The influence of acetazolamide and amlodipine on the intracellular sodium content of rat proximal tubular cells

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1 This investigation set out to use ²³Na n.m.r. spectroscopy to measure changes in intracellular levels of sodium in isolated suspensions of rat proximal tubules. The effects of temperature, an inhibitor of the sodium pump and known natriuretic drugs on intracellular sodium content of such tubular preparations were measured and compared with calcium channel antagonists where action at this level is unclear.

2 Rat kidneys were perfused with collagenase, roughly chopped, subjected to mechanical dispersion and washed to remove all traces of the enzyme. The proximal tubules were then purified and concentrated by Percoll density gradient centrifugation and then resuspended in buffer containing dysprosium tripolyphosphate shift reagent.

3 Distinct peaks corresponding to intracellular and extracellular sodium signals were observed when the tubules were placed into the n.m.r. spectrometer. As the temperature of the suspension rose to 37°C, there was an exponential decrease in sodium content, with a decay constant of $0.15 \pm 0.02 \text{ min}^{-1}$, which reached a stable level within 20 to 25 min. Addition of ouabain, 10^{-3} M , resulted in a significant ($P < 0.01$) 30% increase in intracellular sodium content within 5 min which peaked at 70% 20 min later. Although acetazolamide (10^{-3} M) significantly ($P < 0.01$) increased intracellular sodium content by 45%, amlodipine (10^{-4} M) had no effect.

4 These data show that changes in the activity of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ have a considerable influence on the intracellular levels of sodium in proximal tubule cells. Inhibition of carbonic anhydrase activity resulted in a rise in intracellular sodium content which is compatible with its action to reduce the turnover rate of the $\text{Na}^+/\text{HCO}_3^-$ symporter. The lack of effect of amlodipine was consistent with the suggestion that it does not have a direct action on the sodium handling processes at the level of the proximal tubule.

Keywords: ²³Na n.m.r. spectroscopy; proximal tubules; carbonic anhydrase inhibitor; calcium channel antagonist; sodium transport

Introduction

Currently it is accepted that at the proximal tubule of the kidney, sodium moves from the tubular fluid into the cells by a variety of sodium coupled transport systems (Silbernagl *et al.*, 1975; Krapf, 1989) and leaves at the basolateral surface, mainly facilitated by the sodium pump and $\text{Na}^+/\text{HCO}_3^-$ symporter, into the extracellular space, with water being passively reabsorbed with the sodium. Recent evidence suggests that along the proximal tubule there is a heterogeneity with respect to sodium handling, insofar as the S1 and S2 segments reabsorb the majority of the filtered NaHCO_3 whilst the late S2 and the S3 segments are in the main responsible for the reabsorption of NaCl (Berry, 1982). The characteristics of sodium transport across the tubular cells were elucidated by methods such as measurement of the transepithelial movement of radioactive ²²Na or measurement of potential differences or pH changes between the apical and basolateral sides of the epithelial cells (Baum, 1987; DuBose, 1990; Gesek & Schoolwerth, 1990). Very little is known of the dynamic response of sodium within the cell when challenged with physiological and pharmacological stimuli. The reason for this has been the difficulty with which intracellular sodium (Na_i) can be measured dynamically because of the lack of available methodologies. One method, ion selective microelectrodes, has been utilised to measure intracellular sodium in the face of physiological challenges (Kondo & Frömter, 1990). Another potentially important way in which intracellular sodium content can be measured is by ²³Na n.m.r. spectroscopy.

Dynamic measurement of changes in Na_i content in res-

ponse to physiological challenges, for example temperature changes, and following drug administration has recently been reported in a variety of tissues and cells. ²³Na efflux and influx due to the effect of changing the Na^+ concentration of the incubation medium have been measured in dog and rabbit kidney tubules by Boulanger *et al.* (1987) and the effect of changing temperature on Na_i has been observed on the rabbit proximal tubules (Gullans *et al.*, 1985). ²³Na n.m.r. was also used by Kumar *et al.* (1986) to measure changes in sodium efflux in the rat proximal tubule when the tubular suspension temperature was raised from 4°C to 37°C and Gullans *et al.* (1985) induced sodium influx by using nystatin, a polyene antibiotic.

