

Induction of Tyrosine Aminotransferase in H-35 Hepatoma Cells by cAMP Captured in Phospholipid Vesicles

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ABSTRACT The uptake, metabolism, and action of cAMP, captured within phospholipid vesicles, in H-35 hepatoma cells were studied. Sonication of lipids in buffer containing cAMP resulted in the formation of 300-Å unilamellar lipid vesicles, capturing cAMP in the internal aqueous cavity. Incubation of H-35 hepatoma cells with vesicles containing cAMP (vesicle-cAMP) resulted in rapid incorporation of the vesicle content; apparent saturation of uptake was reached after ~30 min of incubation at 37°C. Uptake of vesicle-cAMP was linear over a 10-fold vesicle concentration range. Pretreatment of cells with combined inhibitors of glycolysis and respiration inhibited vesicle uptake by 27%, suggesting vesicle fusion with the cell membrane as a predominant pathway of vesicle uptake. Studies on the metabolism of incorporated cAMP indicated that >50% of the cell-associated radioactivity, derived from vesicle-³HcAMP, was preserved as cAMP at the end of a 20-min incubation at 37°C.

The incorporation of vesicle-cAMP by H-35 hepatoma cells resulted in increased tyrosine aminotransferase (TAT) activity. The concentration of vesicle-cAMP needed to produce a half-maximal increase in TAT activity was 10 μM, approximately two orders of magnitude lower than that of exogenously added dbcAMP. cAMP was ineffective when added extracellularly.

The kinetic relationship of the cAMP-induced increase in TAT activity and the binding of cAMP to its receptor protein, in intact H-35 cells, was examined using vesicle-trapped 8-N₃-cAMP, a photoaffinity labeling analogue of cAMP. Incubation of H-35 hepatoma cells with vesicle-8-N₃-cAMP resulted in increased TAT activity, preceded by the binding of 8-N₃-cAMP to the regulatory subunit of type II cAMP-dependent protein kinase.

The use of lipid vesicles provides a means of modulating intracellular cAMP concentration without adding cyclic nucleotide in the millimolar concentration range to the extracellular medium. The increased efficiency of intracellular delivery of cyclic nucleotide with retention of biological activity, provides a useful technique in examining the relationship of occupancy of specific cAMP-receptor protein(s) and the occurrence of a cAMP-mediated biological response in intact cells.

Studies from a number of laboratories have demonstrated the stimulatory effect of *N*⁶,*O*^{2'}-dibutyryl cyclic adenosine 3':5'-monophosphate (dbcAMP), a cAMP derivative, on the synthesis of tyrosine aminotransferase (TAT) (EC 2.6.1.5) in rat liver (16, 30, 31) and in cultured rat hepatoma cells (28, 32). Both pretranslational (7, 8, 18) and translational (4, 5, 25) sites of action for cAMP (and its derivatives) have been proposed; however, the biochemical process(es) actually affected by cAMP which leads to an increased rate of TAT synthesis is not known. One attractive hypothesis is that this effect of cAMP is mediated through the activation of cAMP-dependent protein

kinase and that subsequent phosphorylation at some site results in the increased synthesis of TAT (13, 16, 28).

It has proven difficult to establish the involvement of a cAMP-dependent protein kinase in more than a limited number of cAMP-regulated events. The involvement of cAMP-dependent protein kinase in mediating the action of cAMP in TAT induction has been examined; there is a great deal of strong, albeit indirect, evidence obtained in intact animal studies (16) and in homogeneous cultured cell populations (28, 32, 33) that cAMP-dependent protein kinase is involved. In many cases the abilities of various cAMP analogues to induce TAT

were compared with their abilities to activate cAMP-dependent protein kinase. While a correlation between TAT induction and protein kinase activation by various cAMP derivatives has been shown, the use of these derivatives is not without problems. The derivatives must be used in high concentrations because of the impermeability of the cell membrane, and there may be differences in their uptake and/or metabolism. Both of these factors could affect the experimental outcome. Furthermore, the use of cyclic nucleotides at the concentrations commonly used for TAT induction must cause significant changes in the absolute amounts as well as the ratio of adenine nucleotides intracellularly. This could affect a variety of processes, including the uptake and intracellular pool sizes of amino acids and nucleotides, the synthesis of macromolecules, and any number of enzymic reactions. Therefore, it is difficult, based on these studies alone, to draw firm conclusions as to the mechanism of action of cAMP and the role of cAMP-dependent protein kinase in the induction of TAT.

Lipid vesicles can be used to introduce cAMP into intact cells (23). In the past, lipid vesicles (both unilamellar and multilamellar) have been used as carriers of a variety of molecules into living cells. The contents of lipid vesicles are protected from the external environment and can be incorporated intracellularly. The present report is a study of the induction of TAT activity in H-35 hepatoma cells by cAMP captured in lipid vesicles. The role of cAMP-dependent protein kinase in mediating the action of cAMP is discussed.

