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Coordinate Protein Kinase Activation and Specific Enzyme Induction by Cyclic Nucleotide Derivatives in Intact Cultured Hepatoma Cells

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SUMMARY

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A variety of cyclic nucleotide derivatives were tested for their ability to activate cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) and to induce tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5) in intact Reuber H35 rat hepatoma cells. The ratio of protein kinase activity toward a mixed histone preparation ± cAMP in 10,000 × *g* supernatant fractions of H35 cells incubated with or without a number of different cyclic nucleotide derivatives was used as an index of the degree of protein kinase activation in the intact cells. Basal ratios of 0.3–0.4 were observed but the actual ratio of cAMP-dependent protein kinase in untreated cells was estimated to be 0.10–0.15, based on the use of the skeletal muscle inhibitor and the slight stimulatory effect of dilution. The increase in the protein kinase activity ratio generated by each cyclic nucleotide derivative preceded that in aminotransferase activity, a pattern to be expected if a cause-effect relationship exists. The dose-response relationships for a variety of these derivatives revealed a highly significant correlation between kinase activation and enzyme induction. Furthermore, the effects of cAMP itself and two derivatives on both processes were converted from weak to strong by addition of a phosphodiesterase inhibitor. One of the cyclic nucleotide derivatives tested, 8-parachlorophenylthio cAMP, exhibited dramatically higher potency (ED_{50} for kinase activation and enzyme induction ≅ 2–3 μM) than N^6,$O^{2'}$-dibutyryl cAMP (ED_{50} ≅ 100 μM). Sephadex G-100 chromatography of protein kinase activity was used to confirm the results of the ratio method. The results demonstrate that enzyme induction is tightly coupled to protein kinase activation and is consistent with the possibility that these two processes are causally related.

INTRODUCTION

The concept that protein phosphorylation mediates the effects of cyclic AMP (cAMP)[2] on glycogen metabolism and lipolysis is strongly supported by the results of recent research (1). These metabolic effects are caused by rapid activation or in-

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[2] The abbreviations used are: cAMP, cyclic AMP; MIX, 1-methyl, 3-isobutylxanthine; TAT, tyrosine aminotransferase; PK, protein kinase; 6-HScRMP, 6-thiopurine cyclic 3′:5′-monophosphate; Bt_2cAMP, N^6, $O^{2'}$-dibutyryl cyclic AMP; 8-pClC_6H_4ScAMP, 8-parachlorophenylthio cyclic AMP; 8-H_2NcAMP, 8-amino cyclic AMP; TES, *N*-tris(hydroxymethyl 2-aminoethanesulfonic acid; DTT, dithiothreitol; TCA, trichloroacetic acid.

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activation of the appropriate enzymes resulting from phosphorylation catalyzed by a cAMP-dependent protein kinase (1). In contrast, the role of phosphorylation catalyzed by protein kinase in mediating the effects of cAMP on more complex metabolic processes, which may involve selective changes in macromolecular synthesis, is much less well understood. Strong suggestive evidence for a central role of protein phosphorylation in mediating the cytocidal action of cAMP on murine lymphosarcoma cells has been provided. Variant cells that exhibit phenotypic resistance to the lethal effects of cAMP (as the dibutyryl derivative [Bt_2cAMP]) together with structural alterations in cAMP-dependent protein kinase have been isolated (2–4). In addition to resistance to the cytotoxic effects of cAMP, the elevation of phosphodiesterase activity is not as great in these cells. A 2 to 4-fold elevation in the activity of this enzyme is seen in wild-type cells and has all of the hallmarks of an induction[3] phenomenon, although this has not been rigorously established (2, 3).

Elevation of TAT activity by cAMP derivatives in rat liver and cultured hepatoma cells has been established as an induction process requiring protein synthesis (5, 6). The activity of this enzyme is elevated 2 to 5-fold by the administration of various cAMP derivatives and is the result of a selective increase in the rate of synthesis of the enzyme with no apparent change in its rate of degradation (5, 6). All of the cAMP derivatives that have been found to induce TAT in hepatoma cells are capable of activating rat liver protein kinase *in vitro* (6), and preliminary studies of endogenous H_1 histone phosphorylation *in vivo* have suggested that protein kinase activation could be responsible for induction of tyrosine aminotransferase (7). The present report describes more extensive studies using the procedure developed by Soderling and colleagues (8), which has been successfully used by a variety of investigators for analysis of the state of protein kinase activation in intact cells. We have established conditions whereby this assay can be validly applied to cultured hepatoma cells. Our results are consistent with the possibility that protein kinase does mediate the effects of cAMP on TAT synthesis.

[3] Induction is defined here as an increase in the rate of synthesis of a specific protein without regard to the mechanism(s) involved.

MATERIALS AND METHODS

Dibutyryl cAMP was obtained from Boehringer-Mannheim Co. All the other cAMP derivatives were kindly provided by Drs. R. K. Robbins, L. N. Simon, M. Stout, J. Miller and R. Meyer of ICN Nucleic Acid Research Institute, Irvine, California, or were synthesized in our laboratory according to published procedures (9). All other biochemicals were from Sigma Chemical Company except MIX, which was purchased from Aldrich Co. [^{3}H]cAMP (40 Ci/mmole) and [^{32}P]$_i$ were purchased from New England Nuclear Corporation. Tissue culture components were obtained from Grand Island Biological Co. or Microbiological Associates.

