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Alteration in phospholipid methylation and impairment of signal transmission in persistently paramyxovirus-infected C6 rat glioma cells

(virus persistence/β-adrenergic receptor/methylation inhibition)

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ABSTRACT **The paramyxoviruses measles (subacute sclerosing panencephalitis, SSPE) virus and canine distemper virus (CDV) cause an impairment of the catecholamine-induced β-adrenergic receptor-dependent cAMP generation in persistently infected C6 rat glioma cells. In C6 cells persistently infected with CDV the number of receptors is greatly reduced. Hirata and Axelrod have shown that the number of β-adrenergic receptors could be regulated by methylation of phosphatidylethanolamine, resulting in lecithin synthesis [Hirata, F. & Axelrod, J. (1980) *Science* 209, 1082–1090]. We have therefore studied the methylation of phosphatidylethanolamine in persistently infected cells by the incorporation of [^{3}H]methyl groups from [*methyl*-^{3}H]methionine into phosphatidylethanolamine. In both infected systems, C6/SSPE and C6/CDV, we observed a total loss of catecholamine-stimulated β-adrenergic receptor-dependent methylation, whereas the β-receptor-independent methylation of phospholipids was unchanged.**

A rat glioma C6 cell (ATCC CCL107) (1) has about 6,000 β-adrenergic receptors on the cell surface (2). In these cells the addition of catecholamines such as *l*-isoproterenol increases the intracellular cAMP level more than 100-fold. We have established persistent infections of these cells with the neurotropic paramyxoviruses measles virus (subacute sclerosing panencephalitis virus, SSPE) and canine distemper virus (CDV) (3, 4). Both infections resulted in a major impairment of the catecholamine-stimulated β-adrenergic receptor-dependent cAMP synthesis (4). In view of the central role of the neurotransmitter-controlled cAMP levels in cells of the central nervous system, it is possible that such impairments could be significant for cellular dysfunctions in diseases caused by persistent virus infections. In earlier studies we have investigated whether there is an impairment in β receptors alone, in the adenylate cyclase component (EC 4.6.1.1.), or in both components of the signal-transmitting system (4, 5). We have shown a reduction of fluoride-stimulated activity of adenylate cyclase in membrane preparations from C6/SSPE cells without changes of the Michaelis–Menten constant for the substrate ATP (4). In the same C6/SSPE cells, the number of the β-adrenergic receptors and the binding constant for the β-adrenergic receptor antagonist [^{3}H]dihydroalprenolol for the β-receptors were unchanged (5). The two persistently infected C6 cell systems differ only with respect to the number of β-adrenergic receptors. The question therefore remains: How is the number of β-adrenergic receptors in the C6/CDV cells modulated? Strittmatter *et al.* (6) have shown *in vitro*, using reticulocyte ghosts, that the lecithin-producing methylation of phosphatidylethanolamine (PtdEtn) in the cell membrane results in the appearance of previously hidden β-receptors on the cell surface. On the other hand, inhibitors of phospholipid methyltransferases can cause a reduction of the number of β-adrenergic receptors on HeLa cells (7). Therefore it could be possible that in our C6/CDV cells the methylation of PtdEtn is permanently reduced, resulting in a decrease in the number of β-adrenergic receptors. This would also imply that the methylation in C6/SSPE cells should be similar to that in uninfected C6 cells because there is no loss of receptors in these cells.

Furthermore, Strittmatter, Hirata, Tallman, and their colleagues (6, 8, 9) have demonstrated that the stimulation of β-adrenergic receptors by catecholamines increases not only the cAMP level inside the cell but also the specific methylation of PtdEtn to the monomethyl and the dimethyl derivatives and to lecithin (phosphatidylcholine, the trimethyl derivative). This is a sequential reaction which is activated by coupling of β-adrenergic receptors to two phospholipid methyltransferases (7).

In connection with this methylation some of the following important cell biological phenomena are discussed (7): (*i*) the coupling of β-adrenergic receptors to adenylate cyclase, leading to increased cAMP synthesis; (*ii*) the development of the refractory state of the signal-transmitting system; (*iii*) Ca^{2+} influx and therefore a series of connected changes in the cellular biochemistry; (*iv*) the release of arachidonic acid as a precursor of prostaglandins; (*v*) the activation of phospholipase A_2, which can regulate the methylation-dependent processes; and (*vi*) the regulation of membrane fluidity in domains around the β-adrenergic receptors. Therefore it appears important to examine catecholamine-stimulated β-receptor-dependent methylation of phospholipids in our persistently infected C6 cells.

