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Identification and reconstitution of a $Na^+/K^+/Cl^-$ cotransporter and K^+ channel from luminal membranes of renal red outer medulla

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Electrophysiological studies on renal thick ascending limb segments indicate the involvement of a luminal $Na^+/K^+/Cl^-$ cotransport system and a K^+ channel in transepithelial salt transport. Sodium reabsorption across this segment is blocked by the diuretics furosemide and bumetanide. The object of our study has been to identify in intact membranes and reconstitute into phospholipid vesicles the $Na^+/K^+/Cl^-$ cotransporter and K^+ channel, as an essential first step towards purification of the proteins involved and characterization of their roles in the regulation of transepithelial salt transport. Measurements of $^{86}Rb^+$ uptake into membrane vesicles against large opposing KCl gradients greatly magnify the ratio of specific compared to non-specific isotope flux pathways. Using this sensitive procedure, it has proved possible to demonstrate in crude microsomal vesicle preparations from rabbit renal outer medulla two $^{86}Rb^+$ fluxes. (A) A furosemide-inhibited $^{86}Rb^+$ flux in the absence of Na^+ (K^+-K^+ exchange). This flux is stimulated by an inward Na^+ gradient (Na^+/K^+ cotransport) and is inhibited also by bumetanide (B) A Ba^{2+}-inhibited $^{86}Rb^+$ flux, through the K^+ channel. Luminal membranes containing the $Na^+/K^+/Cl^-$ cotransporter and K^+ channels, and basolateral membranes containing the Na^+/K^+ pumps were separated from the bulk of contaminant protein by metrizamide density gradient centrifugation. The $Na^+/K^+/Cl^-$ cotransporter and K^+ channel were reconstituted in a functional state by solubilizing both luminal membranes and soybean phospholipid with octyl glucoside, and then removing detergent on a Sephadex column.

Introduction

The loop-diuretic sensitive $Na^+/K^+/Cl^-$ cotransport system has been detected and studied in various cells [1–8] In kidney, transepithelial NaCl transport across the cells of thick ascending limbs of the loop of Henle has been studied by electrophysiological techniques using isolated tubules [1,2] It is thought to involve the parallel working of an diuretic-sensitive $Na^+/K^+/Cl^-$ cotransporter and K^+ channel at the luminal face and the Na^+/K^+ pump and a net Cl^- conductance at the basolateral surface of the cells [1] Little or no net K^+ reabsorption occurs in the thick ascending limbs and thus K^+ effectively recycles across the luminal membranes of the cells The significance of the luminal K^+ channel is thought to be that, in conjunction with the basolateral Cl^- conductance, it contributes to the characteristic lumen positive transepithelial electrical potential, which acts as a driving force for passive absorption of Na^+ across Na^+-selective tight junctions [1,2]

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Abbreviations Mops, 4-morpholinepropanesulfonic acid, MDCK, Madin-Darby canine kidney, $C_{12}E_8$, octaethylene glycol dodecyl monoether, metrizamide, (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose

In view of the central role of the ascending limb cells in controlling the normal urine concentrating ability of the kidney, it is important to isolate the transport systems of the luminal surface and study their regulation at the molecular level Assays have been developed for [^{3}H]bumetanide binding to renal outer medulla membranes [9] and recently a bumetanide-binding protein of M_r 34000 has been identified in membranes from mammalian renal outer medulla [10] This is presumably one component of the $Na^+/K^+/Cl^-$ cotransporter but it is not known if this protein can sustain cation transport The K^+ channel has not been identified in isolated membrane preparations Identification of these important transport proteins and further characterization require their detection in isotope flux assays, solubilization, isolation, and reconstitution into phospholipid vesicles Furosemide-sensitive cation fluxes have been detected in vesicles derived from isolated cells of thick ascending limb of the loop of Henle [8], but these are not suitable as a starting material for reconstitution and purification due to the low yields

In crude microsomal preparations from outer medulla only a small fraction of the total vesicle volume can be bounded by luminal membranes, which lack brush borders The major problem in measuring specific Na^+ or K^+ isotope fluxes into the heterogeneous population of vesicles is that these are largely obscured by non-specific permeation pathways Furthermore, for a 'channel' type of permeation mechanism, equilibration of an isotope would normally be expected to be complete within fractions of a second and thus be inaccessible to simple manual measurement (see Ref 11)

