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Characterization of the Na^+/H^+ Exchanger in the Luminal Membrane of the Distal Nephron

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Abstract. In the rabbit as well as the rat, a Na^+/H^+ exchanger is expressed in the apical membrane of both the proximal and distal tubules of the renal cortex. Whereas the isoform derived from the proximal tubule has been extensively studied, little information is available concerning the distal luminal membrane isoform. To better characterize the latter isoform, we purified rabbit proximal and distal tubules, and examined the ethylpropylamiloride (EIPA)-sensitive ^{22}Na uptake by the luminal membrane vesicles from the two segments. The presence of 100 μM EIPA in the membrane suspension decreased the 15 sec Na^+ uptake to 75.70 ± 4.70% and 50.30 ± 2.23% of the control values in vesicles from proximal and distal tubules, respectively. The effect of EIPA on 35 mM Na^+ uptake was concentration dependent, with a IC_{50} of 700 μM and 75 μM for the proximal and distal luminal membranes. Whereas the proximal tubule membrane isoform was insensitive to cimetidine and clonidine up to a concentration of 2 mM, the 35 mM Na^+ uptake by the distal membrane was strongly inhibited by cimetidine (IC_{50} 700 μM) and modestly inhibited by clonidine (IC_{50} 1.6 mM).

The incubation of proximal tubule suspensions with 1 mM (Bu_2) cAMP decreased the 15-sec EIPA-sensitive Na^+ uptake by the brush border membranes to 24.1 ± 2.38% of the control values. Unexpectedly, the same treatment of distal tubules enhanced this uptake by 46.5 ± 10.3%. Finally, incubation of tubule suspensions with 100 nM phorbol 12-myristate 13-acetate (PMA) decreased the exchanger activity to 58.6 ± 3.04% and 79.7 ± 3.21% of the control values in the proximal and distal luminal membranes, respectively. In conclusion, the high sensitivity of the distal luminal membrane exchanger to various inhibitors, and its stimulation by cAMP-dependent protein kinase A, indicate that this isoform differs from that of the proximal tubule and probably corresponds to isoform 1.

Key words: Renal Na^+/H^+ exchanger — Distal tubule luminal membrane

Introduction

As early as 1960, Gottschalk, Lassiter and Mylle (1960), in micropuncture experiments, showed a progressive decrease in the tubular fluid pH along the rat proximal convoluted tubule. This observation was subsequently confirmed by Malnic, De Mello-Aires and Giebisch (1972). The role of Na^+ in this acidification was shown in micropuncture as well as in in vivo microperfusion experiments (Bank, 1962; Ullrich, Radtke & Rumrich, 1971; Ullrich, Rumrich & Baumann, 1975; Chantrelle, Cogan & Rector, 1982), and in in vitro microperfusion of rabbit isolated tubules (Burg & Green, 1977; Schwartz, 1981). The technique of isolation of brush border membrane vesicles from rat renal cortex described by Booth and Kenny (1974) allowed Murer, Hopfer and Kinne (1976) and Aronson, Nee and Suhm (1982) to demonstrate more directly the presence of an electroneutral exchange of Na^+ against H^+ through these membrane vesicles. Although the vesicles were prepared from total cortex including distal tubules, the exchange activity was attributed to the proximal tubule. A basolateral Na^+/H^+ exchanger has also been detected in the rat renal cortex, whose role is probably to maintain a normal intracellular pH as found in any other cell (Sabolic & Burckardt, 1983; Seifter & Aronson, 1986; Alpern & Chambers, 1986). More recently, the presence of a luminal Na^+/H^+ exchanger was also described in distal segments. In the rat, this activity was localized in the thick ascending limb by in vitro isolated tubule microperfusion (Good, 1985;

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Watts & Good, 1994), and either in the early distal tubule by in vivo free flow microperfusion (Wang et al., 1993) or along the whole distal tubule by stopflow microperfusion (Fernandez et al., 1994).

Using various techniques such as the measurement of intracellular pH in OK cells and LLC-PK cells, or cDNA cloning from cell cultures and transfection in fibroblasts PS 120 or Chinese hamster ovary cells, several isoforms have been detected. Characterization of these isoforms showed distinct kinetic and pharmacological traits such as the sensitivity to inhibitors, the affinity for H^+ and Na^+ and the regulation by kinases. Immunofluorescence studies localized isoform 3 in the apical membrane of the proximal tubule (Biemesderfer et al., 1993; Amemiya et al., 1995) and isoform 1 in the basolateral membrane of both the late proximal and the distal tubules (Biemesderfer et al., 1992). None of these studies, however, characterized the isoform of the distal luminal membrane.

The present experiments were designed to confirm the presence of Na^+/H^+ exchanger activity in the distal nephron luminal membrane of the rabbit, to characterize this exchanger with specific inhibitors and to determine the effects of protein kinases on this activity.

