

## Na<sup>+</sup>–H<sup>+</sup> exchange in frog early distal tubule: effect of aldosterone on the set-point

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1. Intracellular pH (pH<sub>i</sub>) regulation was investigated in frog early distal tubule. Single tubules were dissected and perfused, such that the compositions of apical and basolateral solutions could be varied independently. pH<sub>i</sub> was measured using the fluorescent probe 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF).
2. Brief exposure to NH<sub>4</sub><sup>+</sup> on the basolateral aspect of the tubules elicited an intracellular acidification, followed by an active recovery. The recovery was inhibited by amiloride and its analogue 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) when added to the basolateral, but not the apical, solution. Omission of Na<sup>+</sup> from the basolateral solution alone completely inhibited pH<sub>i</sub> recovery. Thus the Na<sup>+</sup>–H<sup>+</sup> exchangers appear to be located on the basolateral membrane.
3. Neither amiloride nor EIPA had any effect on pH<sub>i</sub> under control conditions, suggesting that the activity of the Na<sup>+</sup>–H<sup>+</sup> exchangers at the resting pH<sub>i</sub> is low. However, removal of basolateral Na<sup>+</sup> caused an acidification that was blocked by amiloride, indicating that the Na<sup>+</sup>–H<sup>+</sup> exchangers can be activated from the resting state.
4. Intrinsic buffering power (β<sub>i</sub>) was determined by stepwise removal of ammonium from the cells in Na<sup>+</sup>-free conditions, to prevent pH regulation, and in the presence of Ba<sup>2+</sup> and furosemide (frusemide), to inhibit ammonium transport. β<sub>i</sub> was a function of pH<sub>i</sub>, increasing as pH<sub>i</sub> decreased.
5. Proton efflux was calculated during the recovery from an acid load in tubules from normal and K<sup>+</sup>-loaded frogs and in tubules which had been incubated for 30 min with aldosterone. Potassium loading produces a chronic increase in plasma aldosterone. Both acute and chronic aldosterone treatment caused an intracellular alkalinization. This was due to an alkaline shift in the set-point of the basolateral Na<sup>+</sup>–H<sup>+</sup> exchanger, with no change in the density and/or turnover rate.

The early distal tubule of the amphibian reabsorbs sodium chloride in excess of water, thus causing dilution of the tubule fluid (for review see Guggino, Oberleithner & Giebisch, 1988). Therefore this segment, like its mammalian counterpart, the thick ascending limb, is called the diluting segment (Greger, 1985). The energy for NaCl uptake is derived from the inwardly directed Na<sup>+</sup> gradient generated by the Na<sup>+</sup>,K<sup>+</sup>-ATPase located on the basolateral membrane. Sodium chloride movement from the tubule lumen into the cell is via the furosemide (frusemide)-sensitive Na<sup>+</sup>–2Cl<sup>–</sup>–K<sup>+</sup> cotransporter. However, K<sup>+</sup> delivery to this segment is not sufficient to support the levels of NaCl reabsorbed, a problem which is overcome by K<sup>+</sup> recycling back through the apical membrane into the lumen through potassium channels. The supply of K<sup>+</sup> as a substrate for cotransport is a rate-limiting step in the

overall salt transport by this segment, so the apical K<sup>+</sup> conductance is a potential site of transport regulation (Guggino *et al.* 1988).

Several studies have shown that the K<sup>+</sup> conductance of early distal cells is modulated by intracellular pH (pH<sub>i</sub>) (Oberleithner, Dietl, Munich, Weigt & Schwab, 1985*a*; Hunter, Oberleithner, Henderson & Giebisch, 1988; Oberleithner, Kersting & Hunter, 1988; Wang, Henderson, Giebel, White & Giebisch, 1989). The apical K<sup>+</sup> conductance appears to be comprised of a single type of K<sup>+</sup> channel whose activity is increased by cytosolic alkalinization (Hurst & Hunter, 1990). Chronic K<sup>+</sup> loading of amphibians, by elevating the K<sup>+</sup> concentration of the water in which they are maintained, stimulates K<sup>+</sup> secretion (Oberleithner, Guggino & Giebisch, 1983*a*). Potassium loading also causes elevation of plasma aldosterone (Oberleithner, Lang,

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Wang, Messner & Deetjen, 1983*b*). Aldosterone stimulates  $\text{Na}^+-\text{H}^+$  exchange acutely *in vitro*, resulting in an intracellular alkalinization (Oberleithner, Weigt, Westphale & Wang, 1987). Thus transport is stimulated secondary to activation of  $\text{Na}^+-\text{H}^+$  exchange (Oberleithner *et al.* 1983*b*).

$\text{Na}^+-\text{H}^+$  exchangers show a set-point, i.e. once the  $\text{pH}_i$  is above a certain level, activity ceases (Aronson, Nee & Suhm, 1982). Increased  $\text{Na}^+-\text{H}^+$  exchange activity could occur by changes in one or more of the following parameters: (a) the set-point; (b) the turnover rate; or (c) the number of exchangers in the cell membrane. The following experiments were designed to determine which of the above mechanisms are responsible for the observed increase in  $\text{Na}^+-\text{H}^+$  exchange activity following  $\text{K}^+$  loading.

## METHODS

### Animal and tubule preparation

Frogs (*Rana temporaria*) of either sex were used. Control frogs were maintained in tap water. Potassium-adapted animals were obtained by keeping frogs for at least 3 days in water to which 50 mM KCl had been added (Oberleithner, Guggino & Giebisch, 1985*b*). Animals were killed by decapitation and destruction of the spinal cord. The kidneys were removed and cut into slices 1–2 mm thick and stored on ice in Leibovitz 15 medium (Sigma, Poole, UK; prepared to osmolality  $204 \text{ mosmol (kg H}_2\text{O)}^{-1}$ , buffered with 10 mM Hepes, titrated to pH 7.4 with NaOH). Single early distal segments were free-hand dissected in control frog Ringer solution (Table 1). Acute exposure to aldosterone was performed by incubating single tubules in frog Ringer solution containing  $1 \times 10^{-6}$  M aldosterone for 30 min.

