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

(Received for publication, November 27, 1995, and in revised form, January 31, 1996)

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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 of the Na,K-ATPase, as observed 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-
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).

**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), diithreitol, sucrose, ultrapure urea, and Tricine were from Sigma; l-1-lysylamide-2-phenyl-ethylhydrazine methione-substituted trypsin was from Worthington Corporation. β-Mercaptoethanol, sodium dodecyl sul fate, ammonium persulfate, Coomassie R-250, and low molecular weight standards were from Bio-Rad. Acrylamide and bisacrylamide were obtained through Polysciences.

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 Western analysis using a polyclonal antibody. A preliminary report of some of these results has appeared (26).

**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 (NEMBA and bromobenzonil) 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-N,N-cyclohexylamiloride) produced 70–90% inhibition of Na,K-ATPase and cation inhibition of 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: K0.5 for Rb (which is recognized as a cogen for K by the Na pump) is about 1 mM (see Fig. 4A), and for Na is about 7 mM (Fig. 4B), 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
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 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).
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 sometime 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 nonacidic 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 NMBAA) 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 86Rb 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 cogen for K⁰), 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. 4A), and that Rb protects the enzyme with a K₀.₅ 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 K₀.₅ for this protective effect is about 10 mM (Fig. 4B). 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 2001 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 pKa 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. The Enzymes of Biological Membranes (Martonosi, A. N., ed) pp. 35–114, Plenum Publishing Corp., New York

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.

REFERENCES

1. Shull, G. E., Lane, L. K., Schwartz, A., and Lingrel, J. B. (1985) Nature 316, 691–695
2. Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Noma, K., Hagiwara, T., Inayama, S., Hayashida, H., Miyata, T., and Numa, S. (1985) Nature 316, 733–736
3. Ovchinnikov, Y. A., Modyanov, N. N., Broude, N. E., Petrukhin, K. E., Grishin, A. V., Arzamasova, M. N., Aldanov, N. A., Monastyrskaya, G. S., and Sverdlov, E. D. (1986) FEBS Lett. 201, 237–245
4. Kawakami, K., Ohta, T., Noma, K., and Nagaoka, K. (1986) J. Biochem. (Tokyo) 100, 389–397
5. Takeyasu, K., Tanikum, M. M., Renaud, K. J., and Fambrough, D. M. (1989) J. Biol. Chem. 263, 4347–4354
6. Baxter-Lowe, L. A., Gou, J. Z., Bergstrom, E. E., and Hokin, L. E. (1989) FEBS Lett. 257, 181–187
7. Mercer, R. W., Schneider, J. W., Savitz, A., Emanuel, J., Benz, E. J., and Levenson, R. (1986) Mol. Cell. Biol. 6, 3884–3890
8. Verrey, F., Fairs, P., Schaefer, E., Fuentes, P., Gering, K., Rossier, B. C., Thrasher, A., and Krabrhunbl. J.-P. (1989) Am. J. Physiol. 256, F1034–F1042
9. Lebowitz, R. M., Takeyasu, K., and Fambrough, D. M. (1989) EMBO J. 8, 193–202
10. Glynn, I. L. (1985) In The Enzymes of Biological Membranes (Martonosi, A. N., ed) pp. 35–114, Plenum Publishing Corp., New York
11. Kaplan, J. H. (1985) Annu. Rev. Physiol. 47, 535–544
12. Kaplan, J. H. (1989) in Red Blood Cell Membranes: Structure Function and Clinical Implications (Agre, P., and Parker, J. C., eds) pp. 455–480, Marcel Dekker, Inc., New York
13. Pedemonte, C. H., and Kaplan, J. H. (1990) Am. J. Physiol. 258, C1-C23
14. Schoner, W., and Schmidt, H. (1969) FEBS Lett. 5, 285–287
15. Robinson, J. D. (1974) FEBS Lett. 38, 325–328
16. Gora, F. R. (1985) Biochemistry 24, 6783–6788
17. Yamaguchi, M., Sakamoto, J., and Tonomura, Y. (1983) Curr. Top. Membr. Trans. 19, 203–271
18. Pedemonte, C. H., and Kaplan, J. H. (1988) J. Biol. Chem. 261, 3632–3669
19. Pedemonte, C. H., and Kaplan, J. H. (1988) J. Biol. Chem. 261, 16660–16665
20. Arner, E., Svedboda, P., Tegsinge, J., and Zborowski, J. (1988) Biochem. Biophys. Acta 955, 367–370
21. Shani-Seker, M., Goldshleger, R., Tal, D. M., and Karlish, S. J. D. (1988) J. Biol. Chem. 263, 19331–19341
22. Arquello, J. M., and Kaplan, J. H. (1990) J. Biol. Chem. 266, 14627–14635
23. Arquello, J. M., and Kaplan, J. H. (1994) J. Biol. Chem. 269, 6892–6899
24. Kleyman, T. R., and Cragoe, E. J. Jr. (1988) J. Mem. Biol. 105, 1–21
25. Kleyman, T. R., Cragoe, E. J. Jr., and Kraehenbuhl, J. P. (1989) J. Biol. Chem. 264, 11995–12000
26. Ellis-Davies, G. C. R., Kleyman, T. R., and Kaplan, J. H. (1993) Biophys. J. 64, 332a
27. Jørgensen, P. L. (1974) Biochim. Biophys. Acta 356, 36–52
28. Liang, S. M., and Winter, C. G. (1976) Biochim. Biophys. Acta 452, 552–565
29. Brotherus, J. B., Møller, J. V., and Jørgensen, P. L. (1981) Biochem. Biophys. Res. Commun. 100, 146–154
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Kleyman, T. R., Kraehenbuhl, J. P., Rossier, B. C., Cragoe, E. J., and Warnock, D. G. (1989) Am. J. Physiol. 257, 1135–1141
33. Ellis-Davies, G. C. R., and Kaplan, J. H. (1990) J. Biol. Chem. 265, 20570–20576
34. Soffer, S. P., and Mandel, L. J. (1983) Science 220, 957–959
35. Zhuang, Y.-x., Cragoe, E. J., Shaikewitz, T., Glaser, L., and Cassel, D. (1984) Biochemistry 23, 4481–4488
36. Garvin, J. L., Simon, S. A., Cragoe, E. J., and Mandel, L. J. (1985) J. Membr. Biol. 87, 45–54
37. David, P., Mayan, H., Cragoe, E. J., and Karlish, S. J. D. (1993) Biochim. Biophys. Acta 1146, 59–64
38. Glynn, I. M., and Richards, D. E. (1982) J. Physiol. 330, 17–43
39. Glynn, I. M., Howland, L. J., and Richards, D. E. (1982) J. Physiol. 368, 453–569
40. Forbush, B., III (1987) J. Biol. Chem. 262, 11104–11115
41. Forbush, B., III (1988) J. Biol. Chem. 263, 7979–7988
42. Or, E., David, P., Shainskaya, A., Tal, D. M., and Karlish, S. J. D. (1993) J. Biol. Chem. 268, 16929–16937
43. Jelenc, P., Cantor, C. R., and Simon, S. R. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3564–3568
44. Kronenberg, M. E., van den Heyden, A., and Hovinga, E. (1966) Red. Trav. Chim. Pay-Bas 85, 56–58
45. Toney, M. D., Hohenester, E., Cowan, S. W., and Jansonius, J. N. (1993) Science 261, 756–758
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J. Biol. Chem. 1996, 271:10353-10358.
doi: 10.1074/jbc.271.17.10353

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