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Identification of the Renal Na^+/H^+ Exchanger with N,N'-Dicyclohexylcarbodiimide (DCCD) and Amiloride Analogues

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Summary. Dicyclohexylcarbodiimide (DCCD) and the 5-ethyl-isopropyl-6-bromo-derivative of amiloride (Br-EIPA) have been used as affinity and photoaffinity labels of the Na^+/H^+ exchanger in rat renal brush-border membranes. Intravesicular acidification by the Na^+/H^+ exchanger was irreversibly inhibited after incubation of vesicles for 30 min with DCCD. The substrate of the antiporter, Na^+, and the competitive inhibitor, amiloride, protected from irreversible inhibition. The Na^+-dependent transport systems for sulfate, dicarboxylates, and neutral, acidic, and basic amino acids were inhibited by DCCD, but not protected by amiloride. An irreversible inhibition of Na^+/H^+ exchange was also observed when brush-border membrane vesicles were irradiated in the presence of Br-EIPA. Na^+ and Li^+ protected. [^{14}C]-DCCD was mostly incorporated into three brush-border membrane polypeptides with apparent molecular weights of 88,000, 65,000 and 51,000. Na^+ did not protect but rather enhanced labeling. In contrast, amiloride effectively decreased the labeling of the 65,000 molecular weight polypeptide. In basolateral membrane vesicles one band was highly labeled by [^{14}C]-DCCD that was identified as the α-subunit of the Na^+,K^+-ATPase. [^{14}C]-Br-EIPA was mainly incorporated into a brush-border membrane polypeptide with apparent molecular weight of 65,000. Na^+ decreased the labeling of this protein. Similar to the Na^+/H^+ exchanger this Na^+-protectable band was absent in basolateral membrane vesicles. We conclude that a membrane protein with an apparent molecular weight of 65,000 is involved in rat renal Na^+/H^+ exchange.

Key Words irreversible inhibition · affinity labeling · brush-border membranes · kidney · acridine orange

Introduction

The Na^+/H^+ antiporter is an electroneutral cation exchanger of widespread distribution and great physiological significance [1, 2, 35]. It serves to regulate intracellular pH, cell volume and is involved in the initiation of cell growth and proliferation [25]. In the proximal tubule of mammalian kidneys the Na^+/H^+ exchanger is responsible for the bulk of sodium, bicarbonate and water reabsorption. Thereby, secreted protons combine with bicarbonate to form carbonic acid which is converted to CO_2 and water by the aid of carbonic anhydrase. CO_2 is subsequently taken up into the tubule cells. Directly coupled Na^+/H^+ exchange has first been demonstrated in vesicle experiments by Murer et al. [40]. In proximal tubules of the mammalian kidney the antiporter is exclusively located in the luminal (brush-border) membrane [30, 46].

Despite considerable knowledge on the function of the antiporter only a few attempts have been undertaken towards identification of the membrane protein involved in Na^+/H^+ exchange. The activation of the Na^+/H^+ exchanger in lymphocytes by 12-O-tetradecanoylphorbol 13-acetate (TPA) and cell shrinking could be correlated with an increased phosphorylation of a 60-kilodalton polypeptide and of minor polypeptides in the range of 50 to 55 kD [24]. It remained, however, unresolved whether the phosphorylated polypeptides are directly involved in Na^+/H^+ exchange or act as regulatory proteins. In rabbit renal brush-border membrane vesicles the hydrophobic carboxyl group reagent dicyclohexylcarbodiimide (DCCD) caused an irreversible inhibition of Na^+/H^+ exchange [29]. The competitive inhibitor of the Na^+/H^+ antiporter, amiloride, protected from irreversible inhibition. [^{14}C]-DCCD was incorporated into many polypeptides. Amiloride protected one band with apparent molecular weight of 100,000 suggesting that the corresponding protein is involved in Na^+/H^+ exchange in the rabbit kidney [3]. In this contribution we show that DCCD modifies the antiporter also from rat renal brush-border membranes, but identifies an amiloride-protectable protein different from that in rabbit renal brush-border membranes.

In addition to modification with a side group reagent we have applied photoaffinity labeling with an amiloride analog to identify the membrane protein involved in Na^+/H^+ exchange. Although amiloride derivatives with a high affinity towards the antiporter have been developed [51] and applied

[13, 19, 26, 31, 37, 51, 53], an irreversible inhibition of the Na^+/H^+ antiporter with amiloride derivatives has not yet been demonstrated. Investigations on the sodium channel, however, show that 6-bromo-amiloride is a potent competitive inhibitor and, after irradiation with UV or near-UV light, inhibits irreversibly Na^+ transport [7, 15, 38]. Recently, three membrane proteins possibly related to the sodium channel have been identified by photoaffinity labeling with [^{3}H]-bromo-benzamil [34]. Here we report the irreversible inhibition of the rat renal Na^+/H^+ exchanger by N-amidino-3-amino-5-(N-ethyl-N-isopropyl)-6-bromopyrazinecarboxamide (Br-EIPA) and the identification of membrane proteins which are modified by these compounds. Part of the work has been published in preliminary form [20].

Materials and Methods

Vesicle Preparation

Male wistar rats 180 to 200 g, fed on a standard diet of altromin 1324 and distilled water ad libitum, were killed by cervical dislocation. Brush-border membrane vesicles were prepared according to Biber et al. [8] by a Mg^{2+}-precipitation technique. The specific activity of leucine aminopeptidase (E.C. 3.4.11.1), the marker enzyme for brush-border membranes, was enriched 16.2 ± 4.9-fold over that of the starting homogenate. The specific activity of the marker enzyme for basolateral membranes, Na^+, K^+-ATPase, was enriched 0.8 ± 0.9-fold (means ± SD from 16 membrane preparations). Basolateral membrane vesicles were prepared according to Löw et al. [39] by a Percoll® density gradient centrifugation procedure. Brush-border and basolateral membrane vesicles were preloaded with 150 mM KCl, 5 mM HEPES/Tris, pH 7.0, and stored in liquid nitrogen at a protein concentration of 10 mg/ml for up to two weeks. Protein was determined according to Bradford [9] using bovine serum albumin as a standard.

Na^+-H^+ Antiporter Assay

The intravesicular uptake of protons driven by the efflux of sodium ions was visualized by absorption changes of acridine orange. This weak base accumulates in acidic compartments whereby its absorbance and fluorescence are decreased [48]. The absorption of acridine orange was simultaneously monitored at 492 and 540 nm in a Shimadzu UV 300 dual wavelength spectrophotometer at room temperature. The cuvette contained a buffer with 6 μM acridine orange, 100 mM tetramethylammonium chloride, 50 mM potassium chloride, 5 mM HEPES/Tris, pH 7.0, 2.5 μM valinomycin, and inhibitors in appropriate concentrations. Addition of 50 μg brush-border membrane vesicles which were preloaded with 100 mM NaCl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0, usually resulted in a maximal absorption decrease of 0.07 units. The initial absorption decrease, which was proportional to time, was used to estimate the rates of the Na^+/H^+ exchanger and to determine the loss in activity after modification of the membrane proteins. Valinomycin was applied to "clamp" the membrane potential to zero and thus to abolish H^+ uptake driven by a Na^+ diffusion potential.

Covalent Modification of the Na^+/H^+-Exchanger with DCCD

Brush-border membrane vesicles were quickly thawed in a 37°C water bath, diluted threefold with 5 mM HEPES/Tris buffers of pH 7.0 containing 150 mM TMACl ± amiloride, or 150 mM NaCl and equilibrated for 30 min at room temperature. Then they were incubated with appropriate amounts of DCCD per mg protein for 30 min. Thereafter, the vesicles were diluted into 30 ml of 100 mM NaCl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0, and centrifuged in a Sorvall SS 34 rotor for 20 min at 20,000 rpm. The pellet was resuspended and aliquots of 50 μg protein were assayed for Na^+/H^+ exchange as described above.

Covalent Modification of the Na^+/H^+ Exchanger with Br-EIPA

Vesicles were thawed, diluted and equilibrated as for DCCD experiments. Then they were irradiated with various concentrations of Br-EIPA in an RPR 100 Rayonet Photoreactor equipped with sixteen 3500 Å lamps of 8 Watts each (The Southern Ultraviolet Co., Hamden, Conn.). Control vesicles were treated similarly, but in the absence of Br-EIPA. After irradiation the vesicles were processed as described for covalent modification by DCCD. The loss in activity of the Na^+/H^+ exchanger in vesicles irradiated with Br-EIPA was calculated in percent of the activity in vesicles illuminated without Br-EIPA.

