J. Membrane Biol. 71, 39–45 (1983)

The Journal of
Membrane Biology

Sulfhydryl Reagents Affect Na^+ Uptake into Toad Bladder Membrane Vesicles

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Summary. The effect of sulfhydryl reagents on the Na^+ permeability mechanisms of toad urinary bladder vesicles was examined. The reagents 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), iodosobenzoate, and ethylenimine were able to decrease amiloride-inhibited sodium uptake into vesicles when used at low concentrations. When used at higher concentrations these reagents were able to induce large increases in vesicle Na^+ permeability that were not sensitive to amiloride. The reagent *p*-chloro-mercuribenzene sulfonate was able to induce such leaks even at low concentrations. The reagent N-ethylmaleimide was incapable of substantially affecting vesicle Na^+ transport in any way. All of the effects observed could be reversed by removing the reagents from the solution surrounding the vesicles. Our results help explain the varied actions of sulfhydryl reagents on intact epithelial tissue.

Key Words sulfhydryl · sodium · amiloride · toad · bladder · vesicle

Introduction

Many investigators have demonstrated that amiloride-sensitive sodium transport across the apical membrane of intact epithelial tissue is sensitive to agents that can react with protein sulfhydryl groups. These reagents include such ions as Cd^{++} (Stymans, Van Driessche & Borghgraef, 1973; Fleisher, Yorio & Bentley, 1975; Hillyard & Gonick, 1976), Cu^{++} (Ferreira, 1970) and Ag^+ (Li & de Sousa, 1977) and also the organo-mercurial reagent *p*-chloromercuriphenylsulfonic acid (PCMBS) (Frenkel, Ekblad & Edelman, 1975; Spooner & Edelman, 1976; Harms & Fanestil, 1977; Benos, Mandel & Simon, 1980). All of these reagents were shown to significantly increase amiloride-sensitive Na^+ entry into the epithelial cells of either isolated toad urinary bladder or frog skin, which resulted in a stimulation of the short-circuit current across the epithelial tissue (Ferreira, 1970; Stymans et al., 1973; Fleisher et al., 1975; Frenkel et al., 1975; Hillyard & Gonick, 1976; Spooner & Edelman, 1976; Harms & Fanestil, 1977; Li & de Sousa, 1977; Benos et al., 1980). However, when such tissue was exposed to other highly specific sulfhydryl reagents such as PCMB or 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), the amiloride-sensitive Na^+ flux was either inhibited or totally unaffected (Frenkel et al., 1975; Benos et al., 1980). The effect of other sulfhydryl reagents such as N-ethyl-maleimide (NEM), iodosobenzoate, ethylenimine, or tetrathionate on Na^+ transport in epithelial systems has not been examined extensively. In related work, other investigators have determined that the sulfhydryl reagents PCMBS and NEM can inhibit the Na^+-dependent portion of epithelial amino acid or glucose transport (Schaeffer, Preston & Curran, 1973; Will & Hopfer, 1979). Taken together, these results leave a somewhat confusing picture of the effects of sulfhydryl reagents on Na^+ transport in epithelial tissue. Several possibilities may explain the varied effects of the different reagents. First, since in most cases the reagents can react much more slowly or to a lesser degree with other reactive groups, the possibility exists that the observed effects of the presumptive sulfhydryl reagents on epithelial ion transport may have resulted from the interaction of such reagents with nonsulfhydryl groups. Also, since the studies above were conducted on intact tissues, the possibility of complex intracellular effects could not be ruled out. The problem of intracellular effects is complicated by the fact that some of the reagents are membrane permeable while other charged reagents are generally confined to the surface of the cell membrane to which they are originally applied.

We felt that the only way to understand the action of sulfhydryl reagents was to examine their effects in a system somewhat simpler than the whole tissue preparations to which they had been previously applied.

0022-2631/83/0071-0039 $01.40

LaBelle and Valentine have recently reported a preparation of membrane vesicles from toad urinary bladder that is capable of amiloride-inhibited Na^+ uptake (LaBelle & Valentine, 1980). The current studies were conducted to determine what direct effects sulfhydryl reagents could exert on such a simple subcellular system in which the complications associated with intact tissues could be avoided. Additionally, any covalent modifications of the amiloride-sensitive Na^+ transport system might also prove useful in determining some of the characteristic structural features of the proteins involved in transport. Specific irreversible interactions might also prove useful during the isolation of these proteins.

