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A Na-K-Cl cotransporter in isolated rat papillary collecting duct cells

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A Na-K-Cl cotransporter in isolated rat papillary collecting duct cells. Lactate production and ion fluxes were measured in isolated rat papillary collecting duct cells (PCD) to gain further insight into the transport properties of the papillary collecting duct. Lactate production was found to be inhibited by bumetanide in a dose-dependent manner, a maximum inhibition of 22% was obtained at 10^{-4} M bumetanide and an apparent K_i of 10^{-8} M was determined. Bumetanide inhibition of lactate production was dependent on the presence of sodium and chloride. Chloride removal inhibited lactate production also by 20%. Bumetanide (10^{-4} M) inhibited by 35% sodium uptake into PCD cells exposed to 10 mM ouabain and chloride uptake into ion depleted PCD cells by 40%. In addition, this bumetanide-sensitive chloride uptake was dependent on the presence of sodium and potassium in the incubation medium. Furthermore, ^{86}Rb uptake into these cells was significantly reduced in the presence of 10^{-4} M bumetanide. These data provide evidence for the operation of a Na-K-Cl cotransport system in rat papillary collecting duct cells. This transport system might be involved in active chloride transport in the papillary collecting duct and/or volume regulation of the PCD cells.

The papillary collecting duct plays an important role in the final regulation of the ionic composition of the urine and of urine osmolarity. Studies using microcatheterization or micropuncture have demonstrated reabsorption of sodium and chloride, secretion of protons, and potassium transport [1–12]. Sodium reabsorption seems to be active and mediated mainly by an amiloride-sensitive sodium channel [10]. Proton secretion is most probably effected by a proton ATPase located in the luminal membrane [12]. With regard to chloride transport in the papillary collecting duct, there is suggestive evidence that the reabsorption is also active [5, 8] and there are several reports which point to an inhibitory action of furosemide on chloride transport [8, 9]. However, the mechanisms underlying this chloride transport in PCD cells remain unclear.

In a variety of other epithelia it has been demonstrated that a Na-K-2Cl cotransport system is involved in active transepithelial chloride transport [13, 14]. The question arose whether such a transport system might also be operating in the papillary collecting duct. We therefore investigated in highly purified PCD cells [15] the ion dependence and inhibitor sensitivity of ion transport-related lactate production, and of ion fluxes across the plasma membrane. The results suggest that PCD cells contain a Na-K-Cl cotransport system which might be involved in active transepithelial chloride transport or volume regulation in the papillary collecting duct.

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Methods

Preparation of cells from the papillary collecting duct (PCD)

Male Wistar rats (220 to 250 g) received a standard rat chow and had free access to tap water. Cells from the papillary collecting duct were isolated as described previously [15]. In short, the rats were killed by cervical dislocation and the kidneys rapidly removed. Exclusively the white portion of the medulla was excised from the kidney and minced immediately in an ice cold HEPES-Ringer's buffer (118 mM NaCl, 16 mM HEPES, 16 mM Na-HEPES, 14 mM glucose, 3.2 mM KCl, 2.5 mM $CaCl_2$, 1.8 mM $MgSO_4$, 1.8 mM KH_2PO_4; pH 7.4; 285 mOsm). The pieces were then incubated for 60 to 70 minutes at 37°C in HEPES-Ringer's solution containing 0.2% collagenase and 0.2% hyaluronidase (wt/vol) gased with room air. After addition of DNAse the cell suspension was then subjected to three low-speed centrifugations (28 × g; 2 min). The final pellet represented the purified PCD cells.

Purity of the preparation

Enrichment of PCD cells was assessed by determining the binding of Dolichos Biflorus Agglutinin (DBA), a lectin which in rat papilla binds exclusively to PCD cells. As described earlier [15] a small quantity of cells, suspended in HEPES-Ringer's solution was incubated with 0.03 mg/ml FITC labeled DBA (Vector Labs, Burlingame, California, USA) at 25°C in the dark with gentle agitation. After 20 minutes a drop of the cell suspension was examined using phase and fluorescence microscopy. DBA-FITC positive papillary collecting duct cells comprised >90% of the cells in the final preparation. The PCD cell preparation was also screened for the presence of cells of the medullary thick ascending limb of Henle's loop (TALH), which contain high levels of the bumetanide-sensitive Na-K-Cl cotransport system [16–19]. Tamm Horsfall protein (THP) was