The kidney structure in frogs is similar to that of *Amphiuma* (Stanton, Biemesderfer, Stetson, Kashgarian & Giebisch, 1984). There are two, relatively narrow, ciliated regions in the frog nephron; the neck segment, which connects the glomerulus and proximal tubule, and the connecting segment, which lies between the late proximal and early distal tubules. In this study we have used early distal tubules in which we were able to identify positively the connecting segment and to distinguish between the early distal and the relatively thicker proximal tubule.

Following dissection, tubules were transferred into a Perspex perfusion chamber (volume approximately 300  $\mu\text{l}$ ) sited on the stage of an inverted microscope (Nikon,

Kingston, UK). All experiments were carried out at room temperature. During experiments the tubules were either perfused, using standard techniques (Burg, Grantham, Abramow & Orloff, 1966), or positioned on the bottom of the perfusion chamber, depending upon the experimental protocol (see later). Both bath and luminal perfusion solutions could be exchanged for other test solutions by means of hydraulic (Alltech, Cornforth, UK; bath) or solenoid (Lee, Gerrards Cross, UK; lumen) valves.

### Loading of tubule with dye

Tubules were loaded with the acetoxymethyl form of the dye 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM). Dye was dissolved initially in dimethyl sulphoxide (DMSO), 50  $\mu\text{g}$  in 20  $\mu\text{l}$ ; a 1:250 dilution of this initial solution in control Ringer solution was the stock solution. Following transfer of the tubule into the bath, perfusion was begun for about 30 s to remove red cells and other cellular debris. Bath flow was stopped and 100  $\mu\text{l}$  of the stock solution added to the bath to give a final BCECF concentration of approximately 5  $\mu\text{M}$ . Tubules were incubated with dye for 10 min, after which time the bath flow was restarted and the dye fluorescence ratio (see below) allowed to reach a steady state before experiments were begun. The level of fluorescence at the end of the dye-loading period was typically 50–200 times the background level, i.e. the autofluorescence of the tubule in the absence of any dye. Autofluorescence was determined at the beginning of each experiment, before the addition of dye, and was subtracted from all subsequent fluorescence values.

### Fluorescence microscopy

The experimental set-up was housed in a light-proof Faraday cage. The microfluorimeter was purchased from Newcastle Photometric Systems (NPS, Newcastle upon Tyne, UK) and installed on a Nikon Diaphot inverted microscope. The excitation light source was a 100 W xenon arc light (Nikon). This light was passed through a series of neutral density filters (Ealing Electro-optics, Watford, UK) to give 0.1% transmission and then through band-pass interference excitation filters of 440 and 490 nm, which were positioned alternately in the light path using a stepping motor (NPS). This excitation light was reflected using a 510 nm long-pass dichroic mirror onto the tubule via a  $\times 40$  quartz objective lens. Emitted light of a longer wavelength was collected by the objective lens and passed back through the mirror and a 520 nm barrier filter, before being collected by a photomultiplier tube (model 9924B, Thorn EMI, Ruislip, UK). The light reaching the PMT for each excitation–emission

Table 1. Composition of solutions

	Control Ringer solution (mM)	High- $\text{K}^+$ Ringer solution (mM)	5 mM $\text{NH}_4\text{Cl}$ (mM)	0 mM $\text{Na}^+$ (mM)	20 mM $\text{NH}_4\text{Cl}$ in 0 mM $\text{Na}^+$ (mM)
NaCl	97	0	92	0	0
KCl	3	100	3	3	3
$\text{CaCl}_2$	2	2	2	2	2
$\text{MgCl}_2$	1	1	1	1	1
NMDG-Cl	0	0	0	97	77
$\text{NH}_4\text{Cl}$	0	0	5	0	20
Hepes	10*	10**	10*	10**	10**

\*Titrated to pH 7.40 with NaOH. \*\*Titrated to pH 7.40 with KOH.

period (400 ms) at each wavelength was counted by a photon counter (Newcastle Photometrics); the output was digitized by an A/D converter (Newcastle Photometrics) and stored in a computer (Elonex PC486B, Elonex, London). The software controlling data acquisition and display was COUNT (NPS).

### Calibration

The emission ratios (440/490) were converted to pH values using the nigericin technique (Thomas, Buchsbaum, Zimniak & Racker, 1979). At the end of each experiment the bath was perfused with a high-K<sup>+</sup> solution, pH 7.4 (Table 1). Bath flow was then halted and high-K<sup>+</sup> solution containing nigericin (initially dissolved in ethanol, 1 µg ml<sup>-1</sup>; stock solution was a 1 in 50 dilution in high-K<sup>+</sup> Ringer solution) was added to the bath to a final concentration of approximately 7 × 10<sup>-3</sup> mg ml<sup>-1</sup>. After 10 min the bath flow was restarted and the tubule was superfused with three additional high-K<sup>+</sup> solutions with values of pH close to 6, 7 and 8 (the absolute values of these solutions were determined to within 0.01 pH unit) and the fluorescence ratio was allowed to reach a steady state with each solution. Calibration was performed in each tubule.

The experimental data were analysed using GLOW, an analysis program written 'in-house' using AxoBasic (Axon Instruments, Foster City, CA, USA), fitting the following equation to the calibration curve:

$$pK_a = \text{pH} - \log\left(\frac{R - R_{\min}}{R_{\max} - R}\right), \quad (1)$$

where  $R$  is the emission ratio at a given pH, and  $R_{\min}$  and  $R_{\max}$  are the limiting values of the ratio at the extremes of acid or alkaline pH (Graber, Dilillo, Friedman & Pastoriza-Muñoz, 1986). This yields best-fit estimates of  $pK_a$  (-log of the dissociation constant of BCECF for protons),  $R_{\min}$  and  $R_{\max}$  for each tubule, which were then used to convert the measured ratios to pH<sub>i</sub> values.

