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Effects of Chemical Group Specific Reagents on Sodium Entry and the Amiloride Binding Site in Frog Skin: Evidence for Separate Sites

D.J. Benos, L.J. Mandel, and S.A. Simon

Department of Physiology and Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

Summary. Previously we have shown that the inhibition of active transport by amiloride is noncompetitive with sodium in *Rana catesbeiana* skin, suggesting that amiloride acts at a site separate from the sodium entry site (Benos, D.J., Mandel, L.J., Balaban, R.S. 1979, *J. Gen Physiol.* **73**:307). In the present study, the effects of a number of sulfhydryl, amino, and carboxyl group selective reagents were studied on short-circuit current (I_{sc}) as well as the efficacy of amiloride in bullfrog skin, to determine those functional ligands which may be involved with either of these processes.

Addition of the sulfhydryl reagent PCMBS (1 mM) to the outside bathing medium produced biphasic effects, initially reversibly increasing I_{sc} by an average 56% followed by a slower, irreversible decay to levels below baseline. In contrast, the addition of 0.1 mM PCMB always resulted in a rapid, irreversible decrease in I_{sc}. When a 40,000 mol wt dextran molecule was attached to PCMB, a stable, reversible increase in I_{sc} was observed. These observations suggest that at least two populations of $-$SH groups are involved in Na translocation through the entry step. Amiloride was equally effective in inhibiting I_{sc} before and after treatment with PCMBS both during the stimulatory as well as the inhibitory phase. The sulfhydryl reducing agent DTT, and oxidizing agent DTNB had only minor influence on I_{sc} and did not alter the effectiveness of amiloride.

Similarly, the amino reagents, SITS and TNBS did not affect I_{sc}. However, TNBS decreased the ability of amiloride to inhibit Na entry. These results suggest that an amino group may be involved in the interaction of amiloride and its site, without affecting Na entry.

The carboxyl reagents EEDQ, TMO, and three separate carbodiimides were without effect on I_{sc} or amiloride inhibition. Methylene blue (MB), a molecule that interacts with both carboxyl and hydroxyl-specific groups, inhibited I_{sc} by 20% and decreased amiloride's ability to inhibit I_{sc}. These effects, however, are likely to occur from the cytoplasmic side as MB appears to enter into the cells.

These results support the notion that amiloride and Na interact with the entry protein at different regions on the membrane.

Sodium entry into the frog skin epithelium is a facilitated process presumably mediated by specific proteins located within the outer, or apical, membrane (Cereijido & Rotunno, 1968; Biber & Curran, 1970; Biber, 1971; Mandel & Curran, 1973; Benos et al., 1976). Utilizing kinetic techniques, we have previously found that the inhibition of active transport by amiloride is noncompetitive with external sodium in *R. catesbeiana* skins, suggesting that amiloride acts at a site separate from the sodium entry site (Benos et al., 1979). The present study represents an attempt to further characterize the molecular nature of the sodium entry mechanism by the use of group-selective reagents which react with specific chemical groups associated with these entry proteins.

The identification of particular types of components involved in the catalytic function of the entry protein, and possibly, their spatial arrangement may be deduced by observing alterations in transport parameters after reaction. This approach has proven successful in a number of biological systems (*see* Peters & Richards, 1977; and Cabantchik, Knauf & Rothstein, 1978, for reviews). Chemical modification can thus be employed as a probing device for identifying and mapping the structural components of this apical entry process.

The purpose of the present study was to investigate the effects that a number of externally applied sulfhydryl, amino, carboxyl and hydroxyl group-se-

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lective reagents have on short-circuit current (I_{sc}) and amiloride efficacy in the isolated frog skin preparation in an effort to ascertain what functional ligands are involved in both sodium entry and amiloride inhibition in this epithelium. Our results implicate SH groups as components of the Na-translocation pathway through the apical membrane. These groups, however, are not directly involved in the action of amiloride. Although the efficacy of amiloride is inhibited by reagents that modify $-NH_2$ groups, these reagents have no effect on I_{sc}. These results support the notion that amiloride and Na interact with the entry protein(s) at spacially distinct regions.

Materials and Methods

Isolated Anuran Skin Experiments

The abdominal skin of the bullfrog (*Rana catesbeiana*, Jacques Weil Co., Rayne, La.) was mounted as a 3.14 cm^2 flat sheet. The skin was placed between Lucite half-chambers, each equipped with glass bubble-lift solution reservoirs. The solutions in each chamber (10 ml each) were circulated and simultaneously oxygenated by bubbling with air. All experimentation was performed at room temperature (20 °C).

The transepithelial potential difference across the skin was adjusted to 0 mV with an automatic voltage clamp. This circuit also compensated for the series resistance contributed by the solution bathing the tissue. In all experiments, except those in which the external calcium or hydrogen concentration was varied, both sides of the skin were bathed with identical salt solutions. Under the salt concentrations utilized in these experiments, it has been shown that the magnitude of I_{sc} is identical to that of net Na influx (Ussing & Zerahn, 1951; Cereijido et al., 1964; Rawlings et al., 1970; Candia & Reinach, 1977; Mandel, 1978). Since the entry of sodium across the apical membrane is rate-limiting for net transport (Mandel, 1978; Benos et al., 1979), changes in I_{sc} produced by additions or deletions of ions, chemical reagents, or amiloride in the outer bathing solution primarily reflect actions occurring at this membrane, although possible exceptions will be noted.

The composition of the standard Ringer solution was 110 mM NaCl, 2.5 mM $KHCO_3$, and 1 mM $CaCl_2$. The pH of this solution, when equilibrated with room air at room temperature, was 8.4. For those experiments in which the Na concentration was varied, NaCl was replaced with an osmotically equivalent amount of choline chloride while holding the concentrations of all the other ions constant. The concentrations of Na employed were 110, 55, 20, 6 and 3 mM. In experiments in which the external calcium concentration was reduced to zero, $CaCl_2$ was omitted and 0.5 mM ethyleneglycol bis (β-aminoethyl ether)N,N,N′N′-tetraacetic acid (EGTA) was added. The internal solution contained the standard (110 mM Na) Ringer solution under all conditions.

