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Na^+–H^+ exchange in sheep parotid endpieces

Apparent insensitivity to amiloride

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We have used microspectrofluorimetry with the pH-sensitive dye, BCECF, to examine the control of intracellular pH in the secretory endpieces of the sheep parotid gland. Unstimulated endpieces in HCO_3^--free media have a cytosolic pH of 7.5 ± 0.03 (n = 69) which is maintained by a Na^+-dependent proton extrusion process that can be partially supported by Li^+ but not by Cs^+, and is not affected by changes in extracellular Cl^-, HCO_3^- or K^+. It is not blocked by SITS or DIDS, which inhibit Na^+–(n)HCO_3^- co-transport and Cl^-–HCO_3^- exchange, nor is it sensitive to the amiloride analogs, MIA and EIPA, which inhibit Na^+–H^+ exchangers, although very high concentrations of amiloride itself (1 mmol/l) have a (probably non-specific) inhibitory effect. It seems likely that sheep parotid secretory endpieces do contain a Na^+–H^+ exchanger that drives secretion of a HCO_3^--rich juice, and that its insensitivity to amiloride and its analogs explains why these drugs do not block fluid secretion by the intact sheep parotid gland.

Epithelial secretion; Na^+–H^+ exchange; BCECF; Amiloride; Sheep parotid gland

1. INTRODUCTION

Salivary secretion is believed to be driven by the active accumulation of Cl^- and HCO_3^- in the cytosol due to the activity of Na^+–K^+–$2Cl^-$ co-transporters and Na^+–H^+ exchangers in the basolateral membranes of the secretory cells [1], and the secretion process is inhibited by furosemide, an inhibitor of the Na^+–K^+–$2Cl^-$ co-transporter, and amiloride, an inhibitor of the Na^+–H^+ exchanger [1,2]. The predominance of the Na^+–K^+–$2Cl^-$ co-transporter, and the presence in the basolateral membrane of a Cl^-–HCO_3^- exchanger that uses the HCO_3^- gradient to bring Cl^- into the cell, results in the formation of a predominantly Cl^--rich juice.

The sheep parotid differs from the more commonly studied salivary glands in at least three significant respects: (i) it produces an isotonic saliva that, at high secretory rates, is almost entirely made up of $NaHCO_3$ [3], suggesting that the secretory process ought to be driven by HCO_3^- rather than Cl^- transport; (ii) its secretory activity is not blocked by furosemide or amiloride [4], which might suggest that the secretion process is driven by an altogether different mechanism; and (iii) it secretes saliva spontaneously, i.e. in the absence of all neurohormonal stimulation [5], again suggesting that it may employ an atypical secretory mechanism. The sheep parotid is not unique in these respects, however, the parotid gland of the cow (and perhaps other genera in the order Bovidae) appears also to secrete $NaHCO_3$ by an amiloride-insensitive mechanism [6]. Because of the apparent contradiction between the observation that the sheep parotid secretes a HCO_3^--rich saliva, and the observed failure of amiloride to inhibit the secretion process, we decided to investigate the mechanism of proton transport in sheep parotid secretory endpieces by using the pH sensitive dye, BCECF, to study the regulation of cytosolic pH.

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2. MATERIALS AND METHODS

Dispersed secretory endpieces of the sheep parotid were prepared as described previously [7]. For experiments in which was intracellular pH was measured, the endpieces were suspended in a standard Na^+-rich solution containing 2.5 μmol/l of the acetoxymethyl ester of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF-AM). After 15 min incubation at room temperature, the endpieces were centrifuged and resuspended in the bath solution. Typically, this procedure yields clumps of endpieces as well as single endpieces consisting of 10–20 cells and a few individual cells. Since the secretory endpieces of the sheep parotid contain only a single cell type [8], we used single endpieces containing 10–20 cells for BCECF measurements. For experiments in which intracellular Na^+ was measured, the endpieces were suspended in a standard Na^+-rich solution containing 10 μmol/l of the acetoxymethyl ester of SBFI for 30 min at 37°C.

