following composition (mM): NaCl, 113; KCl, 4·5; $MgCl_2$, 1; $CaCl_2$, 1·5; glucose, 6; *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (HEPES), 10; $NaHCO_3$, 20. The AAH was gassed with humidified 5% CO_2:95% O_2 to pH 7·3. Nominally HCO_3^--free solutions (HEPES-AAH) were achieved by replacing 20 mM-$NaHCO_3$ with 20 mM-NaCl and leaving the solution ungassed. For Cl^--free AAH, Cl^- was replaced by equimolar gluconate or isothionate. The low-Na^+ AAH contained 20 mM-$NaHCO_3^-$ with NaCl replaced by *N*-methyl-D-glucamine chloride. In procaine-containing solutions, 5 mM-NaCl was replaced by 5 mM-procaine. Intracellular fluorescence ratios were calibrated by perifusion with high-K^+ buffer solutions containing nigericin (10 μM; Thomas, Buchsbaum, Zimniak & Racker, 1979; Bassnett *et al.* 1990). The composition of high-K^+ buffer was (mM): KCl, 100; NaCl, 25; buffer, 25. These Na^+ and K^+ values were similar to those reported for embryonic chicken lens cells (Shinohara & Piatigorsky, 1977). For calibration solutions of pH 7·0 or less, piperazine-*N*,*N'*-bis(2-ethanesulphonic acid) (PIPES, pK = 6·8 at 25 °C) was used as the buffer. For calibration solutions of higher pH HEPES (pK = 7·5 at 25 °C) was used. Nigericin was added to each buffer from a 4 mM-ethanolic stock immediately before use. Amiloride was obtained from Calbiochem (San Diego, CA, USA) and dissolved directly into the experimental solution. Unless otherwise indicated, all other reagents and drugs used in this study were purchased from Sigma Chemical Co (St Louis, MO, USA).

Dye loading. The present experiments utilized carboxy-seminaphthorhodafluor-1 (Snarf-1) to measure pH_i in lens epithelial explants. Snarf-1 is an emission-shifted, fluorescent pH probe with emission maxima at 587 nm at low pH and 640 nm at high pH. The ratio of these peaks is a sensitive indicator of pH_i (Bassnett *et al.* 1990). The cell-permeant derivative of Snarf-1, Snarf-1 acetoxymethyl acetate (Snarf-1 AM acetate), was purchased from Molecular Probes (Eugene, OR, USA) and stored at 4 °C as a 2 mM stock solution in dimethylsulphoxide for periods of up to 2 weeks. Cells were loaded with 5 μM-Snarf-1 AM acetate in AAH (37 °C) for 30–45 min. Non-specific esterase activity is believed to lead to the intracellular liberation and accumulation of the less-permeant Snarf-1.

Microspectrofluorometry. The optical arrangements for measuring pH_i in lens epithelial explants have been described in detail previously, as has a description of the spectral properties of Snarf-1 (Bassnett *et al.* 1990). Briefly, following the loading period, cells were washed in fresh AAH and positioned on the stage of a scanning laser microscope (ACAS 470; Meridian Instruments, Okemos, MI, USA). Cells were maintained at 37 °C during the experiments by means of an air-stream incubator (Nicholson Instruments, Bethesda, MD, USA). The cells were not continuously perifused. Instead, pre-warmed solutions were exchanged by pipette at the points indicated in the figures. The dye-loaded cells were illuminated using the focused beam (1–2 μm spot size) of an argon laser (514 nm). The emitted fluorescence was measured by photomultiplier tube (PMT) detectors equipped with either a 580±20 nm bandpass filter or a 630 nm longpass filter. For each experiment, the gain on the PMTs was adjusted to give approximately equal signals for both wavelengths at the resting pH_i. For this reason, ratio values between experiments were not strictly comparable and estimates of pH_i were obtained from calibration curves generated at the end of each experiment (see below). The microscope stage was computer-driven and could be moved in pre-set increments of 8–10 μm along the *X*- and *Y*-axes. At each co-ordinate, the laser beam was on for a brief (8 μs) period and the fluorescence was recorded simultaneously at the two emission maxima. By moving the stage, it was possible to sample from an orthogonal array of co-ordinates positioned over the centre of the explant and thereby construct two digital images corresponding

to the two emission maxima. Following background subtraction, the integrated pixel intensity at 640 nm was divided by that at 587 nm to give an emission ratio for defined regions of the image. The scanned region of the explant was approximately 0·06 mm^2 in area and included portions of about 2000 cells (with a distance between sample co-ordinates of 10 μm and a laser spot size of 1–2 μm, only a small fraction of the cytoplasmic area present within the scanned area was probed and some cells were not irradiated at all). At the end of each experiment, ratio data were calibrated by treating the cells with high-K^+ buffer solutions containing the H^+–K^+ exchanger nigericin (10 μM). In general, cells were perifused with high-K^+ buffer at pH 6·5, 7·0, 7·5 and 8·0. Linear interpolation between the ratio values obtained in the four different buffers enabled the construction of a calibration curve. Errors introduced by approximating the full titration curve using four discreet measurements are likely to be small and have been ignored. Following construction of the calibration curve the data could be replotted in terms of pH_i using software included with the ACAS 470.

Measurement of cell height. Changes in cell height were measured during treatment with Cl^--free AAH and taken as an indicator of cell volume changes. Cell height changes have recently been shown to be directly proportional to volume changes in this system (Beebe, Parmelee & Belcher, 1990). Cell height readings were taken approximately every 15 s in the central region of explanted epithelia using the optical measuring system described by Beebe & Feagans (1981). In brief, this involved focusing on the apical and basal surfaces using interference contrast optics and measuring the distance between the two focal planes with a micrometer built into the microscope stage. Measurements from four such experiments were pooled into 1 min intervals and displayed as a cell height histogram.

Results are expressed throughout as mean ± sample standard deviation (S.D.) for n determinations, where each determination represents a single explant. Student's unpaired two-tailed t test was used, where appropriate, to determine whether differences between groups were significant.

RESULTS

Figure 1 shows a digital fluorescence image of an epithelial explant following a 30 min loading period in Snarf-1 AM acetate. The central region of explants from chicken embryos of this age consisted of a pseudostratified epithelium, thickening slightly towards the margin, in the region that will later form the annular pad. At the extreme periphery a few damaged fibre cells and shreds of posterior capsule often remained attached. These cells also accumulated dye. Incisions in the peripheral tissue (arrowed in Fig. 1) resulted from attaching the explant to the Petri dish base (see Methods). The box shown in Fig. 1 indicates the region of the explant in which pH_i measurements were routinely made.

