Intracellular pH and calcium in frog early distal tubule: effects of transport inhibitors

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1. The K^+ channels of the apical membrane of the diluting segment (early distal tubule, EDT) of the frog are involved in the regulation of transepithelial NaCl transport. These channels are sensitive to pH_i and intracellular Ca^{2+} (Ca_i^{2+}). Inhibition of transport by furosemide (frusemide) results in a compensatory increase in K^+ channel activity. The aims of the present study were to determine whether pH_i or Ca_i^{2+} were altered by furosemide, and to identify the means by which such changes were brought about.

2. Experiments were performed using single, microperfused EDT segments. Measurements of pH_i and Ca_i^{2+} were made using the intracellular fluorescent probes, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and fura-2, respectively.

3. Furosemide increased pH_i and Ca_i^{2+}. The intracellular alkalinization was the result of an alkaline shift in the set-point of the basolateral Na^+–H^+ exchanger. This response was dependent upon the increase in Ca_i^{2+}.

4. The increase in Ca_i^{2+} produced by furosemide was due to the release of Ca^{2+} from intracellular stores. Depletion of these stores, by 2,5-di-t-butylhydroquinone (TBQ) and caffeine, prevented the furosemide-induced changes in Ca^{2+} and pH.

5. Furosemide-induced activation of Na^+–H^+ exchange was prevented by the calmodulin antagonist, W-7.

6. Thus furosemide elicits a rise in Ca_i^{2+} which, via calmodulin, results in activation of Na^+–H^+ exchange. The resulting intracellular alkalinization would be expected to increase channel activity.

The amphibian early distal tubule (EDT), like its mammalian counterpart the thick ascending limb, reabsorbs NaCl in preference to water, thus acting to dilute the luminal fluid (Guggino, Oberleithner & Giebisch, 1988). The energy for transport is derived from the hydrolysis of ATP by the basolateral Na^+–K^+-ATPase, which maintains a low intracellular Na^+ concentration. Entry of NaCl into the cell occurs via a furosemide-sensitive, Na^+–$2Cl^-$–K^+ cotransporter located on the apical membrane; there is an obligatory coupling between the inward movement of all four ions. The luminal delivery of K^+ to the diluting segment is not sufficient to support NaCl reabsorption (Guggino *et al.* 1988), a problem that is overcome by the recycling of K^+ through apical K^+ channels. So NaCl reabsorption depends upon the apical recycling of K^+ and thus the apical K^+ conductance is an important regulator of NaCl reabsorption.

The apical recycling of K^+ is also important in pump–leak coupling (Schultz, 1985). K^+ enters the cell across the apical membrane, on the cotransporter, and across the basolateral membrane, via the Na^+–K^+-ATPase. Just as K^+ recycles across the apical membrane via the combined action of the apical K^+ channels and cotransporter, K^+ also recycles across the basolateral membrane via either K^+ channel or KCl cotransporter-mediated K^+ efflux (Guggino, 1986) and the Na^+–K^+ pump. Changes in K^+ entry, due for example to changes in Na^+ load, must be matched by parallel changes in K^+ efflux.

Previous experiments have shown that the apical K^+ channels are sensitive to changes in both pH_i and intracellular Ca^{2+} (Ca_i^{2+}), with an increase in pH_i, or a decrease in Ca_i^{2+}, increasing channel activity (Hurst & Hunter, 1990, 1992). Strophanthidin, which inhibits K^+ entry on the Na^+–K^+ pump, had the predicted effect of reducing the activity of the apical K^+ channels (Hurst & Hunter, 1992). In contrast, and in direct contradiction of the pump–leak hypothesis, inhibition of apical K^+ entry on

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the apical cotransporter, by furosemide, upregulated the apical K^+ channels (Hurst & Hunter, 1992). This stimulation was sensitive to the Na^+–H^+ exchanger inhibitor amiloride, and we postulated that this change in channel activity may be due to an increase in pH_i, mediated by the Na^+–H^+ exchanger which, in this preparation, is located predominantly in the basolateral membrane (Cooper & Hunter, 1994). However, pH_i and Ca_i^{2+} changes often occur together (Vaughan-Jones, Lederer & Eisner, 1983) and both of these potential second messengers have been implicated in channel regulation. Thus we have measured both pH_i and Ca_i^{2+} in an attempt to discern the mechanism(s) underlying the alteration in channel activity produced by furosemide.

METHODS

Tissue preparation

Frogs (*Rana temporaria*) of either sex were maintained in tap water. Animals were killed by decapitation and destruction of the brain and spinal cord. The kidneys were removed and cut into 1–2 mm slices which were stored on ice in Leibovitz L-15 medium (prepared to osmolality 204 mosmol (kg H_2O)$^{-1}$, and buffered with 10 mM Hepes titrated to pH 7·40 with NaOH). Single EDT segments were dissected in control (frog) Ringer solution (solution I, Table 1) as described previously (Cooper & Hunter, 1994) and microperfused using standard techniques (Burg, Grantham, Abramow & Orloff, 1966).

Solutions

The composition of solutions is given in Table 1. Furosemide, bumetanide, amiloride, barium chloride, caffeine, 3-isobutyl-1-methylxanthine (IBMX) and di-butyryl cAMP (db-cAMP) were dissolved directly into control Ringer solution (solution I, Table 1). The other agents were prepared from the following stock solutions: ionomycin, 5 mM in DMSO; strophanthidin, 10 mM in ethanol; ethylisopropyl amiloride (EIPA), 10 mM in DMSO; phorbol 12-myristate 13-acetate (PMA), 1·5 mg ml^{-1} in DMSO; 4-bromo-A23187, 1 mM in ethanol; *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), 50 mM in methanol; and 2,5-di-t-butylhydroquinone (TBQ), 100 mM in methanol.

Fluorescence microscopy

The fluorescence set-up and its use have been described in detail previously (Cooper & Hunter, 1994). Briefly, both 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and fura-2 were used in a dual-excitation, single-emission configuration. Dyes were excited by light from a 100 W xenon source with 0·1 and 1% neutral density transmission filters for BCECF and fura-2, respectively. BCECF was excited alternately by light at 440 and 490 nm for 400 ms, with fura-2 excited at 350 and 380 nm for 600 ms. For both indicators, the light emitted at 520 nm was collected by a single photomultiplier tube. The signal reaching the photomultiplier tube was processed by a photon counter (Newcastle Photometric Systems, Newcastle Upon Tyne, UK) and digitized, prior to storage on computer.

pH_i measurements

Tubules were mounted on microperfusion pipettes and the background autofluorescence measured; the autofluorescence was subtracted from all further signals. Tubules were incubated in 5 μM BCECF-AM (acetoxymethyl ester form; prepared from a stock of 2·5 μg ml^{-1} in DMSO) for about 10 min. Fluorescence signals were calibrated in each tubule at the end of the experiment using the nigericin/high-K^+ method (Thomas, Buchsbaum, Zimniak & Racker, 1979) over a pH range of 6–8 (solution II, Table 1), and then analysed as described previously (Cooper & Hunter, 1994).

