Photoaffinity Labeling by [^{3}H]-N^5-Methyl-N^5-isobutylamiloride of Proteins Which Cofractionate with Na^+/H^+ Antiport Activity†

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ABSTRACT: N^5-Methyl-N^5-isobutylamiloride (MIA) is one of a series of 5-N-substituted amiloride analogues which exhibit high affinity and specificity for inhibition of Na^+/H^+ antiport. Amiloride-sensitive [^{3}H]MIA binding to renal brush border membranes exhibited a K_d of 250 nM and a B_{max} of 8.6 pmol/mg of protein. Specific binding was optimal at pH 7.5 and inhibited in the presence of Na^+ and Li^+. Inhibition by amiloride exhibited biphasic kinetics. After resolution of solubilized membranes by high-pressure liquid chromatography, MIA binding activity cofractionated together with Na^+/H^+ antiport activity, measured after reconstitution in asolectin vesicles, into a major and a minor peak. When fractions containing the major peak of Na^+/H^+ antiport activity were incubated with [^{3}H]MIA and then photolyzed with a mercury arc lamp, covalent incorporation of label into polypeptides of apparent molecular mass 81 and 107 kDa was observed. These photolabeled bands were also observed in intact brush border membranes in addition to labeled polypeptides of apparent molecular mass 60 and 46 kDa, respectively. Labeling was inhibited by amiloride, reduced in the presence of Na^+, and not observed in the absence of photolysis. These data point to the 81- and 107-kDa polypeptides as candidates for identification as components of a Na^+/H^+ antiport system in renal brush border membranes.

A Na^+/H^+ antiporter that catalyzes the obligatory exchange of H^+ for Na^+ is found in the membrane of virtually all vertebrate cells (Boron, 1983). This system is of fundamental importance in the regulation of intracellular pH, cell volume, and response to mitogenic stimuli [reviewed in Moolenaar et al. (1986)].

While the physiological regulation and transport kinetic properties of the Na^+/H^+ antiporter have been extensively investigated, the membrane protein which catalyzes this activity has not yet been identified. Recently, Friedrich et al. (1986) and Igarashi and Aronson (1987) have demonstrated amiloride-protectable labeling by N,N'-dicyclohexylcarbodimide (DCCD) of polypeptides which they tentatively identified as components of the renal Na^+/H^+ exchanger. Since DCCD is a nonspecific chemical modification agent, this identification rested on assumptions concerning the specificity of amiloride binding. However, amiloride is neither a specific nor a high-affinity inhibitor of the Na^+/H^+ antiporter but also inhibits an epithelial Na^+ channel (Cuthbert & Fanelli, 1978), the Na^+,K^+-ATPase (Soltoff & Mandel, 1983), the Na^+/Ca^{2+} antiporter (Kaczorowski et al., 1984), Na^+/glucose and Na^+/amino acid symporters (Harris et al., 1985), and an α_2-adrenergic receptor (Nunnari et al., 1987) in addition to a number of other cellular functions unrelated to membrane transport (Leffert et al., 1982; Besterman et al., 1985).

Replacement of one or both hydrogen atoms of the 5-amino group of amiloride by alkyl or alkenyl groups led to a 10–500-fold increase in potency for inhibition of Na^+/H^+ antiport (Simchowitz & Cragoe, 1986) with corresponding reduction in IC_{50} for inhibition of Na^+/Ca^{2+} antiport (Kaczorowski et al., 1985) and the Na^+ channel (Cuthbert & Fannelli, 1978). The amiloride derivative [^{14}C]-N-amidino-3-amino-5-(N-ethyl-N-isopropylamino)-6-bromopyrazinecarboxamide (Br-EIPA) was used to photolabel a 65-kDa polypeptide in rat renal brush border membranes (Friedrich et al., 1986).

In the present study, we demonstrate that the high-affinity 5-amino-substituted amiloride analogue [^{3}H]-N^5-methyl-N^5-isobutylamiloride ([^{3}H]MIA) is covalently incorporated into renal brush border membrane proteins after photolysis. Labeling is prevented in the presence of amiloride. MIA binding activity and the MIA-photolabeled species cofractionate with Na^+/H^+ antiport activity during HPLC fractionation. Taken together with the recent demonstration that amiloride-sensitive [^{3}H]MIA binding is associated with the Na^+/H^+ exchanger (Dixon et al., 1987), these results implicate the MIA-photolabeled proteins as candidates for identification as subunits of a Na^+/H^+ antiport system in renal brush border membranes. A preliminary account of this work has been presented (Wu & Lever, 1988).

