Inhibition of hormonal induction of tyrosine aminotransferase by polyamines in freshly isolated rat hepatocytes

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We have analysed the effects of natural aliphatic polyamines on hormonal induction of tyrosine aminotransferase (TAT) in suspensions of hepatocytes isolated from adult fed rats. Glucagon or cyclic AMP derivatives (dibutyryl and 8-bromo) used alone caused a 4–5-fold increase in enzyme activity within 4 h. This effect was independent of glucocorticoids, which also increased TAT activity (2.5-fold); when combined, the effects of the two inducers were additive. Spermine and putrescine totally inhibited the hormonally-mediated increase in enzyme activity when added at the onset of incubation with the inducers. Furthermore, polyamines could block the hormonal effect at any time during the course of TAT induction, with, however, a 30 min lag period, suggesting that they must enter the cells. Hepatocytes were indeed shown to take up spermine. At low external concentrations ($<50\mu\text{M}$), a Na^+ -dependent, saturable and concentrative mechanism was predominant; at high concentrations ($>0.5\text{ mM}$) transport occurred mainly through a non-saturable, Na^+ -independent mechanism, building up intracellular concentrations slightly lower than those in the medium. Dose-dependence analysis of the polyamine effect on enzyme induction indicated that half-maximal and maximal inhibition occurred with 0.75 mM- and 2.5 mM-spermine respectively, whereas 2.5 mM- and 7.5 mM-putrescine were required respectively to obtain similar effects. Spermidine was much less effective and cadaverine had virtually no effect. None of the polyamines affected the rate of decay of TAT, nor did they directly or indirectly cause enzyme inactivation, indicating that a post-translational modification was unlikely to account for the polyamine effects. Similarly, these effects could not be ascribed to a non-specific inhibition of overall protein synthesis. We conclude that, in hepatocytes, polyamines (or their metabolites) directly interfere with one or several steps controlled by hormones in the synthesis of tyrosine aminotransferase.

Tyrosine aminotransferase is a highly inducible enzyme in both normal and neoplastic liver-derived tissue. So far, besides amino acids (Andersson *et al.*, 1980), only hormonal factors have been found to stimulate enzyme synthesis *in vivo* and *in vitro*. In hepatoma-culture cells, glucocorticoids (Thompson *et al.*, 1966; Tomkins *et al.*, 1969), cyclic AMP derivatives (Barnett & Wicks, 1971), insulin (Gelehrter & Tomkins, 1969; Heaton *et al.*, 1980; Sorimachi *et al.*, 1981; Sorimachi & Yasumura, 1981), insulin-like growth factors (Heaton *et al.*, 1980) and serum (Gelehrter & Tomkins, 1969;

Sorimachi *et al.*, 1981) were reported to induce the enzyme. In normal liver, glucocorticoids and hormones acting through cyclic AMP (i.e. glucagon and adrenaline) appeared to be the only hormonal positive effectors of TAT synthesis (for reviews see Ernest *et al.*, 1977; Marston *et al.*, 1981). A few examples of factors capable of blocking induction of the enzyme have been described. Thus, the glucocorticoid effect was inhibited by butyrate in hepatoma cells (Tichonicky *et al.*, 1981), by insulin in cultured foetal-rat hepatocytes (Ho *et al.*, 1981) and by somatotropin in primary cultures of adult rat hepatocytes (Shires & Hargrove, 1982). In the last system, we recently found that thrombin inhibited the induction of TAT by glucagon and dexamethasone (Auburger *et al.*, 1983a).

Abbreviations used: TAT, tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5); Bt,cAMP, dibutyryl cyclic AMP.

Although many aspects of the molecular mechanism whereby hormones stimulate the synthesis of specific proteins like TAT remain unclear, phosphoproteins are likely to play an important role, at least for hormones acting through a cyclic AMP-dependent pathway. Accordingly, any factor that will regulate the turnover of phosphoproteins generated by a hormone should interfere with the control of enzyme synthesis by this hormone. We have recently discovered that natural aliphatic polyamines strongly stimulated the dephosphorylation of several proteins that are substrates for cyclic AMP-dependent protein kinases (e.g. ribosomal protein S6) in hepatocytes (Auberger *et al.*, 1983b). We therefore decided to examine the effects of polyamines on hormonal induction of TAT in freshly isolated liver cells. The data in the present paper show that polyamines, more particularly putrescine and spermine, are very potent inhibitors of the induction of TAT by glucagon and glucocorticoids in hepatocytes.

