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A Ca-Dependent K Channel in "Luminal" Membranes from the Renal Outer Medulla

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Summary. This paper describes properties of ^{86}Rb fluxes through K channels in "luminal" membrane vesicles prepared from rabbit renal outer medulla. By measuring ^{86}Rb uptake against an opposing chemical gradient of K ions, using membranes loaded with KCl, a transient accumulation of isotope is observed, which is blocked by Ba ions. This is the behavior expected of a conductive Rb flux through a Ba-sensitive K channel. The ^{86}Rb accumulation is driven by an electrical diffusion potential as shown in experiments using either vesicles loaded with different anions, or an outwardly directed Li gradient with a Li ionophore. The vesicles containing the channel show a cation selectivity with the order Rb > K > Cs > Li > Na > choline. The Ba-sensitive Rb flux is dependent on Ca within the vesicles, with a very high affinity estimated as $K_{0.5}$ 10 to 100 nM. The vesicles appear to be right-side-out. The Ba-sensitive ^{86}Rb uptake is also inhibited by quinine $K_{0.5}$ 30 μM but is insensitive to tetraethyl ammonium ions and apamin. These isotope flux experiments complement electrophysiological experiments in providing independent evidence for the existence of K channels in the luminal surface of cells of this ascending limb of the loop of Henle. The very high Ca affinity suggests that cytoplasmic Ca could play an important role in regulation of transepithelial salt flux in this region of the nephron.

Key Words K channel · Ca activation · renal membranes

Introduction

The mechanism whereby the cells of the mammalian renal thick ascending limb of the loop of Henle (TALH) delivers a dilute fluid to the distal tubule has been studied intensely using isolated TALH tubules and electrophysiological techniques to analyze ion transport pathways (Greger & Schlatter 1983*a*,*b*; Greger, Schlatter & Lang, 1983; Hebert & Andreoli, 1984*b*; Hebert, Friedman & Andreoli, 1984*b*). The entry of NaCl into the TALH cells is facilitated by the working in parallel of an Na,K,Cl-cotransport system and K channel. Exit of Na across the basolateral surface is brought about by the Na/K pump and exit of Cl by a Cl-specific conductance (or, in addition, possibly by a KCl-cotransport system). The most striking of the electrical properties of the TALH is the lumen positive transepithelial potential, which provides a driving force for passive Na reabsorption via relatively Na-selective tight junctions. The sign and magnitude of this transepithelial potential is maintained by the more K-permselective and less Cl-permselective nature of the apical membrane compared to the basolateral membrane. The current through the apical K channel and the chloride current through basolateral Cl channel polarize the epithelium, lumen positive. The putative K channels in the apical membrane can be blocked by Ba ions and such treatment blocks salt reabsorption and eliminates the transepithelial potential. Thus these channels play a key role in the salt absorption process. The net K reabsorbed in this segment of the kidney is at most 10% of chloride reabsorption, and hence the K cycles across the apical membranes.

An important fact is that antidiuretic hormone ADH raises the rate of net salt transport in isolated medulla TALH tubules of mice and rats. It has been suggested that this could involve an increase in the number of functional apical K conductance units and Na,K,Cl cotransport units and or basolateral Cl channels (Hebert, Culpepper & Andreoli, 1981; Hebert & Andreoli, 1984*a*; Greger, 1985).

The present paper is concerned with properties of the apical K channel in membrane vesicles derived from rabbit renal outer medulla, the K conductance being studied by ^{86}Rb flux measurements. This represents an essential first step towards the goal of identification and characterization of the K channel protein.

Two major problems arise in the measurement of isotope fluxes via conductive cation channels in membrane vesicles. Firstly, channels generally have very high turnover rates 10^5 to 10^7 ions per sec and, given the small dimensions of membrane vesicles (0.5 μ diameter) the equilibration time of tracers can be expected to be in a sub-second time range. Secondly, since membrane preparations are

almost always derived from different populations of cells and internal organelles, specific channel densities may be low. Such heterogeneity with respect to the distribution of channels may contribute extensively to nonspecific background isotope fluxes in those preparations. In order to overcome the problem of measuring fast channel-mediated isotope fluxes into heterogeneous membrane populations we have used a simple manual procedure based on the following principles (Garty, Rudy & Karlish, 1983; Garty & Karlish, 1986). The cation isotope flux is measured against a large opposing chemical gradient of the same ion. A diffusion potential, positive outside, is set up across the membranes of the vesicle population of interest due to their selective permeability to the internal cation. The isotope is highly concentrated in those vesicles due to the electrical driving force and high permeability to the isotope. When the chemical gradients and electrical potential dissipate, the isotope flows out again. Because large numbers of isotope atoms accumulate, the uptake usually persists for many minutes. Both selective isotope concentration and the extended time-course greatly magnify the sensitivity of measurements of the selective isotope flux. For a more detailed discussion of the principles involved and alternate ways of imposing the diffusion potential *see* Garty and Karlish (1986).

In a crude membrane preparation from renal outer medulla the vesicle volume bounded by apical membranes of TALH cells can not be more than a few percent of the total vesicle volume. Thus we reasoned that this assay procedure would be applicable in this preparation.