In this paper we describe the changes observed in the specific methylation of PtdEtn in our persistently infected cells and discuss the possible impairments of the regulatory functions of β-adrenergic receptor-dependent processes in persistently infected cells of the central nervous system.

MATERIALS AND METHODS

Cells. C6 rat glioma cells (astrocytoma) (ATCC CCL107) were cultured as described (4).

Virus. SSPE (Lec strain) was propagated in Vero cells and isolated by the method of Kratzsch *et al.* (10). CDV was obtained as the wild type from an infected dog and propagated on Vero cells (11).

Establishment of Persistent Infection with SSPE Virus. C6 cells were persistently infected with the SSPE Lec strain as described (4). The state of infection (presence of cytoplasmic and membrane-associated virus antigen) was monitored by indirect immunofluorescence as described (4).

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Abbreviations: CDV, canine distemper virus; SSPE, subacute sclerosing panencephalitis (measles) virus; PtdEtn, phosphatidylethanolamine.

Establishment of Persistent Infection with CDV. Similar to the establishment of the C6/SSPE virus infection (see above) a persistent infection with the CDV wild type in C6 cells was obtained within a period of 100 days (20 passages). After formation of syncytia, production of cytopathic effect, and virus multiplication, the C6/CDV system reached a permanent state of infection, in which all the cells produced cytoplasmic virus antigens. This was detected in acetone-fixed cells by the indirect immunofluorescence technique either with crossreacting human anti-SSPE antiserum or rabbit anti-CDV antiserum and the corresponding fluorescein isothiocyanate-conjugated anti-IgG antisera. Over a period of 100 days (approximately 20 passages) only 1–10% of the living cells showed cell membrane-associated virus antigens when assayed with human anti-SSPE virus antiserum (3). In these cells there was almost no production of virus and no morphological changes, and the cells had growth properties similar to those of the uninfected C6 cells.

Materials for Methylation Assay. L-[^{3}H]Methionine was purchased from Amersham Buchler (Braunschweig, Federal Republic of Germany) (specific activity 93 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels). Isoproterenol was obtained from Serva (Heidelberg, Federal Republic of Germany). Inorganic and organic substances were obtained from Merck (Darmstadt, Federal Republic of Germany); 3-deazaadenosine was from Southern Research Institute, Kettering-Meyer Laboratories (Birmingham, AL); DL-homocysteine thiolactone was from Serva (Heidelberg, Federal Republic of Germany); *dl*-propranolol was from Sigma; and *dl*-alprenolol was a gift from H. Bönisch (Institute of Pharmacology, University of Würzburg, Federal Republic of Germany).

Materials for Phospholipid Identification. Marker phospholipids (phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine, and phosphatidylcholine) were obtained from Calbiochem–Behring (Giessen, Federal Republic of Germany).

Determination of Protein. Protein concentration was determined according to Lowry *et al.* (12).