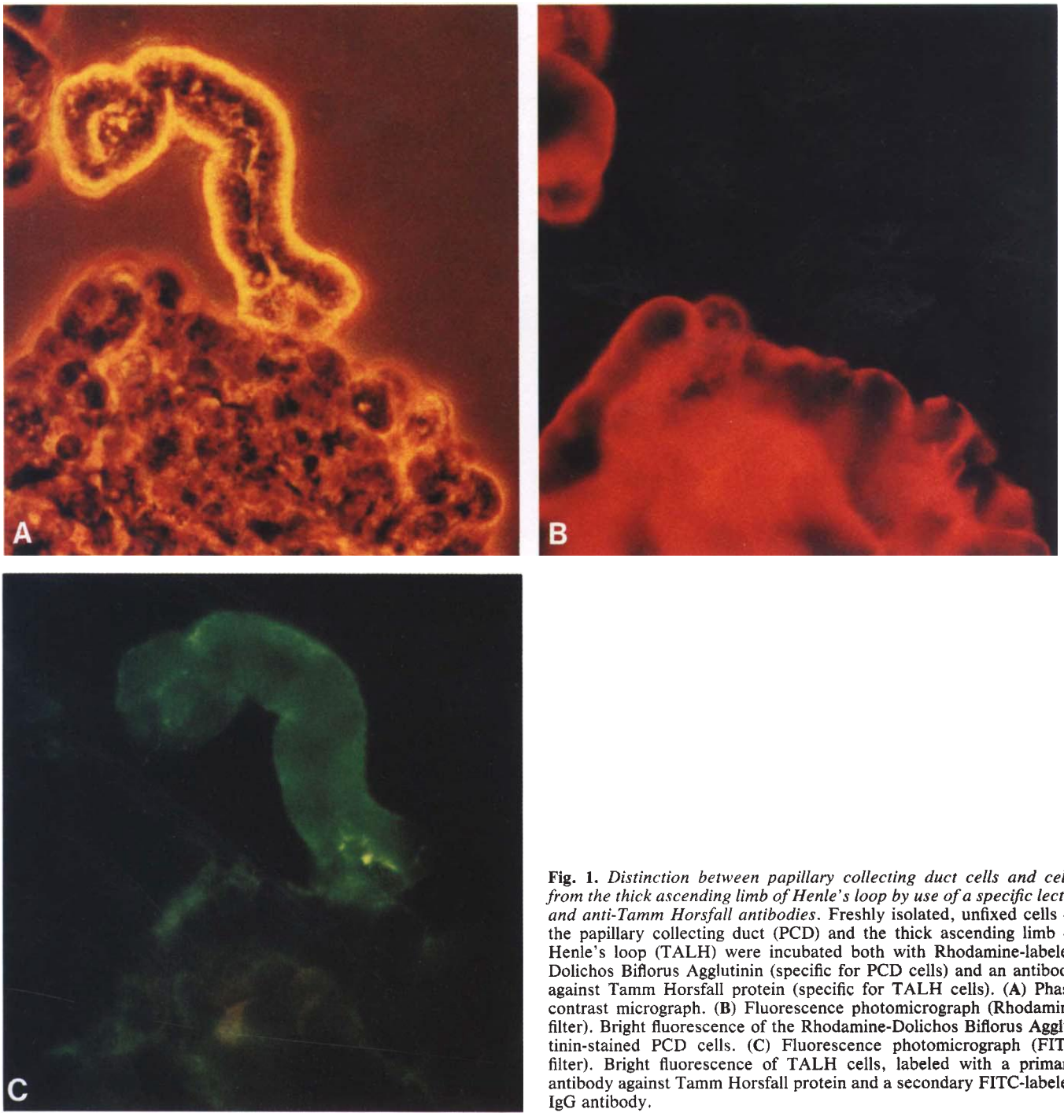

Fig. 1. *Distinction between papillary collecting duct cells and cells from the thick ascending limb of Henle's loop by use of a specific lectin and anti-Tamm Horsfall antibodies.* Freshly isolated, unfixed cells of the papillary collecting duct (PCD) and the thick ascending limb of Henle's loop (TALH) were incubated both with Rhodamine-labeled Dolichos Biflorus Agglutinin (specific for PCD cells) and an antibody against Tamm Horsfall protein (specific for TALH cells). (**A**) Phase contrast micrograph. (**B**) Fluorescence photomicrograph (Rhodamine filter). Bright fluorescence of the Rhodamine-Dolichos Biflorus Agglutinin-stained PCD cells. (**C**) Fluorescence photomicrograph (FITC filter). Bright fluorescence of TALH cells, labeled with a primary antibody against Tamm Horsfall protein and a secondary FITC-labeled IgG antibody.

used as marker for TALH cells since this protein has been demonstrated to be almost exclusively localized in the TALH in kidney sections [20, 21] and can also be detected in isolated TALH cells by immunofluorescence [22]. For this purpose antiserum (Behringwerke, Marburg, FRG) raised in rabbits against human THP and FITC-linked antirabbit Ig(G) from donkey (Amersham, Buckinghamshire, UK) were used. Isolated cells were incubated for 45 minutes at 4°C with the THP antiserum (1:50 diluted with PBS + 10% lamb serum). After two washing steps they were incubated with the FITC-labeled antirabbit Ig(G) (1:10 diluted with PBS + 10% lamb serum) for 30 minutes at 25°C, again followed by a washing step. The cell preparation was then counter-stained with Rhodamine-labeled Dolichos Biflorus Agglutinin. Fluorescence microscopic evaluation of the preparations showed that the purified suspension of PCD cells contained less than 1% TALH cells (Fig. 1).

Lactate production

Lactate production was measured in 1 ml cell suspension (about 0.2 to 0.3 mg cell protein/ml) with 14 mM D-glucose as substrate over an interval of 40 minutes at 37°C with occasional agitation. Lactate production was linear at least up to 60 minutes. HEPES-Ringer's was used for control incubations. The composition of other incubation media is given in the **Results** section. Bumetanide was dissolved in dimethyl-sulfoxide (DMSO, 1 ‰ final concentration), and controls received only the solvent. The reaction was terminated by boiling the samples for three minutes. Lactate was determined in triplicate enzymatically by a commercially available kit (Behringwerke, Marburg, FRG). Protein content of the individual samples was assessed in triplicate by the method of Lowry et al [23].

Measurement of ion fluxes by electronprobe microanalysis

Ion fluxes were estimated from the intracellular element content determined by electronprobe microanalysis as described in detail elsewhere [24]. In short, after the desired incubation time the cell suspension (200 μl) was transferred into a cytocentrifuge and the cells were pelleted by centrifugation (2 min at 32 × g) onto a Thermanox® disc. The extracellular medium was removed from the disc by washings (3 × 1 sec) with ice-cold ammonium acetate buffer (160 mM, pH 7.4 adjusted with 5 mM Tris-HEPES). The sample was then immediately frozen in liquid propane, freeze-dried overnight at −80°C, $P < 10^{-6}$ mbar, and prepared for scanning electron microscopy by coating with carbon. Finally, the PCD cells attached to the Thermanox disc were scanned in an ETEC Autoscan scanning microscope using a primary electron beam of 12.5 keV, 2 nA, and 100 seconds accumulation time. X-rays generated in the specimen were collected by a windowless, energy dispersive detector (Link LZ-5). The peaks of the X-ray spectrum corresponding to sodium, phosphorus, chloride, and potassium were corrected for the background spectrum and the element content was quantified as described previously [24, 25].