Resting pH_i

In control AAH explant pH_i was 7·45 ± 0·15 (mean ± S.D. range = 7·08–7·77; n = 53). Figure 2*A* shows the effect of changing the bathing solution from control AAH (containing CO_2–HCO_3^-) to HEPES-AAH, and illustrates the procedure used throughout this paper for calculating pH_i. On withdrawing the HCO_3^-–CO_2, there was a transient increase in the 640 nm/587 nm ratio, presumably as the highly permeant CO_2 left the cells, followed by a slow decrease in the ratio to below the original value. At the end of the experiment, the ratio values were calibrated by perifusing the cells with high-K^+ buffers containing nigericin (10 μM). It took a few minutes for pH_i to stabilize in each of the calibration buffers. The 640 nm/587 nm emission ratios corresponding to each of the buffer pH values were used to construct a calibration curve (not shown). From the data shown in Fig. 2*A* it can be seen that the response of the calculated ratio was not linear with pH_i. The true pK of Snarf-

1 is 7·4–7·6, although the apparent pK of the 640 nm/587 nm emission ratio is 8·3 (Bassnett *et al.* 1990). Figure 2*B* shows the same data, replotted and calibrated for pH_i. Note that the pH response in the calibration buffers has now been linearized. The same procedure was used in all the experiments reported in this paper but for

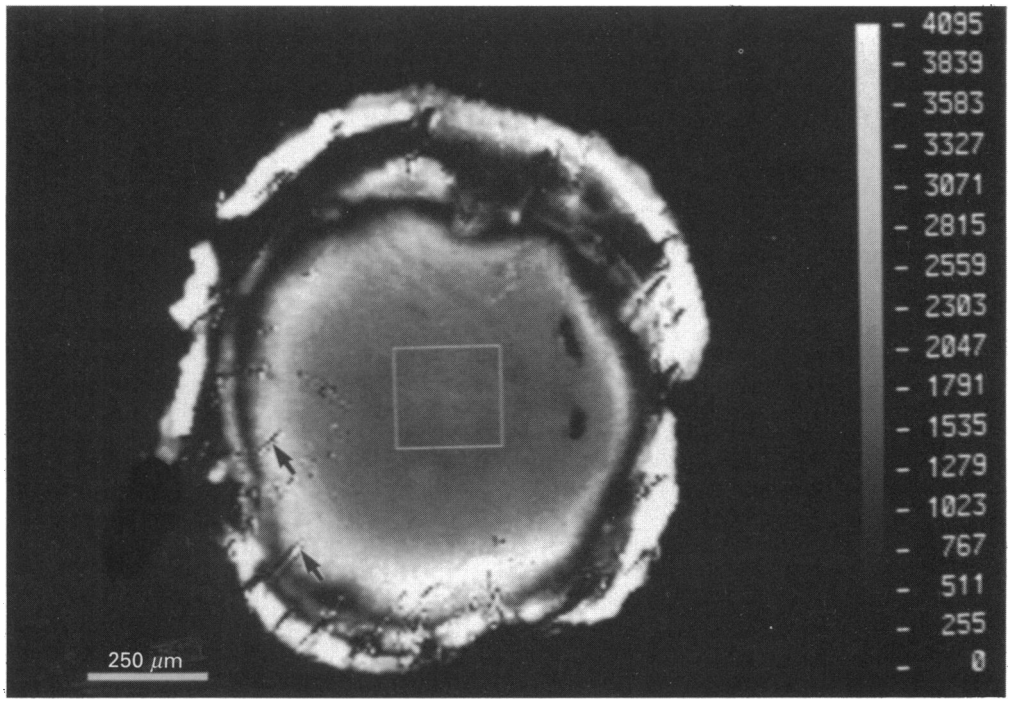

Fig. 1. Digital fluorescence image of a chick lens epithelial explant loaded with Snarf-1. The dye-loaded explant was maintained in control AAH solution and viewed on a scanning laser microscope using the 514 nm line of an argon laser for excitation (see text for details). The box is located over the centre of the epithelium and indicates the region of the explant typically selected for measurement of pH_i. Small incisions used to attach the explant to the base of the Petri dish can be seen in the peripheral tissue (arrows). Box area = 0·06 mm^2.

reasons of space the post-experiment calibration curves have been omitted. In cells maintained in HEPES-AAH during the dye-loading and washing period (30–60 min) pH_i was 7·29$\pm$0·20 (range = 7·02–7·59; n = 17). This value was significantly lower than that obtained from explants in control AAH ($P < 0·01$). The effect of re-introducing CO_2–HCO_3^- to cells previously maintained in HEPES-AAH was essentially the mirror image of that shown in Fig. 2*B*. There was a brief acidification, presumably caused by the rapid re-equilibration of CO_2, followed by a slow increase in pH_i towards control values (data not shown).

The pH_i was sensitive to changes in pH_o of HEPES-AAH (Fig. 3). In the case of increases in pH_o, pH_i showed relatively little response. However, if pH_o was decreased from 7·3 to 6·8 pH_i fell rapidly (n = 4).

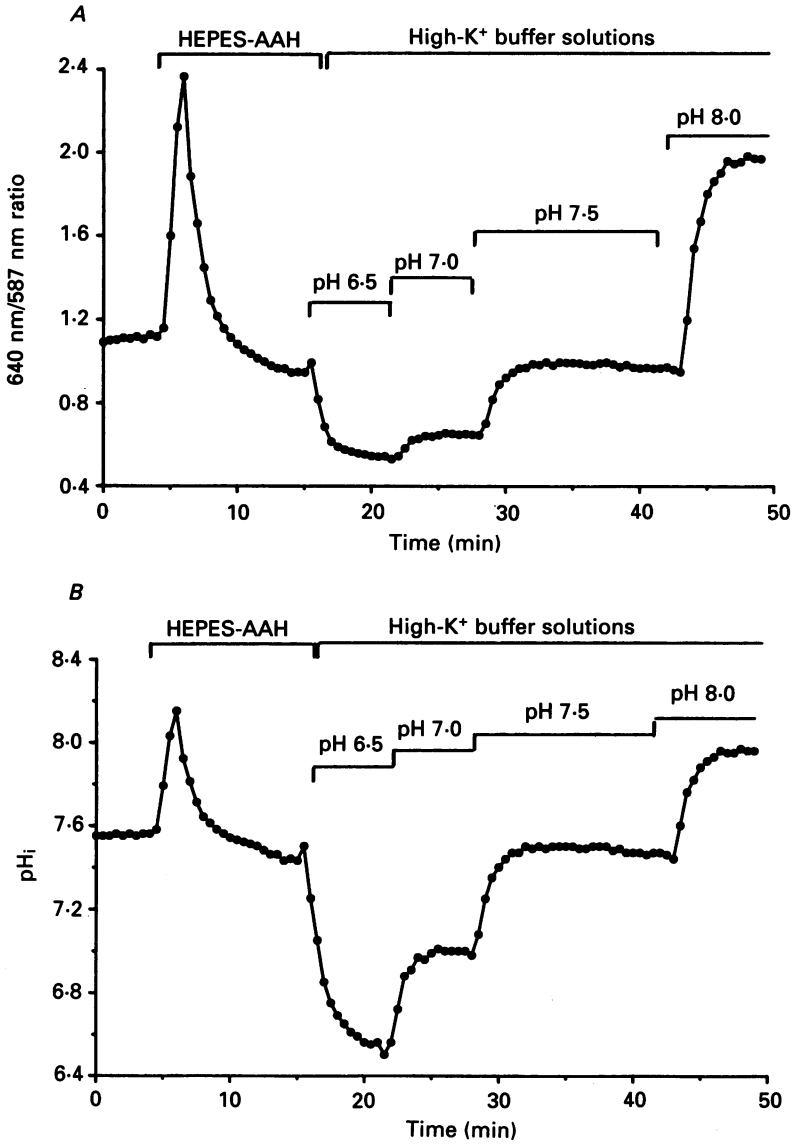

Fig. 2. Effect of HEPES-AAH on calculated 640 nm/587 nm Snarf-1 fluorescence emission ratios and lens epithelial explant pH_i. Cells were perifused with control AAH and then, after 5 min, the solution was changed to HEPES-AAH. Ten minutes later the explant was perifused with a series of calibration solutions containing nigericin (10 μM). Ratio values shown in *A* were used to construct a calibration curve (not shown) which was then used to replot the data in terms of pH_i in *B*.

