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β-Adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and β-adrenergic receptor–adenylate cyclase coupling

(membrane translocation/fluidity/*S*-adenosylmethionine/reticulocytes)

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ABSTRACT **The β-adrenergic agonist L-isoproterenol stimulated the enzymic synthesis of phosphatidyl-*N*-monomethylethanolamine and phosphatidylcholine in rat reticulocyte ghosts containing the methyl donor *S*-adenosyl-L-methionine. The stimulation was stereospecific, dose-dependent, and inhibited by the β-adrenergic agonist propranolol. The addition of GTP inside the resealed ghosts shifted the dose-response of phospholipid methylation by L-isoproterenol to the left by 2 orders of magnitude. Direct stimulation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] with sodium fluoride or cholera toxin did not increase the methylation of phospholipids. At a concentration of *S*-adenosyl-L-methionine that stimulates synthesis of phosphatidyl-*N*-monomethylethanolamine, the activity of isoproterenol-sensitive adenylate cyclase was increased 2-fold without changes in the basal activity of adenylate cyclase and the number of β-adrenergic receptors. The increase of phospholipid methylation by L-isoproterenol decreased membrane viscosity and increased translocation of methylated lipids. These findings indicate that enhancement of phospholipid methylation by L-isoproterenol decreases membrane microviscosity and thus increases lateral movement of the β-adrenergic receptors and coupling with adenylate cyclase.**

Recent work in our laboratory has shown that erythrocyte membranes synthesize phosphatidylcholine from phosphatidylethanolamine by two enzymes (1, 2). The first enzyme methylates phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine, has a high affinity for the methyl donor *S*-adenosyl-L-methionine, and requires Mg^{2+}. The second enzyme transfers two methyl groups to phosphatidyl-*N*-monomethylethanolamine to form phosphatidylcholine via phosphatidyl-*N*,*N*-dimethylethanolamine, has a low affinity for *S*-adenosyl-L-methionine, and does not require Mg^{2+}. These two enzymes, their substrates, and their products, are asymmetrically distributed in the membrane; the first methyltransferase and phosphatidylethanolamine are localized on the cytoplasmic side, whereas the second methyltransferase and phosphatidylcholine are on the exterior surface of the membrane (2). Such spatial segregation of the methyltransferases facilitates the rapid transfer of phospholipids across the membrane by successive methylations (2). The intramembrane synthesis of phosphatidyl-*N*-monomethylethanolamine, an intermediate, causes profound changes in membrane fluidity (3).

The observation that rat reticulocytes have β-adrenergic receptors coupled with adenylate cyclase (4, 5) [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and our findings that the properties of the cell membrane can be altered by phospholipid methylation (2, 3) prompted a study of the role of lipid methylation in the interaction of adenylate cyclase activity and the β-adrenergic receptor. We now report that stimulation of the β-adrenergic receptor increases phospholipid methylation and translocation which, in turn, enhances membrane fluidity and coupling of the β-adrenergic receptor with adenylate cyclase in rat reticulocytes.

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METHODS

Preparation of Rat Reticulocyte Ghosts. Male Sprague–Dawley rats (150–200 g) were injected subcutaneously with 0.5 ml of phenylhydrazine hydrochloride (30 mg/ml) for 3 consecutive days. On day 7, the percentage of reticulocytes was maximal (>85%) as determined by staining with methylene blue. Heparinized blood obtained by cardiac puncture was centrifuged and the pellets were washed three times with 0.9% NaCl. Ghosts were prepared by hemolysis of the cells with 40 vol of 5 mM Tris/phosphate buffer, pH 8.0/1 mM $MgCl_2$ (6, 7). After centrifugation, the pellets were suspended in 10 vol of 0.5 mM Tris/phosphate buffer, pH 8.0/1 mM $MgCl_2$ for 1 hr. The ghosts thus obtained were resuspended in 10 vol of 50 mM Tris/glycylglycine buffer, pH 8.0/5 mM $MgCl_2$. After centrifugation, the packed ghosts (0.3 ml) were suspended in 0.7 ml of the same buffer containing 4 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (22,000 dpm/pmol) or 200 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (800 dpm/pmol). After incubation for approximately 16 hr at 4°C, 9 ml of 0.9% NaCl was added to the mixture and then centrifuged. The pellet was suspended in 0.7 ml of 100 mM Tris/glycylglycine buffer, pH 8.0/10 mM $MgCl_2$. All these procedures were carried out at 0–4°C and are crucial in maintaining the structural integrity of the membranes.