MATERIALS AND METHODS

Cell Culture

Monolayer culture of H-35 hepatoma cells was grown in Eagle's modified minimal essential medium (MEM) supplemented with 10% fetal calf serum at 37°C in a water-jacketed incubator with 5% CO₂. At confluency the cells were subcultured. A solution of 0.25% pancreatin in Earle's balanced salt solution was added to the confluent sheet of H-35 cells and incubated at room temperature for 3 min. The solution was then aspirated off and the cells were flushed off the bottom of the culture flask with a stream of MEM. The cell suspension was then diluted 1:5 to 1:50 in growth medium and cells were plated in petri dishes and incubated as described above.

Cell Counts and Viability

Cells were counted in a hemocytometer chamber, and viability was estimated by the trypan blue dye exclusion method.

Preparation of Lipid Vesicles Containing cAMP

For the preparation of lipid vesicles containing cAMP (vesicle-cAMP), the basic procedure of Papahadjopoulos was used (21). Briefly, 20 μmol of phosphatidylcholine, 5 μmol of stearylamine, and 15 μmol of cholesterol in benzene were mixed and dried under argon into a thin film on the inside of a 15-ml conical centrifuge tube. The lipids were then hydrated with 2.5 ml of a buffer containing 100 mM cAMP or [³H]cAMP (sp act 1.6 Ci/mol), 10 mM NaCl, 2 mM histidine, 0.1 mM EDTA, and 2 mM HEPES, at pH 7.4. The lipid suspension was mixed and sonicated under argon at 40-W power output for 1 h with a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) equipped with a special (stepped) microtip. The sonication procedure was carried out at 4°–10°C. To separate extravascular cAMP from cAMP encapsulated in lipid vesicles, the lipid suspension was passed through a Sephadex G-50 column (25 × 1 cm) equilibrated with MEM. The lipid vesicles eluted in the void volume while the extravascular cAMP was included in the Sephadex G-50. The efficiency of encapsulation (i.e., the ratio of the amount of material remaining with the vesicles after separation from the free material to the amount of material originally present times 100) was generally in the range of 1–2%.

The concentration of vesicle-trapped cAMP in the pooled void volume fraction was determined by measuring the absorbance at 258 nm, correcting for the absorbance attributable to lipid and buffer components. Alternatively, the concentration of vesicle-cAMP in the void volume fraction was calculated from the amount of radioactivity in the pooled fraction and the specific activity of the

[³H]cAMP. Similar results were obtained by these two methods. Various concentrations of vesicle-cAMP were made by diluting the final vesicle preparation with MEM, such that the cAMP concentration per vesicle was constant but that the number of vesicles per unit volume was varied.

Characterization of Vesicle-cAMP

Vesicles containing [³H]cAMP were sized using a modified version of the procedure described by Huang (11). Briefly, the vesicle preparation, obtained from exclusion chromatography over a Sephadex G-50 column, was applied to a Sepharose 2B column (30 × 1 cm) pre-equilibrated in 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl. The column was eluted with the same buffer. Alternate fractions were assayed for total phosphate (2) and [³H]cAMP. Vesicle size was estimated from a standard curve generated from uniformly sized Latex bead standards (24). The elution pattern of the vesicle-cAMP thus prepared was similar in all respects to that reported elsewhere for sonicated phospholipid vesicles. Greater than 95% of the vesicles were unilamellar with an average diameter of 300 Å. Based on the calculation of Huang (11), it was estimated that each 300-Å vesicle contained ~4,700 lipid molecules, and that there were 1.3 × 10¹⁴ vesicles/μmol phospholipid. The molar ratio of encapsulated cAMP to phospholipid was 250 nmol to 1 μmol.

Incorporation of Vesicle-cAMP by H-35 Hepatoma Cells

[³H]cAMP captured in lipid vesicles was used as a marker to monitor the total cellular uptake of vesicle-cAMP. H-35 hepatoma cells at early stationary phase of growth were used. Because of the possible effects of serum components on leakage of material from lipid vesicles (23) and in inhibiting vesicle-cell interactions (1), cells were serum deprived for 0.5–2 h before experimentation. No differences in results were obtained using cells that were serum deprived for 0.5 h vs. cells that were serum deprived for 2 h. Various concentrations of vesicle-cAMP were then added to individual plates of H-35 cells and incubated at 37°C for a predetermined period of time. At the end of this incubation period, cells were quickly rinsed twice in phosphate-buffered saline (PBS) (pH 7.4). To determine the amount of total vesicle-[³H]cAMP incorporated, 0.5 ml of PBS was added to the washed H-35 cells, cells were removed from the substratum by scraping with a rubber policeman. The entire sample was then transferred to a scintillation vial, 10 ml Aquasol was added, and the amount of cell-associated radioactivity was determined by liquid scintillation spectrometry. Identical plates of H-35 cells were used to determine protein content by the method of Lowry et al. (15), using bovine serum albumin as standard. In some experiments, the number of cells was counted in a hemocytometer.

Metabolism of Cell-associated [³H]cAMP

H-35 hepatoma cells at early stationary phase of growth were used. Cells were serum deprived for 0.5–2 h before their incubation with vesicle-[³H]cAMP. At the end of a 20-min incubation period at 37°C, cells were rinsed twice in PBS and 0.7 ml of 6% TCA was added. TCA-precipitable material was removed by centrifugation at 12,000 g for 3 min in an Eppendorf microfuge. The supernatant fractions, containing cAMP and other low molecular weight substances, were extracted three times, each with 5 ml of water-saturated ether. The aqueous phase thus obtained was blown dry with a stream of air at 40°C. The sample was then reconstituted with 100 μl water, and a 20-μl aliquot was used for the determination of total cell-associated radioactivity.