Materials and Methods

Materials. [^{3}H]-N^5-Methyl-N^5-isobutylamiloride, 28 Ci/mmol, was purchased from New England Nuclear Corp. Kidneys from domestic hogs were obtained through the generosity of Texas A&M University, College Station, TX. n-Octyl β-D-glucopyranoside (n-octyl glucoside) was from Boehringer Mannheim. Amiloride and asolectin were from Sigma. All other reagents were of analytical grade.

Centrifugation Assay of MIA Binding. Membranes (80 μg) in buffer B (0.125 M sucrose, 5 mM $MgCl_2$, 10 mM K^+-Hepes, pH 7.2) were first incubated with or without 1 mM amiloride for 20 min at 22 °C. [^{3}H]MIA was then added to a final concentration of 125 nM (except as otherwise noted) in a final volume of 100 μL. After 30 min, incubations were terminated by addition of 1 mL of ice-cold buffer B-1 (50 mM sucrose, 2 mM $MgCl_2$, 4 mM K^+-Hepes, pH 7.2), followed by centrifugation at 12000g for 30 min. Membrane pellets

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were washed once with 1 mL of ice-cold buffer B-1, dissolved in 1 N NaOH, and neutralized with an equal volume of 1 N HCl before liquid scintillation counting.

HPLC Fractionation of Solubilized Brush Border Membranes. Brush border membranes were prepared from pig kidney cortex according to the method of Malathi et al. (1979). Proteins were solubilized from 140 mg of membranes by stirring membranes at 0 °C for 20 min in 24 mL of buffer A (50 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 0.1 mM EDTA, and 4% *n*-octyl glucoside). The unsolubilized materials were removed by centrifugation at 160000*g* for 1 h. Solubilized samples were adjusted to 2% *n*-octyl glucoside and then injected onto a Mono-Q HR 10/10 column (Pharmacia) through a Model 7125 syringe loading sample injector and eluted at a flow rate of 2 mL/min with concentration gradients of 0 to 1 M KCl in 100 mL of buffer A containing 2% *n*-octyl glucoside. Absorbance at 280 nm was monitored with a LKB 2138 Uvicord S UV monitor. Fractions from the Mono-Q column were dialyzed for 24 h against 8 L of 5 mM $MgCl_2$–10 mM K^+-Hepes, pH 7.2, to remove KCl and *n*-octyl glucoside before analysis by SDS/PAGE. Protein was determined by the method of Lowry et al. (1951).

Reconstitution of Na^+/H^+ Antiport Activity and MIA Binding Activity. Asolectin (crude soybean phospholipid), 35 mg/mL, was sonicated to translucency for 10 min at 22 °C. Solubilized brush border membranes or HPLC fractions were mixed with sonicated asolectin to a final concentration of 13.5 mg/mL asolectin–0.3 mg/mL protein in buffer A containing 4.4% *n*-octyl glucoside. Samples were then dialyzed either against buffer C (30 mM potassium gluconate, 190 mM mannitol, 50 mM Tris–MES, pH 6.0) for measurement of Na^+/H^+ antiport activity or against buffer D (125 mM sucrose, 10 mM K^+-Hepes, pH 7.2) for measurement of [^{3}H]-MIA binding activity.

Gel Filtration Assay of MIA Binding to Solubilized Membranes. A gel filtration method described previously (Wu & Lever, 1987a) was used for assaying MIA binding to solubilized membranes and HPLC fractions. Columns containing 1 mL of preswollen Sephadex G-50 (fine) in buffer D were centrifuged at 100*g* for 2 min. Samples reconstituted in asolectin by dialysis in buffer D for 24 h were preincubated for 20 min with or without 1 mM amiloride and then incubated with [^{3}H]MIA. Samples (100 μL) were applied to each column, and columns were centrifuged again at 100*g* for 2 min. Radioactivity in the eluate was determined by liquid scintillation counting.