Transport Studies

The uptake of labeled D-glucose, sulfate, citrate, and amino acids into brush-border membrane vesicles was measured by the rapid filtration technique [28].

Synthesis of Amiloride Derivatives

The synthesis of amiloride derivatives was performed as described earlier [14]. Briefly, starting with 3-aminopyrazinoic acid methyl-3-aminopyrazinecarboxylate was synthetized with methanol and sulfuric acid. After recrystallization this compound was chlorinated in positions 5 and 6 with sulfuryl chloride in dry benzene. Methyl-3-amino-5-(N-ethyl-N-isopropyl)-6-chloropyrazine carboxylate was obtained by refluxing the product with N-ethyl-N-isopropyl amine in dry 2-propanol. The product was extracted with petrol ether (30–60) and was pure as judged from HPLC and thin-layer chromatography. It was then hydrogenated with H_2 in position 6 with palladium catalyst on charcoal (5%) and MgO in methanol. The product was free of educt. Bromine was then introduced into position 6 using Br_2 in acetic acid at 50°C. The obtained oil was reacted with guanidine in sodium methanolate to yield N-amidino-3-amino-5-(N-ethyl-N-isopropyl)-6-bromo-pyrazine carboxamide (Br-EIPA). All other described amiloride derivatives were synthetized by a similar procedure as described in [14]. The pure guanidines were obtained by recrystallization from HCl/NaOH. Purity was checked by thin-layer chromatography and HPLC. Radiolabeled Br-EIPA was synthetized in a microsynthesis from [^{14}C]-guanidine as de-

scribed for N-benzylamidino-3,5-diamino-6-chloropyrazinecarboxamide in [16]. The purification of [^{14}C]-Br-EIPA was performed on HPLC (Millipore) as described in [51]. The HPLC columns (two 25 cm μPorasil silica gel columns in series) were washed with ethanol/triethylamine (9 : 1) and the product was eluted with a gradient of methanol/water (85 : 15). The product appeared after about 25 min at a flow rate of 1 ml/min. The specific activity was 48 Ci/mol.

Labeling of Membrane Vesicles

Labeling of membrane vesicles was performed similar to the covalent modification procedures except that the reaction was stopped by addition of 50 μl of 1 M Na_2 succinate and the wash in sodium-containing buffer was omitted. Instead, vesicles were concentrated by a 20-min spin at 20,000 rpm. After removal of the supernatant, DCCD-labeled membranes were left for 3 hr on ice. The pellet was dissolved in 0.2 ml of an O'Farrell's sample buffer containing 125 mM Tris/HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue [42]. The suspensions were shaken in a 37°C water bath for 1 hr and then applied to gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

Discontinuous gel electrophoresis was performed according to Laemmli [36] in siliconized tubes (6 × 170 mm). Tube gels were preferred over slab gels because greater amounts of protein (0.5 mg/tube) could be applied without a loss in resolution. This enabled us to detect proteins labeled by [^{14}C]-DCCD and [^{14}C]-Br-EIPA despite the low specific radioactivity of these probes. Total acrylamide concentration of separation gels (length: 13 cm) was 8.5% at a ratio of acrylamide/N,N'-methylenebisacrylamide of 27.3 : 2.7. Separation gels contained also 375 mM Tris/HCl, pH 8.8, 0.1% SDS, 0.1% ammonium peroxodisulfate, and 0.033% N,N,N',N'-tetramethylethylenediamine. Stacking gels (length: 2 cm) were of similar composition except that 4.5% acrylamide and 125 mM Tris/HCl, pH 6.8, were used. The electrode buffer contained 50 mM Tris, 384 mM glycine, pH 8.3, and 0.1% SDS. Electrophoresis was run at a constant current of 2 mA/tube for 20 hr. After electrophoresis the gels were fixed in 12.5% TCA, stained with Coomassie Brilliant Blue R250 (0.1% in 250 ml ethanol, 80 ml acetic acid, 670 ml water) and destained.

The molecular weight of membrane proteins was determined by comparing their relative migration distance with that of marker proteins run in a parallel gel. Marker proteins were phosphorylase b (mol wt 94,000), albumin (mol wt 67,000), ovalbumin (mol wt 43,000) and carboanhydrase (mol wt 30,000). The logarithm of the molecular weight was inversely proportional ($r = -0.99$) to the relative migration distance of the marker proteins in the gel.

In order to determine the radioactivity incorporated into membrane proteins the gels were cut into 2-mm slices and treated overnight with 500 μl Protosol per slice. The radioactivity was determined by liquid scintillation counting.

Materials

All chemicals were of analytical grade and obtained from commercial sources. DCCD was obtained from Aldrich. Amiloride was a kind gift of Merck, Sharp and Dohme. Purified Na^+,K^+-ATPase was a gift of Dr. Grell. Dicyclohexyl-[^{14}C]-carbodiimide (54 Ci/mol) and [^{14}C]-guanidine hydrochloride were obtained from Amersham. L-[2,3-^{3}H]-arginine, [1,5-^{14}C]-citric acid, D-[^{14}C-(U)]-glucose, L-[3,4-^{3}H]-glutamic acid, [2-^{3}H]-glycine, [glycine-1-^{14}C]-ethylester hydrochloride, and $Na_2^{35}SO_4$ were purchased from New England Nuclear.

Results

Irreversible Inhibition of Na^+/H^+ Exchange by DCCD

When Na^+-loaded brush-border membrane vesicles are incubated in a Na^+-free buffer, an intravesicular acidification occurs due to the operation of the Na^+-H^+ exchanger. We followed this acidification by measuring the absorbance changes of acridine orange. Determination of absorbance changes in the dual wavelength mode (492 to 540 nm) has an advantage over the previously used fluorescence measurements because absorbance is much less influenced by added compounds for modification of the antiporter.

Figure 1 shows the absorbance decrease of acridine orange which occurs after addition of brush-border membranes to the cuvette and indicates an intravesicular acidification. Within the first 10 to 20 sec the absorbance decrease is proportional to time. Absorption reaches a minimum 1 to 2 min after incubation of vesicles in the Na^+-free buffer. When up to 5 mM DCCD was present in the Na^+-free buffer, no change in the absorbance signal occurred indicating that DCCD does not inhibit Na^+/H^+ exchange during short exposure times (*data not shown*). To test for an irreversible inhibition, brush-border membrane vesicles were incubated for 30 min in buffers containing 150 nmol DCCD per mg protein and tetramethylammonium chloride (TMA Cl) or NaCl. Control vesicles did not receive DCCD. Thereafter the vesicles were washed, loaded with Na^+ and suspended in Na^+-free buffers containing acridine orange. Pretreatment with DCCD caused an inhibition of Na^+/H^+ exchange as estimated from the initial rate of absorbance decrease (Fig. 1, *compare* curves +DCCD and −DCCD). If Na^+ (or Li^+, *data not shown*) was present together with DCCD during the preincubation period, subsequently determined Na^+/H^+ exchange showed a smaller inhibition indicating a protection of the antiporter by its substrates.