Materials and Methods

Materials

Tropical toads (*Bufo marinus*) of Mexican origin were obtained from Nasco, Fort Atkinson, Wisc. Amiloride hydrochloride (N-amidino-3,5-diamino-6-chloropyrazine carboxamide) was the generous gift of Dr. Clement A. Stone of Merck, Sharp & Dohme Research Laboratories, West Point, Pa. Ouabain, Dowex 50X8–100 (H^+ form), DTNB[1], NEM, iodosobenzoate, DTT, HEPS, PCMB, and PCMBS were obtained from Sigma Chemical Co., St. Louis, Mo. ^{22}Na was obtained from Amersham, Arlington Heights, Ill., and Nucleopore filters were obtained from Nucleopore, Inc., Pleasanton, Calif. Ethylenimine was obtained from Pierce Chemical Company, Rockford, Ill.

Methods

Microsomes were made from urinary bladders of the tropical toad *B. marinus*, by the procedure of LaBelle and Valentine (1980), and sodium transport into these microsomes was measured as previously described (LaBelle & Valentine, 1980). Aliquots of the microsomes (30–80 μg) were incubated for 2 min at 22 °C with ^{22}Na phosphate (1 μCi, 1.6 mM), sucrose (0.25 M), ouabain (0.1 mM), Na^+ HEPS buffer (3.3 mM, pH 8.0), and either amiloride HCl (0.6 mM) or NaCl (0.6 mM) substituted for Na^+ phosphate, in a total volume of 0.25 ml. The incubation mixture was applied to a Dowex 50X8 (Tris) column and eluted with 1 ml sucrose (0.25 M) as described previously (LaBelle & Valentine, 1980). Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as standard protein.

Aliquots of toad bladder microsomes (0.6 mg protein) could be separated from specific reagents by filtration on Nucleopore filters (0.05 μm pore size). These microsomes were filtered under vacuum, washed with 0.5 ml sucrose (0.25 M) – HEPS (10 mM, pH 8.0) buffer solution, and then the filters were immersed in 0.5 ml buffer for 20 min, with vortex mixing, in order to recover the microsomes from the filters.

The compound TNB was produced from DTNB by treatment with 10-fold excess DTT, and the concentration of TNB determined spectrophotometrically at 412 nm ($\varepsilon = 1.36 \times 10^4$) Ellman, 1959).

Results

Effect of the Reagents on Influx

In the absence of sulfhydryl reagent, amiloride (10^{-3} M) blocked $45.0 \pm 9.1\%$ of the Na^+ uptake into the vesicles. The component of Na^+ uptake which was blocked by amiloride was denoted the amiloride-sensitive Na^+ uptake. The amiloride-sensitive sodium uptake into toad bladder microsomes was shown to be affected by a number of sulfhydryl reagents.

In preliminary studies, both amiloride-sensitive and amiloride-insensitive sodium uptake processes were significantly inhibited by DTNB, NEM, PCMBS, and iodosobenzoate. Ethylenimine inhibited the amiloride-sensitive sodium uptake but not the amiloride-insensitive uptake, and the other four reagents also inhibited the amiloride-sensitive process more than the amiloride-insensitive process. While DTNB, PCMBS, and ethylenimine could inhibit amiloride-sensitive Na^+ influx by 50% or more, NEM was unable to inhibit by more than 20%. Iodosobenzoate inhibited Na^+ uptake to a greater extent than NEM but not as much as did the other reagents.

Effects of the Reagents on Efflux

Control experiments were performed to assess the ability of the sulfhydryl reagents to produce large leaks in the vesicles. Such leaks could permit a substantial portion of the intravesicular $^{22}Na^+$ to leave the vesicles during the time they were exposed to the ion exchange column. Such leaks would complicate the interpretation of influx experiments. Therefore, we examined the ability of the sulfhydryl reagents to stimulate Na^+ efflux from preloaded vesicles.

