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IN VITRO STIMULATION OF TADPOLE TAIL REGRESSION BY CYCLIC AMP

Elizabeth S. Stuart and Mark S. Fischer
Department of Biochemistry
University of Massachusetts
Amherst, Massachusetts 01003

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SUMMARY: Treatment of Rana catesbeiana tail fin tissue in vitro with 0.1 mM or 0.5 mM cyclic AMP or with triiodothyronine induces an increase in the specific activity of hexosaminidase, a lysosomal marker enzyme, and a decrease in tissue area. Lithium chloride (8 mM), an inhibitor of adenylate cyclase, inhibits these changes when initiated by triiodothyronine but not when initiated by cyclic AMP. The levels of cyclic AMP, determined by radioimmunoassay techniques, increased 110 ± 10% over matched discs in culture after only one day's exposure to triiodothyronine. These results indicate the effect of triiodothyronine on fin resorption may be mediated by cyclic AMP.

INTRODUCTION: The subcellular role of thyroid hormones in amphibian tissues during development has been well studied. One approach which has been emphasized during the last several years is the demonstration of uptake of thyroid hormones into cell nuclei, possibly mediated by a soluble cytoplasmic protein (1,2). At the same time some evidence has been accumulating which suggests a possible involvement of cyclic AMP* in processes induced by thyroid hormones. For example, treatment with thyroid hormones increases intracellular levels of cyclic AMP in mammals (3-5).

The in vitro regression of tadpole fin tissue is a well characterized model system for the analysis of thyroid hormone action (6-9). In previous experiments, we observed a characteristic elevation in the multiple forms of the lysosomal enzyme N-acetyl-β-D hexosaminidase (E.C. 3.2.1.30) during metamorphosis (10). We have used this model system, and hexosaminidase as a marker enzyme, to examine the possible role of cyclic AMP in hormone induced fin regression. Specifically, we tested the following: 1) the ability of cyclic AMP to mimic T_3* effects in cultured tissue, 2) the ability of LiCl, an inhibitor of adenylate cyclase activity in some species (11,12), to inhibit T_3-induced

*Abbreviations: cyclic AMP, adenosine-3',5'-cyclic monophosphate; T_3, 3,5,3'-triiodo-L-thyronine

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fin regression and 3) the ability of exogenous T_3 to induce changes in tissue levels of cyclic AMP. Results from our in vitro experiments and our radioimmunoassays strongly suggest that some aspects of T_3 stimulated fin regression are mediated by cyclic AMP.

METHODS: Premetamorphic Rana catesbeiana tadpoles, caught in local ponds or obtained from Mogul Ed, Oskosh, Wisconsin, were maintained in bins containing 625 mg $CaCl_2$, 1.05 g KCl and 1.125 g NaCl per liter of distilled water. Fresh water and canned spinach were provided daily.

Culture Technique. For in vitro studies dorsal and ventral fins from tadpoles of stages IX-XII (13) were placed in 0.05% sodium sulfadiazene (City Chemical Co., New York) overnight. Using a modification of the methods described by Derby (6), fins were removed, cut into discs of 20-25 mm^2 and placed in 25 ml of modified Hanks Balanced Salt solution (pH 7.2) containing 100 units each of penicillin and streptomycin and 20 mM Hepes buffer. After healing for 24-48 hours, the 8-10 discs from each tail were divided into groups of discs such that fins from the same tadpole provided the discs for both experimental and control groups. The 2-4 discs comprising each group were placed in 60 x 15 mm disposable plastic petri dishes (Falcon Plastics) with 10 ml of modified Hank's solution. Cultures were maintained at 18°C ± 2°C and sterile procedures were used throughout. Solutions of T_3 (Sigma), LiCl (Fisher), or cyclic AMP (Sigma), were added sterily to the cultures with a Swinney adapter. Changes in tissue morphology, resulting from the additives to the culture, were recorded on photographic film.

Biochemical Assays. Hexosaminidase activity was assayed in supernatants prepared from discs minced and homogenized in 0.25 M sucrose (1.0 ml/4 discs) containing 0.1% Triton X-100 and centrifuged at 2000 rpm for 10 min. Both hexosaminidase activity and protein concentrations were determined as described previously (10) using modifications of the methods of Woolen et al. (14) and Lowry et al. (15), respectively. All determinations were done in triplicate.

