***N*-[2-Bromocinnamyl(amino)ethyl]-5-isoquinolinesulphonamide (H-89) inhibits incorporation of choline into phosphatidylcholine via inhibition of choline kinase and has no effect on the phosphorylation of CTP:phosphocholine cytidylyltransferase**

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We have shown previously that *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulphonamide (H-89), a selective inhibitor of cyclic-AMP-dependent protein kinase (PKA), inhibits phosphatidylcholine biosynthesis in HeLa cells. In the present study, we elucidated the mechanism underlying the described inhibition. Treatment of cells with 10 μ M H-89 had no effect on the phosphorylation of CTP:phosphocholine cytidylyltransferase. However, H-89 slightly affected the distribution of cytidylyltransferase between cytosol and membranes, but the cellular 1,2-diacylglycerol content was not influenced. Further-

more, pulse-chase experiments revealed that H-89 did not affect cytidylyltransferase activity. Instead, H-89 inhibited choline kinase, the enzyme catalysing the first step in the CDP-choline pathway. In the presence of 10 μ M H-89, choline kinase activity was inhibited by $36 \pm 7.6\%$ *in vitro*. Additionally, the phosphorylation of choline to phosphocholine was inhibited by $30 \pm 3\%$ in cell-culture experiments. This inhibitory effect could be partly prevented by simultaneous addition of 10 μ M forskolin, indicating that choline kinase is regulated in part by PKA activity.

INTRODUCTION

The CDP-choline pathway is the most important route of phosphatidylcholine (PtdCho) biosynthesis in different cell types, and CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) is the rate-limiting enzyme of this pathway (Pelech and Vance, 1984; Kent, 1990). Its activity seems to be regulated by movement between membranes (active form) and cytosol (inactive form), especially in HeLa cells (Vance and Pelech, 1984). Recently, different cytosolic forms of cytidylyltransferase were described. An inactive L-form can be converted into an active H-form, which represents a cytosolic enzyme-lipid aggregate (Weinhold et al., 1991) and is possibly identical with a complex of cytidylyltransferase in association with a 112 kDa binding protein (Feldman and Weinhold, 1993). The translocation process has been shown to be regulated by various lipids (Jamil et al., 1990; Yao et al., 1990; Cornell, 1991a,b; Weinhold et al., 1991; Utal et al., 1991; Geilen et al., 1992a; Jamil et al., 1993). Furthermore, there are valid indications from studies both *in vivo* and *in vitro* that PtdCho biosynthesis is, at least in part, regulated by phosphorylation/dephosphorylation of cytidylyltransferase (Hatch et al., 1990; Watkins and Kent, 1991; Hatch et al., 1992; Wang et al., 1993), but there exists some controversy concerning the regulation of cytidylyltransferase activity by phosphorylation/dephosphorylation *in vitro* and the regulation of PtdCho biosynthesis in cell-culture experiments. Studies *in vitro* have shown that purified cytidylyltransferase is phosphorylated by cyclic AMP (cAMP)-dependent protein kinase (PKA). This phosphorylation is paralleled by an inhibition of membrane binding of cytidylyltransferase (Sanghera and Vance, 1989).

However, cytidylyltransferase does not seem to be a substrate for PKA (Watkins et al., 1992; Jamil et al., 1992) or protein kinase C (PKC) (Utal et al., 1991) *in vivo*.

Recently, we reported that the potent and selective inhibitor of PKA, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulphonamide (H-89) (Chijiwa et al., 1990), inhibits the incorporation of radiolabelled choline into PtdCho in HeLa cells (Geilen et al., 1992b). This inhibition was prevented by simultaneous addition of forskolin, whereas the choline uptake itself is not affected by H-89.

In the present study, we focused our interest on the mechanism underlying the described inhibition of PtdCho biosynthesis by H-89. Our results show that H-89 inhibits PKA and, at higher concentrations, PKC, in HeLa cells, but has no effect on the phosphorylation of cytidylyltransferase. Furthermore, the distribution of cytidylyltransferase between cytosol and membranes was hardly affected, and the *sn*-1,2-diacylglycerol (DAG) content of the cells was not influenced at all. From the results obtained, we suggest that the main effect of H-89 on PtdCho biosynthesis is mediated by an inhibition of choline kinase (EC 2.7.1.32), the enzyme catalysing the first step in the CDP-choline pathway.

EXPERIMENTAL

Materials

[methyl-³H]Choline chloride (2.8–3.1 TBq/mmol), [methyl-¹⁴C]-phosphocholine (2.04 GBq/mmol) and [γ -³²P]ATP (~ 185 TBq/mmol) were from Amersham (Braunschweig, Germany). H-89 was obtained from Calbiochem (Bad Soden, Germany). Silica-

Abbreviations used: cAMP, cyclic AMP; PtdCho, phosphatidylcholine; H-89, *N*-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulphonamide; DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; PKA, cyclic-AMP-dependent protein kinase; PMSF, phenylmethanesulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate.