In a recent paper we have described partial separation on metrizamide density gradients of "luminal" membranes containing the K channel and Na,K,Cl-cotransporter from basolateral membranes containing Na/K pumps, and the reconstitution into phospholipid vesicles of both K channel and Na,K,Cl-cotransporter (Burnham, Karlish & Jorgensen, 1985). The present experiments have been performed using both crude membrane preparations from the outer medulla as well as preparations enriched in "luminal" membranes. Physical separation of "luminal" membranes from other membranes complements the isotope assay procedure in increasing sensitivity of the K channel-mediated flux due to removal of background isotope permeation pathways.

Materials and Methods

Preparation of Vesicles

Crude microsomes from rabbit kidney outer medulla were prepared by differential centrifugation (Jorgensen, 1984) after homogenizing the dissected tissue in a solution containing (in mM): 250 sucrose, 50 KCl, 2 $MgCl_2$, 1 EGTA, and 10 MOPS, adjusted to pH 7.2 with Tris base. Medullary membrane vesicles enriched in luminal membranes were prepared from crude microsomes as we described recently (Burnham et al., 1985) by centrifuging on a 5 to 15% density gradient of Metrizamide at 25,000 rpm, overnight, in a Beckman SW41 rotor. The Metrizamide was dissolved in a solution containing 250 mM sucrose, 50 mM KCl, 1 mM EGTA, and 25 mM Imidazole, adjusted to pH 7.2 with glacial acetic acid. Variations in the buffer components are noted in the figure legends.

Isotope Flux Measurements

^{86}Rb uptake was measured essentially as described in (Garty et al., 1983; Garty & Karlish, 1986) using columns of Tris-equilibrated Dowex 50 × 8 to exchange external K for Tris immediately prior to the flux assay, and separate vesicles containing isotope from the radioactive medium upon termination of the flux assay. Prior to use the columns were washed first with 500 μl of sucrose 350 mM containing 25 mg/ml bovine serum albumin. The albumin-treated Dowex columns were cooled to 4°C before use. A small volume of microsomes (150 to 200 μ 1, 2 to 5 mg protein/ml) was first placed onto a Dowex column. 350 mM sucrose, 4°C was added after the microsomes had fully entered the resin. The volume of sucrose added was adjusted such that the sum of the volume of vesicles and sucrose was slightly less than the void volume of the column, about 600 μl. The Dowex column was then placed in a clean dry Eppendorf tube, and another 600 μl of 350 mM sucrose (4°C) was added to the column in order to collect the microsomes. By this means, the microsomes were diluted approximately threefold and were suspended in an essentially K-free medium, consisting largely of sucrose and Tris chloride. The Na/K pump was blocked by adding ouabain, $MgCl_2$, and Tris vanadate such that the final concentrations were 500 μM, 2 mM and 100 μM, respectively. Usually, the ouabain, Mg, and vanadate were added to the vesicles prior to the first Dowex treatment, and were allowed to bind for 30 min at room temperature. In some experiments the ouabain, Mg, and vanadate were added after the first, Dowex, immediately prior to the addition of ^{86}Rb. ^{86}Rb uptake was initiated by taking a portion of the vesicles and adding them to buffered $^{86}RbCl$. The usual practice was to add three parts vesicles to one part reaction mixture at room temperature, 20 to 22°C. The final concentrations of components in standard assay conditions were RbCl + ^{86}Rb, 100 to 500 μM, ~ 1 μCi per tube, MOPS (Tris) 10 mM, pH 7.2; Tris · HCl (from the Dowex column) 10 to 12 mM, pH 7.2; sucrose, 250 mM; furosemide, 1 mM and $BaCl_2$ when present, 2 mM. In initial experiments $MgCl_2$ was added to tubes not containing $BaCl_2$. Furosemide was usually included in the reaction mixture to increase the sensitivity of the flux measurements by blocking Na,K,Cl-cotransport (Burnham et al., 1985). A furosemide stock solution was made as 100 mM furosemide and 200 mM Tris base. The ^{86}Rb uptake was stopped by removing 120 to 150 μl of the mixture of vesicles and ^{86}Rb, and pipetting them onto a second Dowex column placed in a scintillation vial. Ice-cold 350 mM sucrose (1.5 ml) was then added to collect the vesicles in the vial.

^{86}Rb was measured by its Cerenkov radiation. Experiments to look at ^{137}Cs or ^{22}Na fluxes were performed exactly like ^{86}Rb flux experiments. ^{137}Cs and ^{22}Na were measured by liquid scintillation counting. In some experiments protein was determined, using the method of Bradford (1976), and results were expressed in terms of pmol Rb taken up per mg protein per time. Protein was always determined in experiments where different preparations of vesicles were to be compared. Ca/EGTA buffers were

calculated according to Pershadsing and McDonald (1980) using a Hewlett–Packard 15C programmable calculator. EGTA ("puriss" grade from Fluka) was used without correction for possible errors in assay by Fluka.

Materials

Dowex 50 × 8, mesh 50 to 100 and quinine sulfate were obtained from Fluka. Li-ionophore AS701 was a gift from A. Shanzer, Organic Chemistry Department, Weizmann Institute, Apamin, Metrizamide and Furoesmide were obtained from Sigma. ^{86}Rb, ^{137}Cs and ^{22}Na were obtained from the Radiochemical Centre, Amersham, U.K. or N.E.N., U.S.