Methylation Assay. For the phospholipid methylation assays, C6 cells were grown in 6-cm-diameter plastic dishes (Nunc, Roskilde, Denmark) for 1 or 2 days until they became a confluent monolayer in 5 ml of Dulbecco's minimal essential medium with 4.5 g of glucose per liter, 5% fetal calf serum, 5% newborn calf serum, and 4 mM L-glutamine. The incorporation of the methyl groups was studied by a modification of the method of Strittmatter *et al.* (13). The volume of medium in each cell culture dish was reduced to 2.5 ml, 20 μCi of [*methyl*-^{3}H]-methionine was added to each well and mixed with the medium, and the mixture was incubated for 35 min. After these incubations different concentrations of *l*-isoproterenol were added to plates of persistently infected and uninfected cells to give concentrations of 0.1, 10, and 100 μM. Controls did not receive isoproterenol. After further incubation for 35 min the stimulation with the hormone was stopped by adding 5 ml of an ice-cold 0.9% sodium chloride solution, washing twice with the same solution, and fixing by the addition of 3 ml of 10% trichloroacetic acid. The cells were scraped with a rubber policeman and then transferred to centrifuge tubes. After centrifugation at 27,000 × *g* for 10 min the supernatant was discarded. The phospholipids from the trichloroacetic acid precipitate were extracted by the addition of 3 ml of chloroform/methanol/hydrochloric acid, 2:1:0.02 (vol/vol). After the addition of 2 ml of 0.1 M KCl in 50% (vol/vol) methanol, the tubes were vigorously shaken in a Vibrax shaker (Jahnke & Kunkel, Type VXR, Staufen im Breisgau, Federal Republic of Germany) for 15 min and then centrifuged at 2,000 × *g* for 10 min. The aqueous phase was aspirated with a pipette, 2 ml of 0.1 M KCl in 50% methanol was added, and the chloroform/methanol phase was washed for 15 min in the Vibrax shaker and centrifuged again. Thereafter the aqueous phase was removed and 0.5 ml of the chloroform/methanol phase was transferred to scintillation vials for measurement of methylation. From the remaining 1.5 ml of the phospholipid-containing phase the chloroform and methanol were evaporated to dryness under a stream of N_2 gas. To separate and quantitate the various methylated phospholipids, the residue was dissolved into a small volume of chloroform/methanol, 1:1 (vol/vol), applied to a silica gel G plate with the unlabeled phospholipids as references, and separated with chloroform/methanol/7 M ammonia, 60:35:5 (vol/vol). The solvent front migrated a distance of approximately 16 cm. The separated phospholipids were visualized and identified by exposure to iodine vapor. Then the spots were scraped off the plate and transferred to counting vials, and ^{3}H radioactivity was measured in a liquid scintillation counter (Packard 460c) with a toluene/1,4-bis[2-(5-phenyloxazolyl)]benzene/2,5-diphenyloxazole mixture.

RESULTS

The methylation of membrane phospholipids in uninfected and persistently virus-infected C6 cells has been studied under unstimulated and hormone-stimulated conditions. Uninfected C6 cells and CDV or measles-SSPE virus-infected C6 cells show the same kinetics of phospholipid methylation in unstimulated cultures. This was shown by the measurement of the radioactivity of [^{3}H]methyl groups found in the extracted phospholipids after 70-min incubation of the C6, C6/CDV, and C6/SSPE cells without isoproterenol stimulation (Fig. 1, zero concentration of isoproterenol). The time dependence of methylation is demonstrated in Fig. 2*A* for uninfected C6 cells and is the same for infected cells. It can be seen that the modulation to a lower number of β-adrenergic receptors on C6/CDV cells that we described earlier (5) is not caused by a permanent inhibition of specific methylation of PtdEtn to lecithin, as could possibly be concluded from the results of Hirata and Axelrod (7). Also, we found no change in the pattern of TLC-separated phospholipids in infected compared to uninfected cells (data not shown). This is similar to the findings of other authors, using monkey kidney BGM cells persistently infected with measles virus (14). Another mechanism must exist to explain this permanent reduction of the receptor numbers in the persistently CDV-infected cells. At the moment, however, we have no data on which to base another hypothesis.

We have also studied the β-adrenergic receptor-specific stimulation of PtdEtn methylation by isoproterenol as described by Strittmatter *et al.* with C6 cells (13). These authors described their results as a direct activation of two specific membrane-located phospholipid methyltransferases by the catecholamine-occupied β-receptors. Subsequent to this event there is a facilitated coupling of the β receptors to the GTP-binding protein of the adenylate cyclase complex, stimulating the catalytic subunit of adenylate cyclase (7). In this way a rapid increase of cAMP is achieved (Fig. 2*B*). On the other hand, when β-adrenergic receptor-dependent methylation is inhibited by methyltransferase inhibitors, the stimulation of cAMP generation by catecholamines is also inhibited (7).

Because we found a strongly reduced cAMP response in our persistently infected C6 cells after addition of *dl*-isoproterenol, we studied the β-adrenergic receptor-dependent stimulation of phospholipid methylation in C6/SSPE and C6/CDV cells compared to uninfected C6 cells.

