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BETA-ADRENERGIC COUPLED PHOSPHOLIPID METHYLATION

A POSSIBLE ROLE IN WITHDRAWAL FROM CHRONIC ETHANOL

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Abstract—Phospholipid methylation was studied in rat brain cortex preparations. Adrenergic agonists stimulated methylation in a dose-dependent manner. The effect was stereospecific and the order of potency of agonists was isoproterenol > norepinephrine ≥ epinephrine. The stimulation could be blocked by propranolol. It was concluded that adrenergic stimulation of phospholipid methylation in these preparations involved a beta-adrenergic receptor and that this response was dependent upon an intact membrane environment. Neither adenosine nor histamine stimulated methylation. In fact, histamine appeared to inhibit methylation. Cleavage of phosphatidylcholine to lyso-phosphatidylcholine occurred in the presence of either adrenergic agonists or histamine, indicating an involvement of phospholipase A_2. Norepinephrine-sensitive methylation in cortex homogenates from rats withdrawn from chronic ethanol administration was double that of controls by 72 hr after the final ethanol dose. Furthermore, basal methylation exhibited a decreasing trend during this period.

Biochemical messengers are recognized and bound by specific receptors located on the outer surface of cell membranes. As a result of these interactions, chemical and physical changes occur within the target cells allowing them to carry out specific biological functions. The methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) has been shown to play an important role in many membrane-mediated events such as beta-adrenergic receptor-adenylate cyclase coupling [1], mast cell secretion of histamine and adenylate cyclase activation [2, 3], chemotaxis [4, 5] and arachidonic acid release by platelets [6].

It has been shown recently that synaptosomes from rat brain are enriched in phospholipid methyltransferases [7–9]. In addition, catecholamines have been reported to have an effect on these enzymes [10–12]. These data suggest that the methylation of phospholipids may play a role in catecholamine-mediated synaptic transmission.

This report describes some of the pharmacological and biochemical aspects of the adrenergic-stimulated methylation of phospholipids in rat brain cortex. Furthermore, withdrawal from chronic ethanol administration is shown to affect this process.

* The data in this report were submitted by John S. Zawad to the Department of Biochemistry of the University of Tennessee in partial fulfillment of the requirement for the Doctorate of Philosophy,.

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MATERIALS AND METHODS

Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine (69.8 Ci/mmole) and Aquasol were purchased from the New England Nuclear Corp. (Boston, MA). L-α-Phosphatidylethanolamine (dipalmitoyl), L-α-phosphatidyl-*N*,*N*-dimethylethanolamine (dipalmitoyl) and L-α-phosphatidylcholine (dipalmitoyl) were purchased from the Sigma Chemical Co. (St. Louis, MO). L-α-Phosphatidyl-*N*-monomethylethanolamine (dipalmitoyl) was obtained from the Calbiochem-Behring Corp. (San Diego, CA). All other reagents were of the highest grade available. Male Sprague–Dawley rats were supplied by Harlan Industries (Indianapolis, IN).

Preparation of tissues

Crude cellular cortex homogenates. Male Sprague–Dawley rats weighing 150–200 g were decapitated and the brains were quickly removed and rinsed in cold Krebs–Ringer bicarbonate buffer (KR buffer), pH 7.5, prepared according to Chasin *et al.* [13]. All subsequent steps were performed at 4°. The cerebral cortex was dissected away as described by Glowinski and Iversen [14], homogenized in 10 vol. of KR buffer according to the procedures of Chasin *et al.* [13], and centrifuged for 10 min at 500 *g*. The supernatant fraction was discarded. The loosely packed pellet was resuspended in the original volume of KR buffer, and the centrifugation step was repeated. The pellet was resuspended in enough KR buffer to give a final volume of 1.0 ml.

Synaptosomes. Synaptosomes were prepared

according to Gray and Whittaker [15]. The crude synaptosomal (P_2) fraction was layered on a discontinuous sucrose gradient consisting of 10 ml of 0.8 M sucrose layered on top of 10 ml of 1.2 M sucrose. After centrifugation at 85,000 *g* for 2 hr, the white fluffy material equilibrating at the 0.8 to 1.2 M sucrose interface was aspirated and diluted (1:2, v/v) with water. This was then centrifuged at 112,000 *g* for 60 min. The pellet (purified synaptosomes) was resuspended in 3 ml of KR buffer and stored at −20° until used.