Cell culture. A clone (KRC-7) of the Reuber H35 hepatoma cell line (H4-EII-C3) was used for all of these studies. The details of propagation and handling of these cells have been described extensively (10, 11). In some cases cells were placed in serum-free medium overnight prior to treatment. No detectable difference in the results obtained was observed when serum was present in the medium (5% calf + 5% fetal calf) and when it was not.

Protein kinase assay. γ-[^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell (12). The assay of protein kinase activity is adapted from that described by Langan (13). The optimal conditions for assay of cAMP-dependent phosphorylation of type IIA histone (Sigma), which is a mixture of all five histones, included the following components in a reaction volume of 250 μl: 5 mM TES buffer, pH 6.5; 0.1 mM DTT; 200 μg histone; 8 mM $MgCl_2$; 0.4 mM γ-[^{32}P]ATP (~10^6 cpm) ± 2 μM cAMP. The reaction was initiated by addition of cell extract and incubation was continued for 4 min at 37°. Trichloroacetic acid was added to a final concentration of 25% and, after 15–30 min at 0–4°, the precipitated histones were collected by filtration using Millipore filters (HAWP 0.45 μ).

Each filter was washed three times with 12–15 ml 25% TCA rinses of the assay tubes and then counted in 0.4% Omnifluor scintillant.

Endogenous phosphorylation varied between 10–20% of total incorporation in the presence of exogenous histone and has been corrected for. Little or no stimulation of endogenous phosphorylation by cAMP was observed. If cell extract is omitted or TCA added first, the amount of ^{32}P retained was less than 5% of that observed with the complete system. Results are expressed as nmoles of ^{32}P incorporated per hr, or as the ratio of activity −cAMP/+cAMP (8).

Tyrosine aminotransferase assay. The activity of this enzyme was assayed by the base-catalyzed oxidative decarboxylation of the initial product p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde as described elsewhere (5, 6). The units of activity are expressed as μg of product formed per mg protein in a 10 min assay at 37°.

Preparation of homogenates. After exposure of cells to various additions, the medium was aspirated and the attached cells washed once with phosphate-buffered saline, scraped and collected by centrifugation. Cells from each 100 mm plastic culture dish (Lux) were suspended in 10 mM Tris-Cl buffer, pH 7.6, 0.5 mM DTT and homogenized by hand with 10–12 strokes in a glass-glass homogenizer. A variety of other methods were tested (sonication, freeze-thawing, nonionic detergents etc.), but all produced variable activation of protein kinase and were discarded. Inclusion of physiological concentrations of KCl was also avoided because they also led to variable activation and total kinase activity was inhibited, although nonspecific trapping of the kinase in the particulate fraction was suppressed (see RESULTS).

After centrifugation at 10,000 × *g* for 10 min at 0–4°, the supernatant fraction was decanted and used as the source of protein kinase. Tyrosine aminotransferase activity was assayed using cells from companion dishes lysed with Non-idet P-40 in 1 ml 0.15 M KCl–1 mM EDTA, pH 7.4 (5, 6). These two procedures provide optimal conditions for the assay of both enzymes and preservation of the state of protein kinase activation *in vivo*. Variations in the activity of either enzyme from dish to dish in any experiment averaged less than 10%.

Protein content was determined by the Lowry method (14). Protein kinase inhibitor was prepared according to the method of Gilman (15). Sephadex G-100 chromatography was performed as described by Corbin et al. (16).

[^3H]cAMP binding was performed using a modification of the procedure described by Kumon et al. (17). The assay was carried out for 10 min at room temperature in a final volume of 210 μl. The reaction mixture contained 72.5 mM NaOAc pH 4.5, 18 mM $Mg(OAc)_2$, 1 mM MIX and 300 nM [^3H]-cAMP. After incubation 5 ml of ice cold 10 mM tris buffer pH containing 40 mM $Mg(OAc)_2$ was added. The solution was filtered through Millipore HAWP 0.45 μ filters and washed with one 15 ml rinse of the assay tubes. The filters were counted in a methyl cellosolve-based scintillation cocktail with a counting efficiency of 25%. Specific binding was defined as the difference between ^3H bound to the filter from duplicate samples incubated ± 10 μM unlabeled cAMP.

RESULTS

Validation of the use of the activity ratio method. The protein kinase activity ratio in untreated H35 cells has been found routinely to be between 0.3 and 0.4, a range similar to or slightly higher than that reported for liver by other groups (17–21). However, H35 cells contain very little cAMP (50–100 fmoles/mg protein ≅ 10–20 nM) (11), much less than is found in rat liver (19, 22). There are at least two factors that contribute to the apparently discrepant kinase ratio. First, about half of the basal protein kinase activity (i.e., −cAMP) is not inhibited by addition of an excess of partially purified protein kinase inhibitor (Fig. 1), suggesting that about 50% of the observed basal activity (toward type IIA histone) is not due to the cAMP-dependent kinase. Second, the large (60–100 fold) dilution involved in cell disruption leads to a small increase in the basal activity ratio. Taking these two factors into account, the apparent basal ratio of 0.3–0.4 can be estimated to be an actual ratio of about 0.10–

