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SODIUM-DEPENDENT POTASSIUM ($^{86}Rb^+$) EFFLUX MODERATES VOLUME REGULATION BY CELLS IN RAT RENAL INNER MEDULLARY SLICES EXPOSED TO STRONGLY HYPEROSMOTIC MEDIA

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The possibility has been examined that Na^+-dependent K^+-conductive pathways, known to exist in certain excitable cell membranes and inhibitable by the drug R56865, may also be present in cells of rat renal inner medulla, and that their activation may explain aspects of volume regulation by these cells in tissue slices exposed to strongly hyperosmotic media, as reflected by the rate of efflux of preloaded $^{86}Rb^+$ (a marker for K^+) and steady-state cell volumes and K^+ contents. Cells incubated in media of 2000 mosmol/kg (400mM Na^+, 1172mM urea) shrink and lose K^+ by comparison with those in 720 mosmol/kg (203mM Na^+, 266mM urea). If 2-aminisobutyric acid (10mM) is added there is partial restoration of cell volume due to inwardly directed Na^+-amino acid cotransport, but $^{86}Rb^+$ efflux is accelerated and cells fail to regain net K^+. R56865 (5μM) completely blocks the increase in efflux and causes marked increases in cell volume and K^+ contents, but only in strongly hyperosmotic media and in the presence of both Na^+ and amino acid. In mildly hyperosmotic media, or media of 2000 mosmol/kg from which Na^+ or amino acid is omitted, R56865 is without effect on these variables.

The degree of shrinkage of cells in slices of rat renal inner medulla exposed to medium made strongly hyperosmotic by high concentrations of NaCl and urea (simulating inner medullary fluids during antidiuresis in vivo) is reduced if amino acid is present in the bathing medium (1). This effect has been attributed to the up-regulatory effect of an inwardly directed Na^+-amino acid symport (1), but is accompanied by a failure of cells to reaccumulate K^+ lost during shrinkage, and by an accelerated efflux of preloaded $^{86}Rb^+$ (tracer for K^+)(2).

Inner medullary cells incubated in high Na^+ media (400mM) attain internal Na^+ concentrations in excess of 100mM(3), and it is possible that the lack of net K^+ re-accumulation following addition of amino acid is due to the activation of Na^+-dependent K^+ efflux. K^+ channels, activated by Na^+ in excess of 20mM, were first

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reported in cardiac myocytes (4), and have subsequently been demonstrated in nerve and muscle cell preparations (5-8, for review see 9). The purpose of the present report is to examine for the first time the possible occurrence of Na^+-dependent K^+ conductive pathways in a non-excitable cell population, the renal inner medulla, using the benzothiazolamine derivative R56865, which has been found to block K^+ channels of this type (7,8,10). Some of these findings have previously been published as an Abstract (11).

METHODS AND MATERIALS

These have been described in detail elsewhere (1-3) and are repeated here only in outline. Slices (4-10 mg, thickness approx. 250 μm) cut from the inner medullas of normally hydrated adult male Wistar rats (250-300g) were loaded with $^{86}Rb^+$ (Amersham International plc) by immersion for 1 h at 37°C in medium of the following composition (mM) - Na^+ 203, K^+ 6, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 167, HCO^-_3 25, $H_2PO_4^-$ 2.2, SO_4^- 1.2, pyruvate 9.6, fumarate 5.3, glucose 10, urea 266, gassed to pH 7.35 with 95% O_2/5% CO_2. The calculated osmolality of this medium is 720 mosmol/kg H_2O, corresponding to that of tissue fluids from the normally hydrated inner medulla in vivo (12). $^{86}Rb^+$ was added to a final activity of 185 kBq/ml. All reagents were of the best commercially available grade. Efflux of $^{86}Rb^+$ into 2ml aliquots of violently agitated unlabelled medium was followed by serial sampling at intervals of 1,2,5,10,15,20,30,40,50 and 60 min from the termination of loading. Temperature was maintained at 37°C by a directed stream of warm air. Residual counts were recovered by extraction for a further 18 h at room temperature. Efflux media had the same composition as the loading medium with the following additions and modifications: (i) Na^+-free media were prepared by equimolar replacement with N-methyl-D-glucamine (NMG), pH adjustment by drop-wise addition of conc. HCl, (ii) 2-aminoisobutyric acid (AIB, 10^{-2}M) was added where appropriate, (iii) osmolality was raised to 2000 mosmol/kg by increasing Na^+ (400mM), Cl^- (367mM) and urea (1172mM) (simulating fluids from the strongly antidiuretic inner medulla (1,12)), (iv) R56865 (N-[1-[4-(4-fluorophenoxy)butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine) (5 x 10^{-6}M) was added at 1 part in 1000 of a 5mM stock solution in dimethylsulphoxide. In the latter experiments R56865 was added 20 min before the termination of $^{86}Rb^+$ loading, since preliminary experiments (not reported in detail here) showed that the effects of R56865 could only be demonstrated if cells were exposed to the drug before the start of efflux.