S-Adenosyl-L-methionine (SAM) loading

To each 0.2 ml of crude cortex homogenate was added 0.3 ml of KR buffer containing [*methyl*-^{3}H]SAM (45.4 μCi, 100 μM). After equilibrating for 20 hr at 4°, 8 ml of KR buffer was added and the mixture was centrifuged at 2500 *g* for 10 min. The supernatant fraction containing excess [*methyl*-^{3}H]SAM not taken up by particulates (approximately 85% of the total labeled materials) was discarded. Aliquots (0.1 ml) of the loosely packed washed pellet were resuspended in KR buffer to give a final volume of 0.5 ml.

In vitro *assay of phospholipid methylation*

Four 0.1-ml aliquots of the final 0.5-ml tissue preparation were taken and transferred to glass-stoppered extraction tubes kept on ice. Methylation was initiated by warming two of these to 37° in a shaking water bath for 30 min. The remaining pair of samples were kept on ice during this time and served as blanks. The reaction was stopped by the addition of 3 ml of a chloroform–methanol–HCl mixture (2:1:0.02, v/v), and the lipids were extracted as described by Hirata *et al.* [1].

During these investigations, a variety of neuroactive substances were tested for their effect(s) on [*methyl*-^{3}H] group incorporation into rat brain phospholipids. These agents (0.01 ml) were added to the final reaction mixture (0.5 ml) just prior to warming at 37°. All subsequent steps were performed as described above.

When synaptosomes were used, the SAM preloading step was omitted. The procedures above were modified slightly. Instead, freeze–thaw lysed synaptosomes were suspended in enough KR buffer to give a final concentration of 1 to 1.5 mg protein/ml. Aliquots (0.5 ml) were then taken, 0.01 ml [*methyl*-^{3}H]SAM (4.54 μCi) was added, and the methylation reaction was assayed as above.

Thin-layer chromatography (TLC)

The chloroform phase retained from above was taken to dryness under a stream of dry nitrogen gas to avoid oxidation of the extracted products. The residue was dissolved in 0.1 ml of chloroform–methanol (2:1, v/v) containing 1 mg/l butylated hydroxytoluene as an anti-oxidant. Authentic phosphatidyl-*N*-monomethylethanolamine (PME) and phosphatidyl-*N,N*-dimethylethanolamine (PDE) were added (10 μg each) as non-radioactive carriers, and the entire mixture was applied to a silica gel plate (Whatman, Inc. LK5D) with a disposable capillary pipette (0.02 ml). One-dimensional TLC was performed using a chloroform–methanol–water mixture (50:50:1, v/v) as an ascending solvent system. Authentic [*methyl*-^{3}H] phospholipid standards were co-chromatographed with the sample. The plates were then developed until the solvent front was within 1–2 cm of the top of the plate.

Phospholipids were visualized by either exposing the air-dried (25°) plates to iodine vapor, spraying with rhodamine-6G (0.01% in water) and viewing under ultraviolet light (280 nm), or by spraying with Zinzadzae reagent [16]. The spots corresponding to authentic phospholipids were scraped directly into scintillation vials and the radioactivity was determined.

Measurement of radioactivity

All samples were taken to dryness in an oven at 90° prior to addition of 5 ml Aquasol. The radioactivity was measured in a Beckman LS 230 liquid scintillation spectrophotometer.

Preparation of tritiated phospholipid standards

The crude synaptosomal pellet (P_2) was sonicated (four times, 15-sec bursts at 60 W) in the presence of 0.3% sodium taurocholate. To 0.1 ml of the solubilized synaptosomes (5.2 mg protein/ml) was added 1.0 ml of 25 mM Trisma–HCl buffer, pH 8, containing 2 mM $MgCl_2$ and 100 μM [*methyl*-^{3}H]SAM (48.7 μCi). The mixture was then divided into 0.2-ml aliquots, to which 0.03 ml of exogenous phospholipid (1 mM, solubilized by sonicating in 1% sodium taurocholate) was added, and incubated at 37° for 30 min. The reaction was stopped and the phospholipid products were extracted as described above.

