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AN X-RAY MICROANALYTICAL STUDY ON THE EFFECTS OF OUABAIN AND N-ETHYL MALEIMIDE ON THE ELEMENTAL CONCENTRATIONS IN MALPIGHIAN TUBULE CELLS OF *LOCUSTA*

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Abstract

X-ray microanalysis was used to study elemental distribution in Malpighian tubule cells of *Locusta migratoria* and how these are affected by the replacement of bathing medium K^+ with Rb^+ and by inclusion of the transport inhibitors ouabain and n-ethyl maleimide (NEM) in standard (K^+-containing) and Rb^+-Ringer (K^+-free) solutions. Incubation of tubules in standard Ringer containing 1 mM ouabain dramatically affected the intracellular levels of K and Na. The intracellular K concentration fell and Na concentration increased in all regions studied. Despite this, a gradient of increasing K concentration from basal to apical cell surface was maintained. Ouabain also reduced the intracellular levels of Rb when applied in Rb^+-Ringer. Cl and P levels were unaffected by ouabain treatment.

Incubation in standard and Rb^+-Ringer solutions containing 1μM NEM caused a significant increase in intracellular K levels in all regions of the cell compared with that observed in the absence of NEM. Rb levels were little affected by NEM except in the apical cytoplasm and microvillar regions where they were significantly reduced compared with Rb^+-Ringer controls. NEM effected a significant increase in cellular levels of Na under Rb^+-Ringer conditions. Intracellular Cl and P were not significantly affected by NEM.

These results are discussed in relation to proposed mechanisms for the transport of ions and water across this secretory epithelium, with particular emphasis on the role of K^+ as the 'prime mover' in this process.

Key Words: X-ray microanalysis, ouabain, n-ethyl maleimide, *Locusta*, Malpighian tubules.

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Introduction

X-ray microanalytical techniques have been successfully used to determine elemental composition and distribution in frozen-hydrated and freeze-dried sections of Malpighian tubules, salivary glands and midgut of various insect species (Gupta *et al.*, 1976, 1977, 1978; Dow *et al.*, 1981; Pivovarova *et al.*, 1993, 1994). Early studies on frozen-hydrated sections revealed that Na, K and Cl were not uniformly distributed in the cytoplasm of Malpighian tubule cells of *Rhodnius* and *Calliphora* (Gupta *et al.*, 1976, 1977), in salivary glands of *Calliphora* (Gupta *et al.*, 1978) with gradients of total elemental concentration being observed between basal and apical surfaces. More recently, Pivovarova *et al.* (1993) have reported a gradient of increasing K concentration from basal to apical surface of Malpighian tubule cells of *Locusta*. Indeed, one of the principal points to emerge from such X-ray microanalytical studies is that neither ionic nor the osmotic concentrations are homogeneous in epithelial cells which support large unidirectional fluxes of ions and water through them (Gupta, 1991).

Malpighian tubules of *Locusta migratoria* consist of two cell types (Martoja, 1959, 1961; Charnley, 1982), the Type 1 cells representing approximately 90% of the total cell number. These cells are characterised by a thick basement membrane beneath which there is an extensively infolded basal plasma membrane and a well-developed apical microvillar border. The cells also possess numerous mitochondria and various electron--dense cytoplasmic inclusions (Anstee and Bell, 1977; Pivovarova *et al.*, 1994). These cells are considered to be responsible for 'urine' secretion; a process dependent on active ion transport with potassium secretion being considered to be the 'prime mover' in fluid production (Ramsey, 1953; Anstee *et al.*, 1979). It has been suggested that K^+ (and perhaps Na^+) movements across the apical plasma membrane is by an electrogenic monovalent cation pump (Berridge, 1968; O'Donnell and Maddrell, 1984; Baldrick *et al.*, 1988). However, recent