The transport of sodium across the proximal tubule is known to be inhibited by a number of drugs which give rise to natriuresis. One well described compound is acetazolamide (ACTZ), which is a mild diuretic and natriuretic agent, and its mechanism of action is inhibition of carbonic anhydrase. The consequence of its action is to reduce bicarbonate reabsorption thereby preventing sodium reabsorption and hence to increase sodium and water excretion (Preisig *et al.*, 1987). Calcium channel antagonists (CCA), especially the dihydropyridine variety, cause hyperpolarization of vascular smooth muscle resulting in vasodilatation. Interestingly, calcium channel antagonists have been reported to have natriuretic and diuretic properties (Struyker-Boudier *et al.*, 1990). There is a possibility that the calcium channel antagonists act directly on tubular transport processes to cause natriuresis, and Rodicio *et al.* (1990) reported that nifedipine increased sodium excretion in man without altering glomerular filtration rate (GFR) and renal plasma flow, suggesting a tubular action. Similarly, in *in vivo* experiments,

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amlodipine has been demonstrated to have natriuretic properties in the absence of changes in renal blood flow or glomerular filtration (Johns, 1988). The dihydropyridine class, calcium channel antagonist, felodipine has also shown direct tubular actions in the kidney of anaesthetized normotensive dogs (DiBona, 1990). However, the site of action along the tubule has yet to be resolved.

The aim of this study was to use ^{23}Na n.m.r. spectroscopy to determine intracellular levels of sodium in suspensions of proximal tubules obtained from rat kidneys and to compare the effect of two natriuretic agents, acetazolamide and the calcium channel antagonist amlodipine on this Na_i content. In this way the action of a compound of known proximal tubular site of action, such as ACTZ, could be compared with that of a compound whose site of action is unknown but which could be acting at the proximal tubule.

Methods

General dissection

The methods used to isolate the proximal tubules were based on techniques described by Balaban *et al.* (1980), Vinay *et al.* (1981) and Gesek *et al.* (1987). Male Wistar rats, 350–400 g, were anaesthetized with Sagatal, 60 mg kg⁻¹, i.p., the kidneys were then exposed via a midline incision and the intestines were displaced to the left. The aorta and vena cava were then cannulated below the level of the kidneys and 500 u heparin was immediately injected intravenously to prevent blood coagulation. Loose ligatures were placed around the aorta and vena cava above the renal vessels and the mesenteric and coeliac arteries were tied off. Prior to the perfusion of the kidneys, the ligatures above the kidneys were tightened to form a closed system.

Both kidneys were then perfused with the physiological buffer solution (containing in mM: NaCl 115, NaHCO₃ 25, KCl 5, glucose 5, MgSO₄ 1, L-alanine 1, sodium lactate 4, mannitol 25, CaCl₂ 2, dextran 0.6%, NaH₂PO₄ 2) which was saturated with 95% O₂/5% CO₂ and kept at 37°C, through the aortic cannula. Perfusion pressure was monitored with an on-line manometer and the perfusion rate adjusted to achieve a perfusion pressure of approximately 120 mmHg. When the venous effluent was seen to be clear of blood, the perfusate was changed to an enzyme solution (containing, in mM: NaCl 115, NaHCO₃ 25, KCl 5, glucose 5, MgSO₄ 1, L-alanine 1, sodium lactate 4, CaCl₂ 2, dextran 0.6%, NaH₂PO₄ 2, collagenase 40 u ml⁻¹). The enzyme solution was collected from the venous cannula and reinfused into the kidneys until the perfusate had infiltrated into surrounding tissue and no perfusate could be collected at the venous end, which was typically achieved after approximately 10–15 min. The kidneys were immediately removed, placed into a beaker of ice-cold buffer (containing, in mM: NaCl 115, NaHCO₃ 25, KCl 5, glucose 5, MgSO₄ 1, L-alanine 1, sodium lactate 4, CaCl₂ 2, dextran 0.6%, NaH₂PO₄ 2). The kidneys were then decapsulated, placed on a glass petri dish, which was kept on ice, and sliced into transverse sections approximately 2 mm thick. The medulla was dissected away and the cortical slices were minced with a razor blade and placed into 15 ml of the enzyme solution. This suspension was incubated at 37°C for 35 min under an atmosphere of 95% O₂/5% CO₂. At 7 min intervals during the incubation, the tubules were gently dispersed by pulling the suspension through a long Pasteur pipette. After 35 min the enzyme solution containing the suspension of dissociated tubules was centrifuged at 120 g at 4°C for 1 min (MSE Mistral 3000). The collagenase solution was decanted away and the tubules were placed into 10 ml of fresh ice-cold buffer solution. The new suspension was filtered through 3 layers of surgical gauze to remove any undissociated tissue and the centrifugation process was repeated 3 times.