Materials and Methods

Tubule Preparation

Rabbit kidneys were obtained immediately following the sacrifice of the animals at the slaughter house. The basic procedure for the purification of the proximal and distal tubules was similar to that described in previous studies (Brunette, Mailloux & Lajeunesse, 1992). Cortex slices were incubated for 20 min at 37°C in a modified Krebs-Henseleit (KH) buffer containing 1 mg/ml of collagenase type V and 0.5 mg/ml bovine serum albumin (BSA). The tissue suspension was filtered through a stainless steel mesh and the filtrate was centrifuged at 200 × *g* for 20 sec. The pellets containing the tubules were washed three times in KH solution supplemented with 0.5 mg/ml BSA, suspended in 40% Percoll (final concentration) previously saturated with 95% O_2, 5% CO_2 for 20 min, and centrifuged for 30 min at 28,000 × *g* at 4°C. The distal and proximal tubule enriched bands were collected, washed three times in KH and suspended either in 250 mM sucrose and 20 mM Tris-HEPES pH 7.4 (for further basolateral membrane preparations) or in hypotonic solution containing 10 mM mannitol and 2 mM Tris-HEPES pH 7.4 (for luminal membrane preparations) and frozen at −80°C. The composition of KH was (in mM): 138 NaCl, 3.8 KCl, 1.4 KH_2PO_4, 1.4 $MgSO_4$, 1.17 $CaCl_2$, 25 Na HCO_3, 60 mannitol, 1 pyruvic acid, 1 glutamic acid, 10 lactic acid and 10 glutamine.

Incubation of the Tubules with Dibutyryl Cyclic AMP (db cAMP) and Phorbol 12 Myristate 13 Acetate (PMA)

The freshly prepared tubules were incubated with either db cAMP (30 min at 37°C) or PMA (20 min at 37°C), or the carrier, in a cell culture medium (DMEM/HAMF-12) containing 2% fetal bovine serum and 0.1 mM phenyl-methylsulfonyl fluoride (PMSF). In the db cAMP experiments, 0.5 mM MIX and 1 mM theophylline were added to the medium. The incubation was stopped by centrifugation, the tubules were washed 3 times, suspended in the solutions described above, and kept frozen at −80°C until the day of the experiment.

Table 1. Enzyme enrichments in luminal and basolateral membrane vesicles from PT and DT compared to their respective tubule suspensions

Preparations	Alk. Phosphatase	Na/K ATPase
PT Lum	5.04 ± 0.43	0.68 ± 0.13
PT BLM	0.73 ± 0.18	6.57 ± 1.20
DT Lum.	1.2 ± 0.08	0.79 ± 0.05
DT BLM	0.86 ± 0.18	2.30 ± 0.05

PT: proximal tubule; DT: distal tubule; Lum: Luminal membrane; BLM: basolateral membrane.

Luminal Membrane Purification

On the day of the experiment, the frozen tubule suspensions were thawed and homogenized with a Potter homogenizer. The luminal membranes were purified using the $MgCl_2$ precipitation technique. Following the addition of 12 mM $MgCl_2$, final concentration, the proximal and distal tubule suspensions were subsequently stirred on ice for 20 and 10 min respectively and centrifuged at 3000 × *g* for 20 min at 4°C. The supernatants were collected and recentrifuged at 28,000 × *g* for 20 min, at 4°C. The membrane-containing sediments were washed twice, suspended in 280 mM mannitol, 20 mM morpholino ethanesulfonic acid (MES) pH 6.0 and allowed to vesiculate at 4°C for 60 min.

The purity of the tubule and membrane suspensions was monitored by the activities of the enzyme markers which are presented in Table 1. Alkaline phosphatase was measured with the technique of Kelly and Hamilton (1970) and Na^+/K^+ ATPase with the technique of Post and Sen (1967). In previous experiments, very low activities of succinate dehydrogenase and glucose-6-phosphatase were detected in our luminal membrane preparations, reflecting negligible contamination with mitochondria or endoplasmic reticulum membranes (Brunette et al., 1992).

Basolateral Membrane (BLM) Purification

The distal tubule suspensions were disrupted with a sonifier cell disrupter for three periods of 10 sec. The homogenates were suspended in 250 mM sucrose, 20 mM Tris-HEPES pH 7.4 containing 20% Percoll and centrifuged at 27,750 × *g* for 30 min at 4°C. After centrifugation, two bands were observed, the top one being enriched in BLM. This band was washed three times in 280 mM mannitol, 20 mM MES pH 6.0, and stored on ice for 1 hr for vesiculization.

Incubation of Membranes with the Various Inhibitors

The membrane vesicles were preincubated with clonidine or cimetidine or the carrier for 10 min at 22°C prior to the uptake measurement. Ethyl isopropyl amiloride (EIPA) or the carrier was added to the vesicle suspensions 2 min prior to the uptake.