Various experiments were performed in which the external pH was varied while the serosal pH was maintained constant. In all of these experiments, the serosal solution was regular Ringer. In the external solution, the bicarbonate buffer was replaced by an equal concentration of potassium phthalate titrated to the desired pH value with either HCl or NaOH.

The following group-selective, chemical-modifying reagents were tested:

a) Sulfhydryl group reagents: dithiothreitol (DTT); 5,5,-dithiobis (2-nitrobenzoic acid) (DTNB); *p*-chloromercuribenzoic acid (PCMB); and *p*-chloromercuribenzene sulfonic acid (PCMBS). PCMB linked a 40,000 mol wt dextran molecule (PCMB-D40) was kindly provided by Dr. Z.I. Cabantchik. The PCMB-dextran molar ratio was determined by spectrophotometric titration with 6 mercaptoguanosine, using a molar absorption coefficient of 21,800 at 342nM (Bibi et al., 1978).

b) Amino group reagents: 2,4,6-trinitrobenzenesulfonic acid (TNBS); and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS).

c) Carboxyl group reagents: 3,9-bis-(dimethylamino)-phenozathionum chloride (methylene blue, MB); N-ethoxycarbonyl-2-ethoxyl-1,2-dihydroquinoline, ethyl 1,2-dihydro-2-ethoxy-1-quinoline-carboxylate (EEDQ), trimethyloxonium tetrafluoroborate (TMO), and two different water-soluble carbodiimides, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CDI) and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (CDII). A more hydrophobic carbodiimide, N-N-dicyclohexylcarbodiimide (CDIII) was also tested. All of the sulfhydryl reagents were obtained from Sigma Chemical Company (St. Louis, Mo.), TNBS from Eastman Chemical Company (Rochester, N.Y.), SITS from ICN Pharmaceuticals (Cleveland, Ohio), EEDQ and the carbodiimides from Aldrich (Milwaukee, Wisc.), and TMO from Wauseka Consulting Company (Wauseka, Wisc.). An appropriate amount of each reagent was dissolved in Ringer to achieve the desired concentration. Fresh solutions were made on the day of an experiment.

The effects of each of these compounds on I_{sc} was assessed by incubating the external surface of the frog skin usually for 30 min in 110 mM NaCl Ringer's solution containing a given concentration of reagent. After this time, the apical surface of the skin was thoroughly washed with reagent-free Ringer and the steady-state level of I_{sc} was compared to that measured prior to compound addition. Amiloride dose-response curves were determined prior to and immediately following reagent treatment.

To insure carboxyl group specificity, the experiments with the water-soluble carbodiimides were performed in accordance with the procedure outlined by Hoare and Koshland (1967). A control amiloride inhibition curve was first obtained on skins bathed with 110 mM NaCl at pH 8.4. Thereafter, the external solution was changed to one containing 1 mM carbodiimide and 3 mM glycine methylester (used as a nucleophile) at pH 3.0 or 4.5, with either 110 mM NaCl or with zero external sodium (choline chloride substitution). In other experiments at zero external sodium, the carbodiimide and glycine methylester concentrations were raised to 25 and 85 mM, respectively, with the choline chloride being omitted. In some experiments, the nucleophile used was taurine rather than glycine methylester. In all of the above cases, the skins were exposed to the carbodiimide and nucleophile for 30 min. After this period, the skins were washed and re-equilibrated with standard Ringer. Amiloride dose-response curves were again determined. The long exposure at low pH did not, in itself, decrease the responsiveness of the skins either in terms of I_{sc} stability or amiloride inhibition. This fact was established in independent experiments.

For the TMO experiments, we essentially followed the procedure of Spaulding (1980). The solution bathing the external surface of the skin was replaced with 50 mM TMO plus 60 mM HEPES (pH 7.4) buffer at 4 °C within 0.3 min after dissolving the TMO. The apical surface of the frog skin was exposed to this solution for 10 min; thereafter, the skin was thoroughly washed with the standard 110 mM NaCl Ringer. Control skins were treated identically except that 50 mM choline chloride was used instead of TMO. Also, TMO was allowed to hydrolyze in buffer for 15 min prior to being placed on the skin. The reaction products themselves produced no change in the transport parameters of the frog skin.

Compensation Potential and Zeta Potential Measurements

Compensation potential measurements were carried out in an 8-ml Teflon trough. The compensation potential was measured between a polonium air electrode and a grounded Ag/AgCl electrode in the aqueous phase. The polonium electrode was connected to the input of a Keithley 602 electrometer, the output of which was connected to a strip-chart recorder. The temperature was maintained constant at 20 ± 0.2 °C with a circulating water bath. The temperature was measured with a thermistor. The entire unit was encased in an aluminum box which provided electrostatic and air current shielding.

The concentration of lipid in the spreading solvent (usually decane) was 10 mg/ml, 10 µl of which was injected along the side of the trough. The lipids used were: phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and glycerolmonooleate (GMO). These lipids were obtained from Avanti Biochemicals (PC, PS and PE), and Supelco (GMO).

The electrophoretic mobility of lipid vesicles was measured at room temperature in a cylindrical microelectrophoresis chamber (Bangham et al., 1958). The zeta potential was computed from the following equation:

$$\zeta = 12.85\, u$$

where u = electrophoretic mobility in volt·cm^{-1}·µsec^{-1} and ζ = the zeta potential in mV (Davies & Rideal, 1963).

Results

Sulfhydryl Group Reagents

a) Short-Circuit Current. Table 1 presents a summary of the effects (and principal mechanism of action) that several externally applied sulfhydryl reagents have upon I_{sc} in isolated bullfrog skin. The results are expressed as the steady-state I_{sc} observed after exposure to the compound normalized to the control value of I_{sc} measured just prior to compound addition.

