S-Adenosyl-L-methionine modulates $\text{Na}^+ + \text{K}^+$ -ATPase activity in rat colonic basolateral membranes

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Rat colonic basolateral membranes were incubated with *S*-adenosyl-L-[methyl- ^3H]methionine (0.3 mM) at 37 °C for 2 h at pH 9.0. This resulted in an increase in the specific activity of $\text{Na}^+ + \text{K}^+$ -ATPase by 60 %. Kinetic parameter analysis revealed a 2-fold increase in the V_{max} of this enzymatic activity, whereas the K_m for ATP was unchanged. The methylation inhibitor *S*-adenosyl-L-homocysteine (2 mM) significantly reduced these *S*-adenosyl-L-methionine-stimulated increases in specific activity and the V_{max} of $\text{Na}^+ + \text{K}^+$ -ATPase. *S*-Adenosyl-L-methionine treatment of basolateral membranes was also found to significantly increase the fluidity of these preparations, as assessed by steady-state fluorescence polarization techniques using the fluorophore 1,6-diphenyl-1,3,5-hexatriene; *S*-adenosyl-L-homocysteine (2 mM) again markedly reduced this *S*-adenosyl-L-methionine-induced increase in fluidity. While transmethylation reactions involving phospholipids, non-polar lipids and proteins were all found to exist in rat colonic basolateral membranes, based on a number of observations, the results of the present studies suggest that transmethylation of membrane phospholipids, but not membrane non-polar lipids or proteins, influenced the fluidity of basolateral membranes which, in turn, modified $\text{Na}^+ + \text{K}^+$ -ATPase activity in these membranes.

INTRODUCTION

Membrane lipids have been shown to play an important role in the regulation of sodium–potassium-dependent adenosine triphosphatase ($\text{Na}^+ + \text{K}^+$ -ATPase) activity (Grisham & Barnett, 1972, 1973; Jarnefelt, 1972; Roelofsen & Van Deenen, 1973; Keefe *et al.*, 1979; Wood *et al.*, 1985). During the past several years, our laboratory has examined the interactions of proteins and lipids of plasma membranes of rat small and large intestinal epithelial cells (Brasitus & Schachter, 1980, 1984; Brasitus, 1983; Brasitus & Keresztes, 1983; Brasitus *et al.*, 1985a,b). In agreement with earlier studies (Grisham & Barnett, 1972, 1973; Jarnefelt, 1972; Roelofsen & Van Deenen, 1973; Keefe *et al.*, 1979; Wood *et al.*, 1985) several of the protein-mediated activities, including $\text{Na}^+ + \text{K}^+$ -ATPase, were found to be influenced by alterations in the composition and physical state of intestinal basolateral membrane lipids (Brasitus & Schachter, 1980; Brasitus, 1983).

In this regard, we have previously demonstrated that transmethylation reactions involved in the conversion of PE to PC (Dudeja *et al.*, 1986a,b), as well as in protein carboxymethylation (Dudeja *et al.*, 1986b) are present in the brush-border membranes of rat colonocytes and, furthermore, appear to modulate electroneutral sodium–proton exchange (Dudeja *et al.*, 1986b). Similar reactions in cultured toad bladder cells (Wisemann *et al.*, 1985) and apical membranes prepared from the cultured amphibian cell line A_6 (Sariban-Sohraby *et al.*, 1984) have also been shown to influence electrogenic sodium transport. *S*-Adenosyl-L-methionine is the methyl donor

for most known biological transmethylation reactions (Zatz *et al.*, 1981; Kloog *et al.*, 1982; Pelech & Vance, 1984), including those involved in the methylation of phospholipids, non-polar lipids and protein. Recently, Hattori & Kanfer (1984) have shown that *S*-adenosyl-L-methionine inhibited $\text{Na}^+ + \text{K}^+$ -ATPase activity in rat brain microsomes.

Accordingly, the present studies were undertaken to determine: (1) whether the enzymes responsible for these transmethylation reactions existed in rat colonic basolateral membranes; and (2) if present, whether these reactions influenced the activity of $\text{Na}^+ + \text{K}^+$ -ATPase. The results of these investigations demonstrate for the first time that enzymes involved in phospholipid methylation, non-polar lipid methylation and protein carboxymethylation exist in rat colonic basolateral membranes. The present data, moreover, demonstrate that *S*-adenosyl-L-methionine appears to modulate $\text{Na}^+ + \text{K}^+$ -ATPase via methylation of phospholipids. Methylation of neither non-polar lipids nor proteins appear to be involved in this phenomenon. These results as well as a discussion of the possible mechanism(s) involved in the regulation of $\text{Na}^+ + \text{K}^+$ -ATPase by *S*-adenosyl-L-methionine serve as the basis for the present report.