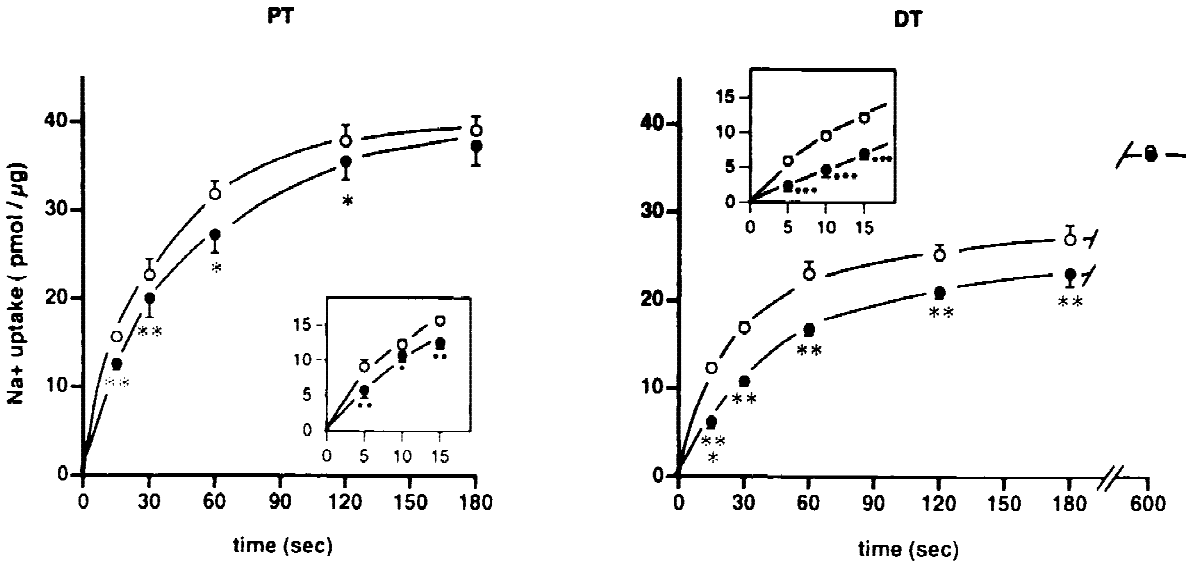

Fig. 1. Effect of 100 μM EIPA on the time course of 35 mM Na^+ uptake by the luminal membrane vesicles from proximal (PT) and distal (DT) tubules. Membrane vesicles were preequilibrated with a medium containing (in mM): 280 mannitol, 20 MES, pH 6.0; Na^+ uptake was measured in 210 mannitol, 20 Tris-HEPES pH 7.4 and 35 ^{22}NaCl in the presence (●) or absence (○) of EIPA. *$P < 0.02$, **$P < 0.01$, ***$P < 0.005$ compared to the control (paired t test). $n = 4$ (PT) and 5 (DT). Insets: uptakes at 5, 10 and 15 sec.

Sodium Uptake Measurement

^{22}Na uptake was measured by the rapid Millipore filtration technique. Samples of 5 μl of membrane suspension, containing approximately 30 μg and 20 μg of proteins for proximal and distal luminal membranes, were added to 15 μl of prewarmed (37%) incubation medium. At the indicated time, the uptakes were stopped with 1 ml of ''stop solution'' (140 mM LiCl, 20 mM Tris-HEPES pH 7.4), the suspensions were filtered, and the filters rinsed with the same stop solution. Unless otherwise stated, the incubation medium for Na^+ uptake measurements contained (in mM): 210 mannitol, 35 ^{22}NaCl and 20 Tris-HEPES, pH 7.4. The concentration of 35 mM Na^+ was chosen to approach the in vivo distal tubular fluid composition. Preliminary experiments also showed that this concentration was below that required for maximal Na^+ uptake.

Materials

Carrier-free ^{22}NaCl (10 mCi/ml) was obtained from NEN Research Products Dupont Canada (Mississauga, Ontario). Cimetidine and clonidine were purchased from ICN (Mississauga, Ontario). All other chemicals and reagents used in these experiments were purchased from Sigma (Mississauga, Ontario).

Results

Effect of EIPA on Na^+ Uptake by the Luminal Membranes from Proximal and Distal Tubules

Figure 1 shows the time course of ^{22}Na uptake by membrane vesicles from proximal and distal tubule origins. As shown in the insets, the slope of initial uptake was linear for the first 15 sec, to slowly decline thereafter. Upon the addition of 100 μM EIPA to the membrane suspensions, the Na^+ uptake decreased from 16.08 ± 0.48 to 12.19 ± 0.97 pmol/μg/15 sec ($P < 0.02$, paired t test) i.e., to 75.7 ± 4.7% of the control values in the proximal membrane vesicles and from 12.17 ± 0.67 to 6.11 ± 0.69 pmol/μg/15 sec ($P < 0.005$) i.e., to 50.3 ± 2.23% of the control values in the distal membrane vesicles.

The effect of EIPA on Na^+ uptake was concentration-dependent. Figure 2 presents the variations of 35 mM Na^+ uptake with increasing concentrations of EIPA. As suggested by the above data, the 15 sec Na^+ uptake by the proximal tubule brush border membrane vesicles was relatively resistant to EIPA, with IC_{50} of 700 μM. In contrast, the IC_{50} EIPA value for the distal luminal membrane was 75 μM. For comparison, a similar dose-response curve was performed with distal tubule basolateral membranes (we were unable to detect any EIPA-sensitive Na^+ uptake in the proximal tubule basolateral membrane: the 35 mM Na^+ uptakes were 5.23 ± 0.26 and 5.3 ± 0.19 pmol/μg 15 sec in the control and treated membranes, respectively). The sensitivity of the distal basolateral membranes to EIPA was similar to that observed with the distal luminal membranes, with a IC_{50} of 70 μM.