Abbreviations

Thick ascending limb of the loop of Henle, TALH; antidiuretic hormone, ADH; 4-Morpholine propanesulfonic acid, MOPS; Ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′tetracetic acid, EGTA; Tris(hydroxymethyl) amino methane, Tris; N,N′-diheptyl-N,N′-didiethylether, 5,5 dimethyl-3,7 dioxanonane diamide, AS701; Tetraethyl ammonium, TEA; (2-3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido-2-deoxy-D-glucose, Metrizamide.

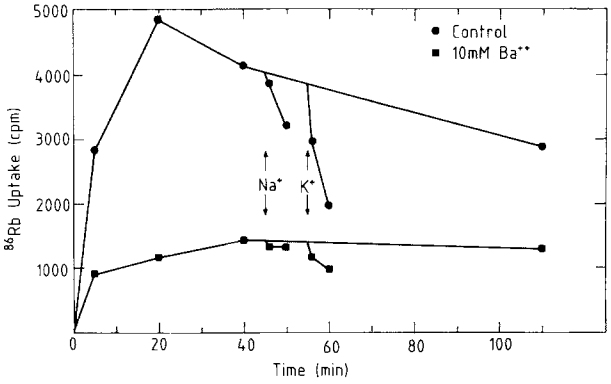

Fig. 1. Time course of ^{86}Rb uptake crude microsomes from outer medulla. Effect of Ba. Crude medullary membrane vesicles, made as described in Materials and Methods, were diluted to a protein concentration of 6.5 mg per ml. 200 μl were passed through a Dowex (Tris) column and collected in 750 μl. 250 μl of ^{86}Rb uptake mixture were added such that the final concentrations of the ingredients were 100 μM RbCl (10 μ Ci/ml), 4 mM $MgCl_2$, 500 μM ouabain, 100 μM Tris vanadate, 10 mM MOPS (Tris) pH 7.2, with (■) or without (●) 10 mM $BaCl_2$. ^{86}Rb uptake was stopped by transferring 100 μl of suspension to a second Dowex column. At the times indicated by the arrows, 240 μl of vesicles and ^{86}Rb were added to 60 μl of 250 mM NaCl or KCl. One minute and five minutes thereafter, 125 μl of this mixture were removed and placed on a Dowex column. The larger volumes (125 μl instead of 100 μl) were used to compensate the dilution of the protein by the salt solution

Results

Figure 1 shows the time-course of ^{86}Rb uptake into crude outer medulla membrane vesicles loaded with KCl, in the absence or presence of Ba ions. In the absence of Ba the uptake of the isotope against the opposing K concentration gradient rose to a peak value at about 20 min and then decreased gradually towards the equilibrium value. In the presence of Ba, isotope uptake was much reduced and the transient isotope-accumulation phenomenon was not observed. These were the features expected for a conductive ^{86}Rb flux via a Ba-inhibited K channel. The maximal level of ^{86}Rb accumulation was three- to fourfold greater than the equilibrium level in the presence of Ba, when the opposing chemical gradient K_{in}/Rb_{out} was about 500-fold. Hence specific channel-mediated ^{86}Rb accumulation was presumably only occurring into a small fraction of the internal vesicle space. The effects of adding K or Na to the medium and inducing Rb efflux (Fig. 1) are discussed below in relation to cation selectivity. The experiment presented in Fig. 1 demonstrates the resolving power of the assay with a crude membrane preparation. Similar phenomena can be observed in preparations enriched with the luminal membranes and the reconstituted proteoliposomes, and here the degree of inhibition of the ^{86}Rb uptake rate by Ba is invariably greater than in crude preparations (*see* Burnham et al., 1985).

Electrical Nature of Rb Uptake

Tables 1 and 2 provide further evidence that the Ba-inhibited ^{86}Rb accumulation is driven by an electrical potential. In the experiment in Table 1 vesicles were prepared containing 25 mM K_2SO_4, 50 mM KCl, or 50 mM KSCN and then Ba- or quinine-sensitive ^{86}Rb uptake was measured over 5 min by the standard procedure. Quinine inhibits the K channel-mediated flux, just like Ba (Fig. 4). This is fortunate, since Ba cannot be used in sulfate media due to precipitation of $BaSO_4$. As seen from Table 1 the rate of Ba- or quinine-sensitive Rb uptake was highest for sulfate, intermediate with the standard chloride medium, and lowest with thiocyanate. The result can be explained simply as follows. It is expected that the passive permeability of the vesicle membrane for the divalent anion SO_4 will be lower than for Cl while the permeability of the relatively lipid-soluble SCN will be greater than for Cl. The K diffusion potential should therefore be progressively short-circuited by the anion of increasing permeability, i.e., in the order SCN > Cl > SO_4, and hence the rate of the ^{86}Rb uptake should follow the observed order SCN < Cl < SO_4. The Ba- or quinine-insensitive flux was not affected by anion and is therefore probably electroneutral.