Determination of Phospholipid Methylation. The methylation reaction was started by warming the test tube containing 50 μl of the ghost suspension from 4°C to 37°C. The [^{3}H]methyl groups incorporated into the lipid fraction were measured by extraction with 3 ml of chloroform/methanol/2 M HCl, 6:3:1 (vol/vol), as described (1, 2). The chloroform phase was washed twice with 1.5 ml of 0.1 M KCl in 50% methanol. For the measurement of phospholipid methylation, 1 ml of chloroform phase was transferred to a counting vial and evaporated to dryness in an oven at 80°C. The residue was taken up in phosphor and the radioactivity was assayed. To separate and quantitate the various methylated phospholipids, the chloroform phase was dehydrated with anhydrous sodium sulfate and evaporated to dryness under a stream of N_2 gas. The residue was dissolved into a small volume of chloroform/methanol, 1:1 (vol/vol), and applied to a silica gel G plate. The development solvent system was propionic acid/*n*-propyl alcohol/chloroform/water, 2:2:1:1 (vol/vol). The front migrated approximately 16 cm.

Measurement of Cyclic AMP. For the measurement of basal and isoproterenol-stimulated cyclic AMP, 20 μl of ghosts was

incubated in the presence of 1 mM isobutylmethylxanthine with and without 0.5 mM L-isoproterenol for 10 min at 37°C. The incubation was stopped by the addition of 500 μl of 6% trichloroacetic acid. Five milliliters of water-saturated ethyl ether was added to each tube and, after a vigorous shaking for 10 sec, the organic phase was discarded. This procedure was repeated twice. The remaining aqueous phase was evaporated to dryness and the residue was resuspended in 1.0 ml of 0.05 M sodium acetate buffer, pH 6.2; 20 μl of this solution was then assayed for cyclic AMP by the radioimmunoassay described by Steiner *et al.* (8). The kit for the radioimmunoassay was obtained from Becton–Dickinson (Orangeburg, NY).

Binding of β-Adrenergic Receptor. For the measurement of β-adrenergic receptor binding sites, 100 μl of reticulocyte ghosts was incubated with 20 nM [^{3}H]dihydroalprenolol in a total volume of 150 μl at 38°C for 10 min (9). After incubation, 5 ml of cold 0.9% NaCl was added, the mixture was filtered through a Whatman GF/B filter, and the filter was washed with additional 10 ml of cold 0.9% NaCl. The filters were dried and the radioactivity was measured in a scintillation counter. Specific binding of [^{3}H]dihydroalprenolol was calculated by subtracting the amount of nonspecific binding in the presence of 5 μM DL-propranolol.

RESULTS

Stimulation of Phospholipid Methylation by Isoproterenol. The effect of the β-adrenergic receptor on phospholipid methylation was examined by treating reticulocyte ghosts with the β-adrenergic agonist L-isoproterenol. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine was previously introduced into the resealed reticulocyte ghosts (2) and the excess [^{3}H]methyl donor present in the medium was removed by centrifugation. After incubation at 37°C the [^{3}H]methyl groups incorporated into phospholipids were measured at various time intervals in the presence and absence of L-isoproterenol. The incorporation of [^{3}H]methyl groups into the lipid fraction was markedly increased by L-isoproterenol. To examine whether one or both methyltransferases of phospholipid synthesis were activated by L-isoproterenol, low and high concentrations of *S*-adenosyl-L-methionine were introduced into the ghosts (Fig. 1 *A* and *B*). Stimulation by L-isoproterenol was observed under both assay conditions but was greater at a high concentration of *S*-adenosyl-L-methionine, indicating that the second methyltransferase was activated by the β-adrenergic agonist to a greater extent than the first enzyme. This conclusion was further supported after the isolation and quantitation of the products by thin-layer chromatography (Figs. 1 *C* and *D*).