Buffering power

The immediate response of pH_i to a change in the acid load on the cell is determined by the activity of the intracellular buffers. The intrinsic (non-CO_2) buffering capacity (β_i) can be estimated by perifusing cells with a weak acid or base.

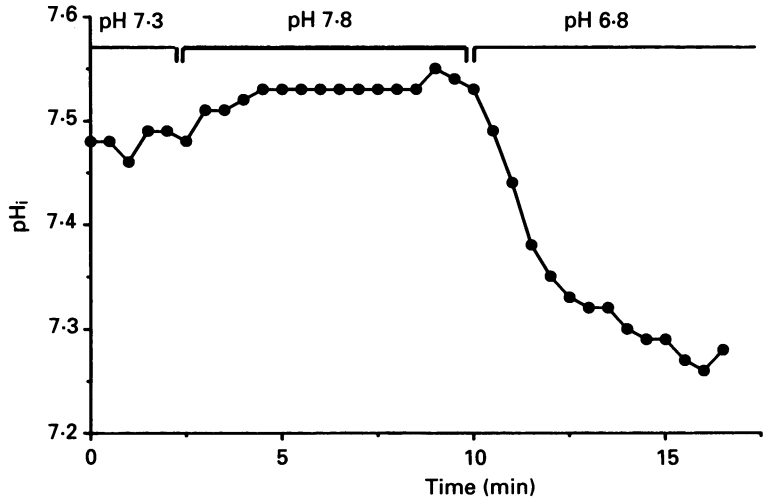

Fig. 3. The effect of changing pH_o on lens epithelial explant pH_i. Explants were maintained in HEPES-AAH at $pH_o = 7{\cdot}3$ and then perifused with HEPES-AAH at either pH 7·8 or 6·8.

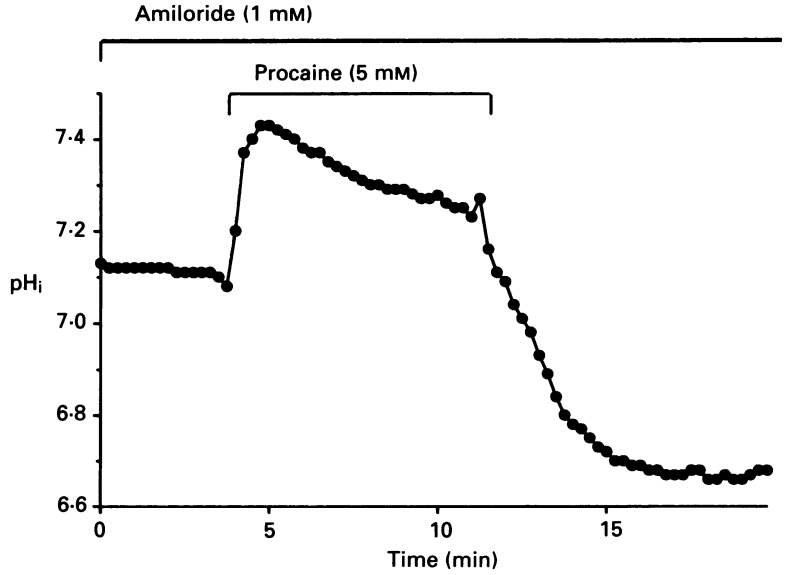

Fig. 4. The effect of procaine (5 mM) on lens epithelial explant pH_i. Explants were maintained in HEPES-AAH containing 1 mM-amiloride. The change in pH_i on removing the procaine was used to calculate the intrinsic cytoplasmic buffering capacity (see text).

In snail neurones, perifusion with procaine has been shown to be a reliable method of increasing pH_i and subsequently calculating β_i (Szatkowski & Thomas, 1989). It should be noted that β_i generally varies with pH_i and that β_i values derived from experimental displacement of pH_i are probably valid only for the narrow range of pH_i in which measurements were taken. Figure 4 shows an experiment in which 5 mM-procaine was applied to lens cells in HEPES-AAH containing 1 mM-amiloride.

Being a weak base (pK = 8·95; Hudgins & Putney, 1972), the uncharged anaesthetic entered the cell causing pH_i to increase rapidly. The pH_i decayed slightly with time, probably indicating that, unlike snail neurones, lens epithelial cells are permeable to the procaine cation. Permeability to the protonated (i.e. cationic) form of procaine is a likely explanation of the mechanism of the plateau-phase acidification. However, Cl^-–base exchange or other acid-loading processes cannot be ruled out *a priori*. Withdrawing the procaine caused pH_i to fall to below the initial value. This effect was much more pronounced in the case of another weak base, NH_4Cl, where it could be utilized experimentally to acidify the cell (see below). The change in pH_i upon withdrawal of procaine can be used to calculate β_i. Following removal of extracellular procaine, intracellular procaine is likely to diffuse from the cell in its uncharged form leading to an acidification that, in HEPES-AAH containing 1 mM-amiloride, will not be blunted by cellular pH regulation. Under these conditions, β_i may be calculated using the equation of Szatkowski & Thomas (1989),

$$\beta_i = \frac{C\,10^{(pK'-pH_i)}}{\Delta pH_i\,(1+10^{(pK'-pH_o)})}, \quad (1)$$

where β_i is the intrinsic buffering capacity (mM), C is the extracellular concentration of procaine (5 mM), pH_i is the value of pH_i taken immediately before removal of procaine, pK' is the pK of procaine (8·95) and ΔpH_i is the change in pH_i on removing the procaine. Using this equation, β_i was 16·5±4·8 mM (n = 4). The additional contribution of the CO_2 buffer system to the total cytoplasmic buffering capacity may also be calculated (see Discussion). In these experiments, cells were pre-equilibrated in HCO_3^--free AAH containing 1 mM-amiloride. Under these conditions pH_i was 6·87±0·17 (n = 4). This value is lower than that observed in cells treated with either amiloride or HCO_3^--free solutions alone, indicating that the effects of these treatments are additive.