Two complementary solutions have been found to the signal to background problem, which now allow detection of $^{86}Rb^+$ fluxes mediated by specific pathways in the vesicles from renal outer medulla

(1) The $^{86}Rb^+$ uptake is measured against a large opposing chemical gradient of K^+ For a detailed description of the principles involved in measuring isotope fluxes through ion channels by this procedure see Ref 11 In short, for that fraction of vesicles containing K^+ channels, the selective K^+ permeability together with an outwardly directed K^+ gradient are expected to produce an electrical diffusion potential, exterior positive, which should cause $^{86}Rb^+$ to be accumulated When the chemical gradient of K^+ is dissipated isotope should then flow out of the vesicle

Similarly it is expected that any carrier type of transport mechanism capable of sustaining a K^+-Rb^+ exchange e g perhaps the $Na^+/K^+/Cl^-$ co-transporter, would show the classical phenomenon of counter-transport or transient accumulation of $^{86}Rb^+$ In either case the specific transport flux should be greatly magnified by comparison with the unspecific isotope equilibration pathways

(2) Luminal membrane vesicles have been separated physically from the bulk of the membrane protein on metrizamide density gradients and the partially purified membranes then used for flux studies and as starting material for reconstitution

With these techniques we have been able to detect Ba^{2+}-sensitive $^{86}Rb^+$ uptake via the K^+ channel and furosemide-sensitive $^{86}Rb^+$ uptake thought to be via the Na^+/K^+Cl^- cotransporter In this paper we demonstrate (a) localization of the Ba^{2+}- and furosemide-sensitive systems to luminal membranes, (b) preliminary kinetic characterization of furosemide-sensitive $^{86}Rb^+$ fluxes and (c) reconstitution of the Ba^{2+}- and furosemide-sensitive systems as a first step towards subsequent attempts to purify the proteins Other experiments (Burnham, Braw and Karlish, unpublished work, manuscript in preparation) provide more detailed characterization of the K^+ channel mediated $^{86}Rb^+$ flux, showing that it is Ca^{2+}-dependent, $K_{0.5}$ 10–100 nM In the conditions of the experiments in this paper the K^+ channel is always Ca^{2+}-activated

Materials and Methods

Preparation of luminal membranes from red outer medulla Crude microsomes from the rabbit renal outer medulla were prepared by differential centrifugation as in Ref 12, except that the buffer used was 250 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM Mops, with the pH adjusted to 7 2 with Tris or triethanolamine The final pellet was resuspended in 250 mM sucrose, 50 mM KCl, 1 mM EGTA and 25 mM imidazole with acetic acid added to adjust the pH to 7 2 (resuspension buffer) Protein concentration was adjusted to between 3 and 4 mg/ml A 5 to

15% (w/v) continuous metrizamide density gradient was prepared in the resuspension buffer on top of a 1 ml cushion of 30% metrizamide (see Ref 13) Approx 1 ml of the microsome suspension was placed on a gradient and centrifuged 16 h at 106 000 × *g* in a Beckman SW 41 swinging bucket rotor at 9°C Fractions of approx 1 ml were removed from the gradient

^{86}Rb flux assay To exchange external K^+ or other cations for Tris or triethanolamine, the vesicles were passed through Tris- or triethanolamine-equilibrated Dowex 50-X8 (50–100 mesh) columns poured in Pasteur pipettes The columns were rinsed with 1 ml of 350 mM sucrose, bovine seerum albumin 25 mg/ml 100–200 μl of suspension containing 30 μg or more vesicle protein were applied and eluted with 300–500 μl of 350 mM sucrose This resulted in a 3–4-fold dilution of the original suspension If desired, the Na^+/K^+ pump inhibitors, ouabain and vanadate were added with $MgCl_2$ so that the final concentrations were ouabain, 500 μM, vanadate, 125 μM and $MgCl_2$, 2 mM The suspension was warmed to room temperature and $^{86}Rb^+$ uptake was initiated by mixing 3 vol of the Dowex-treated vesicle suspension with 1 vol of reaction mixture so that the final concentrations of components present were RbCl, 100 μM or greater plus ^{86}Rb (10^6 cpm per 100 μl) Mops (Tris) or Mops (Triethanolamine) 10 mM (pH 7 2), $MgCl_2$, 2 mM, additional salts such as choline chloride or NaCl where indicated, 50 mM, and inhibitors as indicated in the figure legends $^{86}Rb^+$ uptake was stopped by transferring known volumes of the microsome suspension to ice-cold Dowex columns (see Ref 11), and the vesicles were eluted into counting vials with 1 5 ml of ice-cold 350 mM sucrose solution Radioactivity taken up into the vesicles was measured by Cerenkov radiation Rb^+ fluxes described as furosemide- or Ba^{2+}- or ouabain-sensitive were calculated from the difference of $^{86}Rb^+$ uptake without or with the inhibitor