To determine how efficiently BCECF was taken up by the secretory endpiece cells, we selectively solubilized the plasma membranes of some batches of endpieces with digitonin (20 μmol/l) and measured the extent to which BCECF could be leached from the endpiece cells [9]: we found that more than 90% of the dye was localized in the cytoplasm. We have previously shown that sheep parotid endpieces prepared in this fashion and loaded with the Ca^{2+}-sensitive dye, fura-2, respond to acetylcholine with an increase in cytosolic Ca^{2+} [7].

All experiments were performed at 37°C using a Nikon Diaphot inverted microscope with a ×100 Fluor objective. The dye-loaded endpieces were placed on the coverslip base of a Perspex bath (0.2 ml volume) and a second coverslip was put on top so as to create an enclosed chamber that was then perfused at approximately 1 ml/min. An accumulator was used to dampen the peristaltic pulses. At 5-s intervals, the BCECF-loaded cells were excited alternately at 430 and 490 nm (340 nm and 380 nm in the case of SBFI-loaded cells) for periods of 1 s, and the light emitted by the endpieces was passed through a 530 nm (505 nm for SBFI) bandpass filter to a photomultiplier. A MacLab-4 data acquisition device (ADI Instruments, Sydney, Australia) connected to a MacIntosh IIci computer was used to sample the signal from the photomultiplier so that the relation between the ratio of the emission intensity during illumination at 490 nm to that during illumination at 430 nm, the so-called 490/430 ratio, (or the 340/380 ratio for SBFI) could be calculated. In vivo calibration of BCECF was performed as described by Okada et al. [10] using K^+-rich solutions, adjusted to the appropriate pH, containing 5 μmol/l nigericin to ensure dissipation of pH gradients (Calbiochem, San Diego, CA, USA). The relationship between the 490/430 ratio and the external pH was found to be linear over a pH range from 6.6 to 7.8. Calibrations were performed daily in order to assess day-to-day variability and the significance of our results has been determined by comparison of the mean rate of change in intracellular pH (ΔpH/min) for the test series with the mean control rate for the days on which the particular experiments were performed. The SBFI signal was calibrated using the protocol of Sage et al. [11] using 5 μmol/l gramicidin and 5 μmol/l nigericin. The resultant intracellular Na^+ was calculated from the mean of three calibration curves.

The standard Na^+-rich bath solution contained (in μmol/l): NaCl 145, KCl 5, glucose 10, $CaCl_2$ 1, $MgCl_2$ 1 and H-HEPES 10 adjusted to pH 7.3 at 37°C with NaOH. In cation selectivity studies, Na^+ was replaced by the cation of interest.

Amiloride-hydrochloride, DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid) and SITS (4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid) were obtained from Sigma (St Louis, USA). MIA (5-(*N*-methyl-*N*-isobutyl)-amiloride) was obtained from RBI (Natick, MA, USA) and EIPA (ethyl-isopropyl amiloride) was a gift from Professor B.D. Roufogalis. BCECF-AM and SBFI were obtained from Molecular Probes (Eugene, Oregon, USA). All other chemicals were of AR grade or higher. All results are presented as means ± S.E.M., with the number of observations, *n*, in parentheses; statistical significance was determined using Student's unpaired *t*-test.

3. RESULTS

The resting intracellular pH of sheep parotid endpieces incubated at 37°C in the control (pH 7.3) bath was 7.5 ± 0.03 (n = 69), a value that was significantly higher than the intracellular pH we observed in rat mandibular salivary endpieces (7.28 ± 0.06, n = 18) studied under the same experimental conditions. In contrast to rat mandibular endpieces [10], the intracellular pH of sheep parotid endpieces showed a transient decrease of 0.5 ± 0.05 (n = 7) following exposure to acetylcholine (1 μmol/l).

