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Regulation of Na^+-H^+ exchange by transmethylation reactions in rat colonic brush-border membranes

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Incubation of rat colonic brush-border membrane vesicles with 200 μM *S*-adenosyl-L-[*Me*-^{3}H]methionine resulted in the labeling of both membrane phospholipids and proteins. This labeling was decreased approximately 50% by the methylation inhibitor *S*-adenosyl-L-homocysteine (2 mM). Utilizing the pH-sensitive fluorescent dye, acridine orange, as a means of determining Na^+-H^+ exchange, *S*-adenosyl-L-methionine (200 μM) significantly increased sodium-stimulated proton efflux in these vesicles at all concentrations of sodium (2.5–50 mM) tested. Examination of the kinetic parameters for sodium-stimulated proton efflux in the presence and absence of 200 μM *S*-adenosyl-L-methionine revealed that the methyl donor increased the V_{max} for this exchange mechanism (expressed in arbitrary fluorescence units) by approx. 36% but did not influence its K_m for sodium. *S*-Adenosyl-L-homocysteine (2 mM) inhibited *S*-adenosyl-L-methionine-mediated stimulation of this exchange process. The results demonstrate that methylation of membrane phospholipids and/or proteins can modulate Na^+-H^+ exchange in rat colonic brush-border membrane vesicles.

Introduction

Na^+-H^+ exchange processes have been shown to exist in plasma membranes of several tissues [1–7] and appear to be involved in a number of diverse physiological functions [1–4] Recently, our laboratory has demonstrated that such an exchange process is present in rat colonic brush-border membrane vesicles and exhibits saturation kinetics, amiloride sensitivity and cation specificity similar to other Na^+-H^+ exchange systems [8]

Although evidence supports the existence of regulatory mechanisms for the activity of Na^+-H^+ exchange [9–12], the precise nature of these mechanisms is largely unknown Accordingly, the present studies were initiated to examine the possible role of transmethylation reactions involved in the biosynthesis of phosphatidylcholine and protein carboxymethylation in the regulation of Na^+-H^+ exchange in rat colonic brush-border membrane vesicles Specifically, we explored the hypothesis that Na^+-H^+ exchange in these plasma membrane vesicles is modulated by these transmethylation reactions Aldosterone-stimulated synthesis of phosphatidylcholine via the transmethylation pathway and/or protein carboxymethylation have, in fact, recently been shown to increase electrogenic sodium transport in cultured toad bladder cells [13] and apical vesicles prepared from the cultured amphibian cell line A_6 [14] To date, the possible influence of these transmethylation reactions on the electroneutral Na^+-H^+ exchange process in plasma membranes has not been examined The results of the present experiments demon-

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strate that increases in these transmethylation reactions are associated with increases in Na^+-H^+ exchange in rat colonic brush-border membranes and may be important in the regulation of this electroneutral transport process

Experimental procedures

Materials *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (5–15 Ci/mmol) was purchased from New England Nuclear (Boston, MA) Phosphatidylethanolamine, phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine were obtained from Calbiochim-Behring (San Diego, CA) Non-radioactive *S*-adenosyl-L-methionine was a product of Boehringer-Mannheim (Indianapolis, IN) *S*-Adenosyl-L-homocysteine and all other chemicals were obtained from Sigma Chemical Co (St Louis, MO) and/or Fisher Chemical Co (Fairlawn, NJ), unless otherwise indicated

Preparation of colonic brush-border membrane vesicles Male albino rats of the Sherman strain weighing 250–300 g were fasted 18 h with water ad libitum before being killed The colons were excised and epithelial cells, relatively devoid of globlet cells, were obtained using a technique which combined chelation of divalent cations with mild mechanical dissociation [15]