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gel 60 high-performance t.l.c. 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 t.l.c. scanner (LB2821 HR; Berthold, Wildbad, Germany) was used. Forskolin, PtdCho, protease inhibitors, histone III S, phenylenediamine, peroxidase, choline oxidase, alkaline phosphatase and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (München, Germany). The PKA assay system was from Gibco (Karlsruhe, Germany). The alkaline phosphatase assay kit was from Behring Werke (Marburg, Germany).

Polyclonal peptide-specific antibody against cytidylyltransferase was obtained from D. E. Vance (Edmonton, Canada) and characterized previously (Jamil et al., 1992).

Cell culture

HeLa cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal-calf serum, 0.56 g/l glutamine, 110 000 units/l penicillin and 0.1 g/l streptomycin in plastic culture dishes (Nunc, Roskilde, Denmark). Media and culture reagents were from Gibco, and antibiotics from Boehringer (Mannheim, Germany).

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

Extraction, separation and quantification of lipids and water-soluble metabolites

After incubation with pulse medium, cells were washed twice with ice-cold PBS (pH 7.2) and harvested with a cell lifter (Costar; Cambridge, MA, U.S.A.) followed by modified lipid extraction as described by Bligh and Dyer (1959). After freeze-drying of the cells, 50 μl of methanol, 25 μl of chloroform and 20 μl of water were added. Samples were stirred for 2 min on a vortex mixer and centrifuged at 13 000 g for 10 min. Phase separation was accomplished by addition of 25 μl of chloroform and 25 μl of water. The suspension and centrifugation steps were repeated, and 15 μl samples of the chloroform phases were applied to silica-gel 60 high-performance t.l.c. plates with a high-performance t.l.c. applicator (Linomat III; Camag, Berlin, Germany). Then 70 μl portions of the upper phases were freeze-dried, dissolved in 20 μl of methanol/water (10:9, v/v) and likewise applied to high-performance t.l.c. plates. Lipids were separated as described by Touchstone et al. (1980), in the solvent chloroform/methanol/triethylamine/water (30:35:34:8, by vol.). PtdCho precursors were separated in methanol/0.6% NaCl/aq. 25% NH_3 (8:5:1, by vol.). Radioactivity was quantified by radioscanning. Phospholipids and their precursors were identified by calibrating the scanner with known standards.

Determination of PKA activity

For this, 6×10^6 HeLa cells were collected in 1 ml of extraction buffer containing 50 mM Tris/HCl, pH 7.5, and 5 mM EDTA. The cells were homogenized with 15 strokes in a tight-fitting Dounce homogenizer, and the homogenate was centrifuged at 13 000 g for 2 min. The PKA activity in the supernatant was determined in the presence of different H-89 concentrations, in accordance with the directions of the Gibco PKA assay system.

Briefly, PKA activity was assayed under four different conditions: (I) –inhibitor peptide, –cAMP; (II) +inhibitor peptide, –cAMP; (III) –inhibitor peptide, +cAMP; (IV) +inhibitor peptide, +cAMP. Then PKA activity was calculated as follows: (III – IV) – (I – II). The PKA activity in the presence of H-89 is given as a percentage of activity in the absence of H-89.

Determination of the PKC activity

For this, 2.4×10^7 HeLa cells were collected in 4 ml of extraction buffer containing 20 mM triethanolamine, pH 7.4, 10 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 100 $\mu\text{g}/\text{ml}$ leupeptin. The cells were homogenized with 15 strokes in a tight-fitting Dounce homogenizer, and the homogenate was centrifuged at 150 000 g for 15 min at 4°C . The supernatant was applied to a DEAE-cellulose DE-52 column (1.5 ml), which was previously equilibrated with buffer A, containing 20 mM triethanolamine, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin. The column was washed with 2×4 ml of buffer A and eluted with buffer A containing 0.1 M NaCl. PKC activity was assayed by measuring incorporation of ³²P from [γ -³²P]ATP into histone III S under stimulating and non-stimulating conditions. The assay mixture contained, in a total volume of 50 μl , 20 mM triethanolamine, pH 7.4, 4 mM magnesium acetate, 0.1 mM CaCl_2 , 50 μM histone III S, 50 μM mercaptoethanol, 20 μM ATP supplemented with [γ -³²P]ATP (final radioactivity 2×10^5 c.p.m.), purified PKC and different concentrations of H-89. Stimulation of PKC activity was achieved by adding additionally 50 $\mu\text{g}/\text{ml}$ phosphatidylserine and 5 $\mu\text{g}/\text{ml}$ 1,2-diolein. After incubation at 30°C for 5 min, 50 μl of the reaction mixture was applied to a Whatman P81 filter. The filter was washed three times with 0.05% H_3PO_4 . Radioactivity was determined by measuring Čerenkov radiation in water.