It can be seen from this table that external addition of 1 mM PCMBS caused an initial increase in I_{sc} of $56 \pm 10\%$, usually followed by a decrease in I_{sc}, reaching a new steady-state level $91 \pm 7\%$ below the original baseline. Figure 1 depicts the time course of I_{sc} response to PCMBS in a representative experiment. In the above experiment, the I_{sc} increased to its maximum value 15 min after external addition of PCMBS and decreased thereafter to a new steady-state value 20% below that of the control and 52% below that of the peak approximately 1 hr after peak stimulation. The stimulation of I_{sc} could be reversed by washing with PCMBS-free Ringer solution, whereas the steady-state current could not. This biphasic response of I_{sc} to PCMBS is in contrast to the irreversible decrease in I_{sc} observed subsequent to the external addition of the other mercaptide bond former, PCMB (Table 1 and Fig. 2). Addition of 0.1 mM PCMB to the apical bathing solution resulted in an overall inhibition of I_{sc} of some 60% after 1–1.5 hr. Both of these sulfhydryl reagents were also tested for their effects on the serosal side of the frog skin. These agents produced no initial change

Table 1. Effect of sulfhydryl-group reagents on I_{sc}

Compound	Principal mechanism of action	% Normalized I_{sc}
PCMBS (1 mM) (at peak I_{sc})	Forms mercaptide bonds with SH groups	156 ± 10 ($n=11$)
PCMBS (1 mM) (at steady-state)	Forms mercaptide bonds with SH groups	91 ± 7 ($n=11$)
PCMB (0.1 mM)	Forms mercaptide bonds with SH groups	40 ± 5 ($n=18$)
DTT (1 mM)	Reduces disulfide bonds to SH groups	85 ± 2 ($n=46$)
DTNB (1 mM)	Oxidizes SH groups into disulfide linkages	106 ± 4 ($n=26$)

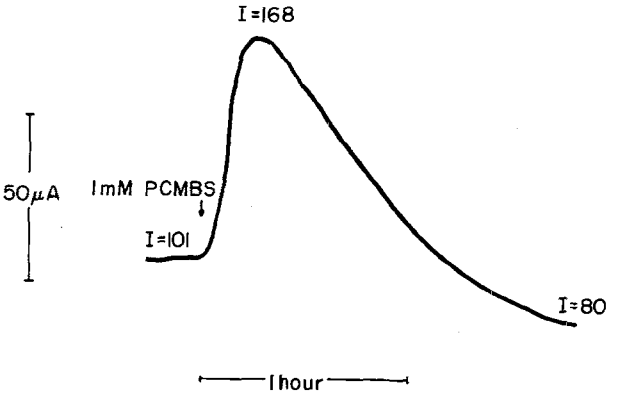

Fig. 1. The effect of 1 mM PCMBS on I_{sc} of isolated bullfrog skin epithelium. PCMBS was added to the solution bathing the external surface of the skin.

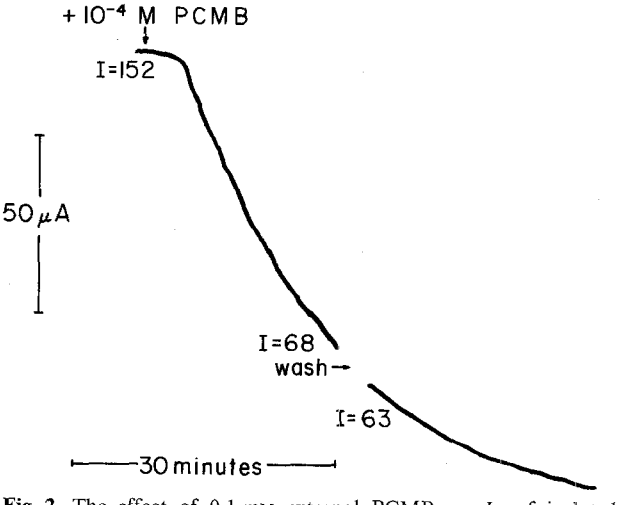

Fig. 2. The effect of 0.1 mM external PCMB on I_{sc} of isolated bullfrog skin epithelium.

in the steady-state levels of I_{sc} for approximately 1 hr after addition, whereupon the I_{sc} showed a distinct, albeit slow, inhibition with time.

The differences in response between PCMBS and PCMB added externally were puzzling because both reagents undergo almost identical chemical reactions, namely, the formation of mercaptide bonds with free $-SH$ groups. One possibility for this difference in behavior could be that each reagent reacts with a separate population of $-SH$ sites because of their differential permeability through the outer membrane of the skin. To test this idea of reagent permeability on the I_{sc} response, PCMB covalently linked to a 40,000 mol wt dextran molecule (assumed to render the mercurial impermeable) was added to the external solution with results shown in Fig. 3. Additions of 40,000 mol wt dextran (purified by dialysis) without the attached mercurial produced no effects up to a concentration of 10^{-4} M. Subsequent addition of 10^{-4} M PCMB-D40 produced a stable, reversible increase of 52% in I_{sc}. This result suggests that reactions with separate populations of sulfhydryl groups cause the reversible increase in I_{sc} and the irreversible decrease in I_{sc}.

Table 1 also summarizes the results of similar experiments performed with the sulfhydryl group reducing agent, DTT, and oxidizing agent, DTNB. DTT is capable of breaking disulfide linkages into separate, reduced sulfhydryl groups (Cleland, 1964) while DTNB oxidizes sulfhydryl groups through formation of a disulfide bond (Ellman, 1959). It can be seen from the table that both of these reagents only had a minor influence on I_{sc}: DTT depressed I_{sc} by 15%, while DTNB did not cause a significant alteration in I_{sc}.

