PREPARATION OF A LIVER PLASMA MEMBRANE WITH AN ADRENALIN-RESPONSIVE ADENYLYL CYCLASE AFTER INHIBITION OF PROSTAGLANDIN SYNTHESIS BY INDOMETHACIN

H. K. WASNER

Department of Physiological Chemistry, University of Würzburg, Koellikerstraße 2, 8700 Würzburg, FRG

Received 3 November 1976

1. Introduction

Sensitivity of liver adenylyl cyclase to glucagon and to a lesser degree to adrenalin was first reported by Murad et al. [1]. In purified liver plasma membrane preparations adenylyl cyclase is mainly sensitive to glucagon as first found by Pohl et al. [2]. Following Bitensky's observation [3] that adrenalectomy increases the sensitivity of rat liver adenylyl cyclase towards adrenalin, F. Lerày [4] demonstrated a weak stimulation of liver plasma membrane adenylyl cyclase by adrenalin.

Cyclic AMP antagonist [5] regulates protein kinase and phosphoprotein phosphatase in an opposite way to cAMP. This antagonist inhibits the adrenalin- but not the glucagon-responsive adenylyl cyclase by phosphorylating this enzyme, most likely in a complex reaction event. [7]. This may be one of the reasons why only very poor responsiveness of adenylyl cyclase to adrenalin has been found in liver plasma membrane preparations. Synthesis of this cAMP antagonist can be inhibited by treatment of rats with indomethacin (H. Wasner, in preparation). The present report shows that liver plasma membrane preparations from indomethacin pretreated rats are highly responsive not only to glucagon but also to adrenalin.

2. Materials and methods

Male Sprague Dawley rats weighing approximately 200 g were used. The rats were fed ad libitum on standard diet and had free access to water. Bilaterally adrenalectomized rats were maintained on physiological saline solution for 5 days. Indomethacin (5 mg/kg body weight) was injected intraperitoneally twice a day for 1–2 days.

The quickly excised livers were immediately homogenized with five to seven strokes of a Dounce homogenizer with a loose fitting pestle in medium I (20 mM Tris–HCl, pH 7.4, 20 mM sucrose, 1 mM EDTA, 1 mM ATP, 2 mM $NaHCO_3$). The homogenate prepared from 4–5 livers was brought to a final volume of 200 ml with medium I, filtered three times through a triple layer of surgical gauze and centrifuged at 1000 × *g* for 10 min. The pellet was suspended in 100 ml medium I and respun. The redispersed pellet was layered on a linear sucrose gradient (20–60% (w/w) sucrose, containing 1 mM EDTA, 1 mM ATP and 10 mM Tris–HCl, pH 7.4) and centrifuged for 60 min at 27 000 rev./min in a SW 27 rotor. The most prominent zone in the sucrose gradient at a density of approximately 1.22 consists primarily of mitochondrial membranes. Above this there is a smaller zone with a density of approximately 1.16, representing the plasma membranes. This zone was aspirated from the top and diluted with 4–5 volumes of medium II (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM sucrose) and centrifuged at 15 000 × *g* for 10 min. The pellet was resuspended in 4–5 ml of medium II and frozen in 0.5 ml aliquots in liquid nitrogen. From five livers (approximately 25 g) 12–20 mg membrane protein was obtained. This preparation could be stored in liquid nitrogen for one year without detectable loss of adrenalin-responsive adenylyl cyclase activity.

Adenylyl cyclase assay was performed essentially

according to Rall and Sutherland [8]. The assay mixture contained in a final volume of 600 μl: 2.4 mM ATP, 4 mM Mg^{2+}, 5 mM theophylline, 30 mM glycylglycine buffer, pH 7.4, 20–50 μg liver plasma membrane proteins. Final concentrations of adrenalin and glucagon were 2.25×10^{-4} M and 5×10^{-8} M respectively. Incubations were carried out at 30°C for 10–20 min and stopped by adding 0.5 ml 0.6 M perchloric acid. [^{3}H]cAMP (6000 cpm) was added for recovery determination. cAMP formed was purified on Dowex 50 WX 8 and estimated by the protein binding assay of Gilman [9].