Brush-border membrane vesicles from epithelial cells were then prepared as described by Brasitus and Keresztes [15] The purity of the membrane suspensions and the degree of contamination with intracellular organelles were assessed by marker enzymes The specific activity ratios [(purified brush-border membrane)/(crude homogenate)] for the brush-border enzyme markers, total alkaline phosphatase (*p*-nitrophenylphosphatase) and cysteine-sensitive alkaline phosphatase, were approximately 15–20 in all membrane preparations The corresponding values for succinate dehydrogenase, NADPH-cytochrome-*c* reductase and sodium-potassium-dependent adenosine triphosphatase, marker enzymes for mitochondrial, microsomal and basolateral membranes, respectively, ranged from 0 40 to 1 50 in all membrane preparations Brush-border membrane vesicles were suspended in appropriate buffer (see below) and used immediately Membrane protein was assessed using bovine serum albumin as standard as described by Lowry et al [16]

Assays of phospholipid methylation The methylation of phospholipids was measured by incorporation of [^{3}H]methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine as previously reported [17] The reaction mixture (500 μl) contained, unless otherwise indicated, 50 mM Tris acetate buffer (pH 8 0), *S*-adenosyl-L-[*methyl*-^{3}H]methionine [200 μM, 2–4 μCi] and plasma membranes (200 μg of protein) The assay was initiated by addition of membranes and incubated at 37°C for 60 min, unless otherwise indicated The reaction was terminated by addition of 3 ml of chloroform/methanol/2 M hydrochloric acid (6 3 1, v/v), followed by addition of 2 ml of 0 1 M KCl in 50% methanol This mixture was vigorously vortexed twice and centrifuged at 200 × *g* for 10 min The aqueous phase was aspirated and the chloroform phase rewashed with 2 ml of 0 1 M KCl in 50% methanol To identify the products of phospholipid methylation, the chloroform phase was evaporated to dryness under nitrogen and the residue dissolved in 100 μl of chloroform The sample was applied on a Silica gel G plate and the chromatogram developed in chloroform/propionic acid/*n*-propyl alcohol/water (2 2 3 1, v/v) The phospholipid standards were simultaneously chromatographed, and their positions were visualized using a saturated solution of iodine in chloroform The areas corresponding to the standard phospholipids, i e, phosphatidylethanolamine (PE), phosphatidyl-*N*-monomethylethanolamine (PME), phosphatidyl-*N*,*N*-dimethylethanolamine (PDE) and phosphatidylcholine (PC) were scraped separately and extracted with chloroform/methanol (2 1, v/v) The radioactivity of each product was then measured separately in a liquid scintillation counter as reported [17]

The chemical identity of the methylated products was further established by two-dimensional chromatography [18] and by hydrolysis of the phospholipids and identification of their free bases as described by Schneider and Vance [19] Additionally, both labeled and unlabeled *S*-adenosyl-L-methionine were routinely purified by ion-exchange before use [20]

Assay of protein carboxymethylation Protein

carboxymethylation of rat colonic brush-border membranes was assayed according to the methods of Diliberto et al [21,22], using bovine serum albumin (20 mg/ml), as an exogenous methyl acceptor For the assay of endogenous protein carboxymethylation, albumin was omitted for the assay Boiled enzyme preparations were used as blanks [21,22]

Na^+-H^+ exchange assay Sodium-stimulated proton efflux was measured in colonic brush-border membrane vesicles, using the pH-sensitive fluorescent dye, acridine orange, as previously described by our laboratory [8] and others [23–25] The fluorescence of acridine orange was measured at 26°C with a Perkin-Elmer 650-40 spectrofluorometer (Perkin-Elmer Corp , Norwalk, CT) (excitation 493 nm, emission 530 nm) equipped with a thermostatted cuvette stirring system and adding port The assay solution contained 6 μM acridine orange, 250 mM sucrose, 100 mM *N*-methylglucamine gluconate and 10 mM Tris-Hepes (pH 7 5) After 2 ml of this buffer reached steady-state fluorescence (approx 90 s), 50 μl of membrane vesicles (100–150 μg protein) preloaded with 250 mM sucrose, 100 mM *N*-methylglucamine gluconate and 10 mM Tris-Hepes (pH 6 0) was added As previously described [24,26], there was a 30–40% quenching in the acridine orange fluorescence signal which reached equilibrium within 2 min Sufficient quantities of sodium gluconate were then added with constant stirring to achieve a final concentration of 2 5 to 50 mM in the external buffer The addition of Na^+ resulted in a collapse of the outwardly directed proton gradient and a reappearance of acridine orange fluorescence [23] The increase in fluorescence after Na^+ addition was linear for greater than 2 s, and the initial rate of acridine orange fluorescence recovery was measured as the initial slope After 300 s the pH gradient was dissipated with 150 mM potassium gluconate and 10 μg nigericin as described by Knickelbein et al [7] The small fluorescence quenching still remaining after nigericin addition was due to binding of the dye to the membranes [25] Appropriate corrections were made for this binding as described by Burnham et al [27] Care was taken to maintain a constant temperature and pH, which are known to influence the reproducibility of these assay results [23]