Permeabilization of cells by digitonin

For this, 6×10^6 confluent HeLa cells were incubated for 1 h in medium containing no supplement (control) or 10 μM H-89. Cells were then washed twice with ice-cold PBS, and all the following steps were carried out at 4°C . Buffer (300 μl) containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 0.025% NaN_3 , 1 mM PMSF and 0.5 mg/ml digitonin was added to the cells. After incubation for 3 min, the digitonin supernatant was carefully removed. Undissolved digitonin was pelleted at 13 000 g for 2 min. The remaining ghosts of the cells in the dishes were washed with 1 ml of PBS, harvested in 50 μl of buffer as described above (the harvesting buffer contained 0.03% Triton X-100 and no digitonin) and then homogenized with a tight-fitting Dounce homogenizer. Cytidylyltransferase activity was measured in the digitonin supernatants and in the cell ghost homogenates as described below. From the data, the ratio of cytosolic to membrane-bound cytidylyltransferase activity was calculated for each dish.

Choline kinase assay

For this, 2×10^7 HeLa cells were collected in 1 ml of extraction buffer, containing 100 mM Tris/HCl (pH 8.0), 10 mM MgCl_2 , 1 mM PMSF and 100 $\mu\text{g}/\text{ml}$ leupeptin. The cells were homogenized with 15 strokes of a tight-fitting Dounce homogenizer and the homogenate was centrifuged at 150 000 g for 15 min at 4°C ; 20 μl of the supernatant was used for the assay.

Choline kinase activity was determined as described by Weinhold and Rethy (1974). The incubation mixtures contained

100 mM Tris/HCl (pH 7.2), 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM [*methyl*- 3H]choline (sp. radioactivity 352 Bq/nmol), 20 μ l of the enzyme preparation and different concentrations of H-89 in a final volume of 105 μ l. The reaction was started by addition of the enzyme and was incubated for 30 min at 37 °C. The reaction was stopped by freezing the samples in liquid nitrogen. Radioactive phosphocholine was separated from [*methyl*- 3H]choline by high-performance t.l.c. as described for the cytidylyltransferase assay. Radioactivity was determined by radioscanning. One unit of enzyme activity is defined as 1 μ mol of phosphocholine formed/min.

Up to 30 min, formation of phosphocholine was linear with incubation time and amount of enzyme.

CTP:phosphocholine cytidylyltransferase assay

Cytidylyltransferase activity was measured by a modified method of Sohal and Cornell (1990). The reaction mixture contained 50 mM Tris/HCl, pH 7.4, 0.03 % Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 3 mM CTP, 1.5 mM [*methyl*- ^{14}C]phosphocholine (sp. radioactivity 20 Bq/nmol), liposomes (400 μ M phosphatidylcholine/400 μ M oleic acid) and 10 μ l of enzyme preparation in a final volume of 55 μ l. After incubation for 30 min at 37 °C the reaction was stopped by freezing the samples in liquid nitrogen. The samples were freeze-dried, dissolved in 20 μ l of methanol/water (1:1, v/v) and applied to high-performance t.l.c. plates. After developing the plates in a solvent system containing methanol/0.6 % NaCl/aq. 25 % NH_3 (8:5:1, by vol.), the radioactivity was determined by radioscanning.

DAG determination

The DAG assay was carried out as described by Preiss et al. (1986). Briefly, H-89-treated and control cells were extracted by the method of Bligh and Dyer (1959) as described above. A sample (35 μ l) of the chloroform phase was evaporated and solubilized in 20 μ l of a detergent solution containing 7.5 % octyl β -D-glucoside and 5 mM cardiolipin in 1 mM diethylenetriamine-pentaacetic acid ('DETAPAc') by sonication. Then the DAG content of the samples was determined as described by Geilen et al. (1992a).

Determination of cellular phosphocholine

For this, 3×10^7 confluent HeLa cells were incubated for 1 h in medium containing 10 μ M H-89 or no supplement as control. Cells were washed twice with 10 mM Tris/HCl (pH 8.0)/150 mM NaCl and collected in 10 mM Tris/HCl (pH 8.0). After freeze-drying and a modified extraction in accordance with Bligh and Dyer (1959), the aqueous phases were evaporated to dryness in a Speed-vac concentrator and resuspended in a buffer containing 100 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$ and 1 mM dithiothreitol. Choline in the sample was determined by a combined enzymic reaction using choline oxidase and peroxidase. Reaction mixtures containing 20 m-units of choline oxidase, 250 m-units of peroxidase, 80 μ g phenylenediamine, 100 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol and a portion of the sample in a total volume of 100 μ l were incubated for 3 h at 37 °C. The reaction product was quantified photometrically at 405 nm. Additionally, the assay was performed in the presence of 2 units of alkaline phosphatase. The amount of phosphocholine present in the sample was calculated as the difference between choline determined in the presence and in the absence of alkaline

phosphatase. Choline and phosphocholine were used as standards. The assay was linear up to 20 nmol, with a detection limit of 1 nmol. Under these conditions there was no spontaneous hydrolysis of the phosphocholine standard.