The effects of 1 mM PCMBS on the external pH dependence of I_{sc} is shown as Fig. 4. These experiments were performed approximately 15 min after the addition of PCMBS. As can be seen in the figure, PCMBS stimulated I_{sc} at pH values above 4.5 but did not otherwise influence the titration curve. This stimulation of I_{sc} was only 10–30% because the experiments were performed during the transient stimulation and, thus, represent a time average over the I_{sc} changes. DTT and DTNB likewise had no effect upon the external pH titration curve of I_{sc}.

The effect of PCMBS on Na transport kinetics was assessed in six additional experiments according to the methods of Benos et al. (1979). Because of the rapid time course of the PCMBS stimulatory effect on I_{sc}, only the steady-state values of K_t (the concentration of Na required to produce an I_{sc} one-half its maximal value) and I_{max} (the maximal I_{sc} observed at saturating [Na]) could be determined. In these experiments, PCMBS (1 mM) decreased I_{max} from 37.8 ± 1.2 μA/cm^2 (control) to 31.5 ± 0.7 μA/cm^2 with no significant change in K_t (9.7 ± 2.0 mM (control) and 13.0 ± 2.3 mM (1 mM PCMBS)).

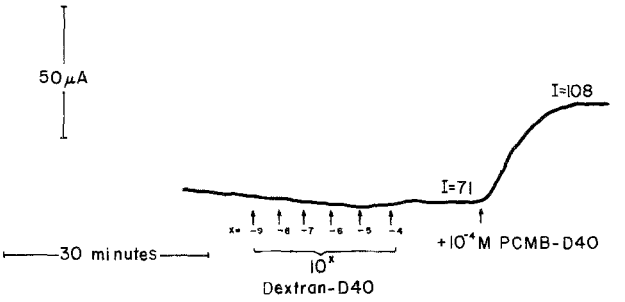

Fig. 3. The effect of increasing concentrations of a 40,000 mol wt dextran (D40) molecule and 0.1 mM PCMB covalently attached to 40,000 mol wt dextran (PCMB-D40) on I_{sc} of isolated bullfrog skin.

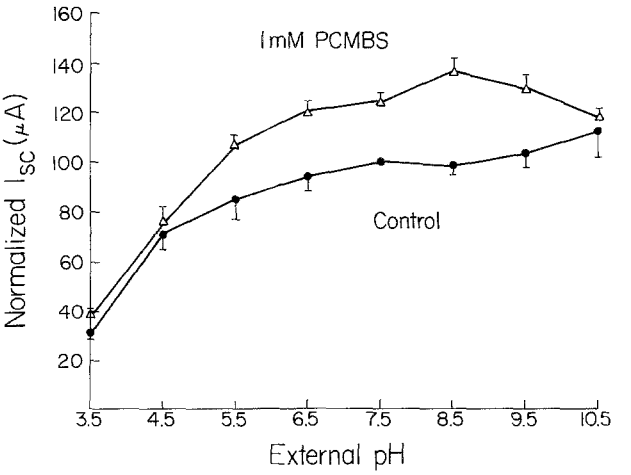

Fig. 4. I_{sc} *vs.* external pH before (solid circles) and after (open triangle) exposure to 1 mM PCMBS in paired bullfrog skins. The serosal solution contained bicarbonate as buffer and always had a pH of 8.4. Each point represents the mean value of eight separate experiments; the vertical bar indicates one SEM.

b) Amiloride-induced Inhibition of I_{sc}. Experiments were also performed to test the influence of these same sulfhydryl reagents upon the ability of amiloride to inhibit I_{sc}. The effectiveness of amiloride was quantitated by computing K_I and I_{max}^{INH} values from amiloride dose-response curves (K_I = the concentration of amiloride which produces a 50% inhibition of I_{sc} and I_{max}^{INH} the percent inhibition of I_{sc} produced by a maximal dose (10^{-4} M) of amiloride). Figure 5 presents log dose-response curves of amiloride inhibition of I_{sc} prior to and after the addition of 1 mM PCMBS to the external bathing solution. The experimental curve was determined during the time interval in which the I_{sc} was maximally stimulated by PCMBS. There was no significant change in the ability of ami-

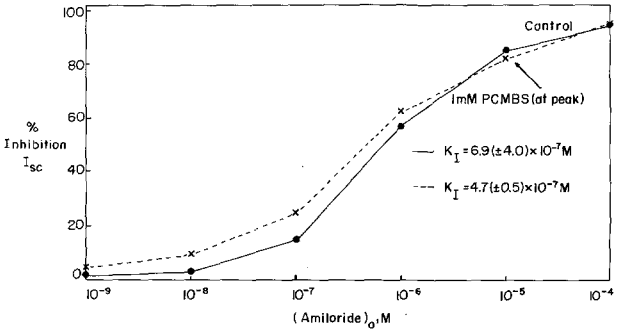

Fig. 5. Log-dose response curves of the amiloride inhibition of I_{sc} in isolated bullfrog skin before (control) and after treatment with 1 mM external PCMBS. The dose-response curve subsequent to PCMBS addition was determined during the time interval in which the I_{sc} was peak stimulated. In these experiments, the I_{sc} was stimulated by PCMBS an average of 66.3% compared to control. Each point represents the mean of six experiments.

Table 2. Effect of sulfhydryl group reagents on K_I and the maximum inhibition of amiloride (I_{max}^{INH})

Condition	K_I($\times 10^{-7}$ M)	I_{max}^{INH} (%)
Control	6.9±4.0	94.8±1.6
1 mM PCMBS (peak of I_{sc})	4.7±0.5	95.6±1.6
(n=6)		
Control	3.4±3.5	95.7±1.1
1 mM PCMBS (steady state)	4.2±4.7	[b]80.6±3.4
(n=5)		
Control	4.3±2.3	93.4±2.8
0.1 mM PCMB	[a]16.6±3.9	[b]73.7±4.1
(n=5)		
Control	3.2±0.6	–
1 mM DTT	4.0±2.6	–
(n=11)		
Control	4.6±1.4	94.8±1.7
1 mM DTNB	2.3±1.0	98.6±0.2
(n=4)		

Paired.
[a] $P < 0.001$.
[b] $P < 0.025$.