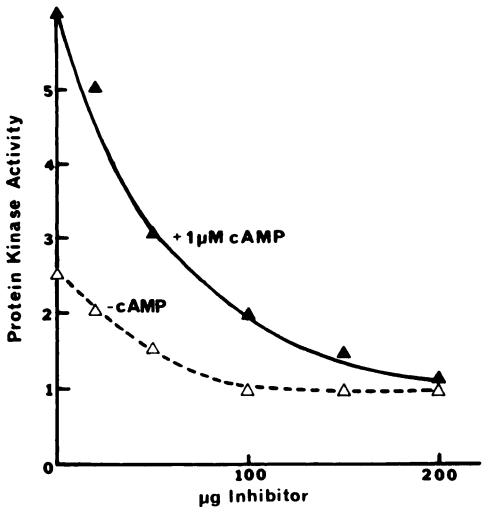

FIG. 1. *Titration of H35 cell protein kinase with skeletal muscle inhibitor*

A 10,000 × *g* supernatant fraction was prepared using untreated H35 cells as described in MATERIALS AND METHODS. Protein kinase activity was measured in the presence and absence of the skeletal muscle inhibitor of protein kinase, as prepared by the method of Gilman (15). Each point is the mean of three separate observations, with a variation of less than 10% for each value from the mean. Protein kinase activity is given as nmoles/hr with a total of 28 μg of supernatant protein per assay tube.

0.20 for the cAMP-dependent protein kinase in the intact cell. These estimations have been borne out by the results of Sephadex G-100 chromatography, as will be detailed below. It seems likely that the very low cAMP concentrations in H35 cells account for the fact that these estimated ratios are lower than those obtained by other workers using liver cells or liver-related cultured cells (17–21). This conclusion is supported by the fact that the kinase is not activated by a moderate elevation of cAMP content (see below). In preliminary experiments H35 cell protein kinase activity eluted from DEAE cellulose in a single peak at ~ 0.2 M NaCl, which is consistent with a type II enzyme.

A variety of additional control experiments were performed to optimize the conditions under which accurate preservation of protein kinase activity changes produced by treatment of cells with the various cAMP derivatives could be achieved during preparation of cells for enzymatic analysis. These are all standard procedures that must be followed in order to validate the use of the ratio method, as discussed by others in detail (8, 19). Consequently most of the data will not be shown but are available (23). The activity ratios from untreated or Bt_2cAMP-treated (and therefore activated) cells are stable for at least 40 min in extracts kept at 0° (by which time all assays are completed). The addition of MIX, a phosphodiesterase inhibitor, to the homogenizing medium at 0° or to the assay itself had no effect on either basal or activated ratios. Addition of charcoal to the homogenizing medium also did not alter basal or stimulated activity ratios. When cAMP (5 mM) was added to cells immediately before harvest and lysis, no activation of protein kinase was detectable, ruling out carryover of extracellular cyclic nucleotide as a problem. Finally, mixing experiments with extracts from control or treated cells yielded expected intermediate kinase ratios.

Activation of protein kinase in H35 cells by cAMP derivatives. Since H35 cells do not respond to hormones that elevate intracellular cAMP and thereby induce tyrosine aminotransferase in rat liver (5, 24), the effects of a series of cAMP derivatives known to induce the aminotransferase were tested in this system for their ability to activate protein kinase in intact cells. In some cases changes in *in vitro* [^{3}H]cAMP binding capacity were also monitored in the same extracts (17). Binding was measured under conditions where it was saturated with respect to cAMP and was proportional both with respect to time and protein concentrations, but exchange of bound cyclic nucleotide with [^{3}H]cAMP was minimal.

Three different cAMP derivatives (Bt_2cAMP, N^6benzylcAMP, 8-pClC_6H_4-ScAMP) added to intact H35 cells all produced large increases in the protein kinase activity ratio and caused a substantial reduction in the amount of [^{3}H]cAMP bound *in vitro* (data not shown). These results are consistent with the conclusion that the cAMP-dependent protein kinase was in fact activated by exposure of intact H35 cells to these cAMP derivatives.

In vivo activation of protein kinase was

found to be routinely accompanied by the dose- and time-dependent loss of 20–40% of total activity from the supernatant fraction to the 10,000 × *g* pellet (see legend to Figure 2), as has been noted by other groups (18, 19, 25). This raised a question as to whether use of the 10,000 × *g* supernatant fraction for estimation of activity ratios would give rise to errors as a result of this phenomenon. It was found that essentially identical dose-response curves for activation by Bt_2cAMP were obtained with the whole homogenate, a 100 × *g* supernatant fraction, a 10,000 × *g* supernatant fraction prepared in 0.15 M KCl or the 10,000 × *g* supernatant fraction prepared as usual in Tris-DTT buffer alone (Fig. 2). No loss of total activity (relative to extracts from untreated cells) was observed with the whole homogenate or KCl-treated preparation. However, since 0.15 M KCl extraction caused both substantial inhibition of protein kinase activity and an increase in the kinase ratio, as also noted by others (16), and because it is difficult to sample accurately the 100 × *g* supernatant fraction or the whole homogenate, the 10,000 × *g* supernatant fraction was used routinely as the source of cAMP-dependent protein kinase.