The inset in Fig. 1 demonstrates the time dependence of irreversible inhibition and its protection by Na^+. Brush-border membrane vesicles were preincubated for the indicated time intervals with 150 nmol DCCD/mg protein with and without Na^+. Three hours later, after washing and loading the

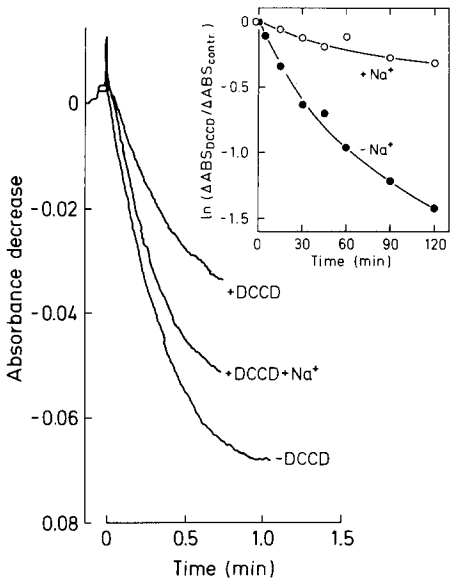


Fig. 1. Irreversible inhibition of Na^+/H^+ exchange by DCCD and protection by sodium. Brush-border membrane vesicles (protein concentration 3.33 mg/ml) were incubated for 30 min in buffers containing 150 nmol DCCD/mg protein, 5 mM HEPES/Tris, pH 7.0, 50 mM KCl, and 100 mM TMACl (+DCCD) or 100 mM NaCl (+DCCD + Na^+). Control vesicles were treated similarly but without DCCD (−DCCD). Thereafter the membranes were washed and loaded with 100 mM NaCl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0. The Figure shows original tracings of absorbance changes of acridine orange caused by dilution of Na^+-loaded brush-border membrane vesicles (50 μg protein) into 1 ml buffer containing 6 μM acridine orange, 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0, and 2.5 μM valinomycin. *Inset:* Brush-border membrane vesicles were incubated for the indicated time intervals with 150 nmol DCCD/mg protein, 5 mM HEPES/Tris, pH 7.0, 50 mM KCl and 100 mM TMACl (−Na^+) or NaCl (+Na^+). Control vesicles were incubated without DCCD in 5 mM HEPES/Tris, pH 7.0, 50 mM KCl, 100 mM TMACl. After washing and Na^+ loading the vesicles were diluted into acridine orange containing buffers as described above. The initial rates of absorbance decrease obtained with DCCD-pretreated vesicles (ΔABS_{DCCD}) were divided by the rates observed with control vesicles ($\Delta ABS_{contr.}$) and were plotted versus preincubation time

vesicles with Na^+, the initial rates of absorbance decrease were determined and normalized to the rate observed with vesicles preincubated without DCCD. The half-time for the inactivation of the Na^+/H^+ exchanger is approximately 30 min. Na^+ increases the half-time considerably to more than 2 hr.

Using 30 min of preincubation we next determined the dependence of irreversible inhibition on the DCCD concentration. As shown in Fig. 2 half-maximal inhibition occurs at 190 nmol DCCD/mg protein. A tenfold greater amount of DCCD almost abolished Na^+/H^+ exchange. The competitive inhibitor, amiloride, protected the Na^+/H^+ exchanger

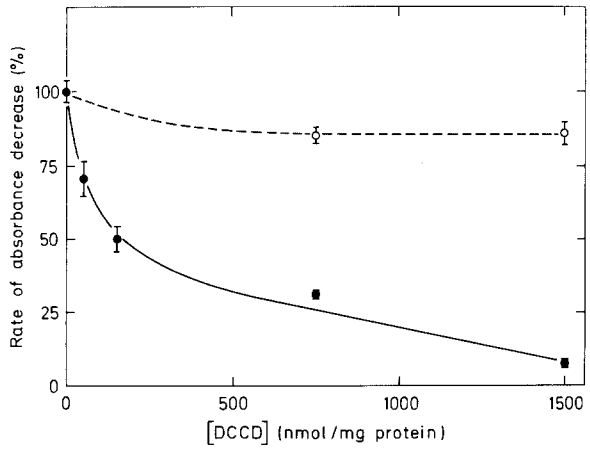


Fig. 2. Concentration dependence of irreversible inhibition of Na^+/H^+ exchange with DCCD. Brush-border membrane vesicles were preincubated for 30 min with different amounts of DCCD ranging from 0 to 1500 nmol/mg protein. The preincubation media contained in addition 5 mM HEPES/Tris, pH 7.0, 50 mM KCl, and 100 mM TMACl with 0 (●) or 4 mM (○) amiloride. After washing and loading the vesicles with Na^+ the absorbance decrease of acridine orange was measured and expressed in percent of the uninhibited control

nearly completely, if present together with DCCD during preincubation. These data indicate that DCCD binds covalently to a side group closely associated with the cation transport site.

Specificity of the Irreversible Inhibition by DCCD

In order to evaluate the specificity of DCCD we tested the influence of this reagent on the uptake of radiolabeled D-glucose, sulfate, citrate, glycine, L-arginine, and L-glutamate into brush-border membrane vesicles. These compounds are handled by separate Na^+-dependent transport systems located in the luminal membrane of the proximal tubule cell. The results are shown in Table 1. Pretreatment of brush-border membrane vesicles with 150 nmol DCCD/mg protein caused a small inhibition of Na^+-driven D-glucose uptake. The uptake of the other substrates was significantly inhibited by up to 84% suggesting that DCCD bound covalently to the respective Na^+-dependent transport systems. Amiloride did not protect any of the investigated transport systems. This finding is in contrast to the protection by amiloride of the Na^+/H^+ exchanger (*see* Fig. 2). Protection of the antiporter occurred in each membrane preparation used for the transport studies in Table 1. Thus, DCCD interacts with several Na^+-dependent transport systems. However, only the antiporter can be protected by amiloride.

Table 1. Irreversible inhibition of Na^+-coupled transport systems by DCCD and lack of protection by amiloride[a]

Substrate	Pretreatment of vesicles			
	−DCCD −amil	−DCCD +amil	+DCCD −amil	+DCCD +amil
	15-sec uptake (% of control)			
D-glucose	100 ± 11.8	112.2 ± 6.4	76.5 ± 16.0	88.0 ± 10.3
Sulfate	100 ± 3.8	90.5 ± 3.0	15.5 ± 1.1	13.3 ± 0.9
Citrate	100 ± 13.2	94.1 ± 16.9	52.2 ± 12.4	44.7 ± 10.1
Glycine	100 ± 7.8	113.9 ± 12.6	40.4 ± 4.1	41.3 ± 12.0
L-Arginine	100 ± 2.7	110.9 ± 16.3	66.8 ± 5.9	69.4 ± 6.2
L-Glutamate	100 ± 2.3	95.0 ± 6.0	45.0 ± 1.4	46.1 ± 0.5

[a] Brush-border membrane vesicles were pretreated for 30 min with 0 or 150 nmol DCCD/mg protein and 0 or 1 mM amiloride in 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0. They were then washed and preloaded with 150 mM KCl, 5 mM HEPES/Tris, pH 7.0, and incubated for 15 sec in buffers containing 150 mM NaCl, 5 mM HEPES/Tris, pH 7.0, and 100 μM of various labeled substrates. The uptake of substrates is expressed in percent of uptake observed with vesicles pretreated without DCCD and without amiloride. Shown are means ± SEM for four determinations. The activity of the Na^+/H^+ exchanger as determined with aliquots was 100% (−DCCD, −amil), 100% (−DCCD, +amil), 30% (+DCCD, −amil) and 90% (+DCCD, +amil), respectively.

Effects of Br-EIPA on the Na^+/H^+ Exchanger

The next series of experiments was performed to find out whether analogues of amiloride are also capable of irreversibly inhibiting the Na^+/H^+ exchanger. In contrast to DCCD, amiloride and its analogues inhibit the antiporter without preincubation. Table 2 shows the concentration of amiloride and its derivatives for half-maximal inhibition of proton uptake. The introduction of the hydrophobic ethylisopropyl-moiety in position 5 increases the apparent affinity sixfold (decrease of apparent $K_{1/2}$ from 43 to 7.1 μM). Replacement of the chlorine at position 6 by hydrogen or bromine decreases the affinity of the compound for the antiporter. Interestingly, the 6-chloro-5-ethylisopropyl-3-amino-pyrazine-O-methyl-ester inhibits Na^+/H^+ exchange although it lacks the positively charged guanidino group. The $K_{1/2}$, however, is considerably higher than that of amiloride analogues. Guanidine itself also causes an inhibition, although approximately 40 mM are required for half-maximal inhibition.