EXPERIMENTAL

Materials

S-Adenosyl-L-[methyl- ^3H]methionine (5–15.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Non-radioactive *S*-adenosyl-L-

Abbreviations used: $\text{Na}^+ + \text{K}^+$ -ATPase, Na^+ and K^+ -dependent adenosine triphosphatase; PE, phosphatidylethanolamine; PME, phosphatidyl-N-monomethylethanolamine; PDE, phosphatidyl-*NN*-dimethylethanolamine; PC, phosphatidylcholine; DPH, 1,6-diphenylhexa-1,3,5-triene.

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methionine, *S*-adenosyl-L-homocysteine, PE, phosphatidyl-*N*-monomethylethanolamine (PME), phosphatidyl-*N,N*-dimethylethanolamine (PDE), and PC were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and obtained from Fisher Chemical Co. (Fairlawn, NJ, U.S.A.) unless otherwise specified.

Preparation of basolateral membranes

Male Sherman rats weighing 250–300 g were fasted for 18 h with water *ad libitum* before being killed. The colons were excised, the caecum discarded, and epithelial cells, relatively devoid of goblet cells, were obtained using a technique which combines chelation of divalent cations with mild mechanical dissociation (Brasitus & Keresztes, 1984). Basolateral membranes were prepared by the method of Brasitus & Keresztes (1983). The purities of the membrane suspensions and resultant contamination from intracellular organelles were assessed by the use of appropriate enzyme markers (Brasitus & Keresztes, 1983). The specific activity ratios (purified basolateral membrane/crude homogenate) for $\text{Na}^+ + \text{K}^+$ -ATPase and ouabain-sensitive K^+ -phosphatase were approximately 15–18 using this preparative technique. As noted previously, specific activity ratios for alkaline phosphatase, succinic dehydrogenase and NADPH: cytochrome *c* reductase, marker enzymes for brush-border, mitochondrial and microsomal membranes, respectively, were 1–2 in these preparations (Brasitus & Keresztes, 1983). The basolateral membranes were suspended in appropriate buffers (see below) and used immediately. Membrane protein was determined, using bovine serum albumin as a standard, as described by Lowry *et al.* (1951).

Enzymic assays

Assay of phospholipid methylation. Phospholipid methylation was assessed by measuring the incorporation of [^3H]methyl groups from *S*-adenosyl-L-[methyl- ^3H]methionine into PME, PDE, and PC (Hirata *et al.*, 1978). The methylation reaction mixture contained, unless otherwise specified, 0.3 mM-*S*-adenosyl-L-[methyl- ^3H]methionine (2–4 μCi) and 50 mM-Tris/acetate buffer (pH 9.0) in a final volume of 0.5 ml. The mixture was preincubated at 37 °C for 10 min and the reaction started by addition of membranes (200 μg) and then allowed to incubate for 120 min, unless otherwise stated. The reaction was terminated by addition of 3 ml of chloroform/methanol/2 M-HCl (6:3:1, by vol.); 2 ml of 0.1 M-KCl in 50 % methanol was then added. The mixture was vortex-mixed and centrifuged at 200 *g* for 10 min. The aqueous phase was removed and discarded, while the chloroform phase was washed twice with 2 ml of 0.1 M-KCl in 50 % methanol. The chloroform phase was then dried down completely under N_2 and resuspended in 100 μl of chloroform. Each sample (80 μl) was applied to a silica-gel G plate and developed in a solution of chloroform/propionic acid/propanol/water (2:2:3:1, by vol.). Standards containing PE, PME, and PC were run simultaneously. The locations of the standards and samples were visualized using a saturated solution of iodine in chloroform. Areas corresponding to the standard phospholipids PE, PME, PDE and PC were scraped separately from the plate and placed in scintillation vials containing Scintiverse E and the activity measured by a Beckman liquid-scintillation counter as

reported previously (Hirata *et al.*, 1978). The levels of PE, PME, PDE and PC were measured as described by Katyal & Lombardi (1974). The chemical identity of the methylated products was further defined by hydrolysis of the phospholipids and identification of their free bases as described by Schneider & Vance (1979). Additionally, both labelled and unlabelled *S*-adenosyl-L-methionine were routinely purified by ion-exchange before use (Audubert & Vance, 1983).

Assay of non-polar lipid methylation. Non-polar lipid methylation was usually assayed in conjunction with phospholipid methylation as described above. Whenever non-polar methylation alone was assessed it was assayed according to the method of Zatz *et al.* (1981) after heptane extraction of the basolateral membranes.

Assay of protein carboxymethylation. Protein carboxymethylation of rat colonic basolateral membranes was assayed according to the method of Axelrod & Daly (1965), unless otherwise specified, at pH 6.0 using bovine serum albumin (20 mg/ml) as an exogenous methyl acceptor. In the assay of endogenous protein carboxymethylation, albumin was omitted. Boiled enzyme preparations were used as blanks (Axelrod & Daly, 1965).