Variations in resting pH_i

A possible criticism of the results presented here is the large variation between the values of resting pH. This may be due to seasonal differences in the circulating levels of aldosterone (Lange & Hanke, 1988), which occur in spite of keeping animals under constant environmental conditions. The resting pH in these cells is modulated by aldosterone (Cooper & Hunter, 1994, 1996). As the experiments reported in this manuscript have been performed over a period of more than 1 year, variations in circulating aldosterone levels will complicate matters. To overcome these problems, each set of experiments was performed and compared with its own control.

Ca_i^{2+} measurements

In the majority of experiments, where the Ca^{2+} ratio was not calibrated, the tubules were first incubated in 5 μM fura-2-AM (acetoxymethyl ester form; prepared from a stock of 2·5 μg ml^{-1} in DMSO, with Pluronic F-127 used as directed by Molecular Probes) for about 1 h before being mounted on the perfusion pipettes. No compensation for background autofluorescence was made, but background fluorescence in unloaded tubules was unaltered by the addition of ionomycin with 2 mM bath Ca^{2+}. In those experiments where the Ca^{2+} ratio was calibrated, the tubules were mounted on the microperfusion pipettes, the background autofluorescence measured and subtracted from all subsequent measurements. Tubules were then loaded with dye for about 1 h. Ca_i^{2+} was measured following the procedure of Grynkiewicz, Poenie & Tsien (1985) using a K_d for Ca^{2+} of 200 nM (Williams, Fogerty, Tsien & Fay, 1988; Frampton, Orchard & Boyett, 1991). Calibration was performed in collapsed tubules by first stopping luminal flow and then exposing the tubule to control solution containing 2 μM ionomycin (prepared from a 5 mM stock in ethanol) at Ca^{2+} concentrations of 2 mM (solution I, Table 1) and 0 Ca^{2+} (solution V, Table 1).

BCECF-AM and fura-2-AM were purchased from Molecular Probes, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) was a gift from Dr Benfield of Merck, Sharp & Dohme (Hoddesdon, Herts, UK). All other chemicals were purchased from Sigma.

All data are presented as means ± S.E.M. with the number of observations (n) in parentheses. Statistical analyses were performed with Student's t tests or one-way ANOVA as appropriate, with significance being assumed at the 5% level.

RESULTS

Effect of furosemide on pH_i and Ca_i^{2+}

Furosemide (50 μM), an inhibitor of Na^+–$2Cl^-$–K^+ cotransport elicited an intracellular alkalinization with pH_i increasing from 7·16 ± 0·05 to 7·30 ± 0·06 ($n = 12$, $P < 0·05$; Fig. 1*A*). The effect of furosemide was reproducible; a second addition of furosemide produced an alkalinization of the same magnitude as the first (ΔpH_i: first addition, 0·12 ± 0·02; second addition, 0·11 ± 0·02; $n = 4$, $P = 0·64$). Bumetanide (1 μM), another inhibitor of Na^+–$2Cl^-$–K^+ cotransport (Hedge & Palfrey, 1992), also elicited an intracellular alkalinization, the magnitude of which was not different from that of furosemide (ΔpH_i: furosemide, 0·14 ± 0·01, $n = 14$; bumetanide, 0·14 ± 0·04, $n = 5$; $P = 0·95$). Furosemide also caused an increase in the

Table 1. Composition of Ringer solutions

Solution	NaCl	KCl	$MgCl_2$	$CaCl_2$	Hepes	NH_4Cl	EGTA
I (control)	100	3	1	2	10*	—	—
II (100 mM K^+)	—	100	1	2	10**	—	—
III (low K^+)	99·9	0·1	1	2	10*	—	—
IV (NH_4Cl)	87	3	1	2	10*	10	—
V (0 Ca^{2+})	97	3	1	—	10*	—	5
VI (high Ca^{2+})	97	3	1	20	10*	—	—
VII (no added Ca^{2+})	97	3	1	—	10*	—	—
VIII (high Ca^{2+} + NH_4)	87	3	1	20	10*	10	—

All values are mM. * Buffered to pH 7·40 with NaOH. ** Buffered to the required pH with HCl/KOH.

350/380 nm ratio from 0·63 ± 0·02 to 0·71 ± 0·02 ($n = 6$, $P < 0·05$), corresponding to an increase in Ca_i^{2+} from 132 ± 24 to 324 ± 35 nM ($n = 6$, $P < 0·05$; Fig. 1*B*).

Role of Na^+–H^+ exchange in furosemide-induced alkalinization

Addition of the Na^+–H^+ exchanger inhibitor amiloride, as in an earlier study (Cooper & Hunter, 1994), had no effect on pH_i (control, 6·93 ± 0·05; amiloride, 6·93 ± 0·06; $n = 5$, $P = 0·72$). Addition of furosemide in the presence of amiloride (Fig. 1*C*) gave an acidification, with pH_i falling from 6·92 ± 0·06 to 6·85 ± 0·07 ($n = 5$, $P < 0·05$).

Effect of membrane potential on pH_i

Furosemide elicits a hyperpolarization (Oberleithner, 1985), raising the possibility that the acidification observed in the presence of furosemide and amiloride may be the result of a cell hyperpolarization. The cells were hyperpolarized by