Na^+–H^+ antiport

The Na^+–H^+ antiporter is a near-ubiquitous transport protein sensitive to the K^+-sparing diuretic amiloride and its analogues. Although a role for the antiporter has been demonstrated in cell volume regulation (see Hoffman & Simonsen, 1989) its primary function is thought to be in relation to pH_i regulation. The effect of amiloride (1 mM) on resting lens cell pH_i is shown in Fig. 5. In four experiments, amiloride caused the pH_i to decrease by 0·23±0·2 over the 10–15 min experimental exposure. The transient alkalinization observed on adding the amiloride was seen in only two of the four experiments and may represent an artifact arising from slight discrepancies in the CO_2 content of the two perifusion solutions. Treatment with low-Na^+ AAH also caused an average decrease in pH_i of 0·23±0·12 (n = 3). A typical experiment illustrating the effect of treatment with low-Na^+ AAH is shown in Fig. 6. Together, these experiments suggest that the antiporter is present in embryonic lens epithelial cells and active at the resting pH_i.

Figure 7 shows a typical experiment in which lens cells were acid-loaded by the NH_4^+ pre-pulse technique (for a full description of this technique see Boron, 1989). Treatment with NH_4^+ (as NH_4Cl) led to a transient increase in pH_i followed by a steady fall to below baseline values (n = 4). In solution, NH_4^+ exists in equilibrium

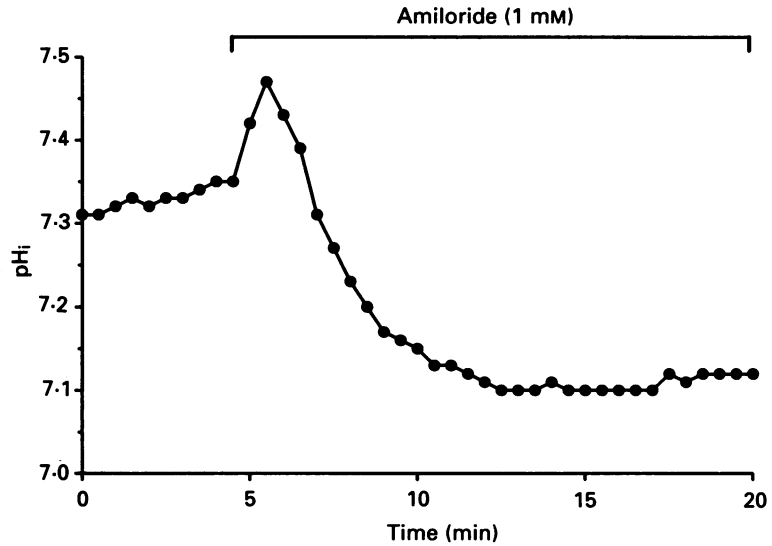

Fig. 5. The effect of amiloride (1 mM) on pH_i of lens epithelial explants. The amiloride was applied in control AAH. The small alkalinization observed on adding the amiloride was seen in two out of four experiments.

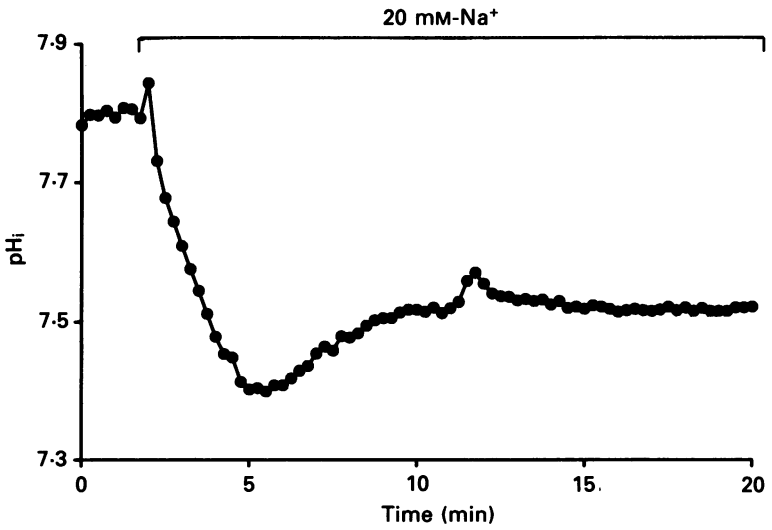

Fig. 6. Effect of low-Na^+ AAH (20 mM-Na^+) on lens epithelial explant pH_i. Na^+ was replaced by *N*-methyl-D-glucamine.

with molecular NH_3. The increase in pH_i is believed to result from the rapid equilibration of NH_3 across the cell membrane. The slower influx of NH_4^+ is responsible for subsequently driving pH_i to lower values. On removing the extracellular NH_4^+, there was a further fall in pH_i as intracellular NH_4^+ converted to NH_3, which diffused from the cell, and H^+, which was trapped. In control conditions (Fig. 7), this period of acidosis following the removal of NH_4^+ was only brief, as the cell actively extruded H^+ equivalents to regulate pH_i back to the initial

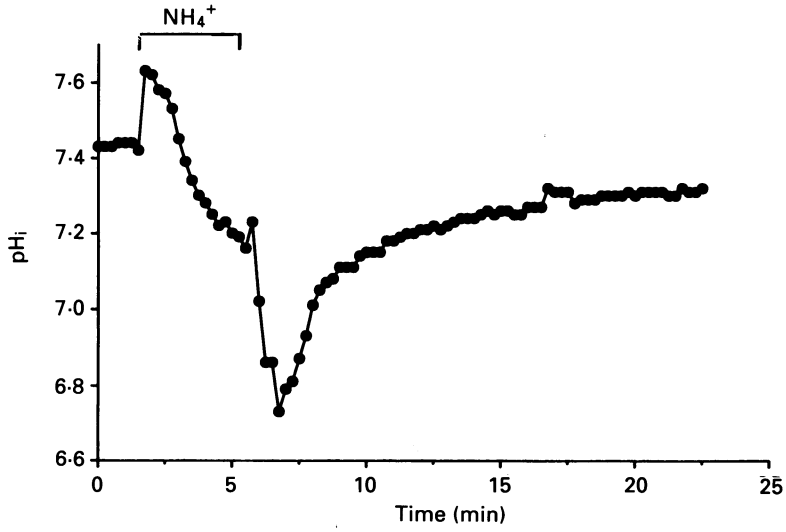

Fig. 7. Effect of NH_4^+ on lens epithelial explant pH_i. 20 mM-NH_4^+ (as NH_4Cl) was substituted for Na^+ and applied in control AAH.