Reconstitution Vesicles from fractions 3–6 of the metrizamide gradient (cf Fig 2) were collected by dilution with 25 mM imidazole/acetate, 1 mM EDTA (pH 7 4), and centrifugation for 90 min at 140 000 × *g* in a Beckman Ti65 ultracentrifuge rotor The vesicles were resuspended in 100 μl of 25 mM imidazole/acetate, 1 mM EDTA (pH 7 2) by repeatedly drawing and expelling the suspension with a micropipette For reconstitution, the vesicles were solubilized by adding, in order, 30 μl 3 M KCl, 6 μl 2% β-mercaptoethanol and 20 μl 0 45 M octylglucoside at 20°C Lipid was solubilized by mixing 100 μl of a suspension of soybean phosphatidylcholine (50 mg lipid/ml in 25 mM imidazole/acetate, 1 mM EDTA (pH 7 2)) with 3 μl 2% β-mercaptoethanol and 50 μl of 0 45 M octylglucoside The solubilized membrane protein was rapidly mixed with the lipid by drawing and expelling the combined solution in a micropipette so as to avoid foaming This resulted in a colorless, transparent solution which was immediately applied to a 1 × 33 cm Sephadex G-50 (coarse) column, equilibrated with 150 mM KCl, 25 mM Tris-HCl (pH 7 2) The column was then eluted with the same buffer and reconstituted vesicles were collected in the void volume

$(Na^+ + K^+)$-ATPase of the microsomal fractions was assayed after treatment with deoxycholate as described by Jørgensen and Skou [14] Protein was assayed by the method of Bradford [15]

Materials Metrizamide, was obtained from Nyegaard and Co A/S, Oslo Dowex 50-X8 (50–100 mesh) and Mops were obtained from Sigma Furosemide and bumetanide were gifts from Merck, Sharpe and Dohm $^{86}Rb^+$ was obtained from New England Nuclear All materials were of the highest analytical grade available

Results

The time-course of $^{86}Rb^+$ uptake into KCl-loaded crude membrane vesicles in the absence of Na^+ ions, and effects of Ba^{2+} ions and furosemide, added together or separately are shown in Fig 1 In the control curve $^{86}Rb^+$ entered the vesicles, reaching a peak at 20 min, and then the level of $^{86}Rb^+$ accumulation declined slowly over the course of 2 h 1 mM Ba^{2+} ions alone greatly inhibited the rate of ^{86}Rb uptake and the isotope accumulation 1 mM furosemide was somewhat effective in slowing the rate of $^{86}Rb^+$ uptake However, the combination of Ba^{2+} and furosemide was synergistic since the Ba^{2+}- or furosemide-inhibited fraction of $^{86}Rb^+$ uptake was much larger when measured in the presence of the second