proposals suggest that this pump is energised by a proton gradient generated by a V-type ATPase which drives a K^+ (or Na^+)/H^+ antiport (Fogg *et al.*, 1991; Zeiske, 1992). The movement of Cl^- into the lumen is thought to be passive resulting from the large and favourable electrical gradient. Electrophysiological studies suggest that the basal membrane is permeable to K^+, but relatively impermeable to both Na^+ and Cl^-. However, the K^+ equilibrium potential is more negative than the membrane potential which argues that K^+ entry cannot be simply passive (Baldrick *et al.*, 1988). The electrochemical gradient also opposed Cl^- entry. Thus, some mechanism must exist to transport both K^+ and Cl^- into the cell from the haemolymph. The presence of an active Na^+K^+ATPase in this preparation (Anstee and Bell, 1978; Anstee *et al.*, 1986; Fogg *et al.*, 1991) identifies one possible entry route for K^+ in exchange for intracellular Na^+. On the basis of work carried out on *Rhodnius* (O'Donnell and Maddrell, 1984) and *Aedes aegypti* (Williams and Beyenbach, 1984) another possible entry route is via an electroneutral co-transport of Na:K:2Cl driven by the favourable inward gradient for Na^+. In *Locusta*, however, it is suggested that K^+ and Cl^- entry may not be coupled with Na^+ but may be by means of a K^+-dependent Cl^- pump, similar to that described for locust rectum (Hanrahan and Phillips, 1983) or by a furosemide-sensitive $\cdot K^+$:Cl- co-transport mechanism (Baldrick *et al.*, 1988; Fogg *et al.*, 1993).

Rubidium is a well-known marker for potassium and can substitute for K^+ in transport pathways because of the close physicochemical relationship between the two elements. It has been successfully used in a number of X-ray microanalytical studies (Beck *et al.*, 1988; Pivovarova and Skul'skii, 1991; Saubermann *et al.*, 1992). Indeed, the use of ion-analogues in conjunction with X-ray microanalysis is a convenient means of elucidating the role of biologically important elements in cell physiology. This approach has been used in an earlier study on Malpighian tubules of *Locusta* in which the intracellular distribution of Rb mimicked that of K in controls, although Rb did not entirely substitute for K loss (Pivovarova *et al.*, 1993). Both Rb and K showed a gradient of increasing concentration from the basal to the apical cell surface. Substantial quantities of K were reported to be present in numerous 'dark bodies' in the cytoplasm of the tubule cells and this was exchangeable for Rb when the latter element replaced K in the bathing medium. Furthermore, incubation in K^+-free, Rb^+-Ringer solution dramatically reduced free cytoplasmic K by substitution with Rb, although K^+ continued to be the dominant cation in the secreted fluid (Pivovarova *et al.*, 1994). Indeed, Rb^+ was a minor constituent of the secreted 'urine' even though it was a major component of the cations present in the cytoplasm. These results were interpreted as suggesting that the apical cation 'pump' had a high affinity for K and that neither Rb nor Na compete effectively at that site for exit. In addition, as Rb^+ extrusion is low and its intracellular concentration is lower than the level of K in control cells, Rb entry at the basal surface must also be reduced as compared with that of K in controls.

The present study concerns the use of selected inhibitors of cation transport processes to determine their effects on the intracellular concentrations of Na, K, Cl and P in type 1 cells of *Locusta migratoria* incubated in standard and Rb-Ringer solutions. This study has provided a further insight into the handling of K^+ (and Rb^+) by these cells.

Materials and Methods

Sexually mature locusts (*Locusta migratoria* L.) were used and these were taken from a population maintained under crowded conditions at 28 ± 0.5°C.

Animals were killed by decapitation and their anterior Malpighian tubules dissected out under standard Ringer solution, at room temperature (about 24°C). The tubules were then transferred to fresh saline which was either the standard Ringer solution or Rb^+-Ringer solution and incubated at 30°C for 30 min. Tubules were then mounted on aluminium pins and immediately frozen in melting Freon 22. The frozen tubules were sectioned at -90°C to -110°C with dry glass knives using an ultramicrotome MT 6000/Cryo Sectioning System FS 1000 (RMC, USA). Sections were cut at 200-300 nm thickness and these were transferred onto Formvar-coated Ni grids and freeze-dried in a conventional freeze-drier equipped with a cold stage, at -50°C and 10^{-2} torr overnight, followed by 6-8h at room temperature. Dried sections were then carbon-coated and stored in a vacuum desiccator. In order to ensure better cell preservation only a superficial layer of the specimen was used for cryosectioning. Ion redistribution caused by ice crystal damage was negligible when compared to probe size. Usually, ice crystals were less than 50-100 nm in diameter. Figure 1 represents a typical section through a Type 1 tubule cell.