Table 1. Effect of different anions on Ba-inhibited ^{86}Rb uptake[a]

Anion	Control	+Ba	+Quinine	Ba-sensitive	Quinine-sensitive
	(^{86}Rb Uptake-cpm per 5 min ± SEM)				
SO_4	4408 ± 207	—	2590 ± 45	—	1818 ± 212
Cl^-	3752 ± 133	2733 ± 86	2533 ± 19	1019 ± 158	1219 ± 154
SCN^-	3073 ± 115	2420 ± 67	2269 ± 23	653 ± 71	804 ± 117

[a] Crude medullary microsomes were prepared as usual except that the medullae were homogenized in a buffer containing 50 mM KCl, 25 mM K_2SO_4, or 50 mM KSCN as indicated. ^{86}Rb uptake was measured in triplicate over 5 min as described in Materials and Methods. 100 μM quinine sulfate or 2 mM $BaCl_2$ were added as indicated. Aliquots of the different vesicles applied to the first Dowex column contained equal quantities of protein.

Table 2. Ba-sensitive ^{86}Rb uptake driven by a lithium diffusion potential[a]

Cation outside	Li ionophore AS701	Control	+Ba	Ba-sensitive
		(^{86}Rb uptake cpm per 5 min)		
Tris	−	1146	1003	143
Tris	+	1602	1202	400
Li	−	704	625	79
Li	+	884	817	67

[a] Crude medullary microsomes were prepared as usual except that the homogenizing buffer contained LiCl, 50 mM instead of KCl. ^{86}Rb uptake was measured for 5 min without or with Ba, in the standard reaction medium with addition of Tris HCl or LiCl to a final concentration of 50 mM. Vesicles were treated with Li ionophore AS701, 125 μM, or 2 μl of ethanol for 1 min prior to mixing with the ^{86}Rb reaction medium. Results are expressed as means of radioactivity taken up in duplicate determinations, the difference between the duplicates being in all cases equal or lower than ten percent.

Table 2 gives details of three similar experiments showing that it is also possible to detect Ba-sensitive ^{86}Rb uptake when the diffusion potential is generated by a Li-specific ionophore, AS701 (Shanzer, Korenstein & Samuel, 1983), and a Li gradient. AS701 is a carrier-type ionophore of the conductive type, like valinomycin. Li is conducted well, and Na to a small degree, but Rb and K are conducted very poorly and hence ^{86}Rb fluxes are measured conveniently in the presence of AS701 (Margalit & Shanzer, 1982). Membrane vesicles loaded with LiCl were passed through a Tris-equilibrated Dowex column as usual, and aliquots were incubated for 60 sec with ionophore, or with ethanol 1% and Rb uptake was then measured in media containing 50 mM Tris-HCl or 50 mM LiCl in addition to the standard components. In the Tris media some Ba-sensitive ^{86}Rb uptake was observed without ionophore, but addition of the ionophore increased the rate by about threefold. In the Li medium, i.e. in the absence of a Li gradient, a very small Ba-sensitive ^{86}Rb uptake was detectable but addition of the ionophore did not affect this rate. The experiment demonstrates conclusively that the Ba-sensitive Rb flux is driven by an electrical diffusion potential. The result also excluded the theoretical possibility that ^{86}Rb accumulation driven by an opposing K gradient as in Fig. 1 is caused by $K/^{86}Rb$ exchange on a carrier mediated K-transport system (i.e. countertransport).

Cation Selectivity

Figure 1 and Tables 3 and 4 provide information on the cation selectivity of the membranes containing the Ba-sensitive K channel. The rate of Ba-sensitive ^{86}Rb uptake is a function of the product of the Rb permeability through the channel and the electrical driving force, and so one must distinguish between effects of agents which reduce the flux by blocking the channel and those which reduce the driving force, such as ions which permeate the membranes. This distinction is made in Fig. 1 showing release of accumulated ^{86}Rb upon addition of K or Na to the medium. Addition of KCl, 50 mM in the absence of Ba caused a rapid release of the accumulated ^{86}Rb, while addition of NaCl, 50 mM, caused a slower release. Efflux of the isotope occurs because the electrical potential driving ^{86}Rb accumulation is rapidly dissipated by addition of external cation. Both cations dissipate the K diffusion potential, but K is seen to be more permeable than Na. The selectivity to cations was tested in a different way in Table 3 by preparing vesicles to contain different cations and looking at the ability of gradients of the different cations to sustain Ba-sensitive ^{86}Rb accumulation. The observed ranking order Rb > K > Cs > Li = Na > choline is a reflection of the decreasing electrical potential driving ^{86}Rb accumulation and therefore of the relative permeability of the membranes of interest to these cations. The result could mean that the K channels themselves show this order of

Table 3. Ba-sensitive ^{86}Rb uptake into membrane vesicles loaded with different monovalent cations[a]

Internal cation 50 mM	Control	+Ba	Ba-sensitive
	(Rb uptake-pmol/5 min/mg protein ± SEM)		
Rb	1800 ± 28	960 ± 20	840 ± 34
K	1760 ± 5	1030 ± 14	730 ± 15
Cs	1410 ± 130	850 ± 5	560 ± 130
Li	1110 ± 26	660 ± 5	450 ± 29
Na	910 ± 30	510 ± 5	400 ± 30
Choline	650 ± 18	420 ± 7	240 ± 19

[a] Crude microsomes containing 50 mM chloride salts of K, Rb, Cs, Li, Na or choline were prepared in the standard way. Ba-sensitive ^{86}Rb uptake was measured in triplicate for 5 min as described in Materials and Methods. Results are expressed as pmol Rb uptake per mg protein per 5 min ± SEM.

