Biochemical Pharmacology, Vol. 33, No. 22, pp. 3547–3551, 1984.
Printed in Great Britain.

0006–2952/84 $3.00 + 0.00

CORRELATIONS BETWEEN PHOSPHOLIPID METHYLATION AND NEURONAL CATECHOLAMINE TRANSPORT*

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(*Received* 6 *July* 1983; *accepted* 13 *February* 1984)

Abstract—A change in the fluidity of biological membranes can be produced by methylation reactions which sequentially transfer methyl groups from phosphatidylethanolamine to phosphatidylcholine. Since the physical properties of membranes may affect the function of membrane-localized transport proteins, the accumulation of norepinephrine (NE) by rat cortical synaptosomes was examined in the presence of *S*-adenosylhomocysteine (AdoHcy) which inhibits the methylation of phospholipids. A concentration-related decrease in the uptake of [^{3}H]NE was produced by AdoHcy with coincident decreases in the *S*-adenosylmethionine (AdoMet)-dependent transmethylation of phospholipids in neuronal membranes. A kinetic analysis for the effects of AdoHcy on the neuronal uptake of NE revealed a significant decrease in both the apparent K_m and V_{max}. Treatment of synaptosomes with adenosine, L-homocysteine thiolactone (HTL), and erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) which leads to the synthesis of intracellular AdoHcy resulted in a decrease in the V_{max} with no significant change in the K_m. Adenosine or EHNA alone had no effect on NE uptake, but HTL alone significantly inhibited NE uptake. The data suggest that the processes of enzymatic methylation of membrane phospholipids and the transport of norepinephrine may be associated within neuronal membranes. Inhibiting phospholipid methylation reactions can reduce the efficiency of neurotransmitter removal and perhaps indirectly alter synaptic function.

The enzymatic methylation of membrane phospholipids has been demonstrated to alter the biophysical as well as the functional properties of various cells [1]. Two methyltransferases involved in converting phosphatidylethanolamine to phosphatidylcholine have been demonstrated in the adrenal medulla [2], erythrocytes [3], and synaptosomes [4]. Stimulation of these enzymatic reactions can induce an increase in membrane fluidity [5] as well as an increase in membrane-localized Ca^{2+}-ATPase activity [6]. Although the function of these enzymes in central neurons is not clear, data involving the use of inhibitors of methylation reaction in other systems, such as mast cells [7] and human basophils [8], have been used to support a general role for phospholipid methylation in secretory processes [1]. A neurosecretory role for phospholipid methylation is also supported by the demonstration that inhibition of transmethylation can increase the depolarization-dependent release of norepinephrine from pheochromocytoma cells [9].

The composition and physical characteristics of biological membranes have been examined extensively in an attempt to correlate biophysical properties with various functional aspects of membrane-localized proteins. The erythrocyte membrane, for example, contains asymmetrically distributed phospholipids and cholesterol arranged in a fluid mosaic, bimolecular leaflet [10]. It has been demonstrated in erythrocyte ghosts that the stimulation of phospholipid methylation reactions can increase membrane fluidity [5]. Since the biophysical properties of a cholesterol-rich membrane can be altered by phospholipid methylation reactions, a carrier-mediated diffusional process such as norepinephrine uptake might also be affected by changes in the activity of AdoMet‡-dependent methylation reactions. The purpose of this study was to correlate inhibition of AdoMet-dependent methylation of phospholipids with inhibition of neuronal uptake of [^{3}H]norepinephrine ([^{3}H]NE). Inhibition of methylation was achieved with *S*-adenosylhomocysteine (AdoHcy) and with the combination of adenosine, erythro-9(2-hydroxy-3-nonyl)adenine (EHNA) and homocysteine thiolactone (HTL) which leads to elevations of endogenous AdoHcy.

MATERIALS AND METHODS

Uptake of [^{3}H]norepinephrine in the presence of AdoHcy. The accumulation of [^{3}H]NE by cortical synaptosomes was measured by the methods of Snyder and Coyle [11]. Male rats (Sprague–Dawley, 140–200 g) were decapitated, and cerebral cortices were placed in iced beakers containing modified

* This study was supported by USPHS-NIH Grant NS 16364 and General Research Support Grant 5606 as well as the Center for Biomedical Research—The University of Kansas.