Assay of Na^+/H^+ Antiport in Reconstituted Fractions. Na^+/H^+ antiport was assayed as described previously (Weinman et al., 1988) in HPLC fractions after reconstitution with asolectin. Samples (50 μL) at pH 6.0 were mixed with 100 μL of a solution containing 7.2 μCi/mL $^{22}Na^+$, 1.56 mM NaCl, 30 mM potassium gluconate, 190 mM mannitol, 10 μg/mL valinomycin, and 50 mM Tris–MES at either pH 8.5 or pH 6.0. After 1-min incubation at room temperature, uptake was terminated by transferring 100 μL from the uptake mixture to a 1-mL Dowex 50X8 (Tris) 100-mesh column followed by rapid elution with 1 mL of 300 mM mannitol, pH 8, at 0 °C with vacuum suction. The H^+-dependent $^{22}Na^+$ uptake was calculated by subtracting the activity observed at pH 6.0 from that observed at pH 8.0.

Photolysis Using [^{3}H]MIA. HPLC fractions were dialyzed against buffer B to remove salt and *n*-octyl glucoside. Samples were incubated with or without 1 mM amiloride (or other drugs as indicated), for 20 min at 22 °C. Then [^{3}H]MIA was added to a final concentration of 250 nM. After 30 min, the mixtures were chilled at 0 °C, and dithiothreitol was added to a final concentration of 50 mM to serve as free radical scavenger. Samples were exposed at 0 °C to light from a 350-W mercury arc lamp at a distance of 25 cm for 45 s. Samples were treated by gel filtration as described above to remove unreacted ligands and then dissolved in gel electrophoresis buffer containing SDS (2% w/v), 5% (w/v) glycerol, 0.02% (w/v) bromophenol blue, 50 mM dithiothreitol, and 50 mM Tris-HCl, pH 6.8, and analyzed by SDS/PAGE (7.5% acrylamide). Radioactivity was determined by liquid scintillation after incubation of 2-mm gel slices overnight at 37 °C with 10 mL of 14% Omnifluor–5% Protosol (New England Nuclear) in toluene.

For experiments using unsolubilized membranes, the above protocol was modified slightly. In order to remove extrinsic membrane proteins, brush border membranes were treated with EGTA at pH 11 as we have described previously (Wu & Lever, 1987b). After photolysis, membranes were solubilized with 2% *n*-octyl glucoside and treated by gel filtration before analysis by SDS/PAGE. This modification reduced the extensive background labeling observed with whole membranes.

Results and Discussion

Binding of MIA to Kidney Brush Border Membranes Is Sensitive to Amiloride. [^{3}H]MIA binding to renal brush border membranes exhibited a saturable amiloride-sensitive component representing about 62% of the total MIA binding activity at 7 nM MIA (Figure 1A). The remainder was insensitive to amiloride concentrations up to 2.5 mM and nonsaturable. A similar high percentage of amiloride-insensitive MIA binding has been noted previously (Dixon et al., 1987). MIA is much more lipophilic than amiloride, with a lipid partition coefficient of 16.9 compared with a value of 0.054 for amiloride (Simchowitz et al., 1987). Thus nonspecific MIA binding may represent lipophilic interaction with membrane phospholipids. On the basis of Scatchard analysis (Figure 1A, inset), values of K_d and B_{max} (maximum binding activity) for the saturable component, after subtraction of the amiloride-insensitive binding, were 249.6 ± 71.5 nM and 8.63 ± 1.10 pmol/mg of protein ($n = 4$), respectively. This K_d value agrees favorably with the K_d of 170 nM observed by Dixon et al. (1987) for the single class of high-affinity MIA binding sites in rat thymocytes. It also correlates with the k_i value of 174 nM noted for MIA inhibition of Na^+/H^+ antiport activity (Dixon et al., 1987).

Inhibition by amiloride was biphasic (Figure 1B). Amiloride concentrations up to 0.14 mM inhibited the specific component of MIA binding by 71% with an IC_{50} value of 58 μM. At concentrations of amiloride above 0.25 mM, further inhibition was observed with an IC_{50} value of 680 μM. These results indicate the existence of at least two types of MIA binding sites with different sensitivities toward amiloride.