The remainder of the sample was spotted onto Whatman 3MM paper and descending chromatography was carried out for 26–28 h in a solvent system of absolute ethanol:0.5 M ammonium acetate (5:2, vol:vol) (9). Adenine nucleoside and nucleotide standards were visualized with ultraviolet light. The R_{Ad} values, defined as the mobility relative to that of adenosine, for ATP + ADP, AMP, and cAMP were 0.40, 0.66, and 0.78, respectively. To determine the extent of metabolism of the cell-incorporated [³H]cAMP, the chromatogram was cut into 1 × 1 inch squares. The paper squares were soaked in 0.5 ml water and placed on a metabolic shaker overnight to elute the nucleotides and/or nucleosides. After the addition of 10 ml Aquasol, the amounts of radioactivity comigrating with authentic ATP + ADP, AMP, or cAMP were determined by liquid scintillation spectrometry.

Induction of TAT Activity by Vesicle-cAMP

For studying the induction of TAT activity, cells at early stationary phase of growth were rinsed with serum-free medium, and incubated in serum-free medium for 0–12 h. (This serum-deprivation period does not appear to have any significant effect on the induction of TAT, but does appear to lower the basal

TAT activity.) Various test agents were then added to the serum-free medium. At designated times, cells were washed in PBS and harvested in 0.7 ml of a buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 μ M pyridoxal phosphate, and 5 mM dithiothreitol. Samples were frozen at -20°C for 24 h, and aliquots of the cell homogenates were then assayed for TAT activity, using a modified version of the method described by Diamondstone (6). The standard assay mixture (final volume 1 ml) contained 100 mM potassium phosphate buffer (pH 7.5), 5 mM tyrosine, 10 mM α -ketoglutarate, 0.1 mM pyridoxal phosphate, 1 mM EDTA, and 1 mM dithiothreitol. The reaction was carried out at 37°C for 45 min, and the absorbance at 331 nm was recorded. *p*-Hydroxyphenylpyruvate (pHPP) was used as standard. Results were expressed as micrograms pHPP formed per minute per milligram protein.

Preparation and Use of Lipid Vesicles Containing 8-azido-adenosine 3':5'-monophosphate

The preparation of vesicle-containing 8- N_3 -cAMP was similar to that described for vesicle-containing cAMP, except that the concentration of 8- N_3 -cAMP present in the sonication buffer was 25 mM instead of 100 mM, as was the case for cAMP. The procedure used for studying the induction of TAT by vesicle-8- N_3 -cAMP was identical to that for vesicle-cAMP. To examine the labeling of intracellular cAMP-binding protein(s) by 8- N_3 -cAMP, monolayer cultures of H-35 hepatoma cells were incubated with vesicle-8- N_3 -cAMP for predetermined periods of time at 37°C . At the end of this incubation period, cells were quickly rinsed twice in PBS, and photolyzed for 10 min with a Mineralite UVS-11 handlamp (Ultra-violet Products, Inc., San Gabriel, Calif.). Cells were removed from the substratum by scraping with a rubber policeman, and were homogenized in 10 mM Tris-HCl (pH 7.4), 50 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride, and 1 mM EDTA. The cell homogenates, or a 150,000 g supernatant fraction of the homogenate, obtained from six 100-mm plates were pooled and were dialyzed exhaustively against 2×4 liters of the homogenizing buffer. The available cAMP-binding sites, of the dialyzed cell extracts from control and vesicle-8- N_3 -cAMP treated H-35 hepatoma cells, were titrated by the addition of 10 μM 8- N_3 - ^{32}P cAMP. Photoactivated incorporation of 8- N_3 - ^{32}P cAMP was carried out as described previously (29). Proteins were separated by SDS-polyacrylamide gel electrophoresis, and autoradiographs prepared. The amount of 8- N_3 - ^{32}P cAMP incorporated into protein bands of interest were quantitated by scanning the autoradiograph with a Schoeffel SD-3000 spectromicrodensitometer (Schoeffel Instruments Div., Kratos, Inc., Westwood, N. J.) and the peak heights of the optical tracings were used as a quantitative measure of the incorporation of ^{32}P . In most experiments, the results obtained by the densitometer method were compared with results obtained by liquid scintillation counting of the dried gel slices. Similar results were obtained by the two methods. Differences in the amount of 8- N_3 - ^{32}P cAMP incorporated into a specific protein between the control (untreated) and vesicle-8- N_3 -cAMP-treated cells were taken as indices of intracellular labeling of that protein by vesicle-8- N_3 -cAMP.