Cell labelling and immunoprecipitation of ^{32}P -labelled cytidylyltransferase

HeLa cells were grown to confluence in 60 mm-diam. dishes. The medium was removed and the cells were starved in phosphate-free DMEM for 30 min. Cells were labelled in phosphate-free medium containing 100 μ Ci/ml [^{32}P]P_i for 2 h. Subsequently the medium was replaced by phosphate-free medium containing 100 μ Ci/ml [^{32}P]P_i and 10 μ M H-89 or no supplement as control for 1 h. Cytosolic proteins were obtained by treatment with 0.3 ml of digitonin buffer for 5 min on ice. The remaining ghosts were scraped into 150 μ l of modified RIPA buffer, containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 10 mM NaF, 100 μ M Na_3VO_4 , 1 mM PMSF, 1 % Nonidet P-40, 0.5 % sodium deoxycholate and 0.1 % SDS with a rubber policeman and homogenized with a tight-fitting Dounce homogenizer. The membrane-bound proteins were obtained as described above.

Immunoprecipitations were performed with Protein A-bearing *Staphylococcus aureus* cells (Pansorbin; Calbiochem). Cytidylyltransferase from cytosol was immunoprecipitated with 5 μ g of anti-cytidylyltransferase antibody, whereas 2 μ g of the antibody was used to precipitate membrane-bound enzyme. Before the immunoprecipitation of cytidylyltransferase, the Pansorbin cells were washed twice with RIPA-washing-buffer, containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS and 1 % sodium deoxycholate, and with pre-wash buffer, containing 10 mM Tris/HCl (pH 7.4), 1 M NaCl and 0.1 % Nonidet P-40. Subsequently the cytosolic and particulate samples were incubated with Pansorbin cells for 30 min on ice to allow unspecific proteins to bind. After centrifuging the cells at 13000 *g* for 2 min, the supernatant was incubated with the anti-cytidylyltransferase antibody for 1 h on ice. Again, Pansorbin cells were added and the incubation was continued for 30 min at 4 °C. The cells were spun down at 13000 *g* for 30 s and the pellet was washed with 3×1 ml of RIPA-washing-buffer, once with 1 ml of prewash buffer and once with 1 ml of PBS. The washed cells were boiled in reducing Laemmli SDS-sample buffer and resolved on an SDS/10 %-polyacrylamide gel (Laemmli, 1970). The dried gel was exposed to Kodak XAR-5 film for 72 h at -80 °C.

Other procedures

Possible cytotoxicity of H-89 and forskolin on HeLa cells was determined by measurement of the release of alkaline phosphatase (Culvenor et al., 1981). Protein was determined by the BCA assay (Smith et al., 1985), with BSA as standard.

Statistical comparisons were made in these studies with Student's *t* test.

RESULTS

Effect of H-89 on the activity of PKC and PKA from HeLa cells

First of all, it was important to know what concentration of H-89 should be used to inhibit PKA selectively, since H-89 also inhibits PKC activity at higher concentrations and PKC activity is also thought to influence PtdCho biosynthesis. Therefore we determined the effect of different H-89 concentrations on PKA and PKC activity in HeLa-cell cytosol *in vitro*. Figure 1 shows

Figure 1 Influence of H-89 on the activity of PKA and PKC from HeLa cells *in vitro*

HeLa cells grown to confluence were homogenized and the kinases were extracted as described in the Experimental section. The activity of PKA (■) and PKC (□) was determined in the presence of different H-89 concentrations. The arrow indicates the H-89 concentration used in all experiments. Values are given as percentages of activity in the absence of H-89 (means $\pm$ S.D., $n = 3$).

Table 1 Effect of H-89 on choline incorporation into PtdCho in HeLa cells with down-regulated PKC

HeLa cells grown to confluence were preincubated with 1 μ M PMA for 24 h. The medium was removed and pulse medium was added containing 7.4×10^4 Bq/ml [*methyl*- 3 H]choline, 10 μ M H-89, 10 μ M H-89 plus 10 μ M forskolin, 125 nM PMA, or no supplements as control. After 1 h the cells were mechanically harvested and PtdCho was analysed as described. The values of incorporated radioactivity are given in d.p.m./ μ g of cellular protein (means $\pm$ S.D., $n = 4$; *significantly different from controls at $P < 0.01$).