The composition of the standard Ringer solution was (mM): NaCl 100, KCl 8.6, $CaCl_2$ 2, $MgCl_2$ 8.5, NaH_2PO_4 4, $NaHCO_3$ 4, glucose 34, HEPES 25, NaOH 11, pH 7.2 (Anstee *et al.*, 1979, 1986). The composition of the Rb^+-Ringer solution was similar except that KCl was replaced by RbCl. Where appropriate the inhibitors ouabain (1mM) and n-ethyl maleimide (NEM, 1 μM) were included in the standard and Rb^+-Ringer solutions.

Sections were imaged and analysed using a transmis-

sion electron microscope (Philips EM 400T) equipped with a Link Analytical AN 10000 energy-dispersive spectrometer (EDS) system, at an accelerating voltage of 100kV and a probe current of 0.3-0.4 nA. The probe current was estimated using an exposure time meter viz.

$$I_p \approx I_s \quad (1)$$

$$I_s = [(53.27s/t-1) * 1.687*10^{-11} + 3.10^{-11})] \quad (2)$$

where I_p = probe current, I_s = screen current (Ampere), s = emulsion setting, and t = exposure time (personal communication from Philips Analytical). The stability of the probe current was checked after each spectrum. Each field was analysed for 100 sec and spectra were recorded at not less that two (usually three) areas for each cell site; probe diameter being 0.3-0.5 μm at magnifications of 10,000 -17,000 fold.

Quantitation was carried out using the Link Quantum/FLS program based on the peak-to-continuum ratio method (Hall, 1979; Gupta and Hall, 1979). Continuum radiation, which was collected over a wide energy range (4-16 keV for samples from standard Ringer and 4-12 keV for samples from Rb^+-Ringer) and represents the mass thickness of the specimen, was used to provide an estimate of the relative density of different cells and cell compartments in the same section.

Previous studies (Burovina and Pivovarova, 1978) showed that bovine serum albumen (BSA) is a suitable organic matrix in which to prepare standards for studies of freeze-dried sections. Thus, calibration constants were established for K, Rb and Na using 20% (w/w) solutions of dialysed BSA containing known concentrations of the appropriate salts. The concentrations of K, Na and Rb in such solutions were confirmed by atomic emission spectroscopy.

The accuracy of the quantitation procedures was estimated by analysis of albumen samples in which the same element was present in different salts. For example, using K,Na-tartrate as standard, the concentration of K (made up as 200 mmol/kg dry wt) in KCl samples was measured as 212 ± 44 mmol/kg dry wt (Mean $\pm$ standard deviation (S.D.); $p > 0.05$). The detection limit with our analytical conditions was in the range of 10 mmol/kg dry wt for K and all elements with atomic number higher than 12; for Na the detection limit was about 50 mmol/kg dry wt.

All data are presented as Mean $\pm$ standard error of the mean (SEM) in mmol/kg dry wt. Significance of difference between means was determined by the application of Student's t-test with reference to statistical tables of Fisher and Yates (1963).

Results

Morphology

Figure 1 shows a transmission electron micrograph of a freeze-dried section through a Type 1 cell and indicates the positions of the different regions analysed; basal infoldings, main cytoplasm, apical cytoplasm and microvilli.

Incubations in standard Ringer solution

Table 1 shows the distribution of K, Na, Cl and P in cells of Malpighian tubules which have been incubated in standard Ringer solution. As can be seen a gradient of K concentration exists from the basal to the apical regions of the cell (Pivovarova *et al.*, 1993). Sodium was present in detectable amounts only in the region of the basal infoldings. No marked gradation in Cl concentration was determined but the value obtained for the main cytoplasm was lower than that for the other regions measured ($p < 0.001$). Phosphorus was more or less evenly distributed throughout the various regions of the cytoplasm.