The [*methyl*-^{3}H]-labeled products were purified by two-dimensional chromatography on silica gel plates (Eastman 6061, without fluorescent indicator). The plates were first developed in chloroform–methanol–water (50:50:1 v/v) and then dried under a stream of dry nitrogen gas and re-chromatographed in the second dimension in *n*-butanol–acetic acid–water (3:1:1, v/v). The spots were visualized by exposure to iodine vapor, scraped into 10-ml extraction tubes and the [*methyl*-^{3}H] phospholipids eluted from the silica gel with chloroform–methanol (2:1, v/v) and stored at −20°.

Protein concentrations were estimated as described by Lowry *et al.* [17] using bovine serum albumin as a standard.

Ethanol studies

In the chronic studies, animals received 20% ethanol (9–15 g/kg body weight in 3–5 fractions per day for 4 days) according to the procedures of Karoum *et al.* [18]. Controls received equal volumes of water. Food and water were available to both groups *ad lib*. At various times after the final dose of ethanol or water (0, 12, 24, 48, 72 hr), the animals were decapitated. The brains were quickly removed and the cortex was analyzed for norepinephrine-stimulated phospholipid methylation as described above.

In some experiments, ethanol (20%, v/v) was administered acutely (2.5 g/kg body weight intraperitoneally) and the animals were killed as above.

RESULTS

In the initial phases of this investigation, the properties and characteristics of the phospholipid methylation reaction were studied. Crude cortex homogenates, prepared as described by Chasin *et al.* [13], were used to study receptor-mediated methylation since they contain intact receptor-coupled adenylate cyclase complexes. Since SAM enters cells and particulates poorly [19, 20], it was introduced by equilibration for 20 hr at 4°. This procedure gave a much lower blank than when SAM was introduced by incubation at 37° even though the initial amount of radioactivity was ten times higher in the former. The ability of SAM preloaded cortex homogenates to methylate membrane lipids is shown in Fig. 1. The reaction was found to be linear with time up to 40 min. No phospholipid methylation occurred either in the cold or in samples that contained heat-inactivated tissues. Recoveries of purified [*methyl*-^{3}H] phospholipids in the final chloroform extract were 95%.

Procedures which disrupt cell membrane structure increased phospholipid methylation activity in SAM preloaded cortex homogenates (Table 1). Basal methylation, for example, was increased by 58% in the presence of Triton X-100. Sonication also increased phospholipid methylation.

The ability of detergent solubilized synaptosomes to methylate phospholipids is shown in Table 2. In the absence of exogenous substrate (i.e. "None"), PC was the major methylated phospholipid while PME and PDE were formed in equal quantities. When PE was added, [*methyl*-^{3}H] group incorporation into PME was increased with no significant changes in either PDE or PC. Likewise, the addition of either PME or PDE resulted in increases in PDE and PC respectively. As with the methylation of PE, PME was not further converted to PC.

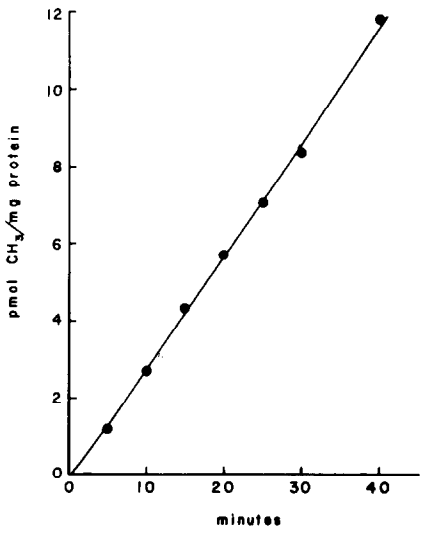

Fig. 1. Effect of time on phospholipid methylation. Phospholipid methylation was assayed in SAM preloaded cortex homogenates as described under Materials and Methods. Data represent basal activity expressed as pmoles [*methyl*-^{3}H] groups transferred per mg protein per 30 min.