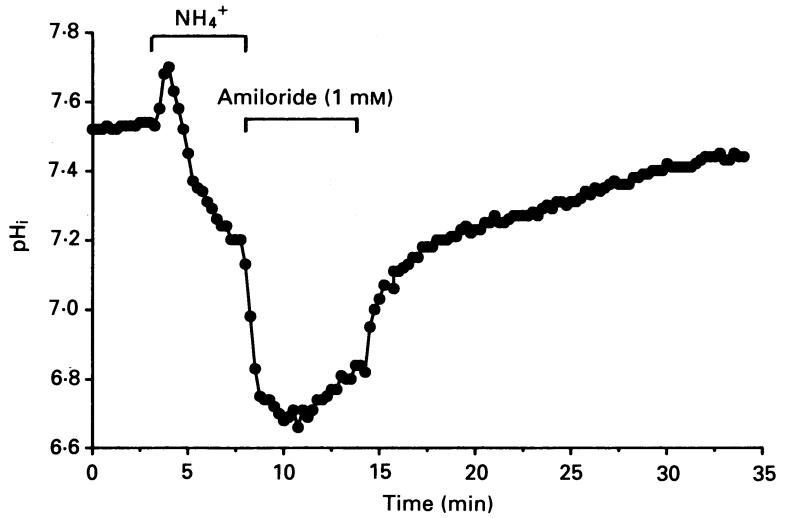

Fig. 8. Effect of amiloride (1 mM) on explant pH_i following NH_4^+-induced acidosis. Both NH_4^+ and amiloride were applied in control AAH ($pH_o = 7{\cdot}3$).

value at a maximum rate of $3{\cdot}8 \pm 2{\cdot}2 \times 10^{-3}$ pH units s^{-1} ($n = 13$). However, in the presence of 1 mM-amiloride (Fig. 8), the rate of recovery from the NH_4^+-induced acidosis was reduced to $1{\cdot}4 \pm 2{\cdot}7 \times 10^{-4}$ pH units s^{-1} ($n = 7$), representing a $96 \pm 6\%$ ($n = 7$) inhibition of the rate of recovery. On withdrawing the amiloride, pH_i quickly returned to the original value. A similar result was obtained by perifusing the lens cells with low-Na^+ AAH following pre-treatment with NH_4^+ (Fig. 9; $n = 4$). Under these conditions, the rate of recovery from acidosis was reduced to $5{\cdot}0 \pm 3{\cdot}6 \times 10^{-4}$ pH units s^{-1} representing an inhibition of the recovery rate of $85 \pm 17\%$ ($n = 4$). On

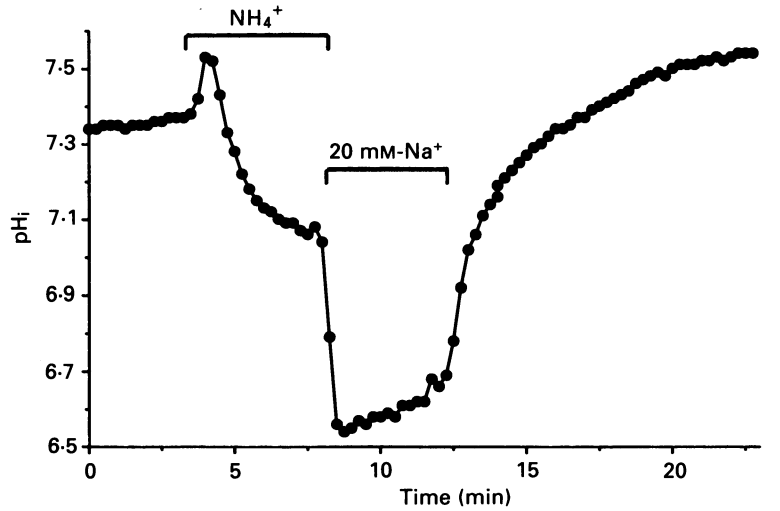

Fig. 9. Effect of low-Na^+ AAH (20 mM) on pH_i recovery following NH_4^+-induced acidosis in lens epithelial explants.

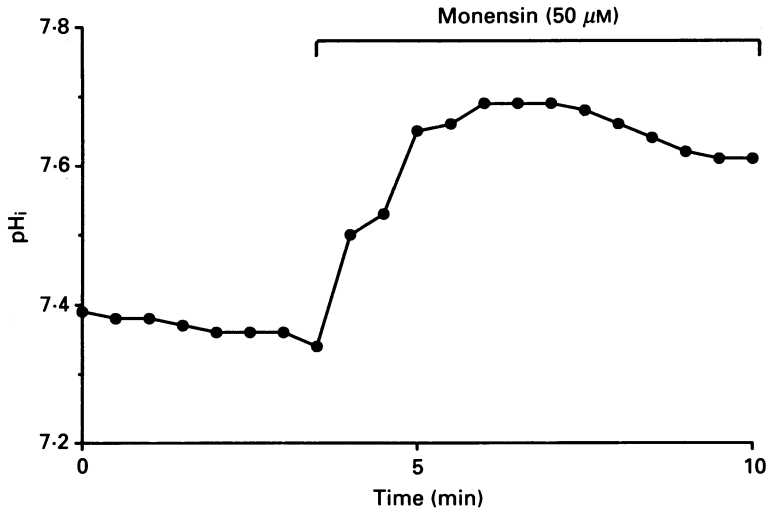

Fig. 10. Effect of monensin on explant pH_i in control AAH.

replacing extracellular Na^+, pH_i rapidly increased to slightly higher than the original value. Treatment with either amiloride or low-Na^+ solutions greatly impeded the recovery of pH_i from an acid load. The percentage inhibition values for the two treatments were not significantly different. However, the rate of recovery in each case, while slow, was not zero. This suggests that either Na^+–H^+ exchange was not completely inhibited under these conditions or the presence of an additional acid-extruding mechanism(s).

In principle, there is enough potential energy stored in the Na^+ gradient to enable the antiporter to elevate pH_i to levels higher than the value of 7·45 observed. The

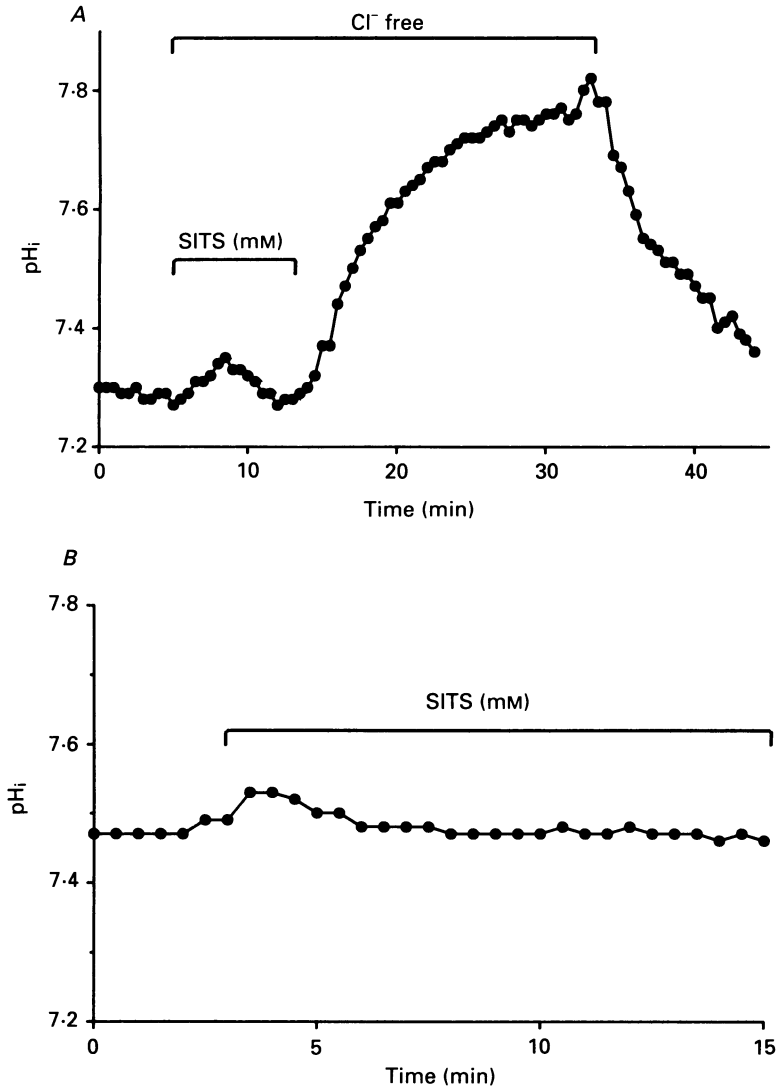

Fig. 11. *A*, effect of Cl^--free AAH on explant pH_i. The cells were initially perifused with control AAH. The bathing solution was then changed to Cl^--free AAH (Cl^- replaced by gluconate) in the presence and then absence of 2 mM-SITS (note that all solutions contained CO_2–HCO_3^-). *B*, the effect of SITS on resting pH_i of lens epithelial explants.

