Role of *de novo* protein synthesis and calmodulin in rapid activation of Na^+–H^+ exchange by aldosterone in frog diluting segment

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1. In the amphibian early distal tubule aldosterone activates the Na^+–H^+ exchangers, resulting in an increase in intracellular pH (pH_i). Since this activation is rapid (within 30 min), it may be mediated by either a genomic or non-genomic pathway.

2. pH_i was measured in single microperfused early distal tubule segments using the fluorescent probe 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF).

3. A 30 min incubation in aldosterone increased both resting pH_i and the setpoint of the Na^+–H^+ exchanger. These changes were prevented by the mineralocorticoid receptor antagonist, spironolactone.

4. Actinomycin D and cycloheximide, inhibitors of transcription and translation, respectively, were without effect on resting pH_i, but inhibited activation of the Na^+–H^+ exchanger by aldosterone.

5. The effect of aldosterone upon pH_i and setpoint was also prevented by the calcium–calmodulin antagonist, W-7.

6. These results indicate that, although the response to aldosterone is rapid, aldosterone binds to a specific mineralocorticoid receptor which then triggers gene activation followed by *de novo* protein synthesis. Furthermore, since calmodulin is a known activator of the Na^+–H^+ exchanger, and the response is inhibited by W-7, it is suggested that this protein may be calmodulin.

The early distal tubule segment of the amphibian kidney functions to reabsorb NaCl in preference to water, resulting in the production of a dilute luminal solution. These transport properties are shared with its mammalian counterpart, the thick ascending loop of Henle, and these nephron regions are collectively known as the diluting segments (Guggino, Oberleithner & Giebisch, 1988). The inwardly directed Na^+ gradient established by the basolateral Na^+,K^+-ATPase provides the energy for the uptake of NaCl by an apical Na^+–$2Cl^-$–K^+ cotransporter. Na^+ leaves the cell via the basolateral Na^+,K^+-ATPase with Cl^- exiting the cells down its electrochemical gradient via a basolateral pathway. Luminal K^+ delivery to this segment is not sufficient to support the levels of NaCl reabsorption observed, a problem which is overcome by the recycling of K^+ ions through an apical K^+ conductance. Inhibition of this apical K^+ conductance leads to impairment of NaCl reabsorption. Several studies have shown that the activity of this apical conductance is modulated by pH_i, and is upregulated by an intracellular alkalinization (Hurst & Hunter, 1990). In the absence of bicarbonate, the regulation of pH_i is governed by a basolateral Na^+–H^+ exchanger (Cooper & Hunter, 1994*b*).

The principal action of the mineralocorticoid hormone, aldosterone, is to regulate ion transport in the distal nephron. These actions are typically mediated by the initiation of gene transcription, followed by production of aldosterone-induced proteins (AIPs), some of which are themselves transport proteins (Horisberger & Rossier, 1992; Rossier & Palmer, 1992). More recently, the ubiquity of this 'classical' mode of action of steroid hormones has been called into question. In the early distal tubule, elevation of aldosterone leads to an increase in the apical K^+ conductance, an event which has been linked to stimulation

This manuscript was accepted as a Short Paper for rapid publication.

of Na^+–H^+ exchange and subsequent alkalinization (Oberleithner, Lang, Messner & Wang, 1984; Oberleithner, Weigt, Westphale & Wang, 1987; Cooper & Hunter, 1994*b*). This activation is rapid and occurs within 20 min (Oberleithner *et al.* 1987). Activation does not involve an increase in the number of Na^+–H^+ exchangers, but rather an alkaline shift in the pH setpoint, where the setpoint is the value of pH_i at which the exchanger switches between an active and quiescent state (Cooper & Hunter, 1994*b*). Rapid activation of Na^+–H^+ exchange has also been observed in human lymphocytes, with aldosterone producing a significant increase in Na^+–H^+ exchanger activity within minutes (Wehling, Kasmayr & Theisen, 1991). In these cells, Na^+–H^+ exchanger activation was still evident in the presence of inhibitors of protein synthesis, and the authors proposed the involvement of a novel, membrane-bound, mineralocorticoid receptor linked to the IP_3–Ca^{2+} second messenger cascade system (Eisen, Meyer, Christ, Theisen & Wehling, 1994).