Materials

cAMP, dbcAMP, AMP, ADP, ATP, adenosine, phosphodiesterase, and other fine biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). 8- N_3 -cAMP was synthesized from cAMP according to the method of Muneyama et al. (17). Stearylamine (octadecylamine) was obtained from Pfaltz & Bauer Inc. (Stamford, Conn.). Cholesterol was obtained from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Phosphatidylcholine, extracted from egg yolk by the method of Singleton et al. (26), was a gift from Dr. W. Veatch, Department of Pharmacology, Harvard Medical School. ^3H cAMP was obtained from New England Nuclear (Boston, Mass.). 8- N_3 - ^{32}P cAMP was obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif.). Tissue culture supplies were obtained from Grand Island Biological Co. (Grand Island, N. Y.).

RESULTS

Incorporation of Vesicle-cAMP by H-35 Hepatoma Cells

The kinetics of cellular uptake of vesicle- ^3H cAMP at 37°C , the temperature used for all the vesicle-cell incubations reported in this study, are shown in Fig. 1. Rapid uptake of vesicle- ^3H cAMP occurred within the first 5–20 min, after which uptake leveled off, reaching a plateau at ~ 30 min and remained at that level for incubation periods up to 2.5 h, the

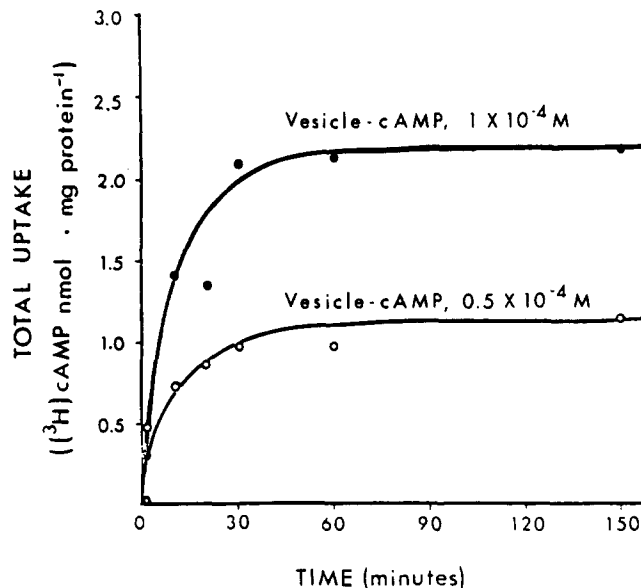


FIGURE 1 Kinetics of uptake of vesicle- ^3H cAMP by H-35 hepatoma cells at 37°C . H-35 hepatoma cells at early stationary phase of growth were serum deprived for 30 min. The preparation of vesicles containing ^3H cAMP, and the dilution of vesicle preparation to yield cAMP concentrations of 0.5×10^{-4} M and 1×10^{-4} M in the external medium were described in detail in Materials and Methods. Results are expressed in nanomoles of ^3H cAMP incorporated per milligram protein.

longest time point studied. The kinetics of uptake of vesicle- ^3H cAMP for two different concentrations of vesicle appeared similar. Because the time-course of increase in the incorporation of vesicle- ^3H cAMP was approximately linear over the first 30 min of incubation, a 20-min incubation period was used to give an approximation of this initial rate of incorporation of vesicle-cAMP. To measure the maximal level of incorporation of vesicle-cAMP and to study the induction of TAT, incubation periods longer than 30 min were used. In some experiments, a second phase of slow increase in the incorporation of vesicle-cAMP was observed at incubation periods > 2 h. This was observed in 5 out of 12 separate experiments carried out over a period of 6 mo. The basis of this phenomenon is not known; the two temporal phases of incorporation of vesicle-cAMP by H-35 hepatoma cells could represent distinct pathways of entry of lipid vesicles. Incubation of H-35 cells with these lipid vesicles had no detectable effects on gross cell morphology or viability as determined by the trypan blue dye exclusion technique.

Fig. 2 compares the total uptake of radioactivity from vesicle- ^3H cAMP and the amount of radioactivity remaining as cAMP at the end of a 20-min incubation. The incorporation of radioactivity by H-35 hepatoma cells was linear over a 10-fold concentration range of vesicle- ^3H cAMP. The metabolism of cAMP, although not extensive, appeared to be more significant at lower concentrations of vesicle-cAMP. In general, $> 50\%$ of the total radioactivity incorporated was recovered as cAMP. In Figs. 1 and 2, no attempts were made to quantitate the amount of radioactivity incorporated at time points shorter than 1 min or at concentrations of vesicle-cAMP lower than $5 \mu\text{M}$, as the amount of radioactivity was too low for quantitation.