As described (3), the first methylation of phospholipids takes place on the cytoplasmic side of the membrane. L-Isoproterenol previously introduced inside the ghosts was ineffective in stimulating phospholipid methylation. Furthermore, when either leaky ghosts or membrane fragments were incubated with *S*-adenosyl-L-methionine in a hypotonic solution or in a detergent-containing buffer, maximal methylation of lipids was observed and no further stimulation by L-isoproterenol occurred. Because heterogeneity of bilayer fluidity in erythrocyte membranes is lost in leaky ghosts (10), these findings suggest that the structural integrity of the membrane is necessary for the stimulation of phospholipid methylation by L-isoproterenol and that the stimulation is not attributable to the direct activation of the phospholipid methyltransferases by L-isoproterenol.

Identity of β-Adrenergic Receptor as the Stimulant for Phospholipid Methylation. To establish that L-isoproterenol acts through the β-adrenergic receptor to increase phospholipid methylation, a number of pharmacological manipulations were

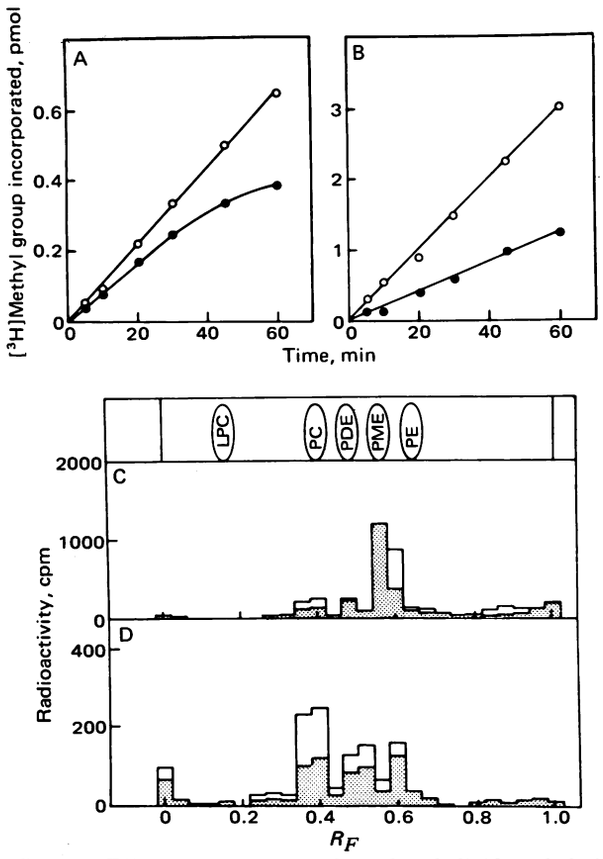

FIG. 1. Effect of L-isoproterenol on phospholipid methylation in reticulocyte membranes. Rat reticulocyte ghosts previously loaded with (*A* and *C*) 4 μM or (*B* and *D*) 200 μM *S*-adenosyl [*methyl*-^{3}H]-methionine were incubated in the absence (●) or presence (○) of 500 μM L-isoproterenol. The lipids isolated after 1 hr of incubation were separated (*C* and *D*) on silica gel G plates with a solvent system of propionic acid/*n*-propyl alcohol/chloroform/water, 2:2:1:1 (vol/vol) in the absence (▤) or presence (□) of 500 μM L-isoproterenol.