Replacement of bath Na^+ with $NMDG^+$ (*N*-methyl-D-glucamine) caused the cytosolic pH to drop by 0.84 ± 0.24 pH units (n = 5) within 215 ± 32 s (Fig. 1) and restoration of Na^+ to the bath caused the cell pH to return to its previous level within 105 ± 18 s (Fig. 1). These results indicate that the cytosolic pH of sheep parotid endpieces is maintained above equilibrium by means of a Na^+-dependent process, such as a Na^+–H^+ exchanger, as has been described in other salivary secretory epithelia [10,12]. Surprisingly, however, the amiloride derivative, methyl-isopropyl-amiloride (MIA), which inhibits Na^+–H^+ exchange with great potency [13], did not inhibit the recovery of the cytosolic pH seen following re-admission of Na^+ to the bath (Fig. 1).

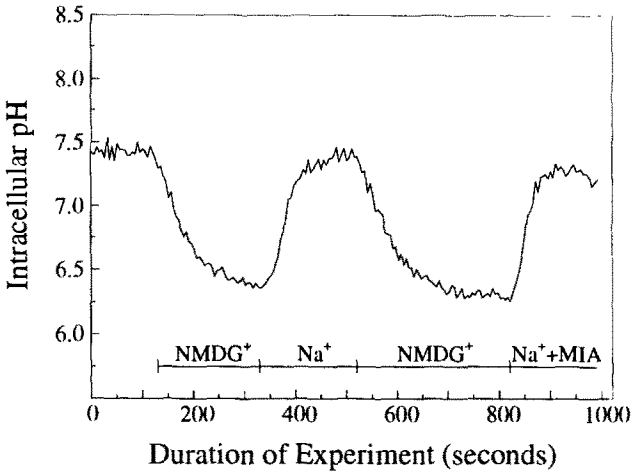

Fig. 1. Intracellular pH in sheep parotid cells. Na^+ in the control bath solution was replaced by $NMDG^+$ during two separate periods. After the first of these periods, the control (Na^+-rich) bath solution was restored. After the second period, the control bath solution plus 100 μmol/l MIA was used.

Acidification of sheep parotid endpieces by exposure to a pulse of NH_4Cl is also followed by Na^+-dependent re-alkalinisation. As shown in Fig. 2A, addition of 20 mmol/l NH_4Cl to the bath caused a transient alkalinisation of the cytosol (due to the movement of NH_3 into the cells), and then, following removal of NH_4Cl from the bath solution, the endpieces rapidly acidified (due to the efflux of NH_3) [14]. This acidification was transient, reaching a maximum 86 ± 14 s (n = 4) after the removal of NH_4Cl. The pH returned to its control level within 266 ± 21 s (n = 4) of the NH_4Cl pulse (Fig. 2A). Just as we had observed for the re-alkalinisation seen following the re-admission of Na^+, we found that the re-alkalinisation following an NH_4Cl pulse was totally dependent on extracellular Na^+: thus, when the NH_4Cl pulse was followed by the substitution of $NMDG^+$ for bath Na^+, the cytosolic pH fell to 6.2 ± 0.03 (n = 57), and for the duration of $NMDG^+$ exposure, re-alkalinisation was negligible (0.0055 ± 0.0058 pH units/min, n = 57) (Fig. 3A). Re-introduction of Na^+ then increased the rate of re-alkalinisation to 0.59 ± 0.056 pH units/min (n = 20) (Fig. 3A). The re-alkalinisation following an NH_4Cl pulse was not noticeably affected by amiloride (1 mmol/l) or MIA (100 μmol/l) (Fig. 2B).