Lipid vesicles can become associated with cells via fusion, endocytosis, or by stable adsorption to the cell surface; with the predominant mechanism depending on the chemical com-

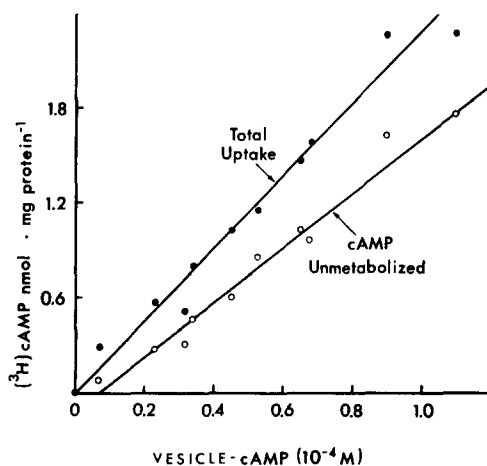


FIGURE 2 Uptake and metabolism of vesicle- ^3H cAMP in H-35 hepatoma cells exposed to different concentrations of vesicles. Various dilutions of vesicle- ^3H cAMP were added to H-35 hepatoma cells. Total uptake represents the amount of radioactivity incorporated at the end of a 20-min incubation at 37°C . The amount of label remaining unmetabolized as cAMP was determined by descending paper chromatography and liquid scintillation spectrometry. Results are presented in nanomoles of radioactivity incorporated per milligram protein.

position of the vesicle, the temperature of incubation, the cell type, and other parameters (23). Studies from a number of laboratories indicated that fusion of vesicles with the plasma membrane is the major pathway in the cellular incorporation of "fluid" charged lipid vesicles, e.g., the vesicle preparation used in this study (20–23). Fusion of the vesicles with cell membrane results in the release of vesicle content to the inside of a cell. On the other hand, endocytosis appears to play a major role in the incorporation of neutral vesicles and solid negatively charged vesicles (21, 23). The intravesicular content of the endocytosed lipid vesicles is bound by the lipid bilayer; its isolation from the intracellular milieu may account for its biological inactivity (21). (It should be noted, however, that fusion of the endocytotic vacuoles with lysosomes may result in vesicle breakdown and release of material trapped inside the vesicle.)

Endocytosis is an energy-dependent process. Inhibitors of glycolysis and respiration have been shown to block endocytosis (22). In this study, the effects of 2-deoxyglucose, an inhibitor of glycolysis, and sodium azide, an inhibitor of respiration, on the incorporation of vesicle-cAMP by H-35 hepatoma cells were investigated (Table I). Results demonstrated that neither 2-deoxyglucose nor sodium azide, when added alone, had any significant effect on the uptake of vesicle-cAMP. The addition of 2-deoxyglucose with sodium azide to H-35 cells resulted in a 27% decrease in the uptake of vesicle-cAMP. The results indicate that at least 70% of the vesicle-cAMP enters H-35 cells by a nonendocytotic mechanism. It also appears likely that the exposure of cells to inhibitors of both glycolysis and respiration is necessary to achieve complete inhibition of endocytosis.

In attempting to dissociate the endocytotic and fusion pathway of incorporation of vesicle-cAMP by H-35 hepatoma cells, the use of lipid vesicles bearing a net charge to entrap soluble markers of opposite charge (in this case positively charged stearylamine and negatively charged cAMP) may raise the question of whether such markers are associated with both

sides of the lipid bilayer via charge-charge interaction. If such an interaction exists, cAMP adsorbed onto the outside of a lipid vesicle could be incorporated into the cytoplasm by some internalization mechanism other than fusion. This possibility was tested by incubating vesicle- ^3H cAMP with phosphodiesterase. Results demonstrated that, in contrast to free cAMP, cAMP captured in lipid vesicles was protected from degradation by phosphodiesterase, suggesting its isolation from the external environment. Control experiments demonstrated that the addition of lipids or lipid vesicles to phosphodiesterase did not result in inactivation of the enzyme.

An important consideration in studies on the mechanism of incorporation of vesicle-cAMP is the possibility of adsorption of lipid vesicle to the cell surface without true intracellular incorporation. The process appears to predominate only at temperatures below the gel-liquid crystalline phase transition temperature of the lipid vesicle; above this temperature different mechanisms of uptake, e.g., endocytosis or fusion, are indicated (20). The adsorption of lipid vesicle to the cell surface appears to involve cell surface proteins, and although these adherent vesicles could not be removed by repeated washings of the treated cells, they could be released by mild trypsinization (20). In this study, the effects of mild proteolytic digestion on the uptake of vesicle-cAMP was investigated. H-35 hepatoma cells were incubated with vesicle- ^3H cAMP for 20 min at 37°C , followed by mild proteolytic digestion of cell surface proteins. The amounts of cell-associated radioactivity with and without the proteolytic digestion were compared. Incubation of H-35 hepatoma cells with a 0.25% solution of pancreatin for 5 min at 25°C resulted in the removal of 30% of total cell-associated radioactivity. It should be noted that the removal, by proteolytic treatment, of 30% of total cell-associated radioactivity represents an upper limit estimate of the extent of cell surface protein-mediated adsorption of lipid vesicles. The possibility exists that this 30% loss of radioactivity may include cell lysis or cell leakage, the release of vesicle-cAMP on the way to fusion with the plasma membrane, in addition to the removal of lipid vesicles adhering to cell surface proteins. Further indication that vesicle- ^3H cAMP was actually incorporated into cells was provided by the recovery of >70% of the total cell-associated radioactivity from the cytosol fraction.