The protocol for the experiment consisted of exposing the membrane vesicles to external ^{22}Na for a period long enough to allow equilibration of the intravesicular volume with the external ^{22}Na (LaBelle & Valentine, 1980). Aliquots of various sulfhydryl reagents were then added at 20 °C to several of the vesicle-containing solutions. After a brief reaction period, all of the vesicle-containing solutions were applied to Dowex columns as described previously. The period of time the vesicles

[1] *Abbreviations:* DTNB – 5,5′-dithiobis-(2-nitrobenzoic acid); DTT – dithiothreitol; NEM – N-ethyl-maleimide; PCMBS – *p*-chloro-mercuriphenylsulfonic acid; TNB – 5-thio-2-nitro-benzoate; HEPS – 4-(2-hydroxy-ethyl)-1-piperazine-propanesulfonic acid; PCMB – *p*-chloro-mercuribenzoate.

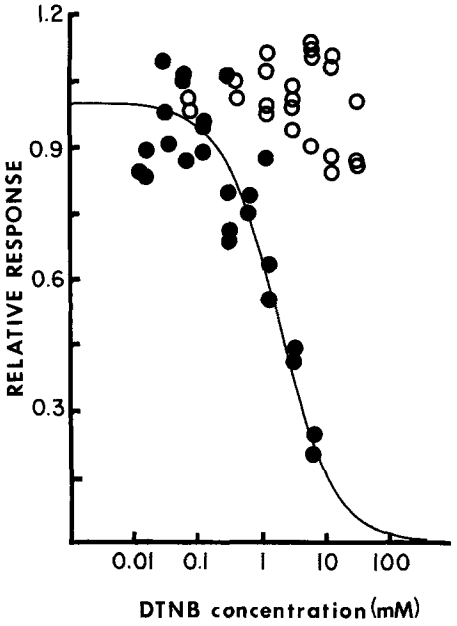

Fig. 1. Effect of DTNB on the Na^+ permeability of toad bladder microsomes. Toad bladder microsomes were incubated with increasing concentrations of DTNB and amiloride-inhibited Na^+ uptake into the microsomes was measured as described in the legend of Table 1. The amount of amiloride-inhibited Na^+ uptake measured relative to uptake in the absence of DTNB is indicated by solid symbols. Toad bladder microsomes (60 μg) were preloaded with ^{22}Na HEPS (0.5 μCi, 9 mM, pH 8.2) in the presence of sucrose (0.25 M) for 30 min at 20 °C in a total volume of 0.25 ml. Increasing amounts of DTNB (dissolved in 6 μl dimethylsulfoxide) were added to the microsomes, the incubations continued for 12 min, and then the microsomes were applied to Dowex columns and eluted as described in Methods. The amount of ^{22}Na remaining in the microsomes relative to the amount remaining in the absence of DTNB is indicated by open symbols.

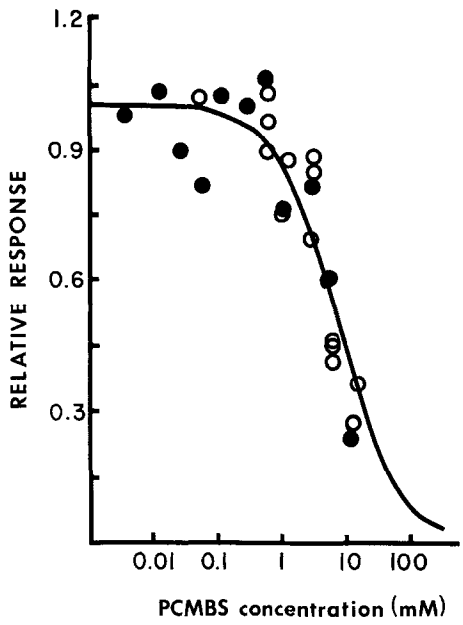

Fig. 2. Effect of PCMBS on Na^+ permeability of toad bladder microsomes. Toad bladder microsomes were incubated with increasing concentrations of PCMBS and amiloride-inhibited Na^+ uptake into the microsomes was measured as described in the legend of Table 1. The amount of amiloride-inhibited Na^+ uptake measured relative to uptake in the absence of PCMBS is indicated by solid symbols. Toad bladder microsomes were preloaded with ^{22}Na, incubated with increasing amounts of PCMBS, and applied to Dowex columns as described in the legend of Fig. 1. The amount of $^{22}Na^+$ remaining in the microsomes relative to the amount remaining in the absence of PCMBS is indicated by open symbols.

