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Inactivation of the Rabbit Parotid Na/K/Cl Cotransporter by N-Ethylmaleimide

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Summary. The inactivation of the rabbit parotid Na/K/Cl cotransporter by the irreversible sulfhydryl reagent N-ethylmaleimide (NEM) is studied by monitoring its effect on high affinity bumetanide binding to the carrier. NEM reduces the number of bumetanide binding sites with no significant change in the affinity of those remaining. NEM also reduces KCl-dependent ^{22}Na flux via the cotransporter by the same factor as the reduction in bumetanide binding sites. Both bumetanide and its analogue furosemide can protect against the effect of NEM. The concentration range over which this protection occurs is in good agreement with affinities of these two compounds for the high affinity bumetanide binding site (2.6 and 85 μM, respectively), indicating an association of this site with the site of action of NEM. Also consistent with this hypothesis are the observations that (i) sodium and potassium, both of which are required for high affinity bumetanide binding, increase the rate of inactivation of binding by NEM and (ii) chloride, at concentrations previously shown to competitively inhibit bumetanide binding, protects the cotransporter against NEM. The effects of NEM on bumetanide binding are mimicked by another highly specific sulfhydryl reagent, methyl methanethiolsulfonate. The apparent rate constant for inactivation of high affinity bumetanide binding by NEM is a hyperbolic function of NEM concentration consistent with a model in which the inactivation reaction is first order in [NEM] and proceeds through an intermediate adsorptive complex. The data indicate that the presence of a reduced sulfhydryl group at or closely related to the bumetanide binding site is essential for the operation of the parotid Na/K/Cl cotransporter.

Key Words loop diuretics · exocrine gland · fluid secretion · parotid · acinar cell · ion transport · chloride secretion

Introduction

Na/K/Cl cotransporters are found in a wide variety of cell types [7, 20, 21]. In many secretory and adsorptive epithelia this transporter plays a major role in driving transepithelial chloride fluxes related to salt and water movements. In nonepithelial cells Na/K/Cl cotransport is frequently involved in volume regulatory ion fluxes. At present it is not clear whether the same transport protein is expressed in all tissues; however, a number of properties, including a high sensitivity to inhibition by loop diuretics and a coupling stoichiometry of $1Na^+ : 1K^+ : 2Cl^-$, are common to all of these systems, indicating that they are at least very similar.

Owing to their importance and ubiquity, considerable recent experimental effort has been devoted to the identification and characterization of Na/K/Cl cotransporters [3, 4, 6, 7–12, 20, 21, 26, 27, and references therein]. Studies from a number of laboratories, including our own, indicate that such a transporter is responsible for driving the bulk of the acinar chloride secretion which drives salivary fluid production [16–19, 22, 27, 28]. We have provided direct evidence for the existence of such a transporter in parotid acinar basolateral membrane vesicles [27]. In addition, we have identified a high affinity ($K_d \simeq 3$ μM) binding site for the loop diuretic bumetanide in this preparation and provided strong evidence that this site is identical with the bumetanide inhibitory site on the cotransporter [26]. Bumetanide binding to this site requires the presence of sodium, potassium and chloride ions. High affinity binding shows a hyperbolic dependence on both sodium and potassium concentration and biphasic dependence on chloride concentration, with binding increasing from 0 to 5 mM chloride and decreasing thereafter (at [Na] = [K] = 100 mM). This latter inhibitory effect of chloride is due to a competitive interaction with bumetanide, consistent with earlier indications that bumetanide inhibits Na/K/Cl cotransport at a chloride site [8].

An essential step toward our eventual understanding of the chemical and physical events associated with any transport process is the identification of the important functional and structural groups on the transporter. In the present paper we study the

effects of the irreversible sulfhydryl reagent NEM[1] on the rabbit parotid Na/K/Cl cotransporter. Our data provide strong evidence for the existence of an essential sulfhydryl group at or closely related to the bumetanide binding site on this protein.

Materials and Methods

Vesicle Preparation

Basolateral membrane vesicles (BLMV) were prepared from rabbit parotid by a Percoll gradient method as previously described [27]. Relative to the starting tissue homogenate the activity of the basolateral membrane marker K-stimulated *p*-nitrophenyl phosphatase is enriched 9–12 times in this membrane vesicle preparation [27]. Freshly prepared vesicles were suspended in Buffer A (10 mM Tris/HEPES plus 100 mM mannitol) containing 100 mM KCl and 1 mM EDTA at a protein concentration of approximately 5 mg/ml. Aliquots (0.75 mg protein) of BLMV were fast frozen and stored above liquid nitrogen.