Materials and methods

Methods

Hepatocytes were isolated from adult (200 g) male Wistar rats fed *ad libitum*, as previously described (Le Cam *et al.*, 1976). Viability of the cell suspensions assessed by membrane refractoriness in phase-contrast microscopy was 90–95% and remained approximately constant throughout the incubations, regardless of the presence of polyamines. Suspensions of hepatocytes (0.25×10^6 cells/ml) were incubated at 37°C in Krebs–Ringer bicarbonate medium (KRB) supplemented with bovine serum albumin (fraction V; 0.5%, w/v), bacitracin (1 mg/ml) and gentamycin (50 µg/ml), pH 7.4, and gassed with CO₂/O₂ (1:19). The effectors (hormones and polyamines) were added as indicated in the legends to the Figures. TAT activity was assayed by the method of Granner & Tomkins (1970), in 20 000 g supernatants prepared from cell homogenates obtained by sonicating (5 s) hepatocytes in a phosphate buffer (125 mM) containing EDTA (1 mM) and dithiothreitol (1 mM) (Auberger *et al.*, 1983a). Protein and RNA synthesis were studied by measuring [³H]leucine and [³H]uridine incorporation into trichloroacetic acid-precipitable material (Fehlmann *et al.*, 1981). Similar results were obtained using low (tracer) or high (saturable, 2.5 mM) concentrations of precursors, for the effects of polyamines on macromolecular synthesis.

The uptake of [¹⁴C]spermine was studied at 37°C by incubating hepatocytes (10⁶ cells/ml) in KRB buffer supplemented with bovine serum albumin (0.5%, w/v), bacitracin (1 mg/ml) and gentamycin (50 µg/ml). Experiments were stopped by adding

5 vol. of chilled buffer to the cell suspensions; hepatocytes were rapidly sedimented by centrifugation (5 s at 3000 rev./min) and the pellets were washed twice in cold buffer. Under these conditions no detectable loss of cell-material-associated ¹⁴C occurred. Hepatocytes were then lysed in scintillation liquid (Unisolve) and the cell-associated radioactivity was measured. The distribution ratio was calculated assuming an average intracellular water space of 3.5 µl/10⁶ cells determined as previously described (Le Cam & Freychet, 1977).

Materials

DL-[4,5-³H]Leucine (sp. radioactivity 45 Ci/mmol), [5-³H]uridine (sp. radioactivity 28 Ci/mmol) and [¹⁴C]spermine tetrahydrochloride (sp. radioactivity 109 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., UK. Spermine tetrahydrochloride, spermidine trihydrochloride, putrescine dihydrochloride and cadaverine dihydrochloride, obtained from Sigma, were dissolved in water and the stock solutions (1 M) were neutralized. The sources of other chemicals and of hormones were as reported previously (Auberger *et al.*, 1983a).

All the experiments were repeated at least three times with different cell preparations. The cyclic AMP analogues used in the present study were dibutyryl cyclic AMP and 8-bromo cyclic AMP; both derivatives gave exactly the same results, which were analogous to those obtained with glucagon.

Results

Time course of TAT induction by Bt₂cAMP: effects of spermine and putrescine (Fig. 1)

After a lag period of 90 min, the enzyme activity, which remained stable in control cells, increased steadily to reach a maximum (4–5-fold stimulation) after 4 h of incubation with Bt₂cAMP. Addition of spermine or putrescine at the onset of incubation concomitantly with the cyclic AMP analogue totally prevented the increase in enzyme activity. Polyamines had little effect if any on basal TAT activity.

When polyamines were added after a 2-h incubation of hepatocytes with Bt₂cAMP, the level of enzyme activity continued to rise for 30 min and then levelled off, indicating a blockade of the effect of the cyclic AMP analogue. In other experiments (results not shown), we observed that polyamines could block the Bt₂cAMP effect at any time during the course of enzyme induction, regardless of the level of enzyme activity.