The proximal tubules were then purified by differential

centrifugation in Percoll. Fresh Percoll was made isotonic with a buffer solution (containing in mM: NaCl 115, NaHCO₃ 25, KCl 5, MgSO₄ 0.5, mannitol 35, NaH₂PO₄ 2) in a 9:1 ratio of undiluted Percoll to buffer. The isotonic Percoll was adjusted to pH 7.4 with HEPES. This isotonic Percoll was taken as 100% and subsequent dilutions were made from this isotonic stock. A 45% Percoll solution was prepared by diluting the 100% stock with a dilution solution (containing, in mM: NaCl 115, NaHCO₃ 25, KCl 5, MgSO₄ 0.05, mannitol 35, NaH₂PO₄ 2). This was kept at 4°C and under an atmosphere of 95% O₂/5% CO₂ for at least 30 min before use.

The tubules were washed twice by low speed centrifugation (500 r.p.m. for 1 min) in 10 ml of the dilution solution and then placed into 30 ml of the 45% Percoll in a 50 ml centrifuge tube. Five millilitres of undiluted isotonic Percoll was then injected carefully into the bottom of the tube. This suspension was then centrifuged at 18,000 g at 4°C for 10 min (8 × 50 fixed head rotor on a MSE superspeed 50 or 17,540 g with a SS-34 rotor head on a Sorvall refrigerated centrifuge). The resulting four to five layers were then removed separately with a Pasteur pipette. The upper two thirds were discarded, whilst the bottom two layers, which contained the proximal tubules, were washed three times by low speed centrifugation (500 r.p.m. for 1 min) in 10 ml solutions of the ice-cold buffer solution to remove any remaining Percoll and then resuspended in the ice-cold buffer and kept on ice until use.

Physiological viability

The viability of the tubular suspensions was estimated by the trypan blue exclusion test and by ATP measurements of the tissues at the end of the preparation procedure. ATP concentrations were determined with an ATP analysis kit (Sigma UK, procedure No. 366-UV). At the same time samples were also taken for protein determination, which was performed with a Sigma kit (procedure No. P5656), utilising the principles of the Lowry procedure (Lowry *et al.*, 1951).

Nuclear magnetic resonance methods

The shift reagent, dysprosium tripolyphosphate, (Dy(PPP)₂)⁷⁻, was prepared, prior to use, by making up 100 mM DyCl₃ in a 50 mM HEPES-buffered solution containing 200 mM sodium tripolyphosphate. The working solution of the shift reagent was prepared by diluting the stock shift reagent with a low sodium buffer (containing, in mM: NaCl 85, NaHCO₃ 25, KCl 5, glucose 5, MgSO₄ 1, L-alanine 1, sodium lactate 4, CaCl₂ 2, dextran 0.6%, NaH₂PO₄ 2) in a ratio of 1:25 with respect to the buffer. This gave a final concentration of 4 mM of Dy(PPP)₂⁷⁻. The proximal tubules were then suspended in the buffer/shift reagent to give a protein concentration of approximately 40 mg protein ml⁻¹.

The n.m.r. spectra were collected using a Bruker 250 spectrometer operating at 66 MHz which accumulated 5000 scans for each spectrum using an acquisition time of 70 ms per scan; a total accumulation time of approximately 5.8 min. Application of 90° pulses every 70 ms ensured that the spectra were fully relaxed. In some experiments a Jeol JNM-GX270 was also used, operating at 71 MHz and 1024 scans were performed for each spectra with an acquisition time of 102 ms, plus a pulse delay of 150 ms, giving an accumulation time of approximately 4.5 min.

Between 1.5–2.0 ml of the suspension was placed in a 10 mm diameter n.m.r. tube. The shift reagent, containing 10% deuterium oxide to act as a frequency lock, and one drop of antifoam reagent B was added to the suspension prior to placing it in the n.m.r. probe. A single bubbling system was arranged to give very gentle agitation of the suspension during the experiment. The probe was kept at 37°C and acquisition was begun immediately after the tubule suspension was placed in the probe. The spectral peaks were

analysed by integration, using Bruker software, which gave an area under the curve for each peak.