Binding and Uptake Measurements

On the day of the experiment aliquots of frozen vesicles were thawed for 30 min at room temperature, diluted 100 times with Buffer A containing 1 mM EDTA, and centrifuged at 48,000 × *g* for 20 min. The resulting pellets were taken up in the same medium and subsequently diluted into appropriate media for the experiments.

Equilibrium bumetanide binding was measured as previously described [26]. Briefly, a 20-μl aliquot of vesicles (1–2 mg protein/ml) was combined with a 20 μl aliquot of incubation medium consisting of Buffer A plus [^{3}H]-bumetanide and other constituents as required (*see below*). After 60 min of incubation the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (Buffer A containing 100 mM NaCl plus 100 mM KCl) and the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter, which retained the BLMV, was then washed with a further 6.0 ml of stop solution, placed in a scintillation vial with 10 ml of ACS (Amersham, Arlington Heights, IL) containing 0.1 ml glacial acetic acid and counted for radioactivity along with samples of the incubation medium and appropriate standards.

In some cases the loss of BLMV bumetanide binding activity was monitored in the presence of NEM. In these experiments vesicles (0.5–1 mg protein/ml) were preincubated for 60 min in an appropriate buffer (*see below*) containing [^{3}H]-bumetanide. The experiment was then begun by the addition of a small volume of NEM stock solution (<5% of the volume of the vesicle mixture). The NEM stock solution was prepared in a medium identical to that of the vesicles (including [^{3}H]-bumetanide). At appropriate times 40–50 μl aliquots of vesicles were removed, added to 1.5 ml of ice-cold stop solution, and filtered and washed as described above.

Unless otherwise stated, bumetanide binding was always determined in Buffer NKC, consisting of Buffer A plus 100 mM sodium gluconate, 95 mM potassium gluconate, 5 mM KCl, 0.5 mM EDTA and appropriate concentrations of [^{3}H]-bumetanide (typically a buffer containing twice these salt concentrations was added to BLMV suspended in Buffer A plus 1 mM EDTA). In previous experiments we have established that these concentrations of sodium, potassium and chloride yield near optimal conditions for high affinity bumetanide binding in this preparation [26]. All bumetanide binding data were corrected for nonspecific effects by subtracting the sodium-independent component of [^{3}H]-bumetanide binding measured by replacing sodium with NMDG in Buffer NKC (*see* Results). Nonspecific binding measured in this way was 6.8 ± 2.2 pmol/mg protein at 1 μM [^{3}H]-bumetanide (n = 27).

^{22}Na uptake measurements were carried out in essentially the same manner as [^{3}H]-bumetanide binding (*see* ref. 27 for details). Uptakes were measured after 15 sec of incubation and represent initial uptake rates [27].

All flux and binding studies were done at 23°C. Equilibrium binding and uptake experiments were carried out in triplicate or quadruplicate. Unless otherwise noted, the errors shown in the Figures (when large enough to illustrate) are standard deviations. When single experiments are shown, these are representative of three or more independent studies yielding similar results.

Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine gamma globulin as the standard.

Materials

[^{3}H]-bumetanide (66.2 Ci/mmol, radiochemical purity 98%) was custom synthesized for us by Amersham (Arlington Heights, IL) from the precursor 3-amino-4-phenoxy-5-sulfamoylbenzoic acid, generously supplied by Dr. P. W. Feit (Leo Pharmaceuticals, Ballerup, Denmark). In preliminary experiments for this study we used [^{3}H]-bumetanide kindly given to us by Drs. B. Forbush, III, and R.W. Mercer, also prepared from starting material supplied by Dr. Feit.

^{22}Na was from New England Nuclear (Boston, MA), NMDG was from Aldrich (Milwaukee, WI), NEM was from Sigma Chemical (St. Louis, MO) and unlabeled bumetanide was a gift from Hoffman-LaRoche (Nutley, NJ). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

Results

Inactivation of the Na/K/Cl Cotransporter by NEM

Figure 1 illustrates the time course of the effect of the irreversible sulfhydryl alkylating reagent NEM on bumetanide binding to rabbit parotid BLMV. In this experiment NEM at a final concentration of 1 mM was added at time zero to BLMV, which had been preincubated with 0.15 μM [^{3}H]-bumetanide.