After 35-min preincubation of the cells with [*methyl*-^{3}H]methionine we added different concentrations of *dl*-isoproterenol and incubated them for an additional 35 min. After ex-

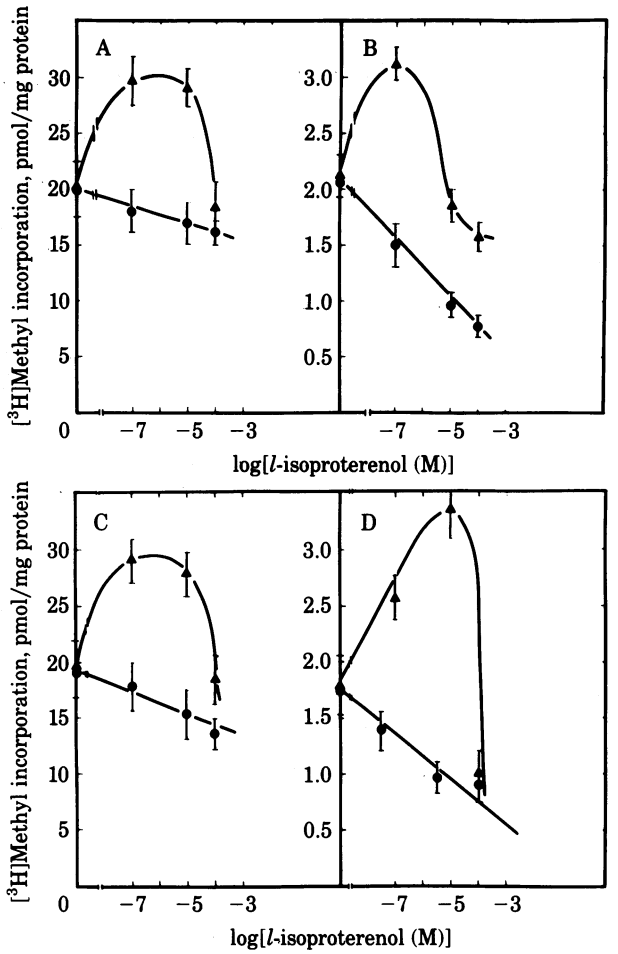

FIG. 1. Isoproterenol stimulation of methylation in uninfected (▲) and persistently infected (●) C6 cells in culture. (*A*) [^{3}H]Methyl group incorporation from [*methyl*-^{3}H]methionine into the total membrane phospholipids extracted from C6/SSPE cells. (*B*) [^{3}H]Methyl group incorporation into lecithin extracted from C6/SSPE cells. (*C*) [^{3}H]Methyl group incorporation from [*methyl*-^{3}H]methionine into the total membrane phospholipids extracted from C6/CDV cells. (*D*) [^{3}H]Methyl group incorporation into lecithin extracted from C6/SSPE cells. The results are obtained from four independent experiments with four individual cell passages from persistently infected cells. The values are calculated with respect to the total cell protein to make the different experiments comparable. Standard deviation is about 14%.

traction and isolation of the phospholipids we determined the [^{3}H]methyl group labeling of the phospholipids. The results are shown for the total phospholipids and also separated lecithin in Fig. 1. It is demonstrated that there is an isoproterenol-dependent stimulation of methylation only in the case of uninfected cells; no stimulation occurs in the infected ones. In addition, the basal methylation of infected cells was depressed in all experiments with increasing concentrations of isoproterenol used. Therefore it was uncertain if this effect was a β-receptor-dependent process. This was further investigated by inhibiting the isoproterenol-dependent effect with the β-receptor antagonist propranolol. This antagonist did not inhibit the decrease of basal methylation (Fig. 3*A*), thus demonstrating that the isoproterenol effect on basal methylation was not a β-receptor-dependent process. The data in Fig. 3*A* further demonstrate that the isoproterenol-stimulated PtdEtn methylation of uninfected C6 cells is totally inhibited by propranolol. Both of the β-adrenergic antagonists propranolol and alprenolol inhibit the isoproterenol-stimulated methylation in the same manner. These