### Determination of buffering power

Non-bicarbonate buffering power ( $\beta_1$ ) was measured using short, open-ended, unperfused tubules, from both control and K<sup>+</sup>-loaded animals. The tubules rested on the bottom of the perfusion chamber and the method used was that of Boyarsky, Ganz, Sterzel & Boron (1988). The technique relies upon the sequential addition of known amounts of acid to the cells, and the resulting measured changes in pH<sub>i</sub>. The experiments must be carried out in the absence of any background pH regulatory mechanisms, so Na<sup>+</sup>-free solutions were used, with Na<sup>+</sup> replaced by *N*-methyl-D-glucamine (NMDG). The NH<sub>4</sub><sup>+</sup> concentration was varied by mixing appropriate amounts of the 0 mM Na<sup>+</sup> solution with the 20 mM NH<sub>4</sub>Cl-0 mM Na<sup>+</sup> solution (Table 1). For determination of  $\beta_1$  it is assumed that NH<sub>4</sub><sup>+</sup> leaves the cells in the form of NH<sub>3</sub> (see Results). The diluting segment contains transporters, such as K<sup>+</sup> channels and the Na<sup>+</sup>-2Cl<sup>-</sup>-K<sup>+</sup> cotransporter, which may also mediate NH<sub>4</sub><sup>+</sup> movement. To minimize such transporter-mediated NH<sub>4</sub><sup>+</sup> fluxes,  $\beta_1$  was determined in the presence of 1 mM BaCl<sub>2</sub> and 50 µM furosemide.

BCECF-AM was purchased from Molecular Probes (Eugene, OR, USA). 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA) was a generous gift from Dr Benfield of Merck Sharp & Dohme (Hoddesdon, UK). All other chemicals were purchased from Sigma (UK).

Data are presented as means ± s.e.m., with the number of observations in parentheses. Statistical analysis was

performed with Student's paired or unpaired *t* test or ANOVA as appropriate; significance was assumed at the  $P < 0.05$  level.

## RESULTS

Initial experiments to confirm the presence of Na<sup>+</sup>-H<sup>+</sup> exchange were carried out by monitoring the recovery of pH<sub>i</sub> following an acid load (Boron & De Weer, 1976). Figure 1A shows the changes in pH<sub>i</sub> during exposure to 5 mM NH<sub>4</sub>Cl and the subsequent recovery upon NH<sub>4</sub><sup>+</sup> removal in the absence of inhibitors. NH<sub>4</sub><sup>+</sup> elicited an initial rapid alkalization, due to the entry of NH<sub>3</sub> and reaction with intracellular protons. This was followed by slower entry of NH<sub>4</sub><sup>+</sup>, resulting in a secondary acidification due to its dissociation to form NH<sub>3</sub> and protons. Upon removal of NH<sub>4</sub><sup>+</sup> from the bath there was an extremely rapid acidification; the cell membranes are apparently more permeable to NH<sub>3</sub> than NH<sub>4</sub><sup>+</sup>, thus NH<sub>4</sub><sup>+</sup> leaves the cell largely as NH<sub>3</sub>, with the resulting protons causing acidification. If protons were distributed passively across the membrane, pH<sub>i</sub> should be around 6.15, assuming a membrane potential of -71 mV (Guggino *et al.* 1988). However, during the exposure to NH<sub>4</sub><sup>+</sup> and in the subsequent recovery period, pH<sub>i</sub> was more alkaline than this value in the majority of experiments. Thus some active transport mechanism, acting effectively to expel acid from the cell, must be involved in both the maintenance of the steady-state pH<sub>i</sub> and the recovery from an acid load.

Na<sup>+</sup>-H<sup>+</sup> exchange is inhibited by the diuretic amiloride and related drugs (Aronson *et al.* 1982; Garritsen, Ijzerman, Tulp, Th, Cragoe & Soudijin, 1991). EIPA is an amiloride analogue with a higher potency and specificity for the Na<sup>+</sup>-H<sup>+</sup> exchanger than amiloride (Garritsen *et al.* 1991). Addition of EIPA to the bath fluid immediately after an NH<sub>4</sub><sup>+</sup> pulse markedly inhibited pH recovery (Fig. 1C). Removal of EIPA relieved inhibition of the Na<sup>+</sup>-H<sup>+</sup> exchangers and pH<sub>i</sub> recovered back towards control levels. When applied to the bath, amiloride was also effective at inhibiting pH<sub>i</sub> recovery (Fig. 1B).

If the Na<sup>+</sup>-H<sup>+</sup> exchangers are to operate in the direction of proton extrusion, their activity should depend upon external Na<sup>+</sup>. Substitution of Na<sup>+</sup> by NMDG in the bath perfusate following an NH<sub>4</sub><sup>+</sup> prepulse also resulted in inhibition of pH<sub>i</sub> recovery (Fig. 1D).

The inhibition of pH<sub>i</sub> recovery, following an NH<sub>4</sub><sup>+</sup> prepulse, by amiloride, EIPA and Na<sup>+</sup> removal was used to determine whether the Na<sup>+</sup>-H<sup>+</sup> exchangers were present on the apical or basolateral membranes (Table 2). The data were compared using ANOVA (degrees of freedom (d.f.) 8, 52;  $F = 11.62$ ) and significance between groups was calculated using treatment comparisons. In all cases there was a significant decrease in recovery rate when inhibitor was present in the bathing solution. There was no change in the rate of recovery when amiloride or its analogue

Table 2. Rate of recovery of intracellular pH after an ammonium pulse

Bath solution	Lumen solution			
	Control	EIPA	Amiloride	0 mM Na <sup>+</sup>
Control	9.28 ± 0.79 (11)	9.02 ± 1.91 (6)	9.48 ± 2.50 (9)	—
EIPA	0.90 ± 0.32* (6)	1.15 ± 0.32* (6)	—	—
Amiloride	0.41 ± 0.05* (5)	—	0.85 ± 0.18* (6)	—
0 mM Na <sup>+</sup>	0.33 ± 0.44*† (6)	—	—	-0.35 ± 0.21*† (6)

Values are given as  $\Delta\text{pH}/\Delta t$  ( $\text{s}^{-1} \times 10^{-3}$ ). \*Significantly different from the control. †Recovery not significantly different from zero. Values of  $n$  are given in parentheses.