[1] Abbreviations: 10 mM Tris/HEPES: 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffered with Tris to pH 7.4; EDTA: ethylenediaminetetraacetic acid; NMDG: N-methyl-D-glucamine; BLMV: basolateral membrane vesicles; NEM: N-ethylmaleimide; MMTS: methyl methanethiolsulfonate.

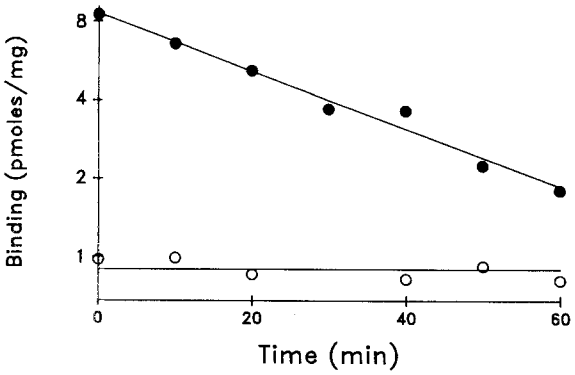

Fig. 1. Inactivation of sodium-dependent bumetanide binding by NEM. BLMV were preincubated for 1 hr in Buffer NKC containing 0.15 μM [^{3}H]-bumetanide (●), or in the same medium with sodium replaced by NMDG (○). At time zero NEM was added to give a final concentration of 1 mM and bumetanide binding was assayed at the times indicated as described in Materials and Methods. The upper plot shows the effect of NEM on the sodium-dependent component of bumetanide binding (i.e., binding measured in the presence of sodium minus that measured with NMDG). The line drawn through these points is a least squares fit yielding $T_{1/2}$ = 27.2 ± 1.8 min. No significant effect of NEM is observed on the sodium-independent component of bumetanide binding (○)

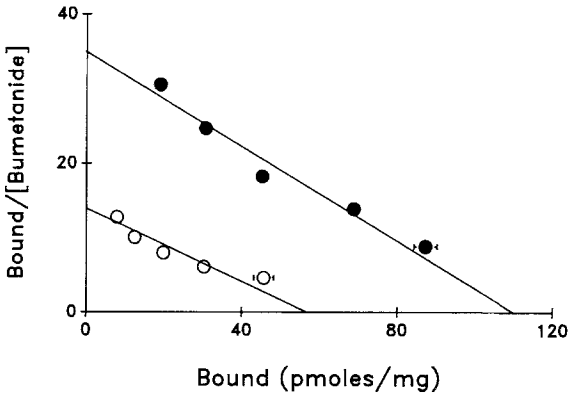

Fig. 2. The nature of the effect of NEM on sodium-dependent bumetanide binding. BLMV were incubated for 15 min at 23°C in Buffer NKC with (○) or without (●) 1 mM NEM. Vesicles were then diluted 35 times in ice-cold Buffer A containing 1 mM EDTA, centrifuged for 20 min at 48,000 × g, resuspended and washed again in the same buffer, and taken up in Buffer A plus 1 mM EDTA for the binding experiment. Equilibrium [^{3}H]-bumetanide binding was measured over the concentration range 0.6–10 μM as described in Materials and Methods. The sodium-dependent component of binding is illustrated. The lines drawn through the data points are least squares fits given by K_d = 3.1 ± 0.4 μM, N_0 = 110 ± 8.1 pmol/mg protein, r = 0.980 (●) and K_d = 4.1 ± 0.9 μM, N_0 = 57 ± 8 pmol/mg protein, r = 0.940 (○)

Samples of membranes were subsequently removed at the times indicated for bumetanide binding determinations (*see* Materials and Methods). A semilogarithmic plot of the sodium-dependent component of bumetanide binding (filled circles) is linear, indicating an exponential loss of binding activity with time ($T_{1/2}$ = 30.1 ± 3.6 min, n = 6). Such behavior is characteristic of a single inactivation process. Figure 1 also shows that the sodium-independent component of bumetanide binding (open circles) is not affected by NEM. Thus NEM specifically inactivates the high affinity sodium-dependent component of bumetanide binding which we have previously associated with the bumetanide inhibitory site on the Na/K/Cl cotransporter [26].