carried out. The increase of phospholipid methylation in reticulocyte membranes was found to be stereospecific and dose-dependent (Fig. 2*A*). The *dextro* isomer of isoproterenol was ineffective at 100 μM. The concentration of L-isoproterenol required for half-maximal activation of lipid methylation was about 30 μM, a value close to that necessary for half-maximal activation of adenylate cyclase in rat reticulocyte ghosts (4). It has been reported that membranes become more sensitive to isoproterenol in the presence of GTP (5, 11). Consistent with this is our finding that GTP markedly increased the ability of isoproterenol to stimulate phospholipid methylation, shifting the K_d value from 30 μM to 0.8 μM (Fig. 2*B*). The order of potency for agonists was isoproterenol > epinephrine > norepinephrine, characteristic of the β_2-adrenergic receptor (4). Furthermore, stimulation of phospholipid methylation by L-isoproterenol was inhibited by the β-adrenergic antagonist propranolol but not by the α-adrenergic antagonist phentolamine (Fig. 2*C*). Tropolone, a catechol-*O*-methyltransferase inhibitor, or pargyline, a monoamine oxidase inhibitor, each at 50 μM, had no effect on the isoproterenol-stimulated phospholipid methylation. All of these results clearly demonstrate that binding of the β-adrenergic receptor to an agonist increases phospholipid methylation in reticulocyte membranes.

A direct relationship appeared to exist between the number of available β-adrenergic receptors and the degree of stimulation of phospholipid methylation. Reticulocytes were found to have 2.5 times as many β-adrenergic receptors (490 fmol/mg