Na^+ uptake by our proximal and distal luminal membranes was saturable and followed Michaelis-Menten kinetics. To determine on which kinetic parameter EIPA was acting, Na^+ uptake at increasing concentrations was measured in the presence and absence of 10^{-4} M EIPA. In these experiments, concentrations of mannitol were

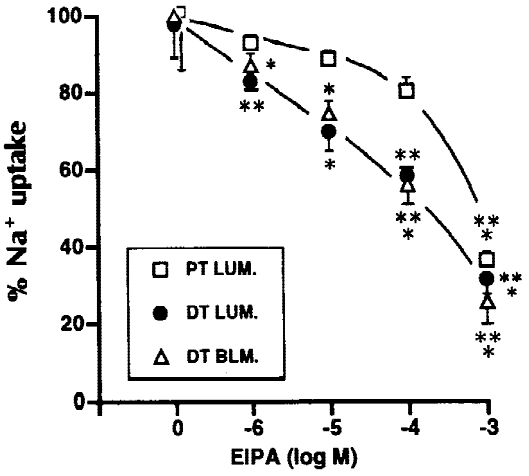

Fig. 2. Dose-response curve of the effect of EIPA on the 15 sec ^{22}Na uptake (Na^+ = 35 mM) by luminal membrane vesicles from proximal (PT) and distal (DT) tubules and by basolateral membranes of distal tubules. The media compositions were the same as in Fig. 1. $^*P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$ compared to control values (paired *t* test). ($n = 3$).

calculated to keep the osmolarity constant. Table 2 presents the kinetic parameters of these uptakes by the distal luminal membranes. In the control and the treated membranes, the maximal velocity of Na^+ uptake was reached with 60 mM Na^+ as the substrate. The inhibitor increased the K_m Na^+ values from 18.88 ± 2.99 to 32.75 ± 5.25 mM ($P < 0.05$, $n = 4$) without significant change in the V_{max} (20.48 ± 2.91 and 21.08 ± 3.05 pmol/μg/15 sec in the control and EIPA-treated distal luminal membranes). The mean K_m and V_{max} values of the EIPA-sensitive Na^+ uptakes were 6.09 ± 1.72 mM and 3.38 ± 0.82 pmol/μg/15 sec, respectively. A similar effect of EIPA was observed with proximal tubule brush border membranes whose K_m values were 11.76 ± 0.6 and 14.91 ± 1.56 mM Na^+ ($P < 0.05$, $n = 6$) in control and treated membranes, with a mean K_m value of the EIPA-sensitive Na^+ uptake of 4.66 ± 1.18 mM (Table 2).

Effects of Cimetidine and Clonidine

In an attempt to further characterize the exchanger isoform present in the distal luminal membranes, we studied the influence of cimetidine and clonidine on Na^+ uptake by these membranes. The membrane vesicles were preincubated with various concentrations of cimetidine for 10 min at room temperature. Figure 3 shows the dose-response curve of the effect of cimetidine on EIPA-sensitive Na^+ uptake by luminal membranes of proximal and distal tubules, and for comparison, by basolateral membranes of distal tubules. Whereas cimetidine had no significant effect on the proximal tubule luminal membranes at any of the concentrations tested, 0.5 mM of the inhibitor strongly decreased the EIPA-sensitive Na^+ uptake by the distal luminal and basolateral membranes, to 59.3 ± 5.3% ($P < 0.01$) and 54.1 ± 22.3% ($P < 0.05$) of their control values (IC_{50} 700 and 600 μM, respectively).

Similar experiments were performed with clonidine. As shown in Fig. 4, the EIPA-sensitive Na^+ uptake by the distal luminal membranes was also sensitive to clonidine, although to a lesser degree than to cimetidine: incubation of the membrane vesicles with 0.5 mM clonidine decreased the 15 sec. Na^+ uptake to 75.7 ± 2.2% of the control values ($P < 0.02$). The IC_{50} was 1.6 mM. Na^+ uptake by the distal basolateral membranes was similarly sensitive to clonidine: 0.5 mM clonidine decreased Na^+ uptake to 67.0 ± 8.1% of the control values ($P < 0.05$, IC_{50} = 1.1 mM). In contrast, the EIPA-sensitive Na^+ uptake by the proximal luminal membranes was resistant to clonidine up to 2 mM.

Regulation of the Luminal Na^+/H^+ Exchangers: Effect of cAMP

It has been previously reported that when proximal tubule cells of various origins are exposed to dibutyryl cAMP (Pollock, Warnock & Strewler, 1986; Helmle-Kolb et al., 1990; Helmle-Kolb et al., 1993; Mrkic et al., 1992; Mrkic et al., 1993; Azarani, Goltzman & Orlowski, 1995), or when brush border membranes are directly incubated with cAMP and a phosphorylating solution (Kahn et al., 1985; Weinman, Shenolikar & Kahn, 1987), the activity of the Na^+/H^+ exchanger in the brush border membrane is significantly decreased. In the following experiments, we investigated the influence of the messenger on the Na^+/H^+ exchanger activity in the distal luminal membranes and compared the results with those obtained with the proximal membranes.

Proximal and distal tubules were incubated for 30 min at 37°C with 1 mM db cAMP or the carrier. Then the suspensions were centrifuged, the tubules were washed and frozen at −80°C. The day of the experiment, the luminal membranes were purified and the EIPA-sensitive ^{22}Na uptake was measured.