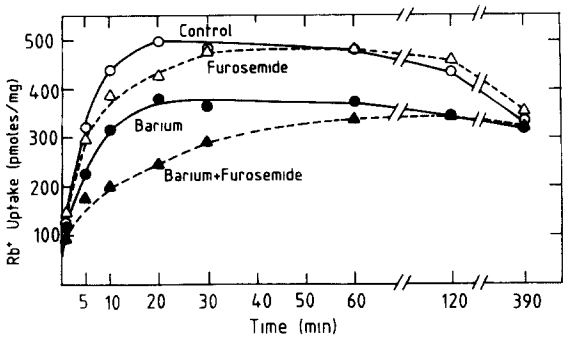

Fig 1 Fig 1 Synergism between Ba^{2+} and furosemide in inhibition of $^{86}Rb^+$ uptake into crude microsomal vesicles $^{86}Rb^+$ uptake into KCl-loaded microsomes was measured in the standard way except that in addition to the standard ingredients, the reaction mixtures contained, as indicated, 1 mM $MgCl_2$ (○——○), 1 mM $BaCl_2$ (●——●), 1 mM furosemide (△------△), or both 1 mM $BaCl_2$ and 1 mM furosemide (▲------▲) All four uptake mixtures contained 0 5 mM ouabain and 100 μM vanadate

inhibitor Several experiments showed that both the Ba^{2+}- and furosemide-sensitive components of the $^{86}Rb^+$ accumulation were dependent on the outwardly directed K^+ gradient since replacement of internal KCl with choline chloride greatly decreased the $^{86}Rb^+$ uptake and inhibition by the Ba^{2+} or furosemide Table I shows results of a more detailed experiment to look at the initial rate of furosemide-inhibited K^+ uptake in microsomes loaded with increasing concentrations of KCl (or choline chloride) The dependence of the furosemide-sensitive $^{86}Rb^+$ flux on the internal K^+ suggests that we are observing a K^+-K^+($^{86}Rb^+$) exchange (see the Discussion) The dependence of Ba^{2+}-sensitive $^{86}Rb^+$ uptake on internal K^+ and other properties of the K^+ channel mediated flux will be described fully in a forthcoming paper (Burnham, Braw and Karlish, unpublished work manuscript in preparation)

TABLE I

DEPENDENCE OF FUROSEMIDE-SENSITIVE $^{86}Rb^+$ UPTAKE ON INTERNAL K^+

Microsomes were prepared after homogenizing outer medulla in the standard medium but containing the indicated KCl plus choline chloride to a total concentration of 75 mM $^{86}Rb^+$ uptake without or with 1 mM furosemide, and in the presence of 1 mM Ba^{2+} was measured over a time-course as in Fig 1 Initial linear rates of uptake per 2 min have been calculated from the three time points between 0 5 and 2 min (uptake between 0 and 0 5 min being fast and furosemide-insensitive and attributable to a small fraction of leaky vesicles) The protein concentration was not measured in this experiment and so rates are given in terms of cpm taken up over the 2 min

KCl (mM)	Initial rate of $^{86}Rb^+$ uptake (cpm in 2 min, ± S E)		
	Control	+1 mM furosemide	furosemide-sensitive flux
0	267 ± 71	322 ± 13	−55 ± 72
25	453 ± 4	348 ± 35	105 ± 35
50	731 ± 16	465 ± 13	266 ± 20
75	709 ± 32	477 ± 6	232 ± 33

As argued in the Discussion the synergism between furosemide and Ba^{2+} in Fig 1 suggests that the furosemide- and Ba^{2+}-sensitive systems are located in the same population of vesicles Supporting evidence for this hypothesis is given in Fig 2, showing the results of two experiments examining the distribution on metrizamide density gradients of furosemide- and Ba^{2+}-inhibited $^{86}Rb^+$ fluxes as well as the Na^+/K^+ pump, and protein concentrations Furosemide-inhibited $^{86}Rb^+$ uptake was measured both in the presence (A) or absence (B) of external Na^+ ions (see also Fig 3) Two assays were used for Na^+/K^+ pump activity, ouabain-sensitive (Na^+-K^+)-ATPase activity of deoxycholate-treated membranes in Fig 2A, and ouabain (plus vanadate) inhibited K^+-$^{86}Rb^+$ exchange in Fig 2B [16] More than 75% of the protein from the crude microsomes was found in the heaviest metrizamide fractions, but much of the specific carrier and channel mediated transport activity was found higher up in the gradient, resulting in a roughly 3-fold increase in specific activities The furosemide-inhibited Rb^+ flux, whether measured in the presence (A) or absence (B) of Na^+, and the Ba^{2+}-inhibited Rb^+ flux were highest in somewhat lighter fractions than the highest activity of the Na^+/K^+ pumps Thus although separation was only partial, two populations of vesicles were distinguishable, which we assume to be 'luminal' membranes containing the furosemide- and Ba^{2+}-inhibited transport systems and 'basolateral' membranes containing the Na^+/K^+ pumps Furosemide- and Ba^{2+}-inhibited Rb^+ fluxes and (Na^+ + K^+)-ATPase activity were also detected in the heaviest fractions, but the specific activity was low due to the preponderance of contaminating proteins These heavy vesicles

