# Isoproterenol stimulates lipid methylation in $C_6$ cells without affecting membrane fluidity

Roderick C. McKenzie and Peter J. Brophy*

*Department of Biological Science, University of Stirling, Stirling FK9 4LA, Scotland*

Received 13 October 1983

$C_6$ glioma cells were stimulated with the $\beta$-adrenergic agonist isoproterenol. The rate of cyclic AMP accumulation and lipid methylation increased. However, the elevated rate of lipid methylation did not affect the fluidity of the plasma membrane. It is concluded that lipid methylation is not responsible for making the plasma membrane more fluid during the $\beta$-adrenergic response.

*$C_6$ cell* *Lipid methylation* *Cyclic AMP* *Membrane fluidity*

## 1. INTRODUCTION

An increased rate of conversion of phosphatidylethanolamine to phosphatidylcholine is associated with a variety of events that are mediated by the plasma membranes of intact cells [1,2]. The role of phospholipid *N*-methylation remains obscure although it has been suggested that the enhanced rate of phosphatidylcholine biosynthesis from phosphatidylethanolamine which accompanies $\beta$-adrenergic stimulation might make the plasma membrane more fluid thereby facilitating coupling between the $\beta$-adrenergic receptor and adenylate cyclase [1]. Authors in [3] have disputed a link between increases in flux through the methylation pathway and increased fluidity, primarily on theoretical grounds. We report here that stimulation of lipid methylation and cyclic AMP accumulation in intact $C_6$ astrocytoma cells by isoproterenol is not accompanied by any change in the molecular order of plasma membrane lipid.

## 2. MATERIALS AND METHODS

### 2.1. *Cell culture*

$C_6$ astrocytoma cells were grown until confluent in Dulbecco's Eagle's Medium supplemented with 5% foetal calf serum, penicillin (100 IU/ml) and streptomycin (100 $\mu$g/ml) (all from Flow Laboratories) in an atmosphere of 5% carbon dioxide, 95% air at 37°C.

### 2.2. *Lipid methylation*

Cells were grown in Linbro multi-dish trays (0.5–0.7 mg protein per well). After the cells had been incubated at 37°C with 20 $\mu$Ci [*methyl*-$^3$H]methionine in 1 ml Medium 199 (Flow Laboratories) for 25 min, the drugs were added and the incubations were continued for a further 30 min. Lipids were extracted from the cells with chloroform/methanol exactly as in [4]. In each 6-well Linbro tray, 4 wells were used for measuring the uptake of [$^3$H]methyl groups into lipid and the remaining two wells were used to determine the protein content of the cells. The results are expressed as pmol [$^3$H]methyl groups incorporated into lipid/55 min incubation period per mg protein.

### 2.3. *Cyclic AMP measurement*

Cells were grown in Petri dishes (4.5 cm diameter, 0.9–1.1 mg protein) and their cyclic AMP content was measured after the same periods of incubation in the presence of Medium 199 (3 ml) and drugs as used in the methylation experiments. Incubations were stopped by washing

* To whom correspondence should be addressed



*Published by Elsevier Science Publishers B.V.*

the cells with ice-cold saline (pH 7.4) and cyclic AMP was extracted with 0.1 M HCl (1 ml). After 30 min the acid extract was removed and its cyclic AMP content was measured by a protein-binding method [5]. The protein content of the cellular residue was measured as in [6].

### 2.4. *Electron spin resonance*

The cells were grown in Petri dishes (9 cm diameter, 1.6–2.0 mg protein) and the incubation protocol was similar to that described for the methylation and cyclic AMP experiments. Cells were incubated in Medium 199 (10 ml) for 25 min and incubated for a further 30 min in the presence of drugs. Ten minutes before the end of the incubation in the presence of the drugs, 3 $\mu$l of an ethanolic solution of 5-doxylstearic acid (5-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearic acid) (50 $\mu$g, Syva) were added. Cells were harvested by scraping with a rubber policeman, centrifuged at 1000 rpm for 5 min and then transferred to a capillary for the electron spin resonance (ESR) measurements. Spectra were recorded with a Jeol JES-PE-1X spectrometer equipped with a temperature control system. To minimize internalization and destruction of the spin-labelled fatty acid, spectra were recorded at 30°C. There was no significant contribution to the spectra from unincorporated label and repeat scans on the same sample showed little diminution in signal intensity over a 15 min period. The molecular order of the spin-labelled fatty acid was assessed by measuring the hyperfine splitting of the outer extrema of the ESR spectra [7,8].