Table 1. Effects of detergents and/or sonication on phospholipid methylation in crude cortex homogenates*

Treatment	N	CH_3 (pmoles/mg protein)
None	10	14.6 ± 0.6
Sonication	4	20.3 ± 1.6
0.1% Triton X-100	4	23.1 ± 2.0
Sonication in 0.1% Triton X-100	4	22.5 ± 1.8

* Assay media contained 0.2 ml of crude cortex homogenate which had been preloaded with SAM. Phospholipid methyltransferase activity was measured as described under Methods and Materials. Treatment indicated by "None" represents control data. Values are means ± S.E. N = number of observations.

Norepinephrine stimulated the incorporation of [*methyl*-^{3}H] groups from SAM into phospholipids of crude cortex membrane preparations (Fig. 2). Stimulation was linear from 10 to 100 μM norepinephrine. Below 10 μM norepinephrine, the response was non-linear and variable. No norepinephrine-sensitive phospholipid methylation could be detected in either fresh or freeze–thawed synaptosomes (data not shown). The distribution of [*methyl*-^{3}H] groups was determined for both basal and stimulated reaction products (Fig. 3). In the absence of any agonist, about 50% of the radioactivity in the chloroform extracts was found to be associated with methylated phospholipids. Approximately 30% of the total labeled material migrated with the solvent front. In the presence of norepinephrine, radioactivity in the phospholipid fractions was increased by about 65%, and significant increases were observed in all three methylated phospholipids. Furthermore, an increased labeling of lyso-phosphatidylcholine (lyso-PC) was observed. This suggested that the breakdown of PC had been increased by norepinephrine.

An increase in radioactive material migrating with the solvent front was also observed in the presence

Table 2. Effects of exogenous substrates on phospholipid methylation in rat brain cortex synaptosomes*

Additives	[*methyl*-^{3}H] group incorporation (pmoles/mg) PME	PDE	PC
None	0.9 ± 0.1	0.9 ± 0.2	3.6 ± 0.5
PE	2.5 ± 0.4	0.8 ± 0.3	3.3 ± 0.6
PME	1.1 ± 0.2	6.9 ± 0.5	3.8 ± 0.3
PDE	1.0 ± 0.1	1.0 ± 0.1	16.8 ± 1.2

* Exogenous phospholipid substrates (1 mM) were solubilized in 1% sodium taurocholate and added in 0.03 ml to the reaction mixture. Methylation in the absence of any exogenous phospholipid ("None") contained an equal amount of detergent. Phospholipid methylation was assayed as described under Methods and Materials as modified for synaptosomes (i.e. no 20 hr SAM loading). Values shown are the mean of four determinations ± S.E.

Table 3. Effects of various neuroactive substances on the methylation of phospholipids in crude cortex preparations*

Additives (50 μM)	N	Methyl groups (pmoles/mg protein)	% Change from basal
None (basal)	10	14.6 ± 0.6†	0
L-Norepinephrine	14	24.5 ± 0.9	67
DL-Norepinephrine	5	19.4 ± 1.3	33
L-Epinephrine	5	22.0 ± 0.7	51
L-Isoproterenol	16	19.1 ± 1.9	31
Adenosine	5	15.5 ± 0.2	6
Histamine (100 μM)	8	10.4 ± 1.4	−29

* Phospholipid methylation was assayed as described under Materials and Methods for SAM preloaded crude cortex homogenates.
† Mean ± S.E. N = number of observations.

Table 4. Inhibition of norepinephrine-stimulated phospholipid methylation by propranolol*

Additives	N	Methyl groups (pmoles/mg protein)	% Change from basal
None	10	14.6 ± 0.6†	0
L-Norepinephrine (40 μM)	14	21.6 ± 1.3	48
L-Norepinephrine (40 μM) + DL-propranolol (100 μM)	5	16.1 ± 1.1	10

* Phospholipid methylation was assayed as described under Materials and Methods for SAM preloaded crude cortex homogenates.
† Mean ± S.E. N = number of observations.

of norepinephrine. This effect has been reported previously [11], but the labeled product(s) have not been identified.