Fluorescence polarization studies Three fluorophores were used 1,6-diphenyl-1,3,5-hexatriene (DPH), DL-2-(9-anthroyl)stearic acid (2-AS) and DL-12-(9-anthroyl)stearic acid (12-AS) All compounds were obtained from Aldrich Chemical Co or Molecular Probes Inc Steady-state fluorescence polarization studies were performed using a Perkin Elmer 650-40 fluorescence spectrofluorometer fitted with an automatic polarizer (C N Wood Mfg , Newtown, PA) The methods used to load the membranes and the quantification of the polarization of fluorescence have been described [28–32] It should be noted, however, that with this instrument fluorescence emission intensities recorded parallel and perpendicular to the excitation plane of each probe could be taken sequentially and the polarization of fluorescence calculated or anisotropy values (see below) could be obtained directly from the instrument These measurements were found to differ by 2% or less The content of each fluorophore in the preparations was estimated fluorometrically as described by Cogan and Schachter [33] Final molar ratios of probe/lipid ranged from 0 001 to 0 002 and the anisotropy differences noted in these studies could not be ascribed to differences in probe concentrations in the membranes Corrections for light scattering (membrane suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were made routinely, the combined corrections were less than 2% of the total fluorescence intensity observed for diphenylhexatriene-loaded membranes and less than 3% of that observed for anthroyloxystearate-loaded suspensions

Fluorescence polarization was expressed as the fluorescence anisotropy, r, which within certain limitations is proportional to the relaxation time of the probe and inversely proportional to the fluidity, as previously discussed [32] The results were obtained according to the modified Perrin relationship [34,35] $r = r_\infty + (r_0 - r_\infty)\ [T_c/(T_c + T_f)]$, where r_0 is the maximal limiting anisotropy (taken as 0 365 for diphenylhexatriene [35] and 0 285 for the anthroyloxy probes [37]), r_∞ is the limiting hindered anisotropy, T_c is the correlation time and T_f is the mean lifetime of the excited state Values for r_∞ of diphenylhexatriene

were calculated from r values as previously described by Van Blitterswijk et al [38] The static component of membrane fluidity was assessed by an order parameter, S, where $S = (r_\infty / r_0)^{1/2}$ as described previously [34,35,38] Possible changes in the excited-state lifetimes of each probe were monitored by calculation of the fluorescence intensity, $F = I_{\parallel} + 2I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities oriented parallel and perpendicular to the direction of polarization of the exciting light, respectively [36] The changes in fluorescence polarization described in these studies were not accounted for by changes in the excited-state lifetimes as assessed by F

Statistical methods The results are expressed as mean values ± S E Paired or unpaired t-tests were used for all statistical analysis A P value < 0.05 was considered significant

Results

Phospholipid methylation

Identification of [^{3}H]methylated phospholipids was determined by thin-layer chromatography as previously described [17,39,40] In agreement with earlier studies by our laboratory [39,40], when colonic plasma membranes were incubated with 200 μM *S*-adenosyl-L-methionine (pH 8 0), three major radioactive peaks with R_F values corresponding to PME, PDE and PC were present (not shown) PME (approx 53%) was the predominant product formed This reaction was found to be linear up to 500 μg of membrane protein As shown in Fig 1, using these incubation conditions, incorporation of [^{3}H]methyl groups into total phospholipids as well as PME, PDE and PC was linear for 120 min