	Choline incorporation into PtdCho (d.p.m./ μ g of cellular protein)
Control	31.1 ± 1.5
H-89 (10 μ M)	$20.1 \pm 3.6^*$
H-89 (10 μ M) + forskolin (10 μ M)	27.8 ± 4.0
PMA	29.2 ± 2.6

that inhibition of PKA activity is achieved at very low H-89 concentrations ($IC_{50} = 0.4 \mu$ M), whereas PKC activity is not affected by concentrations up to 10 μ M. The arrow indicates a concentration of H-89 (10 μ M) where PKC activity is not affected while PKA activity is completely inhibited. Thus we carried out all our experiments in the presence of 10 μ M H-89 to ensure that the effects that we measured were not due to an inhibition of PKC activity.

Effect of H-89 on choline incorporation into PtdCho in cells with down-regulated PKC

In another approach to exclude the possibility that the inhibition of choline incorporation into PtdCho by H-89 was due to inhibition of PKC activity, we determined the choline incorporation into PtdCho in the presence of 10 μ M H-89 in HeLa cells with down-regulated PKC. Down-regulation of PKC in HeLa cells was achieved by treatment with 1 μ M PMA for 24 h as described by Utal et al. (1991). Table 1 shows that H-89 still inhibits choline incorporation into PtdCho by 26.1 % ($P < 0.01$).

Figure 2 Influence of H-89 on the phosphorylation of cytidylyltransferase

HeLa cells were incubated in a phosphate-free pulse medium containing 100 μ Ci/ml [32 P] P_i (carrier-free) for 2 h. Subsequently, the cells were incubated in pulse medium containing 10 μ M H-89 (lanes 2 and 4) or no supplements as control (lanes 1 and 3) for 1 h. The incubation was terminated by washing the monolayers with ice-cold PBS. The cells were incubated with digitonin for 5 min, and digitonin-released protein samples (cytosol, lanes 1 and 2) and membrane cell ghosts (lanes 3 and 4) were prepared. Cytidylyltransferase was immunoprecipitated with antibody to cytidylyltransferase peptide. The immunoprecipitated protein samples were separated on an SDS/10%-polyacrylamide gel and exposed to Kodak XAR-5 film for 72 h. The experiment was repeated and similar results were obtained. Positions of protein standards are shown in kDa.

PMA has no effect on the choline incorporation into PtdCho, which indicates that PKC is down-regulated successfully. The inhibitory effect of H-89 is prevented partly by simultaneous addition of 10 μ M forskolin.

H-89 causes a slight translocation of cytidylyltransferase to the cytosol and has no effect on its phosphorylation

Regulation of cytidylyltransferase is achieved by its translocation from the cytosol to membranes (Vance, 1989). By permeabilizing cells with digitonin, which causes cytosolic contents to leak into the culture medium, it is possible to distinguish between the cytosolic and the membrane-bound form of the enzyme. To examine the effect of H-89 on the translocation process, we compared the distribution of cytidylyltransferase of control and H-89-treated cells by measurement of its activity in the digitonin supernatant and remaining cell ghosts. In order to exclude any possible interference by H-89 in the assay *in vitro*, the cells were washed thoroughly before digitonin treatment. In addition, we used liposomes containing 400 μ M PtdCho and 400 μ M oleic acid. At this concentration of activating lipids, H-89 does not have an inhibitory effect on cytidylyltransferase activity. After treatment of HeLa cells with 10 μ M H-89 for 1 h, the total amount of cytidylyltransferase activity was not altered. It was 1.12 ± 0.12 nmol of CDP-choline formed/min per 6×10^6 cells in control cells and 1.02 ± 0.09 nmol/min per 6×10^6 cells in H-89 treated cells ($n = 3$). H-89 treatment slightly affects the distribution of cytidylyltransferase activity after 3 min of digitonin treatment. Whereas in control cells $40.8 \pm 4.7\%$ of total cytidylyltransferase activity was membrane-bound, the proportion of membrane-bound cytidylyltransferase in H-89 treated cells was $31.2 \pm 3.5\%$. However, this effect was not significant ($P < 0.1$).

Since PKA also has been suggested to regulate cytidylyltransferase activity, we investigated the influence of H-89 on the phosphorylation of cytidylyltransferase *in vivo*. HeLa cells were prelabelled with [32 P] P_i for 2 h and then incubated in the absence or presence of 10 μ M H-89. Cytidylyltransferase was immunoprecipitated from digitonin supernatants (cytosolic) and cell ghosts (membranes) and analysed by SDS/PAGE. Figure 2 shows the autoradiogram of the gel. There was no significant

Figure 3 Dose-dependent inhibitory effect of H-89 on choline kinase activity *in vitro*

HeLa cells were grown to confluence and the cytosolic proteins were prepared as described in the Experimental section. The activity of choline kinase was determined in the presence of [*methyl*-³H]choline (352 Bq/nmol) and different H-89 concentrations *in vitro*. The reaction was terminated and the radioactive phosphocholine was separated from [*methyl*-³H]choline by high-performance t.l.c. Values are given as percentages of activity in the absence of H-89 (means $\pm$ S.D., $n = 3$).