Table 4. A comparison of the effect of Ba ions on uptake of ^{86}Rb, ^{137}Cs and ^{22}Na into membrane vesicles loaded with KCl[a]

	Control cation uptake	+Ba	Ba-sensitive
		(pmol/5 min/mg protein ± SEM)	
^{86}Rb	560 ± 49	292 ± 2	264 ± 49
^{137}Cs	230 ± 14	123 ± 2	107 ± 15
^{22}Na	144 ± 1	91 ± 1	53 ± 2

[a] Crude microsomes containing KCl were prepared in the standard way. Uptake of ^{86}Rb, ^{137}Cs, or ^{22}Na was measured in triplicate in standard conditions except that the media contained, respectively, RbCl + ^{86}Rb, 0.1 mM + 3×10^5 cpm per sample, or CsCl + ^{137}Cs, 0.1 mM + 5.5×10^5 cpm per sample or NaCl or ^{22}Na, 0.1 mM + 3.4×10^5 cpm per sample.

selectivities, but it is more probable that the K channels show a higher selectivity to K than to say Na, and in addition the membranes are somewhat selective for all cations compared to anions, by pathways other than the K channel. The Ba-insensitive flux shows a similar but lower degree of cation selectivity than the Ba-sensitive flux. The nature of this Ba-insensitive flux is unknown. In a complementary experiment (Table 4) the ability of a K diffusion potential to sustain Ba-sensitive uptake of ^{86}Rb or ^{137}Cs or ^{22}Na uptake was compared, and again the order of ranking was Rb > Cs > Na. This gives an indication of the relative order of permeabilities of Rb, Cs and Na through the K channel but again not necessarily in a quantitative way. For example, ^{22}Na could be permeating partially or entirely through a nonselective pathway, but Ba, by blocking the K channel, should reduce the K diffusion potential which is driving ^{22}Na uptake. ^{137}Cs showed a higher rate of Ba-sensitive uptake and hence Cs is presumably able to penetrate the K channel to some extent, a conclusion reached also on the basis of the experiment in Table 3. This is of interest for many K channels are blocked by Cs (Latorre & Miller, 1983). In summary the results of Fig. 1 and Tables 3 and 4 demonstrate that the membranes of interest are more permeable to K than to Na as expected from the presence of K channels, the observed order of cation selectivities reflecting the properties both of the K channels themselves and those of the membranes in which the channels are embedded.

Ca-Dependent of K-Channel Activity

Because the K channel in the thick ascending limb cells is though to be involved in regulation of transepithelial salt transport (Hebert & Andreoli, 1984*b*; Greger, 1985), we have examined the channel-mediated ^{86}Rb flux for Ca dependence, cytoplasmic Ca being a likely candidate for a physiological regulator. These experiments and those in the next section have been conducted with preparations enriched in luminal membranes.

It was found that addition of the Ca-ionophore A23187 to vesicles suspended in a medium containing 1 mM EGTA, caused a reduction in the Ba-sensitive ^{86}Rb uptake (line 2 of expt. 301084, line 3 of expt. 151084). Addition of 1 mM Ca to the medium in the presence of the EGTA and A23187 reactivates the Ba-sensitive ^{86}Rb flux either to the same level (line 3 of expt. 301084) or even higher than that in absence of ionophore (line 4 of expt. 151084). Addition of 1 mM Ca in the absence of the ionophore has no effect (line 2 of expt. 151084). Ba-insensitive ^{86}Rb uptake was hardly affected by the presence or absence of A23187, Ca or EGTA. These findings are consistent with the notion that the K channel is activated by Ca at the inside surface of the vesicles. Under normal conditions sufficient Ca must be trapped within the vesicles during homogenization of the renal tissue to fully activate the K channels. The combination of A23187 and EGTA reduces the concentration of Ca within the vesicles, the ionophore being necessary for egress of the Ca. The lack of effect of EGTA or Ca in the absence of ionophore, and the absence of requirement of ionophore for a full Ba effect suggest that the vesicles containing the K channel are essentially all oriented right-side-out (Ba being effective at the extracellular surface of the kidney tubule). The reversibility of the activation by Ca argues for a direct effect of Ca on the K channel and against Ca dependence of an enzyme which acts upon the K-channel protein and activates it.