EIPA was present in the luminal bathing solution, either in the presence or absence of bath inhibitor. There was no significant recovery in the absence of Na<sup>+</sup> from the bath.

The above inhibitors, as well as reducing the rate of recovery from an acid load, significantly increased the magnitude of the initial acidification on removal of NH<sub>4</sub><sup>+</sup> (d.f. 3, 22;  $F=6.18$ ). Under control conditions the initial

acidification was  $0.39 \pm 0.05$  pH units ( $n=9$ ); this was raised to: EIPA,  $0.68 \pm 0.07$  pH units ( $n=5$ ); amiloride,  $0.52 \pm 0.04$  pH units ( $n=7$ ) and NMDG,  $0.57 \pm 0.02$  pH units ( $n=5$ ).

Removal of Na<sup>+</sup> from the basolateral solution elicited a decrease in the resting pHi, as shown in Fig. 2, from  $7.05 \pm 0.148$  to  $6.85 \pm 0.112$  ( $n=5$ ). This was presumably

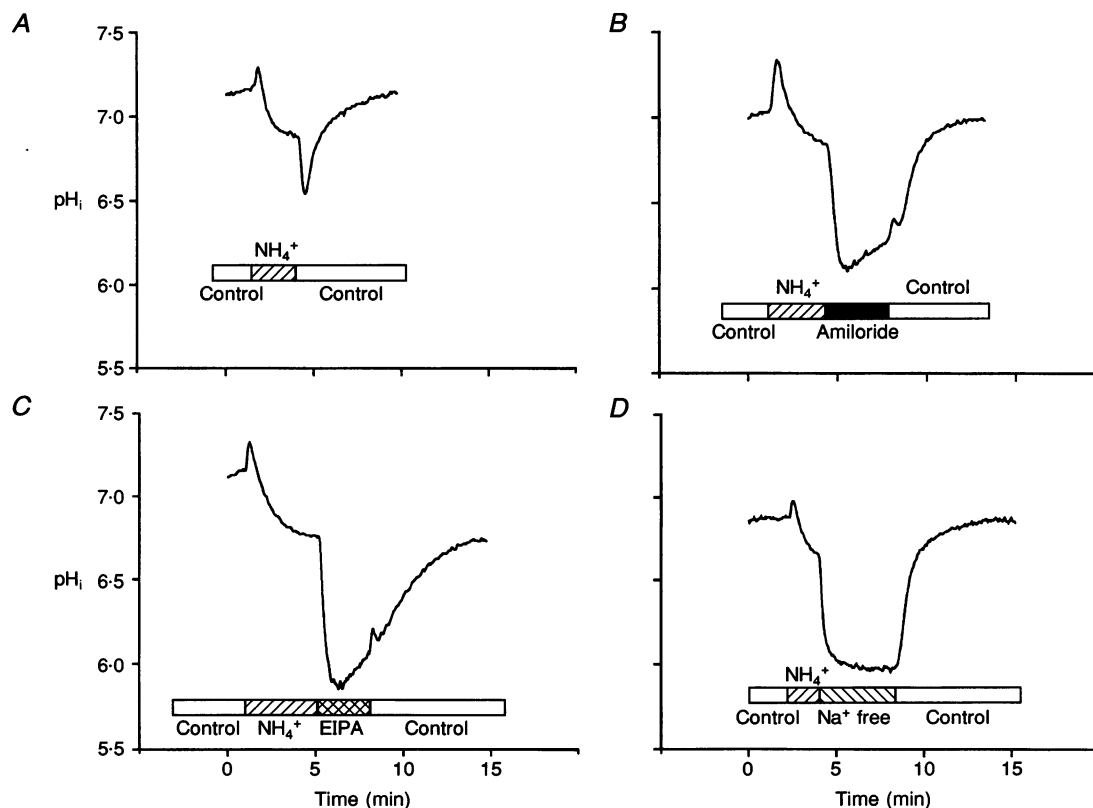
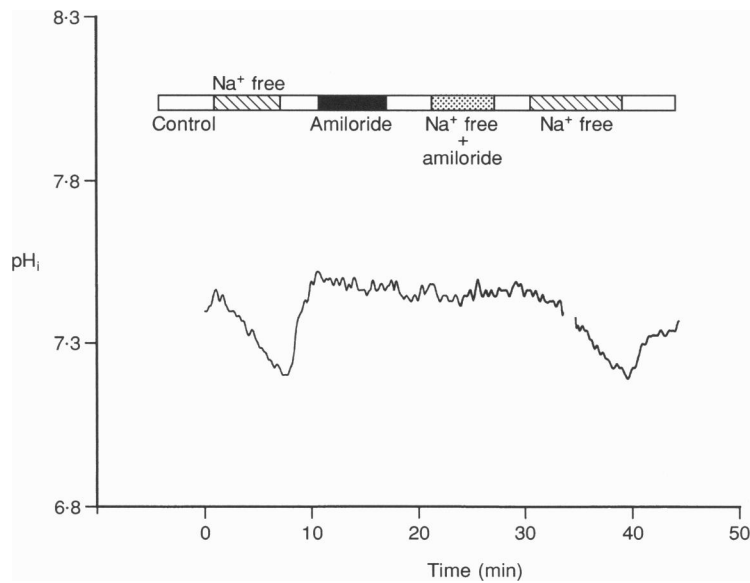


