Volume 309, number 3, 381–384 FEBS 11539 September 1992

A selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide (H-89), inhibits phosphatidylcholine biosynthesis in HeLa cells

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Received 19 July 1992; revised version received 30 July 1992

In this study, we report that the potent and selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide (H-89) interferes with the incorporation of choline into phosphatidylcholine in HeLa cells. Treatment of cells with 10 μM H-89 for 1 h decreases the phosphatidylcholine biosynthesis by 50%. This inhibition is prevented by simultaneous addition of 10 μM forskolin, while the choline uptake itself is not affected by H-89.

Phosphatidylcholine biosynthesis; cAMP-dependent protein kinase; *N*-[2-Bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide (H-89); Forskolin; HeLa cell

1. INTRODUCTION

There are valid indications from both in vitro and in vivo studies that phosphatidylcholine (PC) biosynthesis is at least in part regulated by cAMP. Incubation of fetal lung explants with the cAMP analogue, dibutyryl-cAMP, increased the choline incorporation into PC by 80% [1]. Choline incorporation also was stimulated when an alveolar type 2-like cell line A549 was treated with different cAMP analogues [2]. In contrast, it was reported that in rat hepatocytes PC biosynthesis was inhibited by 30–40% when applying the same cAMP analogues [3]. Furthermore, CTP:choline phosphate cytidylyltransferase (CT) (EC 2.7.7.15) activity was reduced in cells preincubated with these analogues. In vitro studies have shown that purified CT is phosphorylated by cAMP-dependent protein kinase. This phosphorylation is paralelled by an inhibition of membrane-binding of CT. The phosphorylated, inactive, cytosolic form of CT became active after dephosphorylation with alkaline phosphatase [4]. However, it should be pointed out that Watkins and Kent found no effect of cAMP analogues on PC biosynthesis of isolated rat hepatocytes [5]. Besides, cholera toxin had no influence on PC biosynthesis in hepatocytes, although the level of cAMP increased up to 100-fold in these in vivo experiments. Taken together, there exists some controversy concerning the regulation of CT activity by phosphorylation/dephosphorylation in vitro and the regulation of PC biosynthesis in cell culture experiments. In consequence, the possibility of indirect effects on PC biosynthesis, mediated by a cAMP-dependent protein kinase, must be considered.

Recently, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide (H-89) was shown as a new selective inhibitor of cAMP-dependent protein kinase in vivo [6]. The K_i value of this compound for cAMP- dependent protein kinase is 0.048 μM. In the present study, we used this inhibitor to investigate its influence on PC metabolism in HeLa cells. The results show that H-89 inhibits PC biosynthesis. This effect was prevented by forskolin, which is known to increase the cellular cAMP level by activating the adenylate cyclase [7].

2. MATERIALS AND METHODS

2.1. *Materials*

[*methyl*-^{3}H]Choline chloride (2.8–3.1 TBq/mmol) was from Amersham (Braunschweig, Germany). *N*-[2-Bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide (H-89) was obtained from Calbiochem (Bad Soden, Germany). Silica gel 60 HPTLC plates and reagents were purchased from Merck (Darmstadt, Germany). The BCA-kit for protein determination was from Pierce (Weiskirchen, Germany). For quantification of radioactivity a thin-layer chromatography scanner (Berthold LB2821 HR, Wildbad, Germany) was used. Forskolin, hemicholinium-3, phosphatidylcholine and 12-tetradecanoyl-phorbol-13-acetate (TPA) were obtained from Sigma (München, Germany). The alkaline phosphatase assay kit was from Behring Werke (Marburg, Germany).

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Abbreviations: PC, phosphatidylcholine; H-89, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulfonamide; TPA, 12-tetradecanoyl-phorbol-13-acetate; PBS, phosphate-buffered saline.

2.2. *Cell culture*

HeLa cells were grown in Dulbecco minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 0.56 g/l glutamine, 110000 U/l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Denmark). Media and culture reagents were from Gibco (Karlsruhe, Germany), antibiotics were obtained from Boehringer (Mannheim, Germany).

For radiolabelling, medium was removed, and pulse medium ([*methyl*-^{3}H]choline; 7.4×10^4 Bq/ml) supplemented either with H-89, forskolin, or H-89 in combination with forskolin was added. After incubation, cells were washed with ice-cold phosphate-buffered saline (PBS), pH 7.2, harvested and stored at −70°C.