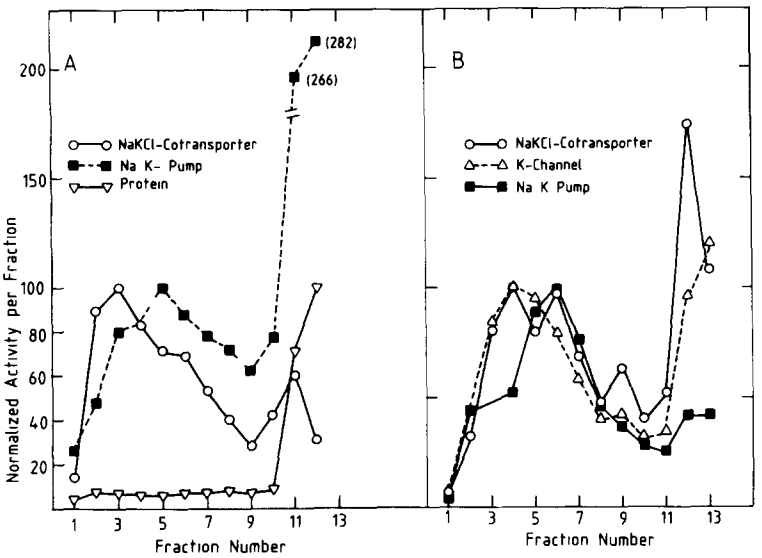

Fig 2 Metrizamide fractionation of medullary microsomal vesicles (A) The difference between Rb^+ flux in the presence and absence of 1 mM furosemide in reaction mixtures containing 50 mM NaCl was measured in each fraction (○———○) as well as the $(Na^+ + K^+)$-ATPase activity (■-----■) and the protein concentration (▽———▽) (B) Furosemide-inhibitable Rb^+ flux in the absence of Na^+ (○———○) 1 mM Ba^{2+}-inhibitable Rb^+ flux (△-----△) and 0 5 mM ouabain-inhibitable Rb^+ flux (■———■) were measured in each fraction Experiments A and B were performed on separate preparations of microsomes on different days In B the vesicles were homogenized in 1 mM ATP, Tris salt, in addition to the usual buffer ingredients

are leaky to and become equilibrated with the metrizamide during centrifugation

The nature of the furosemide-inhibited $^{86}Rb^+$ uptake seem in Figs 1 and 2 and Table I was investigated further The conventional behaviour expected for cotransport of K^+ and Na^+, is that an inwardly directed Na^+ gradient will be coupled to and energise uptake of $K^+(Rb^+)$ Fig 3 shows a time-course of furosemide-inhibited $^{86}Rb^+$ uptake in the luminal membrane vesicles in the absence and presence of external Na^+ ions The flux in the absence of Na^+ is the diuretic-sensitive K^+-Rb^+ exchange observed above The inward gradient of Na^+ ions greatly accelerated the rate of $^{86}Rb^+$ uptake, as expected for a $Na^+/K^+/Cl^-$ cotransport system In the absence of Na^+, increasing the Rb^+ concentration, increased the initial rate of furosemide-sensitive flux as expected for a saturating process (Table II) The presence of the inwardly directed Na^+ gradient increased the Rb^+ uptake at non-saturating Rb^+ concentrations but had no effect at saturating Rb^+ (Table II) Thus external Na^+-stimulated $^{86}Rb^+$ uptake by raising the apparent affinity of the cotransporter for Rb^+