The nature of the effect of NEM on sodium-dependent bumetanide binding is investigated in more detail in the experiment shown in Fig. 2. Here vesicles were incubated in the presence or absence of 1 mM NEM for 15 min then washed and assayed for sodium-dependent bumetanide binding activity over the concentration range 0.6 to 10 μM. The data are presented as a Scatchard plot. The result of NEM treatment is to reduce the number of sodium-dependent bumetanide binding sites (N_0) without significantly affecting the K_d of those remaining (*see* Fig. 2 caption). The averaged results of three independent experiments identical to the one shown in Fig. 2 gave K_d = 2.6 ± 0.5 μM for untreated vesicles and K_d = 3.3 ± 0.9 μM for NEM-treated vesicles, not a statistically significant difference. However, the number of sodium-dependent bumetanide binding sites in untreated vesicles was 2.1 ± 0.3 times that found in NEM-treated membranes, indicating a significant reduction in N_0 by NEM. In vesicles subjected to prolonged treatment with NEM (1 mM for 2 hr), no significant sodium-dependent bumetanide binding could be detected (*data not shown*).

In additional experiments, it has also been confirmed that KCl-dependent sodium transport (via the Na/K/Cl cotransporter, ref. 27) in NEM-treated BLMV is reduced by the same factor as the number of sodium-dependent bumetanide binding sites. For example, in the experiment shown in Fig. 2, the initial rate of KCl-dependent uptake of 1 mM ^{22}Na was 1.61 ± 0.12 pmol/mg · min^{-1} in untreated vesicles and 0.65 ± 0.05 pmol/mg · min^{-1} in NEM-treated vesicles (i.e., a 60 ± 4% loss of flux with NEM *vs.* a 48 ± 9% loss of binding sites). Thus the loss of bumetanide binding activity resulting from NEM treatment represents complete inactivation of the Na/K/Cl cotransporter.

It is interesting to note that the half time for inactivation of sodium-dependent bumetanide binding is approximately 30 min for experiments of the type shown in Fig. 1 (*viz.*, binding measured in the presence of NEM) but approximately 15 min for experiments of the type shown in Fig. 2 (binding

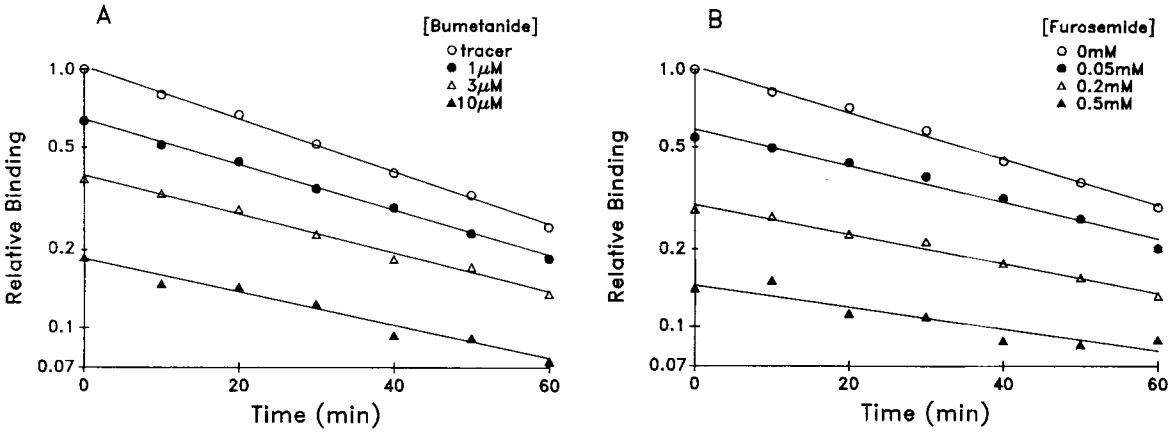

Fig. 3 Effects of bumetanide and furosemide on the inactivation of sodium-dependent bumetanide binding by NEM. BLMV were preincubated for 1 hr in Buffer NKC containing "tracer" [^{3}H]-bumetanide (≤0.12 μM) and the concentrations of unlabeled bumetanide (*A*) or furosemide (*B*) indicated. At time zero NEM was added to give a final concentration of 1 mM and [^{3}H]-bumetanide binding was assayed at the times indicated. The sodium-dependent component of binding is illustrated. The results of three identical independent experiments have been combined by normalizing the results of each experiment to the binding observed at time zero in the absence of added unlabeled bumetanide or furosemide and averaging the resulting normalized results. The line drawn through the data points are least squares fits yielding $T_{1/2}$ values of 29.7 ± 0.7 (○), 34.1 ± 0.8 (●), 39.9 ± 1.8 (△) and 46.7 ± 3.6 min (▲) for *A* and 33.5 ± 1.1 (○), 42.3 ± 3.1 (●), 52.6 ± 2.9 (△) and 70.8 ± 13.1 min (▲) for *B*. All $T_{1/2}$'s in each panel are statistically significantly different from one another