After having demonstrated that 6-bromo-5-ethylisopropyl-amiloride (Br-EIPA) interacts with the Na^+/H^+ exchanger in rat renal brush-border membranes we irradiated this photolabile compound at 350 nm for up to 16 min. This irradiation led to a decrease of the absorbance at 375 and 285 nm (Fig. 3, top panel). Thereby, the absorbance at 375 nm decreased with a half-time of 9.2 min (Fig. 3, bottom panel). The absorbance changes prove that a breakdown of Br-EIPA occurs under our irradiation conditions. Therefore we investigated next whether radicals formed during photodecomposi-

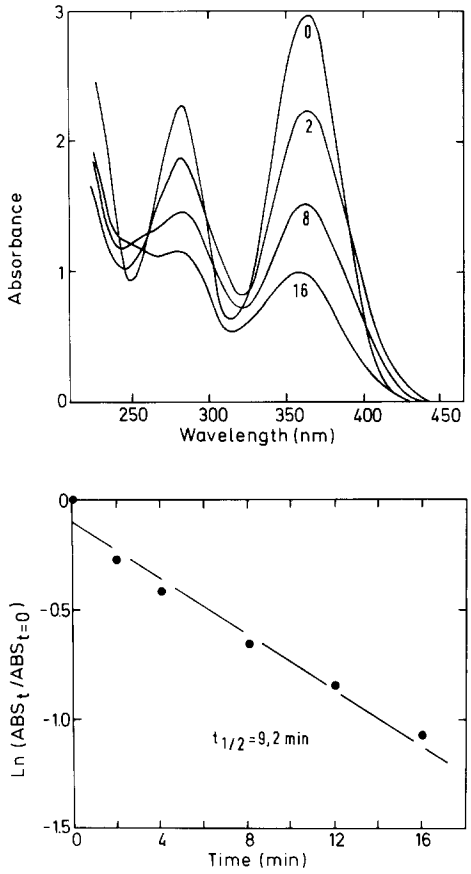
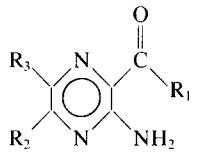
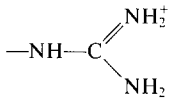
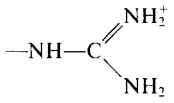
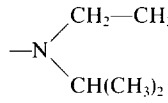
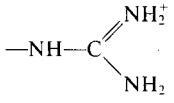
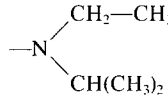
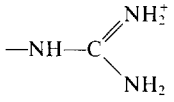
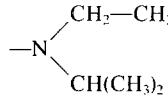
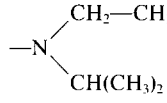


Fig. 3. Effect of irradiation at 350 nm on the absorbance of Br-EIPA. *Top panel:* Fifty μM Br-EIPA in Ringer's were irradiated and the absorbance spectrum was measured after the indicated irradiation times (in minutes). *Bottom panel:* The absorbance at 373 nm was determined after various irradiation times, divided by the absorbance at $t = 0$, and is shown in a semilogarithmic plot as a function of irradiation time

Table 2. Inhibition of Na^+/H^+ exchange by pyrazine compounds and guanidine[a]



R_1	R_2	R_3	$K_{1/2}$ (μM)
	$-NH_2$	$-Cl$	43.0 ± 17.8
		$-H$	26.9 ± 6.0
		$-Cl$	7.1 ± 4.7
		$-Br$	44.7 ± 13.4
$-O-CH_3$		$-Cl$	307.1 ± 63.5
	Guanidine		40.000 ± 25.000

[a] Brush-border membrane vesicles preloaded with Na^+ as described in the legend to Fig. 1 were diluted into 1 ml of buffers containing 6 μM acridine orange, 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0, 2.5 μM valinomycin and the inhibitors at 5 to 10 different concentrations. $K_{1/2}$ values were determined from Dixon plots using the initial rates of acridine orange absorbance decrease as a measure of Na^+/H^+ exchange. The Table shows means ± SD from three membrane preparations.

tion of Br-EIPA bind covalently to the Na^+/H^+ exchanger and inhibit it irreversibly. Brush-border membranes were irradiated without (control) or with 100 μM Br-EIPA at 350 nm for different times. With increasing irradiation time Na^+/H^+ exchange in brush-border membanes is more and more inhibited by Br-EIPA (Fig. 4, filled symbols). This inhibition is not due to an effect of irradiation on the Na^+/H^+ exchanger because irradiation in the absence of Br-EIPA caused no significant loss in antiporter activity (Fig. 4, open symbols). Moreover, the inhibition after irradiation was not caused by an incomplete removal of unbound Br-EIPA. This is demonstrated in Fig. 5. Preincubation of the vesicles with 100 μM Br-EIPA in the dark did not result

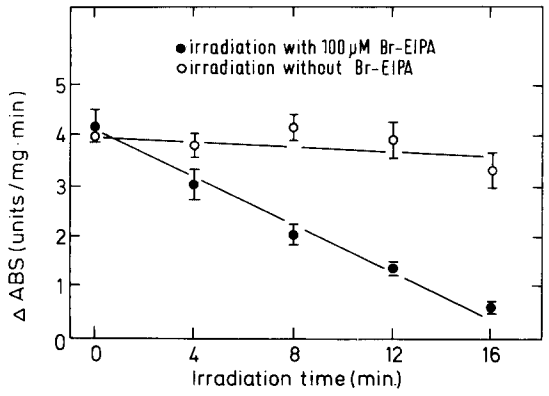


Fig. 4. Dependence of irreversible inhibition of Na^+/H^+ exchange by Br-EIPA on irradiation time. Brush-border membrane vesicles (protein concentration 3.33 mg/ml) were irradiated at 350 nm and 20°C for the indicated times in the presence (●) or absence (○) of 100 μM Br-EIPA in 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0. After irradiation the membranes were washed in the dark and treated as detailed in the legend to Fig. 1

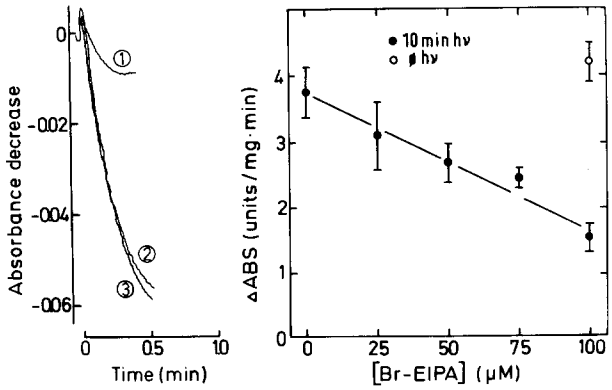


Fig. 5. Dependence of irreversible inhibition of Na^+/H^+ exchange on Br-EIPA concentration. Brush-border membrane vesicles were irradiated for 0 ($\phi h\nu$) or 10 minutes (10 min $h\nu$) at 350 nm with different concentrations of Br-EIPA. *Left panel:* Absorbance decrease of acridine orange after diluting washed and Na^+-loaded vesicles into Na^+-free buffer. Pretreatment of the vesicles: (1) 10-min irradiation with 100 μM Br-EIPA; (2) 10-min irradiation without Br-EIPA; (3) 100-μM Br-EIPA without irradiation. *Right panel:* Dependence of initial absorbance decrease (as a measure of Na^+/H^+ exchanger activity) on Br-EIPA concentration during irradiation

in any inhibition of subsequently determined Na^+/H^+ exchange. Irradiation, however, caused a Br-EIPA concentration-dependent decline in Na^+/H^+ exchanger activity (Fig. 5, right).

Figure 6 illustrates that Na^+ and Li^+ can protect the Na^+/H^+ exchanger from irreversible inhibition when they were present together with 50 μM Br-EIPA during irradiation. This result suggests a binding of photolysis products of Br-EIPA close to the cation transport site of the antiporter.