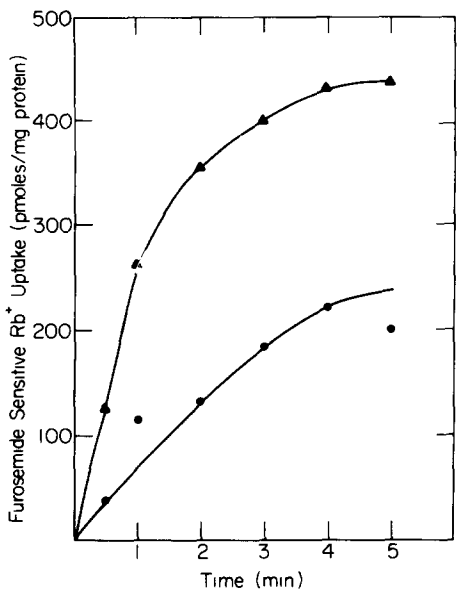

Fig 3 Furosemide-sensitive $^{86}Rb^+$ uptake into luminal membrane vesicles, in the absence or presence of Na^+ ions Vesicles containing KCl were pooled from metrizamide-gradient fractions 2–6 After passage through a triethanolamine equilibrated Dowex column Rb^+ uptake was measured in reaction mixture containing either 50 mM NaCl (▲———▲) or 50 mM choline chloride (●———●) in addition to the standard ingredients

Further evidence that the furosemide-sensitive Rb^+ flux is sustained by the $Na^+/K^+/Cl^-$ cotransporter is the finding that bumetanide, a more selective inhibitor of the system also blocked $^{86}Rb^+$ uptake into the low density vesicles, when measured in the presence of external Na^+ (not shown) The potency of bumetanide in our system was however surprisingly low, $K_{0.5} \sim 100$ μM

TABLE II

THE EFFECT OF AN INWARD Na^+ GRADIENT ON THE RATE OF FUROSEMIDE-INHIBITED Rb^+ UPTAKE AT DIFFERENT Rb^+ CONCENTRATIONS

The experiment was carried out as in Fig 3 except that a fixed amount of $^{86}Rb^+$ was used and the total amount of Rb^+ was altered so as to give the indicated final concentrations The $^{86}Rb^+$ flux was measured in duplicate for 1 min, in the presence and absence of NaCl or choline chloride (50 mM) and presence or absence of 1 mM furosemide Because the flux was measured in duplicate, a S E value could not be calculated However the reproducibility of the duplicates was in all cases better than 10%

Rb^+ concn (mM)	Rate of furosemide-sensitive Rb^+ uptake (pmol/mg protein per min)	
	without Na^+	with Na_o^+ (50 mM)
0 1	103	208
0 2	236	348
0 5	590	720
1	1066	1270
2	1578	1634
5	2613	2674
10	2951	-

compared to other whole cell systems $K_{0.5} \sim 0.1$ μM (see Discussion and Refs 17–19)

For reconstitution a modification of the procedure of Helenius et al [20] was chosen, after preliminary experiments had shown that reconstitution by freeze-thaw sonication [21] was unsuccessful Octyl glucoside was used to dissolve the luminal membranes One important fact already noted is that the solubilization of the cotransporter in octyl glucoside was dependent on salt concentration as observed previously with

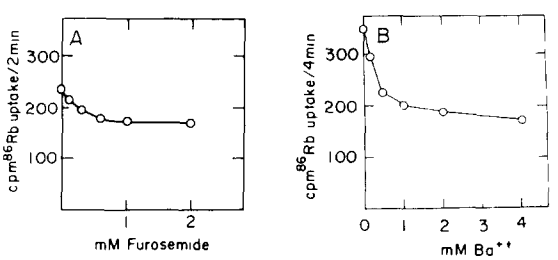

Fig 4 Reconstitution of K^+ channel and $Na^+/K^+/Cl^-$ cotransporter Reconstituted vesicles were prepared and assayed for $^{86}Rb^+$ uptake in the presence of the indicated concentration of $BaCl_2$ (A) or furosemide (B)

$C_{12}E_8$ [10] Only at 0 6 M KCl was full clarification seen of the suspension of luminal vesicles, after addition of detergent

Using this procedure, a small but significant furosemide-sensitive K^+-K^+ exchange could be described in the reconstituted vesicles, Fig 4A The $K_{0.5}$ for furosemide was about 100 μM, the same as in the native membrane vesicles In a series of experiments the results of reconstitution of the furosemide-sensitive flux were variable Further experiments concerning both the solubilization and the reconstitution steps are required to develop a procedure that is satisfactory for further attempts at isolation of the $Na^+/K^+/Cl^-$ cotransport system