It took approximately 10 min for the sample temperature to rise to 37°C after placing the n.m.r. tube, which had been kept at 0–4°C, in the probe. Agents with known effects on the sodium transport in the kidney were then infused into the suspension via an infusion line, 25–30 min after the suspension had been placed into the probe. The temperature of the probe was monitored by an internal thermometer.

Drug treatments

Ouabain (10^{-3} M), acetazolamide (10^{-3} M) or amlodipine (10^{-4} M) or vehicle alone (physiological buffer), was added to the suspension 25–30 min after the tubule suspension had been placed into the probe. The drugs were dissolved in the buffer solution and added to the suspension via the infusion line in a volume of 0.5 ml to give the final concentrations quoted above. After the test drug or vehicle only had been delivered to the suspension, further spectra were acquired over the subsequent 25–30 min to establish whether there was any effect on the intracellular sodium content. Changes were recorded as deviations from the steady state level (100%) just prior to the addition of the drug.

Calculations

Assuming that the intracellular sodium is 100% visible, the sodium content within the cells of the rat proximal tubules can be calculated by comparison of the intracellular sodium peak integral (area underneath the curve) with the integral of the extracellular peak, together with knowledge of the extracellular sodium concentration, which was determined by flame photometry, and the protein content of the sample, as described by Gullans *et al.* (1985). This ensures the derivation of the sodium content of the sample in a specific n.m.r. spectrum, with units of nmol mg⁻¹ protein. Assuming that there is 0.75 mg protein mg⁻¹ dry wt (Balaban *et al.*, 1980) the sodium content can be converted to units of nmol mg⁻¹ dry wt.

Statistical analysis

The n.m.r. data were tested by one way analysis of variance followed by Bonferoni *post-hoc* analysis, comparing steady state intracellular sodium levels with changes due to the effect of the drug over time. All statistical computations were obtained with statistical software, Superanova (Abacus). Significant differences were taken when *P* was less than 5%.

Chemicals

Heparin was supplied as Multiparin (Fisons UK). Antifoam reagent B, ouabain, acetazolamide, collagenase (type IV, 1000 u ml⁻¹) and Percoll, supplied as 23% w/w colloidal solution were purchased from Sigma UK. Amlodipine was supplied by Pfizer UK.

Results

Proximal tubule morphology

Microscopic examination indicated that the fraction retained from the bottom two layers of the 45% Percoll purification step consisted of greater than 95% of proximal tubules. Examination by phase-contrast microscopy indicated that the proximal tubules were 250–400 µm in length and they had a characteristic brush border with open lumens.

Tubular viability

The majority of the tubules sampled excluded trypan blue for up to 15 min which indicated that the cells were still alive.

More accurate measurements were provided by the ATP analysis of the tubule suspensions. The ATP levels for these experiments (Table 1) were between 6–12 nmol ATP mg⁻¹ protein. No treatment group was significantly different from the proximal tubules incubated in buffer alone, which indicated that the tissues were still undergoing active respiration.

n.m.r. results

A typical ²³Na n.m.r. spectrum of a proximal tubule suspension is shown in Figure 1. The smaller peak (A_i) is the intracellular sodium signal and the larger peak (A_e), which has been shifted to the right, is the extracellular sodium signal. The horizontal scale is given in p.p.m. In all n.m.r. experiments, the shift difference between the intra- and extracellular peaks was between 7–10 p.p.m. which provided a good resolution between the two peaks.

Effect of temperature on sodium content in suspensions of proximal tubules

Figure 2 shows the effect on the intracellular sodium content due to warming suspensions of proximal tubules from 4°C to 37°C. On placing the suspension in the probe, which was at 37°C, sodium content fell significantly (*P* < 0.001) over the subsequent 15 min. The sodium efflux followed an exponential rate which gave a value of *k* = 0.15 ± 0.02 min⁻¹. The rate constant, *k*, was calculated from the gradient of the graph by regression analysis. Steady state levels of intracellular sodium, at 37°C, were calculated to be 79.9 ± 5.1 nmol mg⁻¹ protein which was equivalent to 60.0 ± 3.8 nmol mg⁻¹ dry wt and 19.0 ± 1.2 mM (*n* = 57) and this level was chosen as the 100% level from which deviations due to drug application would be recorded.