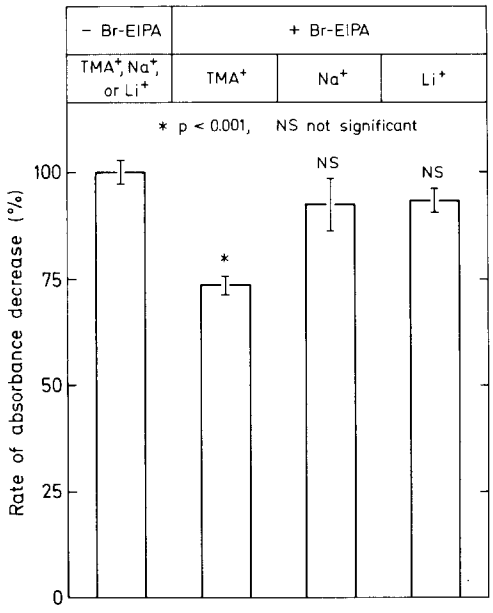


Fig. 6. Irreversible inhibition of Na^+/H^+ exchange by Br-EIPA and protection by sodium and lithium. Brush-border membrane vesicles were irradiated in buffers containing 100 mM TMACl or 100 mM NaCl or 100 mM LiCl and 50 mM KCl, 5 mM HEPES/Tris, pH 7.0, in the presence or absence of 50 μM Br-EIPA. The membranes were washed in the dark and treated as in Fig. 1. The rates of acridine orange absorbance decrease of control vesicles (irradiation without Br-EIPA) were set to 100%. The bars indicate means ± SE for four membrane preparations (six determinations with each preparation)

Labeling of Membrane Proteins with [^{14}C]-DCCD and [^{14}C]-Br-EIPA

Since DCCD and Br-EIPA irreversibly inhibit the Na^+/H^+ exchanger, we used [^{14}C]-labeled DCCD and Br-EIPA to detect the membrane proteins possibly involved in Na^+/H^+ exchange. The polypeptide and [^{14}C]-DCCD-labeling pattern of brush-border membrane proteins is displayed in Fig. 7. Brush-border membranes were reacted with 2.3 nmol [^{14}C]-DCCD/mg protein for 30 min which resulted in the labeling of four bands with apparent molecular weights of (means ± SEM; n: number of membrane preparations): 88,300 ± 840 (n = 8), 65,300 ± 880 (n = 8), 50,500 ± 430 (n = 6) and 34,300 ± 2,300 (n = 3). The maximum incorporation of radioactivity into a band with apparent molecular weight of 88,300 corresponds to a peak in staining. The maximum labeling of the other three bands is, however, not related to intensely stained polypeptides. This result clearly demonstrates that [^{14}C]-DCCD labeling is not proportional to the amount of protein along the gel.

Unexpectedly, Na^+ did not reduce the labeling (Fig. 7, bottom panel) although it protected the anti-

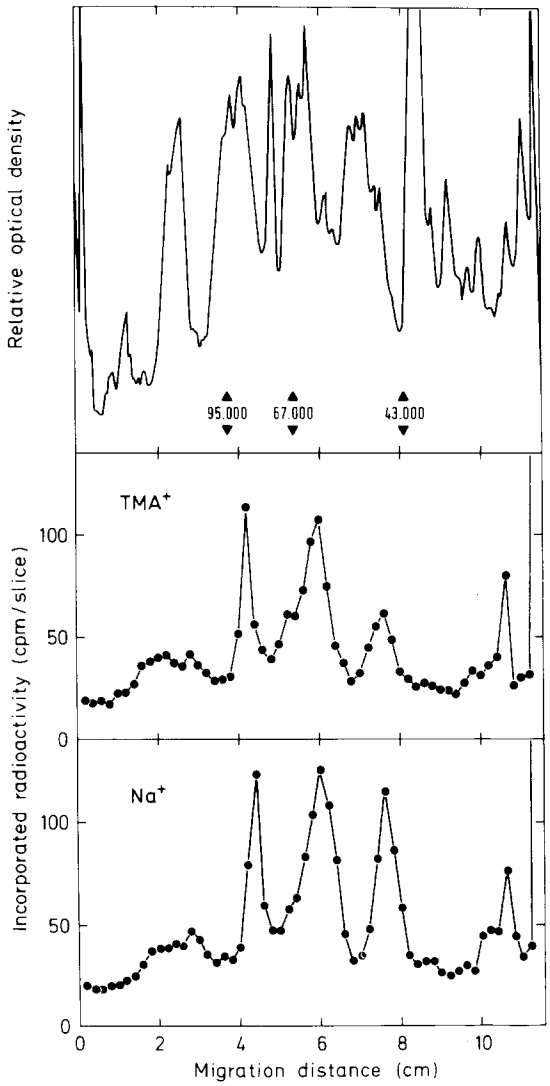


Fig. 7. Affinity labeling of brush-border membranes with [^{14}C]-DCCD and the effect of Na^+. 500 μg of membrane protein (protein concentration of 3.33 mg/ml) were incubated for 30 min with 2.3 nmol [^{14}C]-DCCD/mg protein, 5 mM HEPES/Tris, pH 7.0, 50 mM KCl and 100 mM TMACl (top and middle panel) or 100 mM NaCl (bottom panel). Membrane proteins were separated by SDS gel electrophoresis with 0.5 mg protein applied per gel rod. Total acrylamide concentration of the separation gel was 8.5%. After electrophoresis, staining with Coomassie blue and recording of the polypeptide pattern (top panel), the gels were cut into 2-mm slices and the incorporated radioactivity was determined (middle and bottom panels)

porter from its inactivation by DCCD. The incorporation of [^{14}C]-DCCD into the polypeptide with apparent molecular weight of 65,000 was slightly increased whereas the labeling of the 51,000 band was approximately doubled.

Sodium also increased the labeling of water-exposed carboxyl groups by [^{14}C]-glycineethylester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-

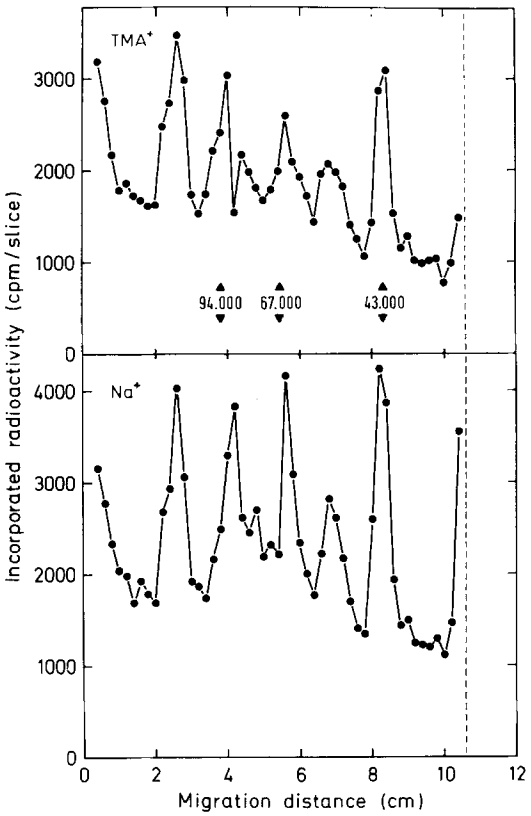


Fig. 8. Labeling of hydrophilic carboxyl groups in brush-border membranes with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide plus [^{14}C]glycine-ethylester and the effect of Na^+. Brush-border membrane vesicles were incubated with 150 nmol 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/mg protein and the same concentration of [^{14}C]glycine-ethylester in buffers containing TMACl (upper panel) or NaCl (lower panel) similar to those used in the experiment shown in Fig. 7. The dashed line represents the position of the tracking dye

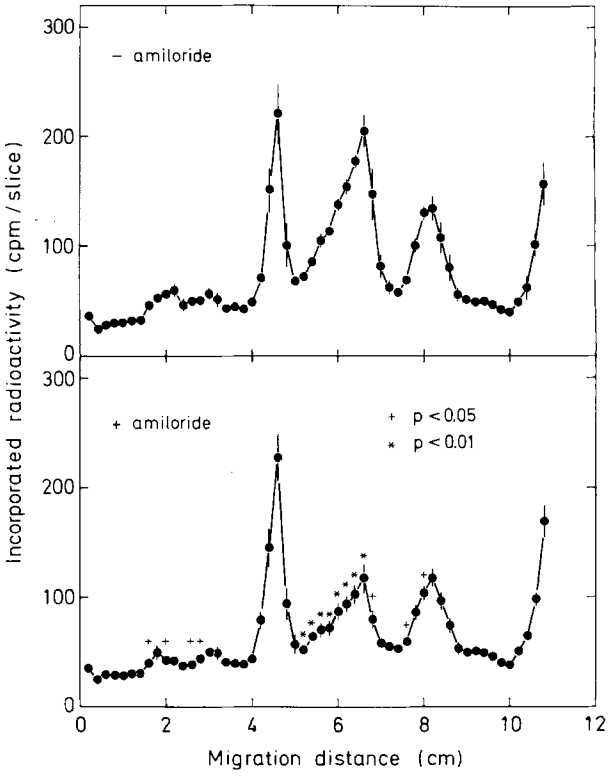


Fig. 9. Affinity labeling of brush-border membranes with [^{14}C]-DCCD and the effect of amiloride. Brush-border membrane vesicles were incubated for 30 min with 2.3 nmol [^{14}C]-DCCD/mg protein, 5 mM HEPES/Tris, pH 7.0, 50 mM KCl, 100 mM TMACl without (top panel) or with 1 mM amiloride (bottom panel). For details of membrane protein separation *see* legend to Fig. 7. Each point is the mean of four determinations from separate gels. Standard errors are indicated whenever they exceed the size of the symbols. Statistical analysis was performed by Student's *t*-test for unpaired data

carbodiimide (Fig. 8). By this technique many more proteins were labeled than with the hydrophobic probe DCCD indicating the abundant presence of hydrophilic carboxyl groups. Yet, the Na^+/H^+ antiporter was not inhibited in membrane vesicles treated with glycineethylester and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (*data not shown*) suggesting that water-exposed carboxyl groups are not involved in the exchange process. The increased labeling of carboxyl groups in the presence of Na^+ suggests a nonspecific effect of Na^+ on the reactivity of carboxyl groups and precludes its use for differential labeling.

