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INVERSE CORRELATION BETWEEN CYCLIC GMP AND TYROSINE AMINOTRANSFERASE DEGRADATION IN RAT HEPATOMA TISSUE CULTURE CELLS

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Several cyclic nucleotide phosphodiesterase inhibitors that increase tyrosine aminotransferase degradation in rat hepatoma tissue culture (HTC) cells—including theophylline, caffeine, theobromine, 1-methyl-3-isobutylxanthine and papaverine—all lower cGMP levels in these cells while exerting variable effects on cAMP levels. In contrast, insulin slows tyrosine aminotransferase degradation and increases cGMP levels when HTC cells are treated with the hormone for 4 hr in the absence of serum. The inverse relationship observed between cGMP and tyrosine aminotransferase degradation is consistent with a role for this cyclic nucleotide in the control of specific protein degradation.

Several compounds frequently used to inhibit cyclic nucleotide phosphodiesterases—including theophylline, 1-methyl-3-isobutylxanthine, and papaverine—lower TAT[2] activity in HTC cells by specifically increasing the rate of TAT degradation (1-3). These compounds do not affect general protein degradation, although they do produce some inhibition of general protein synthesis (2). Our previous studies have shown that the effect of theophylline on TAT degradation in HTC cells is accompanied by an increase in the concentration of cAMP and an unexpected decrease in the level of cGMP (3). Only the latter change appears to correlate with increased TAT degradation in terms of the dose response and time course (3). Other studies have shown that increased cAMP levels have no influence on TAT degradation in hepatoma cells (3-5). Furthermore, when cGMP levels are elevated in HTC cells by adding Mn^{2+} or cGMP derivatives to the cultures, TAT

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[2]Abbreviations used: TAT, tyrosine aminotransferase; HTC, hepatoma tissue culture.

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degradation is slowed (3). Insulin has also been shown to inhibit TAT degradation in HTC cells preferentially (6) compared to its ability to slow general protein degradation (7,8). In addition, insulin has been reported to increase cGMP levels in liver slices (9) and hepatocyte cultures (10,11), although this has not been investigated previously in hepatoma cells.

In the present study, the effects of insulin and several phosphodiesterase inhibitors on cGMP levels in HTC cells have been investigated to determine whether they produce changes in cGMP which correlate with changes in TAT degradation. We have found that in every case, changes in the levels of cGMP, but not cAMP, are inversely related to changes in TAT degradation. These results further support the possibility of a new role for cGMP in controlling specific protein degradation, and they also show that some drugs cause changes in cGMP levels in HTC cells which cannot be explained solely by the inhibition of phosphodiesterases.

EXPERIMENTAL PROCEDURES

HTC cells were grown in spinner or monolayer cultures as previously described (1-3). Logarithmically growing spinner cultures were usually pre-induced overnight with 1 μM dexamethasome. In a typical experiment, a culture having 8×10^5 cells per ml was divided into two or more spinner bottles and incubated for appropriate times in modified Swim's S-77 medium containing 10% bovine serum and appropriate test compounds. The cells were harvested by centrifugation for 2 min at 800 x g (4°) and washed twice by resuspension in cold 0.14 M NaCl-0.01 M potassium phosphate buffer, pH 7.6. Monolayer cultures were maintained in plastic tissue culture dishes with inocula of 1-2 x 10^5 cells/cm^2 in a humidified incubator under 5% CO_2 in air for at least 8 hr before addition of medium containing test substances. The cells were harvested after appropriate incubation times by scraping with a rubber policeman, after which the cells were separated from the medium by centrifugation and washed as described above. In experiments using insulin, HTC cells were collected by centrifugation; resuspended in serum-free medium supplemented with 0.1% bovine serum albumin, 1 μM dexamethasone, 50 U/ml penicillin, and 50 ug/ml streptomycin; and maintained in spinner cultures from 18 hr prior to addition of 1 μM insulin throughout the experiment.

Tyrosine aminotransferase degradation was measured as described earlier (3) either by precipitating radioactive pulse-labeled TAT from cell extracts followed by gel electrophoresis or by monitoring the loss of catalytically active TAT in the presence of 0.2 mM cycloheximide. These two methods yielded similar results (3). Cyclic nucleotide levels were determined as described earlier (3) in 5% trichloroacetic acid-soluble fractions of cell lysates using the ^{125}I-radioimmunoassay procedure reported by Steiner et al. (12) after chromatographic separation of cAMP and cGMP. Protein content and TAT enzymatic activity were measured as reported earlier (1).