Incubation of the tubules in standard Ringer solution containing 1 mM ouabain for 30 min had a profound effect on the levels of intracellular K and Na, but no significant effect on those of Cl or P. Thus, there was a dramatic increase in the concentration of Na in the four regions measured so that it constituted a major portion of the total monovalent metal pool in the cells. In contrast, the level of K fell significantly, by approximately 40%, in all regions as compared with control values ($p < 0.001$); the decrease in the main cytoplasm being about 183 mmol/kg dry wt. Despite this fall, a gradient of K distribution was maintained.

The presence of 1μM NEM in the standard Ringer resulted in a significant increase in the K concentration in all regions except the basal infoldings ($p < 0.01$). In consequence, there was an increase in the K concentration gradient between the basal infoldings and the apical region of the cell. The concentration of Na was unaffected by NEM treatment and, with the exception of the region of the basal infoldings, remained below the detection limit (<50 mmol/kg dry wt). The levels of Cl and P were not significantly different from those observed in controls, in all four regions studied.

Incubations in Rb^+-Ringer

Examination of Table 2 shows that incubation of tubules in Rb^+-Ringer solution (i.e. K^+-free conditions) has a dramatic effect on the elemental composition of the cells. K concentrations were greatly reduced so that levels in the region of the basal infoldings and the main cytoplasm were about 10%, and those in the apical

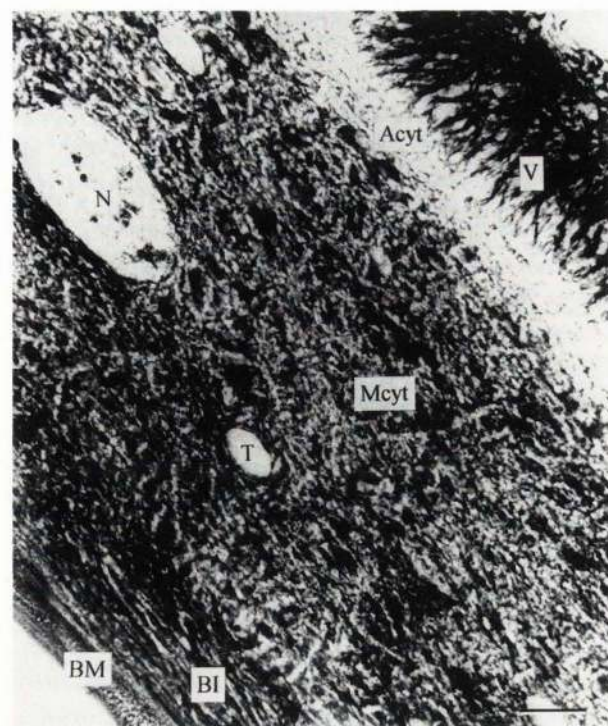

Fig. 1. Transmission electron micrograph of freeze-dried sections of a Malpighian tubule cell of Locusta: BM = basement membrane; BI = Basal infoldings; Mcyt = main cytoplasm; Acyt = apical cytoplasm; V = microvillar border; N = nucleus; T = trachea. Scale bar = 1μm.

region and microvilli about 12% and 15%, respectively, of that seen in standard Ringer controls. Despite this reduction, a gradient of increasing K concentration persisted from basal infoldings to microvilli. The cells contained significant amounts of Rb in all regions of the cytoplasm and this element showed a gradient of concentration from 131 mmol/kg dry wt at the basal infoldings to 249 mmol/kg dry wt at the microvillar border. The amount of Rb accumulated, however, did not compensate for the large reduction in K. However, incubation in Rb^+-Ringer solution caused an increase in Na levels in all regions of the cytoplasm. There were no significant effects of incubation in Rb^+-Ringer on the concentrations of either Cl or P.

Incubation of tubules in Rb^+-Ringer solution containing 1 mM ouabain did not significantly affect K concentrations in the different regions as compared with that observed in Rb^+-Ringer controls (see Table 2). This ouabain treatment significantly increased levels of Na, approximately doubling its concentration in all four regions measured. However, with the exception of the microvillar region ($p < 0.001$) the values obtained for Na were not significantly different from those observed in standard Ringer solution in the presence of 1 mM ouabain. Ouabain treatment significantly reduced the amounts of Rb accumulated ($p < 0.001$) in all regions, when compared to Rb^+-Ringer controls. Despite this, and the absence of K^+ in the bathing medium, it is notable that a gradient of both Rb and K concentration was maintained across these cells. The cellular concentrations of Cl were not significantly affected by the inclusion of ouabain in Rb^+-Ringer solution bathing the tubules nor were they significantly different from values observed in standard Ringer controls containing ouabain (c.f. Table 1 and 2). The levels of P in the four regions were similar to those observed with Rb^+-Ringer controls and in standard Ringer controls with and without ouabain.