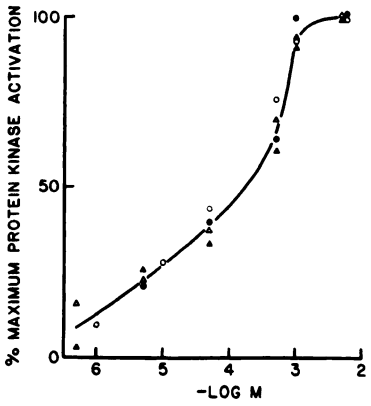

FIG. 2. *Dose response relationship for activation of protein kinase by Bt_2cAMP in different subcellular fractions of H35 cells*

Confluent H35 cells were exposed to the concentrations of Bt_2cAMP indicated for 1 hr prior to harvest. Protein kinase was assayed in the various subcellular fractions, as described in MATERIALS AND METHODS. Each value represents the mean of two separate observations assayed in duplicate.

	% change in total kinase (+cAMP) activity	
	Average	*Range*
△ Whole homogenate	——	——
● 100 × *g* supernatant fraction	−12%	−6 to −26%
▲ 10,000 × *g* supernatant fraction	−21%	−7 to −32%
○ 10,000 × *g* supernatant fraction prepared in 0.15 M KCl	+4%	0 to 13%

Comparison of the kinetics and the dose response relationships of enzyme induction with protein kinase activation by cAMP derivatives. The time course of protein kinase activation by 0.5 mM Bt_2cAMP is shown in the left panel of Figure 3. It can be seen that activation is a rapid process, which is maximal within 20–30 min after exposure of cells to Bt_2cAMP. Removal of the cAMP derivative leads to a rapid reversal of the activation state with a mean half-time for inactivation *in vivo* of about 8 min.

As illustrated in the right panel of Figure 3b, the activation of protein kinase by Bt_2cAMP precedes the induction of TAT, as would be expected if protein kinase mediated the effects of the cAMP derivative on the synthesis of TAT. Removal of Bt_2cAMP from the medium prompts a first-order decay in the activity of the aminotransferase with little or no lag as reported previously (26, 27). The loss of protein kinase activation is more rapid than the decrease in aminotransferase activity, as would be expected, however, given the fact that the latter enzyme has a half-life of 1–2 hr (5, 27). The decay of aminotransferase activity to values somewhat below the original baseline is a consistent finding but the basis for this phenomenon is not known at present.

Analysis of the dose response curves for the ability of Bt_2cAMP to influence TAT induction and protein kinase activation reveals a highly significant correlation with an ED_{50}, approximately 80–120 μM for both processes (calculated by probit analysis) (Fig. 4) ($r = 0.98, p = < 0.005$).

Another cAMP derivative, 8-pClC_6H_4-ScAMP also produced rapid activation of protein kinase that preceded the induction

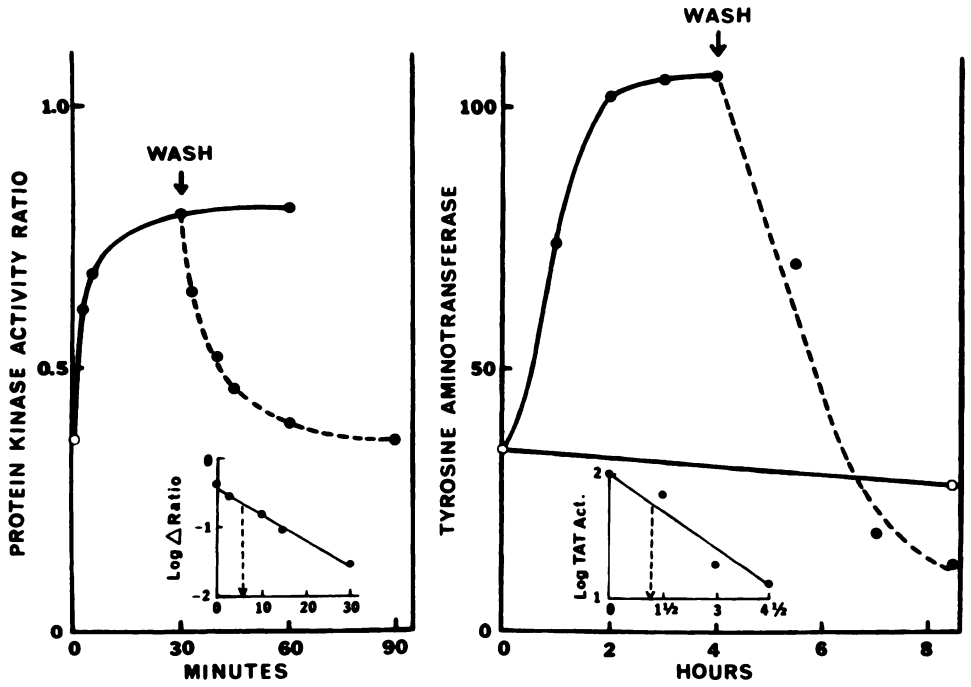

FIG. 3. *Kinetics of changes in protein kinase and tyrosine aminotransferase activities after addition and withdrawal of Bt_2cAMP*