Theophylline, caffeine, theobromine, papaverine, bovine pancreatic insulin, bovine serum albumin, cycloheximide, and dexamethasone were from Sigma Chemical Co.; and 1-methyl-3-isobutylxanthine was from Aldrich Chemical Co. Powdered tissue culture medium and penicillin/streptomycin were purchased from Grand Island Biological Co. Serum was obtained from KC Biologicals.

RESULTS AND DISCUSSION

Theophylline and several other cyclic nucleotide phosphodiesterase inhibitors—including 1-methyl-3-isobutylxanthine, caffeine, and papaverine—were shown previously to lower TAT specific activity in HTC cells by increasing the rate of TAT degradation (1-3). Theobromine, a phosphodiesterase inhibitor similar in structure to theophylline, was also found to increase the rate of TAT degradation with changes identical to those produced by theophylline (13).

Theophylline has been shown to lower cGMP levels in HTC cells at doses and times which correlate with the increase in TAT degradation (3). The generality of this effect on cGMP levels was tested by examining the other phosphodiesterase inhibitors mentioned above. As shown by the data in Table I, all of these compounds decrease cGMP levels in HTC cells; but their effects on cAMP vary. For example, treatment with 0.1 mM papaverine for 1 hr elevates cAMP, while treatment with 0.04 mM papaverine for 3 hr has no significant effect on the level of cAMP. Theobromine at 5 mM does not affect cAMP levels after either 1 or 5 hr. However, all of the drugs result in decreases in cGMP levels in HTC cells under the conditions reported in Table I. Since the rate of TAT degradation is also increased under all of these circumstances (Refs. 1-3, 13), the data support an inverse correlation between cGMP and TAT degradation. Furthermore, the results in Table I provide additional evidence that changes in cAMP do not correlate with changes in TAT degradation.

It is surprising that these drugs, which are commonly used to inhibit cyclic nucleotide phosphodiesterases, lower cGMP levels in HTC cells. We believe this may occur because they also inhibit guanylate cyclase. In the case of theophylline, an inhibitory effect on purified soluble guanylate cyclase from rat liver has been reported by others (16,17); and we have observed that the drug can inhibit crude soluble and particulate guanylate cyclases from HTC cells and several rat tissues (13).

There are several reports that sodium nitroprusside and related compounds can activate guanylate cyclase and elevate cGMP (14,15,16). However, in our system 1 mM sodium nitroprusside had no effect on either the level of cGMP or the rate of TAT degradation (13). Carbachol at a concentration of 10 μM

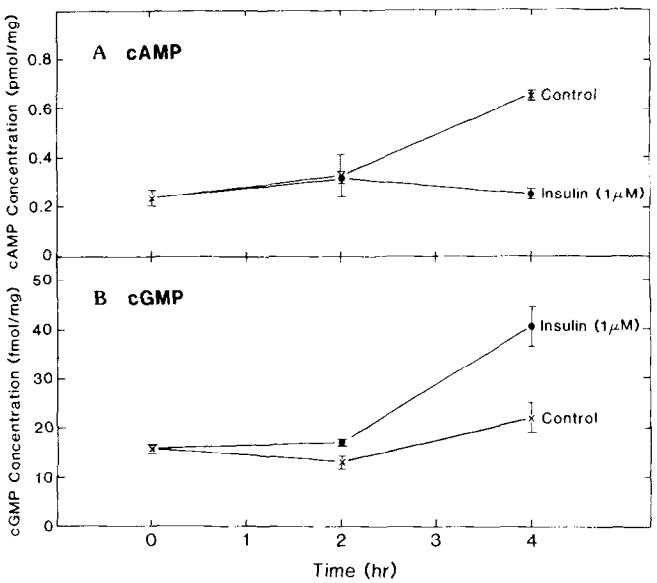

Figure 1. The effects of insulin on cyclic nucleotide levels in HTC cells. The same cultures of HTC cells used for the experiment in Table II were used to determine the changes in cAMP (Part A) and cGMP (Part B) in response to 1 μM insulin. Cyclic nucleotide levels from control (x——x) or insulin-treated (●——●) cells were determined as described previously (3). The results are plotted as the mean ± standard error of duplicate determinations.