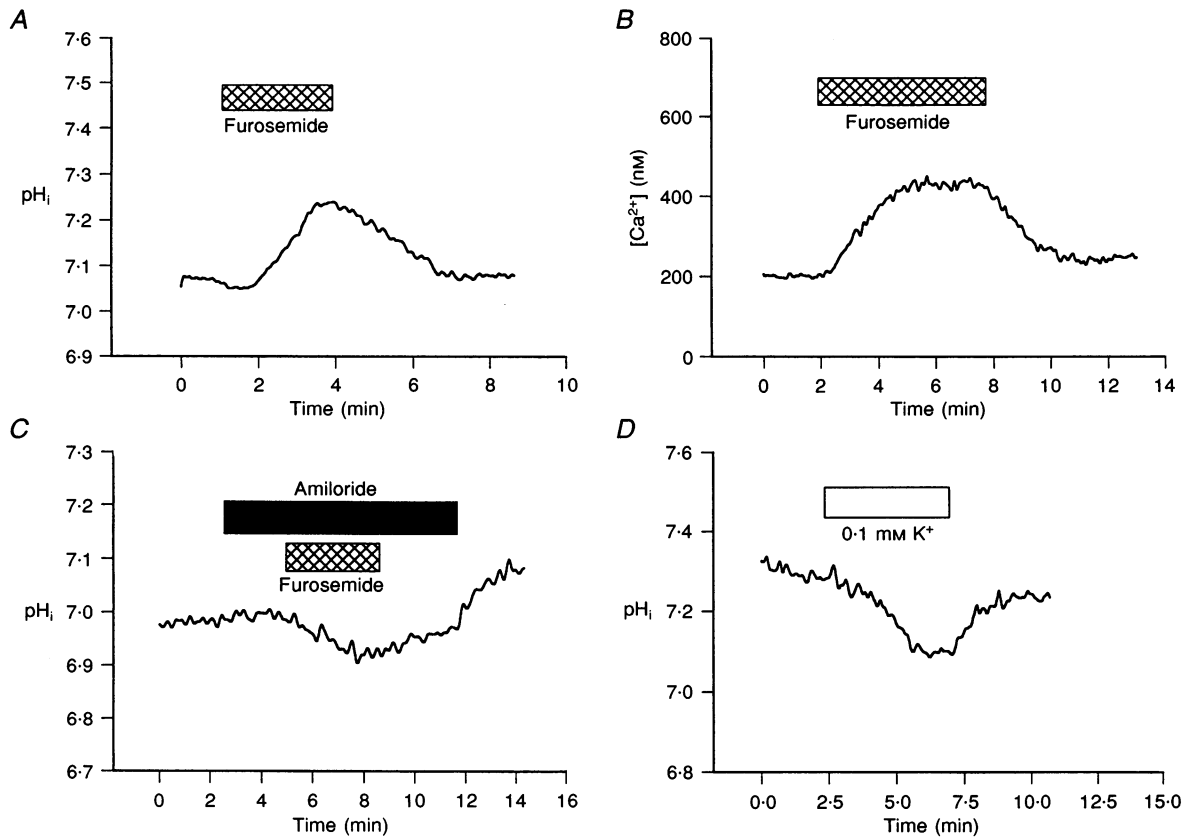

Figure 1. pH_i and Ca_i^{2+} changes

A, effect of furosemide on pH_i. Furosemide (50 μM) was added to the luminal solution for the period indicated by ▩. *B*, effect of furosemide (▩) on Ca_i^{2+}. *C*, effect of furosemide on pH_i in the presence of amiloride. Amiloride (1 mM) was added to the bath solution for the period indicated by ■, followed by the addition of furosemide to the luminal solution (▩). *D*, effect of reducing bath $[K^+]$ to 0·1 mM (□) on pH_i.

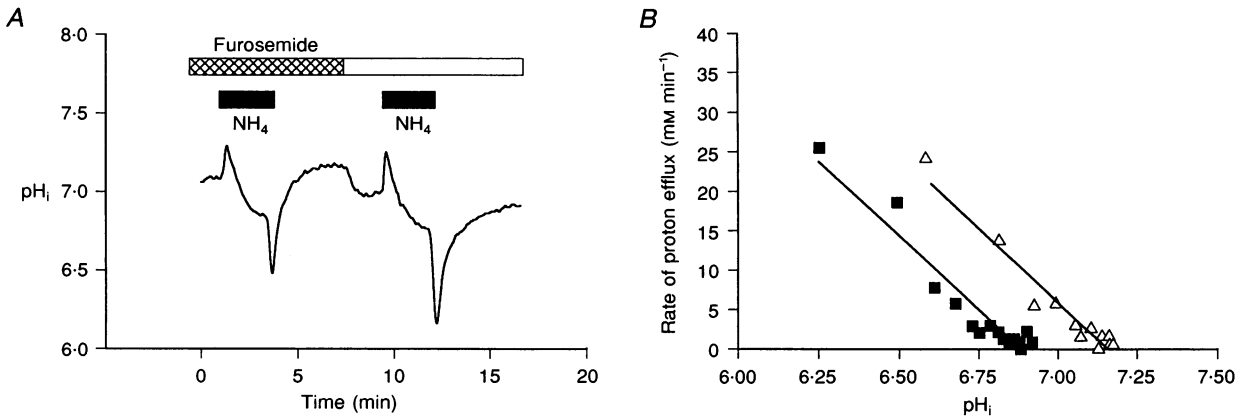

Figure 2. Recovery from acid load

A, the tubule was exposed to two ammonium pulses (10 mM NH_4Cl added to the basolateral solution, ■) in the presence (▩) and absence (□) of luminal furosemide. *B*, rate of proton efflux *versus* pH_i for the recovery phases from acid loading for the experiment shown in *A* (■, control; △, furosemide). Every tenth data point is plotted; regression lines were fitted through all the data points.

lowering bath K^+ concentration (Hurst & Hunter, 1992) (solution III, Table 1). The tubules used in these experiments were not mounted on microperfusion pipettes, but allowed to rest on the bottom of the perfusion chamber. The ends of the tubule were open, allowing the perfusion solution to reach both membranes; we have shown previously that strophanthidin is effective in everted tubules under the above conditions (Hurst & Hunter, 1992). Introduction of low-K^+ Ringer solution (solution III, Table 1) to the bath solution (Fig. 1*D*) gave a reversible acidification, with pH_i falling from 7·24 ± 0·08 to 7·07 ± 0·06 ($n = 4$, $P < 0·05$).

Effect of furosemide on Na^+–H^+ exchange

The rate of proton efflux and set-point of the Na^+–H^+ exchangers were determined as described previously (Roos & Boron, 1981; Cooper & Hunter, 1994). Tubules were exposed to two consecutive acid loads, using the ammonium prepulse method (solution IV, Table 1), in the presence and absence of luminal furosemide, as illustrated in Fig. 2*A*. NH_4Cl removal produced a rapid acidification, followed by recovery of pH_i towards the resting value, as the basolateral Na^+–H^+ exchanger acted to extrude protons (Cooper & Hunter, 1994). In the presence of furosemide the pH_i recovery was mediated solely by Na^+–H^+ exchange; the initial rate of recovery was inhibited by 95 ± 3% ($n = 4$) by 10 μM EIPA as in the absence of furosemide (Cooper & Hunter, 1994). The proton efflux (the rate of pH recovery multiplied by the buffering power, β) was calculated between each consecutive data point during the recovery phase (Cooper & Hunter, 1994). If we plot proton efflux against pH_i we obtain a

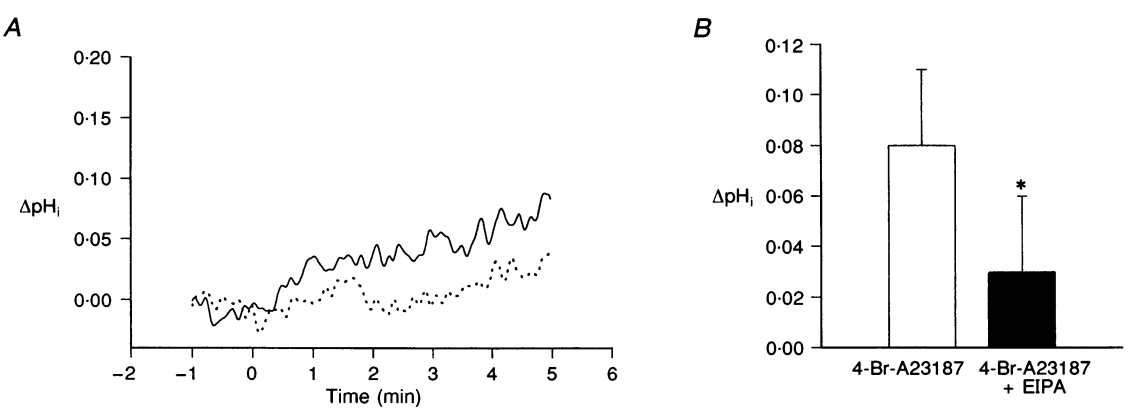

Figure 3. Effect of a Ca^{2+} ionophore

A, effect of 4-Br-A23187 on pH_i in the absence (continuous line) and presence (dotted line) of the Na^+–H^+ exchanger inhibitor EIPA (10 μM). *B*, the mean (± S.E.M.) change in pH_i after 5 min addition of 4-Br-A23187 in the absence (□) and presence (■) of EIPA. * Significant reduction in the magnitude of pH change.

