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Photolabile Amiloride Derivatives as Cation Site Probes of the Na,K-ATPase*

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Treatment of purified canine renal Na,K-ATPase with a range of photoactivatable amiloride derivatives results in inhibition of ATPase activity prior to illumination. Inhibition by amiloride derivatives substituted on a guanidium N could not be prevented by the presence of either K or Na; however, these cations could protect the enzyme against inhibition by derivatives substituted on the 5-position of the pyrazine ring. In the case of 5-(*N*-ethyl-[2′-methoxy-4′-nitrobenzyl])amiloride (NENMBA), the presence of monovalent cations (Na, K, and Rb) protected the enzyme effectively against inhibition, with concentrations in the millimolar range. ATP did not prevent inhibition; furthermore, native and NENMBA-treated enzyme exhibited normal levels of high affinity [^{3}H]ADP (and hence ATP) binding. The rate of inhibition increased with increasing concentrations of NENMBA. Extensive washing of NENMBA-inhibited enzyme did not restore ATPase activity, showing that NENMBA has an extremely slow off-rate for dissociation from its inhibitory site. Partially inhibited enzyme could be rapidly pelleted and resuspended in NENMBA-free buffer and inhibition was observed to continue, albeit at a somewhat diminished rate, suggesting that NENMBA gains access to its inhibitory site after partitioning into the lipid phase rather than directly from the aqueous solution. Photolysis of NENMBA-inhibited enzyme resulted in covalent incorporation of the reagent into the α-subunit of the Na,K-ATPase, as observed by separation of labeled protein on a Laemmli gel and Western analysis using a polyclonal amiloride antibody. Almost all of the covalent labeling could be prevented by the presence of Rb in the incubation and labeling medium. These results suggest that NENMBA inhibits the Na,K-ATPase by disruption of the cation transport domain rather than the catalytic domain of the enzyme and that it promises to be a useful tool for cation site localization.

The sodium pump or Na,K-ATPase (EC 3.6.1.37) is the plasma membrane protein responsible for maintaining the resting concentrations of sodium and potassium in animal cells. It is composed of an α-subunit and a β-subunit. The α-subunit consists of about 1020 amino acid residues and has been cloned from several sources (1–6). The β-subunit is composed of about 300 amino acids (7–9). The enzyme undergoes a series of conformational changes through its catalytic cycle, which couple binding of ATP and enzyme phosphorylation to the active transport of the monovalent cations against their electrochemical potential gradients (for reviews see Refs. 10–12). In its normal transport mode, the enzyme is phosphorylated by ATP when cation binding residues which selectively bind sodium are exposed to the cytosol; the enzyme then undergoes a major conformational transition and transports three sodiums across the cell membrane, where they dissociate to the external medium. The (phospho-) enzyme cation ligating residues are now exposed to the external medium and are more potassium-selective; two potassiums (or cogeners for potassium such as rubidium, cesium, etc.) bind, and the phospho-enzyme bond is hydrolyzed. The potassiums are then occluded within the protein, and the enzyme undergoes the second major conformational transition of its catalytic cycle when ATP binds with low affinity (but does not phosphorylate the protein) and so facilitates the release of potassium to the cytosol. The binding of sodium ions then reinitiates the cycle.

A detailed molecular description of these transport events includes the localization of which amino acid residues are involved in binding and transport of sodium and potassium across the cell membrane. One method that has been used to address this problem is the utilization of residue-selective chemical probes to modify purified sodium pump protein (for review, see Ref. 13). It has been assumed that negatively charged amino acids are involved in cation coordination. This hypothesis has been tested by using aspartate and glutamate-specific carbodiimides which inactivate the sodium pump in a cation-preventable manner (14–21). There are inherent ambiguities in the use of such reagents, as they can also induce the formation of endo-peptide bonds; such crossing linking is also cation-preventable (16, 19). In order to circumvent these problems, 4-(diazomethyl)-7-(diethylamino)-coumarin (DEAC),[1] a stable diazomethane analogue, which reacts specifically and unambiguously with carboxylates to form stable ester derivatives of the protein, has been recently shown to modify Glu-779 in a cation-preventable manner (22, 23). However, by analogy with the naturally occurring ionophores, we would infer that not only glutamate or aspartate side chains are involved but the electron lone pairs of other, neutral residues might also be involved in monovalent cation transport.