Table I. Effects of various phosphodiesterase inhibitors on cyclic nucleotide levels in HTC cells

Experiment Number	Compound Added	Concentration (mM)	Exposure Time (hr)	Cyclic AMP (pmol/mg)	Cyclic GMP (fmol/mg)
1	None	-	1	0.86 ± 0.02	13.0 ± 0.2
	1-Methyl-3-isobutylxanthine	1	1	1.49 ± 0.03	11.7 ± 1.5
	Papaverine	0.1	1	1.04 ± 0.02	2.0 ± 1.0
	Theophylline	1	1	1.22 ± 0.04	4.7 ± 0.7
2	None	-	3	0.60 ± 0.04	12.1 ± 1.2
	1-Methyl-3-isobutylxanthine	1	3	1.70 ± 0.08	9.1 ± 0.7
	Papaverine	0.04	3	0.47 ± 0.08	1.2 ± 1.0
	Caffeine	1	3	1.04 ± 0.02	6.4 ± 3.0
	Theophylline	1	3	0.69 ± 0.01	4.0 ± 0.2
3	None	-	1	0.09 ± 0.003	23.0 ± 3.5
	Theobromine	5	1	0.10 ± 0.05	6.5 ± 2.0
4	None	-	5	0.21 ± 0.01	48.2 ± 2.9
	Theobromine	5	5	0.23 ± 0.02	30.7 ± 0.1

Cyclic nucleotide levels were determined as described in MATERIALS AND METHODS for HTC cells grown in monolayer (Experiment 2) or spinner (Experiments 1, 3 and 4) cultures. The cells were exposed to the indicated drug concentrations for the times shown. Results are presented as averages ± standard errors of duplicate determinations derived from single cultures (Experiments 1 and 2) or two separate cultures (Experiments 3 and 4) for each condition.

Table II. Effects of insulin and theophylline on TAT degradation in HTC cells

Compound Added	Concentration (μM)	TAT Half-life (hr)
None	-	2.1 ± 0.1 (8)
Insulin	1	6.0 ± 1.1 (8)
Theophylline	1000	1.3 ± 0.1 (4)
Insulin plus theophylline	1 1000	3.9 ± 0.6 (4)

Radioactivity in pulse-labeled TAT was determined by antibody precipitation and gel electrophoresis as previously described (3). Half-lives of degradation were calculated from the equation for a first-order process by using duplicate radioactivity measurements made at zero time and at 2 and 4 hr. The results are presented as the mean ± standard error for the number of determinations given in parentheses.

elevates cGMP levels in liver cells (9,18) but had no effect on either cGMP or TAT in HTC cells (13). We have previously reported that Mn^{2+}, an activator of guanylate cyclase (19), elevates cGMP and slows TAT degradation in a dose-dependent manner in HTC cells (3). Derivatives of cyclic GMP, such as 8-bromo-cGMP, also slow TAT degradation (3). All of the above-mentioned results taken together provide strong evidence for a correlation between cGMP and TAT degradation.

One possible physiological function for this phenomenon is that it may play a role in the effect of insulin on TAT. Insulin has been reported by Spencer *et al.* (6) to slow TAT degradation preferentially in HTC cells. Insulin is also known to elevate cGMP levels in hepatocyte cultures (10,11) and liver slices (9). We find that insulin increases cGMP in HTC cells (Figure 1) under the same conditions for which it slows TAT degradation (Table II). Furthermore, insulin reverses the effect of theophylline on TAT degradation. Thus, an increase in cGMP may mediate, at least in part, the effect of insulin on TAT degradation. However, it also seems clear that cGMP cannot account for all actions of insulin (18,20). Even in HTC cells the effect of insulin on general protein degradation probably does not involve cGMP, since neither theophylline, Mn^{2+}, nor 8-bromo-cGMP changes the rate of general protein degradation (1-3, 13). Although the

actions of insulin are complex and may involve a variety of mechanisms, our data suggest that the preferential effect of the hormone on TAT degradation could involve cGMP. The mechanisms by which cGMP may be involved in specific protein degradation are currently being investigated in our laboratory.

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