At an *S*-adenosyl-L-methionine concentration of 200 μM (pH 8 0), 2 mM *S*-adenosyl-L-homocysteine inhibited total phospholipid methylation by 58 3 ± 3 0% ($N = 4$) as well as PME, PDE and PC by approx 58–60% (not shown)

Protein carboxymethylation

Protein carboxymethylation activity was also found to be present in rat colonic brush-border membranes Table I summarizes the data on this activity in the presence and absence of bovine serum albumin This activity was also found to be

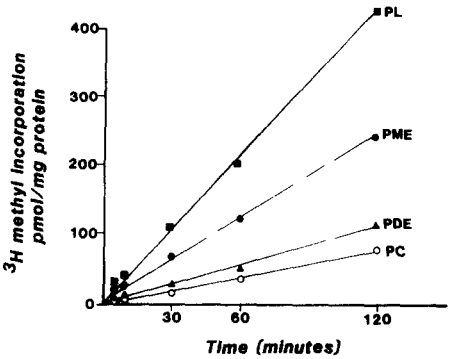

Fig 1 Time-course of phospholipid methylation Colonic brush-border membranes (200 μg protein) were incubated at 37°C with 200 μM S-adenosyl-L-[*methyl*-^{3}H]methionine at pH 8 0 At the indicated time the reaction was terminated, phospholipids extracted and separated by thin-layer chromatography Values are expressed as pmol [^{3}H]methyl groups incorporated into phospholipids per mg protein and represent the mean of three separate determinations at each time point

linear for at least 120 min using 250 μg membrane protein (not shown)

At a concentration of 200 μM *S*-adenosyl-L-methionine, 2 mM *S*-adenosyl-L-homocysteine inhibited endogenous and exogenous protein carboxymethylation activity in rat colonic brush-border membranes by approx 50% (Table I)

Effect of methylation on Na^+-H^+ exchange

To determine if phospholipid methylation and/or protein carboxymethylation influenced Na^+-H^+ exchange, sodium-stimulated proton efflux was assessed in colonic vesicles incubated for 120 min in the presence and absence of 200 μM *S*-adeno-

TABLE I

ENDOGENOUS AND EXOGENOUS PROTEIN CARBOXYMETHYLATION SPECIFIC ACTIVITIES IN RAT COLONIC BRUSH-BORDER MEMBRANES

Values are means ± S E of three different determinations AdoHcy, *S*-adenosyl-L-homocysteine

Incubation conditions	Protein carboxymethylation at 37°C (pmol/mg protein per h)
Endogenous	1327 ± 106
Endogenous + 2 mM AdoHcy	658 ± 62
Exogenous	1648 ± 128
Exogenous + 2 mM AdoHcy	804 ± 71

TABLE II

FLUORESCENCE POLARIZATION STUDIES

Values are mean ± S E of twelve determinations of six different preparations of membranes at 25°C See Methods section for further details * $P < 0.05$ or less compared to control values AdoMet, *S*-adenosyl-L-methionine, AdoHcy, *S*-adenosyl-L-homocysteine DPH, diphenylhexatriene

Probe	Condition	Anisotropy, r	Limiting hindered anisotropy, r_∞	Order parameter S
DPH	Control	0.190 ± 0.002	0.153 ± 0.002	0.647 ± 0.005
	200 μM AdoMet	0.168 ± 0.002 *	0.124 ± 0.002 *	0.581 ± 0.004 *
	2 mM AdoHcy	0.191 ± 0.002	0.155 ± 0.002	0.652 ± 0.004
	200 μM AdoMet + 2 mM AdoHcy	0.192 ± 0.001	0.156 ± 0.002	0.654 ± 0.005
2-AS	Control	0.139 ± 0.003	–	–
	200 μM AdoMet	0.118 ± 0.001 *	–	–
	2 mM AdoHcy	0.138 ± 0.003	–	–
	200 μM AdoMet + 2 mM AdoHcy	0.139 ± 0.004	–	–
12-AS	Control	0.136 ± 0.003	–	–
	200 μM AdoMet	0.116 ± 0.002 *	–	–
	2 mM AdoHcy	0.136 ± 0.002	–	–
	200 μM AdoMet + 2 mM AdoHcy	0.135 ± 0.002	–	–