In this report we have examined the effects of four photoac-

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[1] The abbreviations used are: DEAC, 4-(diazomethyl)-7-(diethylamino)-coumarin; Mes, 2(*N*-morpholino)ethanesulfonic acid; Taps, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; NENMBA, 5-(*N*-ethyl-[2′-methoxy-4′-nitrobenzyl])amiloride.

tivatable amiloride derivatives on the Na,K-ATPase. Amiloride derivatives were chosen because they are known to inhibit other sodium transporting proteins such as epithelial Na channels, the Na/H antiporter, and the Na/Ca exchanger (24, 25). Furthermore, there are well defined structure-activity relationships for amiloride derivatives; guanidium-substituted probes show high affinity for epithelial Na channels, whereas pyrazine ring derivatives evidence a higher affinity for exchangers than channels (25). The four compounds we tested are representative of the two classes of inhibitors. All four reagents inhibit the Na pump, but only the pyrazine ring-substituted derivatives in a cation-preventable manner. The most effective of these probes, NENMBA (Fig. 1), was shown to be covalently incorporated into the α-subunit of the Na pump upon irradiation, as shown by Western analysis using a polyclonal antibody. A preliminary report of some of these results has appeared (26).

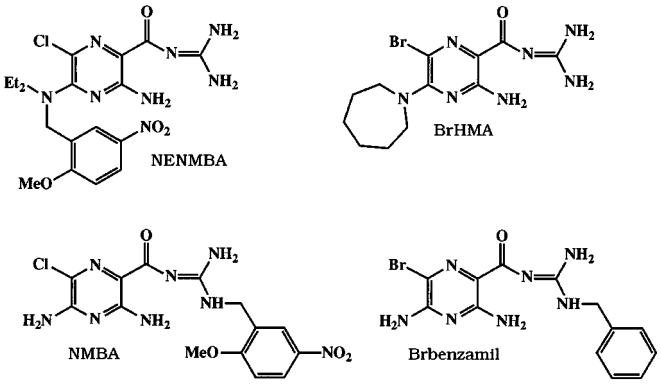

FIG. 1. **Chemical structures of the four photoactivatable, amiloride derivatives.**

EXPERIMENTAL PROCEDURES

Materials—Amiloride derivatives were a gift from Merck, Sharp and Dohme, West Point, PA. ATP, ADP, imidazole, Hepes, Mes, Taps, Ches, Tris, and bovine serum albumin (BSA), dithiothreitol, sucrose, ultrapure urea, and Tricine were from Sigma; L-1-tosylamide-2-phenylethylchloromethyl ketone-treated trypsin was from Worthington Corporation. β-Mercaptoethanol, sodium dodecyl sulfate, ammonium persulfate, Coomassie R-250, and low molecular weight standards were from Bio-Rad. Acrylamide and bisacrylamide were obtained through Boehringer Mannheim. Nitrocellulose was from Millipore.

Enzyme Isolation and Assays—Na,K-ATPase was purified from dog kidneys according to Jørgensen (27) with the modification of Liang and Winter (28). The enzyme was greater than 95% pure as judged by SDS-PAGE (see Fig. 9 in Ref. 33). The standard assay medium for Na,K-ATPase activity was (mM): EDTA, 0.5; NaCl, 130; KCl, 30; $MgCl_2$, 3; ATP, 3: imidazole, 50; pH 7.2 (25 °C); and about 0.8 μg/ml enzyme protein. Unless specified, it also contained 0.3 mg/ml BSA. The suspension was incubated at 37 °C for 15 min and then the P_i released determined as reported by Brotherus *et al.* (29). The Na,K-ATPase activity was the difference between the ATP hydrolysis measured in the presence and absence of 0.5 mM ouabain. The enzyme used in these studies had a specific activity of 20–26 μmol of P_i released mg^{-1} min^{-1}, a negligible ouabain-insensitive ATP hydrolysis and maximal phosphorylation by P_i at 37 °C in the presence of ouabain of about 2.5–3.0 nmol mg $protein^{-1}$. Protein was determined by the method of Lowry *et al.* (30) using BSA as a standard.