The inclusion of 1 μM NEM in Rb^+-Ringer solution bathing the tubules produced a similar effect on the levels of K to that observed in standard Ringer containing NEM. K levels increased significantly in all four regions of the cell, however, they remained substantially below those described for standard Ringer (see Table 2). Indeed, compared with Rb^+-Ringer controls the K concentration effectively doubled in the presence of NEM with the transcellular concentration gradient being maintained. The levels of Rb found were different from those in Rb^+-Ringer controls only in the apical cytoplasm ($p < 0.02$) and microvillar regions ($p < 0.001$). In both cases, Rb levels were significantly reduced. Perhaps the most distinctive effect of NEM on tubules bathed in Rb^+-Ringer solution was the significant increase in cellular levels of Na. This was greatest at the basal infoldings (115 mmol/kg dry wt) and smallest at the microvillar border (70 mmol/kg dry wt). NEM had no significant effect on either the concentration or distribution of Cl or P when compared with Rb^+-Ringer controls.

Discussion

The present study confirms our earlier report (Pivovarova *et al.*, 1993) that there is a variation in K concentration within different cellular compartments in Type 1 cells of Malpighian tubules of *Locusta*; a gradient of increasing K concentration occurs from basal to the luminal side of the cell. This is in agreement with previous reports of variations in intracellular K concentration in Malpighian tubule cells of *Rhodnius* (Gupta *et al.*, 1976). Furthermore, the gradient remained even after incubation in Rb^+-Ringer had resulted in a substantial reduction in K levels in all four compartments measured. The Rb accumulated intracellularly under the

Table 1. The effect of ouabain and NEM on the elemental composition of locust Malpighian tubule Type 1 cells incubated in standard Ringer

Cell Site	n	K	Na	Cl	P
Control					
Basal infoldings	40	385 ± 15	80 ± 5	60 ± 5	603 ± 14
Main cytoplasm	68	463 ± 10	*	27 ± 3	677 ± 14
Apical cytoplasm	31	563 ± 27	*	52 ± 6	700 ± 20
Microvilli	27	658 ± 40	*	55 ± 6	699 ± 27
Ouabain (1 mM)					
Basal infoldings	10	241 ± 19	345 ± 32	90 ± 25	599 ± 20
Main cytoplasm	32	280 ± 20	277 ± 24	30 ± 5	644 ± 22
Apical cytoplasm	8	399 ± 35	250 ± 25	33 ± 8	719 ± 31
Microvilli	21	372 ± 20	239 ± 24	63 ± 10	615 ± 109
NEM (1 μM)					
Basal infoldings	16	405 ± 15	154 ± 30	48 ± 5	576 ± 26
Main cytoplasm	21	529 ± 18	*	33 ± 3	751 ± 24
Apical cytoplasm	12	707 ± 37	*	49 ± 8	691 ± 41
Microvilli	8	795 ± 51	*	53 ± 19	619 ± 19

Mean values ± SEM (mmol/kg dry wt). * Elemental concentration less than the limit of detection (<50mmol/kg dry wt).

Table 2. The effect of ouabain and NEM on the elemental composition of locust Malpighian tubule Type 1 cells incubated in Rb^+- Ringer.