In the present study we have attempted to elucidate whether aldosterone works via a classical genomic action pathway, with the production of a regulatory AIP, or via a non-genomic pathway.

METHODS

Frogs (*Rana temporaria*) of either sex were used. Animals were killed by decapitation and destruction of the spinal cord. The kidneys were removed and cut into slices 1–2 mm in width and stored on ice in Leibovitz 15 culture medium (prepared to an osmolality of 204 mosmol (kg H_2O)$^{-1}$, buffered with 10 mM Hepes titrated to pH 7·40 using NaOH). Single early distal tubule segments were dissected in frog Ringer solution containing (mM): 97 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes; pH 7·40 with NaOH, as described previously (Cooper & Hunter, 1994*b*).

Tubule segments were transferred into a Perspex perfusion chamber (volume, 300 μl) containing Ringer solution plus the appropriate drug(s) (see below). The tubules were incubated under these conditions for 20 min. The perfusion chamber was then transferred to the stage of an inverted microscope (Nikon, Kingston, UK) and the tubule was mounted on microperfusion pipettes with the lumen perfused with control frog Ringer solution. The tubules were then loaded with the fluorescent indicator 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), as described previously (Cooper & Hunter, 1994*b*), in the continued presence of the experimental solution for a further 10 min, giving a total incubation time in the experimental solution of 30 min. At the end of the dye-loading period, bath perfusion was begun. The bath solution contained the inhibitor being tested, but aldosterone was omitted to prevent contamination of the perfusion lines – we have observed previously that the alkalinizing effect of aldosterone is maintained for at least the duration of these experiments (20 min). Aldosterone was used at a concentration of 0·1 μM (prepared from a 1 mM stock in ethanol). The inhibitors used were as follows: spironolactone, 25 μM (from a stock of 10 mM in ethanol); cycloheximide, 20 μg ml^{-1} (from a stock of 2 mg ml^{-1} in water); actinomycin D, 0·1 μg ml^{-1} (from a stock of 0·1 mg ml^{-1} in ethanol); and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), 10 μM (from a stock of 50 mM in methanol).

Dye loading, excitation and calibration were carried out as described previously (Cooper & Hunter, 1994*b*).

Calculation of proton efflux

Tubules were acid loaded using the ammonium prepulse technique (Boron, 1992). Upon removal of 10 mM NH_4Cl (mM: 87 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 NH_4Cl-Hepes; pH 7·40 with NaOH) from the basolateral perfusate there was an intracellular acidification followed by a recovery phase as intracellular pH returned to a steady state (Fig. 1*A*). Proton efflux was measured during this recovery phase. Proton efflux is the rate of change of pH_i multiplied by the intracellular buffering power, which is not constant but varies with pH_i. To take account of this we have used the relationship $\beta = -1{\cdot}454(pH_i)^2 + 2{\cdot}223(pH_i) + 82{\cdot}21$ (Cooper & Hunter, 1994*b*). The rate of change of pH_i was calculated between each consecutive data point and then multiplied by β for the mid-point of the two pH values. This allowed determination of the relationship between pH_i and proton efflux for the duration of the recovery phase. If we plot the rate of proton efflux against pH_i (Fig. 1*B*), a fairly linear relationship is obtained which is well fitted using linear regression. We assume that the slope of this relationship indicates the turnover rate and/or number of Na^+–H^+ exchangers, whilst the intercept with the pH_i axis gives the setpoint.

BCECF-AM was obtained from Molecular Probes. All other chemicals were purchased from Sigma.

Data are presented as means ± S.E.M. with the number of observations in parentheses. Statistical analyses were performed with SigmaStat for Windows (Jandel Scientific, Erkrath, Germany) using one-way ANOVA. Where the test indicated a significant difference between groups, pairwise multiple comparison of groups was performed using the Student–Newman–Keuls method. Significance was assumed at the 5% level.

