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SHORT COMMUNICATION

Effect of actinomycin D, cycloheximide and puromycin on hepatic adenosine 3′,5′-monophosphate in rats treated with glucagon

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ANTIBIOTICS such as actinomycin D, cycloheximide and puromycin have been widely used in clarifying the mechanisms involved in certain biological processes including enzyme induction and hormone action. However, it has been realized that these drugs may also produce undesired and complicating side reactions. In conjunction with studies on the hepatic actions of glucagon, we have found that, in addition to the expected inhibition of protein synthesis, cycloheximide and puromycin interfered with the marked elevation in hepatic cyclic AMP levels seen after treatment of rats with glucagon alone.[1] In contrast, actinomycin D in a dose inhibiting RNA synthesis was without effect on cyclic AMP content.

Weanling, male albino rats (Holtzman) were individually housed in metal cages with wire-mesh bottoms and were maintained on a 12-hr light–12-hr dark cycle, the light period starting at 7:30 a.m. The animals were fed *ad lib.* for about 1 week a diet containing 18 per cent casein[2] prepared as an agar gel.[3] Food was withheld for 23–24 hr before the various experimental procedures were carried out.

Radioactive compounds obtained from New England Nuclear were L-[U-^{14}C]leucine and [^{3}H]cyclic AMP; [6-^{14}C]orotic acid was obtained from Amersham Searle. Other materials were obtained as follows: glucagon, Eli Lilly; cyclic AMP, Sigma; cycloheximide and actinomycin D, CalBiochem; puromycin dihydrochloride, Nutritional Biochemicals. For administration to the rats, the labeled orotate and leucine were diluted in saline, glucagon was prepared in the manufacturer's diluent, cycloheximide and actinomycin D were dissolved in saline, and puromycin was dissolved in water and neutralized with sodium hydroxide.

Cyclic AMP content of liver was determined by the protein binding procedure of Gilman.[4] Frozen tissue[2] was homogenized in 10 vol. of 5 per cent (w/v) trichloroacetic acid containing a tracer amount of [^{3}H]cyclic AMP added to monitor recovery (> 95 per cent). After centrifugation of the homogenates, trichloroacetic acid was removed by extracting five times with 3 vol. each of water-saturated ether. Standards in trichloroacetic acid were extracted in the same manner and the samples and standards (20μl) were analyzed without further treatment. No evidence was obtained to indicate that the antibiotics themselves interfered with the assay, since identical standard curves were obtained in the presence or absence of the drugs. Each antibiotic was examined at two concentrations: (1) approximately that which would be present in the assay if it were uniformly distributed in the rat, and (2) five times this level, since it is likely that accumulation would occur in the liver.

Incorporation of [U-^{14}C]leucine into liver protein was determined by the method of Friedman *et al.*[5] Values were calculated as cpm incorporated/mg total protein; protein content was determined by the procedure of Lowry *et al.*[6] with crystalline bovine serum albumin as the standard. RNA synthesis from [6-^{14}C]orotic acid was measured by the method of Munro and Fleck.[7] Both radioactivity and total amount of RNA present were determined on portions of the hydrolyzed RNA fraction after removal of DNA and protein by acid precipitation; results were expressed as cpm/mg total RNA.

Table 1, column A shows that, within 15 min after the subcutaneous injection of glucagon, there was a 40-fold increase in hepatic cyclic AMP concentration. Neither actinomycin D, cycloheximide nor puromycin administered alone had any statistically significant effect on cyclic AMP concentration. The concentration in livers of rats given actinomycin D 1 hr before glucagon was the same as for rats receiving glucagon alone, indicating that the antibiotic had no influence on the usual glucagon-induced increase in cyclic AMP. However, pretreatment of rats with cycloheximide for 1 hr significantly altered the response to glucagon, since there occurred only a 10-fold increase in cyclic AMP content, one-fourth that obtained with the hormone alone. Treatment with puromycin followed within seconds by glucagon produced rather inconsistent results; in some trials, hepatic cyclic AMP concentrations were barely less than those for rats treated with glucagon alone, while in others a marked reduction was observed. Despite this variable response, the average cyclic AMP concentration was significantly decreased in the presence of the antibiotic (Table 1, A). In another experiment, puromycin was injected 5 min before the glucagon was given. The average content of the nucleotide in livers from these animals was not significantly lower than in experiment A, although the variation was decreased (Table 1, B); thus, a slightly longer exposure to puromycin did not significantly affect the results.