Table 2. Rate of proton efflux and set-point pH during the recovery from an acute acid load under the various treatments

	Rate of proton efflux (mM min^{-1})	Setpoint pH
Control	−31 ± 3·46 (8)	7·28 ± 0·08 (8)
Furosemide	−26·4 ± 3·04 (8)	7·43 ± 0·06 (8)*
Control	−31·1 ± 2·75 (8)	7·12 ± 0·03 (8)
Strophanthidin	−18·2 ± 3·18 (4)*	7·07 ± 0·03 (4)
Strophanthidin + furosemide	−17·3 ± 3·16 (4)*	7·12 ± 0·07 (4)
Control	−45 ± 6·38 (6)	7·06 ± 0·11 (6)
High Ca^{2+}	−30·29 ± 7·5 (6)*	7·23 ± 0·14 (6)*

The number of experiments is given in parentheses. * Significantly different from experimental group control, with different groups separated by lines.

relationship which can be fitted by linear regression (Fig. 2*B*; Cooper & Hunter, 1994). The slope of the regression line is an index of the Na^+–H^+ exchanger turnover rate, whilst the intercept of the regression line with the pH axis gives the set-point. Figure 2*B* shows the regression lines for the experiment shown in Fig. 2*A* and the data for eight such experiments are summarized in Table 2.

Along with other effects, furosemide might be expected to give an increase in intracellular ATP, a reduction in cell volume and a decrease in intracellular Na^+ (Na_i^+). Intracellular ATP and cell volume have been implicated in the regulation of Na^+–H^+ exchange (Cassel, Katz & Rotman, 1986; Grinstein, Cohen, Goetz, Rothstein, Mellors & Gelfand, 1986). In the following experiments we have tried to mimic such changes in the above parameters by means other than furosemide. Additionally, we have investigated which of the second messenger systems known to affect Na^+–H^+ exchange activity, protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}, might be mediating the effects of furosemide (Grinstein *et al.* 1986; Pouyssegur, 1994).

ATP

To elicit a rise in ATP we inhibited the Na^+–K^+ pump directly with strophanthidin (Hurst & Hunter, 1992). Strophanthidin gave an intracellular alkalinization, with pH_i rising from 6·86 ± 0·04 to 7·00 ± 0·06 ($n = 11$, $P < 0·05$). However this alkalinization was not due to activation of the Na^+–H^+ exchanger, as it remained during simultaneous addition of EIPA (ΔpH: strophanthidin, 0·06 ± 0·02; strophanthidin + EIPA, 0·08 ± 0·02; $n = 5$, $P = 0·40$).

Cell volume

To effect a fall in volume we exposed the cells to a hypertonic shock (solution I with 100 mM mannitol). In contrast to furosemide, hypertonic shock elicited an intracellular acidification, with pH_i decreasing from 6·94 ± 0·13 to 6·88 ± 0·13 ($n = 5$, $P < 0·05$).

Intracellular Na^+

If the Na^+–K^+ pump is inhibited, the fall in Na_i^+ produced by furosemide should be prevented. When strophanthidin was added to the bath solution we saw an intracellular alkalinization, as described above, of similar magnitude to that induced by furosemide. Addition of luminal furosemide in the continued presence of strophanthidin, gave no further change in pH_i (strophanthidin, 7·13 ± 0·06; strophanthidin + furosemide, 7·14 ± 0·07; $n = 6$, $P = 0·61$). As strophanthidin produces an alkalinization of similar magnitude to furosemide, the pH_i change associated with the activation of Na^+–H^+ exchange by furosemide may be masked. However, the rate of proton efflux and set-point of the exchangers, determined as described earlier, were not significantly different from control (Table 2).

Protein kinase A and C

Stimulation of PKA with 100 μM IBMX and 100 μM db-cAMP had no effect on pH_i (control, 7·07 ± 0·06; IBMX + db-cAMP, 7·08 ± 0·06; $n = 6$, $P = 0·42$). Similarly, addition of the PKC activator PMA (100 nM) to the bath solution had no effect on pH_i over a 10 min period (control, 7·14 ± 0·10; PMA, 7·16 ± 0·09; $n = 6$, $P = 0·07$).

Ca^{2+}

Elevation of Ca_i^{2+} using the non-fluorescent Ca^{2+} ionophore 4-bromo-A23187 (1 μM), increased pH_i from 7·04 ± 0·09 to 7·12 ± 0·08 ($n = 8$, $P < 0·05$) over a 5 min period (Fig. 3*A* and *B*). In the presence of the Na^+–H^+ exchanger inhibitor EIPA (10 μM, Fig. 3*A* and *B*), 4-bromo-A23187 had no effect upon pH_i (control, 7·02 ± 0·04; 4-bromo-A23187 + EIPA, 7·06 ± 0·04; $n = 8$, $P = 0·31$).