were exposed to the ion exchanger was less than 2 min. Under these conditions the untreated vesicles contained virtually all of the Na^+ with which they were previously loaded, while vesicles treated with all reagents except NEM and iodosobenzoate lost a substantial portion of the preloaded Na^+. Preloaded vesicles treated with DTNB (4 mM) for 30 min lost from 60–95% of their Na^+, vesicles treated with PCMBS (6 mM) for 12 min lost 57% of their Na^+, and vesicles treated with ethylenimine (12 mM) for 12 min lost 30% of their Na^+. Very little, if any, Na^+ was lost from iodosobenzoate-treated vesicles, while NEM produced a small, but statistically significant loss of intravesicular Na^+. We wished to know if this pathway for Na^+ efflux induced by DTNB was sensitive to amiloride. In three experiments, amiloride was incapable of blocking the loss of vesicular Na^+ associated with DTNB treatment.

Separation of Influx Effect from Efflux Effects

In order to determine whether sulfhydryl reagents were capable of inhibiting Na^+ influx into vesicles under conditions wherein nonspecific leaks were not generated, we measured the ability of the reagents to affect Na^+ transport after relatively short incubations (12 min) at low concentrations. The earlier influx experiments had employed longer incubations (30 min) at higher concentrations. In later experiments, the effects of increasing concentrations of DTNB, PCMBS, iodosobenzoate, and ethylenimine on amiloride-sensitive Na^+ influx into vesicles were determined after 12 min exposure (Figs. 1 and 2; Table 1). A nonlinear, least-squares fitting algorithm (Brown & Dennis, 1972) was used to fit the experimental data in Figs. 1 and 2 to expressions for simple single-site binding. The correlation coefficients for curves shown in Figs. 1 and 2 together with correlation coefficients for curves obtained using iodosobenzoate and ethylenimine (data not shown) are given in Table 1.

The concentrations of DTNB, iodosobenzoate and ethylenimine required to inhibit amiloride sensitive Na^+ influx into vesicles by 50% in 12 min ranged from 1.8 to 4.5 mM (Table 1). When vesicles preloaded with $^{22}Na^+$ were exposed for 12 min at 20 °C to concentrations of DTNB, iodosobenzoate or ethylenimine which had previously been shown to inhibit influx by 50%, no significant nonspecific leaks were observed. Only at higher concentrations (6–12 mM) did DTNB and ethylenimine cause some slight leakage of preloaded $^{22}Na^+$ from the vesicles. Iodosobenzoate's effects at higher concentrations could not be observed due to the insolubility of this reagent at such concentrations. Both DTNB and iodosobenzoate inhibited amiloride-insensitive Na^+-influx into vesicles at concentrations that did not produce leaks, but this effect was always less than half of the effect of these reagents on amiloride-sensitive Na^+ influx. Ethylenimine did not inhibit amiloride-insensitive Na^+ influx into the vesicles. PCMBS induced a substantial nonspecific leak in the vesicles after 12 min exposure at 20 °C, and the concentration of PCMBS required to permit the leakage of 50% of the preloaded Na^+ out of the vesicles was nearly the same as the concentration required to inhibit Na^+ "influx" by 50%. NEM failed to either inhibit Na^+ influx into the vesicles or to induce nonspecific leaks in the vesicles even after 12 min exposure to relatively high concentrations (12 mM) of the reagent. PCMB also failed to affect either Na^+ influx into vesicles or to induce nonspecific leaks in the vesicles. Due to the poor solubility of this reagent in aqueous media, no experiments could be performed with more than 0.3 mM PCMB (although the exposure time was increased to 30 min).

Table 1. Inhibition of sodium uptake into toad bladder microsomes by sulfhydryl reagents[a]

Reagent	Reagent concentration required to block amiloride-sensitive uptake by 50% (mM)	Correlation coefficient
DTNB	1.82	0.868
Iodosobenzoate	2.45	0.715
PCMBS	7.6	0.868
Ethylenimine	4.5	0.845

Toad bladder microsomes (120 μg protein) were incubated with sucrose (0.25 M), sodium HEPS (6 mM, pH 8.2) and increasing concentration of the reagents below for 12 min at 20 °C in a total volume of 0.1 ml. Sodium uptake into the vesicles was determined as described in Methods both in the presence and the absence of amiloride (0.6 mM).