## 3. RESULTS AND DISCUSSION

We have measured here changes in the physical properties of plasma membrane lipid in intact $C_6$ cells by studying the motional properties of a spin-label probe. 5-Doxylstearic acid partitions into the plasma membranes of cultured cells and remains there for at least 30 min [9]. Recent studies on Sarcoma 180 cells have shown that changes in the ordering of 5-doxylstearic acid in the plasma membrane are a sensitive indicator of the interaction of certain drugs with the cell surface [10]. The ESR spectrum of 5-doxylstearic acid in biological membranes is characteristic of anisotropic motion about the bilayer normal and the degree of anisotropy is a measure of the average amplitude of motion of the spin label about the long axis of the fatty acid chain. The smaller the angular motion, the more anisotropic is the derived spectrum and the membrane lipid is therefore more ordered. The extent of the lipid ordering is one measure of membrane fluidity [7]. We have measured the order of 5-doxylstearic acid in the plasma membranes of intact $C_6$ astrocytoma cells by measuring the hyperfine splitting ($2T'_{/\!/}$) of the outer extrema of ESR spectra. The larger the splitting, the more ordered, i.e., the less fluid is the membrane. This measurement is a very sensitive indicator of changes in the order of membrane lipid [8].

Table 1 shows that incubation of $C_6$ cells with the $\beta$-adrenergic agonist isoproterenol causes an

Table 1

Effects of isoproterenol and propranolol on lipid methylation, cyclic AMP accumulation and the physical properties of plasma membranes in intact $C_6$ cells

| Drug | [$^3$H]Methyl incorporated into lipid (pmol/mg protein)[a] | Cyclic AMP (pmol/mg protein)[b] | $2T'_{/\!/}$ (G)[c] |
|---|---|---|---|
| None | 32 ± 4 | 14 ± 3 | 52.9 ± 0.1 |
| Isoproterenol (10 $\mu$M) | 75 ± 10 | 806 ± 133 | 52.9 ± 0.1 |
| Isoproterenol (10 $\mu$M) + propranolol (500 $\mu$M) | 36 ± 5 | 27 ± 5 | 53.7 ± 0.1 |
| Propranolol (500 $\mu$M) | 35 ± 11 | 13 ± 1 | 53.9 ± 0.1 |

[a] Means of 4 assays ± SE
[b] Means of 3 assays ± SE
[c] Means of measurements from the spectra of 4 different samples ± SD

intracellular accumulation of cyclic AMP and that this is accompanied by a stimulation in the rate of lipid methylation. Furthermore, propranolol blocks these responses which demonstrates that both effects are mediated by the $\beta$-adrenergic system. These results are consistent with those in [2]. If the increased rate of lipid methylation were to affect the physical properties of plasma membrane lipid, a change in the hyperfine splitting of the ESR spectrum of 5-doxylstearic acid should have been detected: that this is not the case is shown in table 1. Therefore it seems unlikely that lipid methylation influences $\beta$-adrenergic activation of the adenylate cyclase in $C_6$ cells by altering the motional properties of lipid in the plasma membrane. Table 1 also shows that, by contrast, propranolol does affect the physical properties of plasma membrane lipid. Propranolol has been shown to increase the order of pure phospholipid bilayers [11] and it clearly has a similar effect on the plasma membrane of $C_6$ cells.

## ACKNOWLEDGEMENT

R.C.McK. thanks the Science and Engineering Research Council for a studentship.

## REFERENCES

[1] Hirata, F. and Axelrod, J. (1980) Science 209, 1082–1090.
[2] Strittmatter, W.J., Hirata, F., Axelrod, J., Mallorga, P., Tallman, J.F. and Henneberry, R.C. (1980) Nature 282, 857–859.
[3] Vance, D.E. and De Kruijff, B. (1980) Nature 288, 277.
[4] Hirata, F., Viveros, O.H., Diliberto, E.J. jr and Axelrod, J. (1978) Proc. Natl. Acad. Sci. USA 75, 1718–1721.
[5] Brown, B.L., Albano, J.D.M., Ekins, R.P., Sgherzi, A.M. and Tampion, W. (1971) Biochem. J. 121, 561–562.
[6] Lowry, O.H., Rosebrough, N.J., Farr, L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[7] Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326.
[8] Pang, K.-Y. (1980) Mol. Pharmacol. 18, 84–90.
[9] Klausner, R.D., Kleinfield, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295.
[10] Siegfried, J.A., Kennedy, K.A., Sartorelli, A.C. and Tritton, T.R. (1983) J. Biol. Chem. 258, 339–343.
[11] Surewicz, W.K. and Leyko, W. (1981) Biochim. Biophys. Acta 643, 387–397.