High-affinity amiloride-sensitive MIA binding was optimal at pH 7.5 with reduced binding at acid pH (not shown). Amiloride-sensitive MIA binding was competitively inhibited by Na^+, with a K_i value for NaCl of 41.7 mM (not shown). LiCl was almost as effective as NaCl in inhibiting MIA binding, but RbCl, KCl, and choline chloride at 100 mM and $CaCl_2$ at 20 mM had no effect (not shown).

Cofractionation of MIA Binding and Na^+/H^+ Antiport during HPLC Chromatography. After asolectin reconstitution, the initial solubilized membranes contained 17.5 pmol/mg of protein of MIA binding activity. This specific activity represented a 2-fold increase compared with that of intact unsolubilized brush border membranes and an 83-fold increase

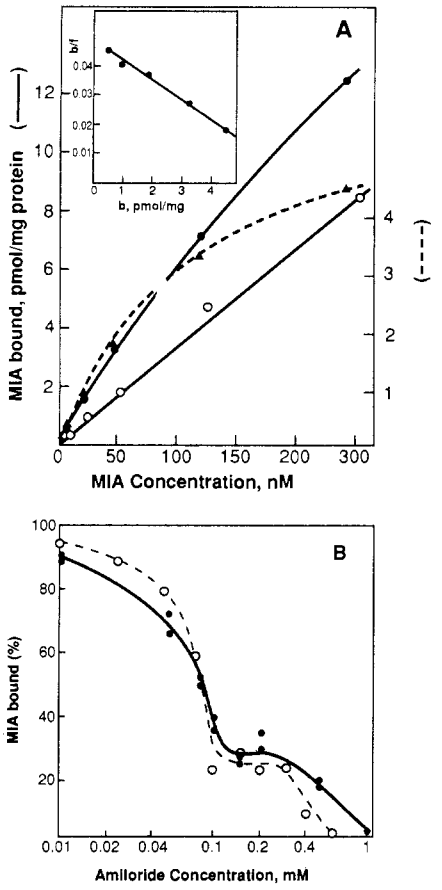

FIGURE 1: Characterization of amiloride-sensitive MIA binding activity in renal brush border membranes. (A) Membranes (70 μg) were incubated with different concentrations of [^{3}H]MIA (10–300 nM) after pretreatment with (○) or without (●) 1 mM amiloride. MIA binding activity was then assayed with the centrifugation assay. The amiloride-sensitive component is shown (▲) after subtraction of amiloride-insensitive nonspecific binding. Apparent K_d and B_{max} values of 160 nM and 7.5 pmol/mg of protein, respectively, were calculated after subtraction of amiloride-insensitive binding, as shown in the Scatchard plot (inset). Data are plotted versus unbound MIA concentration, determined from each supernatant. (B) Membranes (65 μg) were incubated with the indicated concentration of amiloride for 20 min at room temperature, followed by 30-min incubation with 125 nM [^{3}H]MIA, and then binding was quantitated by the centrifugation assay. Results are shown after subtraction of amiloride-insensitive binding (45.2% of total). 100% refers to 4.7 pmol/mg, the activity observed in the absence of amiloride after correction for nonspecific binding. Data from two independent experiments (○, ●) are shown.

compared with that of solubilized membranes dialyzed in the absence of added phospholipids (0.21 pmol/mg of protein).

Figure 2A illustrates the resolution of *n*-octyl glucoside solubilized membranes by HPLC using a Mono-Q column and elution by a KCl gradient. Amiloride-sensitive MIA binding activity of HPLC fractions was assayed after reconstitution into asolectin vesicles. The major MIA binding peak (145 pmol/mg) eluting at 0.34 M KCl represented 15% of total specific MIA binding activity and a 7-fold increase in specific activity compared with the material applied to the column. Activity of the minor peak eluting at 0.29 M KCl ranged between 22 and 24 pmol/mg of protein. Addition of asolectin also increased amiloride-insensitive binding.