The values were calculated as mM of the particular element/kg dry weight dextran. In order to correct for the number of cells, phosphorus content was taken as reference and the results are expressed as element/phosphorus ratios. Cell phosphorus content was constant during the incubation time employed (mean value 785 ± 40 mM/kg dry weight, $N = 8$) and there were no significant differences in cellular phosphorus between cells subjected to the various incubation procedures. For each experimental condition the element content of 20 cell clusters was determined. An area containing approximately 30 cells was scanned in each cluster. For determination of the element content initially present at least ten cell clusters were analyzed.

PCD cells were incubated as indicated under **Results**, usually in a 285 mOsm HEPES-Ringer's solution. In the first experimental series element content was measured after inhibition of the Na-K-ATPase by 10 mM ouabain, either in the presence of the loop diuretic bumetanide or after replacement of potentially cotransported ions. Dimethyl-sulfoxide (DMSO, 1 ‰ final concentration) was used as solvent for bumetanide. In a second series cells were Na-, K-, and Cl-depleted by incubation for 30 minutes at 37°C in a medium devoid of these ions and with the following composition (in mM): 138 N-methyl-D-glucamine, 1 Ca, 1.8 Mg, 142 gluconate, 20 HEPES-Tris, 0.33 dibasic Tris phosphate, 0.44 monobasic Tris phosphate, 14 glucose, 10 ouabain; pH 7.4. The cells were then washed once and kept in this buffer on ice until use. At the beginning of the experiment, cells were centrifuged (1 min/30 × g) and resuspended in a buffer (37°C) of the desired composition. Samples were taken from this suspension at the time points 0 and 15 minutes.

Determination of Rb uptake

The uptake of ^{86}Rb into isolated PCD cells was measured by a centrifugation technique. A 100 μl aliquot of cell suspension was added to a 400 μl microfuge tube containing 50 μl of 10% trichloroacetic acid covered by 100 μl of a silicone oil mixture (1 part AR20 to 2 parts AR200) and placed in a 37°C water bath. Uptake of ^{86}Rb was started by the addition of 100 μl of K-depleted HEPES-Ringer's buffer containing ^{86}Rb (15 μCi/ml), 10 mM ouabain, 2 mM K^+ as KCl, with or without 0.1 mM bumetanide. At the appropriate times, the incubation was terminated by spinning in a Beckman microfuge for 15 seconds. The tubes were cut through the oil layer and the tips were placed in scintillation vials containing 1 ml 4% sodium dodecyl sulfate. After one hour, 7 ml of Ready protein (Beckman) scintillation fluid was added and radioactivity was counted in a Beckman LS 7800 liquid scintillation counter.

Measurements of [^{3}H] water and [^{14}C] sucrose distribution spaces were also performed in parallel with each transport experiment by centrifuging cells through the oil phase as described above. Cells were incubated for 15 minutes at 37°C with [^{3}H] water (15/μCi/ml) and [^{14}C] sucrose (2/μCi/ml). The intracellular space was calculated as the difference between the water and the sucrose distribution spaces in the cell pellet. Cellular ^{86}Rb was determined by subtracting the radioactivity in the extracellular space of the pellet (sucrose space × ^{86}Rb counts/min per μl of uptake media) from total radioactivity in the pellet. The uptake of K^+ was calculated by dividing the cellular ^{86}Rb activity by the medium ^{86}Rb specific activity (cpm ^{86}Rb/potassium content of the medium) and the intracellular volume. Results are expressed as nmoles K^+/μl cell water.

Materials

Collagenase (Worthington, grade II) was purchased from Technicon (Bad Vilbel, FRG), hyaluronidase and DNAse from Boehringer Mannheim (Mannheim, FRG), the kits for lactate determination from Behringwerke (Marburg, FRG). Bumetanide (sodium-free) was from Lövens Kemiske Fabrik (Copenhagen, Denmark). ^{86}Rb (4 mCi/mg Rb) as chloride salt in aqueous solution and [U-^{14}C] sucrose (10 mCi/mmol) were obtained from Amersham Buchler (Braunschweig, FRG) and [^{3}H] water (5 Ci/g) from NEN (Dreieich, FRG). All other chemicals used were of highest purity commercially available.

Statistical analysis

For statistical analysis the Student's t-test for paired or unpaired data or the analysis of variance (ANOV) were employed. Groups found to be different by ANOV were further analyzed by the Newman-Keuls test. Unless stated otherwise a difference was considered as significant at $P < 0.05$. Values are expressed as mean ± SEM.