All others, not significant.

loride to inhibit I_{sc} at any concentration after exposure to PCMBS. Both the maximal percent inhibition of I_{sc} (94.8±1.6% in the control *vs.* 95.6±1.6% at peak stimulation), and K_I ($6.9(\pm 4.0)\times 10^{-7}$ M and $4.7(\pm 0.5)\times 10^{-7}$ M for control and after PCMBS, respectively) were unchanged. Table 2 summarizes these experiments for PCMBS as well as for all of the other sulfhydryl-group reagents. As can be seen from the table, externally applied PCMB decreased both the affinity and maximum inhibition of amiloride, whereas 1 mM PCMBS in the steady-state decreased I_{max}^{INH} without changing K_I. No effects on these amiloride parameters were detected after exposure to DTT, DTNB, or PCMB-D40 (data not shown). Thus, sulfhydryl reagents that reversibly increase or decrease I_{sc} do not alter either K_I or I_{max}^{INH} of amiloride, whereas those reagents that irreversibly decrease I_{sc} inhibit the effectiveness of amiloride.

Table 3. The influence of amino, carboxyl and hydroxyl-group reagents on I_{sc}

Compound	Principle mechanism of action	% Normalized I_{sc}
1 mM SITS	Removes or neutralizes positive charge of amino (–NH) groups	107±12 (n=12)
3 mM TNBS	Trinitrobenzoylates and amino groups	104±9 (n=31)
1 mM MB^+	Associates with $-COO^-$ or –OH groups	80±2 (n=31)
0.1 mM EEDQ	Modifies carboxyl groups by coupling acylamino acids to amino acid esters	110±9 (n=16)
1 mM CD I (Na_o=110 mM)	Modifies carboxyl groups by coupling reaction with nucleophile	102±8 (n=8)
1 mM CD I (Na_o=0 mM; choline)	"	104±15 (n=4)
25 mM CD I (Na_o=0 mM)	"	113±16 (n=4)
1 mM CD II (Na_o=110 mM)	"	121±15 (n=4)
1 mM CD III (Na_o=110 mM)	"	98±14 (n=7)
50 mM TMO	Esterifies carboxyl groups	96±5 (n=4)

Amino and Carboxyl Group Reagents

Table 3 presents a compilation of relevant information concerning mechanism of action of a number of amino and carboxyl group selective reagents. In addition, data concerning the effects that these compounds exerted upon I_{sc} after a 30-min exposure of the apical surface of the frog skin are also included. Neither the amino reagents, SITS or TNBS, nor the carboxyl reagents, EEDQ, the carbodiimides (at low or high concentration), or TMO significantly altered the steady-state level of I_{sc}. The only molecule in this category that had any significant effect on I_{sc} was methylene blue (MB), a carboxyl or hydroxyl reagent (*see* below) which inhibited I_{sc} by 20±2% at 1 mM.

The effect of methylene blue on Na entry transport kinetics was determined in twelve additional bull-

frog skins. Single reciprocal plots of I_{sc} *vs.* I_{sc}/Na showed that methylene blue decreased I_{max} from 38.0 ± 0.8 μA/cm^2 to 22.5 ± 1.5 μA/cm^2 and increased K_t from 9.4 ± 1.1 to $15.4 \pm 0.9 \times 10^{-3}$ M (data not shown). Furthermore, in 18 additional experiments on paired skins, methylene blue did not change the pH dependence of I_{sc} in 110 mM Na.

The effects of these agents on the inhibitory capabilities of amiloride are shown in Table 4. At 3 mM, TNBS depressed both the maximum inhibition of amiloride by 26% and increased K_I from $4.5(\pm 0.5)$ to $15.3(\pm 5.7) \times 10^{-7}$ M. The effect of TNBS was reversible. The other amino reagent, SITS, at 1 mM, did not change either I_{max}^{INH} or K_I. Thus, TNBS did not affect I_{sc} but did change significantly the inhibitory action of amiloride.

The standard carboxyl reagents CDI, CDII, and CDIII under different conditions (*see* Table 4) did not alter amiloride's inhibitory capabilities. In these experiments, glycine methylester was used as the nucleophile. There was no significant change in the observed results when equimolar concentrations of taurine were used in place of glycine methylester. Trimethyloxonium tetrafluoroborate (TMO), effective in esterifying carboxyl groups (Spaulding, 1979), also had no influence on the amiloride inhibitory curves. Likewise, 0.1 mM EEDQ produced no significant alteration of either K_I or I_{max}^{INH}.

Table 4 shows that 1 mM methylene blue significantly depressed the response of the skins to inhibition by amiloride. The apparent K_I for amiloride was shifted to higher values by methylene blue ($4.2(\pm 1.5) \times 10^{-7}$ M to $16.0(\pm 8.8) \times 10^{-7}$ M), and a significant portion of the I_{sc} became amiloride-insensitive after treatment.

Figure 6 illustrates an experiment in which methylene blue (1 mM) was added to the outside bathing solution shortly after I_{sc} was partially (74.6%) inhibited by 10^{-6} M amiloride. Addition of methylene blue slowly reversed the inhibitory effect of amiloride with the I_{sc} reaching a new steady-state level approximately 1 hr subsequent to methylene blue addition. Methylene blue by itself inhibited I_{sc} some 20% at this concentration (Table 3). This observation is consistent with the notion that methylene blue decreases the effectiveness of amiloride, which confirms the result shown in Table 4.

