ACTIVATION OF MONKEY SPERMATOZOAL ADENYL CYCLASE BY THYROXINE AND TRIIODOTHYRONINE

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SUMMARY. Thyroxine and triiodothyronine (T_3) stimulated the conversion of ATP to 3',5'-AMP (cAMP) by ejaculated rhesus monkey (Macaca mulatta) spermatozoa. Compounds structurally related to thyroxine, other than T_3, had no effect on cAMP accumulation. The response to T_3 concentration was biphasic, with maximal stimulation occurring at a hormone level of 4 μM. The action of T_3 on cAMP accumulation appears to be a direct effect on adenyl cyclase since T_3, at concentrations employed in this study, had no effect on the breakdown of cAMP by intact spermatozoa. Anaerobic spermatozoal fructolysis was found to respond to varying concentrations of T_3 in a biphasic manner similar to that found for adenyl cyclase.

INTRODUCTION

Little is known about the influence of hormones on spermatozoa in vitro (1). Only those studies concerning the stimulatory effect of thyroxine on spermatozoa appear to be well documented (1). In 1932, Carter reported that the presence of thyroxine had no effect upon the oxygen consumption of sea urchin or rabbit sperm but prolonged the active life of the spermatozoon (2). Subsequent studies have described effects of thyroxine on sperm respiration (3-6), glycolysis (5), and on the fertility of stored spermatozoa (7). In addition, Sullivan and Menge have studied the active uptake of radioactive triiodothyronine by bull spermatozoa (8).

The role of cAMP as a second messenger in the mechanism of hormone action has been well documented for a variety of hormones (9). In recent studies, Levey has suggested that thyroxine activation of myocardial adenyl cyclase may be involved in the cardiac manifestation of the hyperthyroid state (10,11). We have recently reported the presence of adenyl cyclase in the monkey spermatozoon (12) and Gray, Hardman, Bibring and Sutherland (13) have measured the activity of this enzyme in sea urchin spermatozoa. The present report describes the activation of monkey sperm adenyl cyclase by thyroxine and 3,3',5- L-triiodothyronine and represents the first documentation of an hormonal

activation of this enzyme in mammalian spermatozoa. Evidence for one possible consequence of this activation, enhancement of anaerobic fructolysis, is also presented.

METHODS

Semen was collected from 18 rhesus monkeys (Macaca mulatta) by electroejaculation as previously described (14). Combined sperm-rich exudates from several coagulated ejaculates were suspended in three volumes of a washing media consisting of 20 mM NaH_2PO_4 adjusted to pH 7.0, 119 mM NaCl, 5 mM KCl, and 2 mM $MgCl_2$. Following thorough mixing, the cells were collected by centrifugation and washed twice with the same media. Cells were subsequently washed once with, and suspended in, a solution of the following composition: 40 mM Tris-HCl, pH 7.3; 20 mM KCl; 15 mM theophylline; and 6 mM $MgSO_4$.

Adenyl cyclase was measured by slight modification of recently described procedures (15-17). Each assay tube contained 75 µl of cell suspension, 25 µmoles phosphoenolpyruvate, 7 I.U. pyruvate kinase, 10 µl hormone solution as indicated, and 0.1 µmoles ATP (1-2 µC ^{14}C-ATP or 10 µC ^{3}H-ATP) in a total volume of 110 µl. Control reactions contained, in place of hormone solution, 10 µl of a solution prepared according to the procedure used to prepare the hormone solution. Reagent blank reactions contained an appropriate amount of cell-suspending media or boiled sperm suspension. Under these conditions cAMP accumulation was linear with time for at least 20 min and with sperm concentration between the limits of 2.5×10^7 and 4.0×10^8 cells per tube. Reactions were terminated after 20 min incubation at 30° by addition of 100 µl of a carrier solution containing 20 mM cAMP, 20 mM ATP, and 20 mM 5'AMP followed by immersion in a boiling water bath for two minutes. After addition of 200 µl of water, each sample was mixed and centrifuged at 20,000 x g for five minutes. Supernatant solutions were applied to Dowex-50 columns (0.5 x 5 cm) and eluted with water. The fraction eluting between 4.5 and 7.5 ml was collected and treated with $ZnSO_4$ and $Ba(OH)_2$ as described by Rodbell (18). Cyclic AMP was identified by standard chromatographic techniques as the major radioactive product. Radioactivity in the $BaSO_4$ supernatants was measured by liquid scintillation using Beckman Biosolve III to solubilize the aqueous samples in scintillation cocktail. Recovery of cAMP, as measured with ^{3}H-cAMP, averaged 35-40%.

Analyses were carried out in duplicate. Variation between duplicate determinations was less than 15%.