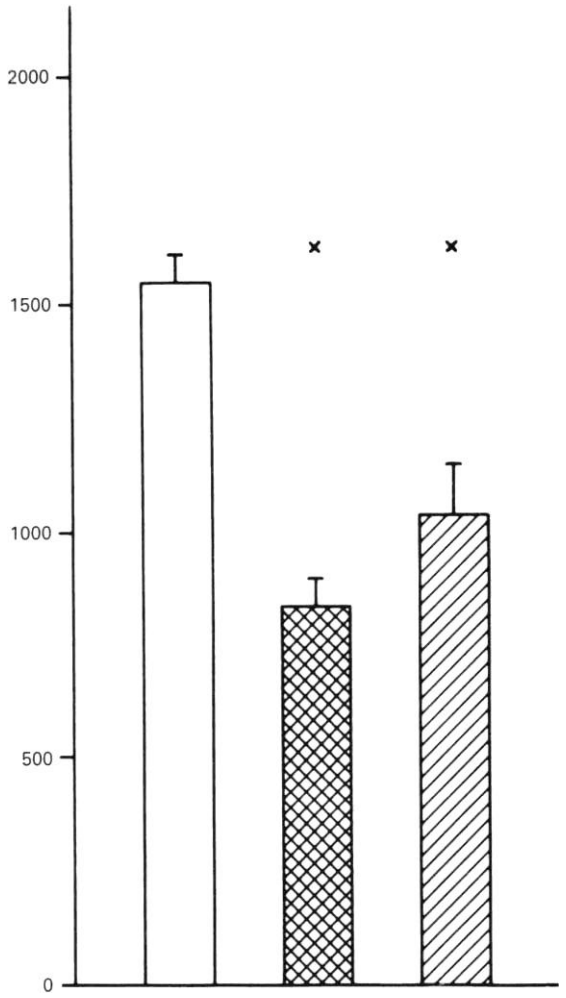

Fig. 2. *Effect of ouabain addition or sodium replacement on lactate production in PCD cells.* PCD cells were incubated for 30 min at 37°C in normal HEPES-Ringer's (□, control), sodium-free HEPES-Ringer's (▩, sodium replaced by choline), or HEPES-Ringer's with 10 mM ouabain (▨). Mean values ± SEM, derived from at least 5 experiments, are given. The asterisk indicates a statistical significance of $P < 0.01$ versus control conditions. Lactate production in the absence of sodium and in the presence of ouabain were not significantly different.

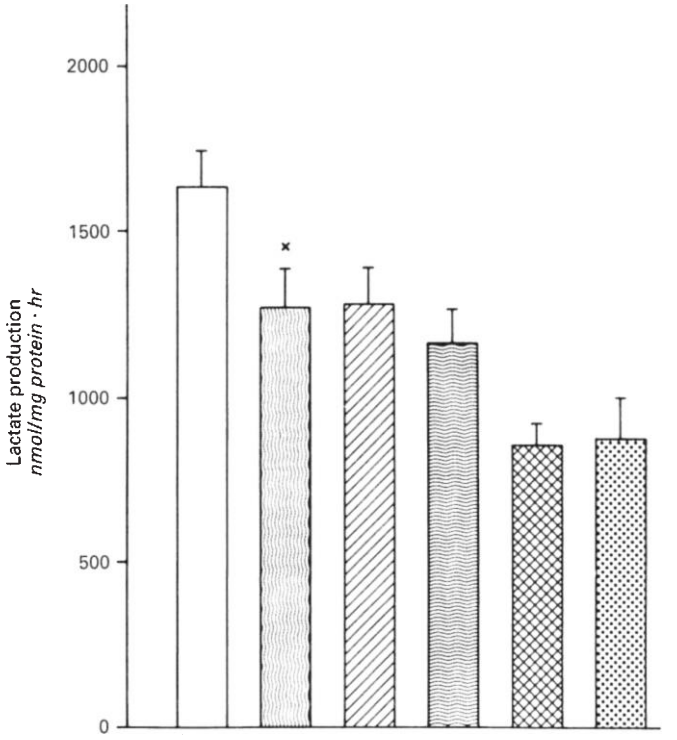

Fig. 3. *Effect of bumetanide, chloride, or sodium replacement on lactate production in PCD cells.* PCD cells were incubated for 30 min at 37°C in normal HEPES-Ringer's (□, control), chloride-free HEPES-Ringer's (▨, chloride replaced by nitrate), or sodium-free HEPES-Ringer's (▩, sodium replaced by choline) in the absence and presence (▥, Co; ▤ Cl^--free; ⊠ Na^+-free; plus bumetanide) of 10^{-4} M bumetanide. Mean values ± SEM derived from at least 5 experiments are given. The asterisk indicates a statistical significance of $P < 0.01$ compared to conditions without bumetanide. In chloride-free and sodium-free HEPES-Ringer's lactate production in the absence or presence of bumetanide was not significantly different.

Results

Lactate production by isolated PCD cells

Effect of sodium replacement and ouabain. In order to evaluate the ion dependence and inhibitor sensitivity of sodium transport we first investigated lactate production of the PCD cells under various conditions. Previous results [15] have suggested that glycolysis is partly determined by the rate of ATP utilization during Na extrusion (Na-K-ATPase activity). As shown in Figure 2, cells incubated in normal HEPES-Ringer solution produce 1550 ± 50 nmol lactate/mg cell protein × hr. Replacement of extracellular sodium by choline or N-methyl-glucamine reduced lactate production by 40% to 853 ± 70 nmol/mg protein × hr. Addition of ouabain in the presence of sodium diminished lactate production by a slightly lesser extent to 1055 ± 80 nmol/mg protein × hr. In the absence of sodium ouabain had no additional effect. These results indicate that about 40% of the lactate production of PCD cells is linked to sodium movement across the cells. The slightly lower effect of ouabain compared to sodium replacement on lactate production is probably due to a small residual activity of the Na-K-ATPase under the experimental conditions employed.

Effect of bumetanide and chloride replacement. In the next experimental series the effect of bumetanide on lactate production was investigated. At low concentrations bumetanide is a rather specific inhibitor of the Na-K-Cl cotransporter. In the presence of 10^{-4} M bumetanide (Fig. 3) lactate production by PCD cells in sodium and chloride containing HEPES-Ringer's was reduced by about 20% (1279 ± 115 vs. 1664 ± 99 nmol lactate/mg protein × hr; $P < 0.05$). When chloride was completely substituted by nitrate also an inhibition of lactate production was observed (third bar, Fig. 3). This inhibition was similar to that found with bumetanide alone. In the absence of chloride (fourth bar, Fig. 3) bumetanide had no additional effect on lactate production, suggesting that the bumetanide action was chloride-dependent. The inhibitory effect of bumetanide also required the presence of sodium. As indicated by the fifth and sixth bar in Figure 3, bumetanide did not affect lactate production in the absence of sodium.