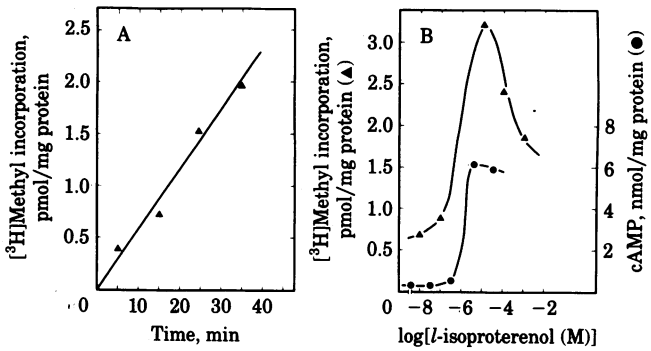

FIG. 2. (*A*) Kinetics of methylation of lecithin in C6 cells incubated with [*methyl*-^{3}H]methionine without stimulation by isoproterenol. Each point was calculated from two independent experiments. (*B*) Concentration dependence of the isoproterenol-stimulated methylation effect and the β-receptor-mediated cAMP response in C6 cells. The cAMP experiment and the determination of cAMP were done as described before (4).

findings demonstrate that the catecholamine-stimulated PtdEtn methylation in C6 cells is β-adrenergic receptor dependent as in reticulocyte ghosts (8). The methylation level of untreated C6 cells is achieved at the antagonist concentration of 10 to 100 μM (Fig. 3*B*). However, the basal methylation without addition of isoproterenol is the same in all cells. It is remarkable that the optimal stimulation of methylation by *dl*-isoproterenol observed in C6 cells is achieved in the same range of concentration as for the stimulation of cAMP (Fig. 2*B*).

Fig. 4 shows the profiles of the TLC-separated [^{3}H]methyl-labeled products from uninfected and infected cells after isoproterenol stimulation. Only the uninfected C6 cells show the changes in methylation, giving a small increase of label in the monomethyl and dimethyl PtdEtn and a larger amount in lecithin. In order to ensure that we were studying the phospholipid methylation and not other reaction products also, we used the phospholipid methyltransferase inhibitors 3-deazaadenosine (1 mM) in the presence of homocysteine thiolactone (0.2 mM) in the incubation. After extraction with organic solvents and separation by TLC the incorporation of [^{3}H]methyl groups into the phospholipids was measured. 3-Deazaadenosine together with

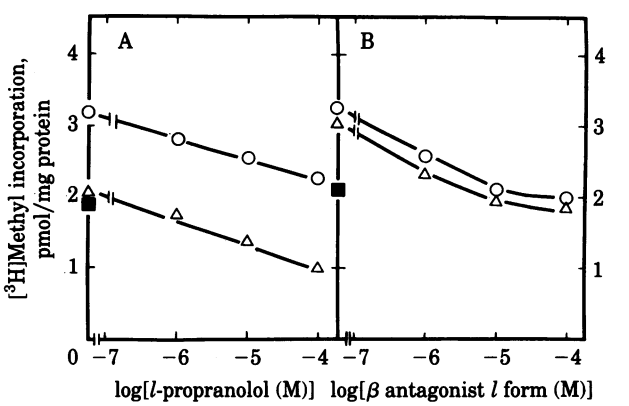

FIG. 3. (*A*) Inhibition of the methylation stimulation induced in the presence of *l*-isoproterenol (10 μM) by different concentrations of *l*-propranolol. [^{3}H]Methyl-labeled lecithin isolated from TLC plates was measured: ○, from uninfected C6 cells; △, from persistently SSPE virus-infected C6 cells; ■, from C6 cells without isoproterenol stimulation and without propranolol. Standard deviation of the measurements was 8%. (*B*) Inhibition of the methylation stimulation induced in uninfected C6 cells in the presence of *l*-isoproterenol (10 μM) by β-receptor antagonists at different concentrations: ○, *l*-propranolol; △, *l*-alprenolol; ■, control without isoproterenol and without antagonists. Standard deviation of the measurements was 8%.