To further investigate the mechanism of re-alkalinisation following an acid load in sheep parotid secretory endpieces, we conducted a series of experiments using the protocol described by Okada et al. for their studies

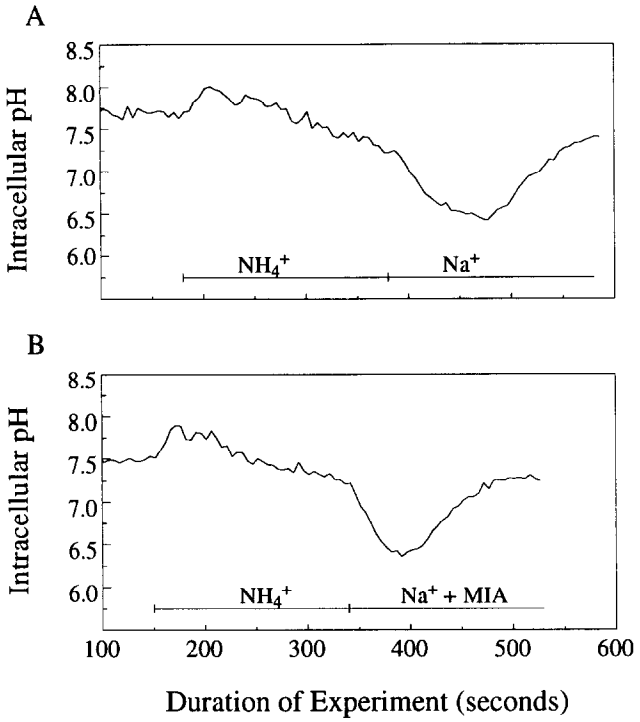

Fig. 2. Intracellular pH in sheep parotid cells. (Upper panel) The pH during and after transient exposure to 20 mmol/l NHCl. (Lower panel) Intracellular pH in an experiment in which 100 μmol/l MIA was added to the bath following the NHCl pulse.

on rat mandibular endpiece cells [10]. This involved performing ion substitution and/or blocker experiments in a test period subsequent to the delivery of an NHCl pulse followed by NMDG substitution. The results of these studies are summarized in Table I. Replacement of bath Na by Cs almost totally abolished re-alkalinisation (Fig. 3B), while Li substitution reduced the rate of re-alkalinisation by 60% ($P < 0.01$; Fig. 3B). Removal of K from the Na-rich bath solution reduced the rate of re-alkalinisation by 50% (Fig. 4A, Table I). (As shown below, this inhibitory effect of K removal is a consequence of inhibition of the Na,K-ATPase leading to dissipation of the Na gradient that drives proton extrusion, rather than the consequence of a requirement for K ions by the proton extrusion process.) Substitution of bath Cl by CHSO had no effect on the rate of re-alkalinisation (Table I). Since patch-clamp experiments indicate that the secretory cells of the sheep parotid gland have a high resting Cl conductance (T. Ishikawa and D.I. Cook, unpublished results), this is good evidence that a Na-dependent Cl–HCO exchanger is not involved in re-alkalinisation [15]. These results are best interpreted as indicating that re-alkalinisation is due to the exchange of internal protons for external Na, with Li being a poor substitute, and they exclude a role for either Cl or K in the transport process. In order to exclude the possible involvement of a Na–(*n*)HCO co-transporter, we examined the effects of both DIDS and SITS on the rate of re-alkalinisation. At 100 μmol/l, neither DIDS nor SITS significantly affected the rate of pH recovery.

Since it thus appeared probable that we were dealing with a Na–H exchange process, we also investigated the effects of MIA and another potent blocker of the Na–H exchanger, ethyl-isopropyl amiloride (EIPA), but even when used in very high concentration (100 μmol/l), neither of these compounds had any effect on the rate of re-alkalinisation (Fig. 3C). When, however,

Table I

Effects of ion substitution and various transport blockers on the rate of re-alkalinisation of sheep parotid secretory endpieces