Treatment with Amiloride Derivatives—Incubation of purified Na,K-ATPase with each reagent (100 μM) was performed at 37 °C by incubating the enzyme (0.05 μg/ml) in a medium containing 50 mM Hepes (except where noted) with 2 mM EDTA. The reagents were initially dissolved in Me_2SO so that the final concentration of Me_2SO in the incubation medium was 2–10%. The reactions were stopped by a 60-fold dilution into ice-cold assay medium. The results presented in Figs. 2–6 are representative of at least three experiments in which all samples were run in duplicate. The range between duplicates was less than 10%. ATPase activity did not vary between experiments by more than 10% about the mean value shown in Figs. 2–6.

Irradiation Procedure—Preparative photolabeling of Na pump protein with NENMBA was performed by incubation of the enzyme (1 mg/ml) with NENMBA (1 mM) for 10 min at 37 °C. The enzyme was then pelleted, washed, and resuspended in NENMBA-free Hepes (50 mM) and EDTA (2 mM). 200-μl aliquots were irradiated in a 1-mm pathlength cuvette for 2 min with a 1000-watt high pressure Hg arc lamp filtered through a 313 ± 10 nm band-pass filter (Oriel) in order to excite specifically the nitroaromatic chomophore. Gel electrophoresis was carried out on intact Na pump using a linear (10%) SDS-PAGE system after Laemmli (31) .

Immunoblots—Serum containing polyclonal antibodies raised against amiloride conjugated to keyhole limpet hemocyanin via the 5-position of the pyrazine ring (32) were purified by twice treating 25 ml of a 1:100 dilution (81 μg) in TBS (200 mM NaCl, 50 mM Tris, pH 7.5) with 100 μg of purified Na pump spotted onto nitrocellulose (and blocked with milk), so removing any cross-reactivity from the serum. After electrophoresis of labeled protein samples and protein transfer to nitrocellulose by electroblotting, the blot was incubated at room temperature for 1 h with 5% milk protein and then incubated with a 1:100,000 dilution of the primary antibody overnight at 4 °C. The blot was washed five times with TBS and then incubated at room temperature for 2 h with a 1:2000 dilution alkaline phosphatase goat anti-rabbit antibodies in buffer, followed by washing five times in TBS. Bound antibodies were then detected with Sigma Fast.

High Affinity ADP Binding—Nucleotide binding was measured as described previously (33) in a medium containing (mM): Hepes/imidazole, 30; pH (25 °C) 7.2; NaEDTA, 0.1; NaCl, 5; [^{3}H]ADP, 0.10; and 0.5 mg/ml protein. The suspension was shaken at 4 °C for 30 s and centrifuged at 400,000 × *g* for 10 min. The pellet was resuspended in 2.5 M NaOH, and radioactivity and protein concentration were determined. Radioactivity bound to the enzyme in the presence of 0.5 mM ATP was subtracted from the experimental values as a correction for nonspecific (or low affinity) binding.

RESULTS

Characteristics of Enzyme Inhibition—Incubation of purified Na,K-ATPase with the four amiloride derivatives shown in Fig. 1 resulted in inhibition of the enzyme activity to varying degrees (Fig. 2). The two "channel-specific" compounds (NMBA and bromobenzomil) inhibited the enzyme (~30–40% under the conditions shown) but the presence of Rb ions in the incubation medium were without effect. Under the same conditions, the "exchanger-specific" compounds (NENMBA and 6-bromo-5(*N*,*N*-cyclohexyl)amiloride) produced 70–90% inhibition of Na,K-ATPase and cation prevention of inhibition was observed. In the case of NENMBA, the protection against inhibition was almost complete. It should be emphasized at this point that even though all these probes can potentially photolabel the Na pump upon illumination (300–360 nm), the inhibition of the Na pump as illustrated in Fig. 2 is occurring in the absence of illumination. The most effective prevention of inhibition by Rb was observed with NENMBA; thus, this probe was examined in more detail.