addition of exogenous Na^+–H^+ exchange activity, in the form of 50 μM-monensin, caused pH_i to increase by $0{\cdot}24 \pm 0{\cdot}14$ ($n = 3$; Fig. 10).

Cl–HCO_3^- exchange

Many cells are known to regulate their pH_i, in part, by HCO_3^--dependent mechanisms (for review see Boron, 1989). These mechanisms often involve Cl^- as a counter ion and are sensitive to disulphonic stilbene derivatives such as 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid (SITS). Figure 11*A* shows an experiment in which the external Cl^- was replaced by gluconate in the presence or

absence of SITS. In control AAH, in the presence of 2 mM-SITS, treatment with Cl^--free solutions had no effect on pH_i. However, as the SITS was withdrawn, the Cl^--free AAH caused pH_i to increase ($n = 4$). On return to control AAH, pH_i immediately returned to baseline values. In experiments where pre-treatment with SITS was

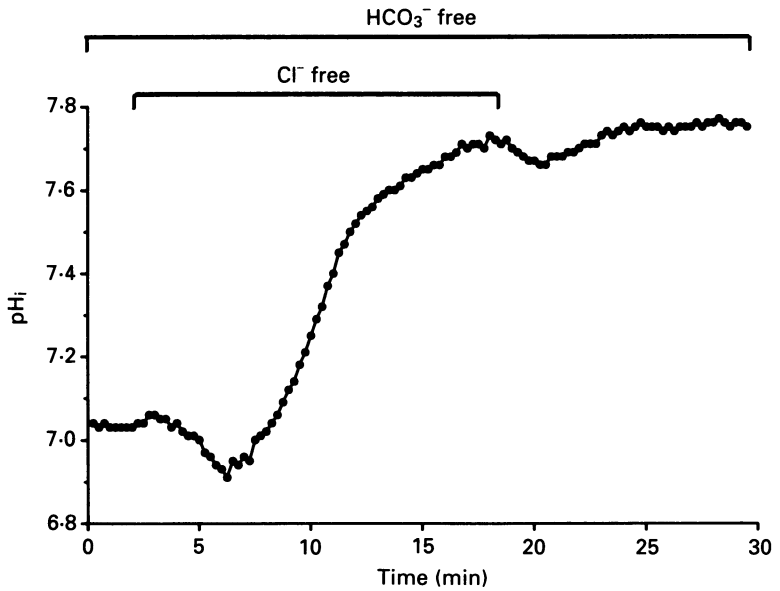

Fig. 12. The effect of Cl^--free AAH (Cl^- replaced by gluconate) on lens epithelial explant pH_i in HCO_3^--free conditions.

omitted, Cl^--free solutions caused an immediate rise in pH_i (data not shown). These observations are consistent with the presence of Cl^-–HCO_3^- exchange. Short-term exposure (< 5 min) to 2 mM-SITS had no measurable effect on pH_i ($n = 4$; Fig. 11*B*). To determine whether longer incubation periods were required to illicit an effect, explants were exposed to SITS during the dye-loading and washing periods (> 45 min). In this case pH_i was $7{\cdot}39 \pm 0{\cdot}12$ ($n = 4$), a value not statistically different from that of control lenses run at the same time ($7{\cdot}53 \pm 0{\cdot}14$, $n = 4$). This suggests that either Cl^-–HCO_3^- exchange was inactive at the resting pH_i or that two or more mutually antagonistic SITS-sensitive processes were blocked resulting in no net change in pH_i.

Under HCO_3^--free conditions, removing Cl^- from the medium had a different effect on pH_i (Fig. 12). Here, treatment with Cl^--free solutions (Cl^- replaced by either gluconate or isothionate) generally caused a small acidification of pH_i followed, after about 5 min, by a large alkalinization ($n = 4$). Return to HEPES-AAH caused a transient fall in pH_i followed by a further alkalinization. The pH_i subsequently returned towards resting values at an extremely slow rate and had not recovered by the end of the experiment shown in Fig. 12. These changes also occurred in the presence of 2 mM-SITS (data not shown). However, if 1 mM-amiloride was added to the Cl^--free solution the large alkalinization was completely blocked, suggesting that it is due to the stimulation of Na^+–H^+ exchange ($n = 3$, Fig. 13). A recent report has suggested that lens Na^+–H^+ exchange can be stimulated by treatment with

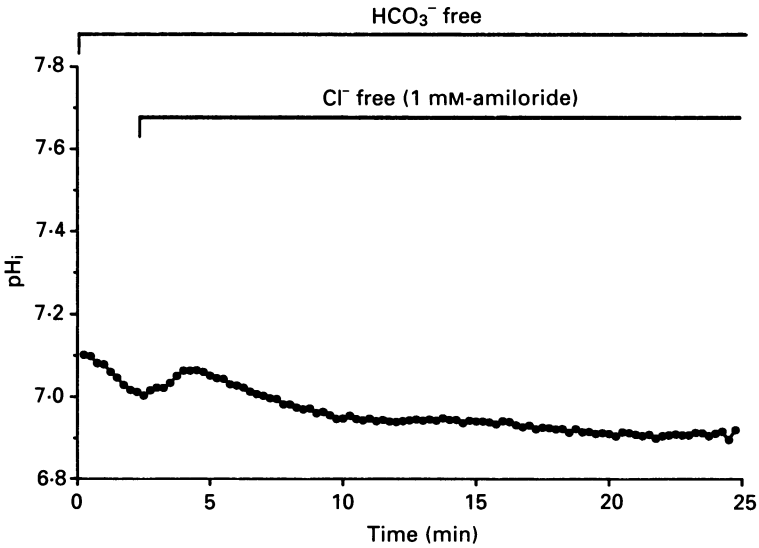

Fig. 13. The effect of Cl^--free AAH (containing 1 mM-amiloride) on lens epithelial explant pH_i in HCO_3^--free conditions.