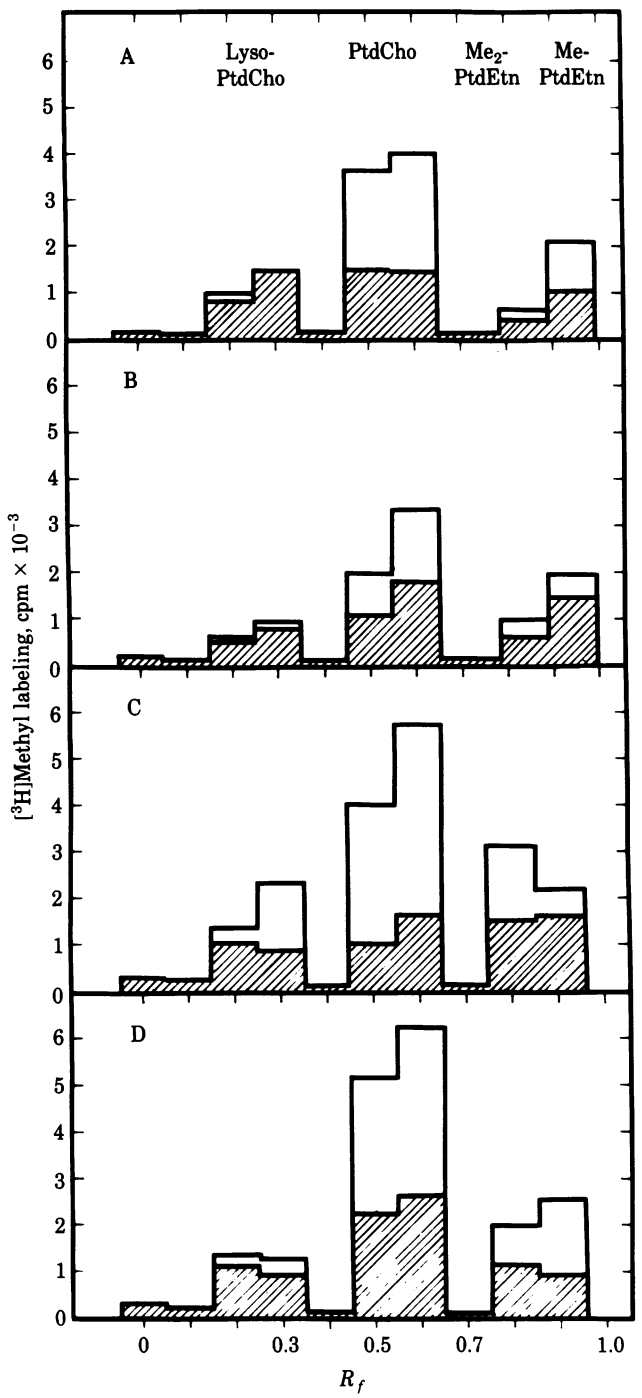

FIG. 4. TLC pattern of [^{3}H]methyl-labeled phospholipids after stimulation with *l*-isoproterenol. Phospholipids were extracted from uninfected (empty bars) and persistently infected C6 cells (hatched bars). (*A*) C6 and C6/SSPE cells, 0.1 μM *l*-isoproterenol; (*B*) C6 and C6/SSPE cells, 10 μM *l*-isoproterenol; (*C*) C6 and C6/CDV cells, 0.1 μM *l*-isoproterenol; (*D*) C6 and C6/CDV cells, 10 μM *l*-isoproterenol. Positions of markers are indicated: lyso-PtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; Me_2PtdEtn, dimethyl PtdEtn; MePtdEtn, monomethyl PtdEtn.

homocysteine thiolactone inhibited the stimulation of methylation at any concentration of isoproterenol used in our experiments (Fig. 5).

DISCUSSION

C6 cells are stimulated by isoproterenol via the β-adrenergic receptors to give high cAMP levels and high methylation of