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‡ Abbreviations: AdoMet, *S*-adenosyl-L-methionine; NE, norepinephrine; AdoHcy, *S*-adenosyl-L-homocysteine; Ado, adenosine; HTL, L-homocysteine thiolactone; EHNA, erythro-9(2-hydroxy-3-nonyl)adenine; and TCA, trichloroacetic acid.

Krebs–Henseleit buffer supplemented with 1.25 μM nialamide. All procedures were performed at 4° except where noted. Cerebral cortical tissues from two rats were combined and homogenized by hand in 0.25 M sucrose using a teflon-on-glass homogenizer. The homogenate was decanted and centrifuged at 1000 *g* for 10 min. The supernatant fraction (S_1) was subsequently decanted into an iced test tube. A 0.3-ml aliquot of the S_1 supernatant was placed into reaction tubes containing 3.0 ml of freshly gassed (95% O_2:5% CO_2) Krebs–Henseleit buffer and the appropriate concentration of AdoHcy. Control samples consisted of co-incubated reaction mixtures maintained at 4°.

All reactions were preincubated for 10 min at 37°, and a 0.2-ml aliquot of [^{3}H]NE (sp. act. 3.2 Ci/mmole; final concentration 70 nM) was added. Incubations were performed for an additional 5 min at 37° and placed on ice. All samples were then centrifuged for 20 min at 20,200 *g*. The resulting pellets were washed once with 3.0 ml of iced saline or Krebs buffer, resuspended in 3.0 ml of ethanol, and recentrifuged for 15 min at 20,200 *g*. A 0.2-ml aliquot of this supernatant fraction was assayed for tritium by liquid scintillation spectrometry, and the remaining ethanol decanted. Protein contents were determined on the resulting pellets by the method of Layne [12].

Phospholipid methylation assay. The protocol utilized was that of Crews *et al.* [4]. Male Sprague–Dawley rats (140–225 g) were decapitated, and the cerebral cortices were removed. Tissues from two rats were immediately placed into iced beakers containing buffer which had been equilibrated with O_2:CO_2 (95%:5%). All tubes and beakers were placed in ice except where noted. The cortices were dissected, and tissues were pooled in a Teflon-on-glass homogenizer containing 10 ml of 0.25 M sucrose. The tissues were gently hand homogenized and the homogenates transferred to polyethylene test tubes. The homogenate was centrifuged at 1000 *g* for 10 min at 2–4°. The resulting supernatant fraction was centrifuged for 20 min at 10,000 *g* to produce a crude synaptosomal suspension (P_2). The P_2 pellet was suspended in freshly gassed (95% O_2:5% CO_2) Krebs–Henseleit buffer (2.4 ml) and gently agitated on a Vortex mixer. To produce labeling of the endogenous Ado Met Pool, L-[^{3}H-*methyl*]-methionine was added. The crude mitochondrial fraction was incubated for an additional 15 min with intermittent agitation on a Vortex mixer to maintain the suspension. The labeling incubation was terminated by centrifugation at 10,000 *g* for 20 min. This procedure resulted in an AdoMet-labeled pellet fraction (P_2'). The P_2' pellet was suspended in 11.0 ml of gassed buffer.

A 0.5-ml aliquot of the P_2' synaptosome suspension was added to capped 6.0 ml polyethylene test tubes containing 3.0 ml of gassed Krebs–Henseleit buffer and the appropriate concentration of drugs(s) or vehicle. The 15-min incubations were terminated by the addition of 3.0 ml of 20% (w/v) neutralized (pH 7) trichloroacetic acid (TCA) containing 30–40 nM methionine as a chemical carrier for subsequent separation procedures. TCA was neutralized to avoid hydrolysis of polar head groups under acid conditions. Controls consisted of co-incubated reaction mixtures to which the TCA solution was added prior to incubation at 37°. Following the incubation, the tubes were placed on ice for 10 min and centrifuged for 15 min at 20,200 *g*. The resulting supernatant fractions were discarded, and the pellets were suspended in 3.0 ml of chloroform–methanol (2:1). Each reaction tube was washed two times with 2.0 ml of 0.1 M KCl in 50% methanol and mixed for 10 min by suspending on a Vortex mixer. Aqueous and organic phases were separated at room temperature by centrifugation for 10 min at 2000 rpm. The aqueous wash was aspirated, and the second, washed organic phase was used for estimation of [^{3}H]methyl group incorporation into membrane phospholipids. A 1.0-ml aliquot of the organic phase was transferred to a scintillation vial and evaporated to dryness under vacuum. Ten milliliters of liquid scintillation counting fluid was added, and the radioactivity was measured by liquid scintillation spectrometry. Counting efficiency for ^{3}H was measured using the external standards ratio method and was found to be approximately 40%.