The norepinephrine-stimulated increase in phospholipid methylation in SAM preloaded cortex homogenates was found to be stereospecific (Table 3). At equal concentrations, racemic norepinephrine was only half as effective as the levo isomer. L-Epinephrine was also equally effective in stimulating methylation. The EC_{50} values (concentration of agonist that produced half-maximal stimulation) were calculated from dose–response curves similar to Fig. 2. Norepinephrine and epinephrine were found to be equipotent (EC_{50} = 38 and 40 μM respectively). Isoproterenol was also stimulatory. At equal concentrations it was about half as effective as either norepinephrine or epinephrine, but was the most potent agonist with respect to its EC_{50} value (12 μM). The order of potency was isoproterenol > norepinephrine ≥ epinephrine. This order of potency has been observed by others for the adrenergic-coupled adenylate cyclase system in rat brain [21, 22] and is characteristic of a $beta_1$-adrenergic receptor subtype.

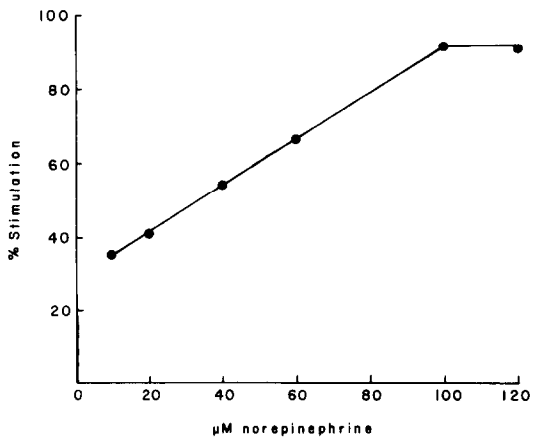

Fig. 2. Effects of L-norepinephrine concentration on phospholipid methylation in cortex homogenates. The reaction mixture contained 0.2 ml of crude cortex homogenate (200 μg protein) preloaded with 100 μM [*methyl*-^{3}H]SAM and 0.3 ml KR buffer. The data are expressed as percent increase over basal methylation activity.

The effects of norepinephrine on phospholipid methylation were blocked by propranolol, a beta-adrenergic antagonist (Table 4). Neither adenosine nor histamine, which are known to have synergistic effects with norepinephrine on adenylate cyclase activation [23], increased basal phospholipid methylation (Table 3). Furthermore, neither altered the effects of norepinephrine (data not shown). In fact, histamine decreased the radioactivity found in PC (Fig. 4), while the label was increased in lyso-PC. These data suggest that histamine increases the turnover of newly formed PC.

In view of the fact that phospholipid methylation can be altered by chronic ethanol administration [10, 24, 25] and that beta-adrenergic receptor density and sensitivity are known to be affected by such treatment, adrenergic-sensitive methylation during withdrawal from chronic ethanol administration was examined. The results of these studies are shown in Fig. 5. By 48 hr after withdrawal of ethanol, there was a significant decrease in basal phospholipid