Reversibility of Reagent Effects

Two of the attractive characteristics of DTNB for the modification of sulfhydryl groups are that the reaction of a sulfhydryl group with DTNB produces a cleavable disulfide bond in the protein reaction product, and the reaction also produces a strongly colored product in solution which can be quantitated spectrophotometrically (5-thio-2-nitro-benzoate anion, TNB) (Ellman, 1959). We hoped to reverse the effects of DTNB by cleaving the disulfide bond which is formed when DTNB reacts with sulfhydryl groups. DTT will react with this disulfide linkage, thus regenerating the original sulfhydryl. Unfortunately, DTT was totally incapable of reversing the inhibitory effects of DTNB. Indeed, DTT seemed to actually increase the effects of DTNB on the microsomes without itself exerting any inhibitory effects. To clarify this issue, we examined the DTNB reaction more carefully.

Toad bladder microsomes are incapable of passing through Nucleopore filters (0.05–0.2 μm pore size) and can be recovered completely from these filters without any loss of activity (Table 2). The recovery of the microsomes from the filters was also shown to be quantitative by the Lowry protein assay (data not shown). Microsomes were treated with DTNB for 2 hr at 4 °C and washed free of unreacted DTNB by filtration. The length of the incubation was set at 2 hr to ensure that

Table 2. Reversibility of DTNB inhibition of toad bladder microsomes[a]

Reagent		Sodium uptake (nmol/min mg ± SD, $n=2$)		
		Uptake in the absence of amiloride	Uptake in the presence of amiloride	Amiloride sensitive uptake
Before filtration	None	5.78 ± 0.61	3.47 ± 0.34	2.31
	DTNB (3.3 mM)	3.17 ± 0.71	2.09 ± 0.01	1.08
After filtration	None	6.01 ± 0.76	3.66 ± 0.08	2.35
	DTNB (3.3 mM)	6.30 ± 0.23	3.86 ± 0.34	2.44

Toad bladder microsomes (0.6 mg) were treated either with or without DTNB for 2 hr at 4 °C as described in the legend of Table 1. Aliquots of the microsomes were collected on Nucleopore filters as described in Methods, washed, and resuspended in control buffer (sucrose 0.25 M), histidine (6 mM), pH 7.0), and then sodium uptake into the washed microsomes and into aliquots of unfiltered microsomes was measured in the presence and absence of amiloride (0.6 mM) as described in Methods.

all of the available – SH groups on the vesicles had the opportunity to react with DTNB, and the temperature was set at 4 °C to ensure that no vesicle deterioration occurred during the relatively long incubation period. Previous experiments had shown that the ability of DTNB to react with vesicle – SH groups was not greatly diminished by the use of lower temperatures (data not shown). Microsomes treated with DTNB and washed free of the reagent were shown to recover all of their initial activity (Table 2). These data seemed to contradict the results of the DTT experiments. The reaction of DTNB with the tissue and with DTT could be followed by measuring the production of TNB spectrophotometrically. After the microsomes had been exposed to 4 mM DTNB for 20 min, a small but significant amount of TNB was present in the incubation mixture (15–25 μM). The addition of excess DTT to the DTNB resulted in the quantitative elimination of free DTNB and the production of TNB equivalent to the original molar concentration of DTNB. However, the reaction product, TNB, was itself shown to be capable of inhibiting amiloride-sensitive sodium transport into the microsomes directly. The inhibitory effect of 2.85 mM TNB on the microsomes was greater than the effect of the same concentration of DTNB, which explained why DTT could transform all of the DTNB in the incubation mixture to TNB without restoring Na^+ transport activity. DTNB, however, does produce a significant effect itself since the small (15–25 μM) amounts of TNB produced by the reaction of DTNB with the microsomes in the absence of DTT were incapable of inhibiting sodium transport significantly (data not shown). Besides DTNB, other sulfhydryl reagents also were reversible. When aliquots of toad bladder microsomes were pretreated with either PCMBS, iodosobenzoate, or ethylenimine at concentrations sufficient to maximally affect Na^+ uptake, and then the reagent was removed from the vesicles by Nucleopore filtration, the Na^+ transport activity of the vesicles was restored to normal (Table 3). The small effect of NEM was, however, not reversible.