Compensation and Zeta Potential Measurements

To better determine the group specificity of methylene blue we measured compensation potentials which are the sum of diffuse double layer and dipole potentials (Bockris & Reddy, 1970) for 1 mM methylene blue

Table 4. The influence of amino, carboxyl and hydroxyl-group reagents on K and I_{max}^{INH} of amiloride

Condition	$K_I(\times 10^{-7}$ M)	I_{max}^{INH} (%)
Control	3.5 ± 0.4	95.8 ± 1.1
1 mM SITS ($n=12$)	4.4 ± 0.8	93.8 ± 4.0
Control	4.5 ± 0.5	96.4 ± 0.6
3 mM TNBS ($n=14$)	[b] 15.3 ± 5.7	[c] 71.1 ± 4.7
Control	4.2 ± 1.5	92.0 ± 1.1
1 mM MB ($n=8$)	[d] 16.0 ± 8.8	[a] 80.2 ± 4.6
Control	2.2 ± 1.3	98.2 ± 1.8
0.1 mM EEDQ ($n=5$)	5.0 ± 3.8	90.3 ± 3.9
Control	4.2 ± 0.6	95.6 ± 0.6
1 mM CD I ($n=8$)	6.2 ± 1.3	96.0 ± 1.0
($Na_o = 110$ mM)		
Control	4.7 ± 1.1	94.6 ± 0.3
1 mM CD I ($n=4$)	4.3 ± 0.8	94.0 ± 2.3
($Na_o = 0$ mM; choline)		
Control	5.2 ± 0.6	95.3 ± 0.3
25 mM CD I ($n=4$)	6.0 ± 1.6	94.2 ± 1.0
($Na_o = 0$ mM)		
Control	1.3 ± 0.8	101 ± 6
1 mM CD II ($n=4$)	1.3 ± 0.5	105 ± 12
($Na_o = 110$ mM)		
Control	2.1 ± 0.9	98.1 ± 0.7
1 mM CD III ($n=8$)	0.9 ± 0.4	98.0 ± 1.0
($Na_o = 110$ mM)		

Paired probabilities: [a] $P < 0.025$; [b] $P < 0.01$; [c] $P < 0.05$; [d] $P < 0.1$; all others, not significant.

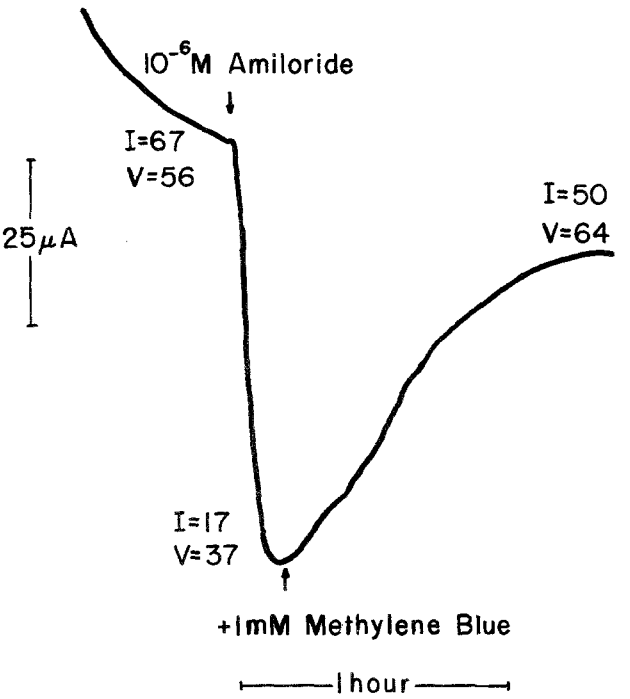

Fig. 6. The ability of 1 mM methylene blue to reverse the inhibitory effect of amiloride in isolated bullfrog skin epithelium as seen in a I_{sc} *vs.* time trace.

Table 5. Effects of methylene blue (1 mM) on the compensation potential (ΔV) of lipid monolayers and the zeta potential of bilayers

Lipid	ΔV(mV)	Exposed groups	Methylene blue	
			$\Delta(\Delta V)$(mV)[a]	$\Delta\zeta$(mV)[b]
Phosphatidyl serine (PS)	310 ± 20 ($n=9$)	PO_4^-, NH_3^+ COO^-	105 ± 12 ($n=5$)	42.1 ± 1.8 ($n=6$)
Glycerolmono-oleate (GMO)[c]	265 ± 17 ($n=9$)	$-OH$	70 ± 8.3 ($n=5$)	0.8 ± 1.3 ($n=6$)
Phosphatidyl-ethanolamine (PE)	426 ± 15 ($n=8$)	PO_4^-, NH_3^+	4.7 ± 2.4 ($n=3$)	3.2 ± 1.3 ($n=3$)
Phosphatidyl choline (PC)	471 ± 18 ($n=8$)	PO_4^-, $N^+(CH_3)_3$	6.3 ± 2.1 ($n=3$)	4.2 ± 1.1 ($n=3$)

[a] 0.1 M NaCl, 10^{-3} M Na phosphate at pH 7.0, 20 ± 0.2 °C.
[b] 0.1 M NaCl, 10^{-3} M Na phosphate at pH 7.0, 25 ± 0.1 °C.
[c] Used monolayer-covered decane droplets.

Table 6. Composite effects of site specific reagents on sodium entry and amiloride efficacy in frog skin epithelium

Class	Reagent	I_{sc}	Amiloride efficacy	
			K_I	I_{max}^{INH}
I	PCMBS (peak)	↑	0	0
	PCMB-D40	↑	0	0
	DTT	↓	0	0
II	TNBS	0	↑	↓
III	DTNB	0	0	0
	SITS	0	0	0
	EEDQ	0	0	0
	Carbodiimides	0	0	0
	TMO	0	0	0
IV	PCMBS (steady-state)	↓	0	↓
	PCMB	↓	↑	0
	Methylene blue	↓	↑	↓

↑ — increase; ↓ — decrease; 0 — no change.

on monolayers comprised of different lipids. Zeta potential (ζ) measurements were also performed. Specific interaction between MB and any of these lipids would be expected to alter one or both of these potentials. These results are presented in Table 5. The numbers in column 2 give the change in compensation potential after lipid addition to the trough and is denoted by ΔV. This number represents the difference between the potential of a clean saline surface without a monolayer and one with a monolayer. The third column gives the primary lipid groups that are exposed to the aqueous phase. The chemical group with which MB appears to interact in PS is the carboxyl group because MB did not cause any appreciable change in potential of either PE or PC which contain only phosphate or amino groups. With the hydroxyl group containing lipid GMO, MB dramatically changed the dipole potential, leaving the surface potential unaffected. With carboxyl groups there seemed to be both a surface and dipole potential change induced by MB. The change in surface potential may be due to the electrostatic interaction between the positively-charged MB molecule and the negatively charged carboxyl group.