Kinetics of norepinephrine uptake. Alterations in the kinetics of uptake produced as a result of inhibiting phospholipid methylation were examined in a crude cytosolic suspension. The S_1 cytosolic suspension containing synaptosomes was prepared as previously described for uptake of [^{3}H]NE. In some experiments utilizing AdoHcy, a 100 μM stock solution was prepared, and both control and AdoHcy-treated synaptosomal suspensions were examined simultaneously for the accumulation of [^{3}H]NE. In experiments with AdoHcy, [^{3}H]NE was utilized at concentrations ranging from 23.5 nM to 0.35 μM. In experiments with EHNA, HTL and adenosine, concentrations of [^{3}H]NE ranged from 30 nM to 0.20 μM. Each experiment was then graphed as a double-reciprocal plot of [^{3}H]NE concentration versus the velocity of uptake. The apparent K_m and V_{max} were mathematically derived from the slope, and the y-intercept was determined by linear regression analysis of individual experiments.

Substances. Nialamide, adenosine, trichloroacetic acid, bovine serum albumin (Fraction V) powder, L-homocysteine thiolactone, and L-methionine were purchased from the Sigma Chemical Co., St. Louis, MO. AdoHcy was obtained from the Calbiochem-Behring Corp., La Jolla, CA. EHNA [erythro-9-(2-hydroxy-3-nonyl)adenine HCl] was obtained from Wellcome Research, Research Triangle Park, NC. Radiochemicals utilized were *levo*-[7-^{3}H(N)]-norepinephrine (3.2 Ci/mmole) from the New England Nuclear Corp., Boston, MA and L-[^{3}H-*methyl*]-methionine (18 Ci/mole) from ICN, Irvine, CA. All other reagents were of the highest purity obtainable, and all were purchased from commercial sources. Male Sprague–Dawley rats were purchased from Sasco, Inc., Omaha, NE.

RESULTS

In Fig. 1, the concentration–effect characteristics for AdoHcy on both phospholipid methylation and [^{3}H]NE uptake are presented. Over a concentration range of 0.1 μM to 1.0 mM, AdoHcy produced a

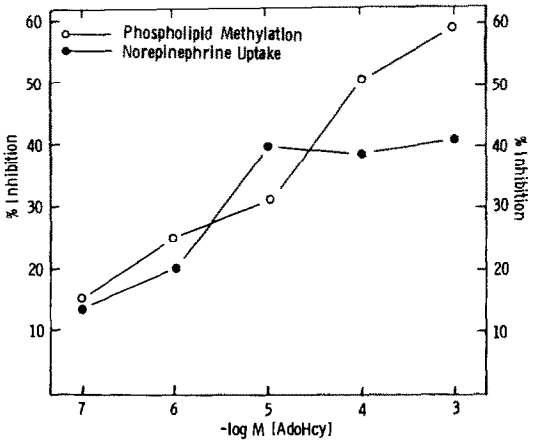

Fig. 1. Concentration-effect characteristics for AdoHcy on phospholipid methylation and [^{3}H]NE uptake. NE uptake was measured in cytosolic fraction containing cortical synaptosomes as described in Materials and Methods. [^{3}H]NE was present at a final concentration of 70 nM for 5 min. Phospholipid methylation was determined in a P_2 fraction after prelabeling with L-[^{3}H-*methyl*]methionine (3 μM). Data represent the mean percent of co-incubated controls from three to five experiments performed in duplicate.