Figure 1. Na<sup>+</sup>-H<sup>+</sup> exchange in early distal tubules

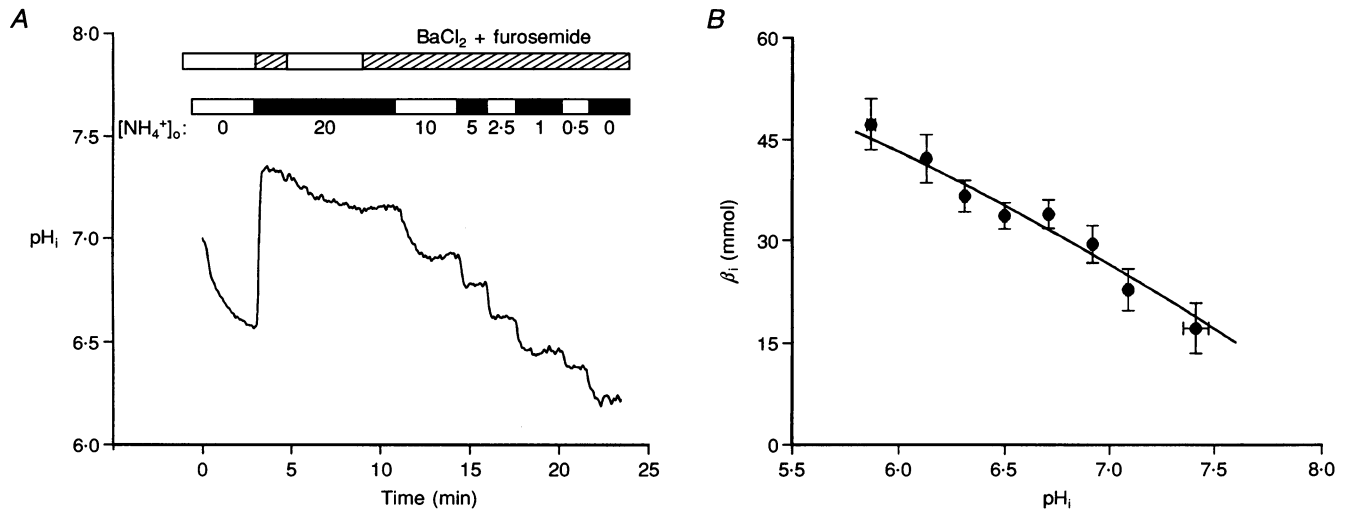
Experimental traces showing acid loading of perfused early distal tubule segments by brief addition of NH<sub>4</sub><sup>+</sup> to the bath solution and the effects of inhibitors on the subsequent recovery rate. Luminal solution: control Ringer solution. *A*, typical response to acid load following an ammonium pulse. On the removal of NH<sub>4</sub><sup>+</sup> from the bathing solution a rapid acidification is seen followed by a recovery of pHi. It is this recovery phase which has been altered by various inhibitors. *B*, *C* and *D*, reduction in recovery rate on removal of NH<sub>4</sub><sup>+</sup> with addition of basolateral  $1 \times 10^{-3}$  M amiloride (*B*),  $1 \times 10^{-5}$  M EIPA (*C*) and Na<sup>+</sup> removal (*D*). The inhibitory effects of EIPA were generally not as reversible as amiloride or Na<sup>+</sup> removal.



**Figure 2. Na<sup>+</sup> removal uncovers Na<sup>+</sup>-H<sup>+</sup> exchange activity**  
Removal of Na<sup>+</sup> from the bath solution in a perfused tubule elicits an intracellular acidification. This effect is inhibited in a reversible manner by amiloride.

due to the reversal of the Na<sup>+</sup>-H<sup>+</sup> exchanger, since removal of Na<sup>+</sup> in the presence of amiloride had no effect upon pHi: control, 7.06 ± 0.132; Na<sup>+</sup>-free solution plus amiloride, 7.07 ± 0.129 (n = 5). Addition of amiloride alone to the bath solution was without effect on the resting pHi: control, 7.05 ± 0.140; amiloride, 7.04 ± 0.136 (n = 5).

Figure 3A shows a typical experiment to estimate intracellular buffering power. Initially the tubule was in a Na<sup>+</sup>-free solution. Introduction of 20 mM NH<sub>4</sub><sup>+</sup> gave the characteristic alkalinization (NH<sub>3</sub> entry). The normal acidification (NH<sub>4</sub><sup>+</sup> entry) was reduced in the presence of Ba<sup>2+</sup> and furosemide, but removal of these blockers allowed



**Figure 3. Determination of intracellular buffering power**  
A, experiment to determine β<sub>i</sub> in a non-perfused tubule. During the period indicated by the bar all solutions were Na<sup>+</sup> free, with varying concentrations of NH<sub>4</sub><sup>+</sup> (mM) as indicated. ▨, 1 mM BaCl<sub>2</sub> and 50 μM furosemide in bath. Reductions in superfusate NH<sub>4</sub><sup>+</sup> elicited stepwise reductions in pHi. From such experiments β<sub>i</sub> can be estimated over a wide range of pHi in a single tubule (see text for details). B, relationship between pHi and intrinsic buffering power. The data are pooled from normal and K<sup>+</sup>-loaded frogs in the presence of furosemide and BaCl<sub>2</sub>. Two hundred and four data points were gathered into pools of 0.2 pH units and are shown as means ± S.E.M. The continuous line is the best-fit polynomial curve (see text).