Confluent H35 cultures were exposed to 0.5 mM Bt_2cAMP and harvested at the times indicated (left panel protein kinase; right panel TAT). In some cases, Bt_2cAMP was removed and the cells washed extensively prior to incubation in fresh medium devoid of Bt_2cAMP. Untreated cultures are designated by O. Each value represents the mean of six observations in three different experiments (left panel) and four observations in one experiment (right panel). The SEMs were less than 10% in all cases. Enzyme assays were performed with 10,000 × *g* supernatant fractions as described in MATERIALS AND METHODS and the values represent the ratio of activity −cAMP/+cAMP for protein kinase and units/mg protein for tyrosine aminotransferase. The insets illustrate a replot in semilog form of the data from the washed cells. In separate experiments the protein kinase activity ratio has been found to remain elevated until the cyclic nucleotide derivative is removed (23).

of tyrosine aminotransferase, as was the case with Bt_2cAMP (data not shown). The dose-response curves demonstrate that 8-$pClC_6H_4ScAMP$ is considerably more potent ($ED_{50} \cong$ 2–3 μM) than Bt_2cAMP in stimulating both processes (data not shown). Once again there is an excellent correlation between the effects of any given concentration of this cAMP analogue on the two processes, as illustrated in summary form in Figure 5. The protein kinase activation results with these two cAMP derivatives have been confirmed by the Sephadex G-100 chromatography method, as will be discussed below.

A third cAMP derivative, N^6-monobutyryl cAMP, proved to be less potent than Bt_2cAMP, with a calculated ED_{50} of approximately 400–500 μM for both processes (data not shown) but, once again, there was an excellent correlation between TAT induction and protein kinase activation (Fig. 5).

Effects of cAMP derivatives which are weak enzyme inducers. As previously reported (6, 7), cAMP itself and certain derivatives have been found to produce only transient or weak induction of tyrosine aminotransferase in rat liver and H35 cells. Since the cyclic nucleotides in this category are excellent substrates for phosphodiesterase (9, 29), the ability of MIX to influence their effectiveness as inducers of the aminotransferase and activators of protein kinase was examined in some detail.

The effects of cAMP by itself (at 1 mM) on both processes proved to be small but significant (Table 1), but no greater effect

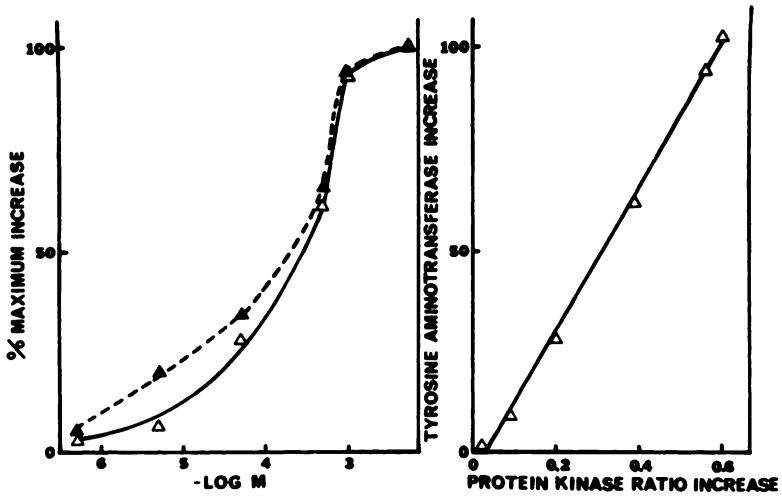

FIG. 4. *Dose-response curves for activation of protein and induction of tyrosine aminotransferase by Bt_2cAMP in H35 cells*

Confluent H35 cells were incubated for 3.5 hr with the concentrations of Bt_2cAMP indicated. After harvest, lysis and preparation of 10,000 × *g* supernatant fractions, enzyme activities were assayed as described in MATERIALS AND METHODS. (Protein kinase ▲, TAT △). The increases in protein kinase are given as the increase in the activity ratio −cAMP/+cAMP and those in TAT as units/mg protein. Each value is the mean of 3–4 separate observations with SEMs of less than 10%. The correlation coefficient for the observed relationship is 0.98 ($p = < 0.005$).

was observed with concentrations as high as 5–20 mM. MIX by itself at 0.25 mM also had essentially no effect on either enzyme induction or protein kinase activation as reported previously (11). This lack of effect is not due to the inability of MIX to inhibit phosphodiesterase activity in H35 cells because the cAMP content is elevated some 20 to 40-fold in H35 cells exposed to 0.25 mM MIX (11). However, the absolute level achieved (~ 1–3 pmoles/mg protein) is still below the basal level reported for rat liver (19, 22), and consequently, might not be expected to have much effect. Inclusion of MIX in the medium together with cAMP produced dramatically greater increases in both enzyme induction and kinase activation than could be achieved with cAMP alone (Table 1).

In the presence of MIX, cAMP is roughly equipotent to Bt_2cAMP ($ED_{50} \cong$ 80–100 μM) (data not shown), a derivative that is relatively resistant to attack by phosphodiesterase (9). Once again an excellent correlation was observed between effects of various concentrations of cAMP (+MIX) on enzyme induction and protein kinase activation (Fig. 5).

A time course of the effects of another weak inducer, 8-H_2N cAMP, on aminotransferase induction and activation of protein kinase is shown in Figure 6. In the absence of MIX, the cAMP derivative exerted transient effects on both parameters, but changes in the kinase activity ratio preceded those in aminotransferase activity. The transient nature of the effects of 8-H_2N cAMP has been observed consistently, as was the decline in tyrosine aminotransferase activity to below the original basal level (6, 7).