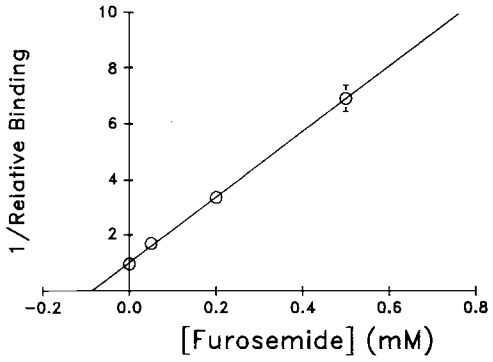

Fig. 4. Effect of furosemide on sodium-dependent bumetanide binding. Relative binding values at time zero were calculated from the least squares fits to the data presented in Fig. 3*B*. The line drawn through the data points is a least squares fit yielding 0.085 ± 0.004 mM for the K_I of furosemide inhibition of sodium-dependent bumetanide binding

measured after NEM removal). The reason for this difference is provided by the results given below.

Effects of Substrates and Inhibitors

The experiments shown in Fig. 3*A* and *B* demonstrate that both bumetanide and its analogue, furosemide, can decrease the rate of inactivation of sodium-dependent bumetanide binding by NEM (*see* Figure caption). The concentration range over which bumetanide is effective is in good agreement with the K_d of the sodium-dependent bumetanide binding site (Fig. 2). Figure 3*B* illustrates that, in addition to its protective effect against NEM, furosemide also inhibits sodium-dependent bumetanide binding. This latter effect is not surprising since bumetanide and furosemide are thought to inhibit Na/K/Cl cotransport by acting at a common site [20]. The K_I for furosemide inhibition of bumetanide binding can be determined from Fig. 3*B* by the analysis shown in Fig. 4. This value (0.085 mM) is also in good agreement with the concentration range over which furosemide protects the bumetanide binding site against NEM. Thus these results indicate that the occupation of the sodium-dependent bumetanide binding site by bumetanide or furosemide can prevent the inactivation of this site by NEM.

The apparent discrepancy mentioned above, between the $T_{1/2}$ values calculated from Figs. 1 and 2, can be accounted for by a combination of the protective effect of bumetanide against NEM and the slow dissociation rate of bumetanide from the high affinity site (the half time for dissociation is 11.8 ± 3.4 min, n = 4, measured in Buffer NKC after 100-fold dilution into bumetanide-free medium; R.J. Turner and J.N. George, *unpublished results*). Thus when the effect of NEM is monitored in the presence of bumetanide, the observed $T_{1/2}$ for inactivation is increased even at low bumetanide concentrations owing to the fact that the sites being monitored (i.e., those occupied by [^{3}H]-bumetanide) are protected against NEM and are only