Figure 5 shows the effect of db cAMP on the EIPA-sensitive ^{22}Na by the proximal and distal tubule luminal membranes. The EIPA-sensitive ^{22}Na uptake by the membranes from proximal tubules treated with 1 mM db cAMP was decreased from 2.90 ± 0.53 to 0.70 ± 0.14 pmol/μg/15 sec ($P < 0.02$, unpaired *t* test), i.e., to 24.1 ± 2.38% of the control values, thus confirming data reported by other groups. In contrast and unexpectedly, incubation of distal tubules with 1 mM db cAMP significantly enhanced the exchanger activity from 6.61 ± 0.07 to 9.69 ± 0.70 pmol/μg/15 sec ($P < 0.02$, unpaired *t* test) i.e., an increase of 46.5 ± 10.3%. For comparison, we prepared basolateral membranes from tubules treated or not with db cAMP, and measured the exchanger activity

Table 2. Effect of 100 μM EIPA on the kinetic parameters of Na^+ uptake by proximal and distal luminal membranes (pH 7.4 in and out)

	Proximal convoluted tubules		Distal convoluted tubules	
	K_m (mM)	V_{max} (pmol/μg/15 sec)	K_m (mM)	V_{max} (pmol/μg/15 sec)
Control	11.76 ± 0.60	12.57 ± 0.59	18.88 ± 2.99	20.48 ± 2.91
EIPA	14.91 ± 1.56*	11.91 ± 1.21	32.75 ± 5.25*	21.08 ± 3.05
EIPA sensitive	4.66 ± 1.18	1.38 ± 0.34	6.09 ± 1.72	3.38 ± 0.82

* $P < 0.05$ compared to the control values. $n = 6$ for proximal tubules and 4 for distal tubules.

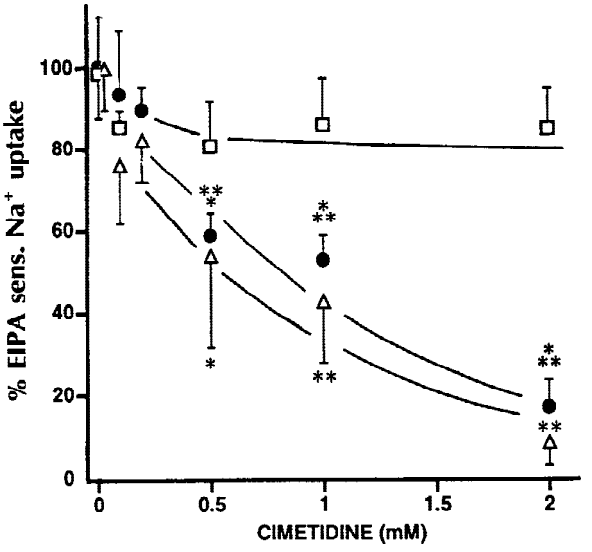

Fig. 3. Dose-response curve of the effect of cimetidine on EIPA-sensitive 15 sec Na^+ uptake (Na^+ = 35 mM) by luminal membranes from proximal (□) and distal (●) tubules, and by basolateral membranes (△) of distal tubules. *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$ compared to control values (paired t test). ($n = 3$).

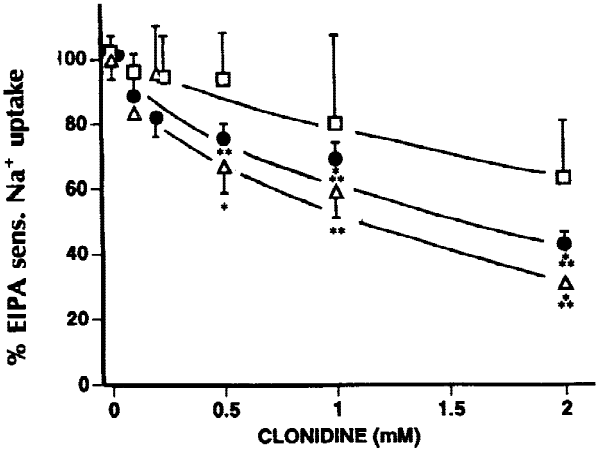

Fig. 4. Dose-response curve of the effect of clonidine on EIPA-sensitive 15 sec Na^+ uptake (Na^+ = 35 mM) by luminal membranes from proximal (□) and distal (●) tubules, and by basolateral membranes (△) of distal tubules. *$P < 0.05$ compared to the control values (paired t test). ($n = 6$).

in these membranes. The messenger enhanced the EIPA-sensitive Na^+ uptake in the basolateral as it did in the luminal membranes of the distal tubules, from 3.43 ± 0.05 to 4.96 ± 0.32 pmol/μg/15 sec in membranes from control and treated tubules, i.e., an increase of 45.5 ± 10.1% ($P < 0.02$, unpaired t test).

Influence of Phorbol Ester (PMA)

We also investigated the influence of PMA on the EIPA-sensitive Na^+ uptake. The proximal and distal tubule suspensions were incubated with 100 nM PMA or the carrier for 20 min at 37°C. PMA significantly decreased the exchanger activity in the proximal tubule brush border from 4.41 ± 0.52 to 2.60 ± 0.38 pmol/μg/15 sec ($P < 0.05$, unpaired t test) and in the distal luminal membranes from 8.01 ± 0.36 to 6.37 ± 0.28 pmol/μg/15 sec ($P < 0.02$) i.e., to 58.6 ± 3.04% and 79.7 ± 3.21% of control values (Fig. 6). For comparison, we studied the effect of 100 nM PMA on the Na^+/H^+ activity of the distal basolateral membrane: the messenger also diminished this activity from 5.35 ± 0.14 to 4.37 ± 0.26 pmol/μg/15 sec ($P < 0.02$) i.e., a decrease to 81.8 ± 5.29% of the control values.