TABLE I
Effect of Metabolic Inhibitors on the Uptake of Vesicle-cAMP by H-35 Cells

	Uptake of vesicle- ^3H -cAMP				Average
	vesicle-cAMP (10^{-4} M)				
	1.00	0.77	0.51	0.36	
	% of control				
Control	100	100	100	100	100
Sodium azide (5×10^{-3} M)	97	100	92	—	96
2-Deoxyglucose (5×10^{-2} M)	90	80	100	—	90
Sodium azide (5×10^{-3} M) plus 2-deoxyglucose (5×10^{-2} M)	70	81	69	71	73

H-35 hepatoma cells were pretreated with metabolic inhibitors for 1 h at 37°C . Various concentrations of vesicle- ^3H cAMP were then added, and incubated for an additional 20 min at 37°C . Results are expressed as percent of control uptake, taken as 100.

Intracellular Metabolism of cAMP

In defining the role of cAMP in hormone action and in studying the action of cAMP in cell function, more often than not the effects of exogenously added cAMP or cAMP derivatives on intact cell preparations were examined. Granner et al. studied the uptake, metabolism, and action of cyclic adenine nucleotides in cultured hepatoma cells; they found that >68% of the label from [³H]cAMP, [³H]mono- or [³H]dibutyryl cAMP was in ADP or ATP, suggesting that these cyclic nucleotides entered the cells as their respective nucleosides (9). This is in contrast with results determined by van Rijn et al. (27). In their study, incubation of rat hepatoma cells with [³H]dbcAMP resulted in the equilibration of intra- and extracellular pools of the cyclic nucleotide within 30–60 min, with >50% of the total cell-associated radioactivity remaining as dbcAMP.

The metabolism of cell-associated cAMP was studied by incubating H-35 hepatoma cells with various concentrations of vesicle-[³H]cAMP. The amount of radioactivity present in the forms of ATP, ADP, AMP, cAMP, and adenosine was determined by descending paper chromatography and liquid scintillation spectrometry. Results are shown in Table II. In all cases, >65% of the total cell-associated radioactivity remained as cAMP at the end of a 20-min incubation period at 37°C. The amounts of radioactivity associated with ATP + ADP (not resolved by paper chromatography), AMP, and adenosine each represented <10% of the total radioactivity recovered. Varying the concentration of vesicle-[³H]cAMP had no dramatic effect on the pattern of distribution of radioactivity in the various nucleosides and nucleotides. In some experiments, the metabolism of internalized cAMP from vesicle-cAMP was studied as a function of incubation time. The preservation of >50% of the total cellular radioactivity in the form of cAMP was observed

TABLE II
Metabolism of Vesicle-[³H]cAMP Incorporated by H-35 Cells

Vesicle-cAMP (10 ⁻⁴ M)	[³ H]cAMP metabolic products				% Radioactivity recovered‡
	ADP + ATP (0.40*)	AMP (0.66*)	cAMP (0.78*)	Adenosine (1.00*)	
	nmol/mg protein				
1.10	0.08 (4)	0.08 (4)	1.76 (87)	0.09 (5)	89
0.90	0.13 (7)	0.16 (8)	1.63 (80)	0.11 (5)	88
0.68	0.13 (10)	0.13 (10)	0.95 (74)	0.08 (6)	81
0.65	0.04 (3)	0.15 (13)	1.01 (78)	0.08 (6)	88
0.53	0.04 (4)	0.06 (10)	0.85 (82)	0.04 (4)	90
0.45	0.09 (11)	0.06 (8)	0.59 (73)	0.06 (8)	78
0.34	0.09 (14)	0.04 (7)	0.46 (71)	0.05 (8)	83
0.32	0.02 (5)	0.02 (5)	0.31 (85)	0.02 (5)	72
0.23	0.09 (20)	0.04 (8)	0.28 (63)	0.04 (9)	76
0.07	0.02 (17)	0.01 (4)	0.10 (68)	0.01 (11)	47

H-35 hepatoma cells were incubated with various concentrations of vesicle-[³H]cAMP for 20 min at 37°C. Adenine nucleotides and nucleoside were separated by descending paper chromatography as described in Materials and Methods. Results are expressed in nanomoles per milligram protein; numbers in parentheses represent the percentages of radioactivity recovered as the individual nucleotides or nucleoside, taking % radioactivity recovered as 100.

* R_{Ad}, defined as the mobility of the nucleotides relative to that of adenosine.

‡ The total amount recovered from the chromatogram, relative to the amount loaded on.

at incubation periods of 10, 20, 30, 60, and 150 min, the longest time point studied.

Parallel experiments on the uptake and metabolism of extracellularly added [³H]cAMP were carried out. Under comparable experimental conditions, the amount of total cell associated radioactivity following incubation of H-35 hepatoma cells in medium containing [³H]cAMP was ~20% of that after incubation in medium containing vesicle-[³H]cAMP. Because of the low amounts of radioactivity recovered, it was not possible to study the metabolism of cAMP of cells incubated in medium containing [³H]cAMP.