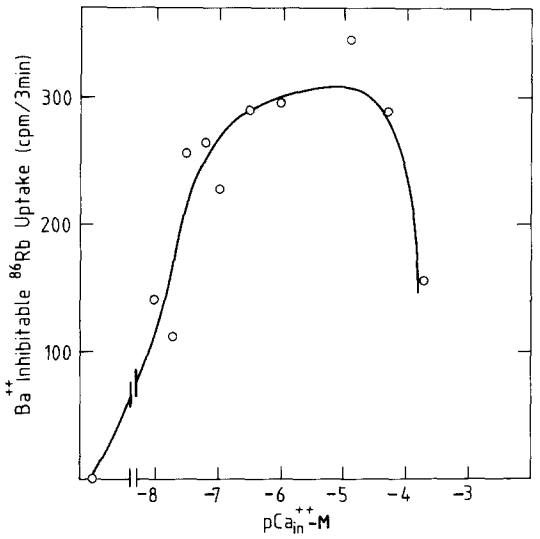

Fig. 2. Effect of different free Ca concentrations on Ba-sensitive ^{86}Rb uptake. Medullary membrane vesicles enriched in luminal membranes were incubated for 30 min of room temperature with 1 μM A23187, EGTA (Tris), 3 mM, 500 μM ouabain 2 mM $MgCl_2$, and 100 μM vanadate (Tris). Then, the vesicles were passed through a Dowex column. It was assumed that all of the EGTA passed through the column, and was diluted threefold. EGTA or Ca/EGTA buffered to pH 7.2 with Tris were readded to give an EGTA concentration of 3 mM and a total added calcium concentration ranging from 0 to 3.2 mM. The calcium was allowed to equilibrate for 5 min at 22°C. The vesicles were than mixed with an ^{86}Rb uptake mixture also containing 3 mM EGTA and the same total Ca concentrations. In addition, 1 mM furosemide and 2 mM $MgCl_2$ were included in the uptake medium. Duplicate determinations of ^{86}Rb uptake were made over 5 min at each Ca concentration, with and without 5 mM $BaCl_2$. The results are expressed as the difference between ^{86}Rb uptake in the absence and in the presence of Ba. Agreement between duplicate samples was better than ten percent

Following this qualitative demonstration of activation by Ca we have attempted to titrate the requirement for cytoplasmic Ca in a more quantitative fashion, using Ca/EGTA buffer plus ionophore A23187, (Fig. 2). First, a preliminary experiment showed that if 3 mM EGTA is used rather than 1 mM EGTA as in Table 5, addition of A23187 without Ca causes Ba-sensitive ^{86}Rb uptake to entirely disappear. For the experiment in Fig. 2 luminal vesicles were thus incubated with 3 mM EGTA + A23187, and were then passed over the first Dowex column as usual. The vesicles were then mixed with the Ca/EGTA buffer, consisting of 3 mM EGTA and total Ca calculated to give free Ca levels of zero (no added Ca) or 10^{-8} to 10^{-4} M in the conditions of the reaction mixture (*see* legend), and Ba-sensitive ^{86}Rb uptake was measured.

The result is complicated by uncertainties and the correctness of a number of assumptions which

Table 5. Stimulation of Ba-sensitive ^{86}Rb uptake by Ca within the membrane vesicles[a]

Expt.	Ca_o	A23187	Control	+Ba	Ba-sensitive
			(Rb uptake-pmol/mg/3 min)		
	−	−	1087	680	407
301804	−	+	675	580	95
	+	+	1054	707	348
151084	−	−	978	706	272
	+	−	1018	724	294
	−	+	883	698	185
	+	+	1108	715	388

[a] Membrane vesicles prepared in the standard way were centrifuged on Metrizamide gradients, and the light fractions used for this experiment (Burnham et al., 1985). EGTA (Tris) 1 mM was added to the vesicle suspension which was then divided into groups to which Ca, 1 mM or A23187, 1 μM (or 1% ethanol) were added as indicated. The vesicles were then incubated for 30 min at room temperature. Aliquots of vesicles were then passed over Dowex columns and sufficient Ca and EGTA were re-added to make final concentrations in the assay, 1 mM, and then ^{86}Rb was measured in the standard way over 3 min.

are difficult to test independently (*see below*). Therefore the titration curve drawn in Fig. 2 can only be considered to give a very rough estimation of the cytoplasmic Ca affinity. Nevertheless the result is quite clear: the Ba-inhibited ^{86}Rb flux is entirely dependent on intravesicular Ca. Ca activates with a very high affinity, at what is normally a cytopasmic oriented site. At high concentrations of Ca there is some inhibition.

The first and most important assumption in the experiment is that in the presence of ionophore the concentrations of free Ca inside and outside the vesicles are equal. A23187 is known to catalyze an exchange of Ca for H which is thought to be largely if not wholly electroneutral, i.e. 1 Ca : 2 H (Pfeiffer, Taylor & Lardy, 1978). For electroneutral exchange the equilibrium ratio of $Ca_{in}/Ca_{out} = (H_{in}/H_{out})^2$. However the kidney vesicles should not be able to maintain a pH gradient due to the presence of high concentrations of Tris which acts as a proton carrier, and in this situation $Ca_{in} = Ca_{out}$. The buffering effect of EGTA inside the vesicles (2 H + CaEGTA $\rightleftharpoons$ Ca + H_2 EGTA) is probably important here. Ca entering via A23187 should liberate 2 H which should exchange for Ca on the A23187. If, however, the ionophore catalyzes a 1 : 1 exchange, Ca : H, the equilibrium level of Ca will be a function of the membrane potential and will be underestimated in our conditions. This will distort the curve to the left of the true Ca activation curve. A second problem arises due to the fact that the electrical potential driving ^{86}Rb uptake must, of course, be lower at sub-optimal concentrations of internal Ca than at