syl-L-methionine As shown in Fig 2, *S*-adenosyl-L-methionine-treated vesicles demonstrated greater proton efflux than control vesicles at all concentrations of sodium (2 5–50 mM) examined Sodium-stimulated proton efflux in vesicles treated

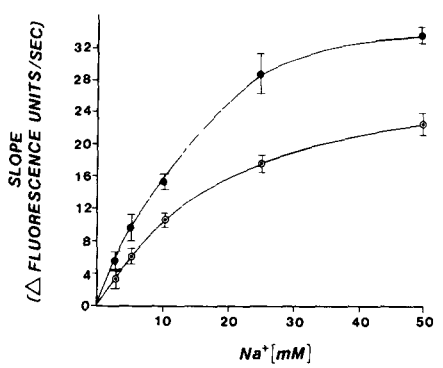

Fig 2 Effect of methylation on sodium-stimulated proton efflux Colonic brush-border membrane vesicles were preincubated with or without 200 μM *S*-adenosyl-L-methionine for 120 min at 37°C and then sodium-stimulated proton efflux measured, using the pH-sensitive fluorescent dye, acridine orange, as described under Experimental Procedures The effect of incubation with (●) and without (○) *S*-adenosyl-L-methionine on the initial slope of fluorescence reappearance (change in fluorescence units per s) as a function of different sodium concentrations (2 5–50 mM) is shown Values represent mean ± S E of three separate preparations of vesicles under each condition

with 2 mM *S*-adenosyl-L-homocysteine plus 200 μM *S*-adenosyl-L-methionine, however, was similar to control values (not shown)

Based on the data obtained in Fig 2, maximal velocity (V_{max}) and K_m kinetic parameters were determined for sodium-stimulated proton efflux in the presence and absence of 200 μM *S*-adenosyl-L-methionine using double-reciprocal plots [41] *S*-Adenosyl-L-methionine increased the V_{max} (expressed in arbitrary fluorescence units) by 36 1 ± 3 2% ($N = 3$) ($P < 0.01$) The K_m for sodium (approx 20 mM) for *S*-adenosyl-L-methionine treated and untreated vesicle preparations was similar (not shown)

Effect of methylation on membrane lipid fluidity *

Using the three fluorophores, steady-state fluo-

* The term 'lipid fluidity' as applied to model bilayer and natural membranes is used throughout this report to express the relative motional freedom of the lipid molecules or substituents thereof More detailed descriptions have been published [31] Briefly, as evaluated by steady-state fluorescence polarization of lipid fluorophores, fluidity is assessed by the parameters of the modified Perrin equation described under Experimental Procedures An increase in fluidity corresponds to a decrease in either the correlation time, T_c, or the hindered anisotropy, r_∞, of the fluorophore thereby combining the concepts of the dynamic and static (lipid order) components of fluidity

rescence polarization studies demonstrated that rat colonic brush-border membranes incubated with 200 μM *S*-adenosyl-L-methionine (pH 8.0) for 120 min were significantly more fluid than control membranes (Table II). *S*-Adenosyl-L-homocysteine (2 mM) alone did not alter fluidity. Incubation of 200 μM *S*-adenosyl-L-methionine with 2 mM *S*-adenosyl-L-homocysteine, however, inhibited the effect of *S*-adenosyl-L-methionine on fluidity (Table II).

Discussion

The mammalian colon is important in the maintenance of normal electrolyte and water balance [42]. In vivo, this organ absorbs sodium, chloride and water and secretes potassium and bicarbonate [42]. In the rat large intestine while electrogenic sodium absorption is present, the predominant sodium absorptive process appears to be electroneutral [42]. Recent sudies [43,44] have suggested that this neutral sodium absorptive mechanism may involve a Na^+-H^+ exchange process.