Treatment	Test		Control		*P*
	ΔpH/min	*n*	ΔpH/min	*n*	
No treatment	–		0.59 ± 0.056	20	–
Li^+ replacement	0.26 ± 0.067	6	0.68 ± 0.108	7	0.010
Cs^+ replacement	0.065 ± 0.013	4	0.67 ± 0.154	4	0.008
$NMDG^+$ replacement	0.003 ± 0.001	78	0.59 ± 0.056	20	0.000
$CH_3SO_4^-$ replacement	0.55 ± 0.120	4	0.70 ± 0.099	6	0.355
K^+ removal	0.24 ± 0.077	5	0.50 ± 0.073	6	0.034
DIDS	0.53 ± 0.075	3	0.59 ± 0.092	6	0.708
SITS	0.63 ± 0.063	3	0.59 ± 0.092	6	0.748
Amiloride (1.0 mmol/l)	0.35 ± 0.050	5	0.69 ± 0.097	8	0.045
Amiloride (0.1 mmol/l)	0.37 ± 0.044	6	0.49 ± 0.101	7	0.311
MIA (0.1 mmol/l)	0.61 ± 0.128	5	0.56 ± 0.089	9	0.783
EIPA (0.1 mmol/l)	0.35 ± 0.030	5	0.40 ± 0.054	6	0.437
Reduced Na^+ (20 mmol/l)	0.19 ± 0.021	7	0.48 ± 0.070	6	0.002
Reduced Na^+ (20 mmol/l)* plus amiloride (0.1 mmol/l)	0.15 ± 0.015	4	0.15* ± 0.014	4	0.996

*In this series (reduced Na^+ in the presence of amiloride), the control solution contained 20, not 150 mmol/l Na^+.

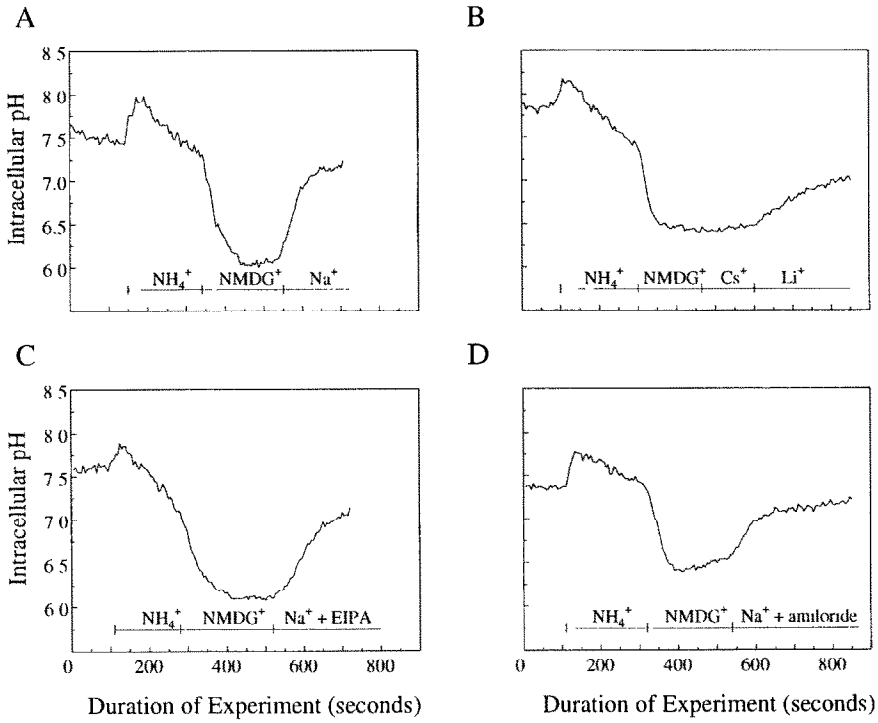

Fig. 3. Intracellular pH in sheep parotid cells. In all four panels, the pH was monitored while the cells were exposed to an NH_4Cl pulse followed by a period in which the Na^+ in the bath was replaced by $NMDG^+$ and then a period in which the bath solution was changed to (A) the control (Na^+-rich) bath solution, (B) a bath solution with Cs^+ substituted for Na^+ followed by a bath solution with Li^+ substituted for Na^+, (C) the control bath solution plus 100 μmol/l EIPA, (D) the control bath solution plus amiloride (1 mmol/l).