The dichotomy in their apparent modes of action made us question whether NEM and DTNB were reacting at the same sites. If the two reagents were exerting their effects by interaction at the same reaction site, then the irreversible agent NEM, might block the effect of DTNB on the amiloride-sensitive uptake. When the microsomes were pretreated with NEM before the addition of DTNB to the incubation mixture, the NEM was totally incapable of preventing DTNB from influencing either Na^+ influx or efflux (data not shown).

Table 3. Reversibility of PCMBS, iodosobenzoate, and ethylenimine effects on toad bladder microsomes[a]

Reagent	Sodium uptake (nmol/min mg ± SD, $n=2$)		
	Uptake in the absence of amiloride	Uptake in the presence of amiloride (0.6 mM)	Amiloride-sensitive uptake
None	5.77 ± 0.45	2.72 ± 0.03	3.05
PCMBS (6 mM)	5.57 ± 0.44	2.57 ± 0.39	3.00
Iodosobenzoate (3 mM)	6.02 ± 0.23	2.71 ± 0.13	3.31
Ethylenimine (12 mM)	6.94 ± 0.81	3.10 ± 0.20	3.84

Toad bladder microsomes (0.7 mg) were treated with the reagents below for 12 min at 20 °C as described in the legend of Table 1. The microsomes were then collected on Nucleopore filters, washed, resuspended in control buffer, and assayed for $^{22}Na^+$ transport activity as described in the legend of Table 2.

Discussion

All of the sulfhydryl reagents with the exception of NEM appeared to substantially modify amiloride-sensitive Na^+ uptake into toad urinary bladder vesicles. This study was complicated by the observed ability of some of these reagents (DTNB, PCMBS, and ethylenimine) to induce large amiloride-insensitive leaks in the vesicles. Since all of the effects of these reagents on the vesicles were reversible, the loss of Na^+ from preloaded vesicles could not simply represent irreversible vesicle deterioration. When both the duration of vesicle exposure to sulfhydryl reagent and the reagent concentration were decreased, the ability of each reagent to produce amiloride-insensitive leaks was eliminated. Yet under conditions wherein such leaks were no longer observed, all of the reagents that had previously shown activity (except PCMBS) were capable of inhibiting Na^+ uptake into vesicles through the amiloride-sensitive channel.

The reasons why NEM fails to affect the amiloride-sensitive channel remains unclear, as does the ability of PCMBS to insert a large leak into the vesicles at a concentration much lower than the concentration at which the other reagents exert such effects. It seems unlikely that DTNB, iodosobenzoate, and ethylenimine can reach –SH groups that are inaccessible to NEM, but this possibility cannot be ruled out. The preponderance of the evidence suggests that NEM does not react with the –SH group that is modified by the other reagents,

for reasons as yet unclear. This conclusion was supported by the demonstration that NEM pretreatment of vesicles could not block the effects of DTNB. Godin and Schrier (1972) have reported that either Mg^{2+} ATPase or Na^+, K^+ATPase activities observed in erythrocyte membranes are about two orders of magnitude more sensitive to DTNB or PCMBS than they are to NEM. The ability of PCMBS to produce a nonspecific leak at very low concentrations compared to the other reagents may simply reflect the exceptionally high affinity of organomercurial reagents for −SH groups (Means & Feeney, 1971).

Ordinarily, if DTNB reacts with a sulfhydryl group, the reaction product should be stable; however, certain protein structures could lead to reversibility. The mechanism of DTNB reversibility would depend upon the availability of one cysteinyl residue, with which DTNB reacts rapidly and a second cysteinyl residue or cystine disulfide with which it reacts slowly or not at all. Under these conditions disulfide interchange could take place with the regeneration of the original protein and the release of thionitrobenzoate anion into solution (Janatova, Fuller & Hunter, 1968; Means & Feeney, 1971). Sutherland, Rothstein and Weed (1967) have shown that the effects of PCMBS on erythrocyte cation permeability could be reversed by a slow exchange process between membrane–SH groups and – SH groups attached to soluble compounds released from the cells.