Fig. 3 shows that increasing [NENMBA] increases the rate of inhibition of the Na pump. If fully inhibited enzyme (<2% residual activity) is pelleted by centrifugation, washed in inhibitor-free buffer, and resuspended in buffer, no recovery of enzyme activity is observed. Thus, the dissociation of the inhibitor appears to be an extremely slow process. The lack of recovery is independent of the composition of the resuspension media (± Rb, ± ATP, ± BSA, ± Na). The presence of monovalent cations (Na, K, or Rb) in the incubation medium can prevent the inhibitory binding of NENMBA to the Na pump. These cations protect the enzyme with concentrations in the mM range: $K_{0.5}$ for Rb (which is recognized as a cogener for K by the Na pump) is about 1 mM (see Fig. 4*A*), and for Na is about 7 mM (Fig. 4*B*), when a concentration of 100 μM NENMBA was used to inhibit the enzyme at 37 °C for 10 min. However, since NENMBA has an extremely slow off-rate, eventually the enzyme is fully inhibited even in the presence of 100 mM monovalent cations.

Effect of pH—When the pH of the medium is varied, the rate of binding of NENMBA to its inhibitory site on the Na pump

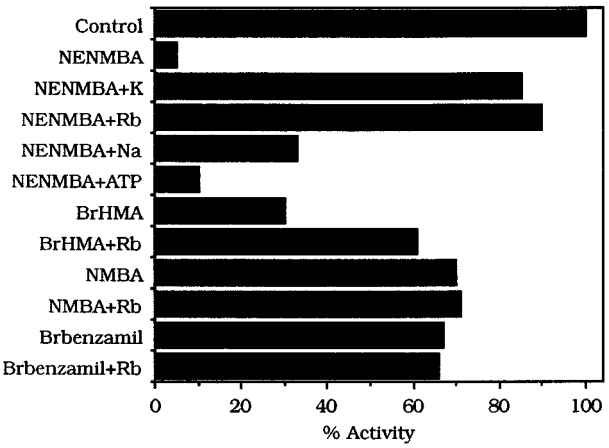

FIG. 2. **Summary of inhibition of purified Na,K-ATPase by the amiloride derivatives.** The enzyme (0.05 mg/ml) was incubated with each compound (100 μM) at 37 °C for 2 min ± Rb, K (10 mM) or Na (60 mM, where applicable) at pH 7.5 (40 mM Hepes) and activity was assayed as described under "Materials and Methods."

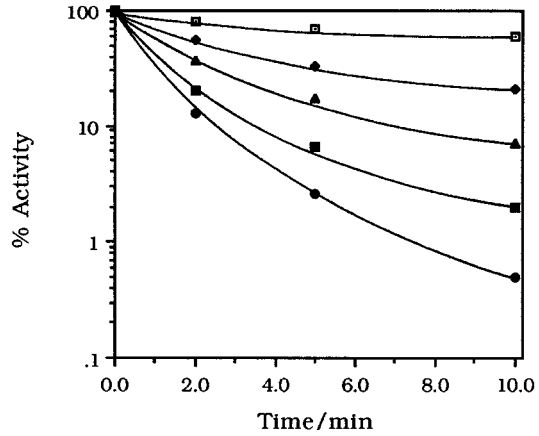

FIG. 3. **Effect of varying [NENMBA] on the time course of Na,K-ATPase activity.** The enzyme (0.05 mg/ml) was incubated with 20 μM (□), 40 μM (♦), 60 μM (▲), 80 μM (■), and 100 μM (●) NENMBA at 37 °C at pH 7.5 (40 mM Hepes), and aliquots were assayed at the time points indicated.

changes. As the pH is increased from 6.5 to 9.0, the rate of development of inhibition increases (Fig. 5). The effect of pH on the inhibitor:protein interaction is probably more complex than simple titration of the inhibitor, the pK_a of which is 8.3 (24). We believe that partitioning into the lipid phase occurs prior to inhibition (see below) and the partition coefficient is likely to increase with increasing pH.

Access Pathway to Inhibitory Site—The slow development of inhibition and its very slow (if any) reversibility, together with the greater effectiveness of the unprotonated reagent, raised the question as to whether NENMBA bound directly to the inhibitory site from the solution or first partitioned into the membrane phase. Experiments were designed to examine these possibilities. A sample of enzyme was incubated with NENMBA at 37 °C and when about 50% inhibition had been attained, half the enzyme was pelleted at 450,000 × g at 4 °C for 5 min and resuspended in inhibitor-free buffer at 37 °C, and the incubation was continued. As shown in Fig. 6, a slower rate of inhibition is seen in the resuspended enzyme than with the uninterrupted incubation.