The effect of bumetanide on lactate production was dose-

Table 1. Effect of bumetanide on the element content of PCD cells in the presence of ouabain

	Sodium (Na/P)			Potassium (K/P)			Chloride (Cl/P)		
	Initial content	Content after 15′	Change	Initial content	Content after 15′	Change	Initial content	Content after 15′	Change
Control ($N = 5$)	0.16 ± 0.05	0.47 ± 0.02	0.31 ± 0.02	0.68 ± 0.01	0.51 ± 0.02	−0.17 ± 0.01	0.62 ± 0.13	0.63 ± 0.1	0.01 ± 0.1
+ 10^{-4} M bumetanide ($N = 5$)		0.36 ± 0.01	0.20 ± 0.02		0.50 ± 0.02	−0.18 ± 0.01		0.58 ± 0.1	−0.05 ± 0.1
P		<0.005	<0.0005		NS	NS		NS	NS

Mean values ± SEM derived from *N* experiments are given. P content of the corresponding samples was not significantly different. Initial value: 597 ± 72; control cells: 593 ± 84; bumetanide-treated cells: 641 ± 48 mM/kg dry weight. Cells were incubated for 15 min at 37°C in HEPES Ringer's containing 10 mM ouabain in the absence (control) or presence of 10^{-4} M bumetanide. *P* values relate to the difference between control and bumetanide-treated cells.

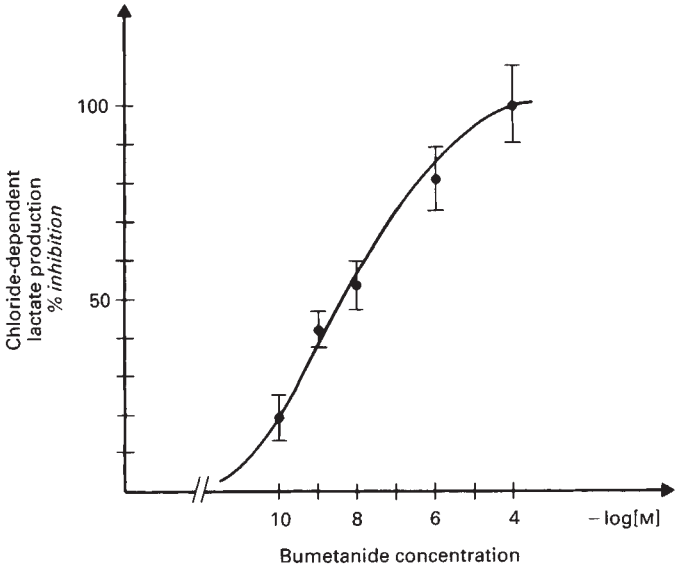

Fig. 4. *Dose dependence of bumetanide effect on chloride-dependent lactate production.* PCD cells were incubated for 30′ at 37°C in normal HEPES-Ringer's (control), and in chloride-free HEPES-Ringer's in the absence or presence of the indicated bumetanide concentration. Lactate production in the absence of chloride was identical at all bumetanide concentrations. Chloride-dependent lactate production was 361 nmol lactate/mg protein × hr in control experiments and decreased with increasing bumetanide concentrations. Mean values ± SEM derived from 3 to 5 experiments are given.

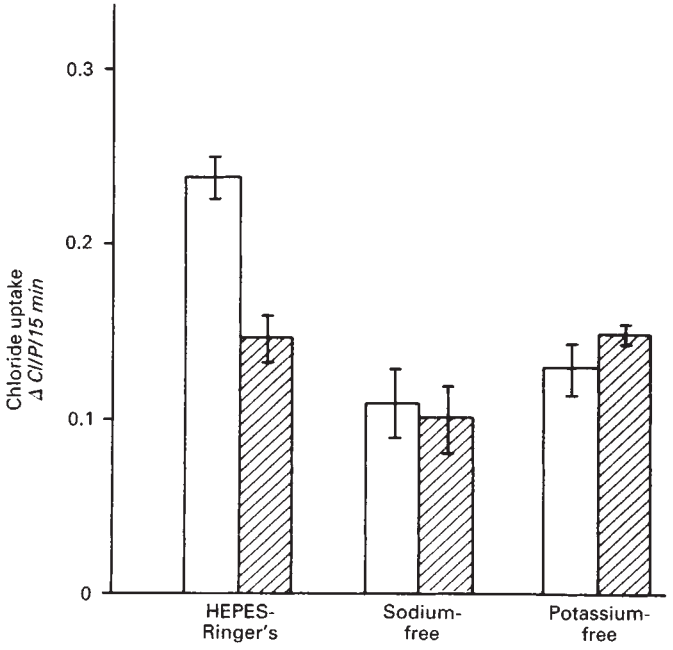

Fig. 5. *Effect of bumetanide and cation replacement on chloride uptake into ion depleted PCD cells.* PCD cells were preincubated for 30 min at 37°C in Na-K-Cl-free HEPES-Ringer's. Subsequently they were incubated for 15 min at 37°C in normal HEPES-Ringer's (control), sodium-free HEPES-Ringer's (sodium replaced by choline), or potassium-free HEPES-Ringer's (potassium replaced by choline) in the absence or presence of 10^{-4} M bumetanide. All incubation media contained in addition 10 mM ouabain. Symbols are: (□) 10 mM ouabain; (▨) 10 mM ouabain + 10^{-4} M bumetanide. Values are given as the change in the intracellular Cl/P molar ratio during the incubation period and represent mean values ± SEM from 5 experiments. Compared to control conditions all values were significantly different at $P < 0.05$; in sodium-free and potassium-free HEPES-Ringer's uptake in the absence or presence of bumetanide was not significantly different.

dependent. Chloride-sensitive lactate production (361 ± 126 nmol lactate/mg protein × hr) was inhibited by 100% in the presence of 10^{-4} M bumetanide, 81% at 10^{-6} M bumetanide, 54% at 10^{-8} M, 41% at 10^{-9} M, and 17% at 10^{-10} M bumetanide (Fig. 4). The apparent K_i of this system is thus slightly below 10^{-8} M bumetanide. These results suggest that PCD cells possess a sodium entry mechanism which is chloride-dependent and bumetanide-sensitive, properties also exhibited by a Na-K-Cl cotransport mechanism.