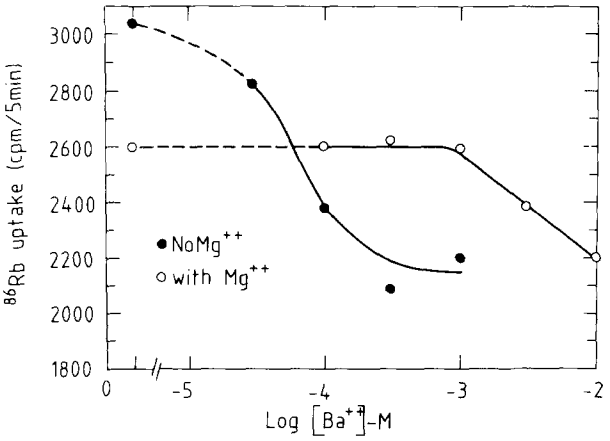

Fig. 3. Dose-response curves for inhibition by Ba of ^{86}Rb uptake. Medullary membrane vesicles enriched in luminal membranes, were incubated with 500 μM ouabain, 2 mM $MgCl_2$, and 100 μM vanadate before passage through a Dowex column. A reaction mixture was added such that the final concentrations of the ingredients were: 195 μM ^{86}RbCl, 11 mM MOPS (Tris), pH 7.2, 1.1 mM furosemide, and the indicated $BaCl_2$ concentration. One set contained 2 mM MgCl (○), the other did not (●). ^{86}Rb uptake was allowed to continue for 5 min when the reaction was stopped as usual. The points represent the means of duplicate determinations

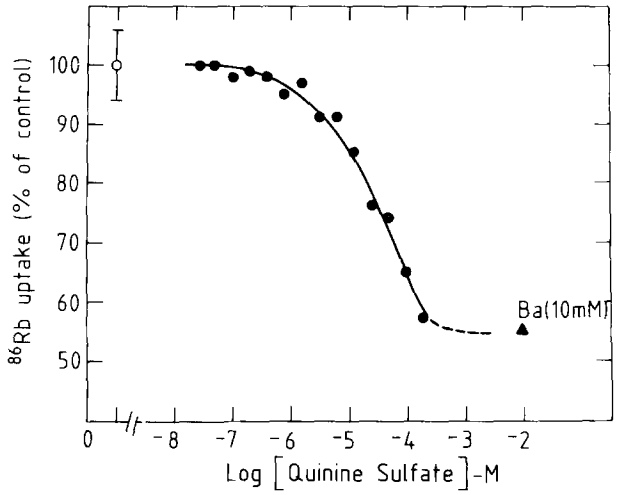

Fig. 4. Dose-response curve for inhibition by quinine of ^{86}Rb uptake. Medullary membrane vesicles enriched in luminal membranes were incubated with 1 μM A23187, 500 μM ouabain, 2 mM $MgCl_2$ 100 μM Vanadate (Tris) and 1 mM Ca/EGTA. After passage through a Dowex column, aliquots were added to an ^{86}Rb reaction mixture such that the final concentration of the ingredients was: 200 μM RbCl, 2 mM $MgCl_2$, 10 mM MOPS (Tris), pH. 7.2, 19 mM choline chloride, 1 mM Ca/EGTA, 500 μM ouabain, 1 mM furosemide, and the indicated concentrations of quinine sulfate or $BaCl_2$. Uptake was allowed to continue for 3 min, when the reaction was stopped, and the ^{86}Rb measured as usual. The results are calculated as percent of control and represent single determinations except for the control which is the mean ± SEM of five determinations

saturating levels. Neglect of this fact leads to underestimation of the flux at sub-optimal Ca and produces a rightward distortion of the curve.

Due to the heterogeneity, even of the "luminal" vesicle preparations there does not seem to be any simple way of taking these factors into account, say by direct measurement of the membrane potential. In view of this situation it seems prudent to estimate a range of Ca concentrations about 10 to 100 nM for half-maximal activation.

Inhibitors

The evidence for Ca-dependence of the Rb uptake suggested that it would be worthwhile to screen for inhibitory effects of agents known to block Ca-dependent K channels in other cells (Lew & Ferreira, 1978). Does-response curves for Ba ions in the absence or presence of Mg ions are shown in Fig. 3. In the absence of Mg, Ba inhibited the flux with a $K_{0.5}$ of 50 to 100 μM. 10 mM Mg itself appears to be a weak channel blocker, but it has a striking effect in reducing the apparent affinity for Ba. The cinchona alkaloids quinine and quinidine have been shown to be quite potent inhibitors of the Ca-dependent K channel in red cells and other cells (Lew & Ferreira, 1978; Burgess, Claret & Jenkins, 1981). Figure 4 shows the effects of increasing concentrations of quinine sulfate. Inhibition of ^{86}Rb uptake to the same level as 10 mM Ba was achieved with 200 μM quinine, ($K_{0.5} \sim 30$ μM). In a separate experiment it was found that a mixture of quinine (200 μM) and Ba (1.8 mM) gave no more inhibition than either inhibitor alone, the concentration of either inhibitor alone being sufficient for a maximal effect (*not shown*). This indicated that the Ba and quinine were interacting with the same system, i.e., the Ca-dependent K channel. Tetraethyl ammonium (TEA), 15 mM, added to the medium had no effect on the Ba-sensitive ^{86}Rb flux. This was not necessarily surprising for different K channels show wide variations in their sensitivity to TEA, and the TEA may also have to obtain access to the inner surface of the cells (Latorre & Miller 1983). A result of more interest was that the bee venom apamin was also ineffective in blocking the Ba-sensitive ^{86}Rb flux even when added to the medium at a concentration of 10 μM. Apamin is a potent blocker of Ca-dependent K channels in liver (Burgess et al., 1981) and brain (Forset et al., 1984) at nanomolar concentration but it is ineffective against K channels of human erythrocytes (Burgess et al., 1981) and MDCK cells (Brown & Simmons, 1982).