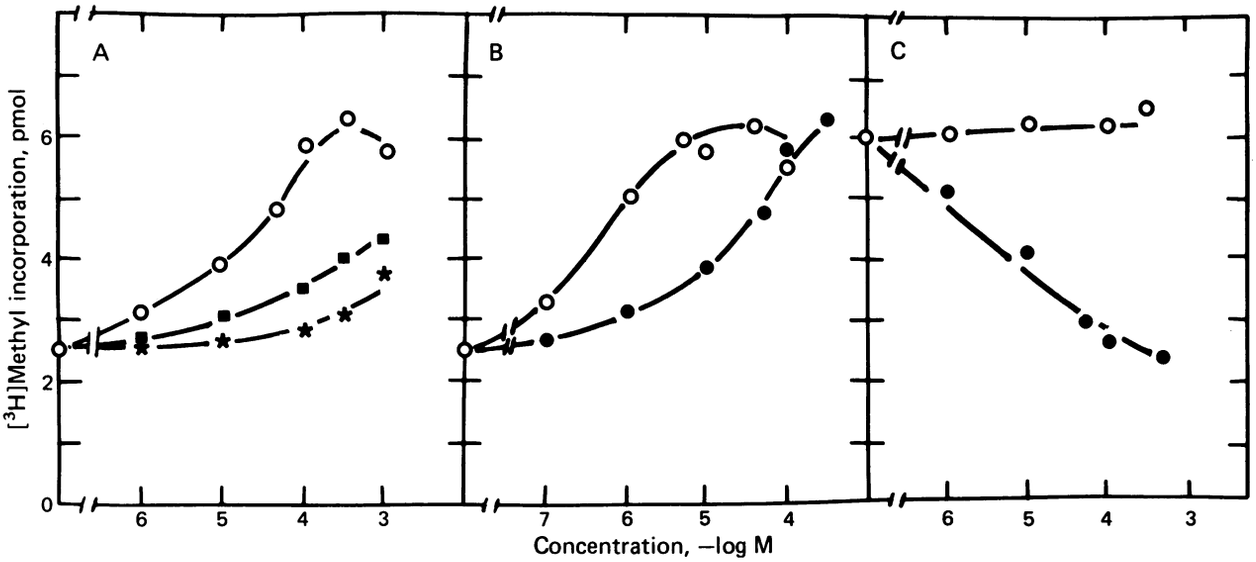

FIG. 2. Effect of adrenergic agonists and antagonists on phospholipid methylation. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (200 μM) was introduced into rat reticulocyte ghosts. Incubation was carried out for 1 hr at 37°C. (*A*) Effect of adrenergic agonists: L-isoproterenol (○), L-epinephrine (■), or L-norepinephrine (✱) was added to the medium as indicated. (*B*) Effect of GTP on the stimulation of phospholipid methylation by L-isoproterenol. Various concentrations of L-isoproterenol were present in the absence (●) or presence (○) of 1 mM GTP. (*C*) Inhibition of L-isoproterenol stimulated phospholipid methylation by adrenergic antagonists. DL-Phentolamine (○) or DL-propranolol (●) was added to the medium together with 500 μM L-isoproterenol as indicated.

of protein) as mature erythrocytes (196 fmol/mg of protein) when measured by [^{3}H]dihydroalprenolol binding (4, 5). Isoproterenol stimulated twice as much phospholipid methylation in reticulocytes (3.2-fold) as in erythrocytes (1.6-fold).

Noninvolvement of Adenylate Cyclase in Phospholipid Methylation. Although cholera toxin and NaF stimulate adenylate cyclase directly, bypassing the β-adrenergic receptor, the addition of these compounds to the reticulocyte ghosts had no effect on phospholipid methylation (Table 1). Theophylline (a phosphodiesterase inhibitor) or 8-bromo cyclic AMP did not increase the methylation of phospholipids. When ATP together with GTP had been previously introduced inside the ghosts, L-isoproterenol increased cyclic AMP formation by 40-fold (100 to 4000 pmol/mg of protein per 10 min). These nucleotides, however, did not further enhance the phospholipid methylation in the presence of 0.1 mM L-isoproterenol. Guanyl nucleotides—GTP and Gpp(NH)p (5′-guanylylimidodiphosphate)—increase hormone-sensitive adenylate cyclase activity but had little effect on the maximal lipid methylation. These results indicate that the stimulation of membrane lipid methylation is mediated by binding of the β-adrenergic receptor to its agonists but not by the activation of the adenylate cyclase system.

Table 1. Effect of adenylate cyclase stimulants and nucleotides on phospholipid methylation

Additions	[^{3}H]Methyl incorporation, pmol/mg protein
None	24.6
NaF (0.01 M)	25.1
Cholera toxin (100 μg/ml)	23.2
Theophylline (5 mM)	25.3
8-Br-cyclic AMP (0.1 mM)	22.8
None	25.2
L-Isoproterenol (0.1 mM)	56.4
+ ATP (1 mM)	58.2
+ GTP (1 mM)	61.6
+ ATP (1 mM)	56.3
+ Gpp(NH)p (0.1 mM)	62.0

The experiments were performed as described in the text except that the compounds indicated were added to the reaction mixture. Nucleotides were previously introduced into the ghosts at 4°C.

Phospholipid Methylation Increases Coupling of β-Adrenergic Receptor with Adenylate Cyclase. Previous reports from our laboratory indicated that methyltransferases play a role in the translocation of phospholipids across the membrane and in membrane fluidity (2, 3). Membrane fluidity influences lateral diffusion and rotation of proteins in the membrane (12). One manifestation of changes in membrane fluidity could be the coupling of the β-adrenergic receptor to adenylate cyclase. To study the role of lipid methylation in the activation of the β-adrenergic receptor–adenylate cyclase complex, varying concentrations of *S*-adenosyl-L-methionine, ATP, and GTP were introduced into reticulocyte ghosts, and basal and isoproterenol-stimulated activities of adenylate cyclase were measured. GTP was necessary for the optimal cyclic AMP formation (11). As shown in Fig. 3, isoproterenol-stimulated adenylate cyclase activity was increased more than 2-fold when the concentration of *S*-adenosyl-L-methionine varied from 0 to 20 μM, and then it reached a plateau. Basal adenylate cyclase activity and the number of β-adrenergic receptors were not changed (Fig. 3). Under these conditions, adenylate cyclase activity in the presence of 5 mM NaF was not affected by *S*-adenosyl-L-methionine (5000 pmol of cyclic AMP formed per mg of protein per 10 min). These results indicate that the increase in cyclic AMP formation in the presence of L-isoproterenol is due to the enhancement of coupling between β-adrenergic receptor and adenylate cyclase. At concentrations of *S*-adenosyl-L-methionine below 20 μM, phosphatidyl-*N*-monomethylethanolamine was mainly synthesized and a nearly maximal change of membrane viscosity (from 1.8 to 0.6 poise at 25°C) was observed by using a fluorescent probe (3, 13). The simultaneous addition of 10 μM *S*-adenosyl-L-homocysteine with 10 μM *S*-adenosyl-L-methionine decreased both the increase in cyclic AMP formation and the microviscosity change by about 50%. These results indicate that phospholipid meth-