In the presence of MIX, however, the response of both enzymes to 8-H_2N cAMP is greater and no longer transient. The new steady-state levels of protein kinase and aminotransferase activities are maintained for at least 2 hr. As with the other derivatives, protein kinase is fully activated before aminotransferase activity reaches steady state.

In the presence of MIX, 8-H_2NcAMP also proved to be about as potent as Bt_2cAMP (ED_{50} = 60–70 μM) (data not shown). A significant correlation between changes in the two enzymes was observed up to a concentration of 1 mM 8-H_2N cAMP

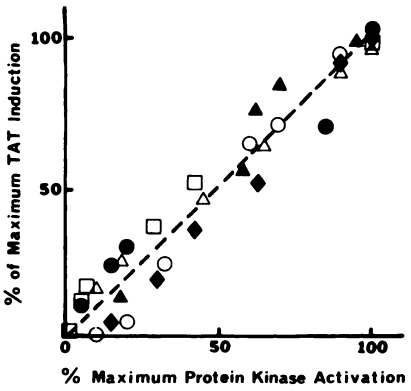

FIG. 5. *Correlation of activation of protein kinase and induction of tyrosine aminotransferase in H35 cells by different cyclic nucleotide derivatives*

The data from Figures 3 and 5 were replotted as percent of the observed maximal increase in each process. Data for 6-HScRMP + MIX are included from an experiment where kinase activity was measured at 1.5 hr and TAT at 2.5 hr (see Fig. 7). In that experiment, the protein kinase activity ratio increased from 0.34 to 0.67 and TAT from 24 to 55 units/mg. Where present, MIX was added at 0.25 mM. ○ Bt_2cAMP: $r = 0.98$, $p = < 0.005$; ◆ 8-pClC_6H_4ScAMP: $r = 0.99$, $p = < 0.005$; □ N^6-monobutyrylcAMP: $r = 1.00$, $p = < 0.005$; △ cAMP + MIX: $r = 0.99$, $p = < 0.005$; ▲ 6-HScRMP + MIX: $r = 0.99$, $p = < 0.005$; ● 8-H_2NcAMP + MIX: $r = 0.98$; $p = < 0.005$.

(Fig. 5). Above 1 mM the changes in both parameters became increasingly smaller but the effect on aminotransferase activity was more dramatic than that on the kinase activity ratio.

6-HScRMP is another derivative which by itself provokes little or no TAT induction (6, 7). A time course of its effects ± MIX is shown in Figure 7. MIX clearly enhances the ability of this analogue to stimulate both processes, but the responses remained transient whether MIX was included or not. The changes in protein kinase activity ratio clearly occurred prior to those in the aminotransferase during both phases of the response. Because of a response with 6-HScRMP that varied more than that of the other analogues, only limited dose-response data were obtained ($ED_{50} \cong$ 100–150 μM). But once again an excellent correlation between the two parameters was observed (Fig. 5).

Sephadex G-100 chromatographic analysis of protein kinase activation. An alternative approach for monitoring the state of protein kinase activation involves estimation of the amount of kinase activity present as the free catalytic subunit as determined by gel filtration (16). This method has been employed as a check on the validity of the results obtained with the ratio method.

TABLE 1

Effects of cAMP ± MIX on enzyme induction and protein kinase activation in H35 cells

Confluent H35 cells were incubated for 2 hr with the additions indicated. cAMP was added at 1 mM and MIX at 0.25 mM. Two groups of cells were incubated in parallel and one group (3 separate plates per group) was used for assay of protein kinase ± cAMP and the other for tyrosine aminotransferase as described in METHODS. Each value represents the mean % of control ± SEM for 3 separate observations assayed in quadruplicate. (For the protein kinase ratio 100% = 0.44; for tyrosine aminotransferase, 100% = 24.0 units/mg protein).

Additions	Protein kinase activity ratio	Tyrosine aminotransferase activity
	(% of control)	
None	100 ± 4	100 ± 3
cAMP	143 ± 5*	164 ± 6*
MIX	92 ± 5	102 ± 2
cAMP + MIX	209 ± 7*	281 ± 14*

* p value for difference from control = <0.001.

Figure 8a illustrates the elution profile of protein kinase activity ± cAMP following chromatography of a 10,000 × g supernatant fraction from untreated cells on Sephadex G-100. Protein kinase activity eluted in a cAMP-stimulatible form between fractions 6–10; this coincided with cAMP-binding activity (data not shown). The remaining protein kinase activity (fractions 11–30) was not stimulated by cAMP. The observed activity has been corrected for cAMP-independent histone kinase activity, which is present in the crude cell extract as in Figure 1. Using this correction it can be seen that 85–90% or more of the inhibitor-suppressible kinase activity is in the holoenzyme or cAMP-dependent form (i.e., the basal activity ≅ 0.10–0.15). In agreement with this result, the activity of protein kinase in the concentrated cell extract used in this experiment was stimulated 7–8 fold by cAMP