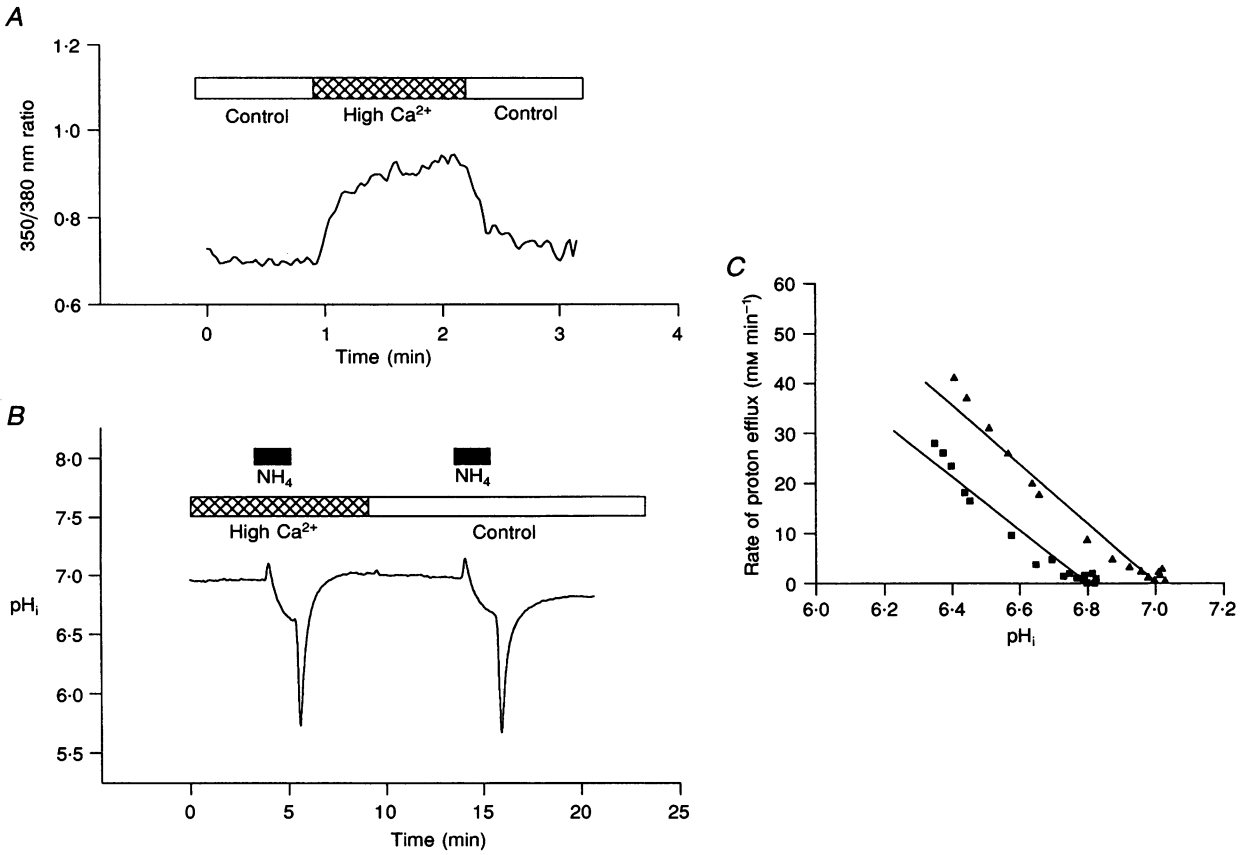

Figure 4. Effect of raising bath Ca^{2+} concentration

A, effect of high bath Ca^{2+} on Ca_i^{2+}. Bath Ca^{2+} was increased to 20 mM for the period indicated by ▩. *B*, recovery from an acid load. Tubules were exposed to ammonium pulses (10 mM NH_4Cl added to the basolateral solution for the period indicated by ■) in 20 mM (▩) and 2 mM bath Ca^{2+} (□). *C*, plot of rate of proton efflux against pH_i for the recovery phases from acid loads for the experiments shown in *B* (control, ■; high Ca^{2+}, ▲). For clarity not all data points are plotted, but the regression lines were calculated for all the data points.

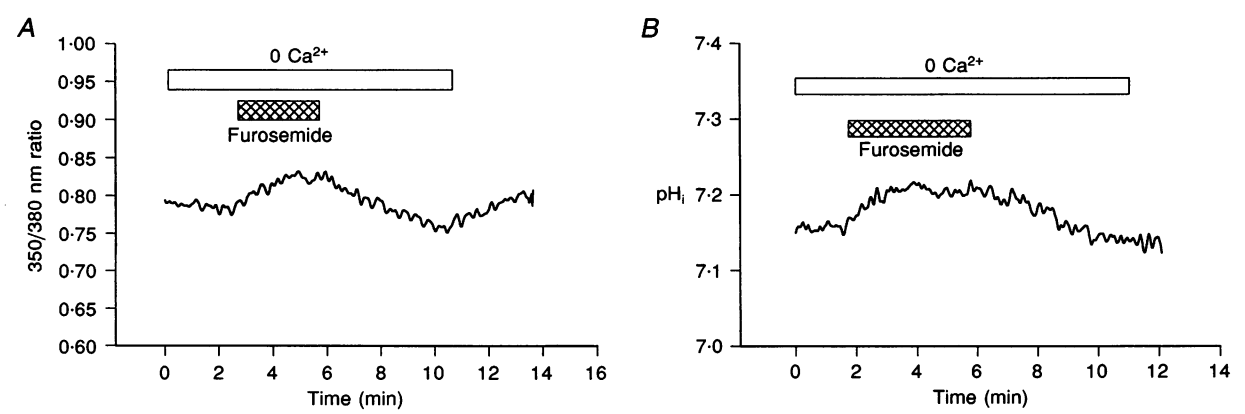

Figure 5. Effect of lowering bath Ca^{2+} concentration

A, effect of furosemide on Ca_i^{2+} in the absence of extracellular Ca^{2+}. Ca^{2+} was removed from the extracellular solutions (solution VII, Table 1) for the period indicated by □ and furosemide (50 μM) was added to the luminal perfusate as indicated (▩). *B*, effect of furosemide on pH_i in the absence of extracellular Ca^{2+}. The experiment was performed as described in *A*.

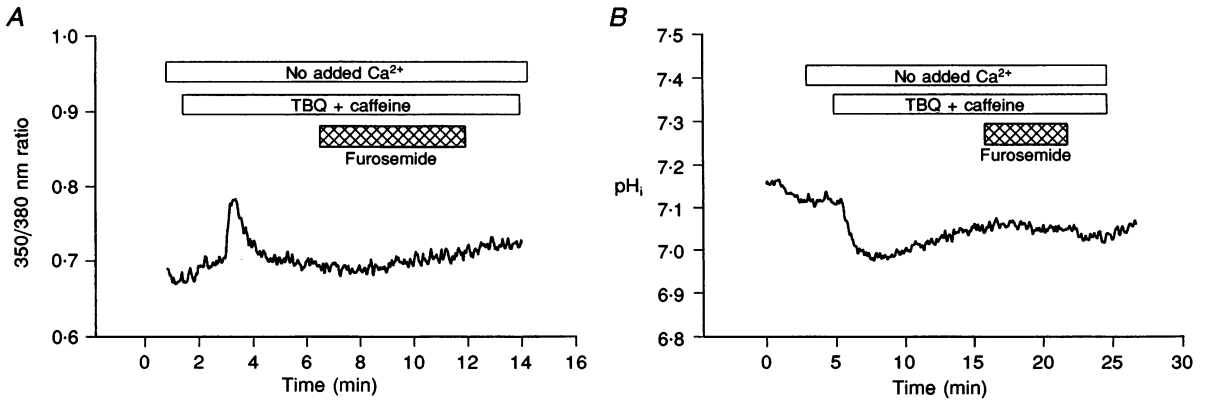

Figure 6. Role of Ca^{2+} stores

A, effect of furosemide on Ca_i^{2+} following depletion of Ca_i^{2+} stores. Ca^{2+} was removed from the extracellular solutions and TBQ (20 μM) and caffeine (10 mM) were added to the basolateral solution as indicated. Furosemide (50 μM) was added to the luminal perfusate for the period indicated by ▩. *B*, effect of furosemide on pH_i following depletion of Ca^{2+} stores. Experiments were performed as described in *A*.