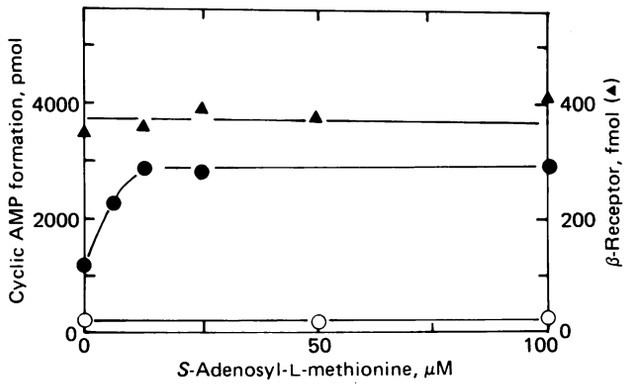

FIG. 3. Effect of phospholipid methylation on coupling of β-adrenergic receptor with adenylate cyclase. In addition to various concentrations of *S*-adenosyl-L-methionine, 10 mM ATP and 1 mM GTP were previously introduced into the resealed reticulocyte ghosts. The hormone-sensitive adenylate cyclase was measured in the absence (○) or presence (●) of 500 μM L-isoproterenol. The data are expressed as pmol/mg of protein per 10 min. An aliquot of each sample was also examined for [^{3}H]dihydroalprenolol binding (▲); these data are expressed as fmol/mg of protein.

ylation enhances the coupling of β-adrenergic receptor with adenylate cyclase by decreasing membrane viscosity.

DISCUSSION

Our experiments show that binding of the β-adrenergic receptor to its agonist, L-isoproterenol, stimulates phospholipid methylation in rat reticulocyte ghosts. The isoproterenol-dependent increase in methylation depends on the available number of β-adrenergic receptors and the structural integrity of the cell membrane. When a low concentration of *S*-adenosyl-methionine is introduced into reticulocyte ghosts, mainly phosphatidyl-*N*-monomethylethanolamine is synthesized. The formation of this compound markedly decreases membrane microviscosity (3). Increasing the concentration of *S*-adenosylmethionine results in further methylation to form phosphatidylcholine and in the translocation of phospholipids from the cytoplasmic side to the outside of the cell membrane (2). After exposure of reticulocyte ghosts to L-isoproterenol, the phospholipid methylation increases the coupling of the β-adrenergic receptor with adenylate cyclase. This increase in coupling can be blocked when the phospholipid methylation is inhibited with *S*-adenosyl-L-homocysteine. Maximal changes in coupling occur at concentrations of *S*-adenosyl-L-methionine that increase membrane fluidity but not phosphatidylcholine formation. Changes in coupling between the hormone receptor and adenylate cyclase due to membrane fluidity have been found in fibroblasts after manipulation of cholesterol content in the membrane (14). Filipin, an antibiotic that interacts with membrane cholesterol, causes uncoupling between adenylate cyclase and β-adrenergic receptors in pigeon erythrocyte membranes (15). These data indicate the importance of lipids in the microenvironment of this receptor–enzyme system.