Cyclic AMP phosphodiesterase activity was measured by following the disappearance of ^{3}H-cAMP from incubation mixtures containing cells and this nucleotide. Conditions for assay, reaction termination, and preparation of cells were the same as those noted above except for replacement of ^{14}C-ATP with ^{3}H-cAMP and omission of theophylline. Supernatant solutions obtained following centrifugation were adjusted to 3.0 ml with water and labeled nucleotides other than cAMP were removed by $BaSO_4$ precipitation. Radioactivity was measured as described above.

Fructolysis was measured as described by Hoskins and Patterson (19). Respiration was inhibited by addition of 2-N-heptyl-4-hydroxyquinoline N-oxide (1 μM) as previously described (19). Oxygen consumption was measured manometrically at 37° using a conventional Warburg apparatus.

RESULTS AND DISCUSSION

General Properties of Monkey Spermatozoal Adenyl Cyclase In the absence of added hormone, rates of adenyl cyclase activity in intact monkey spermatozoa were, in the main, between 2.2 and 3.6 pmoles cAMP formed/min/10^9 spermatozoa. The accumulation of cAMP was stimulated approximately two-fold by 10 mM theophylline and caffeine but was unaffected by 10 mM fluoride. Over 95% of the total cellular adenyl cyclase activity remained associated with the particulate fraction obtained following intense sonication for two minutes and subsequent centrifugation at 25,000 g for ten minutes.

Hormonal Activation Numerous reports have appeared on the effects of thyroxine on viability, respiration, and glycolysis of invertebrate or mammalian spermatozoa (2-7). The effects of L-thyroxine at levels of 0.13 and 1.3 μM on the accumulation of cAMP in intact monkey spermatozoa are shown in Table I. Low, but consistent, stimulations of approximately 50% are noted at the higher level of hormone tested. The effects of compounds structurally related to thyroxine on monkey spermatozoal adenyl cyclase are shown in Table II. Degrees of stimulation by thyroxine and triiodothyronine are comparable to those reported by Levey and Epstein for myocardial adenyl cyclase (10,11). In contrast to their results, however, D-thyroxine had no stimulatory effect on spermatozoal adenyl

Table I

The effect of thyroxine on cAMP accumulation in intact spermatozoa

Addition	cAMP accumulation (pmole/10^9 cells/20min)	Relative Rate
None	57.5 ± 3.0	1.00
Thyroxine (0.13 μM)	69.6 ± 7.1	1.21
Thyroxine (1.3 μM)	88.5 ± 6.9	1.54

Table II

Effect of compounds structurally related to thyroxine on spermatozoal adenyl cyclase

Compound added	Relative Rate
None	1.00
3,3',5, triiodo-L-thyronine	1.90
3,5, diiodo-L-thyronine	1.11
D-thyroxine	0.62
L-thyronine	0.94
3,5, diiodotyrosine	1.22
3, iodotyrosine	0.86

Cyclic AMP accumulation in the absence of added compound was determined in each experiment and the results are expressed relative to this control. Compounds were present in the incubation mixture at a concentration of 1 μg/ml.

cyclase. The most pronounced stimulation of activity, approximately two-fold, was observed with 3,3',5-triiodothyronine. Figure 1, curve A, shows the effect of varying T_3 concentrations on enzyme activity. Hormonal response is seen to be biphasic in nature with maximum stimulation occurring at levels between 1.5 and 7.5 μM. Higher concentrations (15 μM) showed little stimulatory effect.

Since T_3, at much higher concentrations than employed in this study, has been reported to inhibit cAMP-phosphodiesterase (20), the activity of this enzyme in intact sperm cells was measured in the presence and absence of T_3 and caffeine. As shown in Table III, the addition of 10 mM caffeine, a known inhibitor of phosphodiesterase activity, effects a

Table III

The effect of caffeine and T_3 on cAMP phosphodiesterase activity

Contents	^{3}H-cAMP present (cpm/1.5 ml $BaSO_4$ supnt.)	^{3}H-cAMP removed (cpm)	Relative Rate
no sperm	12789 ± 206		
sperm added	2173 ± 436	10616	1.00
sperm + caffeine	8740 ± 30	4049	0.38
sperm + T_3	2779 ± 292	10010	0.94
sperm + T_3 + caffeine	9209 ± 50	3580	0.34

Activity measured as described in the methods in the presence of an ATP-regenerating system. Concentrations of various components are as follows: cAMP, 1.0 mM; cells, 1.72×10^9/tube; caffeine, 10 mM; triiodothyronine, 1.5 μM. Results shown are the average of duplicate determinations.