Iodosobenzoate oxidizes adjacent–SH groups to disulfide linkages, and so the ability of iodosobenzoate to inhibit amiloride-sensitive Na^+ uptake provides more substantive support for the proximity of–SH groups necessary for reversal of DTNB.

PCMBS appears to have been the most thoroughly examined reagent in intact epithelial tissue. The common picture of the action of PCMBS on various epithelial tissues is an initial reversible increase in short-circuit current (I_{sc}) associated with increased apical entry of Na^+. Subsequent to the initial increase in I_{sc} there is usually a slow irreversible decrease in I_{sc} to below control levels (Dick & Lindemann, 1975; Frenkel et al., 1975; Spooner & Edelman, 1976; Harms & Fanestil, 1977; Benos et al., 1980). DTNB has not been examined extensively, but appears to produce very little effect (Frenkel et al., 1975) or an effect similar to PCMBS, but of smaller magnitude (Benos et al., 1980). NEM appears to depress I_{sc} in intact toad bladder (Frenkel et al., 1975).

Many investigators have determined that DTNB and PCMBS are capable of increasing the cation permeability of the red blood cell (Sutherland et al., 1967; Knauf & Rothstein, 1971; Godin & Schrier, 1972). In both red cells and epithelial tissues, the effect of NEM, DTNB and PCMBS is generally interpreted as being due only to the reaction of the reagents with sulfhydryl residues, despite the fact that several investigators have observed in epithelial tissue a rate of reversibility of PCMBS or DTNB action (Benos et al., 1980), that is much more rapid than might be expected based on the rate of reversibility of PCMBS action on the sulfhydryl groups of red cells (Sutherland et al., 1967).

The striking ability of PCMBS to increase the cation permeability of the toad bladder vesicles (Fig. 2) appears consistent with the work of other investigators as does the less dramatic increase in cation permeability exerted by DTNB and the nearly nonexistent increase in permeability exerted by NEM (Sutherland et al., 1967; Knauf & Rothstein, 1971; Godin & Schrier, 1972; Dick & Lindemann, 1975; Frenkel et al., 1975; Spooner & Edelman, 1976; Harms & Fanestil, 1977; Benos et al., 1980).

The ability of DTNB, iodosobenzoate, and ethylenimine to inhibit amiloride-sensitive Na^+ transport into toad bladder vesicles may involve the same phenomenon responsible for the decrease in I_{sc} observed by Benos et al. (1980). The ability of PCMBS to induce a large leak in the vesicles may have decreased our ability to detect an inhibitory effect of PCMBS on Na^+ influx similar to the effect of PCMBS responsible for I_{sc} inhibition (Benos et al., 1980).

Conclusion

Numerous reports have described the effects of sulfhydryl reagents on amiloride-sensitive Na^+ transport in intact epithelial tissue (Ferreira, 1970; Stymans et al., 1973; Fleisher et al., 1975; Frenkel et al., 1975; Hillyard & Gonick, 1976; Spooner & Edelman, 1976; Harms & Fanestil, 1977; Li & de Sousa, 1977; Benos et al., 1980). In general, the effects of these reagents have been varied and the results difficult to interpret in a manner consistent with the known actions of the reagents on simple proteins. We have tried to take an intermediate approach in which we have attempted to alter exposed proteins in membrane vesicles prepared from toad urinary bladder. Our results suggest that substantial care must be exercised in interpreting the effects of group-specific reagents on intact tissues since they may have multiple effects on the preparation being studied. Nonetheless, our results also suggest that alterations of exposed sulfhydryl

residues lead to the loss of activity of the protein component responsible for amiloride-sensitive Na^+ uptake. The possibility of specifically modifying the amiloride-sensitive protein may, in the future, be useful in its isolation.

We would like to acknowledge the expert technical assistance of Ms. Sun O. Lee and Ms. Karen E. Johnson. This work is supported by Grants from the Department of Health and Human Services, AM-25244 to Edward F. LaBelle and AM-20068 and Research Career Development Anward AM-00432 to Douglas C. Eaton.

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Received 20 April 1982; revised 20 July 1982