The mechanism of stimulation of phospholipid methylation by binding of agonists with the β-adrenergic receptor remains unknown. It is possible that, in the unoccupied state, the β-adrenergic receptors interact with the methyltransferases to decrease their activity. Consistent with this is the observation that phospholipid methylation is markedly increased when the enzymes are solubilized by nonionic detergents (unpublished data). When the β-adrenergic receptor is bound to its agonist, L-isoproterenol, the suppression is removed and phospholipid methylation in membranes proceeds at an accelerated rate. The accumulation of phosphatidyl-*N*-monomethylethanolamine

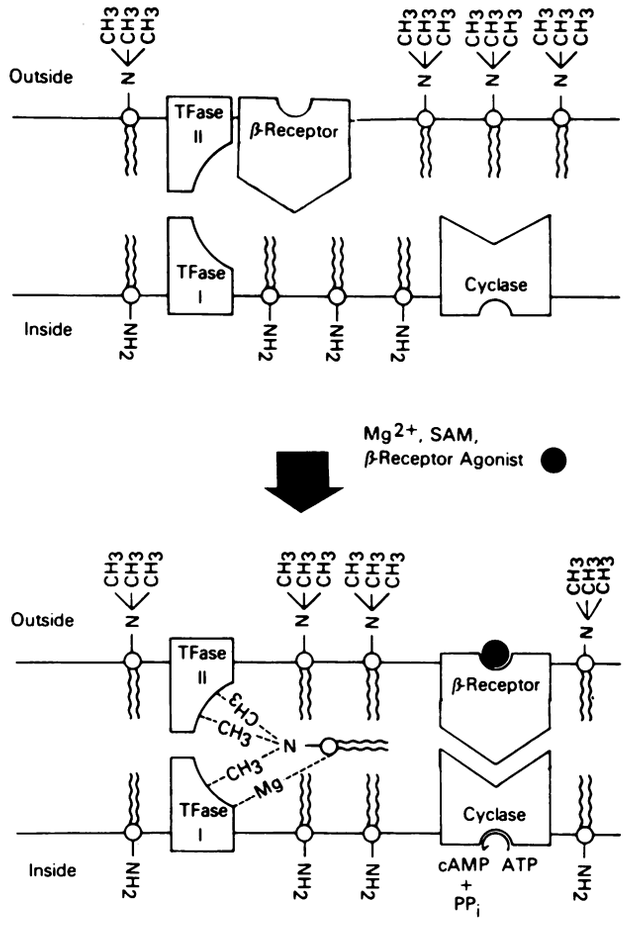

FIG. 4. Proposed mechanism of stimulation of phospholipid methylation by isoproterenol-facilitated coupling of the β-adrenergic receptor and adenylate cyclase. TFase I, phosphatidylethanolamine-*N*-methyltransferase; TFase II, phosphatidylcholine-forming enzyme; SAM, *S*-adenosyl-L-methionine; ●, L-isoproterenol; cAMP, cyclic AMP.

within the membrane decreases membrane viscosity, and this should make possible a more rapid lateral movement of the β-adrenergic receptor–isoproterenol complex (16) and a greater opportunity to couple with adenylate cyclase (Fig. 4). Preliminary results show that the further methylation of phosphatidyl-*N*-monomethylethanolamine to form phosphatidylcholine during longer incubation results in an increased number of available receptor sites that bind with the radioactive ligand [^{3}H]dihydroalprenolol (17). Stimulation of β-adrenergic receptors also increases methylation of phospholipids in other tissues such as HeLa cells (18). Additionally, changes in phospholipid methylation in HeLa cells also affect the function of β-adrenergic receptor adenylate cyclase. Preliminary observations indicate that the methylation of membrane phospholipids affects membrane structure and function such as ion flux, cell fusion, and membrane protein interactions.

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