we used the parent compound, amiloride, in a concentration of 1 mmol/l, we observed a 50% reduction in the rate of re-alkalinisation ($P = 0.045$) (Fig. 3D); when used in a lower concentration (100 μmol/l), amiloride appeared to reduce the rate of re-alkalinisation by 25% although this result failed to reach statistical significance ($P = 0.311$). Since amiloride competes with Na^+ for a common binding site [16], we repeated our experiments with the bath Na^+ reduced to 20 mmol/l (by equimolar replacement of Na^+ with 130 mmol/l $NMDG^+$). In the absence of amiloride, lowering bath Na^+ to 20 mmol/l reduced the rate of re-alkalinisation to 40% of the rate observed when the bath Na^+ concentration was 150 mmol/l. Typically, in the presence of a reduced bath Na^+ concentration, the efficacy of the amiloride block would be greatly enhanced [16] but, in sheep parotid endpieces, we found that amiloride (100 μmol/l) did not block re-alkalinisation under these conditions (Table I).

We used the Na^+-sensitive dye, SBFI, to investigate the mechanism by which removal of extracellular K^+ inhibits re-alkalinisation of sheep parotid secretory endpieces. As shown in Fig. 4A, following delivery of an NH_4Cl pulse and substitution of $NMDG^+$ for Na^+ in the bath, the intracellular pH fell. In the absence of K^+ in the bath the pH rose slowly as soon as Na^+ was re-admitted. Re-admission of K^+ to the bath caused cytosolic pH to return to its control level. When we used SBFI to measure cytosolic Na^+ in such an experiment (Fig. 4B), we found that $NMDG^+$ substitution produced a fall in intracellular Na^+ and that when Na^+ was restored in the absence of extracellular K^+, intracellular free Na^+ rose rapidly to higher than the control level. Re-introduction of K^+ to the bath then caused the intracellular Na^+ to decline to its original level. These findings suggest that K^+ removal inhibits re-alkalinisation by inhibiting the Na^+,K^+-ATPase, rather than by influencing the Na^+-dependent proton extrusion process directly.

4. DISCUSSION

In this paper we have demonstrated the presence of a Na^+-dependent proton extrusion mechanism in sheep parotid secretory cells which is active in unstimulated cells even at cytosolic pH's as high as 7.5. We have also demonstrated that the proton extrusion mechanism is not dependent on the presence of Cl^- extracellularly, indicating that it is not a Na^+-dependent Cl^--HCO_3^- exchanger [17], nor is it dependent on extracellular K^+. It is not inhibited by SITS or DIDS, indicating that it is not attributable to Na^+–$(n)HCO_3^-$ co-transport persisting in the nominally HCO_3^--free conditions. Surprisingly, however, it was totally insensitive to MIA and