2.3. *Extraction of lipids*

Lyophilized cell pellets were extracted according to Bligh and Dyer [8] by adding 50 μl methanol, 25 μl chloroform and 20 μl water. Samples were stirred for 2 min followed by a centrifugation at 13,000 × *g* for 10 min. For phase separation, 25 μl chloroform and 25 μl water were added. Stirring and centrifugation steps were repeated once. For lipid analysis, 20 μl of the chloroform phases were applied to HPTLC plates and the compounds were separated by using chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) [9]. Radioactivity was quantified by radioscanning.

2.4. *Choline uptake*

Choline uptake was measured as described [10]. Briefly, cells grown on collagen-coated plastic sheets, were preincubated for 30 min with medium containing H-89 or no supplements as control. After preincubation, cells were immersed in medium with or without H-89, containing (*methyl*-^{3}H]choline (5 μCi/ml). After different times, cells were washed thoroughly with 58 mmol/l choline chloride in PBS. Finally, uptake and incorporation of [^{3}H]choline were determined by liquid scintillation counting. Background radioactivity was measured by repeating the experiments at 4°C.

2.5. *Other procedures*

Possible cytotoxicity of H-89 and forskolin on HeLa cells was determined by measurement of the release of alkaline phosphatase [11]. Briefly, after incubation for 4 h with different concentrations of H-89 or forskolin, the cells were centrifuged at 1,000 × *g* for 10 min, and the supernatant thus obtained was discarded. The reaction mixture containing 20% diethanolamine, 2 μmol/l $MgCl_2$, 2 μmol/l *p*-nitrophenolphosphate and 10% Triton X-100 was added. After 30 min at 37°C the reaction was stopped with 0.5 mol/l NaOH. The amount of *p*-nitrophenolate was determined photometrically at 410 nm.

Protein determination was carried out by the BCA-assay [12] using bovine serum albumin as standard.

3. RESULTS

First of all, we determined the dose-dependent effects of H-89 and forskolin on cell viability. The highest concentration of H-89 applied (40 μM) had only a minor cytotoxic influence on HeLa cells as measured by the release of alkaline phosphatase. Compared to control, this effect was <10%. Furthermore, the cytotoxic effect of forskolin was in the same range. All experiments in this study were conducted using only 10 μM of these compounds.

The dose-dependent inhibitory effect of H-89 on PC biosynthesis after 1 h of treatment shows that the choline incorporation into PC was decreased by 50% compared to control (Fig. 1). Virtually the same dose-dependent inhibitory effect was seen after an incubation time of 2 h.

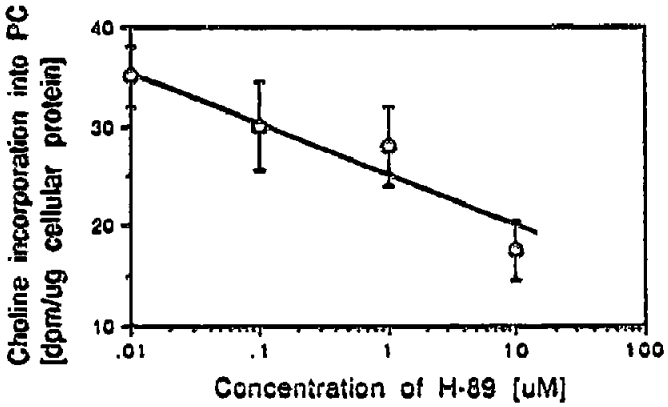

Fig. 1. Dose-dependent effect of H-89 on PC biosynthesis of HeLa cells. HeLa cells were grown to confluence. The medium was removed, and pulse medium was added, containing ([*methyl*-^{3}H]choline (7.4×10^4 Bq/ml) and different concentrations of H-89. After 1 h of incubation, the cells were mechanically harvested and PC was analysed as described in section 2. The amounts of choline incorporated is expressed in dpm/μg of cellular protein ± S.D. ($n = 3$).

To raise the cellular cAMP level, HeLa cells were incubated in the presence of 10 μM forskolin. This treatment resulted only in a slight increase of 12% ± 8 of PC biosynthesis. When HeLa cells were treated simultaneously with H-89 plus forskolin, choline incorporation was the same as in control experiments (Fig. 2). In order to exclude that the effect of H-89 was due to an inhibition of protein kinase C, we stimulated cells with phorbol ester (TPA). It is suggested that in HeLa cells TPA stimulates the incorporation of choline into PC by a protein kinase C dependent mechanism [13]. However, H-89 did not abolish the phorbol ester-induced stimulation of PC biosynthesis (Fig. 2).