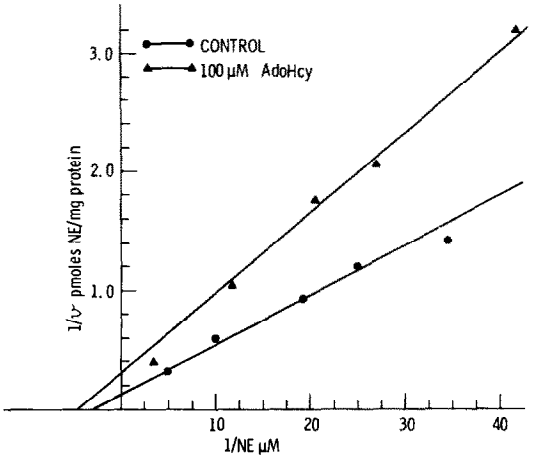

Fig. 2. Lineweaver–Burk plots for the kinetics of norepinephrine (NE) uptake into a cytosolic fraction of cerebral cortex. Data points represent the means of four separate experiments. The final [^{3}H]NE concentrations ranged from 23.5 nM to 0.35 μM. *S*-Adenosylhomocysteine is abbreviated as AdoHcy. Kinetic constants are presented in Table 1.

concentration related decrease in both [^{3}H]NE uptake and [^{3}H]methyl group incorporation into membrane phospholipids. A significant reduction ($P < 0.05$) in [^{3}H]NE uptake was observed at 10 μM AdoHcy, with 1 mM AdoHcy producing an approximately 50% reduction of NE accumulation. The lowest concentration of AdoHcy at which phospholipid methylation was inhibited was 1 μM ($P < 0.05$).

A double-reciprocal plot to examine the kinetic effects of AdoHcy on [^{3}H]NE uptake is illustrated in Fig. 2. The addition of AdoHcy (100 μM) produced a significant ($P < 0.05$) decrease in the apparent K_m for NE uptake while decreasing the maximal velocity by 50% (Table 1). These shifts are consistent with an uncompetitive mechanism for uptake inhibition.

The addition of adenosine, EHNA, and HTL has been shown to decrease AdoMet-dependent transmethylation reactions in human monocytes by producing elevations of cellular AdoHcy [13]. This effect is mediated by *S*-adenosylhomocysteine hydrolase, which catalyzes the synthesis of AdoHcy for adenosine and homocysteine [14–15]. In addition, adenosine and HTL *in vivo* has been shown to increase AdoHcy levels in brain and inhibit transmethylation reactions including phospholipid methylation [16, 17]. Only the combination of drugs was observed in the present study to decrease [^{3}H]methyl group incorporation into membrane phospholipids (Fig. 3). Double-reciprocal plots for examining the inhibition of [^{3}H]NE accumulation demonstrated that only the V_{max} was affected (Table 1 and Fig. 4). There were no significant effects of either adenosine or EHNA on the accumulation of [^{3}H]NE. However, 100 μM HTL alone did produce an inhibition of uptake (Table 2; $P < 0.001$).

DISCUSSION

The present data demonstrate that either AdoHcy or the combination of adenosine, HTL and EHNA can inhibit accumulation of [^{3}H]NE as well as inhibit phospholipid methylation. However, apparent differences in mechanism(s) may be evident since treatment with AdoHcy produces uncompetitive kinetic

Table 1. Kinetics of [^{3}H]NE uptake into cortical synaptosomes in the presence of inhibitors of transmethylation*

Drug	K_m (μM NE)	V_{max} (pmoles NE/mg protein)
Control	0.30 ± 0.02	6.73 ± 0.25
AdoHcy, 100 μM	0.15 ± 0.02†	3.83 ± 0.52†
Ado, 100 μM + EHNA, 10 μM + HTL, 100 μM	0.25 ± 0.04‡	3.40 ± 0.50†

* Values represent mean ± S.E.M. for four experiments. Abbreviations: AdoHcy, *S*-adenosylhomocysteine; Ado, adenosine; EHNA, erythro-9(2-hydroxy-3-nonyl)adenine; HTL, homocysteine thiolactone; NE, and norephrine.

† $P < 0.05$, treated vs control.

‡ $P < 0.05$, Ado + EHNA + HTL vs AdoHcy.