In contrast a large fraction of $^{86}Rb^+$ uptake in reconstituted vesicles was inhibited by Ba^{2+} A typical dose-response curve, Fig 4B, shows a $K_{0.5}$ of 50–100 μM, again similar to that in native membrane vesicles

Discussion

Localization of the $Na^+/K^+/Cl^-$ cotransporter and K^+-channel

The electrophysiological data on isolated segments of thick ascending limbs of the loop of Henle indicate that the $Na^+/K^+/Cl^-$ cotransporter and K^+ channel are located in the luminal surface of these cells [1,2]

Two pieces of evidence from our experiments suggest that the furosemide- and Ba^{2+}-sensitive systems are located in the same population of 'luminal' membrane vesicles Firstly the synergism between Ba^{2+} and furosemide in inhibiting ^{86}Rb uptake (Fig 1) is explained most simply by assuming (a) that upon homogenization of the tissue, the luminal membranes of the cells from the outer medulla form a distinct population of vesicles, and (b) the presence of either inhibitor slows net K^+ efflux and dissipation of the K^+ gradient, and thus allows the gradient-dependent $^{86}Rb^+$ accumulation via the system sensitive to the other inhibitor, to persist for a longer period of time Secondly the physical separation on the metrizamide density gradients, of Ba^{2+}- and furosemide-sensistive fluxes from the ouabain-sensitive flux show that distinct populations of vesicles are present, even though their separation is only partial Palfrey and For-

bush also reported a partial separation of [^{3}H]bumetanide binding sites from ($Na^+ + K^+$)-ATPase on sucrose density gradients of dog kidney outer medulla membranes and suggested, that the lighter fractions were enriched in 'luminal' membranes [9] Because the separation of vesicle populations is only partial it is not possible, on the basis of our experiments to exclude the possibility that K^+ channels and/or $Na^+/K^+/Cl^-$ cotransporter are also located in the 'baso-lateral' surfaces of the cells, although that supposition would not be consistent with the electrophysiological data

It is worth pointing out the utility of the metrizamide for transport studies Due to the high density of the iodinated sugar, the light vesicles reach their equilibrium positions on the density gradient at relatively low concentrations of the metrizamide, and hence are not exposed to the same degree of osmotic shock as would be the case on gradients of say sucrose (see also Ref 22)

The naturee of the furosemide-sensitive K^+ flux

The furosemide-sensitive K^+-K^+ exchange (Table I) and net K^+ efflux implied by the result in Fig 1 are presumed to occur via the $Na^+/K^+/Cl^-$ cotransporter These are obviously abnormal flux modes of the cotransporter Unexpected as this concept may be, there is now evidence for variable coupling ratios of Na^+ and K^+ and for K^+-K^+ exchange via the $Na^+/K^+/Cl^-$ cotransport system in avian [23,24] and human erythrocytes [25] and cultured MDCK [26] and Hela cells [26], and also net uncoupled K^+ movement through the system in MDCK [25] and human [25] and avian red cells [24] The conventional behaviour expected of a Na^+ and K^+ cotransporter, namely mutual dependence of the fluxes of the two cations is apparently not strictly adhered to Rather the inward gradient of Na^+ ions appears to accelerate K^+ movements (Fig 3) but is not absolutely required

One can ask whether the furosemide sensitive K^+ flux in the absence of Na^+ is sustained not by the $Na^+/K^+/Cl^-$ cotransporter, but by the hypothetical KCl cotransporter suggested to exist in the basolateral membranes It is hard to rigorously exclude this possibility although the following points, taken together, support the hypothesis that $^{86}Rb^+$ uptake with or without Na^+ ions represent alternative transport modes of the same system

(1) Furosemide-inhibited $^{86}Rb^+$ fluxes with or without Na^+ are similarly distributed on the metrizamide density gradient and are partially separated from Na^+/K^+ pump-mediated flux Fig 2

(2) Stimulation of Ba^{2+} uptake by furosemide and vice-versa occurs in the absence of Na^+, Fig 1, and is consistent with location of the Ba^{2+}- and furosemide-sensitive systems in the same vesicle population