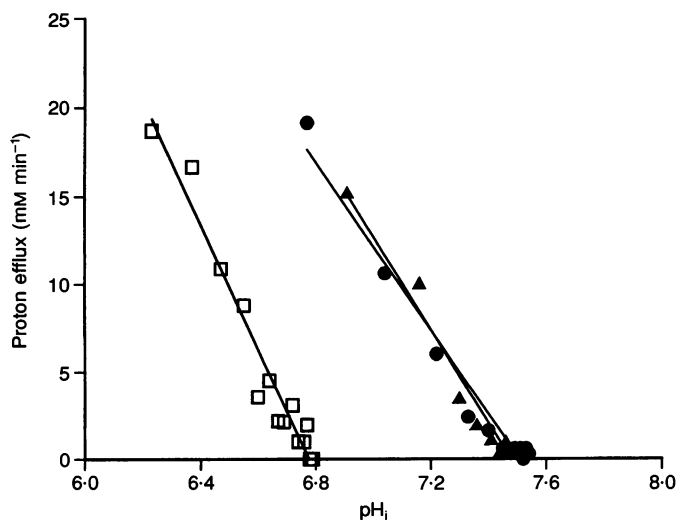
an acidification to a new steady state. At this steady-state point the blockers were reintroduced and it was possible to determine the intracellular  $\text{NH}_4^+$  concentration, by assuming intracellular  $\text{NH}_3$  is equal to extracellular  $\text{NH}_3$  and measuring the  $\text{pH}_i$  (Roos & Boron, 1981). When the bathing solution was changed to a 10 mM  $\text{NH}_4^+$  solution a new equilibrium was established. As  $\text{NH}_3$  leaves, the resulting protons acidify the cell. It was then possible to determine the new steady-state intracellular  $\text{NH}_4^+$  concentration. From these values it was possible to calculate the acid load to the cell, and hence  $\beta_i$  (Boyarsky *et al.* 1988). The buffering power was calculated from the change in  $\text{pH}_i$  for a given acid load (Boron, 1992) and has units of millimolar per pH unit. However, the  $\beta_i$  value obtained is valid only for a particular  $\text{pH}_i$ , which was taken as the mid-point of the two steady-state pH values. Reduction of the  $\text{NH}_4^+$  concentration again caused a further acidification and so on. Thus several values relating  $\beta_i$  to  $\text{pH}_i$  were obtained in a single preparation. The absence of pH recovery during these experiments suggested that, at least in the absence of  $\text{HCO}_3^-$ , there were no significant  $\text{Na}^+$ -independent pH-regulatory mechanisms in this segment.

Buffering power was measured as described above in tubules from normal and  $\text{K}^+$ -loaded frogs. In both cases a relationship between  $\beta_i$  and  $\text{pH}_i$  was obtained, with  $\beta_i$  increasing as  $\text{pH}_i$  decreased. Regression lines were plotted through the data and the regression coefficients were compared. There was no significant difference, so the data were pooled into one group. The 204 data points for  $\beta_i$  against  $\text{pH}_i$  were fitted with a polynomial expression of the form  $\beta_i = -1.454(\text{pH}_i)^2 + 2.223(\text{pH}_i) + 82.21$ , which is shown in Fig. 3B.

### $\text{Na}^+-\text{H}^+$ exchange activity under control and aldosterone-treated conditions

To compare the activity of the  $\text{Na}^+-\text{H}^+$  exchangers under normal conditions and following acute and chronic exposure to aldosterone, proton efflux was measured during the recovery from  $\text{NH}_4^+$  pulses. The complicating factor here is that it is not possible merely to measure the rate of change of  $\text{pH}_i$  during recovery as an index of exchanger activity, since this is affected by  $\beta_i$  and, as shown in Fig. 3B,  $\beta_i$  is itself a function of  $\text{pH}_i$ ; this was the reason why  $\beta(\text{pH}_i)$  was determined. However, once  $\beta(\text{pH}_i)$  is known, proton efflux can be calculated for any given change in  $\text{pH}_i$  as the product of  $\beta(\text{pH}_i)$  (mM/pH) and the rate of change of  $\text{pH}_i$  (dpH/dt). Proton efflux thus has units of millimolar per minute. Proton efflux was measured as a function of  $\text{pH}_i$  during the recovery from an  $\text{NH}_4^+$  pulse. The slope and mid-point pH between sequential points was determined and proton efflux was calculated for the mid-point pH. In this way the raw data records ( $\text{pH}_i$  vs. time) were transformed to show the relationship between proton efflux and  $\text{pH}_i$  throughout the recovery phase, as shown in Fig. 4.

Figure 4 shows proton efflux as a function of  $\text{pH}_i$  in tubules from the three conditions. The relationship is fairly linear and the intercept of this line with the  $x$ -axis was taken as the set-point of the  $\text{Na}^+-\text{H}^+$  exchanger (Stewart, 1988). The slope of the line is an indication of the number of exchangers present or the turnover rate of the exchangers; if the number of transporters, or their activity, increased at any  $\text{pH}_i$  on the acid side of the set-point, this would result in an increased proton flux and



**Figure 4.** Effect of aldosterone on  $\text{pH}_i$  recovery

Proton efflux as a function of  $\text{pH}_i$  in tubules from normal (□) and  $\text{K}^+$ -loaded frogs (▲) and aldosterone-treated tubules (●). Proton efflux was measured during the recovery phase following an acid load (see text). Regression lines are drawn through the data points.

Table 3. Properties of Na<sup>+</sup>-H<sup>+</sup> exchange in control and K<sup>+</sup>-adapted animals

	Rate of proton efflux (mm min <sup>-1</sup> )	Set-point pH <sub>i</sub>	Resting pH <sub>i</sub>
Control	21.86 ± 2.91 (10)	6.99 ± 0.05 (10)	7.09 ± 0.04 (15)
K <sup>+</sup> -loaded frogs	33.91 ± 3.45 (11)	7.30 ± 0.05* (11)	7.35 ± 0.04* (9)
Acute aldosterone	30.58 ± 5.88 (6)	7.33 ± 0.08* (6)	7.42 ± 0.07* (6)

\*Significantly different from control. Values of *n* are given in parentheses.

consequently an increased slope. The slope, set-point and resting pH<sub>i</sub> between the three groups were compared using ANOVA followed by treatment comparison. There was a significant increase in the set-point upon K<sup>+</sup> loading and acute aldosterone addition (d.f. 2,18; *F* = 13.36) in comparison with controls, but there was no significant change in the slope of the lines (d.f. 2,18; *F* = 1.67). Resting pH<sub>i</sub> was significantly increased compared with controls in K<sup>+</sup>-loaded and aldosterone-treated tubules (d.f. 2,18; *F* = 9.50). There was no significant difference in set-point and resting pH<sub>i</sub> between K<sup>+</sup>-loaded and aldosterone-treated tubules (Table 3).