As amiloride protected the Na^+/H^+ exchanger, but not the Na^+-coupled transport systems, from irreversible inhibition by DCCD, we next looked for amiloride-protectable, [^{14}C]-DCCD-binding proteins. Figure 9, bottom panel, shows that amiloride decreased highly significantly the labeling of the band with apparent molecular weight of 65,000. A smaller decrease was seen with weakly labeled polypeptides in the molecular weight range of 120,000 to 140,000 and with the band of M_r 51,000. The incorporation of [^{14}C]-DCCD into the polypeptide with apparent molecular weight of 88,000 was completely unchanged raising doubts about its relation to Na^+/H^+ exchange. One or more of the amiloride-protectable membrane proteins, however, may be considered as candidates for the Na^+/H^+ antiporter.

Basolateral membranes from rat kidney cortex do not contain an Na^+/H^+ exchanger [30, 46]. Therefore, they should lack those [^{14}C]-DCCD-labeled proteins which are involved in Na^+/H^+ exchange. Figure 10, top, shows that [^{14}C]-DCCD was nearly exclusively incorporated into a polypeptide with an apparent molecular weight of 88,300 ± 333 (mean ± SEM, three preparations). The labeling of

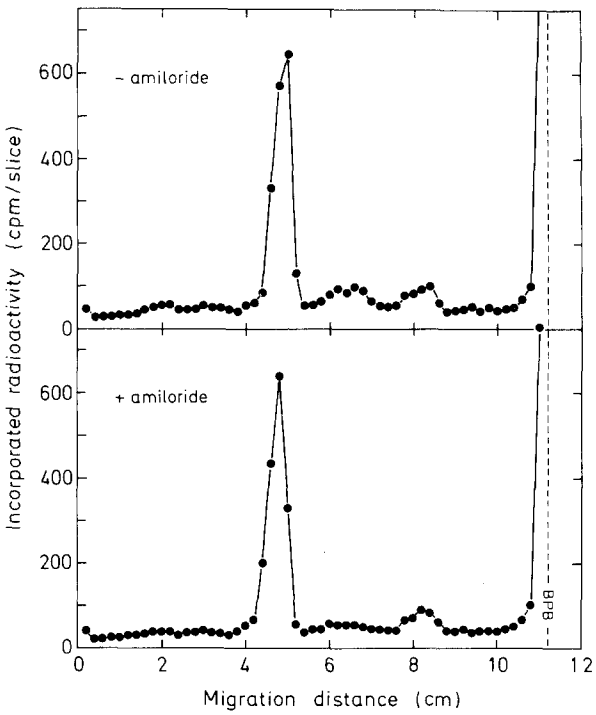


Fig. 10. Labeling of rat renal basolateral membrane vesicles with [^{14}C]-DCCD and the effect of amiloride. Basolateral membrane vesicles (500 μg protein) were reacted with 2.3 nmol [^{14}C]-DCCD/mg protein in 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0 without (top panel) or with 1 mM amiloride (bottom panel). For further details *see* legend to Fig. 7. The dashed line represents the position of the tracking dye bromophenol blue (BPB)

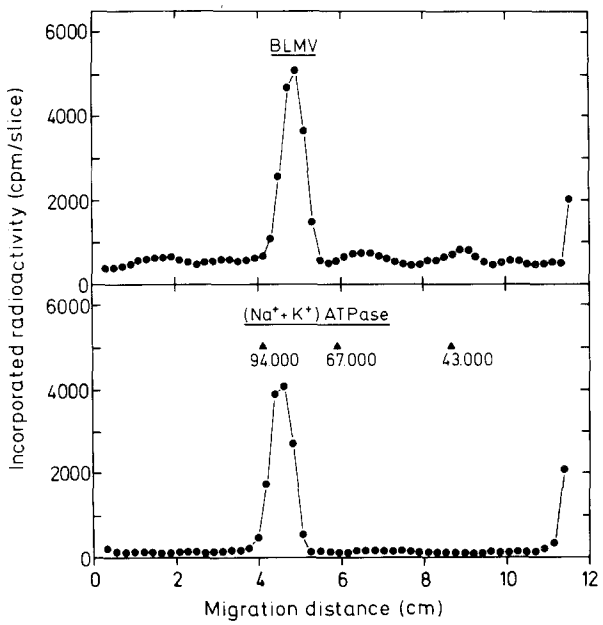


Fig. 11. Labeling of basolateral membrane vesicles (top) and purified Na^+,K^+-ATPase (bottom) with [^{14}C]-DCCD. Basolateral membrane vesicles (500 μg protein) or purified Na^+,K^+-ATPase (250 μg protein) were reacted with 150 nmol [^{14}C]-DCCD/mg protein in 100 mM TMACl, 50 mM KCl, 5 mM HEPES/Tris, pH 7.0. For further details *see* legend to Fig. 7

this band was insensitive to amiloride (bottom panel). Polypeptides with apparent molecular weights of 65,000 and 51,000 showed only minor incorporation of [^{14}C]-DCCD. Amiloride decreased the labeling of the 65-kD polypeptide. In basolateral membranes (Fig. 10) labeling of the 88,000 molecular weight band was much higher and that of the 65,000-kD polypeptide smaller than in brush-border membranes (Figs. 7, 9). This result suggests that a DCCD-binding protein of apparent molecular weight of 88,000 is genuine to basolateral membranes whereas the amiloride-protectable polypeptide with an apparent molecular weight of 65,000 belongs to the brush-border membrane.

Labeling studies with purified Na^+,K^+-ATPase resulted in a labeling pattern (Fig. 11, bottom) comparable to that of basolateral membranes (Fig. 11, top). This result suggests strongly that the 88,000 molecular weight polypeptide labeled in basolateral membranes is the α-subunit of the Na^+,K^+-ATPase.

Next we applied photoaffinity labeling of brush-border membranes with [^{14}C]-Br-EIPA to identify proteins possibly related to the antiporter. Photoaffinity labeling resulted in the most effective incorporation of ^{14}C into a band corresponding to an apparent molecular weight of 65,200 ± 80 (Fig. 12). Na^+ reduced the labeling of this band. In basolateral membranes radioactivity was incorporated into three bands with apparent molecular weights of 88,500, 72,000 and 53,000 after photoaffinity labeling with [^{14}C]-Br-EIPA (Fig. 13). The molecular weights of these labeled polypeptides are clearly different from those of the [^{14}C]-Br-EIPA-labeled brush-border proteins (Fig. 12) which have been separated by SDS gel electrophoresis in the same chamber. Moreover, sodium had no effect on the labeling pattern in basolateral membranes. Thus, the [^{14}C]-Br-EIPA-binding, Na^+-protectable membrane protein of apparent molecular weight of 65,000 is restricted to the brush-border membrane similar to the Na^+/H^+ exchanger.