Ca_i^{2+} was also elevated by increasing the bath Ca^{2+} concentration to 20 mM (solution VI, Table 1). There was an increase in the 350/380 nm ratio (Fig. 4*A*) from 0·68 ± 0·03 to 0·92 ± 0·06 ($n = 5$, $P < 0·05$). The rate of proton efflux during the recovery from an acid load was measured in 2 mM and 20 mM Ca^{2+} (Fig. 4*B* and *C*) and the data are summarized in Table 2. The set-point of the Na^+–H^+ exchangers was elevated, and the turnover rate decreased, by raising Ca^{2+} to 20 mM.

Release of Ca^{2+} stores by furosemide

In the absence of extracellular Ca^{2+} (solution VII, Table 1), furosemide still increased intracellular Ca^{2+} (Fig. 5*A*), a change not significantly different to that seen with furosemide alone (Δ350/380 nm ratio: furosemide, 0·08 ± 0·01, $n = 6$; no added Ca^{2+} + furosemide, 0·06 ± 0·004, $n = 9$; $P = 0·09$). When pH_i was measured under the same conditions there was still an alkalinization (Fig. 5*B*, ΔpH_i: furosemide, 0·14 ± 0·01, $n = 14$; no added Ca^{2+} + furosemide, 0·12 ± 0·03, $n = 6$; d.f. = 3,28, $F = 19·232$, $t = 0·894$). The initial removal of extracellular Ca^{2+} had no effect on pH_i (control, 7·12 ± 0·06; no added Ca^{2+}, 7·12 ± 0·05; $n = 12$, $P = 1·0$).

Ca^{2+} stores were depleted using caffeine and TBQ. In the absence of Ca^{2+} (solution VII, Table 1), 10 mM caffeine and 20 μM TBQ were added to the basolateral solution. There was a transient increase in Ca^{2+} (peak ratio: 0·80 ± 0·07, $n = 5$; $P < 0·05$ when compared with no added Ca^{2+}; Fig. 6*A*) followed by a return to its initial level (350/380 nm ratio: no added Ca^{2+}, 0·71 ± 0·05; TBQ and caffeine, 0·71 ± 0·05; $n = 5$, $P = 0·62$). Subsequent addition of furosemide (Fig. 6*A*) had no effect upon the Ca^{2+} ratio (350/380 nm ratio: TBQ + Caffeine, 0·71 ± 0·05; TBQ + caffeine + furosemide, 0·70 ± 0·04; $n = 5$, $P = 0·28$) or pH_i (Fig. 6*B*: TBQ + caffeine, 7·11 ± 0·05; TBQ + caffeine + furosemide, 7·13 ± 0·06; $n = 6$, $P = 0·475$).

Effect of furosemide on pH_i: role of calmodulin

Addition of the Ca^{2+}–calmodulin (Ca–CaM) antagonist W-7 (10 μM) to the basolateral solution produced an alkalinization (pH_i: control, 7·07 ± 0·07; W-7, 7·12 ± 0·06; $n = 6$,

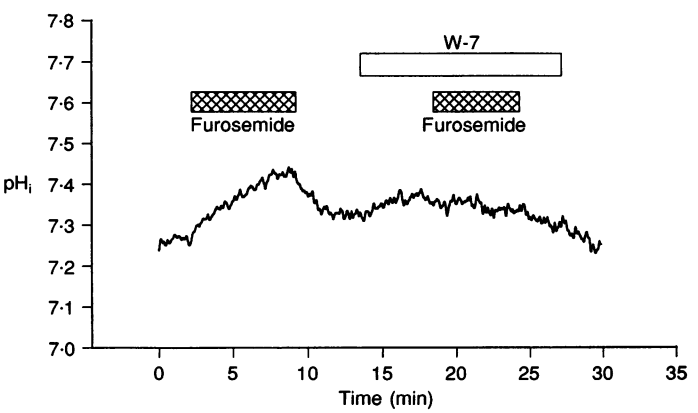

Figure 7. Effect of furosemide on pH_i in the presence of W-7

The Ca^{2+}–calmodulin antagonist W-7 (10 μM) was added to the basolateral perfusate for the period indicated by □. Furosemide (50 μM) was added to the luminal perfusate as indicated.

$P < 0{\cdot}05$), with addition of furosemide (Fig. 7) having no further effect upon pH_i (W-7, $7{\cdot}12 \pm 0{\cdot}06$; W-7 + furosemide, $7{\cdot}12 \pm 0{\cdot}06$; $n = 6$, $P = 0{\cdot}76$). The alkalinization produced by W-7 addition was significantly smaller than that produced by furosemide (ΔpH_i: W-7, $0{\cdot}05 \pm 0{\cdot}02$, $n = 6$; furosemide, $0{\cdot}14 \pm 0{\cdot}01$, $n = 14$; $P < 0{\cdot}05$).

DISCUSSION

Furosemide produced increases in both pH_i and Ca_i^{2+}. Given the sensitivity of the apical potassium channels to pH_i and Ca_i^{2+} (see Introduction) it would appear that pH_i rather than Ca_i^{2+} is responsible for the observed increase in channel activity elicited by furosemide. However, it is also apparent that whilst the change in Ca_i^{2+} is not directly responsible for the upregulation of channel activity, it is necessary to give exchanger activation. As predicted by our initial hypothesis, the furosemide-induced alkalinization was sensitive to amiloride; this result is in keeping with the patch clamp data, where furosemide increased channel activity in an amiloride-sensitive manner. Thus addition of furosemide activates the Na^+–H^+ exchanger, resulting in intracellular alkalinization.

Effects of amiloride

When tubules were exposed to furosemide in the presence of amiloride the pH change was not only prevented, but reversed, resulting in a significant acidification. This reversal cannot be explained as an effect of amiloride *per se*, since amiloride itself has no effect on pH_i (Cooper & Hunter, 1994). Another candidate mechanism could be a change in background acid loading, due either to alteration of metabolic state or proton flux. Changes in pH_i due to alterations in metabolic state seem unlikely since, if anything, the reduced Na^+ load to the cell via the Na^+–H^+ exchangers would result in a reduction in cell metabolism, with a consequent reduction in cellular metabolic acid production. Perhaps a proton conductance is involved? Furosemide elicits a hyperpolarization, (Oberleithner, 1985; Hurst & Hunter, 1992). In our experiments, with an extracellular pH of 7·40, an intracellular pH of about 7, and a membrane potential of −60 mV (Hurst & Hunter, 1992), the electrochemical gradient favours inward proton movement. Although there is no other published evidence for such a conductance in these cells, lowering extracellular K^+, which hyperpolarizes the cells, also yields an acidification. Another possibility is that the changes in membrane potential may affect pH_i by modulating HCO^-_3 fluxes through the basolateral sodium bicarbonate transporter (Wang, Dietl, Silbernagel & Oberleithner, 1987). However, all experiments were carried out using Hepes-buffered solutions, and so the contribution of any bicarbonate transport mechanisms should be minimized. In addition, in HCO^-_3-buffered solutions (6·2 mM HCO^-_3, 1% CO_2, pH 7·4) furosemide produced a change in pH of the same magnitude as that seen in Hepes-based solutions, and so it would appear that the Na^+–H^+ exchanger is still the principal mechanism involved in the change of pH.