Finally, no effect of H-89 on choline uptake was measured (Fig. 3). In contrast, 200 μmol/l hemicholinium-3, a known inhibitor of choline transport into cells, did block the choline uptake (Fig. 3).

4. DISCUSSION

We report the inhibition of PC biosynthesis in vivo by a new selective inhibitor of cAMP-dependent protein kinase, H-89. This inhibitory effect is prevented by forskolin which activates cAMP-dependent protein kinase by increasing the cAMP level. Based on the background of recent in vitro studies on phosphorylation/dephosphorylation of the rate-limiting enzyme of the CDP-choline pathway, CTP:choline phosphate cytidylyltransferase [3,4,14,15], we expected that H-89 will activate PC biosynthesis by inhibiting cAMP-dependent protein kinase. On the other hand, forskolin, an activator of adenylate cyclase which elevates cellular cAMP level should inhibit PC biosynthesis. Surprisingly, the data obtained show the opposite effect indicating that inhibition of cAMP-dependent protein kinase inhibits

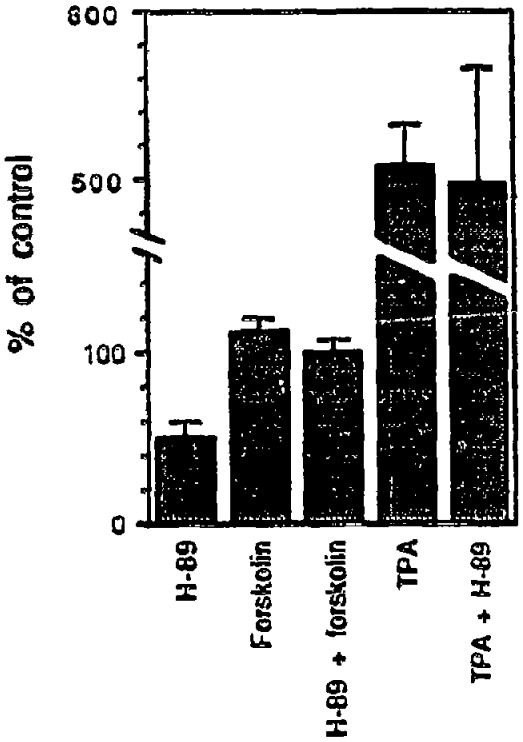

Fig. 2. Effect of H-89, forskolin and TPA on PC biosynthesis of HeLa cells. HeLa cells were grown to confluence. The medium was removed, and pulse medium was added containing [*methyl*-^{3}H]choline (7.4×10^4 Bq/ml) and 10 μmol/l H-89, 10 μmol/l forskolin, 10 μmol/l H-89 plus 10 μmol/l forskolin, 125 nmol/l TPA or 10 μmol/l H-89 plus 125 nmol/l TPA. For controls no supplements were added. After 2 h of incubation, the cells were mechanically harvested and PC was analysed as described in section 2. The values are given in % of choline incorporation into PC compared to controls ($n = 3$).

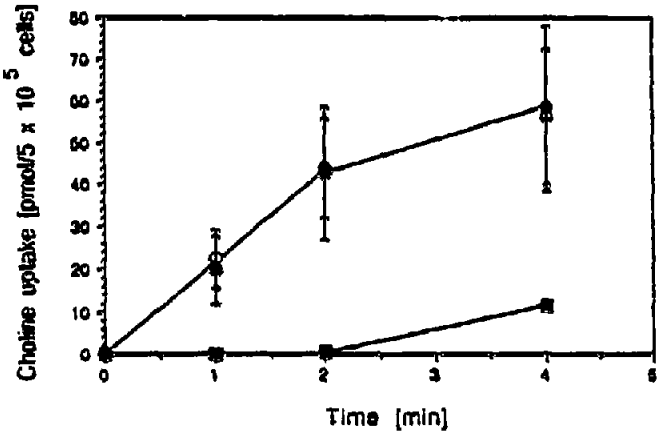

Fig. 3. Choline uptake of HeLa cells treated with H-89 or hemicholinum-3. HeLa cells grown on collagen-coated plastic sheets (35 mm diameter), were preincubated for 30 min with medium containing 10 μmol/l H-89 (●), 200 μmol/l hemicholinum-3 (■), or no supplements as control (○). Choline uptake was determined at 37°C as described in section 2 and calculated as the difference between the uptake at 37°C and 4°C. Values are given in pmol/5×10^5 cells ± S.D. ($n = 3$).