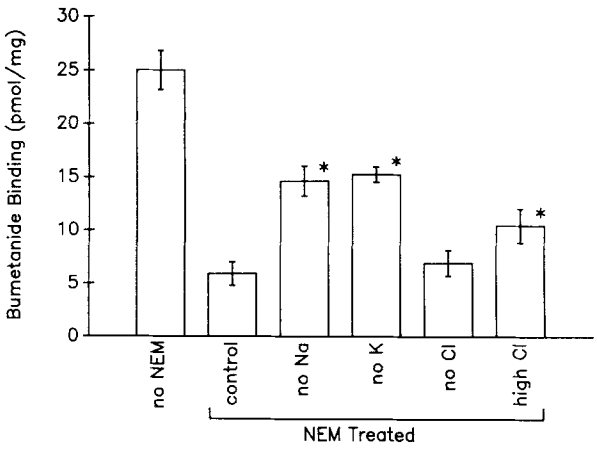

Fig. 5. Effects of substrates of the Na/K/Cl cotransporter on inactivation of sodium-dependent bumetanide binding by NEM. BLMV were suspended in Buffer NKC ("control"; Buffer NKC contains 100 mM sodium, 100 mM potassium, 5 mM chloride and 195 mM gluconate plus other additions noted in Materials and Methods) or in the same buffer with sodium replaced by NMDG (*no Na*), potassium replaced by NMDG (*no K*), chloride replaced by gluconate (*no Cl*), or gluconate replaced by chloride (*high Cl*). These vesicles were incubated with 1 mM NEM for 20 min at 23°C then washed as described in the caption of Fig. 2. An aliquot of BLMV in Buffer NKC was also subjected to the washing procedure without NEM treatment (*no NEM*). Sodium-dependent bumetanide binding (1 μM) was measured on the final pellets as described in Materials and Methods. The results shown are averages ± SD of five independent experiments. Bumetanide binding values that are significantly different from that of "control" vesicles treated with NEM in Buffer NKC are marked with an asterisk

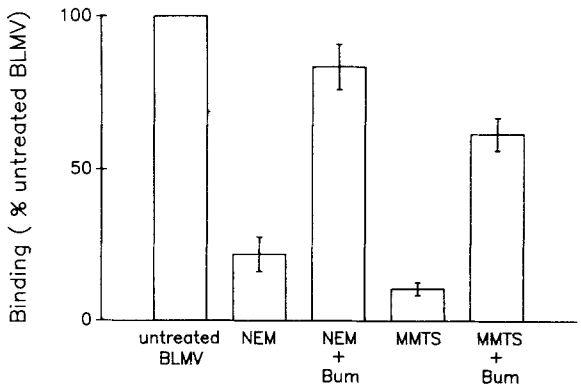

Fig. 6 Inactivation of sodium-dependent bumetanide binding by NEM and MMTS. BLMV were preincubated for 1 hr at 23°C in Buffer NKC with or without 10 μM unlabeled bumetanide, then 1 mM NEM or 0.1 mM MMTS were added. Twenty minutes later the vesicles were diluted 35 times in ice-cold Buffer A containing 1 mM EDTA, centrifuged for 20 min at 48,000 × *g* and resuspended in 5 ml of the same buffer. This material was left for 1 hr at room temperature to allow for complete dissociation of bound bumetanide, then diluted with 8 ml of ice-cold buffer and centrifuged as before. The final pellet was taken up in Buffer A plus 1 mM EDTA for the determination of sodium-dependent bumetanide binding (1 μM; *see* Materials and Methods). *Untreated BLMV* were subjected to the washing procedure but not to NEM or MMTS. Bumetanide binding to vesicles treated with 10 μM unlabeled bumetanide without NEM or MMTS was identical to that measured with *untreated BLMV* (*data not shown*). The results shown are the averages ± SD of three independent experiments for each reagent. The results of each experiment have been normalized to the binding measured with *untreated BLMV*

slowly exposed. A theoretical analysis of this process incorporating both dissociation and reassociation of labeled ligand and inactivation of unoccupied sites (*not shown*) indicates that the observed $T_{1/2}$ under these conditions should be approximately equal to the sum of the $T_{1/2}$'s for dissociation and inactivation, in agreement with our observations.

In the experiments illustrated in Fig. 5 we have examined the effects of the presence of sodium, potassium and chloride, the substrates of the cotransporter, on the inactivation of sodium-dependent bumetanide binding by NEM. In these studies vesicles were treated with NEM in various salt solutions *in the absence of bumetanide* and then washed free of NEM before bumetanide binding determinations. These results show clearly that the inactivation of sodium-dependent bumetanide binding by NEM is significantly reduced by the removal of either sodium and potassium, as well as by the presence of high concentrations of chloride (200 mM). No significant effect of the removal of 5 mM chloride on the inactivation is detectable under these experimental conditions. Since high affinity bumetanide binding requires the presence of both sodium and potassium and is inhibited by high chloride concentrations [26], these results indicate a strong correlation between the reactivities of bumetanide and NEM for their sites on the Na/K/Cl cotransporter.