These results show that methylene blue preferentially interacts with lipids containing exposed carboxyl and hydroxyl groups, and less so with HPO_4^-, $-N(CH_3)_3^+$, and $-NH_3^+$ groups.

Discussion

We have previously demonstrated that in the isolated skin epithelium of *Rana catesbeiana* the inhibition of active sodium transport by amiloride is through a noncompetitive mechanism (Benos et al., 1979). This finding suggests that amiloride acts at a site separate from the Na entry site, possibly through an allosteric interaction. We undertook the present study with the intention of identifying chemical residues essential to the operation of both the Na entry mechanism and the amiloride binding site. This was accomplished by monitoring the response of I_{sc} (used as an index for the rate of Na entry) and the inhibition of I_{sc} produced by amiloride after addition of site-specific reagents to the external bathing solution. Alterations in either parameter subsequent to modification of specific chemical residues would implicate involvement of such groups in the function of the site. On the other hand, if chemical modification produced no effect on either property, it is reasonable to conclude that either the modified residues are not essential for the activity in question or that these groups are not accessible to the reagent for reaction. The application of chemical reagents to intact biological tissues is not without difficulty, especially from the standpoint of ligand group specificity and accessibility. Consequently, caution must be exercized in interpretating data obtained from such studies. This is especially true in epithelia where transport parameters can be affected in a multitude of ways, and at a multitude of different sites.

Table 6 shows the composite results of all the experiments regarding how these chemical reagents affected sodium transport and amiloride induced inhibition of I_{sc}. In this table, the magnitudes of the changes were not considered but only whether these reagents increased (↑), decreased (↓) or did not change (0) the variable of interest. The reagents, fall into four general classes:

Class I: Those that are reversible and affect only Na transport;
Class II: Those that affect only the ability of amiloride to inhibit I_{sc};
Class III: Those that have no discernible effect on either Na transport or amiloride efficacy;
Class IV: Those that are irreversible and affect both Na transport and amiloride efficacy.

The class I compounds are only found among the sulfhydryl group reagents. They are PCMB-D40, PCMBS (at peak), and DTT. From these experiments it is apparent that there is a class of SH groups controlling I_{sc} which is in contact with the external solution. The interaction of these groups with these reagents may either increase or decrease I_{sc}, depending upon the nature of the reaction. This deduction is reasonable because PCMB-D40 is probably not permeable, and hence can only interact with the membrane from the external solution. This same class of SH groups may also be involved in the transient stimulation of I_{sc} by external PCMBS (Fig. 1) since this effect can also be rapidly reversed by washing. Also, since the effects of DTT on I_{sc} are reversible, an external site of action may be implicated.

The lack of effect of these components on the external pH dependence of I_{sc} and, possibly, the lack of effect on Na transport kinetics seems to indicate that this class of SH group-modifying reagents does not change the manner with which Na binds to its site, but does alter the actual rate determining step of Na translocation across the apical border.

Similarly, we may conclude that the amiloride binding site does not have SH groups facing the external solution. This is in direct contrast to the situation which obtains in the isolated rabbit colon preparation (Gottleib et al., 1978). These authors demonstrated that the effectiveness of amiloride as an inhibitor of Na entry was greatly diminished after treatment of the colon with PCMBS. Indeed, they found that PCMBS could even reverse the effect of amiloride. However, it is not clear whether this reversal phenomenon was due to the displacement of bound drug or to a dissociation of the entry process from the inhibitory one.

The only substance in class II is the amino reagent, TNBS. This molecule, at 3 mM, did not alter Na transport but did increase the K_I and decreased I_{max}^{INH} of amiloride. It is of interest to note that the other amino reagent, SITS, had no effect upon either of these parameters. This difference may reflect either different reaction mechanisms or different membrane permeabilities. There are conflicting reports in the literature concerning the reaction of TNBS with amino groups. Means and Feeney (1971) claim that TNBS reacts with protonated amino groups while Freedman and Radda (1968) and Goldfarb (1970) claim that reaction can occur with the unprotonated moiety. SITS, on the other hand, reacts electrostatically only with charged amino groups (Cabantchik & Rothstein, 1972; Castranova & Miles, 1977). SITS is virtually impermeable, whereas TNBS has significant finite permeability through cell membranes (Castranova & Miles, 1977). The results presented in this paper indicate that the Na entry pathway in bullfrog skin does not contain an amino group directly responsible for transport but that amino groups may be involved in the interaction between amiloride and its receptor site. It is not presently clear whether these groups exist directly at the amiloride site or whether reactions with these groups produce allosteric interactions that affect this site.

The effects of TNBS upon amiloride sensitivity were anomalous in that they were reversible, contrasting with TNBS' known covalent trinitrobenzylation reaction with primary amino groups. The reasons for this are unknown, but several possibilities exist. First, TNBS may be forming reversible acceptor-donor complexes with primary or secondary amines of apical membrane proteins. Second, trinitrobenzylation may be proceeding but the complex may initially be reversible due to differential reactivities and/or the very short reaction time allotted (Means, Congdon & Bender, 1972). Finally, TNBS can also react with SH groups, but only at a much slower rate. This reaction product, however, is very labile (Haynes, Osuga & Feeney, 1967). It is therefore possible that TNBS is reacting with a class of sulfhydryl groups located at or near the amiloride receptor to produce its observed effects. These SH groups must, however, be inaccessible to attack by PCMB, PCMBS, DTT, or DTNB.