High Affinity Nucleotide Binding—Although the greatest protection against inhibition was shown by cations and only little (if any) protection by ATP (see Fig. 2), it was important to see whether or not the ATP binding domain was greatly affected by the presence of the inhibitor. This experiment was

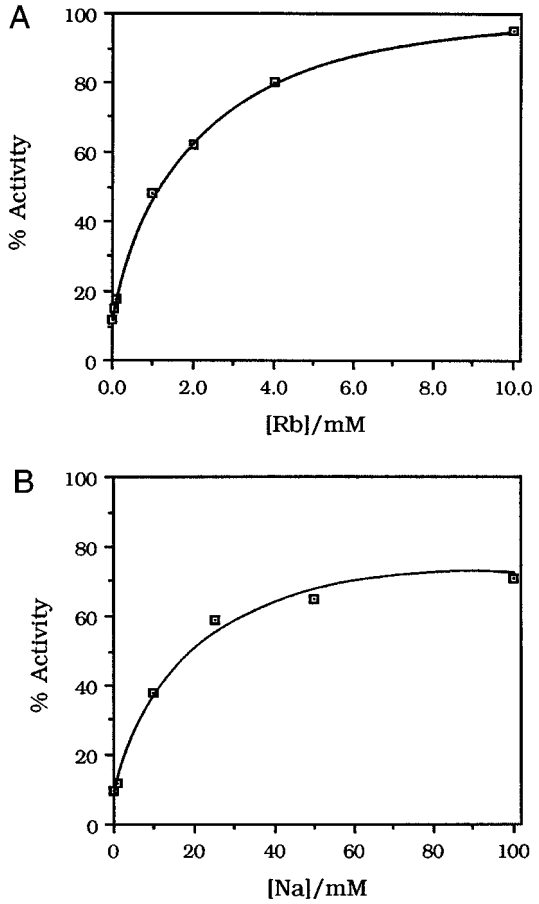

FIG. 4. **Protective effect of Rb (*A*) and Na (*B*) on the inhibition of purified Na,K-ATPase by NENMBA (Rb is used as a cogener for K in these experiments).** The enzyme (0.05 mg/ml) was incubated with NENMBA (100 μM with Rb, 40 μM with Na) at 37 °C at pH 7.5 (40 mM Hepes) for 2 min with the concentrations of cations indicated and activity was assayed as described under "Materials and Methods."

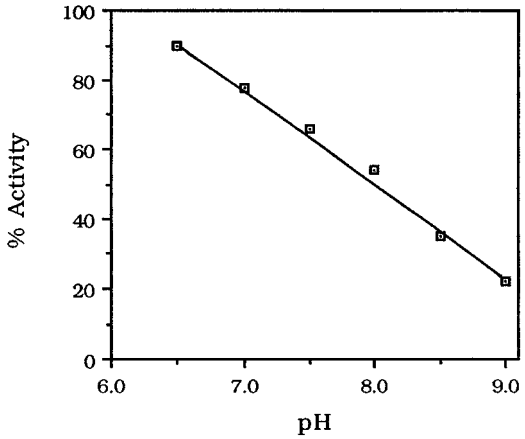

FIG. 5. **Effect of varying pH on the inhibition of purified Na,K-ATPase by NENMBA.** The enzyme (0.05 mg/ml) was incubated with NENMBA (100 μM) at 37 °C for 2 min, and activity was assayed as described under "Materials and Methods." The buffers used were: Mes (6.5), Hepes (7.0, 7.5), Taps (8.0, 8.5), and Ches (9.0).

possible because of the extremely slow reversibility of the inhibition due to NENMBA addition. It was found that both native and NENMBA-treated enzyme could bind [^{3}H]ADP to the same extent; about 2.5 nmol of ADP was specifically bound per milligram of enzyme. This level of binding is very similar to that previously observed for native enzyme (33) and DEAC-treated enzyme (22).