RESULTS

Effect of aldosterone

After a 30 min incubation in aldosterone, there was an increase in the resting pH_i compared with time-matched controls (Table 1). As in our previous study (Cooper & Hunter, 1994*b*), this was accompanied by a significant increase in the setpoint of the Na^+–H^+ exchanger with no effect on the rate of proton efflux (Table 1).

Effect of inhibitors

The aldosterone-induced changes in pH_i were prevented by all of the inhibitors used in the present study; spironolactone, cycloheximide, actinomycin D and W-7 (Table 1). By themselves, these inhibitors were without effect upon the resting pH_i, setpoint pH, or rate of proton efflux (Table 1).

Table 1. Effect of inhibitors on Na^+–H^+ exchanger activity

	pH_i	Setpoint pH_i	Proton efflux (mM min^{-1})
Control	7·07 ± 0·03 (21)	7·04 ± 0·02 (21)	−26·75 ± 2·78 (21)
Aldosterone	7·30 ± 0·03 (20)*	7·32 ± 0·04 (21)*	−27·50 ± 2·79 (21)
Spironolactone	6·95 ± 0·08 (6)	6·94 ± 0·09 (6)	−19·54 ± 4·16 (6)
Spironolactone + aldosterone	7·10 ± 0·06 (6)	7·07 ± 0·07 (6)	−25·83 ± 6·13 (6)
Cycloheximide	7·02 ± 0·08 (5)	7·02 ± 0·08 (5)	−24·88 ± 6·25 (5)
Cycloheximide + aldosterone	7·09 ± 0·13 (5)	7·06 ± 0·11 (5)	−35·67 ± 11·4 (5)
Actinomycin D	7·03 ± 0·03 (5)	7·03 ± 0·03 (5)	−26·75 ± 4·6 (5)
Actinomycin D + aldosterone	6·95 ± 0·05 (5)	7·05 ± 0·02 (5)	−19·83 ± 2·56 (5)
W-7	7·10 ± 0·06 (5)	7·09 ± 0·06 (6)	−18·46 ± 2·46 (6)
W-7 + aldosterone	7·09 ± 0·09 (6)	7·08 ± 0·05 (6)	−28·96 ± 3·70 (6)

All values are quotes as means ± S.E.M. with number of observations in parentheses. * Denotes significantly different from control. Statistics were performed as described in Methods (pH: d.f. 9,84, $F = 5·41$; setpoint pH: d.f. 9,85, $F = 5·99$; rate of proton efflux: d.f. 9,85, $F = 0·909$).