Discussion

Irreversible Inhibition of the Na^+/H^+ Exchanger

Identification of the membrane protein involved in Na^+/H^+ exchange requires the covalent binding of a

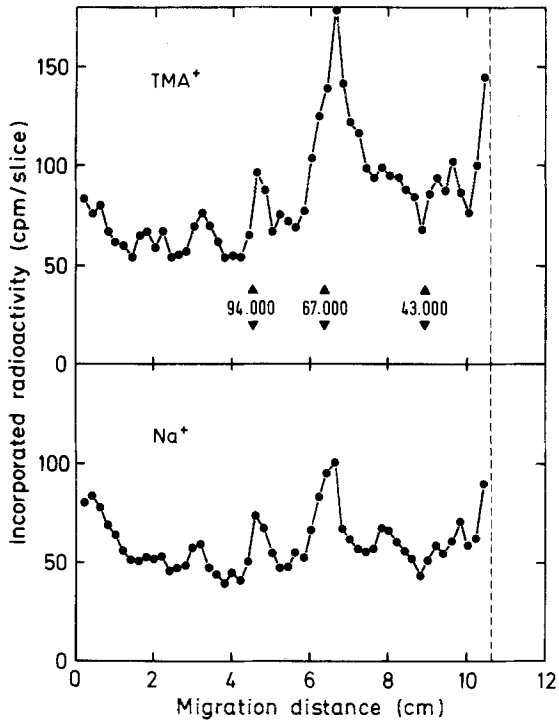


Fig. 12. Photoaffinity labeling of brush-border membrane vesicles with [^{14}C]-Br-EIPA. Brush-border membrane vesicles (500 μg protein, protein concentration 3.33 mg/ml) in 100 mM TMACl or 100 mM NaCl, and 50 KCl, 5 mM HEPES/Tris, pH 7.0, were irradiated with 100 μM [^{14}C]-Br-EIPA for 10 min at 350 nm. The membranes were washed in the dark and the pellets were treated as described in the legend of Fig. 7. The position of the tracking dye is indicated by the vertical dashed line

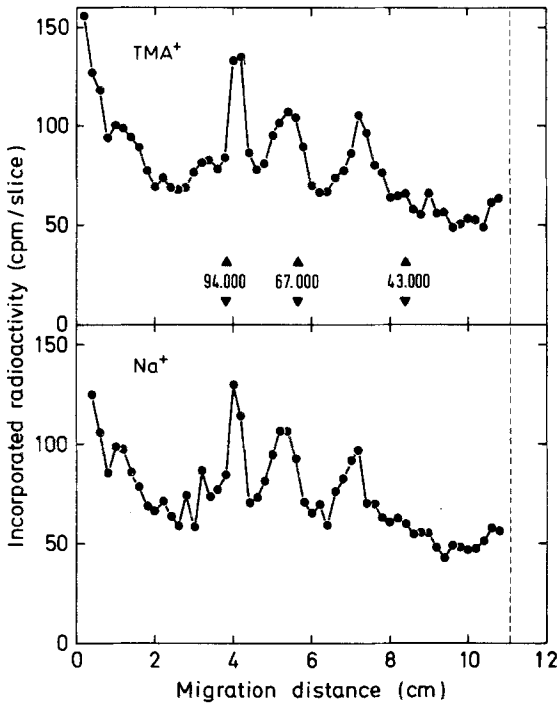


Fig. 13. Photoaffinity labeling of basolateral membrane vesicles with [^{14}C]-Br-EIPA. The labeling conditions were similar to those applied to brush-border membrane vesicles (Fig. 12). Dashed line represents the position of the tracking dye

radiolabeled probe to the antiporter. This probe may be a reagent that specifically reacts with side groups involved in cation translocation or an appropriately modified inhibitor of the antiporter. Covalent modification of the antiporter at the cation transport site must result in an irreversible inhibition of Na^+/H^+ exchange. In this contribution we have demonstrated an inhibition of the Na^+/H^+ exchanger in rat renal brush-border membrane vesicles pretreated with the side group reagent DCCD or irradiated with the inhibitor Br-EIPA. The following arguments strongly suggest that the observed inhibition was irreversible and not due to incomplete removal of reagents or a loss of intact vesicles. First, no inhibition of Na^+/H^+ exchange occurred when DCCD was added acutely, i.e. during Na^+-driven intravesicular acidification. Rather a preincubation was necessary to inhibit Na^+/H^+ exchange. Incompletely removed DCCD could therefore not account for the observed inhibition. Second, Na^+ and amiloride protected from the inhibition by DCCD. The possibility that Na^+ and amiloride decreased the loss in intact vesicles is remote. A loss in intact vesicles can be further excluded, since the artificial Na^+/H^+ exchanger, monensin, caused a rapid intravesicular acidification even at near-complete inhibition of the intrinsic Na^+/H^+ exchanger (*data not shown*). Third, Br-EIPA caused an inhibition of Na^+/H^+ exchange only after irradiation. The degree of inhibition dependend on irradiation time similar to the photodecomposition as visualized by changes in the absorbance spectra. Preincubation of vesicles with 100 μM Br-EIPA in the dark did not affect the subsequently determined antiporter activity. This finding clearly excludes an incomplete removal of Br-EIPA. Fourth, Na^+ efflux-driven H^+ uptake was not affected by irradiation itself. This result excludes a damage of the exchanger and increased leakiness of irradiated vesicles.

Taken together the results show an irreversible inhibition of the Na^+/H^+ exchanger and strongly suggest a covalent binding of the reagents to the antiporter protein. Our data are compatible with previously published reports showing an irreversible inhibition of the Na^+/H^+ exchanger in rabbit renal brush-border membrane vesicles by carbodi-

imides [10, 29, 33, 45]. In contrast, the Na^+/H^+ exchangers in *E. coli* [17] and thymic lymphocytes [23] were not sensitive to carbodiimides. It needs to be clarified whether these differences reflect different types of antiporters.

Mechanism of Irreversible Inhibition

DCCD and Br-EIPA inhibit the antiporter irreversibly by two completely different mechanisms. DCCD binding to the antiporter requires the presence of a mercapto-, tyrosyl-, or carboxyl-group in a hydrophobic environment [4–6, 11, 12, 23, 27, 32, 43, 49]. In contrast, the binding of radicals formed during irradiation of Br-EIPA does not require a specific side group. Nevertheless, the binding site for Br-EIPA has to fulfill two criteria. First, a negative charge at the binding site is required to accommodate amiloride and its analogues. This is concluded from the relatively low affinity of the antiporter for the uncharged ethylisopropyl-pyrazine ester as compared to the positively charged amiloride derivatives (Table 2, and ref. 37). Second, the binding site must allow for a hydrophobic interaction with amiloride and its derivatives. We conclude this from the higher affinity for analogues with the hydrophobic ethylisopropyl moiety at position 5 of the pyrazine ring (refs. 18, 19, 26, 37, 41, 50–52, and this study) and from the negligible affinity of the antiporter for the positively charged guanidine.

As amiloride and its derivatives protected the antiporter from the attack by DCCD (Figs. 1, 2, and ref. 29, 45) it is likely that both bind to closely associated domains within the Na^+/H^+ exchanger. This conclusion is supported by the fact that Na^+ and Li^+ protect the exchanger from irreversible inhibitions by both, DCCD and Br-EIPA. The location of the negatively charged side group in a hydrophobic environment is further suggested by the lack of inhibition of the Na^+/H^+ exchanger by modification of water-exposed side groups by up to 5 mM 1-ethyl-3-(3-diaminopropyl)carbodiimide in the presence of glycine ethylester. From these observations we would like to postulate that the binding site of the Na^+/H^+ exchanger has a negatively charged, DCCD-sensitive side group, e.g. a carboxyl group, in a hydrophobic pocket. The pyrazine ring of amiloride fits very well into this pocket and is thus by itself able to inhibit the antiporter. Sodium ions and protons interact only with the negatively charged side group. The hydrophobic pocket may strip the water shell. Thereby, the binding sites for cations and amiloride overlap but are not completely identical as discussed previously [44].

The irreversible inhibition of the rabbit renal Na^+/H^+ exchanger by diethylpyrocarbonate, a reagent for histidyl groups, was protected by amiloride and protons, but, in contrast to our studies with DCCD, not by sodium [22]. Similarly, the irreversible inhibition of the antiporter in thymic lymphocytes by N-ethylmaleimide [23] was not prevented by the presence of sodium. These data indicate that the modified histidyl- and sulfhydryl side groups may not be directly involved in binding and translocation of sodium. They may, however, serve as an acceptor for the translocated protons [3]. Protection of irreversible inhibition by amiloride indeed suggests that these groups are in close neighborhood of the Na^+-transport site.