Mechanism of Action of NEM

NEM is known to react covalently with the sulfhydryl groups of proteins with high specificity, converting them to N-ethyl succinimides [1]. Figure 6 shows the results of an experiment where the effects of NEM on bumetanide binding are compared to those of MMTS, another highly specific sulfhydryl reagent [23]. MMTS also inactivates sodium-dependent bumetanide binding and its effect, like that of NEM, is markedly blunted when bumetanide is present during its application (Fig. 6). In this experiment bumetanide was employed at a concentration of 10 μM at which approximately 80% of the high affinity sodium-dependent sites are occupied (*cf.* Fig. 2). Assuming that bumetanide provides total protection against NEM, it can be shown that

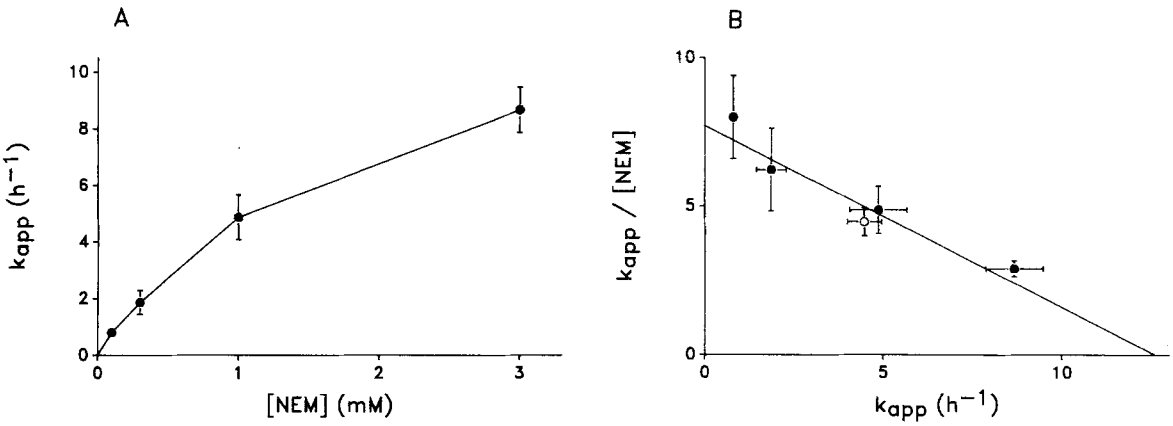

Fig. 7. Effect of NEM concentration on the rate of inactivation of sodium-dependent bumetanide binding. (*A*) BLMV suspended in Buffer NKC were incubated with 0, 0.1, 0.3, 1.0 or 3.0 mM NEM for 20 min at 23°C then washed as described in the caption of Fig. 2. Sodium-dependent bumetanide binding (1 μM) was measured on the final pellets and from these values the apparent rate constant (k_{app}) for inactivation of bumetanide binding was calculated ($k_{app} = \ln 2/T_{1/2}$). The results shown are averages ± SE from three independent experiments. (*B*) The data in *A* are replotted as described in the text (●). The open circle (○) represents the k_{app} calculated from the "control" run of Fig. 5. The line drawn through the data points a least squares fit of the data to the equation discussed in the text. The least squares parameters are $k_2 = 7.7 \pm 0.6$ hr^{-1} and $K_i = 1.64 \pm 0.32$ mM (*see* text)

the presence of this concentration of bumetanide should decrease the rate of inactivation of the transporter by NEM by a factor of five [25]. The results shown in Fig. 6 are in agreement with this prediction.

The effect of NEM concentration on the inactivation of sodium-dependent bumetanide binding is illustrated in Fig. 7. Here BLMV were incubated with various concentrations of NEM in the absence of bumetanide for 20 min, then washed before binding determinations. The apparent first-order rate constant, k_{app}, at each NEM concentration was then calculated from the bumetanide binding data. As shown in Fig. 7*A* a plot of k_{app} *vs.* [NEM] is nonlinear. This type of kinetic behavior in protein modification reactions is commonly taken to indicate that the reaction proceeds through the intermediacy of an adsorptive complex [2, 13, 15], i.e., that the reaction is described by the equation

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E^* \qquad (1)$$

where E is the native enzyme (the bumetanide-binding protein), I is the site-specific reagent (NEM), EI is the adsorptive complex and E^* is the modified (inactivated) enzyme. Under steady-state conditions this reaction scheme predicts an exponential loss of enzyme activity with time [15] with

$$k_{app} = k_2\,[I]/(K_i + [I]) \qquad (2)$$

where

$$K_i = (k_2 + k_{-1})/k_1\,. \qquad (3)$$

Thus k_{app} is expected to be a hyperbolic function of $[I]$ and a plot of $k_{app}/[I]$ *vs.* k_{app} should be linear. Figure 7*B* illustrates that the dependence of k_{app} on [NEM] does indeed conform to the predictions of this model.