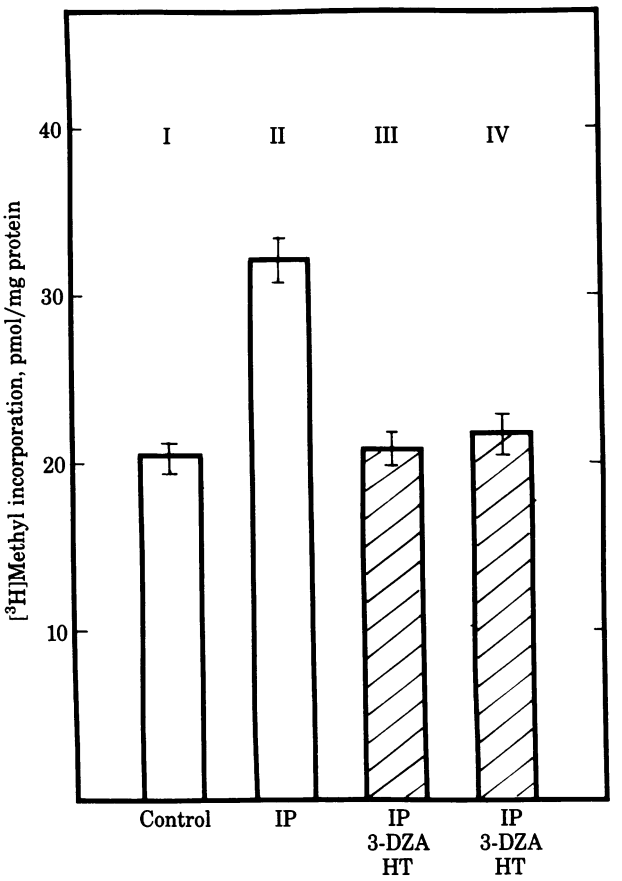

FIG. 5. Inhibition of phospholipid methylation (lecithin) by methyltransferase inhibitors in C6 cells. Inhibitors were 3-deazaadenosine (3-DZA) at 1 mM and homocysteine thiolactone (HT) at 0.1 mM. Cells were preincubated for 35 min with [*methyl*-^{3}H]methionine, then inhibitors were added (bars III and IV); 5 min later *l*-isoproterenol (IP) (bar III, 10 μM; bar IV, 0.1 μM) was added and cells were further incubated for 30 min. Bar I shows methylation of unstimulated, uninhibited cells and bar II shows the isoproterenol-stimulated uninhibited cells. Values were calculated from duplicate samples. The deviation from the mean is about 5%.

PtdEtn, yielding monomethyl, dimethyl, and trimethyl PtdEtn (lecithin). In C6/CDV and C6/SSPE cells we have observed a total loss of β-receptor-dependent phospholipid methylation and a strong reduction of the cAMP stimulation by isoproterenol. Uninfected and persistently CDV- and SSPE-infected C6 cells show the same kinetics of unstimulated methylation and the same methylation products. We conclude that the reduced number of β-adrenergic receptors observed only in the C6/CDV system is not caused by a permanent reduction in methylation of phospholipids in these persistently infected cells.

Hirata and Axelrod have postulated that methylation of PtdEtn facilitates the coupling of β-adrenergic receptors to adenylate cyclase. Inhibition of these methylation reactions makes this event more difficult, resulting in a strongly reduced stimulation of cAMP synthesis by catecholamines. In both cases of persistent infections we have observed such a reduced cAMP response. Therefore, it is possible that the observed impairment of signal transmission to the adenylate cyclase is primarily caused by the inhibition of methylation stimulation in our infected cells. At the moment the mechanism for this loss of β-adrenergic receptor-dependent catecholamine stimulation of the PtdEtn methylation is unknown. It is possible that there is an inhibition of interactions between the β receptor and the stimulation-sensitive methyltransferases or that these enzymes

are inactivated in the infected cells. The normal PtdEtn methylation without stimulation does not invalidate such an idea because there may be stimulation-insensitive methyltransferases that are not influenced by the virus infection.

Furthermore, it is necessary to discuss the drop of basal methylation with increasing isoproterenol concentration shown in Fig. 1. We have investigated the possibility that this phenomenon might be β-adrenergic receptor dependent. But our results (Fig. 3A) clearly show that the isoproterenol-induced drop cannot be inhibited by the antagonist propranolol. The observed increase in basal methylation must be due to other as yet unknown mechanisms. Our results (Fig. 4) demonstrate that there is no enhanced degradation of methylated PtdEtn. An increased degradation should result in an increase in the lysophosphatidylcholine concentration, which does not occur. Because only the amount of phosphatidylcholine is reduced we can conclude that the methylation of PtdEtn giving phosphatidylcholine was affected.

Our persistently infected cell systems could also be used to examine some of the other proposals of Hirata and Axelrod. These authors postulated a function for catecholamine-stimulated PtdEtn methylation in the desensitization or refractory state of the β-adrenergic receptor/adenylate cyclase system of C6 cells (7, 15). This seems to be mediated by an activation of phospholipase A_2 after methylation. These authors have carried out experiments with inhibitors and activators of phospholipase A_2 with respect to the formation of refractoriness. Because our infected cells cannot be stimulated to further methylate PtdEtn they should show a strong inhibition of desensitization of the β-adrenergic receptor/adenylate cyclase system.

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