If fractions were dialyzed without addition of phospholipids and assayed for amiloride-sensitive MIA binding, the two peaks of binding activity were also observed (not shown). In this case, activity of the pooled major peak eluted at 0.34 M KCl exhibited a 17-fold increase in specific activity (3.6 pmol/mg) compared with the material applied to the column (0.21 pmol/mg of protein) and 25% recovery of activity. A smaller peak of binding activity (0.51 pmol/mg) was observed to elute at 0.29 M KCl. These values may not reflect the actual increase in specific activity since samples before and after column fractionation may differ in endogenous lipid content.

Na^+/H^+ antiport activity accompanied MIA binding activity during HPLC fractionation. Antiport activity was assayed as $^{22}Na^+$ influx driven by an outwardly directed proton gradient in proteoliposomes after reconstitution of aliquots of the same HPLC fractions with asolectin. By use of this assay with reconstituted renal membranes, a rank order of potency of ethyisopropylamiloride > 6-iodoamiloride > amiloride > phenamil was observed for inhibition of Na^+ flux (Weinman et al., 1988). Figure 2A shows that a major peak of antiport activity (open circles) corresponded to the major peak of MIA binding activity (filled circles) eluting at 0.34 M KCl. A second peak of antiport activity was observed to elute at 0.29 M KCl, corresponding to the minor peak of MIA binding activity (n = 3). These observations strongly support previous conclusions that MIA binding activity is associated with the Na^+/H^+ antiporter (Dixon et al., 1987). The finding of two peaks of Na^+/H^+ antiport activity is unexpected and suggests that the proximal tubule may contain two distinct forms of the antiporter.

Photolysis Studies with MIA. When [^{3}H]MIA was incubated with HPLC fractions containing the major peak of MIA binding and Na^+/H^+ activity and then exposed to high-intensity UV light from a mercury arc lamp, label was covalently incorporated into specific membrane proteins (Figure 2B). Incorporation was partially blocked by 1 mM amiloride (Figure 2B). No inhibition was observed in the presence of 0.1 mM amiloride (not shown). Two species, with apparent molecular masses of 107 and 81 kDa, were labeled in eight independent experiments. In the representative experiment shown in Figure 2B, the two peaks incorporated 5% and 10%, respectively, of total added radioactivity, after correction for nonspecific basal labeling. The large number of other proteins contained in this fraction as detected by silver stain (inset of Figure 2B) were not specifically labeled by MIA.

The two photolabeled species differed in sensitivity to amiloride and Na^+ inhibition of incorporation. In the presence of 1 mM amiloride, incorporation into the 107-kDa region was reduced by 45% and that in the 81-kDa region by 76% (Figure 2B). Addition of 100 mM NaCl inhibited incorporation into the 107-kDa peak by 40% and into the 81-kDa peak by 20% (not shown). This result is consistent with our observation that Na^+ inhibits specific MIA binding to brush border membranes. If NaCl was substituted for KCl in HPLC gradient elution, no effect of Na^+ on MIA photolabeling was detected (not shown). It seems likely that sufficient Na^+ to effect full inhibition remained trapped in the sample even after dialysis.

When samples incubated with MIA were directly analyzed by SDS/PAGE without prior exposure to UV light, no covalent incorporation of radioactivity could be detected (Figure 2B). Dithiothreitol was required as a free-radical scavenger in order to observe specific photolabeling. In the absence of dithiothreitol, a complicated labeling pattern without any recognizable peak was observed (data not shown). It was necessary to use samples reconstituted by dialysis without addition of phospholipids in order to observe specific photolabeling, due to the large increase in nonspecific background labeling in the presence of exogenous phospholipids.

A broad photolabeled peak which occupied the 73–115-kDa region was observed when depleted brush border membranes were subjected to photolysis in the presence of [^{3}H]MIA