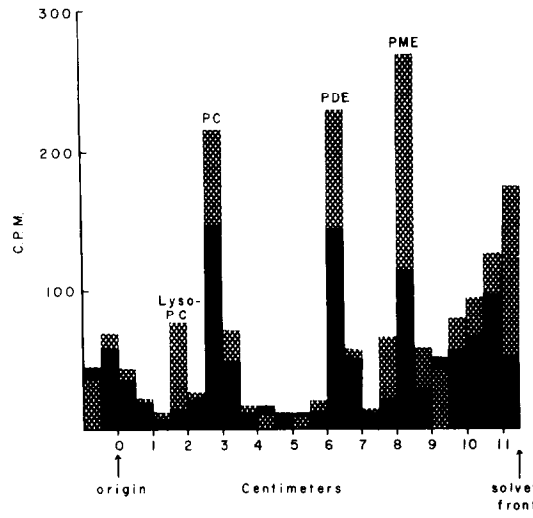

Fig. 3. TLC chromatography of L-norepinephrine-stimulated phospholipid methylation reaction products. Phospholipids were extracted with chloroform–methanol (2:1, v/v). One ml of the chloroform extract was dried under N_2 and taken up in a small volume of chloroform–methanol (2:1, v/v), and the entire mixture was chromatographed in chloroform–methanol–water (50:50:1, by vol.) on silica gel plates. Cortex methylation was assayed in the absence (solid bars) and the presence (hatched bars) of 40 μM L-norepinephrine. Positions of authentic phospholipid markers are indicated by: Lyso-PC, PC, PDE and PME. Abbreviations are defined in the text.

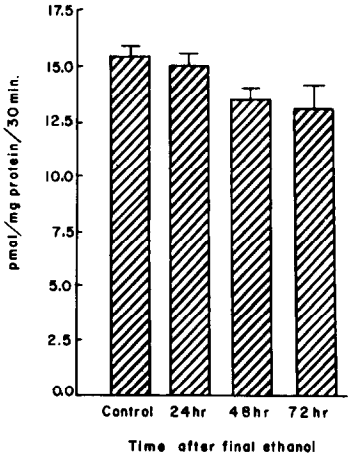

Fig. 5. Effects of withdrawal from chronic ethanol administration on basal phospholipid methyltransferase activity in crude cortex preparations. The experimental details and procedures are presented under Materials and Methods Data are expressed as pmoles methyl groups incorporated into cortex phospholipids per mg protein per 30 min. Bars represent the means ± S.E. for six animals in each group. The values at 48 and 72 hr were found to be significantly different from the control group by one-way analysis of variance and subsequent Neuman–Kuel analysis ($\alpha = 0.01$).

methylation activity as compared to control (i.e. animals that receive no ethanol). Table 5 shows the effects of withdrawal on norepinephrine-sensitive phospholipid methylation. As in Fig. 5, there was no significant difference in the methylation in control animals as compared to those on chronic ethanol. However, during withdrawal norepinephrine-stimulated methylation increased from 46 to 80% by 72 hr. No effect was observed after a single dose of ethanol (data not shown).

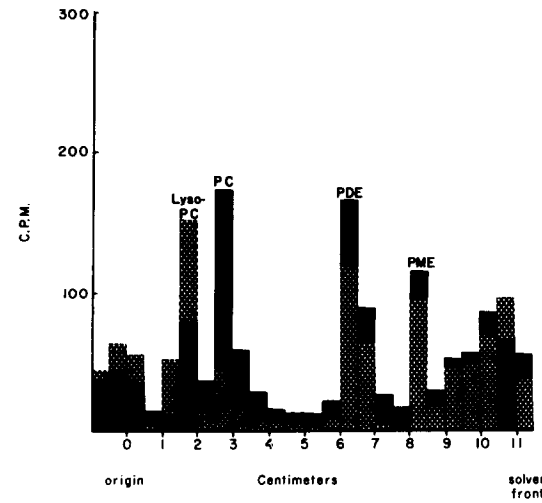

Fig. 4. Effects of histamine on the TLC pattern of labeled phospholipids. Crude cortex homogenates were assayed in the absence (solid bars) and in the presence (hatched bars) of 100 μM histamine. Phospholipids were chromatographed as in Fig. 3. Positions of authentic phospholipid markers are indicated by: Lyso-PC, PC, PDE and PME. Abbreviations are defined in the text.

DISCUSSION

Synthesis of PC from PE occurred in both intact and cell-free cortex preparations. However, beta-adrenergic sensitive phospholipid methylation could be detected only in carefully prepared homogenates. The apparent lack of adrenergic-stimulated methylation in synaptosomes (resealed pinched off pre-

Table 5. Effects of chronic ethanol administration on norepinephrine-stimulated phospholipid methylation in crude cortex homogenates*

Time (hr) after final dose	No. of animals	Percent stimulation
Control	8	46.1 ± 4.2
0	4	41.3 ± 5.0
12	4	48.1 ± 6.0
24	5	65.0 ± 6.2†
48	3	70.0 ± 8.0†
72	6	80.3 ± 11.2†

* The experimental details and procedures are presented under Materials and Methods. Controls received water instead of ethanol. Basal methylation activity was 15.4 ± 0.5 pmoles/mg protein for the controls. Norepinephrine concentration in the assay media was 40 μM.