Assay of $\text{Na}^+ + \text{K}^+$ -ATPase. $\text{Na}^+ + \text{K}^+$ -ATPase was assayed according to method of Siegel & Goodwin (1972) as previously described (Brasitus & Schachter, 1980). The reaction mixture contained 10 mM-imidazole/HCl (pH 7.1), 5 mM- MgCl_2 , 2.5 mM-ATP, 120 mM-NaCl, 20 mM-KCl $\pm$ 1.25 mM ouabain, and basolateral membrane (50–100 μg), in a total volume of 80 μl . The reaction was started by the addition of the ATP/ MgCl_2 . After 15 min at 37 °C, the reaction was terminated by addition of 20 μl of 5 % SDS. The samples were then centrifuged at 2000 *g* for 2 min. The amount of P_i liberated from the substrate into the supernatant was assayed by the method of Gomori (1942). $\text{Na}^+ + \text{K}^+$ -ATPase activity was calculated as the difference between ouabain-inhibitable ATPase activity and total ATPase activity (Siegel & Goodwin, 1972). The reaction was linear with respect to time and protein.

Liposomes

Liposomes were prepared as previously described (Brasitus *et al.*, 1979). Briefly, to prepare the liposomes, suspensions of lipids extracted from basolateral membrane preparations were placed in phosphate-buffered saline and sonicated for 10 min, under N_2 , at 5 °C. Thereafter, the preparations were centrifuged at 10000 *g* for 10 min and the resulting supernatant used for fluorescence polarization experiments (see below). In certain experiments, non-polar lipids were first separated from phospholipids (Katz *et al.*, 1976) and liposomes then prepared from the extracted basolateral membrane phospholipids as described above.

Fluorescence polarization studies

Steady-state fluorescence polarization studies were performed using a Perkin-Elmer 650–40 fluorescence spectrofluorometer (Perkin-Elmer, Norwalk, CT, U.S.A.) fitted with an automatic polarizer (C. N. Wood, Newtown, PA, U.S.A.). The methods used to load the membranes and liposomes and the quantification of the

polarization of fluorescence have been discussed previously (Schachter & Shinitzky, 1977; Brasitus *et al.*, 1979; Brasitus *et al.*, 1980). Using this instrument, fluorescence emission intensities recorded parallel and perpendicular to the excitation plane of the probe could be taken sequentially and the polarization of the fluorescence calculated or anisotropy values could be obtained directly from the instrument. These measurements were found to differ by 2 % or less. The content of the fluorophore in the preparations was estimated fluorimetrically as described by Cogan & Schachter (1981). Final molar ratios of probe/lipid ranged from 0.001 to 0.002 and the anisotropy difference noted in these studies could not be ascribed to differences in probe concentration in the membranes. Corrections for light scattering (suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the preparations after each estimation) were made routinely, and the combined corrections were less than 2 % of the total fluorescence intensity observed for the 1,6-diphenyl-1,3,5-hexatriene (DPH)-loaded preparations.

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 (Brasitus *et al.*, 1979). [The term 'lipid fluidity' as applied to anisotropic bilayer membranes is used to denote the relative motional freedom of the lipid molecules or substituents thereof. A more detailed description of the sense in which the term is used is given in Brasitus & Schachter (1982). Briefly, as evaluated by steady-state fluorescence polarization of lipid fluorophores, fluidity is assessed via the parameters of the modified Perrin equation described in the Experimental section. An increase in fluidity corresponds to either a decrease in the correlation time, T_c , or the hindered anisotropy, r_∞ , of the fluorophore. Hence, the term combines the concept of the dynamic and static (lipid order) components of fluidity.] The results were obtained according to the modified Perrin relationship (Heyn, 1979; Jahnig, 1979);

$$r = r_\infty + (r_0 - r_\infty) [T_c / (T_c + T_f)]$$

where r_0 is the maximal limiting anisotropy, taken as 0.365 for DPH (Shinitzky & Barenholz, 1974), 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_∞ for DPH were calculated from r values as previously described by Van Blitterswijk *et al.* (1981). The 'static' component of membrane fluidity was assessed by an order parameter, S , where

$$S = (r_\infty / r_0)^{1/2}$$

as described previously (Heyn, 1979; Jahnig, 1979). Possible changes in the excited-state lifetimes of DPH were monitored by calculation of the total fluorescence intensity (F), $F = I_{\parallel} + I_{\perp}$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities oriented parallel and perpendicular to the direction of polarization of the exciting light, respectively (Shinitzky & Barenholz, 1974; Brasitus *et al.*, 1979). The changes in fluorescence polarization described in these studies were not accounted for by the changes in the excited-state lifetimes assessed by F .

Statistical methods

All results are expressed as the mean values $\pm$ s.e. Paired or unpaired t -tests were used for all statistical analyses. A P value of < 0.05 was considered significant.