Element content of isolated PCD cells

Effect of bumetanide on ouabain-induced sodium influx. In Table 1 the sodium, potassium, and chloride content of isolated PCD cell clusters, as determined by electronprobe microanalysis, are compiled. Initially the PCD cells which had been kept on ice had a K/Na ratio of 4.25; this value is similar to the value reported previously where cryosections of PCD cells had been analyzed [15].

When the cells were incubated at 37°C in the presence of ouabain the Na/K ratio decreased by 75% to 1.09. This decrease was mainly due to a threefold increase in intracellular sodium, whereas the reduction in intracellular potassium was only 25%. The increase in intracellular sodium was partly inhibited by

Table 2. Effect of bumetanide and ion replacement on element uptake into sodium, potassium, and chloride-depleted PCD cells

		Sodium (Na/P)			Potassium (K/P)			Chloride (Cl/P)			Phosphorus (mM/kg dry weight)
		Initial content	Content after 15′	Change	Initial content	Content after 15′	Change	Initial content	Content after 15′	Change	Content after 15′
Series 1	Control		0.404 ±0.016	0.347 ±0.020		0.491 ±0.015	−0.065 ±0.030		0.309 ±0.013	0.223 ±0.010	793 ±48
		0.066 ±0.007			0.559 ±0.01			0.088 ±0.005			
	10^{-4} M bumetanide ($N = 6$)		0.357 ±0.019 $P < 0.05$	0.282 ±0.023 $P < 0.05$		0.459 ±0.017 NS	−0.104 ±0.025 NS		0.237 ±0.013 $P < 0.01$	0.146 ±0.012 $P < 0.005$	818 ±76 NS
Series 2	Control		0.429 ±0.031	0.375 ±0.031		0.438 ±0.065	−0.094 ±0.096		0.329 ±0.015	0.243 ±0.016	814 ±71
		0.054 ±0.01			0.526 ±0.02			0.093 ±0.005			
	sodium free ($N = 3$)		0.054 ±0.005 $P < 0.005$			0.448 ±0.037 NS	−0.073 ±0.054 NS		0.211 ±0.014 $P < 0.05$	0.110 ±0.021 $P < 0.05$	946 ±101 NS
Series 3	Control		0.415 ±0.020	0.353 ±0.036		0.446 ±0.047	−0.125 ±0.056		0.304 ±0.014	0.220 ±0.015	823 ±45
		0.059 ±0.01			0.557 ±0.02			0.084 ±0.007			
	potassium free ($N = 5$)		0.410 ±0.017 NS	0.355 ±0.011 NS		0.435 ±0.022 NS	−0.108 ±0.038 NS		0.212 ±0.017 $P < 0.01$	0.129 ±0.020 $P < 0.025$	863 ±75 NS

The corresponding values for non-depleted cells ($N = 22$) were Na/P = 0.161 ± 0.005; K/P = 0.708 ± 0.01; Cl/P = 0.382 ± 0.01. Mean values ± SEM derived from paired experiments are given. PCD cells were preincubated for 30 min at 37°C in a Na-K-Cl-free HEPES-Ringer's. Subsequently, they were incubated for 15 min at 37°C in normal HEPES-Ringer's (control), in the presence of 10^{-4} M bumetanide, in sodium-free HEPES-Ringer's (sodium replaced by choline), or in potassium-free HEPES-Ringer's (potassium replaced by choline). All incubation media contained in addition 10 mM ouabain. *P* values compared to the paired controls by ANOV and the Newman-Keuls test.

10^{-4} M bumetanide, that is, intracellular sodium increased only 2.3-fold. The effect on Na influx is most evident when the increments in cell Na concentration are compared. In the absence of bumetanide, cell Na/P increased by 0.31 while in the presence of bumetanide cell Na/P increased by 0.20. Thus, bumetanide reduced Na influx by 35%. Under these experimental conditions bumetanide did not affect the potassium or the chloride content of the cell clusters.

These results—in agreement with the results obtained measuring lactate production—indicate that part of the sodium entry into PCD cells occurs by a bumetanide-sensitive mechanism, a finding consistent with the operation of a Na-K-Cl cotransporter. Since, however, neither potassium nor chloride content were affected by bumetanide the experiments provide no evidence for the coupled entry of the three ions.

Studies on ion depleted cells. In order to optimize the driving forces for a putative Na-K-Cl cotransport system we used the experimental conditions described by Geck et al in their studies in Ehrlich' ascites tumor cells [26]. As shown in Table 2 we preincubated PCD cells in sodium-, chloride-, and potassium-free medium and followed the reuptake of these ions into the cells. These maneuvers produced no obvious change in cell morphology. Preincubation of PCD cells in Na-K-Cl-free buffer decreased the chloride content by 74%, the sodium content by 56% and the K content by 25%.

After incubation of the ion depleted cells in normal HEPES-Ringer's containing 10^{-4} M ouabain, sodium content increased 5.5 fold, chloride content tripled, and the potassium content did not change. In the presence of bumetanide both sodium and chloride fluxes were reduced. Sodium uptake decreased significantly by 16% and chloride uptake by 39%, suggesting coupling between sodium and chloride flux via a bumetanide-sensitive system. It is interesting to note that bumetanide-sensitive chloride uptake was about 1.5 times higher than bumetanide-sensitive sodium uptake. Flux coupling also became evident in experiments where sodium was absent from the reuptake solutions. Chloride uptake amounted to only 50% of the uptake observed in the presence of sodium and was no longer sensitive to bumetanide (Fig. 5).