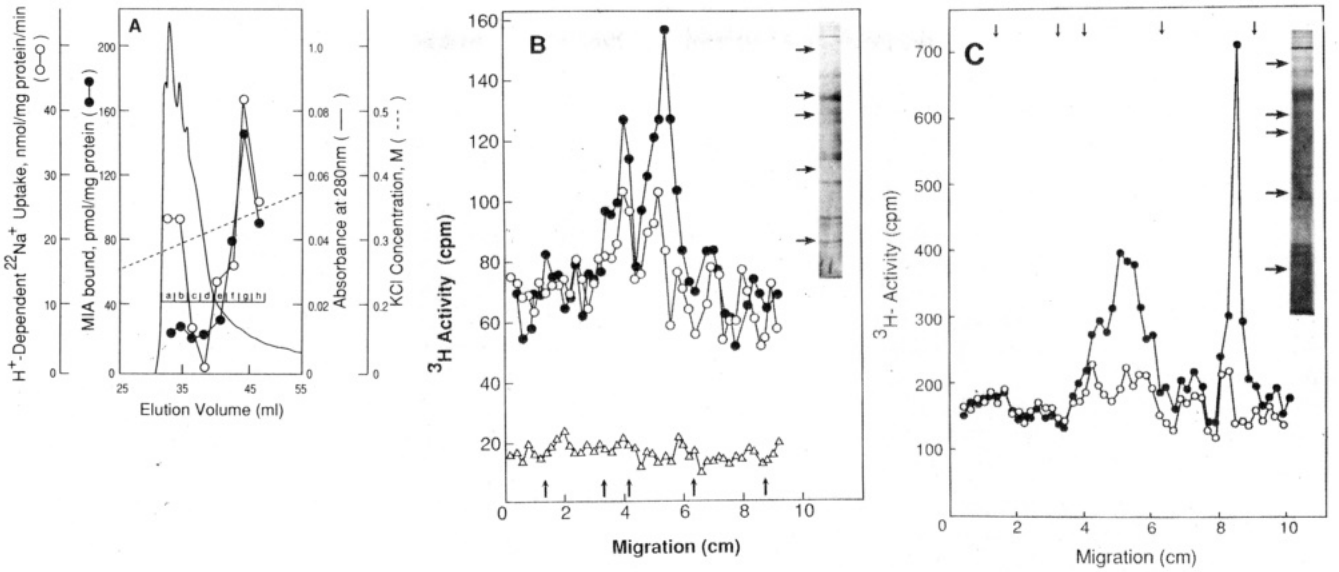

FIGURE 2: Incorporation of [^{3}H]MIA into HPLC-fractionated membrane proteins after photolysis. (A) Fractionation of MIA binding activity by HPLC. Samples solubilized from 140 mg of pig kidney brush border membranes were applied to a Mono-Q column and eluted with a KCl gradient indicated by the dashed line. Aliquots of fractions a–h were reconstituted with asolectin and assayed for amiloride-sensitive MIA binding activity at 125 nM [^{3}H]MIA with the gel filtration binding assay. (●) MIA binding activity shown is the amiloride-sensitive component after subtraction of the amiloride-insensitive component (81%) from total binding activity. (○) Na^+/H^+ antiport activity of aliquots of the same material after reconstitution with asolectin. (B) Photoaffinity labeling of pooled HPLC fractions f–h. Aliquots of pooled fractions were dialyzed to remove salt and detergent, and samples (80 μg) were pretreated with (○) or without (●, △) 1 mM amiloride followed by addition of [^{3}H]MIA (250 nM) as described. Samples were then either exposed to UV light (○, ●) before SDS/PAGE or directly subjected to electrophoresis without exposure to UV light (△). (C) Photoaffinity labeling of depleted brush border membranes. Depleted brush border membranes (500 μg) were preincubated with (○) or without (●) 1 mM amiloride followed by addition of [^{3}H]MIA (250 nM) as described. The insets show the silver staining pattern of the samples. In (B) and (C) the arrows indicate the position of molecular mass markers of 200, 130, 92.5, 66, and 45 kDa. After resolution of photolyzed samples by SDS/PAGE, radioactivity of 2-mm gel slices is shown as a function of distance of migration.

(Figure 2C). This broad peak representing 15% of total added radioactivity presumably contains both the 107- and 81-kDa MIA-labeled proteins eluting at 0.34 M KCl after HPLC fractionation. However, in brush border membranes, two additional peaks of apparent molecular mass 60 and 46 kDa, containing 2% and 9% of total added radioactivity, respectively, were also observed. In the presence of 1 mM amiloride, labeling of the broad 73–115-kDa peak was reduced by 66% while the 60- and 46-kDa peaks were reduced by 10% and 77%, respectively. No photolabeling by MIA was detectable with apical membranes from LLC-PK_1 cells (not shown).