Discussion

The Presence of a Na^+/H^+ Exchanger in the Distal Luminal Membrane

Although a number of micropuncture studies have established that a substantial amount of bicarbonate reabsorption with a concomitant decrease in tubular fluid pH, occurs along the distal tubule (Malnic et al., 1972; Levine, 1985; Levine & Jacobson, 1986; Capasso et al., 1987; Chan, Malnic & Giebisch, 1989), the contribution of each type of acidification mechanism i.e., the proton

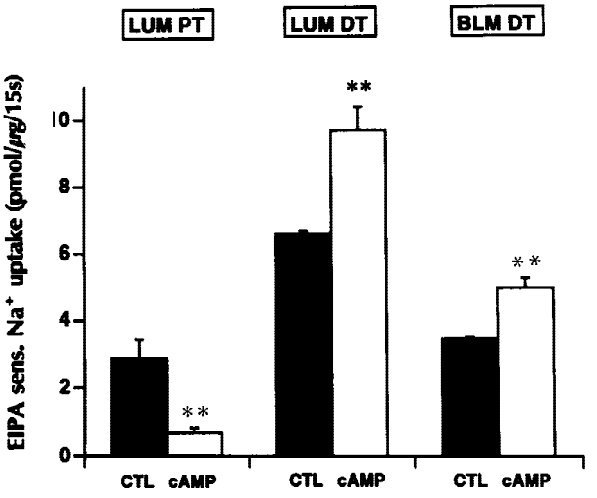

Fig. 5. Effect of cAMP on the EIPA-sensitive 35 mM Na^+ uptake by luminal membranes from proximal (PT) and distal (DT) tubules, and for comparison, basolateral membranes of distal tubules (BLM DT). Tubule suspensions were incubated with 1 mM of $(Bu)_2$ cAMP or the carrier for 30 min at 37°C. Then the tubules were collected by centrifugation, suspended in a hypotonic solution and frozen. The day of the experiment, the membranes were prepared as described in the methods section. $^{**}P < 0.02$ compared to Na^+ uptake by membranes from untreated tubules (unpaired *t* test) ($n = 3$).

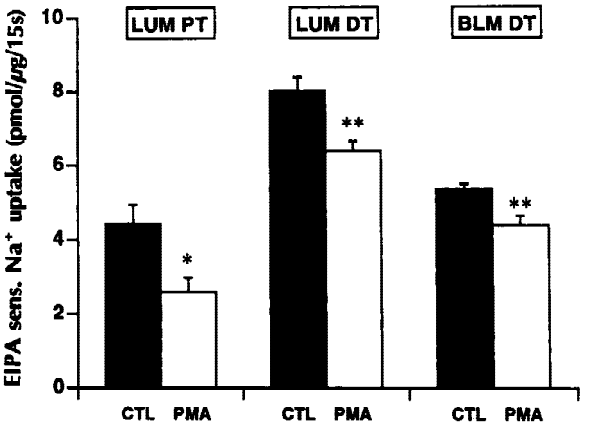

Fig. 6. Effect of 100 nM PMA on the 35 mM Na^+ uptake by luminal membranes from proximal (PT) and distal (DT) tubules, and by basolateral membranes of distal tubules (BLM DT). The tubules were incubated with PMA or the carrier for 20 min. at 37°C. The day of the experiment, the membranes were prepared as described in the methods section. $^{*}P < 0.05$, $^{**}P < 0.02$ compared to Na^+ uptake by membranes from untreated tubules (unpaired *t* test) ($n = 3$).

pump (Khadouri et al., 1991), the H^+/K^+ ATPase (Planelles et al., 1991), and finally the Na^+/H^+ exchanger (Wang, Orlowski & Shull, 1993; Fernandez et al., 1994), has been determined only recently. All of these mechanisms have specific inhibitors: Bafilomycin A for the H^+ ATPase, Schering compound 28080 for H^+/K^+ ATPase and amiloride derivatives such as EIPA or Hexamethylene-Amiloride (HMA) for the Na^+/H^+ exchanger. Using these inhibitors in separate microperfusion experiments of early and late distal tubules, Wang et al. (1993) reported that in the rat, EIPA inhibited bicarbonate reabsorption in the early distal tubule, while Bafilomycin A inhibited this transport in both the early and late segments, and Schering compound 28080 was active exclusively in the late distal tubule. These findings were confirmed in stop-flow experiments by Fernandez et al. (1994) although these authors also observed an exchanger activity in the late segments of the distal nephron.

Our distal tubule suspension constitutes a mixed population containing all of the segments of the cortical distal nephron, i.e., from the cortical ascending limb to the cortical collecting tubule. When evaluated as a function of protein content, and using 35 mM Na^+ as the substrate, the luminal membranes of these segments exhibited a higher EIPA-sensitive Na^+ uptake than those of the proximal tubules. Inhibition by EIPA was competitive, as was previously reported (Aronson et al., 1982). The K_m values of the EIPA-sensitive Na^+ uptakes (4.66 ± 1.18 and 6.09 ± 1.72 mM Na^+ for the proximal and the distal membranes) are similar to those reported by Burnham et al. (1982) and Warnock, Reenstra and Yee, (1982) with brush border membrane preparations (10 and 13 mM Na^+), by Levine et al. (1993) with PS 120 fibroblasts transfected with cloned intestinal isoforms 1, 2 or 3 (15, 18 and 17 mM Na^+), or by Orlowski (1993) in Chinese hamster ovary cells transfected with isoforms 1 and 3 (10 mM Na^+ and 4.7 mM Na^+). These K_m values are relatively low compared to the Na^+ content of tubular fluid, suggesting that in vivo, the mechanism is saturated.