Table 2 Effect of H-89 and forskolin on choline incorporation into phosphocholine and PtdCho

HeLa cells were grown to confluence. The medium was removed, and pulse medium was added containing [*methyl*-³H]choline (7.4×10^4 Bq/ml) and 1 μ M H-89, 10 μ M H-89, 10 μ M H-89 plus 10 μ M forskolin or 10 μ M forskolin alone. For controls, no supplements were added. After 1 h of incubation, the cells were mechanically harvested and PtdCho and phosphocholine were analysed as described in the Experimental section. The values are given in d.p.m./ μ g of cellular protein (means $\pm$ S.D., $n = 3$; * significantly different from controls at $P < 0.001$).

	Choline incorporation (d.p.m./ μ g of cellular protein) into:	
	Phosphocholine	PtdCho
Control	983 $\pm$ 10	35 $\pm$ 1
H-89 (1 μ M)	908 $\pm$ 89	28 $\pm$ 4
H-89 (10 μ M)	682 $\pm$ 29*	17 $\pm$ 3*
H-89 (10 μ M) + forskolin (10 μ M)	888 $\pm$ 124	34 $\pm$ 4
Forskolin (10 μ M)	1113 $\pm$ 376	39 $\pm$ 3

difference observed in the phosphorylation levels of cytosolic cytidyltransferase after incubation of HeLa cells with or without H-89. This was confirmed by quantifying radioactivity by using a PhosphorImager (Molecular Dynamics G.m.b.H., Krefeld, Germany), which revealed that the extent of phosphorylation of cytidyltransferase immunoprecipitated from the cyto-

Table 3 Influence of 10 μ M H-89 on choline incorporation into PtdCho in pulse-chase experiments

HeLa cells were labelled with [*methyl*-³H]choline (2.5 μ Ci/ml) for 2 h. Pulse medium was removed, the cells were washed three times with PBS and chase medium was added containing unlabelled choline (0.58 mM). Additionally, the chase media contained 10 μ M H-89 or no supplements as control. The radioactivity incorporated into PtdCho was quantified as described in the Experimental section ($n = 3$).

Incubation time (min)	Radioactivity incorporated into PtdCho (d.p.m./ 10^6 cells)	
	Control	H-89
0	3850 $\pm$ 560	3850 $\pm$ 560
30	4020 $\pm$ 290	5000 $\pm$ 1100
90	6400 $\pm$ 700	7030 $\pm$ 700
120	12300 $\pm$ 2400	12140 $\pm$ 1300

sol of H-89-treated cells was 6% less than the phosphorylation of cytidyltransferase from untreated cells (136 c.p.m. for controls and 128 c.p.m. for H-89-treated cells). These data indicate that H-89-mediated inhibition of choline incorporation into PtdCho was not due to a change in the phosphorylation of cytidyltransferase.

A 37 kDa phosphoprotein is precipitated from the samples of membrane-associated proteins, whereas no band is seen in the 43 kDa range, where cytidyltransferase migrates on the gel. The phosphorylation of this 37 kDa fragment is also not affected by treatment with H-89.

H-89 does not influence the cellular DAG content

DAG is known to signal the translocation of cytidyltransferase to membranes (Utal et al., 1991). Therefore, we determined the cellular DAG level in untreated and H-89-treated cells after 1 h of incubation. These experiments revealed that H-89 has no effect on the cellular DAG level (4.3 ± 0.48 nmol/ 10^6 cells for controls and 4.2 ± 0.39 nmol/ 10^6 cells for H-89-treated cells). Thus the slight influence of H-89 on the translocation of cytidyltransferase can not be explained by a decrease in the cellular DAG level.

H-89 inhibits choline kinase activity

In search of the mechanism for the H-89-mediated inhibition of choline incorporation into PtdCho, we finally found that H-89 inhibits choline kinase activity, the enzyme catalysing the first step in the CDP-choline pathway. Figure 3 shows the dose-dependent inhibitory effect of H-89 on choline kinase activity *in vitro*. The IC_{50} is approx. 35 μ M. At concentrations > 200 μ M and pH 7.2 the solution of H-89 seems to be saturated. Therefore, inhibition at 1000 μ M is not complete. In the presence of 10 μ M H-89, choline kinase activity is inhibited by $36 \pm 7.6\%$ ($P < 0.01$).