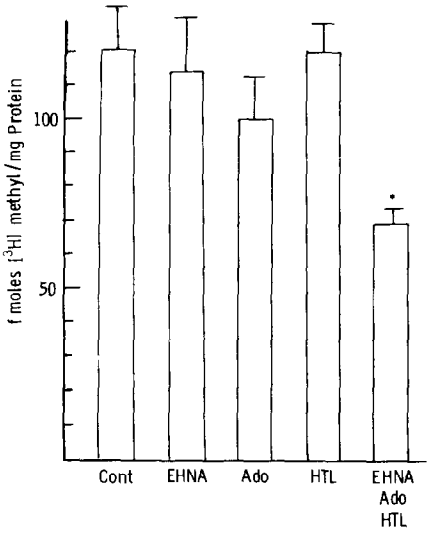

Fig. 3. Effects of EHNA, adenosine and HTL on [³H]methyl group incorporation into a crude suspension of cortical synaptosomes. Cortical tissues were prelabeled with L-[³H-*methyl*]methionine (3 μM). Data represent femtomoles (fmoles) of [³H]methyl incorporated/mg protein during a 15-min incubation. Abbreviations: EHNA, erythro-9(2-hydroxy-3-nonyl)adenine (20 μM); Ado, adenosine (100 μM); cont, control; and HTL, homocysteine thiolactone (100 μM). Data represent the mean ± S.E.M. for five to six experiments performed in duplicate. Key: (*)P < 0.001, treated vs control by unpaired Student's *t*-test.

changes, but the combination of adenosine, HTL and EHNA produces noncompetitive changes.

Apparent differences in the kinetics of uptake inhibition between these two treatments may involve the subcellular loci at which elevated AdoHcy levels are produced. Treatment of synaptosomes with adenosine, HTL and EHNA will elevate cytoplasmic

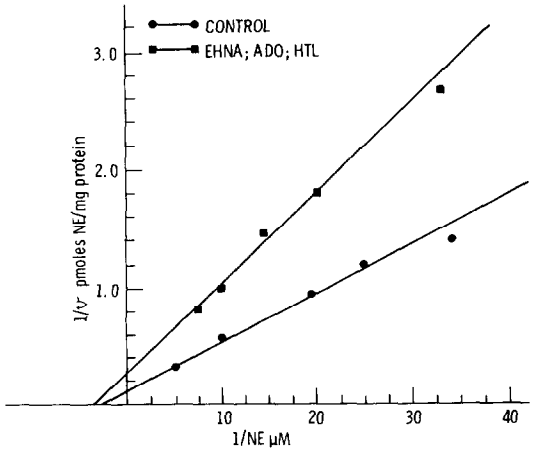

Fig. 4. Lineweaver–Burk analysis for [³H]NE uptake into a crude suspension of cortical synaptosomes. The crude synaptosomal suspension was incubated with [³H]NE concentrations ranging from 30 nM to 0.2 μM in the presence or absence of EHNA (20 μM), adenosine (100 μM) and HTL (100 μM). Kinetic constants are presented in Table 1.

Table 2. Effects of adenosine, HTL and EHNA on [³H]NE accumulation in cortical synaptosomes*

Drug	[³H]NE uptake (pmoles/mg protein)
Control	0.61 ± 0.08
Ado, 100 μM	0.64 ± 0.08
HTL, 100 μM	0.40 ± 0.04†
EHNA, 10 μM	0.60 ± 0.08
Ado, 100 μM + EHNA, 10 μM + HTL, 100 μM	0.42 ± 0.05†

* Values represent mean ± S.E.M. for three to five experiments performed in duplicate. Abbreviations: Ado, adenosine; HTL, homocysteine thiolactone; and EHNA, erythro-9-(2-hydroxy-3-nonyl)adenosine.

† $P < 0.001$, treated vs control.

AdoHcy because the hydrolase responsible for AdoHcy synthesis is a soluble enzyme [18, 19]. Adenosine is accumulated in synaptosomes by a facilitated diffusion process which has broad substrate specificity [20, 21]. Cytoplasmic elevations of AdoHcy will necessitate movements of AdoHcy to the membrane for inhibition of phospholipid methylation reactions. Alternatively, the incubation of synaptosomes with elevated extraneuronal AdoHcy will have direct effects on membrane-localized transmethylation reactions. These differences in the subcellular localization of AdoHcy may be, in part, responsible for differences in apparent affinities for the uptake carrier for NE. The locus for oxidative reduction of the thiolactone is unknown. Potential membrane sites are possible since HTL alone can inhibit NE uptake. Cytoplasmic sites of reduction are also likely since the thiolactone must be reduced prior to incorporation into AdoHcy. Since no experiments were performed in which the kinetic effects of HTL alone on [³H]NE uptake were examined, it is not possible to determine whether the combination of drugs and HTL are inhibiting uptake in a similar manner. HTL did differ from the drug combination in that the drug combination inhibited transmethylation whereas HTL was without effect. Therefore, it is possible that uptake inhibition by HTL treatment alone occurs independent of methylation-dependent effects.