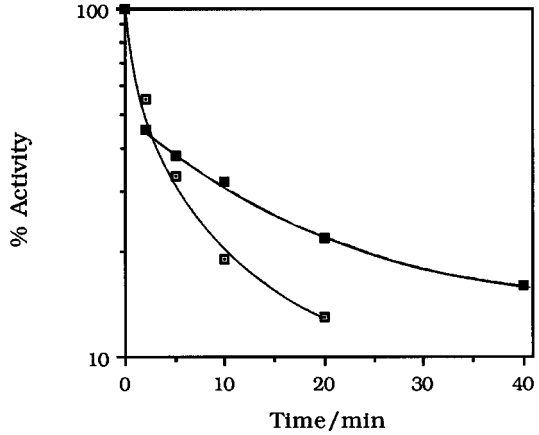

FIG. 6. **NENMBA access to inhibitory site.** The enzyme (0.05 mg/ml) was incubated with NENMBA (100 μM) at 37 °C for 2 min at pH 7.5 (40 mM Hepes), and then half the sample was pelleted rapidly (450,000 × *g*, 4 °C, 2 min) and resuspended in NENMBA-free buffer. Aliquots were taken at the time points indicated, and activity was assayed as described under "Materials and Methods."

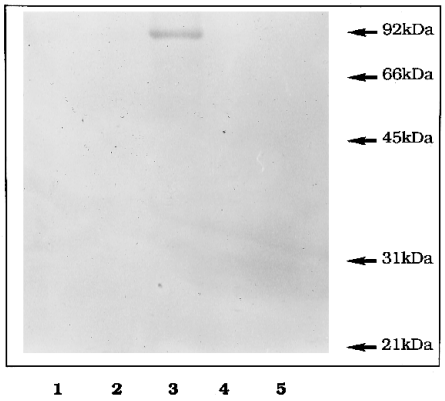

FIG. 7. **Photoincorporation of NENMBA into Na,K-ATPase α-subunit.** The enzyme (0.05 mg/ml) was incubated with NENMBA (100 μM) at 37 °C for 5 min ± Rb (20 mM) at pH 7.5 (40 mM Hepes) and irradiated with a high pressure Hg arc lamp for 2 min through a 313 nm narrow band-pass filter, separated using a Laemmli gel (10%), and transferred to nitrocellulose. Covalent incorporation of NENMBA was tested using a polyclonal amiloride antibody. No signal was observed in the Western analysis from NENMBA (*lane 1*), Na pump (*lane 2*), Na pump and NENMBA without irradiation (*lane 4*). Irradiation produces a stable chemical bond between NENMBA and Na pump α-subunit (*lane 3*); the presence of Rb (*lane 5*) prevents most of this incorporation.

Photoincorporation and Location of Labeling—Irradiation of fully inhibited enzyme at 313 ± 10 nm with the output from a high pressure Hg arc lamp (only exciting the nitroaromatic chromophore; Ref. 25) results in the covalent incorporation of the probe specifically into the α-subunit of the Na pump (see Fig. 7). Western analysis of Na pump, after separation by SDS-PAGE and transfer to nitrocellulose, using an amiloride polyclonal antibody, shows that irradiation is necessary for covalent incorporation (Fig. 7, *lanes 3* and *4*; there is no cross-reactivity of the antibody with the Na pump, *lane 2*). Irradiation of Na pump in the presence of NENMBA and Rb shows that there is very little, if any, nonspecific photoincorporation of the probe into the protein (*lane 5*). Staining of the nitrocellulose membrane after transfer but prior to blocking with milk with the reversible dye, Ponceau Red, showed that approximately the same amount of protein had been transferred in each lane of Fig. 7 (not shown).

DISCUSSION

In the present work we have shown that NENMBA, a pyrazine-substituted amiloride derivative, is a potent inhibitor of the Na,K-ATPase. The inhibition is prevented by the simultaneous presence of monovalent cations, which are transported by the Na pump, and the inhibitor is covalently coupled to the protein upon irradiation at 313 nm.

It has been known for some time that amiloride itself inhibits the Na pump, albeit with low affinity, and that pyrazine-ring substituted amiloride derivatives exhibit an enhanced affinity for the Na pump compared to the parent compound (34, 35). However, these studies were performed either on various cell lines or on Na pump preparations which had 1.5% the specific activity of the enzyme used in this report. The intent of the present studies was to exploit the binding of *photoactivatable* amiloride derivatives to the Na pump, so as to localize non-acidic amino acid residues in the cation transport domain of the enzyme. The fundamental requirement for the success of this strategy is that the cation photoaffinity probes bind with high affinity to Na pump prior to photolysis, in a cation-preventable manner, so that reasonably specific covalent incorporation of the probe into the protein can subsequently be achieved.