The Isoform of the Distal Luminal Na^+/H^+ Exchanger: Sensitivity to Inhibitors

The main purpose of our experiments was to characterize the Na^+/H^+ exchanger in the distal luminal membrane by studying the sensitivity of this exchanger to inhibitors and messengers, and eventually to identify the isoform.

In the mammalian kidney, at least four isoforms have been successfully cloned and expressed in several cell types including PS 120 fibroblasts (Tse et al., 1993*a,b;* Levine et al., 1993; Honda et al., 1993), Chinese hamster ovary cells (Orlowski et al., 1993; Wang et al., 1993; Yu, Shull & Orlowski, 1993), Mardin-Darby canine kidney cells (Noel, Roux & Pouyssegur, 1993), and OK cells (Helmle-Kolb et al., 1993). These techniques allowed, to some extent, the characterization of these isoforms: their sensitivity to amiloride and its derived substances, to cimetidine and clonidine, and their modulation by different kinases. Using these techniques, it was shown by several laboratories that isoform 1 was strongly inhibited by amiloride and EIPA, while isoform

3 was resistant to both inhibitors (Orlowski, 1993; Tse et al., 1993*a;* Tse et al., 1994; Yu et al., 1993). Characterization of isoform 2 is far less established: Tse et al. (1993*b*) and Kuwahara et al. (1994) qualify the isoform as resistant to EIPA, with a relatively high IC_{50} of 500 nM (compared to 20 nM for isoform 1). In contrast, Yu et al. (1993) reported a strong inhibitory effect of EIPA on isoform 2 with a IC_{50} of 79 nM (compared to 2.4 μM for isoform 3 and 15 nM for isoform 1). Finally, sensitivity to cimetidine and clonidine also varies with the isoform: whereas the sensitivity to clonidine is maximal for isoform 2, with the following order: 2 > 1 > 3, sensitivity to cimetidine is highest for isoform 1 (1 > 2 > 3) (Kulanthaivel et al., 1990; Yu et al., 1993).

Immunocytochemical techniques have been used to localize isoforms along the kidney tubule. In the rabbit and the rat, isoform 1 was found in the basolateral membrane of most of the nephron segments of the superficial cortex except S1 and S2 (Biemesderfer et al., 1992), confirming molecular biology experiments showing the absence of any human growth factor-activated Na^+/H^+ antiporter in S1 and S2 (Krapfs & Solioz, 1991). In contrast, isoform 3 was reported to be exclusively within the brush border membrane of the proximal tubule (Biemesderfer et al., 1993; Amemiya et al., 1995). The isoform 2 has been less clearly localized. Haggerty et al. (1988) and Casavola, Helmle-Kolb and Murer (1989), using intracellular pH fluorescence techniques, reported in LLC-PK1/C14 cells, i.e., a pig renal cortex cell culture containing a mixture of proximal and distal tubule cells, the presence of this exchanger isoform in the luminal membranes. The authors attributed the activity to the proximal tubule cells. Wang, Orlowski and Shull (1993) using Northern hybridization technique also showed the presence of isoform 2 mRNA in the kidney, but in very low concentration compared to intestine. However, recent studies using Northern blot analysis and *in situ* hybridization detected isoform 2 mRNA in the distal convoluted but not in the proximal tubules of the rat kidney (Ghishan et al., 1995), and in preliminary experiments, Chambray et al. (1997) reported the presence of this isoform in the rat distal tubule luminal membrane. Finally, Northern hybridization experiments rather detected isoform 2 in the medullary collecting ducts (Soleimani et al., 1994) and, to further complicate the matter, Bookstein et al. (1997) was unable to demonstrate any isoform 2 mRNA in the rat kidney.

Our results demonstrate that the Na^+/H^+ exchanger present in the distal luminal membrane is very sensitive to EIPA and cimetidine, and relatively sensitive to clonidine compared to the brush border membrane of proximal tubules which was resistant to the various inhibitors, thus confirming the data of Biemesderfer et al. (1992, 1993) and Orlowski et al. (1993). Our failure to detect any exchanger activity in the proximal basolateral membrane is probably due to the fact that only superficial cortex was selected in our experiments excluding S_3. The high sensitivity of the distal luminal isoform to EIPA, cimetidine and to a lesser degree clonidine, eliminates the possibility that this membrane is the site of isoforms 3, but rather suggests an isoform 1. This hypothesis is also supported by the similarity of sensitivity to inhibitors between the enzymes of distal luminal and basolateral membranes. However, because of the lack of consensus concerning the pharmacological properties of isoform 2, its presence in the distal luminal membrane is not excluded.