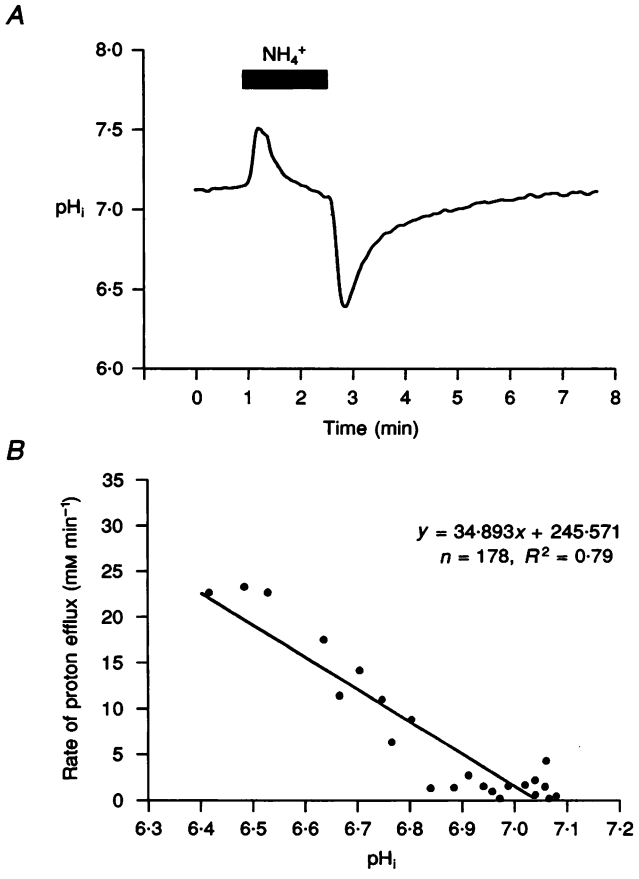

Figure 1. pH sensitivity of proton efflux

A, experimental trace showing the changes in pH_i during acid loading of the cells. Tubules were exposed to 10 mM NH_4Cl for the duration of the filled bar. Upon removal of NH_4Cl from the perfusate there was a rapid acidification followed by an active recovery phase as intracellular pH returned towards the initial level. *B*, rate of proton efflux as a function of intracellular pH during the recovery phase for the experiment shown in *A*. The regression line and equation were calculated from all the data points, but for the sake of clarity not all the points are plotted.

DISCUSSION

The 'classical' mineralocorticoid action involves aldosterone binding to a cytoplasmic receptor, which then migrates into the nucleus where it either activates or represses the promoters of several genes (Horisberger & Rossier, 1992). The mRNA resulting from the transcription of activated genes results in the production of proteins of varying molecular weights, the AIPs (Geheb, Hercker, Singer & Cox, 1981), which result in the modulation of electrolyte transport (Horisberger & Rossier, 1992). The above responses are observed in a number of epithelia, including the early distal tubule, cortical collecting tubule and urinary bladder (reviewed in Rossier & Palmer, 1992). However, in human lymphocytes aldosterone causes a rapid activation of the Na^+–H^+ exchanger, which is insensitive to the mineralocorticoid receptor antagonist, spironolactone (Wehling *et al.* 1991). Although dampened, this activation still occurred in the presence of protein synthesis inhibitors. These spironolactone-insensitive events seem to be mediated via a high affinity, membrane-bound aldosterone receptor, activation of which leads to stimulation of the IP_3–Ca^{2+} cascade system, thus accounting for the rapid effect of aldosterone in lymphocytes (reviewed in Wehling, 1995).

In the present study we have sought evidence for involvement of the 'classical' mineralocorticoid pathway and attempted inhibition at three points: receptor binding, DNA transcription and RNA translation. In agreement with a previous study (Oberleithner *et al.* 1987), inhibition of the mineralocorticoid receptor with a 250-fold excess of the competitive inhibitor spironolactone prevented activation of Na^+–H^+ exchange by aldosterone. The importance of gene activation, followed by subsequent AIP production, was shown by the effects of actinomycin D and cycloheximide. Actinomycin D inhibits the transcription of DNA into mRNA, and fully inhibited the stimulatory effect of aldosterone. Thus aldosterone initiates the production of mRNA. Similarly, Na^+–H^+ exchange activation was prevented by the RNA translation inhibitor cycloheximide, implying that aldosterone's action involves *de novo* protein synthesis. Since both actinomycin D and cycloheximide abolish the aldosterone response, we can rule out the possibility that aldosterone increases the translation of a mRNA species which was present prior to aldosterone treatment. Thus, although Na^+–H^+ exchange activation occurs within 30 min, it is mediated by the classical mineralocorticoid pathway.

We do not think it likely that there are even more rapid effects than those shown above. In lymphocytes, the very rapid, spironolactone-insensitive, non-genomic, stimulation of Na^+–H^+ exchange was detectable within 2 min (Wehling *et al.* 1991). In the early distal tubule, aldosterone causes a monophasic increase in pH_i following a lag period of about 10–20 min (Fig. 2 of Oberleithner *et al.* 1987 and own pilot experiments). The effect on pH_i is maximal within 30 min (Cooper & Hunter, 1994*b*). Thus the very rapid (within 2 min) effects which were reported by Wehling are not present in this preparation.