Table 1 ATP levels in proximal tubule suspensions

Control	Ouabain	ACTZ	Amlodipine
11.1 ± 0.9 <i>n</i> = 22	10.7 ± 1.9 <i>n</i> = 3	13.1 ± 2.7 <i>n</i> = 3	10.4 ± 1.5 <i>n</i> = 4

All ATP concentrations given as nmol ATP mg⁻¹ protein. No treatment group was significantly different from the proximal tubules incubated in buffer alone when tested using analysis of variance. ACTZ = acetazolamide.

Figure 1 A typical ²³Na n.m.r. spectra of intra- and extracellular sodium intensities in a suspension of rat proximal tubules. A_i represents the intracellular sodium peak and A_e represents the extracellular sodium peak. The horizontal scale is given in p.p.m. In all n.m.r. experiments, the shift difference between the intra- and extracellular peaks was between 7–10 p.p.m. which provided a good resolution between the two peaks.

The effect of pharmacological agents on $[Na]_i$ using n.m.r.

In the 7 experiments performed for the control study, in which the vehicle was added once steady state was reached, the intracellular sodium content of the proximal tubules did not change significantly from the baseline level over the subsequent 20–30 min (Figure 3).

In a further set of 4 experiments, addition of ouabain 10^{-3} M, once steady state had been reached, caused a rapid and significant ($P < 0.01$) increase in intracellular sodium content, by approximately 30% of the steady state, after 5 min and the effect was seen to plateau at about 70% after 30 min (Figure 3). The increase in $[Na]_i$ after addition of ouabain, over 30 min was significantly different from the steady state level ($P < 0.01$).

Following the addition of ACTZ 10^{-3} M, in 5 experiments, the steady state intracellular sodium content of the proximal tubule suspension was seen to increase by approximately 35% within 5 min and thereafter increased in a time-dependent manner, peaking at about 45% after 30 min (Figure 4). The rise in intracellular sodium content due to ACTZ was significantly different from the steady state level ($P < 0.001$) but the magnitude of increase was smaller than that seen with ouabain after 30 min ($P < 0.05$).

Figure 2 The effect of increasing temperature on intracellular sodium content in proximal tubules. The points are means of 17 experiments. At time 0 the tubule suspension was at $0-4^{\circ}\text{C}$ and when placed in the n.m.r. probe it was warmed to the ambient temperature of the probe which was kept at 37°C . Steady state was reached after 15–20 min and was designated 100%. * $P < 0.001$ for the decrease in intracellular sodium.

Figure 3 The effect of vehicle alone, in seven experiments (□) and ouabain 10^{-3} M, four experiments (●) on intracellular sodium content in proximal tubules. Steady state was reached after 15–20 min and was designated 100% and intracellular sodium was plotted as a percentage of this steady state. The profile of response seen over 30 min in the presence of vehicle alone was significantly different from that seen in the presence of ouabain ($P < 0.01$).

Figure 4 The effect of acetazolamide (ACTZ, 10^{-3} M) in five experiments (●) and amlodipine 10^{-4} M, in four experiments (□) on intracellular sodium content in proximal tubules. Intracellular sodium was plotted as percentage change in steady state, which was designated 100%. The profile of response seen in the presence of ACTZ was significantly different from that seen with the control study ($P < 0.001$). The response seen with amlodipine was not significantly different from the baseline levels.

In a group of 4 experiments, addition of amlodipine to a final concentration of 10^{-4} M to the proximal tubule suspension appeared to have no effect on the steady state intracellular sodium level (Figure 4). The intracellular sodium content remained relatively constant after the addition of amlodipine over 30 min and did not vary by any significant amount from the level prior to drug treatment, nor was it significantly different from that observed when the vehicle was given alone.

Discussion

The purpose of this study was to follow changes in intracellular sodium content within the epithelial cells of suspensions of rat proximal tubules using ^{23}Na n.m.r. spectroscopy. The paramagnetic shift reagent, $\text{Dy}(\text{PPP})_2^{7-}$, permitted the resolution of intra- and extracellular sodium in the n.m.r. spectrum (Figure 1), thus providing the possibility of assessing changes in intracellular sodium due to changes in the external environment and application of drugs. The concentration of $\text{Dy}(\text{PPP})_2^{7-}$ chosen, 4 mM, had been previously reported to be non-toxic (Gullans *et al.*, 1985) and the initial trypan blue exclusion test supported this. Moreover, ATP levels in the tubules (Table 1) were similar to or greater than levels reported in the literature (Gesek *et al.*, 1987) and this provided further evidence to support the view that the tubules were functionally normal. Thus the tubules were respiring and able to generate and retain their intracellular ATP content at a level comparable to other studies (Balaban *et al.*, 1980). At 4 mM, $\text{Dy}(\text{PPP})_2^{7-}$ also provided a shift between the two sodium peaks (about 8 p.p.m.) which was sufficient to permit accurate resolution of the intra- and extracellular sodium signals.