RESULTS

Characterization of basolateral membrane phospholipid methylation

Identification of [³H]methylated phospholipids was determined by t.l.c. as previously described (Hirata *et al.*, 1978; Dudeja & Brasitus, 1985). Incubation of basolateral membranes with 0.3 mM-S-adenosyl-L-(methyl-³H)methionine yielded three major radioactive peaks corresponding to the methylated phospholipids PME, PDE, and PC which were identified at the R_F values noted in Fig. 1. The major product of this reaction was PME. The optimal pH for basolateral phospholipid methylation was determined by incubating the membranes and S-adenosyl-L-[methyl-³H]methionine in the following buffers: 50 mM-sodium acetate (pH 5.0 and 6.0); 50 mM-Tris/acetate (pH 7.0, 8.0 and 9.0); 50 mM-sodium borate (pH 10.0). Optimal activity was obtained at a pH of 9.0 (not shown) and all subsequent methylation reactions were carried out at this pH. In agreement with previous studies performed in rat colonic brush-border membranes (Dudeja *et al.*, 1986b), the incorporation of [³H]methyl groups into total phospholipids, PME, PDE and PC was linear for 120 min. The reaction was also found to be linear for up to 500 μ g of membrane protein (not shown).

The kinetic parameters of this enzyme then were determined by incubating basolateral membranes with

Fig. 1. Colonic basolateral membranes (0.3 mg of protein) incubated at 37 °C with 0.3 mM-S-adenosyl-L-[methyl-³H]methionine at pH 9.0

After 60 min the reaction was terminated by the addition of chloroform/methanol/2 M-HCl (6:3:1, by vol.). The reaction products were extracted and separated by t.l.c. Values represent the means $\pm$ s.e. of six separate preparations. Abbreviation: NPL, methylated non-polar lipids.

various concentrations of *S*-adenosyl-L-[methyl-³H]-methionine ranging from 0.025 mM to 0.4 mM. A double-reciprocal plot was obtained (Lineweaver & Burk, 1934) and yielded a straight line with an apparent K_m of 0.2 mM and a V_{max} of 1.82 nmol/h per mg of protein; accordingly all reactions were performed using 0.3 mM-*S*-adenosyl-L-[methyl-³H]methionine (pH 9.0). At an *S*-adenosyl-L-[methyl-³H]methionine concentration of 0.3 mM (pH 9.0), 2 mM-*S*-adenosyl-L-homocysteine was found to inhibit total phospholipid methylation by $45.0 \pm 5.0\%$ ($n = 6$). Formation of PME, PDE, and PC was also each inhibited by approximately 40–50% (not shown). Formation of methylated phospholipids was completely inhibited at 0 °C and when heat-denatured membranes were used.

Characterization of non-polar lipid methylation

In agreement with previous studies performed on rat lung membranes (Zatz *et al.*, 1981) as well as rat basophilic leukaemic cells, rat retinal cells, and rat parotid gland cells (Kloog *et al.*, 1982), incubation of 0.3 mM-*S*-adenosyl-L-[methyl-³H]methionine with basolateral membranes for 2 h at 37 °C and pH 9.0 led to the incorporation of [³H]methyl groups into non-polar lipids (Fig. 1). Non-polar lipids accounted for $39 \pm 5\%$ ($n = 4$) of the total methylated lipids present in these membranes. This reaction was found to be linear for 2 h with up to 500 µg of membrane protein (not shown). Formation of methylated non-polar lipids was also completely inhibited at 0 °C and when heat-denatured membranes were used. Additionally, *S*-adenosyl-L-homocysteine (2 mM) in the presence of 0.3 mM-*S*-adenosyl-L-methionine, inhibited non-polar lipid methylation by approx. 45%.

Characterization of protein carboxymethylation

Endogenous and exogenous protein carboxymethylation activities were also found to exist in rat colonic basolateral membranes. In agreement with earlier studies performed in the bovine pituitary gland (Diliberto & Axelrod, 1974), optimal activity in rat colonic basolateral membranes was obtained at pH 6.0 and virtually no endogenous or exogenous activity was seen at pH 9.0 (not shown). These results are summarized in Table 1. Additionally, as shown in Table 1, *S*-adenosyl-L-homocysteine (2 mM), in the presence of 0.3 mM-*S*-adenosyl-L-methionine, inhibited endogenous and exogenous protein carboxymethylation, respectively, by approx. 66% and 36%.

Effect of *S*-adenosyl-L-methionine on lipid composition

The lipid composition of basolateral membranes was examined in the presence and absence of 0.3 mM-*S*-adenosyl-L-methionine. Basal levels of PC, PME, PDE, and PE in colonic basolateral membranes were 51.9 ± 4.6 , 3.9 ± 0.1 , 2.1 ± 0.1 and 33.0 ± 4.6 µg/100 µg of protein respectively ($n = 6$). The latter values are much higher than previously reported in rat liver and lung (Katyal & Lombardi, 1974) and may explain, at least in part, why the present system demonstrates linear accumulation of reaction products for 120 min, unlike liver membranes (Vance & deKruiff, 1980; Audubert & Vance, 1983). These levels were not found to be significantly altered by *S*-adenosyl-L-methionine after 2 h (not shown).

Incubation of 0.3 mM-*S*-adenosyl-L-methionine was also not found to significantly influence the neutral or

other phospholipids of rat colonic basolateral membranes (not shown).