Discussion

The results presented here demonstrate that the high affinity sodium-dependent bumetanide binding site we have previously identified as the bumetanide inhibitory site on the rabbit parotid acinar basolateral membrane Na/K/Cl cotransporter [26] is irreversibly inactivated by the covalent sulfhydryl reagent NEM. NEM also blocks KCl-dependent ^{22}Na transport in the same preparation, indicating a complete inactivation of the Na/K/Cl cotransporter. Both bumetanide and its analogue furosemide can protect against the effect of NEM (Figs. 3 and 6). The concentration range over which this protection occurs is in good agreement with affinities of these two compounds for the high affinity bumetanide-binding site (Figs. 2 and 4), indicating that this protective effect is directly due to the occupation of this site. Also consistent with this hypothesis is the observation that chloride, at concentrations previ-

ously shown to competitively inhibit bumetanide binding [26], likewise protects the cotransporter against NEM (Fig. 5).

It is particularly interesting that the absence of either sodium or potassium results in both the loss of high affinity bumetanide binding [26] and a substantial reduction of the rate of inactivation of bumetanide binding by NEM (Fig. 5). Thus the conformational or other change(s) induced by the binding of these cations to the cotransporter apparently simultaneously alters the availability of the bumetanide-binding site and the reactivity of the group attacked by NEM. The effect of NEM is to reduce the number of bumetanide-binding sites with no significant effect on binding affinity (Fig. 2). Taken together, the above results indicate that the site of action of NEM is at or closely related to the bumetanide binding site on the Na/K/Cl cotransporter.

NEM is known to be highly specific for sulhydryl groups under our experimental conditions (*viz.*, physiological pH and NEM concentrations ≤ 1 mM, ref. 1); however, reactions with amino and imidazole groups have occasionally been observed [1, 24]. We demonstrate here that a second highly specific sulfhydryl reagent, MMTS [23], inactivates sodium-dependent bumetanide binding to the parotid Na/K/Cl cotransporter in a manner which closely parallels the effects of NEM (Fig. 6). This result, together with the specificity of both NEM and MMTS for sulfhydryls and their otherwise marked structural and chemical differences, provides strong evidence that the site of action of both these compounds is the same essential sulfhydryl group on the cotransporter.

The apparent rate constant for inactivation of sodium-dependent bumetanide binding by NEM does not increase linearly with NEM concentration as one would expect for a simple first-order reaction (Fig. 7*A*). However, these data are consistent with a model in which the inactivation reaction is first order in [NEM] but proceeds through an intermediate adsorptive complex (Fig. 7*B*). Other explanations for this apparent saturation of reaction rate with increasing [NEM] are, of course, also conceivable. An interesting possibility is that the group attacked by NEM is alternately made accessible or inaccessible to the reagent as a result of stochastic conformational changes, perhaps those related to the transmembrane cycling of cotransporter. Thus at high NEM concentrations the inactivation rate would be limited by the rate at which this group was presented for attack. At the present time we cannot distinguish between these, and possibly other, kinetic mechanisms.

Several comments about the possible role of the sulfhydryl group attacked by NEM in the operation of the cotransporter are also appropriate here. It seems reasonable to conclude that the presence of this group in a reduced state is essential to both high affinity bumetanide binding and Na/K/Cl cotransport, since even the addition of the relatively small methanethio group (CH_3S-) by MMTS results in inactivation. Konings, Robillard and collaborators [5, 14] have suggested that the transport cycle of a number of bacterial cotransport systems involves a series of dithio-disulfide interchanges. Thus the reaction of these sulfhydryls with exogenous reagents (including transport inhibitors) would result in the inhibition of transport. Further experiments are required to establish whether the sulfhydryl group identified here is involved in dithio-disulfide exchange. In additional experiments not presented here we have been unable to reverse the inactivation of bumetanide binding by MMTS using the potent disulfide-reducing reagents dithiothreitol and β-mercaptoethanol. This result may indicate that the cotransporter undergoes a conformational change after reaction with MMTS which results in the occlusion of the MMTS reactive site and/or irreversible inactivation. Thus the sulfhydryl group studied here may also play a role in stabilizing the configuration of the active transporter.

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