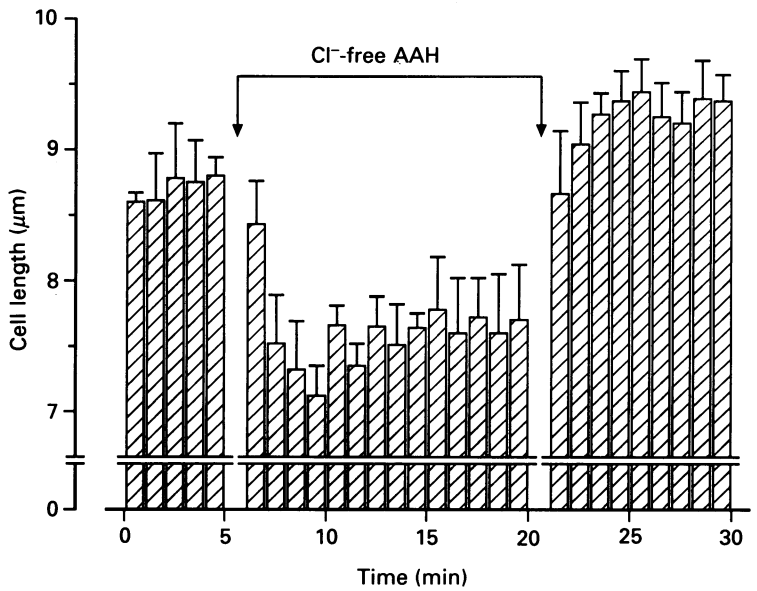

Fig. 14. The effect of Cl^--free AAH on lens epithelial cell height in HCO_3^--free conditions. Cell height was measured optically (see text for details) and taken as an indicator of cell volume. The measurements represent the mean ± S.E.M. ($n = 4$).

hypertonic solutions functioning as part of the cellular volume regulatory response (Wolosin *et al.* 1989). To investigate whether treatment with Cl^-–HCO_3^--free solutions caused a change in cell volume, cell height was measured using the optical technique of Beebe & Feagans (1981). Fig. 14 shows that during application of Cl^-–HCO_3^--free AAH there was a significant decrease in cell height and, presumably,

volume which may have served to trigger the stimulated activity of the Na^+–H^+ antiporter. The measured changes in cell height probably represented an underestimate of cell volume changes as, under Cl^--free conditions, gaps were observed to form between the shrinking cells.

The interconversion of CO_2 and HCO_3^- is catalysed by the activity of carbonic anhydrase (CA). Relatively high (3·4 μM) levels of CA have been measured in the vertebrate lens (Friedland & Maren, 1981) although the role of the enzyme in this tissue remains obscure. Unlike most lens enzymes, CA is present at high concentration in the centre of the tissue. To investigate the role of CA in determining pH_i in the lens epithelium, I applied 1 mM-acetazolamide, a CA inhibitor, in control AAH to explanted epithelia. In four experiments there was no significant effect on pH_i of short-term exposure (5–10 min) to acetazolamide (data not shown).

DISCUSSION

As in most cell types studied (for reviews see Roos & Boron, 1981; Hoffman & Simosen, 1989), the pH_i of embryonic lens epithelial cells is maintained at a level too high to be explained by passive ionic distribution. The equilibrium distribution for H^+ ions can be calculated from the Nernst relation,

$$pH_i = pH_o + (E_m/60), \quad (2)$$

where E_m = membrane potential. The membrane potential of chick lens cells is approximately −25 mV (S. Bassnett, unpublished observations). Thus, at 37 °C with $pH_o = 7{\cdot}3$, the equilibrium value for pH_i is 6·88. In fact, in common with a number of cell types, embryonic chick lens epithelial cells maintain their pH_i at values more alkaline even than the surrounding medium ($pH_o = 7{\cdot}3$). Lens cell pH_i is sensitive to pH_o, however, and is influenced, at least in the absence of CO_2–HCO_3^-, by acidification of the bathing medium. This supports earlier observations in the whole rat lens, where both pH_i and membrane potential were sensitive to acidification of the medium but where even prolonged incubation at higher pH_o had little or no effect (Bassnett & Duncan, 1985). In this regard, it is interesting to note that conditions such as severe diarrhoeal diseases, which frequently result in systemic acidosis, are well-known risk factors for human cataract formation (Clayton, Cuthbert, Seth, Phillips, Bartholomew & McK. Reid, 1984). The relatively small membrane potential of the chick lens epithelium (−25 mV) ensures that, in this tissue, the equilibrium value for pH_i is high in comparison to many other cells. This may explain why pre-treatment with NH_4^+ does not reduce pH_i to the low levels seen in other preparations. Also the slight increase in pH_i observed following NH_4^+ pre-treatment in the presence of amiloride or low-Na^+ solutions might represent passive movement of H^+ towards equilibrium rather than active pH_i regulation. In the rat lens, Bassnett & Duncan (1987) measured membrane depolarization following NH_4^+ treatment which they attributed to reduction of a pH_i-sensitive K^+ conductance. If similar changes occur in chicken lens cells this would increase the tendency for pH_i to passively increase following NH_4^+ treatment. Interestingly, simultaneous treatment of lens epithelial cells with both amiloride and HCO_3^--free solutions produced a fall in pH_i to 6·87, very close to the calculated equilibrium value.

The immediate intracellular response to a change in the acid load imposed by the environment is determined by the magnitude of the intracellular buffering capacity. The buffering capacity of the cytoplasm has two major components, the intrinsic buffering capacity (β_i), which arises from the contributions of proteins and other impermeant species, and the CO_2 buffering capacity (Roos & Boron, 1981; Szatkowski & Thomas, 1989). In this study, β_i was calculated from the change in pH_i observed on removing procaine while blocking Na^+–H^+ antiport. The value of 16·5 mM reported here for β_i is close to that of several recent estimates (including the value of 11 mM obtained by Szatkowski & Thomas (1989) using the same technique) but somewhat lower than that reported previously for a number of other cell types (see Roos & Boron, 1981). Bassnett *et al.* (1987) obtained a value for β_i of 40 mmol H^+ kg water^{-1} pH unit^{-1} by direct strong-acid titration of adult rat lens homogenates. The discrepancy between this and the present value may reflect the fact that the earlier measurements were made on whole lens rather than isolated epithelia. Fibre cells constitute the majority of the lens volume and contain crystallin proteins at high concentration. The β_i value for fibre cell cytoplasm would, therefore, be expected to be higher than that of the isolated epithelium. Cells represent open buffer systems where the principle permeant species is likely to be CO_2 (Boron, 1989). The contribution of the CO_2 buffer (β_{CO_2}) can be estimated using eqn (39) of Roos & Boron (1981),

$$\beta_{CO_2} = 2{\cdot}3[HCO_3^-]_i. \quad (3)$$

Assuming that $[CO_2]_i = [CO_2]_o$, $[HCO_3^-]_i$ can be calculated using the Henderson–Hasselbalch equation. At a pH_i of 7·45, this gives a value for $[HCO_3^-]$ of 25 mM and, from eqn (3), a value of β_{CO_2} of about 58 mM It can be seen that the expected contribution of the open, CO_2 buffer system is large compared to β_i.