Discussion

The ^{86}Rb flux measurements presented here illustrate the utility of the assay procedure for detecting the functioning K channel in a very minor component of the vesicle population. The K channel is a transport "enzyme" and the specific value of the flux measurement is therefore that it provides a very simple biochemical screening assay for (a) inhibitors and activators of the channel, (b) location of the channel in different vesicle populations and (c) detection of function of reconstituted channels (Burnham et al., 1985). The latter is an essential prerequisite for isolating the K-channel protein, a task now being undertaken.

The findings of a conductive ^{86}Rb flux, inhibition by Ba and relative selectivity of the luminal vesicles to K compared to Na are consistent with published electrophysiological measurements demonstrating a Ba-inhibited K-selective channel in the luminal surface of the TALH cells (Greger, 1985).

Ca Dependence

The requirement for Ca within the vesicles and the high Ca affinity for activating Rb flux (Table 5 and Fig. 2) suggest strongly that we are looking at a cytoplasmic Ca binding site in vesicles oriented right-side-out. If the Ca-dependent K channel rectified, so that ^{86}Rb could move in only one direction or faster in one than the other direction, the conclusion concerning the orientation of the luminal vesicles would not be valid, and a random vesicle orientation would not be excluded. Since the assay procedure itself depends on permeation of K from one face and of ^{86}Rb from the other face of the channel, strict rectification is ruled out. More complicated flux interactions including partial rectification or single file effects cannot be excluded, but in this case a higher permeability, of ^{86}Rb into right-side compared to inside-out vesicles would be at least partially compensated by a higher K permeability out of the inside-out vesicles and thus a higher driving force for ^{86}Rb uptake. It therefore seems more likely that the simple conclusion concerning vesicle orientation from the experiments of Table 5 and Fig. 2 is correct. It is worth pointing out that basolateral membranes from dog kidney outer-medulla isolated using density gradients of Hypaque (3,5-diacetamido-2,4,6-triiodobenzoate) have also been shown to be oriented right-side-out (Forbush, 1982).

Inhibition of K channels by high concentrations of Ca (Fig. 2) has also been observed in squid axon (Eaton & Brodwick, 1980) and chromaffin cells (Marty, 1981).

Ca-dependent K channels have now been identified in many cell types including red cells, neurones, muscle, endocrine and exocrine glands (Petersen & Maruyama, 1984). The use of patch-clamp technique has allowed workers to distinguish three different categories of Ca-activated K channels two of which are K selective and voltage sensitive, but differ in the single-channel conductance (low, 20 pS and high, 150 to 200 pS) and a third class which appears to be voltage-insensitive and unselective to cations. ^{86}Rb flux measurements provide very little information on single-channel properties, which is necessary in order to assign the renal K channel to one of these classes. One hint that the renal channel may bear similarity to the erythrocyte K channel is the finding (Table 1) that the renal luminal vesicles are at least as permeable to Rb as to K. Petersen and Maruyama (1984) have pointed out that high conductance channels, typical of exocrine and endocrine glands and neurones discriminate between K and Rb ions in favor of K. This is not the case with the erythrocyte Gardos channel (Schwartz & Passow, 1983). The lack of sensitivity of the renal K channel to apamin does not distinguish between the classes for both erythrocyte (low conductance) (Burgess et al., 1981) and muscle (high conductance) (Romey & Lazdunski, 1984) K channels are insensitive to the peptide, while the liver K channel is sensitive (Burgess et al., 1981). Ba and quinine appear to inhibit all types of Ca-activated K channels (Lew & Ferreira, 1978; Schwartz & Passow, 1983).

Physiological Role of the Ca-Dependent K Channel

The finding of Ca dependence with a very high affinity for Ca, is highly suggestive of a role for cytoplasmic-free Ca in controlling the apical K conductance in intact TALH cells. One important role of the apical K conductance is maintenance of the lumen positive transepithelial potential which provides the driving force for the passive component of Na reabsorption (Hebert & Andreoli, 1984*b*; Greger, 1985). In addition, activation of apical K channels and K efflux could lead to a significant rise in the K concentration of the tubular fluid thereby increasing the driving force for net Na, Cl and K entry into the TALH cells via the Na,K,Cl-cotransport system. This is similar to the mechanism proposed for secretory epithelia (Petersen & Maruyama, 1984).

Antidiuretic hormone (ADH) or permeable cAMP analogues have been shown to raise the transepithelial electrical potential and net salt reabsorption in isolated tubules from mouse and rat kidney (Hebert et al., 1981). Recently, these effects

have been suggested to derive from an increase in the density or unit conductance (or both) of the K channels (Hebert et al., 1984). If cytoplasmic Ca is a physiological effector, the hormone could cause cytoplasmic-free Ca concentrations to rise, or the sensitivity of the K channels to Ca to rise without a change in Ca concentration. Either of these events effectively increase the density of functional K channels provided that Ca activation is not complete in the absence of hormone. However, this simple scenario can be only one part of a complex picture, for ADH effects involve cAMP-dependent phosphorylation of target proteins, and these are unknown (Andreoli & Schafer, 1976).

Conclusion

Characterization of the K-channel mediated ^{86}Rb fluxes represents important steps towards identification and purification of the K-channel protein and the longer-term objective of studying regulation of salt transport across renal tubules.

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