The above results beg the question of the identity of the AIP which causes activation of the Na^+–H^+ exchanger. The NHE-1 isoform of the Na^+–H^+ exchanger contains binding sites for the calcium–calmodulin complex (Ca^{2+}–CAM), binding of which results in exchanger activation without phosphorylation (Wakabayashi, Bertrand, Ikeda, Pouyssegur & Shigekawa, 1994). We have seen recently that frusemide (furosemide) causes a rapid upregulation of the Na^+–H^+ exchanger, which is prevented by W-7 (Cooper & Hunter, 1995). W-7 binds to the hydrophobic active site of Ca^{2+}–CAM, therefore preventing activation of Ca^{2+}–CAM-mediated target proteins (Tanaka & Hidaka, 1980). Calmodulin is a relatively small protein of 148 amino acids with a molecular weight of about 17 kDa (Vogel, 1994). In toad urinary bladder, aldosterone induced the production of several AIPs, including an as yet unidentified protein with a molecular weight in the region of 15 kDa (Geheb *et al.* 1981), which may be calmodulin. Although it is difficult to state unequivocally, since transcription and translation rates depend upon many factors, it is conceivable that calmodulin is produced within the 30 min incubation period. In prokaryotes, transcription proceeds at about 500 nucleotides per second, whereas translation occurs at around 20 amino acids per second (Alberts, Bray, Lewis, Raff, Roverts & Watson, 1994). Therefore the combined times for the production of a 150 amino acid protein, with a corresponding mRNA coding sequence of 450 base pairs, would be less than 10 s. Although this is probably a lower estimate, this figure does give us some idea of the minimum time necessary for protein expression. Thus the circumstantial evidence does not rule out the notion that calmodulin synthesis may mediate aldosterone's effects in our experiments. Calmodulin is certainly involved, since the effects of aldosterone were abolished by W-7. Also, our results indicate clearly that the full response, from gene activation to protein translation, is completed within 30 min.

The idea that aldosterone may induce the synthesis of calmodulin also fits with our experimental observations concerning the difference between the time courses of Na^+–H^+ exchange activation with frusemide and aldosterone. Given that it is the Ca^{2+}–CAM complex that is responsible for activation of the Na^+–H^+ exchanger, it follows, by the law of mass action, that raising the concentration of either intracellular calcium (Ca^{2+}_i) or calmodulin would increase the levels of the activated Ca^{2+}–CAM complex. Frusemide causes a sustained increase in intracellular calcium, which returns rapidly to normal following removal of frusemide (Cooper & Hunter, 1994*a*). This increase in Ca^{2+}_i may be expected to activate pre-existing calmodulin, which would then stimulate the Na^+–H^+ exchanger. In keeping with this, frusemide produces a rapid, W-7 sensitive, alkalinization, which is the result of activation of the Na^+–H^+ exchanger

(Cooper & Hunter, 1995). On the other hand, with aldosterone we may speculate that there is an increase in the cytoplasmic calmodulin concentration, leading to activation of the Na^+–H^+ exchanger, with or without a change in Ca^{2+}_i. In this scheme, the stimulatory effect upon the Na^+–H^+ exchanger of a 30 min exposure to aldosterone would continue until the cytoplasmic calmodulin levels returned to their initial values, which we would suggest has not occurred within 20 min, i.e. the duration of our experimental protocol. Similarly, in other tissues, for example rabbit collecting tubule (Sansom & O'Neil, 1985), as well as in the amphibian early distal tubule (Cooper & Hunter, 1994*b*), the changes in membrane transport characteristics upon chronic mineralocorticoid stimulation are evident for several hours following tissue isolation. We would suggest that calmodulin mRNA levels are elevated following aldosterone treatment, but not with frusemide.

In conclusion, the rapid activation of the Na^+–H^+ exchanger produced by aldosterone in the diluting segment of the frog kidney is the result of a genomic action, involving *de novo* protein synthesis. We would like to propose that this protein may be calmodulin, a known activator of the ubiquitous Na^+–H^+ exchanger, NHE-1.

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Acknowledgements

This work was supported by The Wellcome Trust.

Received 30 October 1995; accepted 11 December 1995.