Cell site	n	Rb	K	Na	Cl	P
Control						
Basal infoldings	21	131 ± 10	36 ± 6	170 ± 17	53 ± 6	571 ± 28
Main cytoplasm	36	148 ± 8	51 ± 6	132 ± 10	35 ± 4	656 ± 21
Apical cytoplasm	30	201 ± 13	71 ± 7	176 ± 14	48 ± 5	691 ± 20
Microvilli	24	249 ± 11	98 ± 14	193 ± 20	54 ± 6	704 ± 19
Ouabain (1 mM)						
Basal infoldings	9	41 ± 3	30 ± 3	317 ± 34	47 ± 6	536 ± 29
Main cytoplasm	19	54 ± 4	51 ± 5	245 ± 21	29 ± 3	688 ± 20
Apical cytoplasm	13	80 ± 3	71 ± 4	284 ± 30	53 ± 5	730 ± 31
Microvilli	17	102 ± 6	94 ± 7	395 ± 43	59 ± 7	594 ± 34
NEM (1μM)						
Basal infoldings	10	103 ± 21	79 ± 17	285 ± 35	65 ± 13	621 ± 42
Main cytoplasm	24	154 ± 15	116 ± 10	231 ± 16	42 ± 6	696 ± 27
Apical cytoplasm	24	141 ± 20	164 ± 16	257 ± 15	58 ± 6	684 ± 24
Microvilli	19	122 ± 19	184 ± 18	263 ± 26	66 ± 5	629 ± 22

Mean values ± SEM (mmol/kg dry wt). * Elemental concentration less than the limit of detection (<50mmol/kg dry wt).

latter conditions also showed a gradient in concentration similar to that of K. The significance of such gradients is uncertain. However, it is clear that this gradient can be maintained under a variety of experimental conditions. These include treatment with ouabain, a specific inhibitor of Na^+K^+ATPase activity (Anstee and Bowler, 1984), and NEM, a putative inhibitor of V-type ATPases (Forgac, 1989; Wieczorek *et al.*, 1989; Bertram *et al.*, 1991), conditions that compromise the normal functioning of these cells (Zeiske, 1992; Al-fifi, personal communication).

The present study showed that treatment with ouabain resulted in significant reduction in intracellular K and this was accompanied by a substantial increase in cellular Na concentration, under standard Ringer conditions. This observation was predictable, being consistent with the established role of the Na^+K^+ATPase in the counter transport of Na^+ out of and K^+ into the cell. Previous work from our laboratory shows that this enzyme is present in membrane fractions obtained from this tissue (Anstee and Bell, 1975, 1978; Anstee and Bowler, 1984; Fogg *et al.*, 1991). Furthermore, this enzyme has been implicated in the secretion of cations and fluid by these cells, as well as by the Malpighian tubules of other insect species (Anstee and Bowler, 1984; Maddrell and Overton, 1988; Nicolson, 1993). Indeed, Anstee *et al.* (1986) estimate, on the basis of ouabain-binding studies on Malpighian tubules of *Locusta*, that the turnover of the sodium pump is adequate to account for substantial K^+ transport by these cells. In Rb^+-Ringer studies, ouabain did not significantly change the measured levels of intracellular K beyond the marked reduction observed with Rb^+-Ringer alone. It would appear, that there is a component of the intracellular pool of K which is not readily exchangeable. However, ouabain did significantly reduce the Rb content in all regions studied ($p < 0.001$) and, as under standard Ringer conditions, resulted in an increase in the levels of cellular Na ($p < 0.001$). This reduction in intracellular Rb and the rise in intracellular Na due to ouabain suggests that significant amounts of Rb^+ enter the cells by the Na^+K^+ATPase. Indeed, this may be one of the main means of Rb^+ entry as preliminary electrophysiological studies, using ion substitution, suggest that the basal plasma membrane is relatively impermeable to Rb^+. In this study, Rb plus K content of the main cytoplasm was about 199 mmol/kg dry wt in Rb^+-Ringer controls which compares with a value for K of 463 mmol/kg dry wt in standard Ringer controls. This implies a much reduced uptake/retention of the K^+ mimic as compared with K^+ itself. This may be partially accounted for if the activity of the Na^+K^+ATPase is reduced under Rb^+-Ringer conditions; a suggestion that is consistent with the increase in cell Na reported in Rb^+-Ringer controls. Further support for this suggestion comes from recent work showing that the ouabain-sensitive activity of a membrane preparation in the presence of Rb^+ is only 80% of that determined with K^+ (Marshall, personal communication). Nevertheless, some Rb^+ is likely to enter the cells via a route(s) other than the Na^+K^+ATPase, although the mechanisms for K^+ entry across the basal membrane do not appear to be equally accessible to Rb^+.