The model proposed for the arrangement of methyltransferases in membranes is that methylase I is on the internal (cytoplasmic) membrane while methylase II faces the extracellular environment on the outer half of the membrane [1, 2]. Phospholipid methylase I requires Mg^{2+} for activity and has a high affinity for AdoMet [2, 4]. Elevations of AdoHcy outside of the nerve ending might be expected to alter only methylase II activity, whereas adenosine and homocysteine might produce elevations of intracellular AdoHcy accessible only to methylase I. Since methylase II has a low affinity for AdoMet [4], a high concentration of a competitive inhibitor would be required to alter AdoMet-dependent transmethylation reactions involving this enzyme.

The addition of AdoHcy or treatments with adenosine and homocysteine can inhibit phospholipid methyltransferases in brain [22, 23]. However, dif-

ferences in methyltransferase inhibition profiles are reported. In brain membranes or synaptosomes, AdoHcy inhibits formation of phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*'-dimethylethanolamine [22]. Alternatively, treatment with adenosine and homocysteine inhibits only phosphatidyl-*N*,*N*'-dimethylethanolamine formation [17]. These findings suggest that exogenous AdoHcy can inhibit both methylase I and methylase II while AdoHcy synthesized from adenosine and HTL inhibits only methylase II. Differences in the inhibition profiles of phospholipid methylation may explain the apparent discrepancies of kinetic effects observed in the present experiments on NE uptake between exogenously added AdoHcy and the drug combination.

It has been observed that high concentrations of AdoMet can stimulate [^{3}H]NE uptake [24]. Methylase II is stimulated under these conditions since high concentrations of [^{3}H]AdoMet resulted in [^{3}H]methyl group incorporation only into phosphatidyl-*N*,*N*'-dimethylethanolamine and phosphatidylcholine. These findings support the view of direct methylase II stimulation by exogenously added AdoMet. Further support for this hypothesis is that no increase in the products of methylase I activity was reported, suggesting that [^{3}H]AdoMet did not cross the neuronal membrane. It is plausible that since AdoHcy inhibits both methylase I and methylase II [24], a stimulation by AdoMet of norepinephrine uptake could be correlated with increases in either phosphatidyl-*N*,*N*'-dimethylethanolamine formation or phosphatidylcholine formation. These events are the result of methylase II activity and are localized to the outer membrane leaflet. Conversely, reductions of [^{3}H]NE uptake could be correlated with inhibition of formation of both monomethyl and dimethyl derivatives of phosphatidylethanolamine, a result of inhibition of methylase I and methylase II activities.

The sequential methylation of phosphatidylethanolamine to phosphatidylcholine is an event associated with both methylase I and methylase II activities. These enzymes have been reported to alter the fluidity properties of membranes from erythrocytes [5]. Exogenous AdoHcy can decrease both the apparent affinity and maximum velocity of the carrier molecule for norepinephrine. It is plausible that kinetic alterations of norepinephrine uptake are a direct manifestation of phospholipid methylation which maintains particular fluidity characteristics at localized membrane sites. The amount of methylating activity observed in cerebral cortical synaptosomes is very low. Furthermore, the production of phosphatidylcholine during the time course of the present experiments is small compared to the total content of choline-containing phospholipids. Phosphatidylcholine is believed to be primarily distributed on the outer membrane leaflet [10]. It would seem likely that alterations in norepinephrine accumulation produced by inhibiting membrane methylation reactions most probably occur in close proximity to carrier molecules. Since the composition of the membrane component(s) responsible for catecholamine uptake is unknown, an additional possibility is that direct methylation of the carrier molecule can result in the inhibition of NE accumulation reported in the present experiments. Indeed, evidence for AdoMet-dependent methylation of nonpolar membrane constituents and aspartate residues of membrane proteins has been reported [25–27]. Consequently, both membrane proteins and lipids can serve as methylation substrates. At present, it is not possible to distinguish between these possibilities. Although the data demonstrate that inhibition of phospholipid methylation covaries with inhibition of norepinephrine accumulation, the precise connection between these events has yet to be established.

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