Total counts per slice were calculated, and the rate of efflux of $^{86}Rb^+$ expressed on a semi-logarithmic basis as percentage counts remaining (initially 100%) with respect to time.

For each incubatory medium in which $^{86}Rb^+$ efflux was studied, steady-state cell volumes (water contents) and K^+ contents were measured in a separate series of experiments. Fresh slices, weighed to the nearest 50μg, were incubated in appropriate media for 100 min at 37°C. [^{14}C] sucrose (Amersham International plc) was added to the media after 75 min, at a final activity of 20 kBq/ml. The 25 min volume of distribution of sucrose within inner medullary slices is regarded as a quantitative delineation of slice extracellular space (3). Slices were then briefly rinsed, blotted and re-weighed, and leached for 20 h at room temperature in 1.2 ml deionized water, in order to extract sucrose and K^+.

Analytical Procedures. Radioactivity was measured using a Packard TriCarb Liquid Scintillation Spectrometer Model 2405. K^+ was estimated by flame photometry. Cell volume (µl fluid/mg solute-free dry weight (s.f.d.w.)) and K^+ contents (nmol/mg s.f.d.w.) were calculated as previously described (1). The s.f.d.w. of each slice was taken as 0.088 x fresh weight (1). Results were expressed as mean ± s.e.m. (n). Statistical comparisons were made on the basis of Student's unpaired t-test, with $P<0.05$ or better being considered significant.

RESULTS

Efflux of $^{86}Rb^+$ from inner medullary slices can be quantified as two mono-exponential curves (2). The initial rapid washout probably represents mainly loss of $^{86}Rb^+$ from the extracellular compartment, and will not be further considered here. The 2nd, slower, phase is regarded as reflecting cellular loss.

The results of the present study are shown in Table 1, and may be summarized as follows: (i) When slices were incubated in media of 720 mosmol/kg (containing 203 mM Na^+ - see Methods and Materials) the addition of AIB or R56865 had no significant effect on any of the 3 variables measured (lines A, B and C in Table 1): (ii) In media of 2000 mosmol/kg (400 mM Na^+) the presence of AIB led to (a) a marked increase in the rate of $^{86}Rb^+$ efflux ($P<0.001$), (b) moderate cell shrinkage ($P<0.005$), and (c) loss of cell K^+ ($P<0.001$) (comparisons made between data in lines B and D): (iii) If R56865 was incorporated in the medium, (a) this

TABLE 1. Rate constants for the 2nd phase of $^{86}Rb^+$ efflux from renal inner medullary slices into media of varying composition, and corresponding steady-state cell volumes and K^+ contents. Concentrations of AIB and R56865 were 10^{-3}M and 5 x 10^{-6}M respectively. Values are mean ± s.e.m.(n).