Structure-Activity Relationships—We examined the effects of four amiloride derivatives, all of which are potentially photoactivatable, on the ability of purified renal Na,K-ATPase to hydrolyze ATP. All four compounds inhibited ATPase activity (Fig. 2). However, we found that it was only the "exchanger-specific" probes, NENMBA and 6-bromo-5(*N*,*N*-cyclohexyl)-amiloride, which could be prevented from binding to and inhibiting the Na pump by the presence of monovalent cation ligands. It seems from this pharmacological profile that the Na pump interacts with amiloride derivatives in a manner which is more like other transport proteins such as the Na/Ca and Na/H exchangers than epithelial Na channels. The Na/Ca and Na/H exchangers are like the Na pump in that they undergo a series of conformational transitions during their transport cycle in which the cation binding sites are alternately exposed to each side of the membrane. However, there is no homology in the primary structures or evidence of similarity of cation binding site structure between these exchangers and the Na,K-ATPase. Tight binding of one amiloride derivative (phenamil) has also been previously observed on epithelial Na channels (36). David *et al.* (37) have recently surveyed 15 amiloride derivatives (including NMBA) for their inhibitory activity against the Na pump. They observed that pyrazine-substituted amilorides were effective competitive inhibitors of Rb occlusion, with concentrations in the tens of micromolar range, whereas guanidine-substituted amilorides only seemed to prevent Rb occlusion at very high concentrations. The rates of development or reversal of inhibition were not reported, as the assay used in their study was performed by incubating purified Na pump at room temperature for 3 min with ^{86}Rb in the absence and presence of varying concentrations of amiloride derivatives.

Ligand Prevention of NENMBA Inhibition—Since cation prevention of inhibition of the Na,K-ATPase was clearest in the case of NENMBA (Fig. 2), it was decided to examine these effects in more detail (Fig. 4). Both Na^+ and Rb^+ (as a cogener for K^+), protected the enzyme against inhibition of NENMBA. We have found that almost all (90%) the inhibition by NENMBA can be prevented when 10 mM K or Rb is included in the incubation medium (Fig. 4*A*), and that Rb protects the enzyme with a $K_{0.5}$ in the 1–2 mM range. Sodium does not prevent binding quite so effectively; nevertheless, about 60–70% of the enzyme activity can be preserved when about 60 mM Na is included in the incubation medium. The $K_{0.5}$ for this protective effect is about 10 mM (Fig. 4*B*). Protection against covalent inhibition by DEAC, a cation-site directed reagent, has also been reported to be more effective and complete with K than Na (22). However, these protective effects of ligand

cations can be overcome simply by prolonging the incubation period. Once the probe is bound to its inhibitory site, it exhibits an extremely slow dissociation rate from this complex, as extensive washing of the enzyme with ligands (Na,K-ATP) does not restore ATPase activity. Furthermore, native and NENMBA-inhibited Na pump exhibited the same level of high affinity nucleotide binding, suggesting that NENMBA does not perturb greatly the catalytic domain of the enzyme. This is encouraging for the use of NENMBA as a cation-site probe, since it is desirable that such a reagent should have effects which, as far as possible, are limited to the cation-binding domain.

The tight binding or "occlusion" of both Na^+ and K^+ have been well characterized kinetically and it is thought that these intermediates do in fact form part of the normal catalytic cycle of the Na pump (10, 38–40). An important characteristic of the cation occlusion phenomenon is the slow association and dissociation of the cations. It is tempting to speculate that since NENMBA does not seem to dissociate from the enzyme it also becomes occluded by the Na pump; however, since other substituted amines (41), including guanidines (42), do not seem to be occluded by the Na,K-ATPase, we deem it unlikely that NENMBA is exceptional in this regard.