Dose-dependence relationships for polyamine effects on TAT induction by glucagon

The dose–response curves shown in Fig. 2 reveal that spermine was a much better effector than

Fig. 1. Time course of TAT induction by Bt_2cAMP : effects of spermine (SPE) and putrescine (PUT) Hepatocytes were incubated in suspension during 4 h, in the absence (open symbols) or in the presence (filled symbols) of Bt_2cAMP (0.1 mM). Spermine (5 mM) or putrescine (10 mM) were added either at the onset of the incubation (lower two broken lines) or after 2 h (upper two broken lines). At each time indicated, portions of cell suspensions were taken to prepare 20000 g supernatants for enzyme assay. Each bar represents $\pm$ S.E.M. of means obtained in three experiments performed with different cell preparations. The effects of polyamines on basal TAT activity were minor (see Fig. 4) and were not plotted.

putrescine. Half-maximal and maximal inhibitions of glucagon stimulation of TAT activity occurred with 0.75 mM- and 2.5–3 mM-spermine respectively, whereas 2.5 mM- and 7.5 mM-putrescine were required to observe similar effects. Spermidine at 10 mM caused a 40–50% inhibition and cadaverine was totally ineffective.

Effect of a submaximally effective concentration of polyamines on the dose-dependence relationship for glucagon stimulation of TAT

To test the possibility that polyamines altered cell sensitivity toward glucagon, we studied the dose-dependence relationship for the effect of the hormone in cells incubated without or with a submaximally effective concentration of polyamines. The inhibitory effect of polyamines was observed at

Fig. 2. Dose-dependence relationships of polyamine effects on Bt_2cAMP induction of TAT Hepatocytes were incubated during 4 h in the absence (open symbols) or in the presence (filled symbols) of Bt_2cAMP (0.1 mM). Polyamines were present throughout the incubation. Each value is the mean of triplicate determinations, within one experiment.

Fig. 3. Effects of a submaximally effective concentration of polyamines on the dose-dependence relationship for glucagon induction of TAT Hepatocytes were incubated for 4 h with various concentrations of glucagon, in the absence or in the presence of putrescine (3 mM) or spermine (0.75 mM). Each point is the mean of triplicate determinations within one experiment.

all glucagon concentrations, with roughly the same magnitude (Fig. 3). Neither spermine nor putrescine affected the EC_{50} (concentration producing half-

maximal stimulation) for glucagon (5 nM). Similarly, maximal stimulation of TAT activity required 50 nM hormone, irrespective of the presence of polyamines.

Effect of various polyamines on the induction of TAT by glucagon and dexamethasone used alone or in combination

When used at a maximally effective concentration dexamethasone caused a 2.5-fold increase in TAT activity after 4 h and was about half as effective as glucagon (Fig. 4). Both hormonal effects were totally blocked by spermine and putrescine; spermidine, which inhibited the glucagon effect by about 40%, did not seem to affect the stimulation of TAT by dexamethasone. Cadaverine was ineffective, regardless of the hormone used.

When used in combination, glucagon and dexamethasone effects were additive and, as expected, were completely inhibited by spermine and putrescine (Fig. 4).

Dose-dependence analysis of polyamine effects on overall RNA and protein synthesis

None of the polyamines tested significantly affected RNA synthesis (Fig. 5a). In contrast, protein synthesis was partially inhibited (maximal inhibition of 30–40%) in a dose-dependent manner by spermine and to a lesser extent (15–20%) by the other polyamines (Fig. 5b). It should be noted that

Fig. 4. Effects of polyamines on basal and hormonal stimulation of TAT

Hepatocytes were incubated for 4 h without (basal) or with a maximally effective concentration of glucagon (GLU) (100 nM) or dexamethasone (DEX) (10 μM), used alone. Polyamines (10 mM) were omitted (control) or added at the beginning of the incubation with the inducer. Each column represents the mean + S.E.M. (shown by the bar), of five determinations. □, Control; ▨, spermine; ■, putrescine; ▩, spermidine; ░, cadaverine.

the concentrations of spermine (1–2 mM) and putrescine (5–7.5 mM) that were found to block by more than 90% hormonal TAT induction had little effect (approx. 15%) on overall protein synthesis.