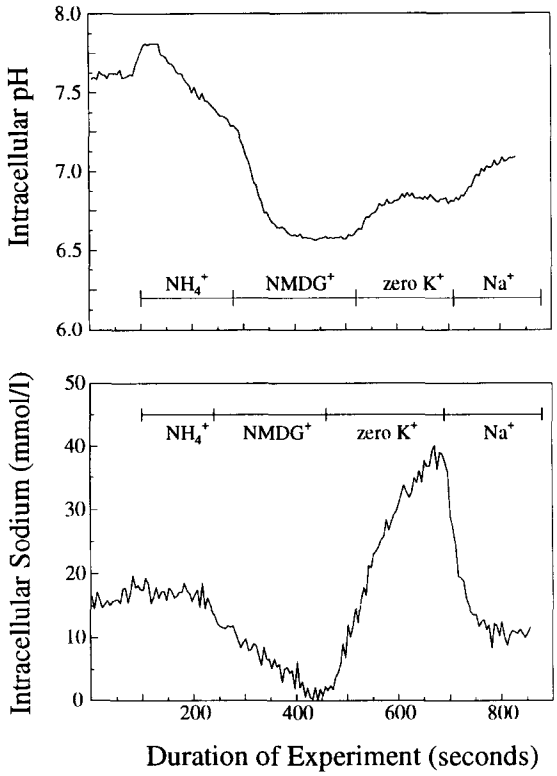

Fig. 4. Intracellular pH and Na^+ in sheep parotid cells. In both panels, the cells have been first exposed to an NH_4Cl pulse, followed by a period in which extracellular Na^+ was replaced by $NMDG^+$. The bath was then changed to a control solution from which K^+ had been omitted; finally, the control solution was restored. (Upper panel) Intracellular pH. (Lower panel) Intracellular Na^+.

EIPA, which are very potent blockers of the Na^+–H^+ exchanger [13], although the parent compound, amiloride, in a concentration of 1 mmol/l, did reduce the rate of re-alkalinisation. At such a high concentration, amiloride is known to have non-specific actions on the Na^+,K^+-ATPase and on cellular energy metabolism [13]. Our failure to observe the expected increase in amiloride affinity as extracellular Na^+ was decreased [16] reinforces the view that the action of amiloride seen in our studies was non-specific. It is not possible, using the techniques employed in this study, to demonstrate unequivocally that this Na^+-dependent proton extrusion is mediated by a Na^+–H^+ exchange protein. However, based on the criteria widely used in the literature, the behaviour is consistent with a Na^+–H^+ exchanger.

Genetic studies show that the external Na^+ site and the amiloride binding site on the Na^+–H^+ exchanger are not identical [18], and there have been several recent reports of Na^+–H^+ exchangers that are insensitive to amiloride (see [19] for a review). This insensitivity is usually relative, blockers such as MIA and EIPA being effective in doses around 1 μmol/l, and amiloride only above 100 μmol/l [19], but in some tissues, such as rat thymocytes [20] and hippocampal neurons [21], the exchanger appears to be totally insensitive to amiloride and its analogs. Interestingly, many of the Na^+–H^+ exchangers reported to be insensitive to amiloride are derived from the luminal membranes of HCO_3^- transporting epithelia, such as the renal proximal tubule [22] and the small intestinal mucosa [23], where it is a feature of the exchanger that it remains active at cytosolic pH's higher than is the case for the amiloride-sensitive 'house-keeping' exchanger seen in so many other tissues.

It is thus of considerable interest that the secretory cells of the sheep parotid gland, which appear able to secrete HCO_3^-, have a resting pH in excess of 7.5, and that they have a Na^+–H^+ exchanger that operates in unstimulated cells and is insensitive to potent amiloride analogs. These secretory cells thus contrast with the Cl^--secreting cells of other salivary glands, such as the mandibular glands of the rat and rabbit, which have cytosolic pHs in the range 7.1–7.2 [2,10,24] and a Na^+–H^+ exchanger that is relatively inactive in resting cells and is quite sensitive to amiloride [2,10] and its analogs [25]. The amiloride insensitivity of the sheep parotid is not due to a species difference, since the re-alkalinisation following a NH_4^+ pulse in the sheep mandibular gland is blocked by 300 μmol/l amiloride (Poronnik and Cook, unpublished data).

Our observation that the Na^+–H^+ exchanger in sheep parotid cells is insensitive to potent amiloride analogs provides an explanation for the puzzling observation of Wright et al. [4] that the secretion of the sheep parotid is not inhibited by amiloride. In their in vivo perfusion studies, using amiloride concentrations of 300 μmol/l or less, Wright et al. failed to observe any effect on the production of saliva by the sheep parotid, an observation that they interpreted as providing evidence that secretion by the sheep parotid was not driven by Na^+–H^+ exchange. From our present studies, however, it becomes clear that a Na^+–H^+ exchanger could still be present, the only difference between the sheep parotid and other salivary glands being that the exchanger in the sheep parotid is insensitive to amiloride in the concentration used by Wright et al. [4].

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