Fig. 6 shows that when partially inhibited Na pump is pelleted and resuspended in NENMBA-free buffer, the observed rate of inhibition is reduced 2–3-fold. Several conclusions can be drawn from this observation. First, as observed previously and mentioned above, inhibition is not relieved by resuspension in inhibitor-free medium, *i.e.* there is a very slow off-rate from the inhibitory site. Second, it seems highly likely that inhibitor in the membrane phase is associated with the slow development of inhibition. NENMBA has been found to be highly hydrophobic, having a partition coefficient of 200:1 between chloroform and 0.1 M phosphate buffer at pH 7.4 (24). We estimate that the volume ratio of the pellet/resuspension medium is about 1:100. We would expect the partition equilibrium to be re-established very rapidly on resuspension, and thus the concentration of NENMBA in the membrane following resuspension would be about one-half of its value prior to resuspension, and the rate of development of inhibition would fall by about 2-fold, which is what is observed (Fig. 6). Therefore, the probe gains access to its inhibitory site *after* it has partitioned into the lipid environment. If NENMBA bound to the Na pump directly from the aqueous solution, pelleting and resuspension of the partially inhibited protein should effectively stop any further inhibition; such is not the case. NENMBA has a pK_a of about 8.3 (24); thus, as the pH is raised from 6.5 to 9.0 (Fig. 5), the reagent deprotonates and partitions more completely into the membrane. Entry to the inhibitory site seems to occur from the membrane phase, so that the rate of inhibition thus increases as the pH is raised.

Localization of Labeling—One of the potential disadvantages of photoaffinity labeling in general is the possibility of producing nonspecific photo-incorporation of a probe, as irradiation normally produces a hyper-reactive chemical species (nitrene or carbene), which can even insert into C-H bonds. NENMBA is an attractive chemical probe for monovalent cation binding residues in the Na,K-ATPase for at least two reasons. First, the tight binding and slow dissociation of NENMBA from the Na pump enabled fully inhibited enzyme to be pelleted, washed, and resuspended in NENMBA-free buffer and irradiated to produce cation-preventable, specific photolabeling of the Na pump α-subunit (Fig. 7, compare *lanes 3* and *5*). Radioactive NENMBA was not available; therefore, covalent incorporation was probed by Western analysis of labeled Na pump transferred onto nitrocellulose, and probed using the amiloride polyclonal antibody. Even though NENMBA binding can completely prevent ATP hydrolysis by the Na pump, this inhibition does not result in a positive signal in the Western blot (*lane 4*), *i.e.*. irradiation *is* necessary for covalent incorporation of the probe into the α-subunit.

Second, NENMBA promises to be a useful cation binding site photo-activated probe since the nitroanisole chromophore reacts by displacement of the methoxy group through a photoaromatic nucleophilic substitution reaction (43). These types of chromophores are stable upon irradiation in the *absence of nucleophiles* (44). This property makes them rather different from the more widely used aromatic azides, which produce reactive nitrenes upon illumination. If these nitrenes do not react with the target protein, they decompose to inert side products; for this reason, observed yields of covalent incorporation of such photoaffinity probes are often very low. In principle, the efficiency of covalent modification of the target enzyme can be much higher with the chromophore used in NENMBA, since it returns to ground state starting materials, if it does not encounter a nearby nucleophile, and so can be re-excited repeatedly during irradiation until a productive encounter occurs.

Two other types of cation binding site chemical probes have been used with the Na pump. The most extensively used are carbodiimides, which react with carboxyls (*i.e.* glutamates and aspartates) to produce an activated ester, which can react with a nucleophile to produce an amide; thus, intramolecular crosslinking is possible with these probes (16, 19). This disadvantage has been recently circumvented by use of a cationic substituted diazomethane (DEAC), which also reacts chemoselectively with carboxylates to form stable ester derivatives of amino acids directly (22, 23). These reagents have been used because it has been thought for some time that negatively charged amino acid residues might be involved in cation binding and transport. However, inspection of the structures of naturally occurring monovalent cation ionophores suggests that serines, threonines, and tyrosines could also be involved in cation co-ordination of Na and K by the Na pump. Furthermore, the first atomic-resolution crystal structure of a Na/K binding enzyme (the dialkylglycine decarboxylase) shows *no* Asp or Glu involved in Na/K binding at one of its two cation co-ordination centers (45).

In summary, we found that four photoactivatable amiloride analogues inhibit the Na,K-ATPase and monovalent cations protect the enzyme only when the photolabile substituent is on the pyrazine ring. Upon illumination, NENMBA is covalently incorporated into the α-subunit as shown by Western analysis using an amiloride specific antibody. Further studies are under way to localize the amino acid residue labeled by NENMBA.

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