The class III reagents, none of which affect Na entry nor amiloride efficacy, are the sulfhydryl oxidizing agent DTNB, the carboxyl reagents EEDQ, the water soluble carbodiimides CDI and CDII, the lipid soluble carbodiimide CDIII, and TMO. This lack of effect of all these carboxyl reagents on I_{sc} is in agreement with similar experiments performed on toad bladder (Harms & Fanestil, 1977), but does not agree with the results reported in isolated *R. temporaria* skin by Zeiske and Lindemann (1975) and Lindemann and Voute (1977). These investigators found that both EEDQ and the water-soluble carbodiimides *completely* abolished I_{sc}. Consequently, they attributed the external pH dependence of I_{sc} to titration of ionized acidic groups located at or near the orifice of the Na entry site. Hence, abolition of I_{sc} subsequent to exposure of the apical surface of the skin to these specific carboxyl reagents was taken as evidence that these acidic ligands were intact carboxyl residues. We

have qualitatively corroborated these results on *R. temporaria* in our laboratory. We found that a 30-min exposure of the apical surface to 1 mM CDII inhibited I_{sc} by $49.7 \pm 9.6\%$ ($n=7$) and 1 mM CDIII inhibited I_{sc} by $34.0 \pm 6.9\%$ ($n=4$). The amiloride inhibition curves were left unaffected. However, in bullfrog skin, we found no evidence that externally located carboxyl groups are integral elements of the Na entry mechanism.

The class IV reagents include the sulfhydryl reagents PCMBS (in the steady state) and PCMB, and the carboxyl and hydroxyl-interacting molecule, methylene blue. All of these compounds decreased I_{sc} and reduced the effectiveness of amiloride as an inhibitor of net Na transport. We believe that the decline in the short-circuit current seen with PCMBS and PCMB is due to the penetration of these compounds into the cell. This contention is based on the following reasoning: (i) the biphasic response of I_{sc} subsequent to PCMBS addition (Fig. 1); (ii) the differential membrane permeabilities of PCMBS and PCMB, PCMB being faster (Sutherland, Rothstein & Weed, 1967; Frenkel, Ekblad & Edelman, 1975); and (iii) the stimulation of I_{sc} seen with PCMB rendered impermeable by attachment to a large dextran molecule (Fig. 3). This dual effect of activation followed by inactivation of Na entry by PCMBS is not without precedent. This has been observed in the isolated toad bladder (Frenkel et al., 1975; Spooner & Edelman, 1976; Harms & Fanestil, 1977). Likewise, Janacek (1962) reported that very low concentrations of PCMB (0.04 mM) increased the permeability of the apical membrane of *R. temporaria* skin, and that higher concentrations (0.1–1.0 mM) resulted in a rapid decrease in Na permeability. It is noteworthy that Lindemann and Voute (1977) and Dick (1977) found that both PCMB and PCMBS stimulated I_{sc} in *R. temporaria* skin. Although the time course of their experiments was much shorter, they never commented upon seeing a decrease in I_{sc} with either agent.

The irreversible, slow effects observed in our experiments may thus be assumed to occur from an intracellular site. Hence, it would be very difficult to localize a site(s) of action. These could act directly on the cytoplasmic aspect of the entry site or, indirectly, through metabolism or pump inhibition. If these agents are acting on the entry site, cytoplasmic SH group modification would cause a general conformational change decreasing both I_{sc} and amiloride efficacy.

The positively-charged agent, methylene blue, was initially chosen as a carboxyl-specific reagent on the basis of its use in the quantification of the carboxyl content of oxidized starches and cellulose (Davidson, 1948; Cheung, Carroll & Weill, 1960), and because of its affinity for carboxyl groups in the saxitoxin receptor (D'Arrigo, 1976). Its specificity for various chemical groups was assessed by measuring the changes it produced in total surface and dipole potentials in monolayers and liposomes constructed from lipids with various groups (Table 5). Methylene blue, at 1 mM, clearly associates with and produces a +40 mV change in the zeta potential of phosphatidylserine liposomes. In addition, methylene blue induces a large (+70 mV) dipole potential change in the hydroxyl group amphiphile glycerolmonooleate. Either one of these effects may induce alterations in the biological activity of membrane proteins.

Nonetheless, it would be very difficult to ascribe an external site of action to methylene blue. Methylene blue is known to permeate biological membranes (Sass, Caruso & Axelrod, 1967; Ehrenfeld, Masoni & Garcia-Romeu, 1976), and the fact that MB inhibited I_{sc} and "displaced" amiloride (Fig. 6) with a relatively slow time course may indicate an internal site of action. The complex inhibitory kinetics observed between MB and Na also complicate localization of MB's site of action.

The lack of effect of either MB or PCMBS on the external pH dependence of I_{sc}, as well as the lack of effect of any of the carboxyl group specific reagents on I_{sc}, make it unlikely that the chemical group or groups titrated by external pH face the external solution. This finding supports the proposal of Mandel (1978) who suggested that external H^+ ions affect I_{sc} by permeating through the apical border and interacting with intracellular binding sites.

In conclusion, the results of the present study confirm our previous observations that the amiloride and Na entry site are separate and distinct in bullfrog skin epithelium because the various site-selective reagents tested affect the two sites differently. Sulfhydryl groups are important in modulating the rate of Na entry, whereas externally located amino, carboxyl, and hydroxyl groups are not. Externally located amino groups may be involved in the interaction between amiloride and its receptor site. It is not presently clear whether these groups exist directly at the Na entry and/or amiloride sites or whether reactions with these ligands cause allosteric interactions that in turn affect these sites.

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