The study with ACTZ was aimed at examining whether it was able to change intracellular sodium and to this end a high dose was used which would completely block both luminal and cytosolic carbonic anhydrase. ACTZ increased intracellular sodium content in suspensions of proximal tubules by 45% compared to the steady state level after 30 min of incubation with ACTZ, which was taken to indicate a reduction in the rate of sodium flux. This action of ACTZ was probably achieved as the consequence of the blockade of the enzyme, carbonic anhydrase. Carbonic anhydrase (CA) has been positively identified by histological staining throughout the kidney and extensively in the proximal tubule in both the luminal membrane and cytosol (Lönnerholm & Ridderstale, 1980; Dobyan *et al.*, 1982). The enzyme facilitates the conversion of HCO_3^- and H^+ to H_2O and CO_2 at the luminal membrane and the regeneration of intracellular HCO_3^- and H^+ . The consequence of the luminal

action of ACTZ would be to inhibit CA-catalysed HCO_3^- transport across the luminal membrane. In the cytosol, CA inhibition by ACTZ would reduce the intracellular levels of HCO_3^- and hence the total basolateral NaHCO_3 transport via the $\text{Na}^+(\text{HCO}_3^-)_3$ symporter (Preisig *et al.*, 1987). Moreover, in other studies, ACTZ has been shown to decrease the activity of the $\text{Na}^+(\text{HCO}_3^-)_3$ symporter on the basolateral membrane of rabbit isolated perfused S2 proximal tubules (Sasaki & Marumo, 1989). This could result in a reduction in the rate of total intracellular sodium efflux since it has been suggested that the $\text{Na}^+(\text{HCO}_3^-)_3$ symporter is responsible for up to $\frac{1}{3}$ of the total sodium transported across the basolateral membrane (Moe *et al.*, 1990) while Sasaki & Marumo (1989) suggested that the symporter could account for 50% of the sodium removed from the proximal tubule cell at the basolateral membrane. Thus, in this present study the basolateral sodium exit could have been inhibited by ACTZ reducing the activity of the $\text{Na}^+(\text{HCO}_3^-)_3$ symporter, the outcome of which was an increase in intracellular sodium content. NaHCO_3 transport into the cell would also have been inhibited but sodium entry would still occur via other transporters. The observed increase in intracellular sodium due to ACTZ was of a smaller magnitude than that seen by ouabain after 30 min ($P < 0.05$) which suggests that the $\text{Na}^+/\text{K}^+/\text{ATPase}$ provides the greater driving force for basolateral Na^+ transport.

Increasingly, calcium channel antagonists (CCA), especially the dihydropyridine variety, have been reported to have natriuretic and diuretic properties (Struyker-Boudier *et al.*, 1990). There is a possibility that the calcium channel antagonists act directly on tubular transport processes to cause natriuresis and Rodicio *et al.* (1990) reported that nifedipine increased sodium excretion in man without altering GFR and renal plasma flow, which suggested a tubular action. Figueiredo *et al.* (1982) also demonstrated that verapamil, a phenylalkylamine CCA, attenuated sodium reabsorption in rabbit isolated perfused proximal tubules when it was added to the bathing solution and felodipine has been shown to cause natriuresis and diuresis, without affecting renal haemodynamics, in anaesthetized normotensive dogs when administered via the renal artery (DiBona, 1990). Amlodipine is a recently developed dihydropyridine class CCA (Burges *et al.*, 1987) which has been demonstrated to have natriuretic properties *in vivo* (Johns, 1988). In this present study, amlodipine 10^{-4} M, a concentration which has been shown to block Ca^{2+} -induced contractions in rat isolated aorta (Burges *et al.*, 1987), was utilised to determine whether it had effects at the level of the proximal tubules. The addition of amlodipine to the proximal tubule suspension did not alter the Na^+ content from the steady state level which remained constant throughout the experiment, even 30 min after amlodipine was added. The present observations were in agreement with functional studies on rats, using lithium clearance as a measure of proximal Na^+ reabsorption (Johns, 1988) which found that amlodipine at concentrations of $200 \mu\text{g kg}^{-1}$ and $400 \mu\text{g kg}^{-1}$ did not have any significant action on Na^+ transport at the proximal tubule segment which suggested that the natriuretic action was due to an effect further along the tubule. This conclusion supports the findings that in this preparation amlodipine does not act directly at the proximal tubule to alter intracellular sodium content. Conversely studies have been conducted which indicate that the dihydropyridine class of calcium channel antagonists do have an influence on proximal tubule function. McCarty & O'Neil (1991) determined that rabbit proximal straight tubule had nifedipine-sensitive Ca^{2+} channels on the basolateral membrane which were activated during cell swelling. The reasons for these conflicting findings are not clear but it may be that these channels only become