Preliminary studies indicate that the 60-kDa MIA-labeled species fractionates in the minor peak eluting at 0.295 M KCl after HPLC chromatography (Wu & Lever, 1988). This peak also contains a 100-kDa and a 150-kDa MIA-labeled species (Wu & Lever, 1988). Friedrich et al. (1986) labeled a 65-kDa band in intact rat renal brush border membranes using the N^5-ethyl-N^5-isopropyl-6-bromo derivative of amiloride.

MIA photolabeling was not affected by 20 mM Ca^{2+}, by 1 mM ouabain, or by 5 μM phenamil, an inhibitor of the epthelial Na^+ channel (Barbry et al., 1986). Inhibition of incorporation into both peaks was observed at 10 μM phenamil (not shown). Barbry et al. (1987) have purified a 105-kDa protein from pig kidney identified as the epithelial Na^+ channel on the basis of [^{3}H]phenamil binding. We have observed that [^{3}H]phenamil binding activity elutes as a symmetrical peak at a 0.01 M lower salt concentration than the major peak of [^{3}H]MIA binding activity (not shown). While it is possible that a small fraction of the phenamil binding protein was pooled with the MIA binding fraction used for photolysis, it seems unlikely that the phenamil binding protein was photolabeled by MIA since labeling was not inhibited by 5 μM phenamil or by 0.1 mM amiloride. By contrast, phenamil binding to its receptor solubilized from the same tissue, pig kidney, exhibited a K_d value of 20 nM while amiloride inhibition of phenamil photolabeling exhibited a $K_{0.5}$ of 10 μM (Barbry et al., 1987). Inhibition of MIA photolabeling by Na^+ also distinguishes between the two systems. Different candidate polypeptides for the Na^+ channel have been identified with different photoreactive ligands. Using benzamil derivatives, Kleyman et al. (1986) labeled peptides of 176, 77, and 47 kDa, and Benos et al. (1986) have identified a 700-kDa protein, as candidates for the epithelial Na^+ channel.

MIA also binds the α_2-adrenergic receptor (Nunnari et al., 1987). Addition of the α_2-adrenergic receptor agonist yohimbine, 10 μM, did not inhibit MIA binding or MIA photolabeling (not shown).

While our results do not directly reveal the functional identity of these MIA-labeled species, several considerations strongly implicate one or more of them as candidates for identification as a subunit of the Na^+/H^+ antiporter. The photolabeled species cofractionated with Na^+/H^+ antiport activity and MIA binding during HPLC chromatography. Photolabeling was inhibited by 1 mM amiloride, a concentration which inhibits the antiporter, but was not sensitive to concentrations of amiloride (0.1 mM) or phenamil (5 μM) which inhibit the Na^+ channel. Covalent incorporation was inhibited by concentrations of Na^+ which inhibit specific MIA binding. High-affinity amiloride-sensitive MIA binding has been previously shown to quantitatively correspond to inhibition of Na^+/H^+ antiport in thymocytes (Dixon et al., 1987).

Using renal microvillus membranes, Vigne et al. (1984) have reported that the closely similar analogue ethylpropylamiloride binds with a K_d of 17 nM and inhibits antiport with a $K_{0.5}$ of 90 nM. However, binding was measured in the absence of Na^+, but antiport was measured in the presence of Na^+, which may influence binding as we have noted in the case of MIA. The importance of testing these inhibitors with Na^+-free media

to assay Na^+ flux for this reason has been emphasized by O'Donnell et al. (1986).

The finding of two resolvable HPLC peaks of Na^+/H^+ antiport activity, each containing different MIA-photolabeled species, raises the question of whether these represent different proteins, related proteins, or artifacts of proteolysis. Since amiloride inhibition of MIA binding to brush border membranes exhibits biphasic kinetics, it is possible that there are at least two distinct MIA receptors. Our preliminary results suggest that the 81- and 107-kDa bands in the major peak are immunologically related to each other but do not cross-react with proteins in the minor peak. It is important to note that the apparent molecular weights of MIA-labeled proteins may not correspond to their actual molecular weights due to the possibility of cross-linking during photolysis.

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