RESULTS: The possible involvement of cyclic AMP in T_3-initiated tail resorption was examined in experiments in which discs were treated with cyclic AMP instead of T_3 or in which LiCl, a known inhibitor of adenylate cyclase activity, was used to interfere with the adenylate cyclase system. In initial experiments, the specific activity of hexosaminidase obtained from fins of eight different premetamorphic tadpoles varied by as much as 35%. However, the specific activity determined in matched discs from the same tadpole differed by less than 5%. Thus, any increase of 20% or more above control levels would be significant at greater than the 99.9% confidence level. For this reason, matched discs were used in all the experiments presented below.

Discs cultured with T_3 undergo a characteristic regression and darkening as shown in Fig. 1a (top vs. bottom) and a progressive increase in the specific

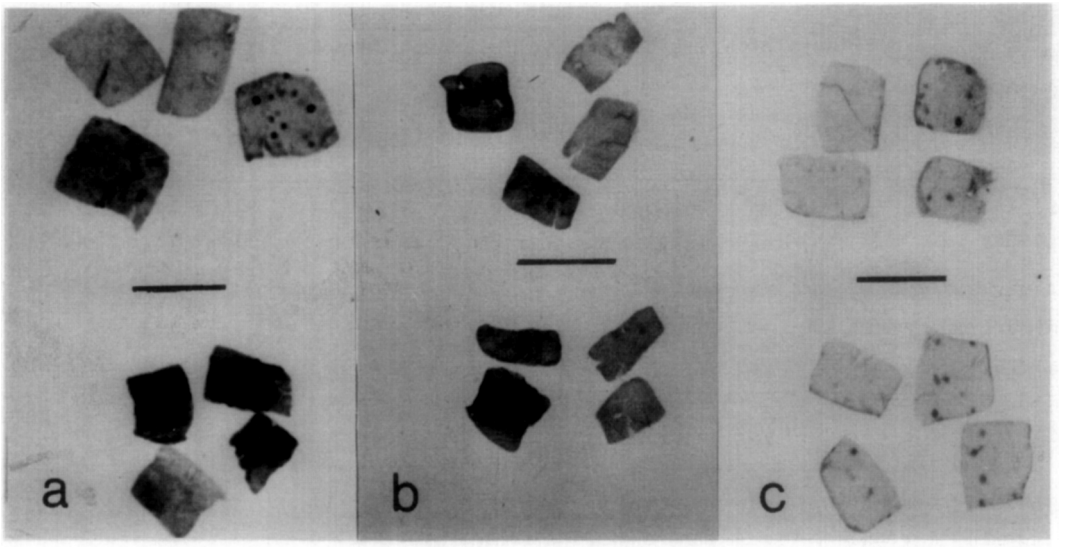

Figure 1. Effects of different culture additives on fin disc morphology after cultivation for five days. Bar = 5 mm. a) Control media (top) vs. 154 nM T_3. b) 154 nM T_3 (top) vs. 0.5 mM cyclic AMP. c) 8 mM LiCl (top) vs. 8 mM LiCl + 154 nM T_3.

activity of hexosaminidase which reaches the 35 ± 4% level by five days (Table I. a,b). Discs cultured with 0.5 or 0.1 mM cyclic AMP undergo an increase in the specific activity of hexosaminidase of 23-25% in 4 days (Table I. c,d). That the magnitude of increase is the same after treatment with T_3 or cyclic AMP was demonstrated by culturing matched discs with either T_3 or cyclic AMP for 5 days. As shown by Table I. e, the specific activities differ by only 3 ± 3%. Therefore the magnitude of the stimulation is essentially the same. Although the morphological changes accompanying treatment cannot be quantitated precisely by simple visual inspection, Fig. 1b shows that T_3 (top) and cyclic AMP (bottom) appear to produce comparable morphological changes.

If increased levels of cyclic AMP in fin tissue do mediate some aspects of T_3-stimulated metamorphosis, then interference with the production of cyclic AMP should inhibit metamorphosis. At a concentration of 8 mM, LiCl inhibits 89 ± 15% of the elevation of hexosaminidase activity normally induced by T_3; this is shown in Table I. b,f. In addition, the regression characteristic of T_3 treat-