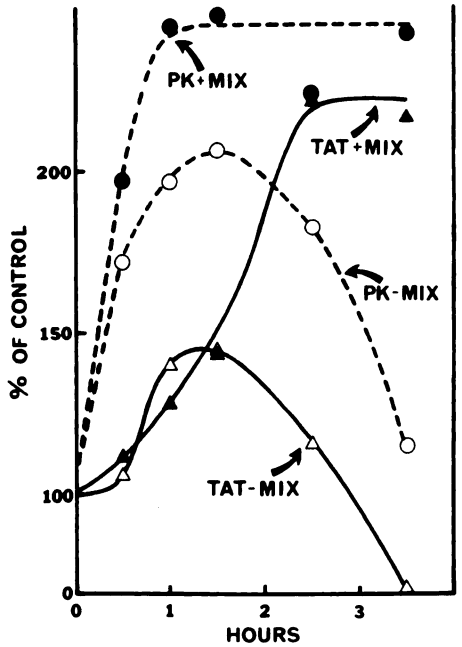

FIG. 6. *Kinetics of effects of 8-H_2NcAMP ± MIX on protein kinase and tyrosine aminotransferase activities in H35 cells*

Confluent H35 cells were incubated with 1 mM 8-H_2NcAMP + 0.25 mM MIX for the times indicated. Enzyme assays were performed as described in MATERIALS AND METHODS. Other details as in Figure 2. Each value represents the mean of three separate observations with SEMs of less than 5%. The protein kinase ratio and TAT activity in the untreated cells was 0.38 and 32 units/mg protein ± MIX, respectively.

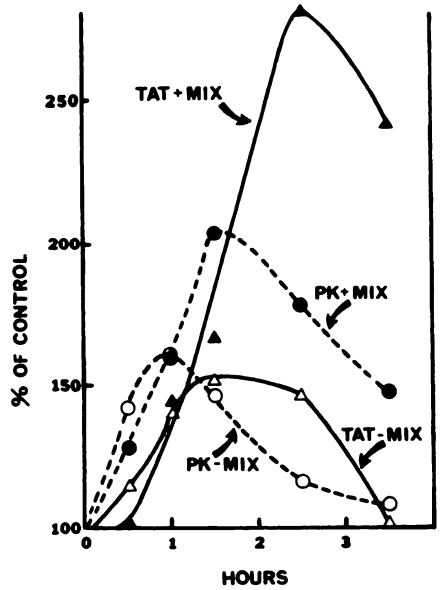

FIG. 7. *Kinetics of effects of 6-HScRMP ± MIX on protein kinase and tyrosine aminotransferase activities in H35 cells*

Confluent H35 cells were incubated with 1 mM 6-HScRMP ± 0.25 mM MIX for the times indicated. Enzyme assays were performed as described in MATERIALS AND METHODS. Other details as in Figure 2. Each value represents the mean of three separate observations with SEMs of less than 5%. The protein kinase ratio and TAT activity in the untreated cells was 0.37 and 23 units/mg protein ± MIX, respectively.

(after elimination of inhibitor resistant activity).

Exposure of cells to 0.5 mM Bt_2cAMP (a suboptimal dose) led to partial activation of protein kinase as demonstrated by a decrease in both the total activity in the holoenzyme peak together with a reduction in the degree of stimulation by cAMP and binding of [^{3}H]cAMP in the excluded fraction (data not shown). Stimulation of cells with a maximally effective dose of Bt_2cAMP (2 mM) completely abolished the cAMP dependency of protein kinase (Fig. 8b) and the bulk of the catalytic activity was included in the column. [^{3}H]cAMP binding activity was also markedly reduced and shifted to a lower molecular weight form (data not shown). Similar chromatographic profiles were observed with 0.2 mM 8-pClC_6H_4ScAMP, which causes maximal activation of the kinase of the aminotransferase.

DISCUSSION

The control experiments performed with H35 lysates suggest that the modified ratio method provides a valid and useful means of assessing the state of protein kinase activation in these intact cultured cells. One of the advantages of a cell culture system for these studies is that the cell population is a homogeneous cloned one in which changes in protein kinase activation are occurring in the same cells as those being provoked to a biological response by external stimulation. The results obtained with the use of the ratio method provide strong suggestive evidence that protein kinase mediates the effects of cAMP on TAT synthesis. Thus, kinase activation precedes increases in TAT activity. This is expected because the increase in TAT activity is

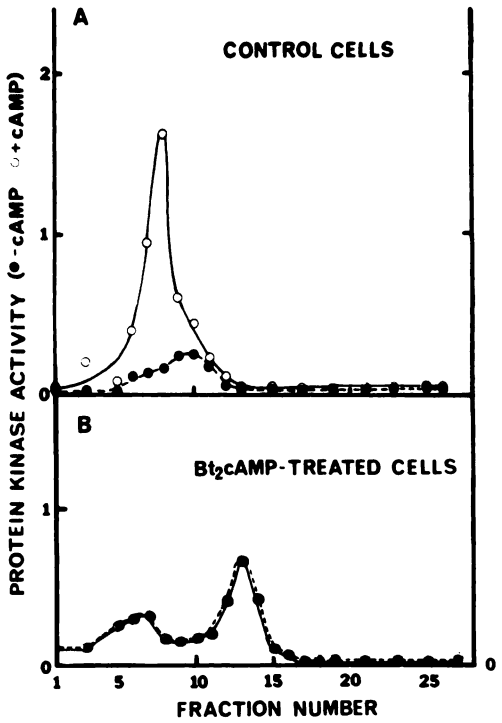

FIG. 8. *Gel filtration analysis of protein kinase activation by Bt_2cAMP in H35 cells*