So furosemide has the potential to increase or decrease pH_i. Under normal conditions, when the Na^+–H^+ exchangers are active, there is an intracellular alkalinization. However, in the presence of amiloride an acidifying capacity is unmasked.

Effect of furosemide on Na^+–H^+ exchange

The above results beg the question of how furosemide might stimulate Na^+–H^+ exchange. Our initial hypothesis for this action of furosemide revolved around the change in Na_i^+. Furosemide causes Na_i^+ to fall from 12 to 4 mM (Oberleithner, Lang, Wang & Giebisch, 1982), thus increasing the driving force for Na^+ entry on the Na^+–H^+ exchanger, which may lead to increased proton efflux and an intracellular alkalinization. This change in Na^+ gradient could, in theory, drive a 0·5 unit change in pH_i, with the pH_i change we observed falling within this range. However, the exchangers appear not to be active at the resting pH_i (Cooper & Hunter, 1994). So, enhancing the gradients favouring Na^+–H^+ exchange would be expected to have no effect on pH_i. Thus the alkalinizing effect of furosemide must be due to activation of the exchangers. Na^+–H^+ exchangers show a set-point, i.e. once the pH_i is above a certain level, activity becomes negligible (Aronson, Nee & Suhm, 1982). An alkaline shift in the set-point would elicit an alkaline shift in pH_i (Cooper & Hunter, 1994). In a plot of proton efflux against pH_i (Fig. 2*B*) a change in the turnover rate of the exchanger, which we may expect if the Na^+gradient were increased, would manifest as a steepening of the flux–pH_i relationship. However, we do not see such a change in the slope (Table 2) but, rather, a shift in the set-point by 0·15 pH unit. This shift in set-point effectively means that the exchangers are switched on at the resting pH_i, start to alkalinize the cells, and shift pH_i towards the new set-point value. Such selective changes in the set-point of the Na^+–H^+ exchanger have been observed previously in this tissue in response to aldosterone and in hepatocytes in response to phorbol esters (Stewart, 1988; Cooper & Hunter, 1994).

As in Fig. 1*A*, activation and deactivation of the Na^+–H^+ exchanger upon furosemide addition and removal are rapid, mediated presumably by an intracellular agent or second messenger. As mentioned in the results, furosemide might be expected to alter several cell properties, including cell volume, as well as the intracellular concentrations of ATP, Na^+ and Ca^{2+}. We tried to mimic as many of these changes as possible in order to determine if any of these factors could cause activation of the exchanger, and thus possibly mediate the stimulatory action of furosemide. As well as manipulating these variables we have also considered the roles of PKA, PKC and calmodulin. Upregulation of these pathways has been implicated in the stimulation of Na^+–H^+ exchange (Grinstein *et al.* 1986; Pouyssegur, 1994; Wakabayashi, Bertrand, Ikeda, Pouyssegur & Shigekawa, 1994).

Intracellular ATP

Oberleithner (1982) showed that furosemide caused a rapid fall in Na_i^+ from 12 to 4 mM. Intracellular ATP levels have been shown to play a role in regulating Na^+–H^+ exchange (Goss *et al.* 1994). Furosemide inhibits the entry of Na^+ to the cells but the Na^+–K^+ pump continues to expel Na^+, thus the Na_i^+ concentration falls. Intracellular sodium is the prime determinant of Na^+–K^+ pump turnover rate (Lewis & Wills, 1983), and so the fall in Na_i^+ will reduce pump turnover. In the thick ascending limb, this reduction in pump turnover reduces the energy demand of the cell and so intracellular ATP rises (Jung & Endou, 1990). In the present study a rise in ATP was mimicked by inhibiting the pump directly with strophanthidin, which also resulted in an alkalinization, but which was not sensitive to amiloride. Thus direct pump inhibition, which should lead to an increase in ATP, does not activate the exchangers.

Cell volume

Several studies have demonstrated activation of Na^+–H^+ exchange in response to cell volume changes (Grinstein *et al.* 1986). The Na^+–$2Cl^-$–K^+ cotransporter is the major solute influx pathway in these cells. In the absence of compensatory volume regulatory mechanisms, cotransporter inhibition would be expected to cause cell shrinkage. In contrast to furosemide, hypertonic shock yielded an acidification. Thus any reduction in cell volume in response to furosemide would be unlikely to lead to activation of the Na^+–H^+ exchangers.

Protein kinase A and C

Both PKA and PKC have been implicated in the regulation of Na^+–H^+ exchange (reviewed by Pouyssegur, 1994). Stimulation of either of these second messenger pathways was without effect upon pH_i, suggesting that PKA and PKC are not involved in the regulation of Na^+–H^+ exchange in the amphibian diluting segment.

Intracellular Ca^{2+}

Since Ca^{2+} inhibits channel activity, the elevation of Ca_i^{2+} cannot be directly responsible for channel stimulation. However, the rise in Ca_i^{2+} was mandatory for Na^+–H^+ exchanger activation. Thus when intracellular Ca^{2+} stores were depleted, and the rise in intracellular Ca^{2+} prevented, furosemide failed to elicit an alkalinization. Direct elevation of intracellular Ca^{2+}, by either a Ca^{2+} ionophore or by raising bath Ca^{2+} concentration, gave a significant increase in pH_i. The alkalinization produced by the Ca^{2+} ionophore 4-bromo-A23187 was inhibited by EIPA, implicating the Na^+–H^+ exchanger in the pH change. Similarly, with high bath Ca^{2+} concentrations, which gave a concomitant increase in Ca_i^{2+}, there was a significant increase in set-point, yielding a cellular alkalinization. Thus raising Ca_i^{2+} leads to an alkaline shift in the set-point of the Na^+–H^+ exchanger.

The increase in Ca_i^{2+} associated with furosemide treatment persisted even after removal of extracellular Ca^{2+}, as did the pH_i changes. So, the furosemide-induced rise in Ca^{2+} is not the result of a transmembrane Ca^{2+} flux, but instead involves mobilization of Ca^{2+} from internal stores. To deplete Ca_i^{2+} stores we used caffeine, which opens the ryanodine-gated Ca^{2+} channel, and TBQ, which inhibits organellar Ca^{2+} pumps (Frampton *et al.* 1991; Inesi & Sagara, 1994). Although we do not know the nature of the Ca^{2+} stores in these cells, this combination of inhibitors, in the absence of extracellular Ca^{2+}, produced a transient increase in Ca_i^{2+}, presumably as Ca^{2+} was released from the stores and then removed from the cells. Subsequent addition of furosemide had no further influence on Ca_i^{2+} or pH_i.