We also investigated the role of potassium in sodium and chloride uptake by the PCD cells. In potassium-free medium chloride uptake was reduced by 45% compared to control and bumetanide had no effect. No significant changes in sodium movement could be detected, although sodium uptake tended to be lower in potassium-free medium.

Thus in this experimental series sodium and potassium-dependent chloride uptake into PCD cells was clearly demonstrable. Bumetanide reduced Cl uptake to the same values as those measured in the absence of Na or K (Fig. 5). These data, therefore, strongly suggest that PCD cells possess a bumetanide-sensitive Na-K-Cl cotransport system.

In order to further substantiate this suggestion we also investigated rubidium uptake into these ion-depleted cells. As can be seen in Figure 6, bumetanide significantly inhibits rubidium uptake into these cells in the presence of ouabain, indicating coupling of sodium, chloride, and rubidium (potassium) flux via a common transporter.

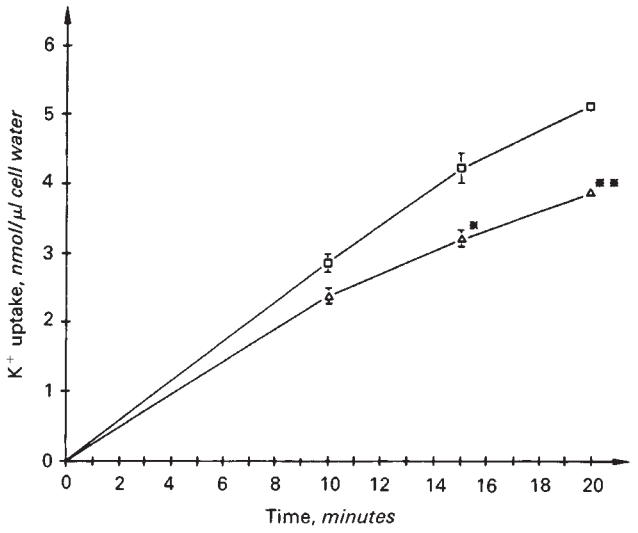

Fig. 6. *Effect of bumetanide on rubidium uptake by ion-depleted PCD cells.* PCD cells were preincubated for 30 min at 37°C in Na-K-Cl-free HEPES-Ringer's. Subsequently, they were incubated at 37°C for the time indicated in the figure in modified HEPES-Ringer's (only containing 1 mM K^+ and 15 μCi ^{86}Rb/ml) in the presence (△) or absence (□) of 10^{-4} M bumetanide. Details are in **Methods.** Values represent mean values ± SEM from 4 experiments. * $P < 0.02$; ** $P < 0.01$.

Discussion

Determination of intracellular element content by electron probe microanalysis of cell clusters

In the present study, element content of isolated PCD cells was determined by analyzing clusters of cells in a scanning electron microscope rather than in cryosections of PCD cells, as done in a previous publication [15]. Almost identical Na/P, K/P, and Cl/P ratios were obtained under control conditions in both studies, suggesting that the method applied here accurately mirrors the intracellular ion content of the cells during the incubation. The similarity of the values also indicates that contamination of the cell clusters with extracellular medium is minimal. The method of analyzing cell clusters as compared to cryosections has the advantage of being less time consuming and the cells of interest can be positively and easily identified by their typical morphology exhibited in the scanning electronmicroscope. One disadvantage is that it is not possible to obtain information on the water content of the cells. This drawback does not affect the conclusions drawn in this study because changes of element content per dry weight and phosphorus content are adequate indicators of solute movement. The consistency of the phosphorus/dry weight ratios under the various experimental conditions provides good evidence that the experimental maneuvers do not alter cell phosphorus content significantly. Thus, changes in Na, K, and Cl content factored for phosphorus likely reflect changes occurring in a single cell.

The ion content under control conditions translates into an intracellular sodium concentration of 24 mmol, an intracellular potassium concentration of 123 mmol, and an intracellular chloride concentration of 63 mmol (assuming previously-determined wet/dry weight ratios [15]). The values for sodium and chloride are quite similar to those found by Beck et al [27, 28] in sections of collecting duct cells prepared from the papilla of rats during antidiuresis. The potassium content is similar to the values obtained from diuretic rats [28]. The general agreement between these values obtained under very different conditions is good evidence supporting the viability of the in vitro preparation. However, we are aware of disagreements regarding the methods used to determine cell ion content in papillary tissue [29]. Until these disagreements can be resolved, comparisons between in vivo and in vitro PCD cells must be made with substantial circumspection.

It is important to note that the phosphorus content of the cells remained constant under the various experimental maneuvers. The cells do respond, however, to the different experimental maneuvers with a change in their total ion content. Exposure to ouabain for 15 minutes at 37°C leads to an increase in total intracellular ion content of 10%, indicating as expected some swelling of the cells. Ion depleted cells contain 50% less ions than control cells, suggesting some cell shrinkage. Since thus far only gross morphology of these cells in the scanning EM has been investigated which appears not to be significantly altered by these two maneuvers, the question of volume changes of the cells remains to be further elucidated.

Lactate production in PCD cells

In the current investigation lactate production provided a sensitive indicator for ion transport related metabolic activity of the PCD cells. The experiments using sodium replacement and ouabain treatment indicate that 40% of the lactate production of isolated PCD cells is linked to ion transport. These results in Wistar rats are somewhat different than those we have previously reported with Sprague-Dawley rats [15]. Absolute values of lactate production are almost twice as great in the Wistar rats. In addition, there is a greater inhibition of lactate production with ouabain or Na removal. The reason(s) for these differences is not clear, but may be owing to a tighter coupling of glycolysis and Na-K-ATPase activity in Wistar PCD cells than in Sprague-Dawley PCD cells. Such tight coupling has recently been proposed for MDCK cells [30]. This phenomenon might also explain the minimal effect of bumetanide on O_2 consumption in Wistar PCD cells (data not shown). Furthermore, the method used to determine lactate was different in the two studies. With the former method ouabain was found to sometimes yield lactate levels which were too high.