To verify that pH<sub>i</sub> regulation under stimulated conditions was due to activation of the Na<sup>+</sup>-H<sup>+</sup> exchanger, rather than some other transport process, the degree of inhibition of the recovery from an acid load by basolateral EIPA was measured. In control tubules, EIPA reduced the rate of recovery by 88.7 ± 4.49% (*n* = 6). In K<sup>+</sup>-loaded frogs recovery was reduced by 90.5 ± 2.20% (*n* = 6) and in aldosterone-treated tubules by 92.7 ± 2.51% (*n* = 6). There was no significant difference within these three treatments (d.f. 2,15; *F* = 0.39).

## DISCUSSION

### Location of the Na<sup>+</sup>-H<sup>+</sup> exchangers

In agreement with a previous study, cells of the early distal tubule of the frog are able to recover their pH<sub>i</sub> following an acid load (Oberleithner *et al.* 1987). This pH recovery is inhibited by Na<sup>+</sup> removal and by amiloride and EIPA, drugs which have both been shown to inhibit Na<sup>+</sup>-H<sup>+</sup> exchange (Boron & Boulpaep, 1983; Boyarsky *et al.* 1988). A major difference between this and previous studies concerns the location of the exchangers. The current cell model, based upon work by Oberleithner and co-workers (Oberleithner, Lang, Messner & Wang, 1984; Oberleithner, 1985) suggests that the Na<sup>+</sup>-H<sup>+</sup> exchanger is located on the luminal membrane. In the present study we have been unable to find evidence of apical Na<sup>+</sup>-H<sup>+</sup> exchange. Amiloride and EIPA both inhibited pH recovery, but only when added to the basolateral solution. There was no significant difference in the rate of pH recovery with luminal EIPA or amiloride, either in the presence or absence of the inhibitor in the bath (Table 2). The most

striking evidence for the absence of apical Na<sup>+</sup>-H<sup>+</sup> exchange is the lack of pH recovery in the absence of bath Na<sup>+</sup> (Fig. 1D and Table 2). In this condition the rate of recovery is reduced by 97% compared with controls, a recovery rate which is not significantly different from zero. This suggests that apical Na<sup>+</sup>-H<sup>+</sup> exchange could comprise, at most, 3% of the total Na<sup>+</sup>-H<sup>+</sup> exchange activity. This is similar to rat thick ascending limb, in which basolateral Na<sup>+</sup>-H<sup>+</sup> exchange accounts for about 88% of the recovery from an acid load (Krapf, 1988).

As mentioned above, these results are in direct contrast to earlier reports by Oberleithner and colleagues, who located the Na<sup>+</sup>-H<sup>+</sup> exchangers in the apical membrane of the frog diluting segment (Oberleithner *et al.* 1984; Oberleithner, 1985). The reasons for this discrepancy are not clear, although they did use a different technique, i.e. the doubly perfused kidney in conjunction with ion-sensitive microelectrodes, and a different species of frog, *Rana esculenta*. Perhaps the most plausible reason is that of species difference. However, our results are unequivocal; one of the main advantages of the isolated perfused tubule preparation is the ability to alter independently the composition of the solutions on either side of the tubule cells, thus enabling localization of membrane transport processes. Using this approach we find, within the resolution of our methods, that the exchangers are located exclusively on the basolateral membrane.

### Are the exchangers active at resting pH<sub>i</sub>?

The absence of any acidification following acute amiloride administration suggests that the Na<sup>+</sup>-H<sup>+</sup> exchanger is not active at the resting pH<sub>i</sub> and that there are no significant acid loading mechanisms in the cell, e.g. due to metabolism (Boron, 1992). Perhaps the pH<sub>i</sub> is at the set-point or, if an alkalinizing pH regulatory mechanism exists, more alkaline than the set-point pH. The only other documented pH regulatory mechanism in these cells is the electrogenic Na<sup>+</sup>-HCO<sub>3</sub><sup>-</sup> cotransporter (Wang, Dietl & Oberleithner, 1987). However, the present studies were performed in the absence of HCO<sub>3</sub><sup>-</sup>, with Hepes as a buffer, so we would expect little contribution from this transporter. Additionally, in the experiments to determine β<sub>1</sub>, which were carried out in Na<sup>+</sup>-free Ringer solution (Fig. 3A), for any NH<sub>4</sub><sup>+</sup> concentration there was no detectable change in

pH<sub>i</sub> other than the monotonic decrease to a new steady state. This was evident over a wide pH range.

This lack of effect of amiloride is at variance with the observed acidification upon the removal of Na<sup>+</sup> from the bath, which was completely inhibited by amiloride, indicating that acidification was due to reversal of the Na<sup>+</sup>-H<sup>+</sup> exchanger. Perhaps there is a small turnover of the exchangers under control conditions in the presence of amiloride and high bath Na<sup>+</sup>. Upon Na<sup>+</sup> removal the exchanger may be expected to reverse; the influx of protons would lead to progressive stimulation at the internal regulatory site (Aronson *et al.* 1982). The efficacy of amiloride may also be different under the two experimental circumstances. Amiloride exerts its effect at the Na<sup>+</sup>-binding site of the Na<sup>+</sup>-H<sup>+</sup> exchanger, acting as a competitive inhibitor (Aronson & Igarashi, 1986), thus its effectiveness depends on the Na<sup>+</sup> concentration. Some exchanger activity may well have remained at the relatively high Na<sup>+</sup> concentrations used in this study. As we saw, amiloride completely inhibited the reversal of the Na<sup>+</sup>-H<sup>+</sup> exchanger induced by Na<sup>+</sup> removal, whereas it only partially inhibited activity with 97 mM Na<sup>+</sup> (Fig. 1B and Table 2). Also, amiloride was not as effective as Na<sup>+</sup> removal in inhibiting pH<sub>i</sub> recovery (Fig. 1B and D). We suggest that in the absence of HCO<sub>3</sub><sup>-</sup>, basolateral Na<sup>+</sup>-H<sup>+</sup> exchange is the only significant pH regulatory mechanism in the early distal tubule. Following an acidification, these exchangers return the pH<sub>i</sub> to resting levels, close to the set-point. There is a low background activity, which is revealed by reversing the direction of transport.