Table I

In vitro Effects of T_3, LiCl and Cyclic AMP on Tadpole Tail Fin Discs

Cutlure		Time (Days)	Tissue Regression (1)	% Change in Hexosaminidase Activity
a) 154 nM T_3	vs. control	3	-	6 ± 2%
b) 154 nM T_3	vs. control	5	++	35 ± 4
c) 0.5 mM cyclic AMP	vs. control	4	+	23 ± 5
d) 0.1 mM cyclic AMP	vs. control	4	+	25 ± 3
e) 154 nM T_3	vs. 0.5 mM cyclic AMP	5	++(2)	-3 ± 2
f) 8 mM LiCl + 154 nM T_3	vs. control	5	-	4 ± 3
g) 6 mM LiCl + 154 nM T_3	vs. control	5	-	11 ± 4
h) 8 mM LiCl	vs. control	5	-	3 ± 3
i) 8 mM LiCl + 154 nM T_3 + 0.5 mM cyclic AMP	vs. control	5	++	39 ± 3

(1) Regression was scored as follows: ++ > 35% decrease in area; + 20-30% decrease in area;-little or no regression.

(2) There is no difference in regression for T_3 vs. cyclic AMP; both show shrinkage and darkening of the tissue.

ment also is inhibited as shown by Fig. 1c (bottom vs 1a bottom or 1b top). As shown in Table I. h, 8 mM LiCl, alone does not alter the specific activity of hexosaminidase in cultured discs nor does it cause morphological changes suggestive of toxicity (Fig. 1c [top]). At a concentration of 6 mM, LiCl inhibits only 69 ± 20% of the hexosaminidase increase (Table I. g). In another experiment, 0.5 mM cyclic AMP was shown to overcome the LiCl inhibition, i.e., the hexosaminidase activity of discs cultured in cyclic AMP + LiCl + T_3 showed an increase of 39 ± 3% in five days, a change comparable to the 35 ± 4% characteristic of T_3 treatment alone (see Table I. b,i).

The in vitro experiments presented above strongly implicate increases in cyclic AMP as a factor in T_3-stimulated fin regression. If cyclic AMP were to mediate enzymatic and morphological changes, its level would increase after hor-

mone treatment of the tissue. The amount of cyclic AMP was determined in aliquots from six different preparations of control and experimental cultures; levels ranged from 3.7 to 7.7 f mole/μg protein. Culturing discs for 24 hours with T_3 (154 nM) resulted in a 110 ± 10% stimulation in the level of cyclic AMP as compared with matched but untreated discs cultured for the same period. After four days *in vitro* with T_3 (154 nM), the level of cyclic AMP in treated tissue was only 51 ± 15% higher than that of untreated, matched controls. It is thus evident that T_3 treatment of the tissue is associated with an early and dramatic increase in the level of cyclic AMP.

DISCUSSION: The experiments presented above on the stimulation and inhibition of fin regression provide data which strongly implicate increases in the level of cyclic AMP as a factor in the initiation of these events by T_3. In the first set of experiments, the results demonstrate that cyclic AMP can stimulate increases in hexosaminidase activity which are comparable to those initiated by treatment with T_3 (Table I. e). These changes produce a level of activity significantly above the levels in matched, control tissue ($p > 99.9\%$). In addition, the morphology of the tissues treated with either substance is strikingly similar (Fig. 1b) although the precise degree of regression and of tissue darkening have not been quantitated.

Results from the second group of *in vitro* experiments also suggest cyclic AMP may be involved in T_3-stimulated regression. In these experiments, the presence of 8 mM LiCl inhibited the enzymatic increases and morphological changes characteristic of T_3 treatment without altering the base level of enzyme activity or the morphology (Table I. h; Fig. 1c bottom vs 1a, bottom). Furthermore, the results show that the addition of cyclic AMP to cultures containing LiCl overcomes the inhibition and characteristic increases in enzyme activity occur (Table I. i). If LiCl acts in amphibians as it does in mammals, then our findings suggest T_3 may be stimulating the adenylate cyclase system of fin tissue.

A mediation of T_3 stimulation by cyclic AMP should produce an increase in

cyclic AMP prior to changes in enzyme activity and tissue morphology. Our results from radioimmunoassays show such an increase. After only 24 hours of treatment with the hormone, there is a significant increase in the level of cyclic AMP in the treated tissues as compared with matched controls.

Studies of other systems provide evidence which support the suggestion that some effects of T_3 may be mediated by increases in cyclic AMP. In mammalian systems, for example, investigators (3,4) have shown that T_3 and thyroxine both can increase the levels of cyclic AMP by stimulating adenyl cyclase activity. In addition, increasing the levels of cyclic AMP *in vitro* can stimulate other tissues to undergo specific courses of differentiation (9,17). The precise mechanisms by which altered levels of cyclic AMP initiate different tissue responses still requires considerable clarification but the present studies suggest that the role of cyclic AMP in development should be further investigated.

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