Additionally, we investigated the effect of H-89 on choline kinase activity in cell-culture experiments. Table 2 shows that H-89 inhibits the incorporation of choline not only into PtdCho but also into phosphocholine. Simultaneous addition of forskolin prevented the inhibition of choline incorporation into both phosphocholine and PtdCho, whereas forskolin alone had no effect. In these experiments it was not possible to detect significant changes in the choline and CDP-choline pool. The specific radioactivity of phosphocholine in H-89-treated cells is not decreased as compared with control cells, since the decrease in

radioactivity incorporated into the phosphocholine pool is accompanied with a decrease in the actual pool size of phosphocholine in these cells. The amount of phosphocholine in control cells was 6.6 ± 0.7 nmol/ 10^6 cells, which is in the same range as reported previously (Vance et al., 1980). In HeLa cells treated with $10 \mu\text{M}$ H-89 for 1 h the pool size was decreased by $50 \pm 10\%$ ($n = 3$).

Furthermore, pulse-chase experiments, where H-89 has no inhibitory effect on the formation of PtdCho from phosphocholine (Table 3), revealed that cytidylyltransferase is not a target of H-89 *in vivo*.

DISCUSSION

We used an inhibitor of PKA, H-89, as a tool to investigate the role of PKA in the regulation of PtdCho biosynthesis in HeLa cells. Surprisingly, we found that treatment of cells with $10 \mu\text{M}$ H-89 leads to a decreased choline incorporation into PtdCho (Geilen et al., 1992b).

In the present study we addressed the question of how H-89 mediates the inhibition of choline incorporation into PtdCho. Since H-89 is known to inhibit not only PKA but, in higher concentrations, also PKC, we showed that the inhibition of choline incorporation into PtdCho was not due to inhibition of PKC activity. The studies *in vitro* of PKA and PKC activities from HeLa cells in the presence of different H-89 concentrations clearly demonstrate that PKC activity is not influenced in the presence of $10 \mu\text{M}$ H-89, whereas PKA activity is completely inhibited. Furthermore, in HeLa cells with down-regulated PKC, H-89 still inhibits choline incorporation into PtdCho.

In order to determine the block of choline incorporation, the choline labelling of the different precursors of PtdCho was measured. H-89 causes a decrease of label not only in PtdCho, but also in phosphocholine. Additionally, we determined the actual pool sizes of phosphocholine in control and H-89-treated cells, using a rapid and non-radioactive assay system. These experiments showed that the phosphocholine pool of H-89-treated cells is decreased by 50%. Since we have ruled out previously that H-89 inhibits choline uptake (Geilen et al., 1992b), it seems reasonable that choline kinase is the main target of H-89, thereby decreasing the substrate level for cytidylyltransferase. Experiments *in vitro* and *in vivo* revealed that choline kinase activity is inhibited in the presence of $10 \mu\text{M}$ H-89, indicating that H-89 is not so selective as promised.

Choline kinase has been shown to be stimulated by hormones, such as insulin and glucocorticoids (Oka and Perry, 1979; Ulane and Ulane, 1980) and by cAMP analogues (Pelech et al., 1981). Furthermore, the protein corresponding to the yeast choline kinase gene (CKI) contains two putative phosphorylation sites for PKA (Hosaka et al., 1988). Since the formation of phosphocholine is inhibited by H-89 *in vivo*, a regulation of choline kinase by PKA is possible. This is supported by the observation that the inhibition of choline incorporation into phosphocholine and PtdCho can be prevented by simultaneous addition of forskolin, raising the intracellular cAMP level. Thus a higher cAMP level may overcome the H-89-mediated inhibition of choline kinase.

The next question was: Does an inhibition of PKA affect the phosphorylation of cytidylyltransferase? Immunoprecipitation of ^{32}P -labelled cytidylyltransferase from H-89-treated and untreated cells shows that the phosphorylation state of cytidylyltransferase is not influenced by inhibition of PKA, indicating that H-89 does not influence cytidylyltransferase activity via phosphorylation/dephosphorylation. These experiments provide further evidence that cytidylyltransferase is not a substrate for PKA *in vivo*. This is in agreement with previous observations by

other groups, who found that treatment of hepatocytes with cAMP analogues or cholera toxin has no effect on the phosphorylation of cytidylyltransferase (Watkins et al., 1992; Jamil et al., 1992).