Protein was measured by the procedure of Lowry et al. [10] using bovine serum albumin as standard. [^{3}H]cAMP was obtained from Amersham-Buchler; ATP and cAMP were from Boehringer, Mannheim; adrenalin was a gift from Farbwerke Hoechst; glucagon from Eli Lily; and indomethacin from Dr C. A. Stone, Merck Sharp and Dohme. Dowex 50 was obtained from Bio-Rad Lab.; all other chemicals were from E. Merck, Darmstadt.

3. Results

3.1. *Plasma membrane of normal rats*

In liver plasma membranes prepared from normal, untreated rats basal, as well as glucagon and adrenalin stimulated cAMP synthesis by adenylyl cyclase was determined. Mainly glucagon-responsive adenylyl cyclase was found as demonstrated also by others [2,4]. The average glucagon stimulation was at least 10-fold over the basal level (fig.1).

3.2. *Plasma membrane of indomethacin treated rats*

Indomethacin, known as an inhibitor of prostaglandin synthetase [11], also inhibits the synthesis of the cAMP antagonist (H. Wasner, in preparation). Rats were usually subjected to 4 injections of indomethacin within 2 days. Liver plasma membranes were prepared, and basal, glucagon and adrenalin stimulated adenylyl cyclase activity was measured. As illustrated in fig.2 the membranes of indomethacin treated rats exhibited, in addition to their glucagon responsiveness a marked increase in adrenalin-stimulated adenylyl cyclase activity. Glucagon stimulation was at least 10-fold, and stimulation by adrenalin about 4-fold over the basal level.

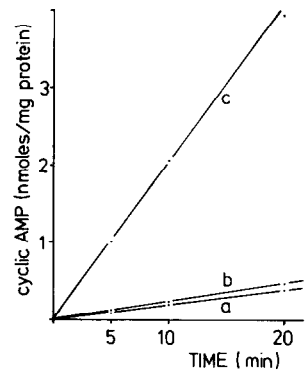

Fig.1. Time course of cAMP synthesis by adenylyl cyclase from a purified liver plasma membrane of normal, untreated rats. Curve (a) basal level of cAMP synthesis, curve (b) adrenalin-stimulated cAMP synthesis (2.25×10^{-4} M) and curve (c) glucagon-stimulated cAMP synthesis (5×10^{-8} M).

3.3. *Plasma membrane of indomethacin treated, adrenalectomized rats*

Bilaterally adrenalectomized rats were also treated with indomethacin. A first injection was made at the end of the fourth and a second on the fifth day after adrenalectomy. A third injection was omitted, because the survival rate of adrenalectomized rats is much smaller on indomethacin treatment, when compared with normal rats. At the end of the fifth day after adrenalectomy liver plasma membranes were prepared

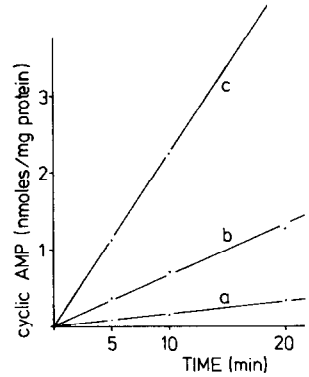

Fig.2. Time course of cAMP synthesis by adenylyl cyclase from a purified liver plasma membrane of indomethacin treated rats. Curve (a) basal level of cAMP synthesis, curve (b) adrenalin-stimulated (2.25×10^{-4} M) and curve (c) glucagon-stimulated cAMP synthesis (5×10^{-8} M).

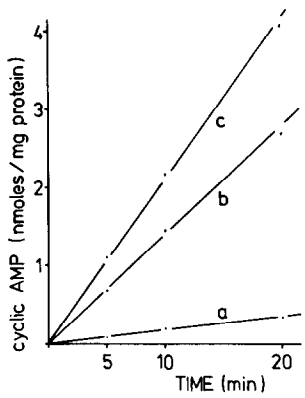

Fig.3. Time course of cAMP synthesis by adenylyl cyclase from a purified liver plasma membrane of indomethacin treated, adrenalectomized rats. Curve (a) basal level, curve (b) adrenalin-stimulated (2.25×10^{-4} M) and curve (c) glucagon-stimulated cAMP synthesis (5×10^{-8} M).

and assayed for adenylyl cyclase activity (fig.3). Glucagon stimulation of adenylyl cyclase was approximately 10-fold over the basal level, but on indomethacin treatment the responsiveness of adenylyl cyclase to adrenalin was largely increased in adrenalectomized rats, resulting in an 8-fold stimulation.