### Role of the exchangers

Obviously, one of the primary roles of the Na<sup>+</sup>-H<sup>+</sup> exchangers is to remove acid from the cell. Another is to regulate transepithelial transport of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> by modulating the pH<sub>i</sub> (Oberleithner *et al.* 1983a; Munich, Dietl & Oberleithner, 1986). However, closer inspection of Fig. 1 shows another very interesting feature: the degree of acidification following an NH<sub>4</sub><sup>+</sup> pulse is much smaller when the Na<sup>+</sup>-H<sup>+</sup> exchangers are active. This means that not only do the exchangers restore pH<sub>i</sub> following an acid load, they also *prevent* large changes in pH<sub>i</sub>. The activity of the exchangers increases with acidification (Fig. 4), and thus the resulting proton extrusion limits the degree of acidification. In this respect the exchangers are *effectively* acting as buffers by minimizing the change in pH<sub>i</sub> for a given acid load.

### Buffering power

The ability of a cell to minimize changes in pH<sub>i</sub> in the absence of any active pH regulatory mechanisms is given by the buffering power. This is due to simple physicochemical buffering by intracellular proteins and other buffers, and is defined as the change in pH<sub>i</sub> for a known acid load (Van Slyke, 1922). If a cell has a high buffering power, then there will be a relatively small change in pH<sub>i</sub>

for a given acid load. However, it appears that for most cell types studied so far, β<sub>i</sub> is itself a function of pH<sub>i</sub>, with β<sub>i</sub> increasing as the pH falls (Boron, 1977; Boyarsky *et al.* 1988; Bonanno & Machen, 1989). This means that the values of pK<sub>a</sub> of the fixed buffers within the cell are generally lower than pH 7.

At first sight it would seem that the cell is relatively unprotected against alkaline swings in pH<sub>i</sub>. However, it must be stressed that the current experiments have all been carried out in the absence of HCO<sub>3</sub><sup>-</sup>, which is an extremely important physiological buffer, even in the amphibian. There are two types of buffers in cells; open, like HCO<sub>3</sub><sup>-</sup>, and closed. The closed type comprises molecules that do not readily cross the cell membrane, like phosphate. This latter group are also referred to as intrinsic buffers (Boron, 1992). In the present study we have determined only the intrinsic buffering power. However, we can calculate the buffering power of the open-ended HCO<sub>3</sub><sup>-</sup>-buffer system as 2.3[HCO<sub>3</sub><sup>-</sup>] (Boron, 1992). Total buffering power (β<sub>T</sub>) is the sum of these two buffer systems. In the presence of HCO<sub>3</sub><sup>-</sup>, β<sub>T</sub> would be relatively constant in the physiological range, with a value of around 35 mM (pH unit)<sup>-1</sup>.

### Change in set-point or number of transporters?

Comparison of the relationship between proton efflux and pH<sub>i</sub> in normal and K<sup>+</sup>-loaded frogs (chronic aldosterone) and with acute aldosterone treatment reveals that aldosterone increases the set-point of the Na<sup>+</sup>-H<sup>+</sup> exchanger. It also seems that aldosterone exerts its full effect on the Na<sup>+</sup>-H<sup>+</sup> exchanger within 30 min, since there was no difference between acute and chronic aldosterone treatment. There was no detectable change in the turnover rate or the number of exchangers present in the cell, as judged by the slope of the relationship between pH<sub>i</sub> and proton efflux. From the present study, we would suggest that both the acute and chronic stimulation of Na<sup>+</sup>-H<sup>+</sup> exchange is due to activation by a change in set-point, rather than by the insertion of new or preformed transporters.

Several agents or treatments are known to alter the set-point of Na<sup>+</sup>-H<sup>+</sup> exchangers (Grinstein, Cohen, Goetz, Rothstein, Mellors & Gelfand, 1986; Grinstein & Rothstein, 1986). Phorbol esters are the most commonly quoted activators of Na<sup>+</sup>-H<sup>+</sup> exchange, although this is not a general phenomenon (Chang, Musch, Drabik-Evans & Rao, 1991). Osmotic shrinkage of cells often results in a volume regulatory increase, which in part depends upon activation of Na<sup>+</sup>-H<sup>+</sup> exchange, perhaps mediated by protein kinase C (Grinstein *et al.* 1986). Volume changes may also be responsible for the observed change in set-point following replacement of Na<sup>+</sup> by NMDG in rabbit corneal cells (Bonanno & Machen, 1989). The stimulatory effect of aldosterone on Na<sup>+</sup>-H<sup>+</sup> exchange in fused early distal tubule cells was inhibited by spironolactone; this



suggests that the binding of aldosterone to its cytosolic receptor is an initial event. This is thought to be followed by the induction of the synthesis of proteins which regulate various transport mechanisms (Minuth, Steckelings & Gross, 1987). However, the effect of aldosterone on Na<sup>+</sup>-H<sup>+</sup> exchange is very rapid in human lymphocytes, occurring within 3 min (Wehling, Kasmayr & Theisen, 1989). The initial stimulation of Na<sup>+</sup>-H<sup>+</sup> exchange is too rapid to involve protein synthesis and a high-affinity membrane-resident aldosterone receptor has been implicated. Stimulation of the membrane receptor may lead to activation of Na<sup>+</sup>-H<sup>+</sup> exchange via a second messenger system (Wehling, Eisen & Christ, 1992). The results obtained in the present study show an increased set-point of the Na<sup>+</sup>-H<sup>+</sup> exchanger following both acute and chronic aldosterone addition, and are in agreement with the hypothesis of a rapid aldosterone action involving activation of membrane-resident exchangers, perhaps via a second messenger pathway. At present, these pathways remain unknown.

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