Effect of *S*-adenosyl-L-methionine on Na⁺ + K⁺-ATPase activity

As shown in Table 2, incubation of membranes with 0.3 mM-*S*-adenosyl-L-methionine at 37 °C for 1 and 2 h led to increases of approx. 30% and 60%, respectively, of the specific activity of Na⁺ + K⁺-ATPase when compared with control values. Addition of 2 mM-*S*-adenosyl-L-homocysteine reduced *S*-adenosyl-L-methionine-stimulated Na⁺ + K⁺-ATPase activity after 2 h to a level comparable with that seen in membranes incubated with *S*-adenosyl-L-methionine alone for 1 h (Table 2). *S*-Adenosyl-L-homocysteine (2 mM) alone, however, had no effect on this enzymatic activity (Table 2).

Analysis of the kinetic parameters of Na⁺ + K⁺-ATPase in the presence and absence of *S*-adenosyl-L-methionine by double-reciprocal plots (Lineweaver & Burk, 1934), moreover, demonstrated that this methyl donor significantly increased the V_{max} but did not alter the K_m for ATP of this enzymatic activity (Fig. 2; Table 3).

Effect of *S*-adenosyl-L-methionine on basolateral membrane and liposome fluidity

Previous studies on red blood cell membranes (Hirata & Axelrod, 1978) had demonstrated that incubation of

Table 1. Endogenous and exogenous protein carboxymethylation specific activities in rat colonic basolateral membranes

Values are means $\pm$ S.E. of four different determinations, * $P < 0.05$ or less compared with endogenous or exogenous control values. Abbreviation: AdoHcy, *S*-adenosyl-L-homocysteine.

Incubation conditions	Protein carboxymethylation at 37 °C (pmol/h per mg of protein)
Endogenous	675.3 $\pm$ 112.8
Endogenous + 2 mM-AdoHcy	231.7 $\pm$ 10.2*
Exogenous	741.3 $\pm$ 90.6
Exogenous + 2 mM-AdoHcy	475.9 $\pm$ 20.2*

Table 2. Na⁺ + K⁺-ATPase specific activity

Values represent means $\pm$ S.E. of the specified number of determinations (n). * $P < 0.05$ or less compared with control value. Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine.

Conditions	n	Specific activity (nmol of p _i /h per mg of protein)
Control	(4)	1212.3 $\pm$ 49.4
0.3 mM-AdoMet (1 h)	(4)	1581.5 $\pm$ 27.3*
0.3 mM-AdoMet (2 h)	(4)	1984.6 $\pm$ 85.7*
2.0 mM-AdoHcy (2 h)	(3)	1180.7 $\pm$ 31.8
0.3 mM-AdoMet + 2.0 mM-AdoHcy (2 h)	(4)	1484.7 $\pm$ 36.2*

these membranes with S-adenosyl-L-methionine could significantly increase their lipid fluidity, as assessed by steady-state fluorescence polarization techniques using the lipid-soluble fluorophore DPH. Recently, our laboratory has also demonstrated similar effects of this methyl donor on the fluidity of rat colonic brush-border membranes (Dudeja & Brasitus, 1985; Dudeja *et al.*, 1986b). It was, therefore, of interest to examine the effects of S-adenosyl-L-methionine on the fluidity of rat colonic basolateral membranes. As shown in Table 4, when these plasma membranes were incubated with 0.3 mM-S-adenosyl-L-methionine at 37 °C for 120 min, their lipid fluidity was found to be significantly increased. Liposomes prepared from the total extracted lipid of these membranes showed similar alterations (Table 4), as did phospholipid liposomes devoid of non-polar lipids (control, $r = 0.171 \pm 0.001$; treated $r = 0.154 \pm 0.001$, $n = 3$, $P < 0.05$). The S-adenosyl-L-methionine-induced changes in fluidity seen in the membranes were significantly reduced when 2 mM-S-adenosyl-L-homocysteine was added to the incubation mixture (Table 4). The latter agent alone had no effect on basolateral membrane fluidity (Table 4).

Effects of benzyl alcohol and methanol on membrane fluidity and Na⁺ + K⁺-ATPase kinetics