(3) The result of (Table II) that Na^+ ions increase the apparent affinity for Rb^+ but not the maximal rate of Rb^+ uptake, is not consistent with two entirely separate transport systems ((a) a KCl cotransporter independent of Na^+ and (b) a Na^+/KCl cotransporter in which the K^+ fluxes are entirely dependent on Na^+) for in this case we should have observed an effect of Na^+ even at saturating Rb^+ concentrations

The low potency of bumetanide in inhibiting $^{86}Rb^+$ fluxes is partly attributable to the lack of optimal conditions for binding, in our assay conditions These are known to include the presence of both Na^+ and K^+ ions at tens of millimolar concentrations, and the absence of Cl^- ions at high concentrations which antagonize binding [9] Direct measurement of bumetanide binding to dog kidney outer medulla membranes showed the presence of a class of high-affinity sites ($K_d \approx 30$ nM), but of very low capacity, 2 pmol/mg protein [9] Conversely a much higher binding capacity, 226 (pmol/mg protein, but relatively low affinity site $K_d \approx 5$ μM has been observed using pig kidney outer medulla membranes [10] These results may not be contradictory for in isolated bovine kidney and flounder intestine membranes both high- and low-affinity sites are detectable, the latter in at least 10-fold excess over the former (Palfrey, H C , personal communication) It is therefore possible that in intact cells the state of the system is different (high affinity) from that in isolated membranes (mainly low affinity)

Reconstitution

The experiments in Fig 4 represents a first step and necessary precondition for further attempts to purify and characterize the cotransporter and K^+ channel The experiments show that the principle of measuring isotope uptake against the opposing

chemical gradient, as developed for plasma membrane vesicles provides a sensitive method for detection of both furosemide- and Ba^{2+}-inhibited fluxes in reconstituted vesicles

Physiological regulation

Fig 5 indicates the present concept of the location of the important transport systems involved in transepithelial salt transport across the cells of the thick ascending limb and some possibilities for regulation at the molecular level The bumetanide-binding protein can be bound tightly to cytoskeletal proteins [10] and as mentioned in the Introduction the Ba^{2+}-inhibited K^+ channel is sensitive to Ca^{2+} in the physiological range (Burham, Braw and Karlish, unpublished work) In avian erythrocytes the cotransporter is greatly activated by catecholamines [27,28] and this is accompanied by phosphorylation of a cytoskeletal proteins of 240 kDa [29], an ankyrin like molecule In mouse and rat, antidiuretic hormone or permeable cAMP analogues increase the rate of transepithelial salt reabsorption across cells of the thick ascending limb [30,31] Short-term kinetic regulation should involve changes in turnover rates or surface density of transport systems which are limiting for overall salt reabsorption In the present context these are the luminal systems, i e the $Na^+/K^+/Cl^-$ cotransporter and K^+ channel, but possibly also basolateral systems such as the Cl^- conductance The rate of the basolateral Na^+/K^+ pump is limited mainly by the concentration of cytoplasmic Na^+ On the basis of the preliminary

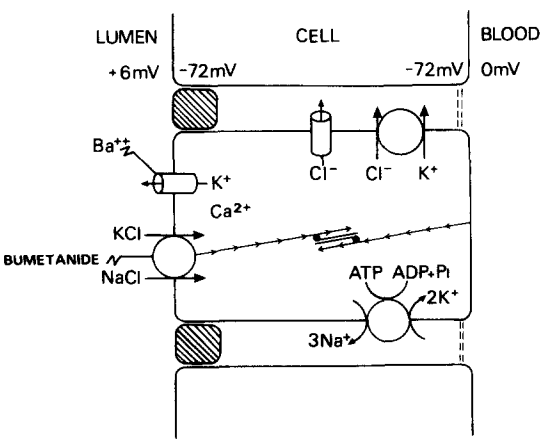

Fig 5 Model for TALH salt transport

data our working hypothesis is that in the cells of the thick ascending limb as in other epithelia [32,33] the levels of cytoplasmic Ca^{2+} and the state of phosphorylation of cytoskeleton and/or the transport system themselves, play a central role in the coordinated control of transepithelial NaCl reabsorption The techniques we have developed should now allow an investigation of the hypothesis at the level of the isolated membrane transport systems

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