Induction of TAT by Vesicle-cAMP

The induction of TAT activity by vesicle-cAMP was studied (a) as a function of the concentration of vesicle-cAMP (Fig. 3), and (b) as a function of incubation time (Fig. 4). In both cases results obtained were compared to those of dbcAMP and cAMP. In agreement with previous observations (27, 28, 32),

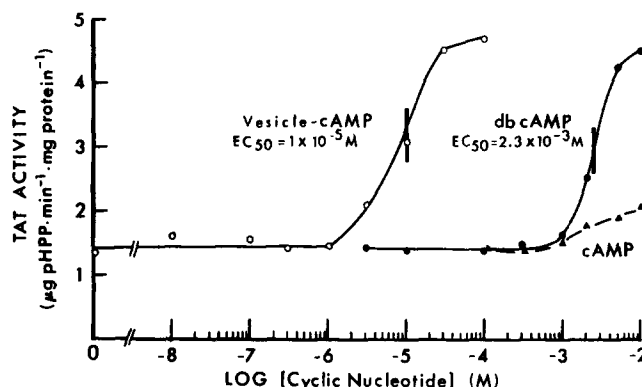


FIGURE 3 Induction of TAT in H-35 hepatoma cells by cAMP captured in phospholipid vesicles (vesicle-cAMP) (○), by exogenously added dbcAMP (●) or cAMP (▲). H-35 hepatoma cells at early stationary phase of growth were serum deprived for 10 h. Various concentrations of vesicle-cAMP, dbcAMP, and cAMP were added to the cells, and incubated at 37°C for 2.5 h. Cells were then harvested and assayed for TAT activity according to methods described in detail in text. Results are expressed in micrograms of pPPP formed per minute per milligram protein.

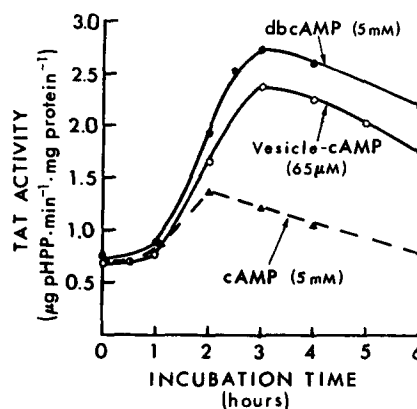


FIGURE 4 Kinetics of induction of TAT in H-35 hepatoma cells. Vesicle-cAMP (50 µM), dbcAMP (5 mM), and cAMP (5 mM) were added to H-35 hepatoma cells, and incubated at 37°C for predetermined periods of time. Results are expressed in micrograms of pPPP formed per minute per milligram protein.

dbcAMP, added to the extracellular medium, caused an increase in TAT activity of H-35 hepatoma cells. The concentration of dbcAMP required to produce a half-maximal increase (EC_{50}) in TAT activity was 2.3 mM; the maximal TAT activity achieved was 3.2 times that of basal activity. Under identical experimental conditions, the concentration of vesicle-cAMP needed to produce a half-maximal increase in TAT activity was 10 μ M, approximately two orders of magnitude lower than that of dbcAMP. The magnitudes of increase in TAT activity produced by vesicle-cAMP and dbcAMP were comparable. In contrast to cAMP captured in lipid vesicles, free cAMP added to H-35 hepatoma cells had little effect on TAT activity, even at a concentration as high as 1 mM. The kinetics of TAT induction produced by dbcAMP and vesicle-cAMP appeared similar (Fig. 4). Significant increase in TAT activity was detected at 2 h, and proceeded at a rapid rate for the next 60 min, after which the activity declined.

Labeling of Intracellular cAMP-binding Protein(s) by Vesicle-8- N_3 -cAMP

cAMP-dependent protein kinase has been identified as a major, if not the only, mediator of cAMP action in eukaryotic cells (13). The occurrence of a type II cAMP-dependent protein kinase in H-35 hepatoma cells has been described (14). The relationship of the binding of cAMP to the regulatory subunit of the type II cAMP-dependent protein kinase (R_{II}) and the increase in TAT activity in intact H-35 hepatoma cells was examined using 8- N_3 -cAMP, a photoaffinity labeling analogue of cAMP, and lipid vesicles as carriers for the cyclic nucleotide (Fig. 5). Incubation of H-35 hepatoma cells with vesicle-8- N_3 -cAMP (40 μ M) resulted in a time-dependent increase in TAT activity. The increase in TAT activity correlated well with the binding of 8- N_3 -cAMP to R_{II} , a protein with an apparent mol wt of 54,000 on SDS-polyacrylamide gel electrophoresis (14, 29).

DISCUSSION

Numerous cellular functions, including the induction of TAT in rat liver and cultured rat hepatoma cells, have been found

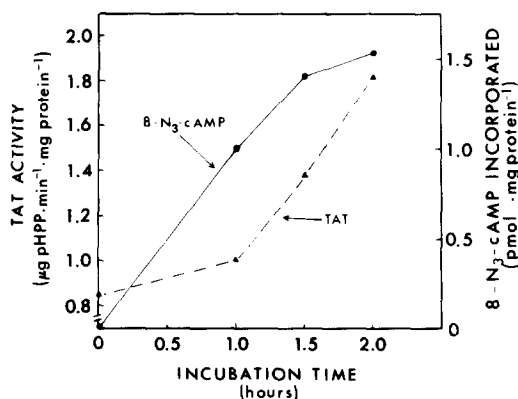


FIGURE 5 Kinetics of induction of TAT and labeling of the regulatory subunit of type II cAMP-dependent protein kinase (R_{II}) by vesicle-8- N_3 -cAMP. The preparation of vesicle-8- N_3 -cAMP (40 μ M) was described in detail in the text. Vesicle-8- N_3 -cAMP was added to serum-deprived H-35 hepatoma cells, and incubated at 37°C for predetermined periods of time. Results for the TAT activity are expressed in micrograms of pHPP formed per minute per milligram protein. Results for the labeling of R_{II} are expressed in picomoles of 8- N_3 -cAMP incorporated per milligram protein.