apparent under conditions of chemiosmotic stress and were not functional under the conditions of the present study.

We have used ^{23}Na n.m.r. spectroscopy to follow changes in intracellular sodium content in rat proximal tubular cells. The results presented in this study demonstrate that intracellular sodium levels are temperature-dependent, which probably reflects a dependency on the activity of $\text{Na}^+/\text{K}^+/\text{ATPase}$ enzyme. This view was supported by the observation that, following blockade of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ with ouabain, sodium content gradually rose. Administration of the natriuretic agent ACTZ also increased intracellular sodium which was consistent with ACTZ's known action of blocking carbonic anhydrase activity. By contrast, the dihydropyridine calcium channel antagonist, amlodipine, appeared to have no effect on sodium transport at the proximal tubule suggesting that the compound does not act at this site.

The tubular cells became sodium-loaded during the preparation at $0-4^\circ\text{C}$ and when allowed to warm to 37°C intracellular sodium decreased in an exponential way (Figure 4). The sodium efflux followed an exponential decay over 15–25 min. The rate constant, $k = 0.15 \pm 0.02 \text{ min}^{-1}$, was similar to that reported by Gullans *et al.* (1985) ($k = 0.14 \text{ min}^{-1}$) whose team used a similar approach, but with rabbit proximal tubules. In their case, the suspension warmed to 37°C over approximately 10 min whereas the $[\text{Na}]_i$ reached steady state after approximately 20 min which was similar to the results reported here. Gullans *et al.* (1985) undertook detailed metabolic studies to provide evidence that suggested that this time course and rate of efflux of intracellular sodium corresponded closely with the rate of ATP regeneration, which indicated that the re-energization of the cells involved an increase in ATP at the same time as a decline in intracellular sodium content.

The steady state intracellular sodium content of the proximal tubules prepared in the present study was about 80 nmol mg^{-1} protein, which corresponded to approximately 60 nmol mg^{-1} dry wt and by using cell volume estimation (Grantham *et al.*, 1977) gave a real intracellular sodium concentration of 19 mM . This value of intracellular sodium derived in the present study corresponded closely to those obtained by Kumar *et al.* (1986), 17 mM versus 19 mM , who also used suspensions of rat proximal tubules and Rayson & Gupta (1985) reported a n.m.r. visible $[\text{Na}]_i$ of 23 mM at 37°C in rat outer medullary tubules. In tests by Beck *et al.* (1980) using electron probe microanalysis, *in vitro*, reported values were 22.4 mM for the intracellular sodium content in the rat proximal tubule, which corresponds closely with the values obtained in the current study.

Ouabain was chosen as an accepted tool for blockade of the sodium pump in this tissue and was used at a high concentration, comparable to that utilised by Gullans *et al.* (1985) and by Kumar *et al.* (1986). Incubation with ouabain, a sodium pump inhibitor, rapidly increased $[\text{Na}]_i$ by 30% in the first 5 min and finally reached a plateau at 70% over the next 25 min. This pattern of response was comparable to results obtained by Gullans *et al.* (1985), who reported a rapid increase of 45% over the initial 2.5 min and then a more steady rise to 78% over the next 30 min and those observed by Boulanger *et al.* (1987), using the dog cortical tubules. The results of the present study clearly showed application of a known inhibitor of the sodium pump lead to appropriate and meaningful changes in sodium content in the proximal tubular epithelial cell.

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