pronounced inhibition of enzyme activity while 1.5 μM T_3 had no significant effect. It is of interest that, in the presence of inhibitor, the apparent phosphodiesterase activity, 20 nmoles/min/10^9 cells, was several orders of magnitude greater than that of adenyl cyclase. Dowex-50 chromatography of a phosphodiesterase assay mixture, in the presence of theophylline, followed by paper chromatography of the concentrated fractions, indicated that ATP was the predominant radioactive product. Apparently, this result was due to the presence of the ATP-regenerating system in the incubation mixture. When the phosphodiesterase assay was repeated in the absence of the regenerating system, enzyme activity was decreased by a factor of ten. A possible explanation for these results is that the product of the phosphodiesterase reaction, 5'-AMP, is rapidly converted to ATP in the presence of the ATP-regenerating system. Presumably, rapid removal of 5'-AMP enhances the phosphodiesterase activity. In light of these results, the effect of various T_3 concentrations on spermatozoal adenyl cyclase was reinvestigated in the absence of the ATP-regenerating system (Figure 1, curve B). In these experiments, at least 3 mM ATP was necessary in order to produce linear results over the 20 min incubation period. Dose-response curves in the presence and absence of an ATP-regenerating system are seen to be qualitatively similar; maximal stimulation with T_3, however, is about two-fold greater in

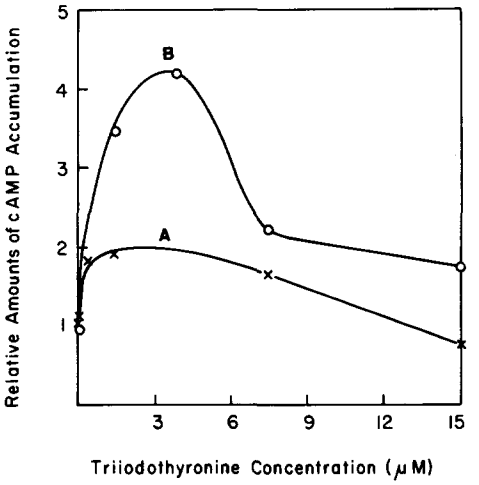

Figure 1. The effect of triiodothyronine concentration on cAMP accumulation by intact spermatozoa. Results are expressed relative to the amount of cAMP accumulated in the absence of added T_3. Curve A represents results obtained in presence of an ATP-regenerating system. Curve B, those obtained in absence of the regenerating system.

the latter case. Maximum stimulation of adenyl cyclase activity, four-fold greater than controls, occurs at a level of 3.8 μM T_3.

Effect of Triiodothyronine on Fructolysis and Respiration The effect of various concentrations of T_3 upon anaerobic fructolysis of washed monkey spermatozoa are shown in Figure 2. It is noteworthy that the dose-response curve for fructolysis parallels closely the curve obtained for adenyl cyclase (Figure 1). Maximal stimulation (33%) was obtained with 7.5 μM T_3. In similar studies, in the absence of quinoline N-oxide, T_3 was without effect. The effect of T_3 on the respiration of washed monkey spermatozoa was also studied. No effect of 3.8 μM T_3 was noted on endogenous respiration during the first 30 min of incubation, but a slight inhibition (10-15%) was observed during the next 2 hours. No significant effect of T_3 on the respiration of cells incubated in the presence of 10 mM fructose was observed. These results are similar to those reported by Lardy and Philips (5) who have reported a slight enhancement of the respiration of washed bull spermatozoa.

The results presented in this paper demonstrate the activation of monkey spermatozoal adenyl cyclase by thyroxine and triiodothyronine. The failure of adenyl cyclase to respond maximally to high levels (15 μM) of T_3 is inexplicable at present. It is of interest,

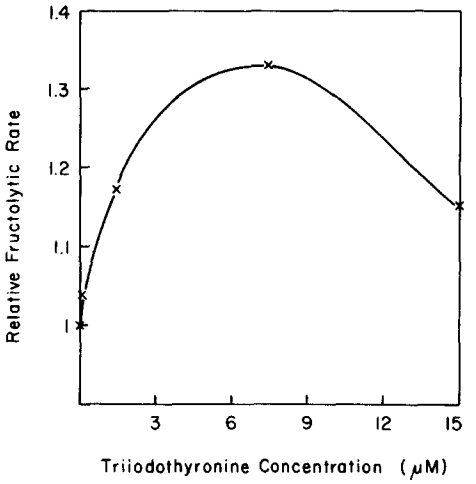

Figure 2. The effect of triiodothyronine concentration on anaerobic fructolysis of washed spermatozoa. Respiration was inhibited by 1 μM 2-N-heptyl-4-hydroxyquinoline N-oxide. Results are expressed relative to the fructolytic rate found in the absence of T_3.

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however, that rates of spermatozoal fructolysis similarly fail to respond to this same level of hormone. Previous reports from this laboratory have demonstrated a potent activating effect of cAMP on partially purified monkey spermatozoal phosphofructokinase (12) and on the conversion of fructose-6-P to lactate by cell-free extracts (21). In view of these findings, it is interesting to speculate that the observed effectsof triiodothyronine on sperm fructolysis may occur via a direct effect of cAMP on spermatozoal phospho-fructokinase.

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