to be affected by dbcAMP or agents that increase cellular cAMP levels (3). In many of these studies, exogenously added dbcAMP has been demonstrated to be effective while equimolar concentrations of cAMP added to the extracellular medium are either ineffective or much less effective. The reason for the greater potency of the dibutyryl derivative is not clear. There is no direct evidence that it penetrates cell membranes more readily than cAMP, although this may occur and may in some cases be the explanation for the greater potency. It is also possible that it is more potent because it is more resistant to inactivation by phosphodiesterase than cAMP (10, 19). Based on the unifying hypothesis for the mechanism of action of cAMP (13), namely, that its many and diverse effects are mediated through activation of cAMP-dependent protein kinase(s), it is generally assumed that the effects of dbcAMP on cell function are also mediated through the activation of cAMP-dependent protein kinase. The abilities of dbcAMP, mbcAMP, and cAMP to activate cytosolic cAMP-dependent protein kinase obtained from rat hepatoma cells have been studied and compared to their abilities to induce TAT activity in intact cells (9). Results demonstrated that while cAMP is the most effective both in competing with [3 H]cAMP for binding to the regulatory subunit and in stimulating catalytic activity of cAMP-dependent protein kinase, it is not effective in inducing TAT activity in intact hepatoma cells. dbcAMP, while being the least effective analogue in activating cAMP-dependent protein kinase, gave maximal increase in TAT activity. It is therefore apparent that the uptake and metabolism of, as well as the spectrum of effects produced by dbcAMP and cAMP are not identical. In fact, apparently diametrically opposed effects of cAMP and dbcAMP on glycogen metabolism in HeLa-S3 cells have been reported (12). In this study, the induction of TAT activity in H-35 hepatoma cells by cAMP was reported, phospholipid vesicles were used as carriers to introduce cAMP intracellularly.

Liposomes (phospholipid vesicles) have proved to be suitable carriers for many biologically active molecules (23). In general, liposomes form when water-insoluble polar lipids (e.g., phospholipids) are confronted with water. The multilamellar liposomes thus formed consist of concentric lipid bilayers. Upon sonication, these multilamellar liposomes break up to form smaller unilamellar lipid vesicles. Before closed structures form, there is unrestricted entry of water and solutes; thus, water-soluble substances can be entrapped in the aqueous compartments. In this study, we found that cAMP captured in lipid vesicles stimulates the enzyme TAT of H-35 hepatoma cells.

There are several mechanisms by which lipid vesicles can become associated with cells, namely, fusion, endocytosis, stable adsorption to the cell surface, and the exchange of lipid components between the vesicle and cell membrane. The results obtained in this study suggested that a major pathway of entry of the vesicle-cAMP is via fusion of the lipid vesicles with the plasma membrane of H-35 cells. Further indication that the vesicle-cAMP was actually incorporated into cells was provided by the recovery of >70% of [3 H]cAMP from the cytosol fraction of the cell homogenate. Observations on the induction of TAT by vesicle-cAMP also support the notion that some cyclic nucleotide was released into the cytoplasmic space.

The participation of a cAMP-dependent protein kinase in the induction of TAT by cAMP has been strongly implicated by a number of previous studies. Thus, the addition of cholera toxin to H-35 hepatoma cells resulted in a dose-dependent increase in adenyl cyclase, protein kinase, and TAT activities

(32). Furthermore, the abilities of various cyclic nucleotide derivatives in activating cAMP-dependent protein kinase correlate well with their abilities to induce TAT (28, 31, 33). These results are consistent with but do not prove a mediatory role for cAMP-dependent protein kinase in regulating TAT synthesis. In this report, the relationship of occupancy of specific cAMP-receptor protein and TAT induction was examined in intact H-35 cells. 8-N₃-cAMP was used to covalently label cAMP-binding sites; phospholipid vesicles were used as carriers for the delivery of 8-N₃-cAMP intracellularly. Results demonstrated the binding of 8-N₃-cAMP to the regulatory subunit of cAMP-dependent protein kinase, and the induction of TAT activity under the same experimental conditions.

The identification of >95% of the cAMP-binding activity as the regulatory subunit of the type II cAMP-dependent protein kinase in extracts of H-35 hepatoma cells (14), the existence of the regulatory and the catalytic subunits of cAMP-dependent protein kinase in equimolar ratio (14), together with the observation of labeling of R_{II} and induction of TAT under the same experimental condition, certainly indicate the mediatory role of cAMP-dependent protein kinase in the induction of TAT by cAMP. The use of lipid vesicles as carriers for cAMP may provide a powerful tool in the analysis of the mechanisms of action of cAMP.

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