Confluent H35 cells were incubated in the absence (A) or presence (B) of 2 mM Bt_2cAMP for 1 hr. Cells were harvested by scraping and homogenized in 10 mM KPO_4–10 mM EDTA, pH 7.0, containing 2 mg/ml bovine serum albumin. The 10,000 × *g* supernatant fraction was prepared and applied to a 1 × 20 cm column containing degassed Sephadex G-100 equilibrated with the KPO_4–EDTA buffer. The column was developed with KPO_4–EDTA buffer at 0–4° and 0.5 ml fractions were collected and analyzed for protein kinase activity ± cAMP as described in MATERIALS AND METHODS. −cAMP ●– – –●; + cAMP ○——○.

caused by a change in rate of synthesis and consequently movement toward a new steady state occurs. This steady state is dictated by the rate of enzyme degradation (30), which is 70–90 min (5, 28). Removal of Bt_2cAMP prompts a first-order decay in kinase activation, with a half-time of about 6–8 min, and TAT also exhibits a first-order loss of activity with a half-time of about 75 min, as reported previously (26, 27). Because this is well within the reported values for the half-life of TAT as measured immunochemically (5, 28), the elevated rate of TAT synthesis provoked by Bt_2cAMP must be expected to return very rapidly to the basal rate, following removal of the cyclic nucleotide derivative. The rapid inactivation of protein kinase is entirely consistent with this sequence of events. The only other requirement would be rapid dephosphorylation of the putative (but currently unknown) protein kinase substrate; this is a reasonable suggestion but cannot be evaluated as yet.

Keely *et al.* also observed rapid reversal of protein kinase and phosphorylase activation in perfused rat heart following removal of epinephrine and MIX from the perfusion medium (31). The half-time for decay of kinase activation back to the basal level was 1–2 min. These results coupled with ours indicate that in these two systems, at least, reassociation of protein kinase occurs rather rapidly upon abrupt reduction of the level of cAMP. This response stands in contrast to studies with the adrenal medulla in which apparently prolonged activation of kinase occurs even after return of cAMP levels to the unstimulated value (32).

The dose-response relationships for activation of protein kinase and induction of TAT are highly correlated for all of the cyclic nucleotide derivatives tested. These results are consistent with but do not prove a mediatory role for protein kinase in regulating TAT synthesis. The ability of MIX to convert cAMP, 8-H_2NcAMP, and 6-HScRMP concomitantly into effective inducers of TAT activity and activators of protein kinase provides strong further support for this conclusion. The kinetics of activation of the kinase for these three compounds differed from all of the other derivatives, which did not require MIX for effective action. In each case, however, the response of TAT followed that of the kinase, as would be expected in a cause-effect relationship. The basis for the decline in TAT activity to values below those in untreated cells with 8-H_2NcAMP and 6-HScRMP is not understood at present. These two derivatives are cytotoxic to growing H35 cells but do not inhibit protein synthesis appreciably during a period of exposure from 3–6 hr (33).[4] Evaluation of the rates of TAT

[4] Koontz, J. and Wicks W. D., in revision.

synthesis and degradation under these conditions will be undertaken in an effort to address this question. The explanation for the transient effect of 6-HScRMP even in the presence of MIX is also not clear but may have happened because the presumed competitive inhibition of phosphodiesterase exerted by MIX was partially surmounted.

We have recently found that cholera toxin produces irreversible activation of protein kinase and induction of TAT.[5] The kinetics and concentration-dependency relationships are fully consistent with a cause-effect relationship, as will be described elsewhere.

The ED_{50} of 2–3 μM for 8-pClC_6H_4ScAMP in provoking a biological response in these cells is remarkably low. Indeed, this value overlaps the range (0.5–2 μM) of internal cAMP concentrations that produce half-maximal induction of TAT with cholera toxin.[5] These results suggest that 8-pClC_6H_4ScAMP readily penetrates intact cells and must be highly resistant to phosphodiesterase attack, as has already been reported (29). Miller and co-workers also found that 8-pClC_6H_4ScAMP is the most potent inducer of TAT in rat liver (29). Interestingly, this derivative is roughly 50 times more potent than Bt_2cAMP in both rat liver and H35 cells. This derivative would appear to be the one of choice in most systems and deserves to replace Bt_2cAMP in view of the complications produced by butyrate effects, which are independent of cyclic nucleotide actions (34–38).

The nature of the putative substrate for protein kinase that might influence TAT synthesis is unknown at present. Based upon a variety of data obtained in the H35 cell system, we have proposed a translational site of action for cAMP (39). Thus, one would expect that a polysome-associated protein(s) or the nascent TAT chains themselves might well be logical candidates for the kinase substrate. In fact, TAT has been found to be phosphorylated but it appears to represent a post-synthetic, cAMP-independent process (10). The possibility that nascent TAT chains are subject to transient phosphorylation still remains open and is currently under investigation. Ribosomal protein S_6 is also a possible candidate, although no functional consequence(s) of this modification has been reported (40). It is quite possible, however, that the synthesis of only a few (and perhaps minor) proteins is productively influenced by S_6 protein phosphorylation. The validity of this suggestion remains to be tested by additional work, however. Finally, as pointed out previously (6, 7), the possibility cannot be ruled out that upon binding of a cAMP derivative the regulatory subunit of protein kinase is the active component involved in modulating TAT synthesis.

[5] Wimalasena, J., Leichtling, B. H., and Wicks, W. D., in preparation.

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