mosmol/kg H_2O	k ($\times 10^{-4}$ sec^{-1})	Cell volume (µl/mg s.f.d.w.)	Cell K^+ content (nmol/mg s.f.d.w.)
A. 720	1.55±0.03(6)	4.05±0.11(12)	488±15(12)
B. 720 + AIB	1.63±0.02(6)	3.97±0.12(12)	508±17(12)
C. 720 + AIB + R56865	1.59±0.02(6)	3.91±0.11(14)	490±18(15)
D. 2000 + AIB	1.91±0.04(12)	3.42±0.09(12)	424±13(12)
E. 2000 + AIB + R56865	1.57±0.03(12)	4.74±0.21(23)	483±10(23)
F. 2000	1.24±0.02(6)	2.76±0.14(12)	405±14(12)
G. 2000 + R56865	1.29±0.03(6)	2.67±0.16(12)	395±11(12)
H. 2000 +AIB NMG-for-Na^+	2.11±0.03(6)	4.72±0.33(12)	278±14(12)
I. 200 + AIB NMG-for-Na^+ + R56865	2.15±0.04(6)	4.57±0.28(12)	291±10(12)

enhancement of $^{86}Rb^+$ efflux was abolished $P<0.001$, D vs E, not significant (n.s.), C vs E), (b) there was a marked increase in cell volume ($P<0.001$, D vs E, <0.01, C vs E, and (c) there was net restoration of cell K^+ ($P<0.005$, D vs E, n.s., C vs E): (iv) Conversely, when slices were incubated in media of 2000 mosmol/kg from which AIB was omitted (a) not only was $^{86}Rb^+$ efflux markedly depressed ($P<0.001$), with (b) a further decrease in cell volume ($P<0.001$) and (c) no parallel decrease in cell K^+ contents (comparisons D vs F), but furthermore R56865 failed significantly to affect any of these variables (F vs G): (v) When Na^+ was replaced by NMG (in the presence of AIB) (a) efflux of $^{86}Rb^+$ was accelerated, (b) cell volume increased and (c) cell K^+ greatly depressed ($P<0.001$ for each comparison, D vs H) but, as in AIB-free media, R56865 failed significantly to influence any of these variables (H vs I).

DISCUSSION

The inhibitory effect of R56865 on medullary cell $^{86}Rb^+$ efflux, with associated increases in cell volume and K^+ contents, and the finding that these effects are nullified by omission of Na^+ from the bathing medium, suggests for the first time that a non-excitable cell population may contain Na^+-dependent K^+ efflux pathways comparable with those which have been found in excitable cells. Activation appears to occur only in strongly hyperosmotic media, and it has been suggested (1) that loss of K^+ may serve to prevent inner medullary cells from too-rigorously maintaining their volume under conditions in which the whole tissue is required to undergo a marked fall in hydration (viz severe antidiuresis in vivo). Although replacement of Na^+ by NMG caused enhancement of $^{86}Rb^+$ efflux, a possible reason for this may have been increased cell volume due to entry of NMG (13) and activation of volume- (or stretch-) activated K^+ channels such as have been shown in certain renal cells (14). Depression of cell K^+ presumably reflects Na^+-pump deactivation. In the present context, the relevant consideration is that R56865 was ineffective in the presence of NMG. As suggested below, cell volume itself may be a factor influencing $^{86}Rb^+$ efflux, but inspection of the data in Table 1 makes it clear that the rate of efflux is not directly related to volume - e.g. R56865 in media containing both Na^+ and AIB causes these two variables to alter in opposite directions.

A relevant consideration is the level of Na^+ within the inner medullary cells used in this study. This is difficult to measure accurately by chemical means, due to the high but variable amounts which are bound by medullary macromolecules (15). However, in experiments closely resembling those reported here, it was shown that increasing external Na^+ from 203 mM to 400 mM caused internal free Na^+ to rise from 11 to 108 mM (3), thus spanning the concentration (20-40mM) found to activate K^+ pathways in excitable cells (4, 7, 16, 17).

Significant levels of amino acids are believed to be present in renal medullary interstitial fluids (18, 19). The apparent necessity for external AIB as well as Na^+ in activating R56865-inhibitable $^{86}Rb^+$ efflux remains to be explained, because although AIB causes cellular Na^+ entry, presumably by means of Na^+-amino acid cotransport (1), leading to increased cell Na^+ **content**, the **concentration** of Na^+ remains unchanged due to the entry pari passu of osmotically obligated water. A possibility to be investigated is that there exists a critical conjunction between cellular Na^+ concentration and volume (or membrane stretch) which is required to activate K^+ loss, and hence permit demonstration of the inhibitory effect of R56865 on $^{86}Rb^+$ efflux.

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