In *Locusta*, K^+ secretion is believed to be the 'prime mover' in generating fluid secretion and this cation is electrogenically driven across the apical plasma membrane from the cytoplasm to the lumen (Anstee *et al.*, 1979; Baldrick *et al.*, 1988; Fogg *et al.*, 1991). Thus the effect of NEM in increasing intracellular K levels in the main cytoplasm ($p < 0.01$), the apical cytoplasm ($p < 0.001$) and the microvilli ($p < 0.05$) but not the basal infoldings, and the consequent increase in the K gradient between the basal infoldings and apical cytoplasm, is consistent with inhibition of apical extrusion of K^+ into the lumen. Since NEM is a known inhibitor of V-type ATPase activity, these results support the suggestion that this enzyme is an integral part of the apical plasma membrane transport of K^+ (Zeiske, 1992). In contrast to the effects of NEM on cellular K levels no effect on Na content was seen when tubules were incubated in standard Ringer containing NEM, except for an increase in the region of the basal infoldings. This might suggest that Na^+ secretion at the apical plasma membrane is less (or not) dependent on V-type ATPase activity. However, Na levels were elevated following NEM treatment in Rb^+-Ringer even when compared to Rb^+-Ringer controls, although to a lesser extent than was observed with ouabain treatment. This rise may be partially accounted for by the effects of Rb^+ on (Na^+ $+K^+$)-ATPase activity. However, the additional rise in intracellular Na might be accounted for if some apical Na^+ extrusion is NEM sensitive or if the transport of Na^+ across the basal cell membrane is also compromised by NEM. Indeed, the elevation of Na levels in the basal infoldings under standard Ringer plus NEM conditions, which was also most marked in Rb^+-Ringer plus NEM, may be an indication of an impaired handling of Na^+ at this membrane.

Rb^+-Ringer containing NEM also caused an increase in K levels compared with Rb^+-Ringer controls whilst apical cytoplasmic and microvillar Rb levels were reduced compared with Rb^+-Ringer controls. This might indicate that in the absence of apical K^+ extrusion from the cell, Rb^+ loss is increased. If this is so, it implies that Rb^+ movement across the apical plasma membrane is by a different mechanism to K^+. The question also

arises as to how cellular levels can increase in the absence of K^+ in the bathing medium. Pivovarova *et al.* (1993; 1994) reported that substantial quantities of K were present in numerous 'dark bodies' in the cytoplasm of the tubule cells and that this K was exchangeable for Rb under K^+-free Rb^+-Ringer incubation conditions. Furthermore, whilst incubation under these conditions dramatically reduced free cytoplasmic concentration by substitution with Rb, K^+ continued to be a substantial cation in the secreted 'urine' (Pivovarova *et al.*, 1994). Rb^+ was a minor constituent of the secreted 'urine' even when it represented a major component of the monovalent metal elements present in the cytoplasm. It is tempting to suggest that the increase in cellular K observed in Rb^+-Ringer containing NEM is due to the continued loss of K from the 'dark bodies'. If this is confirmed in future studies, it might also suggest that sequestration of K in these organelles involves an NEM-sensitive mechanism, possibly a V-type ATPase. This would certainly be consistent with the known association of such enzymes with intracellular vacuoles (Forgac, 1989; Nelson, 1987). Alternatively, the observed accumulation of K may be explained by the K loss from 'dark bodies' not being matched by transport from the cells into the tubule lumen due to the apical V-type ATPase activity, alone, being inhibited by NEM.

The origin of the gradient for K across these cells and the mechanism of its maintenance is also unclear. The gradient is maintained under conditions that inhibit ion transport and affect fluid production by these cells and so it is unlikely to be the product simply of a differential uptake of K^+ at one surface and extrusion at the other. Furthermore, a similar gradient is established for Rb in K^+-free Ringer. In this respect it is interesting that Rb replaces K in the 'dark bodies' (Pivovarova *et al.*, 1994) and so the dynamics of loading into these stores might be a factor in determining these concentration gradients.

In conclusion, this X-ray electron probe microanalytical study has shown that a significant uptake of K^+ is dependent on a basal Na^+K^+ATPase, and that Rb^+ is not a perfect mimic for K^+. NEM affects K^+ handling by these cells which supports the idea that K^+ extrusion is linked to the activity of a V-type ATPase at the apical plasma membrane.