PC biosynthesis in HeLa cells. This is in agreement with results obtained when cells treated with H-89 plus forskolin. However, phorbol ester-stimulated PC biosynthesis was not affected by H-89.

Recently, the cDNA of CT was reported for rat liver [16] which showed that there is only one potential phosphorylation site of cAMP-dependent protein kinase (^{61}RVTM^{64}). This motif contained threonine and not serine as possible phosphorylation sites as reported for the in vitro phosphorylation of CT by cAMP-dependent protein kinase [4].

Since the decrease of choline incorporation into PC could be due to an inhibition of the choline uptake, we studied the effect of H-89. Our data clearly demonstrate that H-89 has no negative influence on the choline uptake in HeLa cells.

Furthermore, it was suggested that cAMP-dependent protein kinase acts via phosphorylation of acetyl-CoA carboxylase thus affecting the biosynthesis of fatty acids. Consequently PC biosynthesis may be impaired by a decreased diacylglycerol formation [17,18]. In a different study it was reported recently that cAMP analogues enhance PC catabolism in choline-deficient rat hepatocytes [19].

Finally, our results indicate that cAMP-dependent protein kinase does not have the same effect on PC biosynthesis in vivo as expected by recent in vitro studies [5,18]. We will focus our interest on this in vivo effect of H-89 on PC biosynthesis to elucidate the mechanism underlying the described inhibition of PC biosynthesis.

Acknowledgements: The work was supported by a grant of the Freie Universität Berlin (Forschungsgebietsschwerpunkt 'Zelloberflächen und Erkennungsprozesse'). The authors thank Dr. Ch. Bauer, Dr. D. James Morre and Mark Paulik for helpful discussions and proofreading the manuscript.

REFERENCES

[1] Gross, I. and Rooney, S.A. (1977) Biochim. Biophys. Acta 488, 263–269.
[2] Niles, R.M. and Makarski, J.S. (1979) J. Biol. Chem. 254, 4324–4326.
[3] Pelech, S.L., Pritchard, P.H. and Vance, D.E. (1981) J. Biol. Chem. 256, 8283–8286.
[4] Sanghera, J.S. and Vance, D.E. (1989) J. Biol. Chem. 264, 1215–1223.
[5] Watkins, J.D., Wang, Y. and Kent, C. (1992) Arch. Biochem. Biophys. 292, 360–367.
[6] Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990) J. Biol. Chem. 265, 5267–5272.
[7] Seamon, K.B. and Daly, J.W. (1981) J. Biol. Chem. 256, 9799–9805.
[8] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[9] Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374–378.
[10] Geilen, C.C., Wieder, Th. and Reutter, W. (1992) J. Biol. Chem. 267, 6719–6724.

[11] Culvenor, J.G., Harris, A.W., Mandel, T.E., Whitelaw, A. and Ferber, E. (1981) J. Immunol. 126, 1974–1977.
[12] Smith, P.K., Krohn, R.I., Hermanson, G.T., Malia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[13] Utal, A.K., Jamil, H. and Vance, D.E. (1991) J. Biol. Chem. 266, 24084–24091.
[14] Hatch, G.M., Lam, T.-S., Tsukitani, Y. and Vance, D.E. (1990) Biochim. Biophys. Acta 1042, 374–379.
[15] Pelech, S.L. and Vance, D.E. (1982) J. Biol. Chem. 257, 14198–14202.
[16] Kalmar, G.B., Kay, R.J., Lachance, A., Aebersold, R. and Cornell, R.B. (1990) Proc. Natl. Acad. Sci. USA 87, 6029–6033.
[17] Vance, D.E., in: Biochemistry of lipids, lipoproteins and membranes (D.E. Vance and J.E. Vance, Eds.) Elsevier, Amsterdam, 1991, pp. 205–240.
[18] Jamil, H., Utal, A.K. and Vance, D.E. (1992) J. Biol. Chem. 267, 1752–1760.
[19] Hatch, G.M. and Vance, D.E. (1991) Biochem. Cell Biol. 69, 515–522.