The observations described above suggested that at least one possible mechanism, whereby S-adenosyl-L-methionine-induced alterations in Na⁺ + K⁺-ATPase activity in basolateral membranes, was via stimulation of transmethylation reaction(s), leading to an increase in fluidity, which in turn increased the activity of Na⁺ + K⁺-ATPase. We reasoned that if this were true, other manipulations that 'fluidized' these colonic membranes should also lead to increases in Na⁺ + K⁺-ATPase activity. To test this hypothesis, basolateral membranes were incubated with various concentrations of benzyl alcohol (25 and 50 mM) and methanol (50 mM) and their effects on fluidity and Na⁺ + K⁺-ATPase activity were examined. The former agent is a known 'fluidizer' (Seeman, 1972; Brasitus & Schachter, 1980; Gordon *et al.*, 1980), whereas, the latter alcohol is not (Fernandez *et al.*, 1984). Presumably their disparate effects on fluidity are due to differences in the ability of these alcohols to partition into the membrane (Seeman, 1972; Gordon *et al.*, 1980). As shown in Table 5, 25 and 50 mM-benzyl alcohol increased basolateral membrane fluidity by approx. 10% and 16%, respectively, whereas, 50 mM-methanol had no effect on fluidity. Furthermore, addition of 25 and 50 mM-benzyl alcohol increased the $V_{\max}$ of Na⁺ + K⁺-ATPase by $56.1 \pm 3.7\%$ and $77 \pm 4.8\%$, respectively, compared with control membranes ($P < 0.05$) but did not affect this enzyme's K_m for ATP (Fig. 2; Table 3). In contrast, methanol did not affect either of these kinetic parameters for Na⁺ + K⁺-ATPase (Table 3).

Since Na⁺ + K⁺-ATPase present in sealed vesicles could not be measured due to the inaccessibility of ATP and/or accumulation or depletion of ions inside the vesicles; the findings noted above could theoretically be due to disruption of such vesicles by benzyl alcohol. This, in turn, might lead to an increase in assayable enzymic activities. To test this possibility, Na⁺ + K⁺-ATPase was assayed in the presence and absence of 0.05% Triton X-100. This detergent has previously been shown to open 'sealed' vesicles (Chauhan *et al.*, 1982). The specific

Fig. 2. S-Adenosyl-L-methionine-stimulated Na⁺ + K⁺-ATPase activity

Measured at 37 °C in rat colonic basolateral membrane using the assay described in the Experimental section. Representative double reciprocal plots of three separate experiments under control (■), 0.3 mM-S-adenosyl-L-methionine (○), 50 mM-benzyl alcohol (□), and 25 mM-benzyl alcohol (▲) conditions are shown. Studies using 2 mM-S-adenosyl-L-homocysteine + 0.3 mM-S-adenosyl-L-methionine as well as 50 mM-methanol yielded plots which were superimposable on the control plot and are therefore not shown (see Table 3).

Table 3. Kinetic parameters for Na⁺ + K⁺-ATPase of basolateral membranes of rat colon

Values represent means $\pm$ S.E. of four separate preparations. * $P < 0.05$ or less compared with control values. Abbreviations: AdoMet, S-adenosyl-L-methionine; Ado-Hcy, S-adenosyl-L-homocysteine.

Conditions	K_m (mM)	$V_{\max}$ ($\mu\text{mol/h per mg protein}$)
Control	4.1 ± 0.3	3.1 ± 0.1
0.3 mM-AdoMet (1 h)	4.0 ± 0.2	$3.9 \pm 0.2^*$
0.3 mM-AdoMet (2 h)	3.7 ± 0.5	$6.4 \pm 0.1^*$
0.3 mM-AdoMet + 2.0 mM-AdoHcy (2 h)	4.0 ± 0.4	$3.6 \pm 0.2^*$
25 mM-Benzyl alcohol	3.8 ± 0.3	$6.7 \pm 0.1^*$
50 mM-Benzyl alcohol	3.9 ± 0.5	$13.1 \pm 0.1^*$
50 mM-Methanol	3.8 ± 0.3	3.0 ± 0.1

activities of Na⁺ + K⁺-ATPase in these preparations were similar (not shown). It is, therefore, unlikely that benzyl alcohol increased this enzymic activity via this mechanism.

DISCUSSION

Phosphatidylcholine is a major phospholipid of many plasma membranes including rat colonic basolateral membranes (Brasitus & Keresztes, 1983). The enzymic synthesis of PC from PE via a methylation pathway was previously shown to be present in several plasma membranes (Diliberto & Axelrod, 1974; Crews *et al.*,

Table 4. Basolateral membrane and liposome fluorescence polarization studies using DPH

Values represent means $\pm$ S.E. of four different preparations of membranes at 25 °C and four determinations of two separate preparations of liposomes at 25 °C. * $P < 0.05$ or less compared with control values. Abbreviations: BLM, basolateral membranes; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine.

Conditions	Preparations	Anisotropy (r)	Limiting hindered anisotropy (r_{∞})	Order parameter (S)
Control	BLM	0.230 ± 0.001	0.207 ± 0.002	0.752 ± 0.004
	Liposomes	0.219 ± 0.002	0.192 ± 0.003	0.725 ± 0.006
0.3 mM-AdoMet (1 h)	BLM	$0.221 \pm 0.002^*$	$0.194 \pm 0.002^*$	$0.729 \pm 0.004^*$
	Liposomes	$0.202 \pm 0.003^*$	$0.169 \pm 0.004^*$	$0.680 \pm 0.007^*$
0.3 mM-AdoMet (2 h)	BLM	$0.214 \pm 0.001^*$	$0.185 \pm 0.002^*$	$0.712 \pm 0.004^*$
	Liposomes	$0.191 \pm 0.002^*$	$0.155 \pm 0.003^*$	$0.650 \pm 0.006^*$
2.0 mM-AdoHcy (2 h)	BLM	0.234 ± 0.003	0.212 ± 0.004	0.760 ± 0.007
0.3 mM-AdoMet + 2.0 mM-AdoHcy (2 h)	BLM	$0.224 \pm 0.001^*$	$0.199 \pm 0.002^*$	$0.737 \pm 0.003^*$

Table 5. Benzyl alcohol- and methanol-treated basolateral membrane fluorescence polarization studies using DPH

Values are means $\pm$ S.E. of six determinations of two different preparations of membranes at 25 °C. * $P < 0.05$ or less compared to control values.