Role of calmodulin

Ca^{2+} can upregulate many different second messenger pathways, including PKC and calmodulin. As stated earlier, the lack of effect of PMA suggests that PKC is not involved. By itself W-7 caused an alkalinization, but this was less than that elicited by furosemide. Such an effect has been observed in other tissues (Shen, 1989), although the mechanism of action remains unresolved. The NHE-1 isoform of the Na^+–H^+ exchanger contains a consensus sequence for calmodulin binding. Calmodulin upregulates the exchanger by a mechanism which does not involve Ca–CaM kinases but, rather, a direct interaction of the Ca–CaM complex with the exchanger (Bertrand, Wakabayashi, Ikeda, Pouyssegur & Shigekawa, 1994; Wakabayashi *et al.* 1994). In the present study the calmodulin antagonist W-7 abolished the furosemide-induced alkalinization. Thus activation may be brought about via the Ca–CaM complex.

Role of intracellular Na^+

As mentioned above, furosemide addition rapidly lowers cell Na^+ (Oberleithner *et al.* 1982) due to the continued action of the Na^+–K^+ pump which, in the absence of HCO^-_3, is the only Na^+ exit pathway (Guggino *et al.* 1988). If the pump is inhibited then the fall in Na^+ should be minimized. Addition of furosemide in the presence of strophanthidin produced no change in pH_i. However, strophanthidin by itself had already alkalinized the cells to an extent comparable to that of furosemide. Thus any stimulation of the Na^+–H^+ exchangers, and subsequent change in pH_i, by furosemide could have been masked by the action of strophanthidin on pH_i. However, the set-point of the Na^+–H^+ exchangers with strophanthidin alone or strophanthidin and furosemide together was not different from control (Table 2). This indicates: (a) that by preventing the fall in Na_i^+, the furosemide-induced activation of Na^+–H^+ exchange was prevented; and (b) that the strophanthidin-induced alkalinization is not mediated by the Na^+–H^+ exchangers, reinforcing our previous results. From Table 2 we see that the slope of the proton flux–pH_i relationship has been reduced, indicating a reduction in the turnover rate of the exchangers. The mechanism underlying this change is unknown. Nonetheless, the Na^+–H^+ exchanger set-point

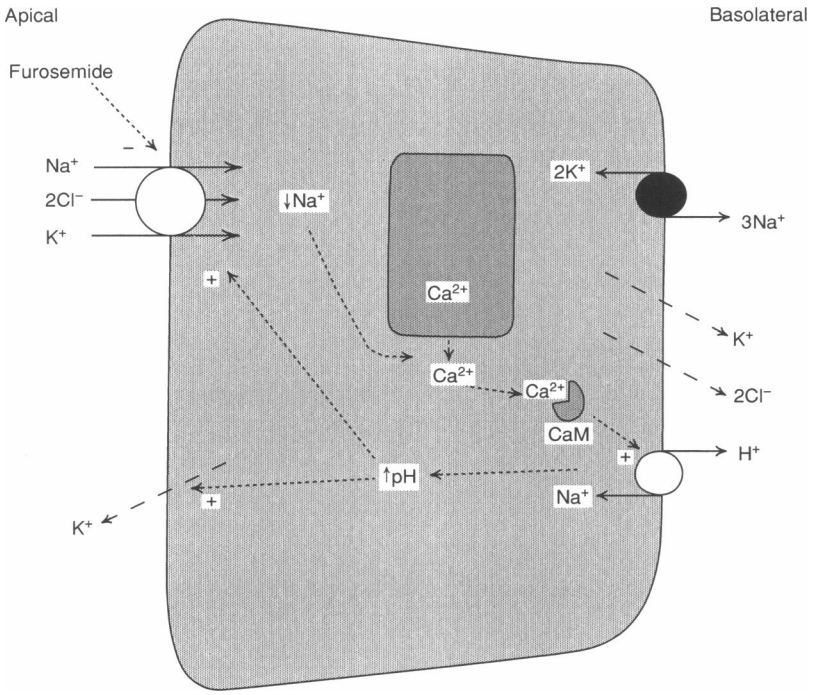

Figure 8. Chain of events leading to activation of apical transport following inhibition of Na^+–$2Cl^-$–K^+ cotransport

See text for details. CaM, calmodulin.

change normally associated with furosemide addition is prevented by strophanthidin.

Cell model: maintenance of cell function *versus* pump–leak coupling

As mentioned earlier the primary function of the diluting segment is NaCl reabsorption. The limiting factor in this process is the luminal K^+ concentration, which is itself determined by the amount of K^+ leaking into the lumen through the apical K^+ channels. The process of NaCl reabsorption is pH sensitive, with the activity of both the apical potassium channels and Na^+–$2Cl^-$–K^+ cotransporters being upregulated by an intracellular alkalinization (Hurst & Hunter, 1992; Palfrey & O'Donnell, 1992). When the apical cotransporter is inhibited Na_i^+ falls, due to the continued action of the basolateral pump. This fall in Na_i^+ may trigger the release of Ca^{2+} from intracellular stores, raising Ca_i^{2+}. This elevation of Ca_i^{2+} increases the concentration of the active Ca–CaM complex, in turn activating Na^+–H^+ exchange by means of an alkaline shift in set-point. The resulting alkalinization will stimulate both apical K^+ channels and Na^+–$2Cl^-$–K^+ cotransport, as the cell attempts to re-establish normal levels of NaCl reabsorption (as summarized in Fig. 8).

This is an extreme example of cross-talk between the apical and basolateral membranes. In this case, an initial event at the apical membrane, the reduction in Na^+, Cl^- and K^+ uptake via the apical cotransporter, has led to activation of the Na^+–H^+ exchanger in the basolateral membrane, thus there is information flow between the apical and basolateral membranes. In turn, the increased activity of the Na^+–H^+ exchanger results in an intracellular alkalinization, which stimulates the apical K^+ channels; this time information is relayed back across the cell to the apical membrane. The enhanced K^+ leak will tend to promote salt reabsorption on the apical cotransporter. In the present experiments, inhibition has been brought about by addition of an exogenous drug, furosemide. However, the above sequence of events may represent a physiological mechanism to regulate salt reabsorption under circumstances in which it may be compromised; for example, following a fall in glomerular filtration rate or increased reabsorption by the proximal tubule, where the delivery of K^+ to the distal tubule will be reduced.

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Acknowledgements

The support of The Wellcome Trust is gratefully acknowledged.

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Received 16 May 1996; accepted 26 September 1996.