Spermine uptake by intact hepatocytes

As illustrated in Fig. 6, spermine was taken up by hepatocytes through both Na⁺-dependent and Na⁺-independent mechanisms. Time-course experiments (Fig. 6a) showed that transport increased linearly with time in the presence or in the absence of Na⁺ for more than 1 h; furthermore, the internal concentration became rapidly higher than the incubation medium (distribution ratio > 1), suggesting an active-transport process. However, this concentrative uptake might also result from intracellular binding of spermine (e.g. to nucleic acids).

Fig. 5. Effects of polyamines on macromolecular synthesis: dose-dependence analysis

Hepatocytes were incubated for 3 h with [³H]-uridine (5 μCi/ml) (a) or [³H]-leucine (5 μCi/ml) (b) and 2.5 mM of unlabelled precursor. At the end of incubation, cells were washed twice in chilled KRB medium and lysed in 250 μl of hot 10% (w/v) trichloroacetic acid. The pellets of acid-insoluble material were washed twice with 5% trichloroacetic acid, dissolved in 100 μl of 0.1 M-NaOH and counted for radioactivity. Each value is the mean of triplicate determinations within one experiment. Symbols in (a) are defined in (b).

Fig. 6. Spermine-transport experiments

(a) shows time course of $[^{14}\text{C}]$ spermine (100 μM) uptake in Na^+ -containing and Na^+ -free (Na^+ replaced by choline) media. In (b) transport was measured over 60 min, as a function of the external concentration. The broken line represents the Na^+ -dependent, saturable component of spermine transport, obtained by subtracting the Na^+ -independent component from the total transport. Each value is the mean of triplicate determinations within one experiment. The distribution ratio is the ratio of the intracellular to the extracellular concentrations of spermine. V_i refers to the initial rate of total spermine uptake.

Kinetic analysis was carried out in an attempt to evaluate the contribution of the two transport processes at various concentrations (Fig. 6b). Only the Na^+ -dependent component appeared to be saturable and to follow Michaelis–Menten kinetics (K_m approx. 20 μM ; V_{max} . 0.25 $\text{nmol}/10^6$ cells per h). The Na^+ -independent transport was not saturable for up to 10 mM polyamine. This component, which contributed to spermine transport to the extent of about 2.5 $\text{nmol}/10^6$ cells per h per mM, became the major route of polyamine transport for external concentrations higher than 0.5 mM. It could be calculated that for these concentrations, the intracellular spermine was about 0.7 times the extracellular concentration after 1 h incubation.

Discussion

Isolated hepatocytes represent a valuable system to study *in vitro* regulatory mechanisms like the induction of specific proteins by hormones. Concerning tyrosine aminotransferase, it has been reported that glucocorticoids and cyclic AMP derivatives used alone could not stimulate the enzyme synthesis in hepatocytes isolated from normal fed rats (Ernest *et al.*, 1977; Marston *et al.*, 1981). Our results clearly differ from those of these latter workers, since we obtained a 4–5-fold and a

2.5-fold increase in TAT activity with glucagon and dexamethasone respectively after a 4 h incubation. This discrepancy cannot be explained by differences in basal enzyme activity in hepatocytes, since a rough calculation indicated that basal levels in our preparations were similar to those reported by Marston *et al.*, (1981). A partial explanation can be probably found in differences in composition of the incubation medium, more particularly the absence (the present study) or the presence (the other studies) of serum. Indeed, in Reuber H35 hepatoma cells, Bt₂cAMP induction of TAT was markedly reduced in cells cultured with serum (Snoek *et al.*, 1981a). Nonetheless, our results strongly argue for an effect of cyclic AMP independent of pre-exposure of hepatocytes to glucocorticoids, which is in agreement with the conclusion reached by Marston *et al.* (1981), using cells from adrenalectomized rats.

The discovery that spermine and putrescine could totally inhibit the induction of TAT by hormones in hepatocytes was unexpected. Indeed natural aliphatic polyamines have been shown to stimulate macromolecular synthesis *in vitro* (for review see Jänne *et al.*, 1978; Abraham & Pihl, 1981). However an inhibition of RNA and protein synthesis by polyamines has recently been reported in apple (Apelbaum *et al.*, 1982). In our studies, we also found a partial inhibition of protein synthesis by spermine and putrescine. Nonetheless, it seems unlikely that this could account for the total inhibition of enzyme induction, since the spermine concentration (approx. 2.5 mM) causing maximal inhibition of hormonal TAT induction had only a slight (10–15%) inhibitory effect on $[^3\text{H}]$ leucine incorporation into the bulk of cellular proteins.