† One-way analysis of variance and subsequent Neuman–Keul analysis showed samples to be significantly different from controls ($\alpha = 0.05$).

synaptic nerve terminals) could be due to the fact that adrenergic-sensitive phospholipid methylation is a post-synaptic phenomenon. On the other hand, the receptor–methyltransferase complex could have been destroyed during the isolation of the synaptosomes. Synaptosomes did not form PC from exogenous PE or PME. A possible explanation is that both PME and PDE are competing for the same active site and, since PME is in excess, the binding of PDE would be inhibited. An alternate explanation is that three different enzymes are involved in the conversion of PE to PC. Both explanations are consistent with the model proposed by Crews *et al.* [7]. They suggest that the methyltransferases are asymmetrically oriented in the membrane and are in close proximity to one another. Thus, any procedure which would disrupt the arrangement may interfere with the conversion of PE to PC.

Phospholipid methyltransferases have been shown to have a marked effect on cellular responsiveness to catecholamines [1, 12, 26–28]. These data and the demonstration of the presence of two phospholipid methyltransferases arranged asymetrically in the synaptosomal membrane [29] imply a role for phospholipid methylation in synaptic transmission. It has been proposed that, in some membrane systems, unoccupied beta-receptors interact with the methyltransferases to suppress their activity and that binding of the receptor with agonist relieves this suppression [30]. In the studies presented here, norepinephrine-stimulated methylation was readily apparent in cortex preparations prepared as described by Chasin *et al.* [13] which contain intact receptor-coupled adenylate cyclase. It could not be detected, however, in cell-free or membrane-disrupted preparations. Thus, it appears that an intact membrane environment is essential to beta-receptor coupled phospholipid methylation in cortex.

Beta-adrenergic receptor subtypes are unevenly distributed in brain [31]. Cortex is especially rich in $beta_1$-receptor subtypes. Additionally, it has been demonstrated recently that binding of beta-adrenergic ligand in cortex is affected by phospholipid methyltransferase activity [28]. We report that beta-adrenergic agonists stimulate the methylation of phospholipids in rat brain cortex preparations. The order of potency of agonists suggests the involvement of a $beta_1$-adrenergic receptor subtype. However, further characterization with other $beta_1$ and $beta_2$ selective agonists and antagonists must be evaluated before this process can unequivocally be identified as an exclusively $beta_1$-adrenergically mediated event.

The inability of adenosine or histamine to stimulate methyltransferase activity argues against a direct coupling between adenylate cyclase and phospholipid methyltransferases. In fact, the decreased formation of labeled PC with histamine was accompanied by an increased formation of lyso-PC via the methylation pathway. This suggests a direct effect of histamine rather than an indirect competitive-type inhibition by histamine-*N*-methyltransferase for available SAM. Perhaps histamine affects phospholipid methylation by activating phospholipase A_2.

Alterations in beta-adrenergic receptor density [32] and sensitivity [33] have been shown to be associated with the development of tolerance to ethanol. Furthermore, propranolol, which has been used to control some of the clinical signs of ethanol withdrawal [34, 35] is known to alter the metabolism of phospholipids [36]. Our study demonstrates a relationship between beta-adrenergic agents and phospholipid methyltransferase activity during withdrawal from chronic ethanol ingestion. The observed changes are accentuated by the decrease in basal methylation during this time. The change in basal methylation was not unexpected, since Banerjee *et al.* [32] reported increased beta-receptor density following withdrawal from chronic ethanol treatment. This would increase the suppression of basal methylation activity and result in an overall greater response to agonist. These results suggest, therefore, a role for both beta-adrenergic receptor and phospholipid methyltransferases in ethanol-induced changes in membrane functions.

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