The antibody also precipitates a membrane-associated phosphoprotein with an apparent molecular mass of 37 kDa. We suggest that the 37 kDa band represents a membrane-bound protein that is co-precipitated by the antibody. Since other groups also detected a 37 kDa band with a different anti-cytidylyltransferase antibody in extracts of insect cells infected with a recombinant baculovirus containing rat cytidylyltransferase cDNA (Luche et al., 1993), it is possible that the 37 kDa band is a fragment or a precursor of cytidylyltransferase. However, the phosphorylation of the 37 kDa protein was also not altered. As a possible mechanism for H-89-mediated inhibition of choline incorporation into PtdCho, we investigated the influence of H-89 on the distribution of cytidylyltransferase between cytosol and membranes. Our data show that H-89 causes a slight translocation of the enzyme from membranes to the cytosol, but the decrease in membrane-bound cytidylyltransferase of about 20% does not correlate with an inhibition of choline incorporation into PtdCho of 50%. These data are in agreement with the results concerning the DAG content of the cells. DAG is known to signal translocation of cytidylyltransferase to membranes (Utal et al., 1991) and a decrease in the cellular DAG level could be responsible for the observed translocation of cytidylyltransferase to the cytosol. Since the DAG content in the cells is not altered after H-89 treatment, we think that the slight translocation of cytidylyltransferase might result from detergent-like effects of H-89. The hypothesis that cytidylyltransferase is not a target of H-89 is supported by pulse-chase experiments, where H-89 has no effect on choline incorporation into PtdCho.

In conclusion, in experiments using H-89 as an inhibitor of PKA, we found that other kinases, such as choline kinase, might also be targets of H-89.

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REFERENCES

- Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
- 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
- Cornell, R. B. (1991a) Biochemistry **30**, 5873–5880
- Cornell, R. B. (1991b) Biochemistry **30**, 5881–5888
- Culvenor, J. G., Harris, A. W., Mandel, T. E., Whitelaw, A. and Ferber, E. (1981) J. Immunol. **126**, 1974–1977
- Feldman, D. A. and Weinhold, P. A. (1993) J. Biol. Chem. **268**, 3127–3135
- Geilen, C. C., Wieder, Th. and Reutter, W. (1992a) J. Biol. Chem. **267**, 6719–6724
- Geilen, C. C., Wieprecht, M., Wieder, Th. and Reutter, W. (1992b) FEBS Lett. **309**, 381–384
- Hatch, G. M., Iam, T.-S., Tsukitani, Y. and Vance, D. E. (1990) Biochim. Biophys. Acta **1042**, 374–379
- Hatch, G. M., Jamil, H., Utal, A. K. and Vance, D. E. (1992) J. Biol. Chem. **267**, 15751–15758
- Hosaka, K., Kodaki, T. and Yamashita, S. (1988) Abstr. Int. Conf. Biochem. Lipids 29th, Tokyo
- Jamil, H., Yao, Z. and Vance, D. E. (1990) J. Biol. Chem. **265**, 4332–4339
- Jamil, H., Utal, A. K. and Vance, D. E. (1992) J. Biol. Chem. **267**, 1752–1760
- Jamil, H., Hatch, G. M. and Vance, D. E. (1993) Biochem. J. **291**, 419–427
- Kent, C. (1990) Prog. Lipid Res. **29**, 87–105
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Luche, M. M., Rock, C. O. and Jackowski, S. (1993) Arch. Biochem. Biophys. **301**, 114–118

- Oka, T. and Perry, J. W. (1979) *Dev. Biol.* **68**, 311–318
- Pelech, S. L. and Vance, D. E. (1984) *Biochim. Biophys. Acta* **779**, 217–251
- Pelech, S. L., Pritchard, P. H. and Vance, D. E. (1981) *J. Biol. Chem.* **256**, 8283–8286
- Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. and Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- Sanghera, J. S. and Vance, D. E. (1989) *J. Biol. Chem.* **264**, 1215–1223
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, 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
- Sohal, P. S. and Cornell, R. B. (1990) *J. Biol. Chem.* **265**, 11746–11750
- Touchstone, J. C., Chen, J. C. and Beaver, K. M. (1980) *Lipids* **15**, 61–62
- Ulane, R. E. and Ulane, M. M. (1980) *Life Sci.* **26**, 2143–2149
- Utal, A. K., Jamil, H. and Vance, D. E. (1991) *J. Biol. Chem.* **266**, 24084–24091
- Vance, D. E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed.), pp. 33–45, CRC Press, Boca Raton, FL
- Vance, D. E. and Pelech, S. L. (1984) *Trends Biochem. Sci.* **9**, 17–20
- Vance, D. E., Trip, E. M. and Paddon, H. B. (1980) *J. Biol. Chem.* **255**, 1064–1069
- Wang, Y., MacDonald, J. I. S. and Kent, C. (1993) *J. Biol. Chem.* **268**, 5512–5518
- Watkins, J. D. and Kent, C. (1991) *J. Biol. Chem.* **266**, 21113–21117
- Watkins, J. D., Wang, Y. and Kent, C. (1992) *Arch. Biochem. Biophys.* **292**, 360–367
- Weinhold, P. A. and Rethy, V. B. (1974) *Biochemistry* **13**, 5135–5141
- Weinhold, P. A., Charles, L., Rounsifer, M. E. and Feldman, D. A. (1991) *J. Biol. Chem.* **266**, 6093–6100
- Yao, Z., Jamil, H. and Vance, D. E. (1990) *J. Biol. Chem.* **265**, 4326–4331