Conditions	Anisotropy (r)	Limiting hindered anisotropy (r_{∞})	Order parameter (S)
Control	0.232 ± 0.002	0.209 ± 0.002	0.757 ± 0.004
25 mM-Benzyl alcohol	$0.209 \pm 0.002^*$	$0.179 \pm 0.002^*$	$0.700 \pm 0.003^*$
50 mM-Benzyl alcohol	$0.191 \pm 0.003^*$	$0.155 \pm 0.004^{**}$	$0.652 \pm 0.007^*$
50 mM-Methanol	0.230 ± 0.002	0.206 ± 0.003	0.751 ± 0.006

1978; Hirata *et al.*, 1978; Gordon *et al.*, 1980; Chauhan *et al.*, 1982; Fernandez *et al.*, 1984; Sariban-Sohraby *et al.*, 1984). This synthetic pathway for PC was not found to be present in the rat small intestine (Bremer & Greenberg, 1961) and had, therefore, been presumed to be absent from the entire gut. Recently, however, our laboratory has documented the existence of this transmethylation pathway for PC synthesis in rat colonic brush-border membranes (Dudeja *et al.*, 1986b; Dudeja & Brasitus, 1985). The present studies demonstrate for the first time that enzymic activities are also present in rat colonic basolateral membranes for transmethylation of phospholipids and non-polar lipids as well as carboxymethylation of proteins.

In agreement with earlier studies performed in rat colonic brush-border membranes (Dudeja *et al.*, 1986b), phospholipid methylation intermediates were found to accumulate linearly for up to 2 h in rat colonic basolateral membranes. Based on a number of criteria, it would appear that colonic brush-border membranes, unlike liver plasma membranes (Vance & deKruiff, 1980; Audubert & Vance, 1983), synthesize PC from PE by at least two distinct methyltransferases (Dudeja *et al.*, 1986a). By analogy with their luminal counterparts, colonic basolateral membranes may, therefore, possess

more than one methyltransferase. Clarification of the issue will, however, require further studies.

Earlier studies by our laboratory also localized $\text{Na}^+ + \text{K}^+$ -ATPase activity to the basolateral membrane of rat colonocytes (Brasitus & Keresztes, 1983). Furthermore, in agreement with studies performed in other membranes (Jarnefelt, 1972; Grisham & Barnett, 1972, 1973; Roelofsen & Van Deenen, 1973; Keefe *et al.*, 1979; Strittmatter *et al.*, 1979; Abeywardena *et al.*, 1984; Wood *et al.*, 1985), the lipid composition and fluidity of this plasma membrane was shown to be capable of altering the activity of this enzyme (Brasitus, 1983). Recently, Hattori & Kanfer (1984) found that S-adenosyl-L-methionine appeared to inhibit $\text{Na}^+ + \text{K}^+$ -ATPase in rat brain microsomes. In contrast with these prior observations (Hattori & Kanfer, 1984), the present results demonstrate that incubation of rat colonic basolateral membranes with S-adenosyl-L-methionine for 1 and 2 h increases the specific activity of $\text{Na}^+ + \text{K}^+$ -ATPase by approx. 30% and 60%, respectively, compared with control membrane values. Analysis of the kinetic parameters of $\text{Na}^+ + \text{K}^+$ -ATPase by double-reciprocal plots revealed that the $V_{\max}$ of this enzyme was significantly increased, whereas, the K_m for ATP was unchanged. Moreover, addition of S-adenosyl-L-homo-

cysteine (2 mM), which decreased transmethylation reactions by approx. 50%, was also found to decrease the S-adenosyl-L-methionine-stimulated $\text{Na}^+ + \text{K}^+$ -ATPase activity, seen after 2 h of incubation, by one-half. These observations support the contention that the effect of S-adenosyl-L-methionine on this basolateral membrane's enzymic activity is via transmethylation reaction(s).