Specificity of the Irreversible Inhibition

A probe for the identification of a transporter should ideally react covalently only with the transport system under investigation. This is clearly not fulfilled for DCCD which inhibited various Na^+-dependent transport systems in the brush-border membrane. Among these systems the Na^+-D-glucose cotransporter was least affected by DCCD. The greatest inhibition was observed with the Na^+-sulfate cotransporter, intermediate inhibitions with the Na^+-dependent transport systems for di- and tricarboxylates (tested substrate: citrate), and neutral, acidic and basic amino acids (tested substrates: glycine, L-glutamate, and L-arginine, respectively). The irreversible inhibition may indicate that negatively charged tyrosyl-, carboxyl-, or mercapto groups may be involved also in Na^+-coupled cotransport of various substrates. These groups, however, differ from those related to Na^+/H^+ exchange, because amiloride cannot protect from irreversible inhibition. This result is of great importance for the interpretation of the [^{14}C]-DCCD labeling experiments: Amiloride is expected to protect the labeling of proteins involved in Na^+/H^+ exchange, but not that of proteins involved in Na^+-coupled substrate transport.

The specificity of the irreversible inhibition by Br-EIPA could not be studied, because the Na^+-dependent cotransport systems are highly sensitive to irradiation. Therefore we tested Br-EIPA as a possible reversible inhibitor of the above-mentioned Na^+-coupled transport systems. At 50 μM, i.e. a concentration sufficient to block Na^+/H^+ exchange, Na^+-dependent transport of D-glucose, sulfate, citrate, and amino acids was not significantly affected (*data not shown*). Thus, the possibility of an irreversible inhibition of various Na^+-dependent transport systems by photoaffinity labeling with Br-EIPA seems remote.

Labeling of Brush-Border Membranes

Since DCCD and Br-EIPA bind covalently to membrane proteins involved in Na^+/H^+ exchange it must be possible to identify these proteins by radiolabeled DCCD and Br-EIPA. A rather selective labeling was obtained with [^{14}C]-DCCD suggesting that only a few membrane proteins contain hydrophobic DCCD-reactive side groups. In contrast, labeling of rabbit renal brush-border membrane vesicles resulted in the incorporation of radioactivity into many membrane proteins [3]. It should be pointed out, however, that these results were obtained after an exposure of membranes to [^{14}C]-DCCD for 8 hr at 0°C whereas we incubated the membranes for only 30 min at room temperature with the label. We preferred labeling at room temperature because pretreatment of rat renal brush-border membranes with DCCD for 30 min at 0°C caused a much smaller irreversible inhibition of Na^+/H^+ exchange than a similar pretreatment at room temperature (*data not shown*).

In brush-border membranes [^{14}C]-DCCD was incorporated with comparable efficiency into three polypeptides with apparent molecular weights of 88,000, 65,000 and 51,000. Photoaffinity labeling with [^{14}C]-Br-EIPA resulted in the most efficient incorporation of radioactivity into a 65-kD polypeptide. In order to find out which of the DCCD- and Br-EIPA-binding proteins may be involved in Na^+/H^+ exchange we performed differential labeling and compared the results on brush-border membranes with those on basolateral membranes which lack the antiporter. Na^+ which protected the antiporter from irreversible inhibition by DCCD and Br-EIPA, did not protect any membrane protein from labeling with [^{14}C]-DCCD. In contrast, Na^+ decreased the labeling of the 65-kD polypeptide by [^{14}C]-Br-EIPA. The reason for the different effects of Na^+ on DCCD and Br-EIPA labeling is not obvious. Na^+ may nonspecifically increase labeling of carboxyl groups not related to Na^+/H^+ exchange. This assumption is supported by the increased labeling of water-exposed carboxyl groups by [^{14}C]-glycineethylester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. A nonspecific increase in carboxyl group reactivity towards carbodiimides may have hidden a possible protective effect of Na^+ on side groups involved in cation antiport. Thus, differential labeling with Na^+ and DCCD did not allow to assign any membrane protein a role in Na^+/H^+ exchange. The effect of Na^+ on the incorporation of radioactivity from [^{14}C]-Br-EIPA into the 65-kD band, however, suggested a possible relation of this protein to Na^+/H^+ exchange.

Amiloride, which protected the Na^+/H^+ exchanger from irreversible inhibition by DCCD, reduced weakly the labeling of membrane proteins with apparent molecular weights between 120,000 and 140,000 and of a band of M_r 51,000. The predominant effect, however, was observed on a 65-kD polypeptide. This polypeptide is identical to that identified with [^{14}C]-Br-EIPA because prior photoaffinity labeling with cold Br-EIPA decreased subsequent labeling of the 65-kD protein by [^{14}C]-DCCD (*data not shown*). Thus, this protein binds DCCD and—in a Na^+-protectable way—Br-EIPA suggesting once more a relation to Na^+/H^+ exchange.

In rabbit renal brush-border membrane vesicles a membrane protein with apparent molecular weight of 100,000 showed an amiloride-sensitive incorporation of [^{14}C]-DCCD [3, 29]. Similar to those studies we observed a narrow DCCD-labeled band which in our hands showed an apparent molecular weight of 88,000. However, amiloride exerted no effect on the labeling of this band. Moreover, this polypeptide was present in basolateral membranes to a higher extent than in brush-border membranes which is opposed to the localization of the Na^+/H^+ exchanger. It remains to be elucidated whether species differences can account for the different results obtained with rat and rabbit renal brush-border membrane vesicles.

The molecular weights of two of the [^{14}C]-DCCD-labeled polypeptides in rat renal brush-border membrane vesicles (M_r 65,000 and 51,000) compare well with those of proteins phosphorylated in lymphocytes by phorbol esters (65, 60, 55–50 kD) and by cell shrinking (60, 55–50 kD, refs. 24, 25). Although it is tempting to speculate that phosphorylation of the antiporter is involved in its activation by tumor promoters and cell volume changes, demonstration of $^{32}P_i$ incorporation into a DCCD or Br-EIPA-labeled membrane protein is ultimately needed to support this hypothesis.

Vigne et al. [51] titrated [^{3}H]ethylpropylamiloride binding sites in brush-border membranes from rabbit kidney. They estimated 2.4 pmol of binding sites per mg protein. In our protection experiments amiloride decreased the radioactivity of incorporated [^{14}C]-DCCD by a total of 500 cpm per M_r 65,000 band, when 0.5 mg protein were applied per gel. Taking into account a specific activity of 54 Ci/mol we arrive at 8 pmol DCCD binding sites per mg protein that were protected by amiloride. This figure underestimates the number of all Na^+/H^+ ex-

changers in the membrane because the DCCD concentration used in the labeling experiments was insufficient to block all antiporters. Thus, brush-border membrane vesicles from the rat kidney cortex seem to contain a much greater amount of antiporters than brush-border membranes from the rabbit kidney.

Labeling of Basolateral Membranes

In basolateral membranes which lack the Na^+/H^+ exchanger [30, 46] a polypeptide with apparent molecular weight of 88,000 was predominantly labeled by DCCD. A similar labeling pattern was obtained when purified Na^+,K^+-ATPase was exposed to [^{14}C]-DCCD. The α-subunit was highly labeled whereas the β-subunit showed a very weak, if any, incorporation of [^{14}C]-DCCD. We therefore conclude that the highly labeled band in basolateral membrane vesicles is the α-subunit of the Na^+,K^+-ATPase. The predominant occurrence of this band in basolateral membranes and data showing an inhibition of the Na^+,K^+-ATPase by DCCD [21, 47] are in accordance with this interpretation.

The occurrence of a DCCD-labeled band with a similar molecular weight in the brush-border membrane was surprising. A possible explanation is a contamination of brush-border membranes with basolateral membranes. Accordingly, preparations of basolateral membranes seem also to be cross-contaminated with brush-border membranes, because an amiloride-protectable labeling in the 65,000-molecular weight range was visible. However, in brush-border membranes the 88-kD and the 65-kD polypeptides were labeled by [^{14}C]-DCCD to a comparable extent, whereas basolateral membranes exhibited an approximately sixfold higher incorporation of radioactivity into the 88-kD protein as compared to the 65-kD polypeptide. Thus, the amiloride-protectable DCCD-labeled band with an apparent molecular weight of 65,000 corresponds most likely to a genuine brush-border membrane protein. The apparent absence in basolateral membranes of the Na^+-protectable Br-EIPA-binding polypeptide of similar molecular weight supports this conclusion. As this protein shows the same distribution as the Na^+/H^+ exchanger and binds DCCD and Br-EIPA we think that in brush-border membranes from rat kidney cortex a protein of M_r 65,000 is a part of the Na^+/H^+ exchanger. Future attempts towards isolation and purification of the Na^+/H^+ exchanger should therefore concentrate on this protein.

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