An interesting feature of the polyamine effect was that, in cells pre-incubated for a given period (e.g. 2 h) with the inducer, inhibition of enzyme induction still occurred, but after a lag-period of about 30 min. Such a delay suggested that polyamines had to enter the hepatocytes, which implied that uptake occurred. Transport experiments clearly demonstrated that spermine was taken up by hepatocytes in a time- and concentration-dependent manner, like different polyamines in other cell types (Clark & Fuller, 1975; Mandel & Flintoff, 1978; Alhonen-Hongisto *et al.*, 1980). For concentrations effective in inhibiting TAT induction (>0.2 mM), spermine entered the cells mostly through an Na^+ -independent, non-saturable process giving rise to intracellular levels slightly lower (approx. 70%) than that in the outside. These results are consistent with an intracellular site of action of the polyamine (or its metabolites).

How did polyamines prevent hormonal induction of TAT in hepatocytes? Several observations support the view that they did not directly or indirectly inactivate the enzyme. (1) The blockade of hormonal

effect on TAT induction in cells exposed to the inducers before adding the polyamines was never followed by a rapid decrease in enzyme activity. (2) The de-induction of the enzyme occurred at similar rates without and with spermine or putrescine (P. Auberger, M. Samson & A. Le Cam, unpublished work). (3) We failed to demonstrate the presence of an inhibitor in extracts of hepatocytes incubated with spermine and we did not find any direct effect of polyamines on enzyme activity (P. Auberger, M. Samson & A. Le Cam, unpublished work). Thus, despite the fact that TAT has been shown to undergo several types of post-translational modifications (for review see Hargrove & Granner, 1981), such a mechanism is unlikely to account for the polyamine effect.

Alternatively, an inhibition of enzyme synthesis could be involved. This would be consistent with the fact that in all the biological systems that have been studied, a change in TAT activity was always accounted for by a variation in the amount of enzyme. Hormonal induction of TAT clearly involves various mechanisms that are not fully understood. If it is generally admitted that steroid hormones act predominantly if not exclusively at the level of transcription by stimulating the synthesis of specific messenger RNA (Nickol *et al.*, 1978), the way cyclic AMP acts appears more complex. Indeed, evidence has been presented to support both transcriptional (Nogushi *et al.*, 1978; Scherer *et al.*, 1982) and post-transcriptional (Roper & Wicks, 1978; Snoek *et al.*, 1981a,b) sites of cyclic AMP action. In addition, it was recently suggested that the two inducers might directly interact (Lewis *et al.*, 1982). At the present time, the site(s) of action of polyamines leading to the blockade of both glucocorticoid and cyclic AMP induction of TAT in normal hepatocytes is totally unknown. Direct measurement of net synthesis of the enzyme using selective immunoprecipitation techniques along with translation experiments in cell-free systems are being carried out and should help answering this question.

Although factors that control the translation step in protein synthesis in eukaryotic cells are largely unknown, variations in the degree of phosphorylation of ribosomal protein S6 have been postulated to play a role (for review, see Leader, 1980). In isolated liver cells this protein becomes highly phosphorylated in response to glucagon (Le Cam, 1982; Wettenhall *et al.*, 1982); however, there is presently no evidence showing that this increase in S6 phosphorylation is a mandatory event in the cascade of reactions leading to the induction of a specific protein-like TAT, by glucagon. To our knowledge, aliphatic polyamines, especially spermine, are the first example of compounds capable of eliciting dephosphorylation of ribosomal protein S6 (Auberger *et al.*, 1983b) and concomitantly of

inhibiting hormonal induction of TAT in an intact living cell. Although this can be taken as an argument to relate the two processes causally, these observations do not eliminate the possibility that other cyclic AMP-mediated events [like phosphorylation of nascent TAT chains, as suggested by Roper & Wicks (1978)] might be involved in the regulation of TAT synthesis. Clearly, elucidation of the site(s) of polyamine action should help in clarifying the mechanism of hormonal induction of the enzyme.

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