The large intestine assumes added importance in view of the increased incidence of malignant transformation that occurs in this organ [45]. In this regard, prior studies have implicated stimulation of Na^+-H^+ exchange as an initial event in differentiation and/or proliferation of normal [4] and malignant [3] cells. Na^+-H^+ exchange may, therefore, be involved in a number of important processes in the colon.

Prior in vitro studies in brush-border membrane vesicles prepared from rabbit proximal tubules have demonstrated that Na^+-H^+ exchange activity could be increased by such in vivo perturbations as (1) thyroparathyroidectomy [11], (2) dexamethasone administration [10], and (3) metabolic acidosis [10,11]. Furthermore, the severity of the metabolic acidosis was shown to correlate well with increases in the maximal velocity (V_{max}) of this exchange activity without changing the affinity (K_m) of the exchanger for sodium in these vesicles [12].

Recent experiments in our laboratory using benzyl alcohol, a known fluidizer [46], have, in fact, revealed a relationship between rat colonic brush-border membrane vesicular fluidity and Na^+-H^+ exchange [47]. Increases in fluidity were correlated with increases in the V_{max} without affecting the K_m for sodium of this process [47].

The present studies demonstrate for the first time that transmethylation of phospholipids and/or protein can also influence this exchange process in rat colonic brush-border membrane vesicles. Under conditions where phospholipid methylation and carboxymethylation were increased, the V_{max} of sodium-stimulated proton efflux was also increased by approximately 36% with no change in the K_m for sodium. The addition of *S*-adenosyl-L-homocysteine inhibited phospholipid methylation and protein carboxymethylation and also inhibited stimulation of proton efflux by *S*-adenosyl-L-methionine.

Methylation of membrane phospholipids has been noted to influence the function of carriers and receptors in membranes [48], although recently these observations have been questioned [20,49,50]. Carboxymethylation of proteins has also been reported to modify enzyme activities [14]. Since both activities were stimulated by *S*-adenosyl-L-methionine and inhibited to approximately the same extent by *S*-adenosyl-L-homocysteine, it is not clear which of these methylation reactions is responsible for modulating Na^+-H^+ exchange activity in rat colonic brush-border membrane vesicles.

In 1978, Hirata and Axelrod [51] suggested that enzymatic methylation of phosphatidylethanolamine in erythrocyte membranes of the rat increased the lipid fluidity of the membranes. Subsequent studies in other plasma membranes, however, have failed to support the contention that phospholipid methylation modulates membrane fluidity [20,49]. In view of our previous studies which demonstrated that changes in fluidity induced by benzyl alcohol could influence Na^+-H^+ exchange in rat colonic brush-border membrane vesicles, it was of interest to determine whether methylation influenced colonic plasma membrane fluidity. As shown in Table II, incubation of colonic plasma membranes with 200 μM *S*-adenosyl-L-methionine (pH 8.0) at 37°C for 120 min significantly increased their fluidity, as assessed by steady-state fluorescence polarization techniques using three different fluorophores. While 2 mM *S*-adenosyl-L-homocysteine alone did not alter fluidity, incubation of 200 μM *S*-adenosyl-L-

methionine with 2 mM *S*-adenosyl-L-homocysteine inhibited the effect of *S*-adenosyl-L-methionine on fluidity The results, taken together with our previous studies [47], suggest that methylation of phospholipid and/or protein may influence membrane fluidity which, in turn, may modify Na^+-H^+ exchange These experiments, however, do not exclude other possibilities such as direct influence of the products of methylation on the exchange process or changes in lipid/protein charge following a redistribution of lipid/protein species Further studies will be necessary to elucidate the exact mechanism(s) whereby methylation of phospholipids and/or proteins modulate Na^+-H^+ exchange Regardless of the mechanism(s), however, it does appear that methylation of membrane constituents can influence Na^+-H^+ exchange in rat colonic brush-border membrane vesicles

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