Lactate production was also inhibited by chloride removal and by bumetanide. The inhibition was, however, smaller than the inhibition found after sodium removal. This result suggests that rat PCD cells contain an additional pathway for sodium entry which is chloride-independent and bumetanide-insensitive. One possible pathway is an amiloride-sensitive sodium channel. Such a pathway would be consistent with in vivo experiments demonstrating amiloride inhibition of Na absorption in Wistar rat papillary collecting ducts [10]. An amiloride-sensitive Na pathway has also been proposed by Zeidel et al [31] to explain the amiloride-induced reduction in O_2 consumption by rabbit PCD cells. In the rabbit, furosemide had no effect on PCD O_2 consumption [31].

The reduction in lactate production by bumetanide is most likely the result of a reduction in Na entry into the cell and the subsequent reduction in energy demand from the Na-K pump. Another possibility is that bumetanide reduced Na-K-ATPase activity directly. We think such an explanation is unlikely

because concentrations as low as 10^{-8} M bumetanide inhibited lactate production. Such a high affinity most likely represents an effect on the Na-K-Cl cotransporter. In addition to this dose response evidence, direct measurements of Na-K-ATPase activity in these PCD cells showed no effect of 10^{-4} M bumetanide (data not shown).

Evidence for the presence of a Na-K-Cl cotransporter

Four different experimental approaches were employed to obtain evidence for the operation of a Na-K-Cl cotransporter in PCD cells. First, as has been done with isolated TALH cells [16], a link between cell metabolism and the presence of sodium and chloride in the incubation medium was established. As discussed above, the reduction in lactate production by bumetanide is most likely the result of the inhibition of a Na-K-Cl cotransporter. A second approach utilized the measurement of cell Na, K, and Cl following exposure to bumetanide. In cells incubated in HEPES-Ringers and ouabain, clear evidence for an inhibition of ion uptake was found only for Na (Table 1). The reason that cell K and Cl were not significantly altered may be because other translocation systems exist for these ions or that under these circumstances the bumetanide-sensitive Na-K-Cl cotransporter operates in a KCl or NaCl mode. Reductions in influx via a Na-K-Cl cotransporter may secondarily cause reductions in flux via these other transport systems. The resulting change in cell K and Cl content might have been too small to detect. More convincing evidence regarding an effect of bumetanide on a Na-K-Cl cotransporter was obtained by using ion depleted cells (Table 2). Under these conditions, bumetanide reduced the influx of Na and Cl but not K. The reason for this minimal change in cell K may also be related to the presence of other K translocation pathways.

The third approach to demonstrate a Na-K-Cl cotransporter utilized ion substitutions. Removing either Na or K caused the increase in Cl content to fall to values not different from that with bumetanide. The results, when taken together, provide strong evidence for the presence of a Na-K-Cl cotransporter in the rat PCD cell.

Furthermore, studies on rubidium uptake into the ion-depleted PCD cells were performed and the effect of bumetanide in the presence of ouabain was investigated. These experiments clearly showed that bumetanide inhibited rubidium uptake, a criterion frequently employed to demonstrate Na-K-Cl cotransport in epithelia [32].

It is unlikely that the present results can be explained by the presence of a Na-K-Cl cotransporter in a cell other than the PCD. The possibility of contamination by cells of the medullary thick ascending limb was seriously considered and eliminated for three reasons: (a) Extreme care was taken to avoid contamination by the red medulla when dissecting the papilla. (b) Searching for cells containing Tamm Horsfall protein, a marker for TALH cells, consistently yielded low or absent numbers. (c) Finally, each cell cluster examined by scanning electron microscopy displayed the typical appearance of papillary collecting duct cells and not TALH cells.

Another candidate for contamination is the papillary surface epithelial cell. In the rabbit, these cells appear to have a Na-K-Cl cotransporter on the apical membrane with a very high affinity for bumetanide [33]. It is possible that rat papillary surface epithelial cells also contain a Na-K-Cl cotransporter. However, surface epithelial cells comprise only a small fraction of the number of PCD cells. If only these cells possess a Na-K-Cl cotransporter, we should not have measured bumetanide-sensitive fluxes in virtually every cell cluster. Whether rat papillary surface epithelial cells also possess a Na-K-Cl cotransporter cannot be answered by the present data.

Physiological role of the cotransporter

Two major functions have been ascribed to the Na-K-Cl cotransporter. In a variety of nonpolarized cells as well as some epithelial cells [13, 34], the cotransporter is involved in cell volume regulation. We have no direct information on whether the cotransporter identified in the present experiments operates in this fashion. However, the well known variability in interstitial osmolality of the papilla makes it likely that cell volume regulating systems are of critical importance to PCD cell survival.

The second major function of the Na-K-Cl cotransporter involves the mediation of Cl entry into the cell during transepithelial Cl transport [14]. However, in view of the lack of knowledge on the cellular distribution of the transporter and in view of the disagreement about the intracellular ion concentrations [27–29] under various experimental conditions, it is currently not possible to state when absorption of Na and Cl via the Na-K-Cl cotransporter is thermodynamically possible.

In summary, the present experiments provide evidence for a Na-K-Cl cotransporter in rat papillary collecting duct cells. There is good reason to believe that its operation contributes to a significant amount of ion influx and that a portion of the cell's energy production is devoted to extrusion of the ions entering the cell via this pathway. It is likely that other transporters also provide entry pathways for ions. The contribution of each pathway and its role in cell function remains to be determined.

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