Maintenance of the resting pH_i appears, in large part at least, to reflect the activity of the Na^+–H^+ antiporter. In many preparations, antiport activity is believed to approach zero at the resting pH_i. However, amiloride added to control solutions caused the resting pH_i of lens epithelial cells to fall. If amiloride specifically inhibited Na^+–H^+ antiport then this suggests that the antiporter was active at the resting pH_i, presumably counteracting some persistent acid load on the cell. Following NH_4^+-induced acidification, the antiporter was also capable of regulating pH_i back to resting levels in the presence or absence (Bassnett *et al.* 1990) of CO_2–HCO_3^-. In the presence of CO_2–HCO_3^-, recovery was largely, although not completely, inhibited by amiloride or low (20 mM) extracellular Na^+. Typical of experiments in which regulation was inhibited by low-Na^+ AAH was an increase in pH_i observed on return to control AAH. The increase may have resulted from a depletion of intracellular Na^+ during exposure to low-Na^+ AAH. Such a depletion would lead to an increase in $[Na^+]_o/[Na^+]_i$ on return to control AAH, possibly driving pH_i to higher values by Na^+–H^+ antiport. Addition of monensin caused an increase in pH_i towards the maximum theoretically obtainable value. This suggests spare capacity in the Na^+–H^+ antiporter and confirms that its activity is regulated under normal conditions.

In the rat lens, Bassnett *et al.* (1987) have measured intracellular lactate levels of about 0·3 μmol lens^{-1} (13 mM) and have shown that an amount approximately equivalent to this is secreted by the lens every hour. For pH_i to be as high as observed, this persistent metabolic acid load must presumably be actively

counteracted by some mechanism. A high capacity Na^+–H^+ antiport system located in the anterior epithelium would be a good candidate for such a mechanism. Fröhlich (1989) has suggested, on the basis of affinity for amiloride and its analogues, the co-existence of two types of antiporter: a ubiquitous 'house-keeping' protein and an epithelial-specific protein, specialized for transepithelial transport of Na^+ and acid equivalents. Further study of both the amiloride affinity and precise tissue distribution of the Na^+–H^+ antiporter will be necessary to clarify these points in the lens.

In contrast to the toad lens (Alvarez, Wolosin & Candia, 1989), treating the chicken lens epithelium with Cl^--free solutions led to a SITS- and HCO_3^--sensitive increase in pH_i suggesting the presence of some form of anion exchange protein. Allen, Low, Dola & Maisel (1987) recently identified a protein homologous to the erythrocyte band 3 protein (the red cell anion exchanger) in human lens membrane fractions. In addition, Bassnett, Stewart, Duncan & Croghan (1988) identified a SITS-sensitive component of the Cl^- efflux that was activated by pre-treatment of rat lenses with NH_4^+.

At least two types of Cl^-–HCO_3^- exchange are known to exist in vertebrate cells: Na^+-dependent (believed to be an acid-extrusion mechanism) and Na^+-independent exchange (believed to be an acid-loading mechanism). The Na^+ dependence of lens cell Cl^-–HCO_3^- exchange was not investigated in this work. Na^+-dependent Cl–HCO_3^- exchange generally acts to alkalinize the cell and can, in some cases, be the major mechanism by which cells recover from acidosis (Boyarsky, Ganz, Sterzel & Boron, 1988*a*). In lens epithelia, the fact that recovery from NH_4^+-induced acidosis occurs readily in the absence of HCO_3^- (Bassnett *et al.* 1990) and is almost completely inhibited by amiloride suggests that Na^+-dependent Cl^-–HCO_3^- exchange, if present, plays a relatively small role in recovery from severe acidification. It is possible that, as the slow recovery from induced acidosis seen in the presence of amiloride was also seen under low-Na^+ conditions (where both Na^+–H^+ antiport and Na^+-dependent Cl^-–HCO_3^- exchange would be inhibited), other mechanisms may be involved, including a drift of pH_i towards its equilibrium value (discussed above). There is some evidence, however, that a HCO_3^--dependent acid-extruding mechanism plays a significant role at values close to the resting pH_i because although SITS had no effect on the resting pH_i, removing HCO_3^- from the medium caused pH_i to fall. One explanation which is consistent with these observations is that both types of exchanger are present and acting antagonistically at the resting pH_i. They would both be affected by SITS treatment whereas removing external HCO_3^- would only disable the acid-extruding exchanger. Further experiments will be needed to clarify the role of putative Na^+-dependent anion exchange in this tissue.

An unexpected finding in the present experiments was the large delayed increase in pH_i observed, in the absence of HCO_3^-, on replacement of Cl^- by gluconate. The magnitude of these transients may have reflected the reduced intracellular buffering in the absence of CO_2–HCO_3^-. That the increase was observed on replacing Cl^- with either gluconate or isothionate suggests that the effect was due to Cl^- removal and not to the presence of a specific substitute. Measurements of cell height indicated that the lens cells lost 10–20% of their volume during treatment with Cl^-–HCO_3^--free solutions. This was probably due to the depletion of intracellular KCl and subsequent osmotic water loss. There was some slight indication in the cell height

measurements that cell volume recovery took place in the continued presence of Cl^--free solution. The onset of recovery coincided with the rapid alkalinization suggesting that the increase in pH_i might be involved in the volume regulatory process. Na^+–H^+ exchange has previously been implicated in the control of cell volume in the lens (Wolosin *et al.* 1989) and other tissues (see Hoffman & Simonsen, 1989). The fact that amiloride blocked the increase in pH_i caused by treatment with Cl^-–HCO_3^--free AAH indicates that a similar mechanism may be operating here.

The avian lens differs from the amphibian lens in terms of its response to CO_2–HCO_3^--free media. The fall in pH_i of about 0·15 observed in the avian lens on removing CO_2–HCO_3^- from the AAH has been reported for a number of cell types but is in marked contrast to the results of Wolosin *et al.* (1988) who have reported an increase in pH_i in toad lens under these conditions. In glomerular mesangial cells this decrease is thought to be mediated by the inactivation of SITS-sensitive Na^+-dependent anion exchange (Boyarsky, Ganz, Sterzel & Boron, 1988*b*). Although the presence of CO_2–HCO_3^- is essential for the maintenance of normal pH_i in the lens epithelium, CA did not appear to play an active role in the process. Possibly the enzyme works to allow the rapid interconversion of CO_2 and HCO_3^-, thereby increasing the effective intracellular buffering capacity in a similar fashion to that described by Thomas (1976).

In the present study, the pH_i of chicken lens epithelial cells was determined to be 7·45. It is conceivable that this relatively high value was the result of measuring pH_i in tissue that had been disconnected from the acidified lens interior. However, Wolosin *et al.* (1988) obtained a similar value of 7·4 for pH_i of the toad lens epithelium measured on the intact lens in CO_2–HCO_3^--containing solutions. The pH_i in the bulk of the lens has been measured by a number of workers using ion-sensitive microelectrodes and values of about 6·8 have been obtained for the rat (Bassnett & Duncan, 1985) and frog lenses (Mathias *et al.* 1988). This raises the intriguing possibility that as maturing lens cells move from the epithelial population into the fibre population they experience changes in pH_i of $> 0·5$ units. If this is correct, it would be expected to have dramatic consequences for many aspects of the physiology and biochemistry of the developing lens cell.

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