In a control experiment purified liver plasma membrane preparations of adrenalectomized rats without indomethacin treatment showed a twofold stimulation of adenylyl cyclase by adrenalin, confirming the results of Lerày [4] and Hanoune [13].

4. Discussion

Glucagon- and adrenalin-responsive adenylyl cyclase have different stabilities. Thus, adrenalin sensitivity is much more labile against dilution with buffer during the preparation. In plasma membranes prepared according to Neville [12] from livers of adrenalectomized, indomethacin treated rats, mainly elevated basal cAMP synthesis rather than a hormonal stimulation of adenylyl cyclase could be observed.

It appears that the increase in responsiveness of adenylyl cyclase to adrenalin after adrenalectomy represents an increase in enzyme protein levels [3]. It may also be the result of sensibilisation of adenylyl cyclase towards adrenalin because of adrenalin deficiency. The effect of indomethacin on adrenalin stimulated adenylyl cyclase is independent from adrenalectomy. An often observed post mortem effect and result of tissue trauma is that tissue prostaglandin concentrations rise rapidly [14,15].

Hepatocytes, which were prepared under mild conditions and are kept in well oxygenated media still respond to adrenalin with an increased cAMP synthesis. In contrast the rigorous conditions for the plasma membrane preparations may lead to an increase not only in the prostaglandin but also in the cAMP antagonist levels. Evidence has been gained that this antagonist seems to regulate the inhibition of the adrenalin-responsive adenylyl cyclase indirectly by activating a membrane bound protein kinase, which then inhibits the adenylyl cyclase by phosphorylation [7]. This concept is supported by the present finding that it is possible to prepare a liver plasma membrane with an adrenalin-responsive adenylyl cyclase after indomethacin treatment of the donor rats. Furthermore it is suggested that there may exist a correlation between prostaglandin formation and the synthesis of a cAMP antagonist [6].

Acknowledgements

This work was initiated in the laboratory of E. W. Sutherland (Department of Physiology, Vanderbilt University and Department of Biochemistry, University of Miami) and was supported by NIH grant HL 16671-01. H. Wasner was recipient of the fellowships WA 297/1, 2 and 3 from the Deutsche Forschungsgemeinschaft. The work is now supported by grant WA 297/4 from the Deutsche Forschungsgemeinschaft.

References

[1] Murad, F., Chi, Y. M., Rall, T. W. and Sutherland, E. W. (1962) J. Biol. Chem. 237, 1233–1238.
[2] Pohl, S. L., Birnbaumer, L. and Rodbell, M. (1969) Science 164, 566–567.
[3] Bitensky, M. W., Russel, V. and Blanco, M. (1970) Endocrinology 86, 154–159.
[4] Lerày, F., Chambaut, A. M. and Hanoune, J. (1972) Biochem. Biophys. Res. Commun. 48, 1385–1391.
[5] Wasner, H. K. (1975) FEBS Lett. 57, 60–63.
[6] Wasner, H. K. (1975) FEBS Meet. Abstract Commun., No. 1367.

[7] Wasner, H. K. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 285.
[8] Rall, T. W. and Sutherland, E. W. (1962) J. Biol. Chem. 237, 1228–1232.
[9] Gilman, A. G. (1970) Proc. Natl. Acad. Sci. USA 67, 305–312.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Vane, J. R. (1971) Nature New Biol. 231, 232–235.
[12] Neville, D. M. (1968) Biochim. Biophys. Acta 154, 540–552.
[13] Hanoune, J., Lacombe, M. L. and Pecker, F. (1975) J. Biol. Chem. 250, 4569–4574.
[14] Wolfe, L. S., Pappius, H. M. and Marion, J. (1976) in: Advances in Prostaglandin and Thromboxane Research (Samuelsson, B. and Paoletti, R., eds) Vol. 1, pp. 345–355, Raven Press, New York.
[15] Änggard, E., Bohman, S. O., Griffin, J. E., Larsson, C. and Maunsbach, A. B. (1972) Acta Physiol. Scand. 84, 231–246.