Regulation of Na^+/H^+ Isoforms

Protein kinase A (PKA)

A large number of studies have investigated the regulation of the isoform activities by hormones and their messengers. Most of them concluded that PKA stimulation decreases the Na^+/H^+ exchanger activity in the apical membranes of a variety of renal cell lines: LLC-PK/PKE 20 originating from pig kidney (Casavola et al., 1989; Casavola et al., 1992), MCT (Mrkic et al., 1992) which is an immortalized mouse cortical tubule cell line, RKPC-2 (Mrkic et al., 1993), a rabbit renal cortical cell line or OK cells originating from opossum kidney cortex (Pollock et al., 1986; Helmle-Kolb et al., 1993; Azarani et al., 1995). Similarly, direct application of cAMP associated with a phosphorylating solution, to rabbit brush border membranes, was reported to decrease the enzyme activity (Weinman et al., 1987). Although all of these experiments were performed with cells from total cortex, i.e., proximal and distal tubule cells, the effects were attributed to the predominant proximal tubule luminal membranes, whose isoform is type 3 (Biemesderfer et al., 1993). Therefore, our finding of a stimulatory effect of cAMP on the distal luminal isoform again excludes the hypothesis of an isoform 3.

Studies concerning the influence of PKA on the basolateral membrane isoform are more confusing. In experiments performed in LLC-PK cells (Casavola et al., 1989, 1992), in MCT cells (Mrkic et al., 1992) or RKPC-2 cells (Mrkic et al., 1993), cAMP decreased the basolateral membrane exchanger activity, suggesting that its isoform, like isoform 3, is downregulated by this messenger. There is one exception, however: Kandasamy et al. (1995) reported recently that choleratoxin stimulates isoforms 1 and 2 stably transfected into mutant Chinese hamster ovary cells.

Our experiments clearly showed that db cAMP enhances the exchanger activity in both the basolateral and the luminal membranes of the distal tubule. If, as reported in immunofluorescence experiments (Biemesderfer et al., 1992), the basolateral isoform is type 1, then

our results not only confirm the conclusions of Kandasamy (1995), but again suggest that the distal luminal membrane also contains type 1.

Protein Kinase C (PKC)

The effect of protein kinase C on the Na^+/H^+ exchanger activity has been the matter of discrepancies, probably due to the variations of the cells from which the isoforms originated or variations of the cells receiving the transfected isoforms. Aside from two exceptions (Weinman & Shenolikar, 1986; Mrkic et al., 1993), both in the rabbit, PKC stimulation with PMA was reported to decrease the activity of the luminal membrane isoform 3 of in the proximal tubule. Indeed, an inhibition of the activity has been observed in OK cells which only contain isoform 3 (Helmle-Kolb et al., 1990, 1993; Azarani et al., 1995), in luminal membranes of LLC-PK cells (Casavola et al., 1989, 1992), in primary cell cultures of medullary thick ascending limb (Bichara et al., 1995), in immortalized mouse cortical tubule (MCT) cells (Mrkic et al., 1992), and in PS 120 cells (Tse et al., 1993*a;* Levine et al., 1993) or Chinese hamster ovary cells (Kandasamy et al., 1995) transfected with the cloned isoform. Weinman & Shenolikar (1986) using a different technique, i.e., the direct contact of PKC and ATP with the internal surface of rabbit brush border membrane vesicles, reported, on the contrary, a stimulation of the apical exchanger by the kinase. Such a stimulation was again observed with solubilized membrane proteins incubated with kinases and reconstituted in liposomes (Weinman et al., 1988).

The action of PKC on the basolateral Na^+/H^+ exchanger, i.e., isoform 1, is also a debated topic although to a lesser extent. The kinase has been reported to stimulate the basolateral membrane isoform in MCT and RKPC-2 cells from mouse and rabbit kidney (Mrkic et al., 1992, 1993), in LLC PK-20 cells (Casavola et al., 1992), and in fibroblasts (Levine et al., 1993) or Chinese hamster ovary cells (Kandasamy et al., 1995) transfected with cloned isoform 1. On the contrary, Helmle-Kolb (1993) observed a slight decrease of the exchanger activity in OK cells transfected with isoform 1, and treated with phorbol ester. Finally, the effect of the protein kinases on isoform 2 has not been clearly defined. One of the reasons is probably that, as mentioned previously, the exact site of isoform 2 has not been definitely established. Whereas Casavola et al. (1992) reported in LLC-PK1/PKE20 that both cAMP and PMA were decreasing the activity of the apical isoform (thought to be isoform 2 by the authors), Mrkic et al. (1993), in PKPC2 cells and Levine et al. (1993) in PS120 transfected with the intestinal isoform, observed an increase in the activity following incubation with PMA.

Our experiments showed that PKC decreases the Na^+/H^+ exchanger activity in the distal luminal as in the basolateral membranes. Due to the lack of consensus upon the effects of PKC on the various isoforms, our results do not contribute to the identification the distal luminal membrane exchanger. However, since once again the basolateral and luminal membranes respond similarly to the messenger stimulation, it is tempting to assert that their isoforms are the same.

Conclusions

Our results confirm that the Na^+/H^+ exchanger activity of the proximal tubule brush border membrane is resistant to various inhibitors and is decreased by both cAMP and PMA. In the distal tubule, the response of the luminal and the basolateral exchangers to EIPA, cimetidine and clonidine are similar. They are both very sensitive to these inhibitors. Finally, whereas PMA decreases these exchanger activities, cAMP on the contrary, enhances them at the two sites. If the basolateral membrane isoform is type 1, and if, as proposed by the group of Orlowski (Kandasamy et al., 1995), isoform 1 is stimulated by protein kinase A, then this isoform could be a good candidate for the distal luminal membrane exchanger. However, the presence of isoform 2 in this membrane cannot be definitely eliminated. Further immunohistologic experiments should confirm this hypothesis.

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