TABLE 1. CYCLIC AMP CONTENT IN LIVERS OF RATS TREATED WITH GLUCAGON AND ANTIBIOTICS*

Treatment	Cyclic AMP concentration (nmoles/g liver)	
	A	B
Control	1·41 ± 0·08 (17)	
Glucagon (2 mg/kg)	60·8 ± 4·4 (18)	
Actinomycin D (1 mg/kg)	1·52 ± 0·13 (9)	
Cycloheximide (1 mg/kg)	1·19 ± 0·08 (9)	
Puromycin (100 mg/kg)	1·26 ± 0·07 (14)	
Actinomycin D + glucagon	57·6 ± 3·8 (8)	55·7 ± 4·2 (4)
Cycloheximide + glucagon	14·7 ± 1·7 (9)†	17·0 ± 1·9 (5)†
Puromycin + glucagon (a)	37·7 ± 4·0 (15)†	
Puromycin + glucagon (b)	36·2 ± 3·6 (12)†	50·2 ± 4·2 (5)

* In column A, rats were fasted 23 hr (wt 75–85 g) and injected with actinomycin D, cycloheximide or saline (i.p.) 1 hr before injection with glucagon or diluent (s.c.). Rats given puromycin + glucagon (a) were fasted 24 hr, injected with puromycin or saline (i.p.) and with glucagon or diluent at the same time; those given puromycin + glucagon (b) received puromycin 5 min before glucagon. In column B, actinomycin D or cycloheximide was administered 5 min before, and puromycin 1 hr before, glucagon. All animals were killed 15 min after being injected with glucagon or diluent. Values are for mean ± S. E.; number of animals is shown in parentheses. Statistical significance was determined by Student's *t*-test.[8]

† Significantly different from glucagon alone: $P < 0{\cdot}001$.

Since these results with the antibiotics were obtained with two very different pretreatment times, limited experiments were also carried out to examine the opposite effects of administering puromycin at 1 hr, and cycloheximide or actinomycin D at 5 min, before injecting glucagon. Table 1, column B, shows that, under these conditions, actinomycin D was still without effect on the glucagon-induced elevation of cyclic AMP, whereas cycloheximide again caused marked interference. However, the results suggest that the effect of puromycin may be relatively short-lived since, after an exposure period of 1 hr, it no longer significantly interfered with the normal response to glucagon.

Doses of the antibiotics used in these experiments effectively inhibited incorporation of radioactive orotic acid or leucine into total liver RNA or protein respectively (Table 2).

It is not known why puromycin or cycloheximide partially prevented the massive increase in hepatic cyclic AMP concentration induced by glucagon alone. The antibiotics may have delayed the normally rapid elevation in nucleotide content. It is possible that with smaller doses of the hormone even lower levels of cyclic AMP might have been found after treatment with the antibiotics; conversely, larger amounts of glucagon might obscure this effect. It also appears, from the puromycin experiments at least, that the relative times of administering the antibiotic and the hormone are important. If adenylate cyclase has a short half-life, inhibition of protein synthesis could then conceivably affect cyclic AMP concentrations relatively quickly. Direct effects of the antibiotics on this enzyme could be involved as well.

TABLE 2. EFFECT OF ANTIBIOTICS ON INCORPORATION OF PRECURSORS INTO TOTAL LIVER RNA OR PROTEIN *in vivo**

Treatment	Incorporation of	
	Leucine (cpm/mg protein)	Orotic acid (cpm/mg RNA)
Control	530 ± 20 (12)	8530 ± 370 (5)
Actinomycin D	510 ± 20 (6)	4610 ± 330 (5)
Cycloheximide	70 ± 3 (6)	
Puromycin	90 ± 3 (4)	

* Rats were treated as described in Table 1, column A; puromycin was injected at the same time as the leucine. Rats were killed 1 hr after i.p. administration of precursor ([6-^{14}C]orotic acid, 5 μCi/rat, 61 mCi/m-mole; [U-^{14}C]leucine, 2·5 μCi/rat, 327 mCi/m-mole). Results are expressed as mean ± S.E.; number of animals is shown in parentheses.

However, the drugs were not found to interfere with the usual increase in cyclic AMP concentration seen in corpus luteum[9] or adrenal cortex[10] incubated *in vitro* with the appropriate hormone, or in isolated fat cells incubated with norepinephrine.[11] Puromycin alone did not affect cyclic AMP content in fat cells *in vitro*[11], while its concentration in diaphragm was increased after incubation with puromycin, possibly as a result of inhibition of phosphodiesterase.[12] In contrast, cycloheximide has been found to decrease cyclic AMP levels in cultured mouse fibroblasts.[13]

These various results may be related to the nutritional state of the animal, to tissue specificity, or to differences in systems *in vitro* and *in vivo*. However, our finding that both cycloheximide and puromycin can interfere with the well-known glucagon-induced elevation in hepatic cyclic AMP emphasizes the need for caution in interpretation of studies using such inhibitors.

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