In view of previous studies from our laboratory which demonstrated that $\text{Na}^+ + \text{K}^+$ -ATPase activity could be increased by increases in fluidity (Brasitus, 1983), it was of interest to determine whether methylation reactions in these basolateral membranes influenced colonic plasma membrane fluidity. As shown in Table 4, incubation of colonic basolateral membrane with 0.3 mM-S-adenosyl-L-methionine at 37 °C for 120 min, significantly increased their fluidity, as assessed by steady-state fluorescence polarization using DPH, while 2 mM-S-adenosyl-L-homocysteine significantly reduced the latter's effect on fluidity. Furthermore, in agreement with prior studies performed in rat small intestinal basolateral membranes (Brasitus & Schachter, 1980), incubation of rat colonic basolateral membranes with the known fluidizer, benzyl alcohol (Seeman, 1972; Gordon *et al.*, 1980), also stimulated $\text{Na}^+ + \text{K}^+$ -ATPase activity, whereas, incubation with methanol, which did not influence fluidity, also did not alter $\text{Na}^+ + \text{K}^+$ -ATPase activity. These results, therefore, suggest that transmethylation reaction(s) may influence basolateral membrane fluidity, which in turn, stimulates $\text{Na}^+ + \text{K}^+$ -ATPase activity.

Methylation of membrane phospholipids has been noted to influence many important physiological functions including certain membrane-bound enzymic activities (Hirata & Axelrod, 1980; Aloia *et al.*, 1983), although recently these observations have been questioned (Chauhan *et al.*, 1982; Audubert & Vance, 1983; Moore *et al.*, 1984). Carboxymethylation of proteins has also been reported to modify enzyme activities (Sariban-Sohraby *et al.*, 1984). Previous studies in rat erythrocyte membranes suggested that enzymic methylation of PE to form PME increased this membrane's fluidity (Hirata & Axelrod, 1978). Subsequent studies in other plasma membranes, however, have failed to support this finding (Chauhan *et al.*, 1982; Audubert & Vance, 1983; Moore *et al.*, 1984). In the present studies, although measurable increases in PME, PDE or PC could not be detected under incubation conditions in which membrane fluidity was increased, a role for phospholipid methylation in the observed fluidity changes induced by S-adenosyl-L-methionine is not necessarily precluded. In this regard, it should be noted that previous studies from our laboratory have indicated that various microdomains of lipids may exist in rat colonic (Brasitus, 1983) and small intestinal (Brasitus *et al.*, 1980) basolateral membranes. Moreover, the activity of $\text{Na}^+ + \text{K}^+$ -ATPase appears to reside in a more fluid microdomain than other rat, large or small intestinal, basolateral membrane enzymic activities such as adenylate cyclase (Brasitus *et al.*, 1980; Brasitus, 1983). These observations, taken together with the present results, suggest that the fluorescent probe used in the current experiments, DPH, might monitor local changes in fluidity which correlate with increased phospholipid methylation and increased $\text{Na}^+ + \text{K}^+$ -ATPase activity. Previous studies (Barrow & Lentz, 1985) have, in fact, suggested that DPH may indeed localize into different microdomains of lipid present in various plasma mem-

branes. Along these same lines the present experiments, which demonstrate that liposomes prepared from phospholipids of S-adenosyl-L-methionine-treated basolateral membranes are more fluid than their control counterparts (see the Results section), lend additional support to the contention that phospholipid methylation increases membrane fluidity which, in turn, increases $\text{Na}^+ + \text{K}^+$ -ATPase.

Although, to date, no studies have shown that methylation of non-polar lipids or carboxymethylation of proteins could influence membrane fluidity, it was thought to be theoretically possible that the S-adenosyl-L-methionine-induced changes in membrane fluidity were secondary to these transmethylation reactions. In this regard, however, the virtual absence of carboxymethylation activity at pH 9.0 rules out the possibility that methylation of proteins was responsible for the S-adenosyl-L-methionine-induced fluidity changes seen at that pH.

Similarly, the present experiments using phospholipid liposomes devoid of non-polar lipids would also suggest that the methylation of non-polar lipids was not responsible for the S-adenosyl-L-methionine-induced basolateral membrane fluidity changes. Despite this latter observation, however, the finding of significant quantities of methylated non-polar lipids in rat colonic basolateral membranes incubated with S-adenosyl-L-methionine is still interesting. In agreement with the present results, previous studies (Zatz *et al.*, 1981; Kloog *et al.*, 1982) have demonstrated the presence of non-polar lipid methylation reactions in the membranes of a number of different cell types. In rat lung membranes these non-polar lipid products have been identified as fatty acid methyl esters (Zatz *et al.*, 1981), while in rat basophilic leukaemic cells, rat retinal cells and parotid gland cells, both ubiquinone-9(Q9) and fatty acid methyl esters were found (Kloog *et al.*, 1982). While the exact identity of these methylated non-polar lipids in rat colonic basolateral membranes remains unclear, preliminary studies by our laboratory suggest that they are not fatty acid methyl esters (unpublished work). Identification of these methylated products as well as clarification of their possible role in the various physiological processes that occur in rat colonic basolateral membranes, will, however, require further studies.

In summary, the present results suggest that S-adenosyl-L-methionine increases colonic basolateral membrane fluidity via phospholipid methylation reactions which, in turn, lead to an increase in $\text{Na}^+ + \text{K}^+$ -ATPase activity in rat colonic basolateral membranes.

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