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Discussion with Reviewers

B.L. Gupta: Your results again confirm the existence of gradients of ionic concentrations inside the cells under all tested conditions of transport. However, the concentrations are given in mmol/kg dry mass and therefore also influenced by the local variations in the dry mass. Was it not possible to estimate the local dry mass by comparing the continuum counts of the specimen fields with the counts from the albumin standard?

Authors: The local dry mass of different cell compartments was estimated by continuum radiation as compared to the main cytoplasm. The relative values obtained were as follows: basement membrane, 0.97; basal infoldings, 1.13; apical cytoplasm, 0.93; microvilli, 0.68. Only the microvillar values were significantly different from those recorded from the main cytoplasm. Thus, the relative values for K concentrations when expressed in terms of mmol/wet weight or cell water will remain essentially as presented with the exception of the microvillar region.

B.L.Gupta: Did you measure the nucleus? If so, how do the ionic changes in the nucleus compare with those in the cytoplasm?

Authors: Yes, this was done when the section to be

analysed included the nucleus, but it was possible to make only a relatively small number of such measurements (see also Pivovarova *et al.*, 1994). However, these were too few to permit a definitive answer to the question. In general, the changes in the nucleus were consistent with those observed in the main cytoplasm, so that under all experimental conditions the concentration of K in the nucleus was higher than in the main cytoplasm whilst the dry mass was lower. However, only following treatment with NEM were the differences likely to be significant.

T. von Zglinicki: Are the measurements truly cytoplasmic or are other compartments, like basal lamina, dark bodies, or luminal precipitates on the outer side of microvilli included? If yes, please indicate the ion concentrations in the respective compartments. Especially, can those overlap effects be excluded as the source of low K at the basolateral side and high K in the apical microvilli?

Authors: The measurements in the main cytoplasm included organelles such as the endoplasmic reticulum which could not be resolved independently. Mitochondria and dark bodies were excluded from such measurements. The basal lamina was easily resolved and so was analysed separately (Pivovarova *et al.*, 1994). In the regions of the basal infoldings and the microvilli, it was not possible to discriminate between the cytoplasmic processes and the extracellular spaces between them. It is possible, therefore, that this may contribute to the low K values in the region of the basal infoldings. However, it is less certain that this could account for the high K in the region of the apical microvilli as the luminal K concentration is unlikely to be substantially different from that of the apical cytoplasm (Pivovarova *et al.*, 1994). Luminal precipitates were not found on the microvilli and are not considered to have contributed significantly to the measurements made. This is supported by the fact that low Ca values were recorded and such precipitates to which you refer typically show high Ca concentrations.

T. von Zglinicki: It is suggested in the discussion that intracellular ion concentrations might not yet be in "equilibrium" at the measuring point, i.e., 30 min after the exchange of K by Rb. Are there any dynamic experiments providing proof for this hypothesis?

Authors: Previous studies (Pivovarova *et al.*, 1994) support this suggestion, because incubations for 45 min in Rb^+-Ringer significantly increased concentrations of Rb in all compartments.

T. von Zglinicki: Is the dry mass gradient across the cells in parallel to the K gradient? As beam current stability was carefully controlled in your experiments, the background readings obtained are well suited to answer this question.

Authors: The estimations of dry mass in the various compartments have been referred to in a reply above. It is clear that there is not a dry mass gradient across the cell which is in parallel to the K gradient.

G.M. Roomans: What Rb line was chosen for analysis? If the L-line was used, was there any interference from (instrumental) Al?

Authors: The Rb concentration was determined mainly by use of the K-line. However, concentrations calculated using both the K and L-lines were consistent. There were no noticeable extraneous peaks (Al or Si) in the position of the Rb L-line (see Pivovarova *et al.*, 1993, 1994). Moreover, the spectra collected were processed using a computer programme that separated overlapping peaks and ignored extraneous peaks.

G.M. Roomans: After ouabain treatment in control Ringer, the cation content (K+Na) is increased, but Cl is not increased. What compensates for the increase in cation concentration?

Authors: It is possible that electroneutrality is maintained by phosphates and/or by bicarbonate, but we cannot provide a definitive answer to this question from the existing evidence available.