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EFFECTS OF CHOLERA TOXIN AND 5'-GUANYLYLIMIDODIPHOSPHATE ON HUMAN SPERMATOZOAL ADENYLATE CYCLASE ACTIVITY

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SUMMARY

The effects of cholera toxin and 5'-guanylylimidodiphosphate (Gpp(NH)p) on human spermatozoal adenylate cyclase activity were tested. Cholera toxin had no demonstrable effect on adenylate cyclase activity in human spermatozoa at concentrations between 5 and 20 µg/ml, whether the toxin was preincubated with intact spermatozoa between 5 min and 5 h prior the adenylate cyclase assay, or was added to lysed spermatozoa, where the adenylate cyclase would be accessible to the toxin. In contrast, Gpp(NH)p at concentrations between 10 and 100 µM was effective in activating human spermatozoal adenylate cyclase activity.

INTRODUCTION

The presence of adenylate cyclase (ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1.) in human spermatozoa was first demonstrated in 1971 (1,2). A search for specific hormonal activators of adenylate cyclase has been carried out with spermatozoa from the monkey (3,4), bull (5) and man (1). The attempts can be judged to have been only marginally successful (6). Mammalian spermatozoal adenylate cyclases seem to be insensitive to steroid hormones (for review, see 6-8). However, monkey spermatozoal adenylate cyclase can be activated by thyroxine and triiodothyronine (3,4), but such activation cannot be demonstrated in bull spermatozoa (5). It has been demonstrated that adenylate cyclase from ejaculated human spermatozoa is inhibited by sodium fluoride, a number of metallic ions and carboxylic acids (9), but this could not be demonstrated in spermatozoa of a number of other species (10,11). This report presents results of studies on the effects of cholera toxin and 5'-guanylylimidodiphosphate (Gpp(NH)p)* on

Abbreviations: Gpp(NH)p, 5'-guanylylimidodiphosphate; KRP, Krebs-Ringer-Phosphate buffer (Ca^{++}-free).

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associated constituents induced by numerous stimuli. Nevertheless, by utilizing a secretagogue such as PMA, whose mode of action is independent of extracellular calcium, we are able to employ an agent such as TMB-8 as a pharmacologic tool which will aid in the elucidation of the true relevance of calcium to the granule enzyme secretory process which heretofore has not been possible.

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The activity of adenylate cyclase was measured by Method C (in "Note Added in Proof") of Salomon et al. (19). This is a labelled substrate assay method, based on the conversion of [α^{32}P]-ATP to [α^{32}P]-cAMP by adenylate cyclase, with isolation of [α^{32}P]-cAMP by passing the reaction product through Dowex cation-exchange and alumina columns. Each assay tube contained 50 µl of either erythrocyte or sperm suspension which had (test) or had not (control) been treated with cholera toxin for various incubation periods; 50 mM Tris-HCl, pH 7.4; 10 mM KCl; 3 x 10^6 cpm [α^{32}P]-ATP (20-26.3 Ci/mmole), (The Radiochemical Centre, Amersham, England); 1 mM ATP; 1 mM cAMP (A grade, Calbiochem); 6 mM $MgCl_2$; 5 mM 2-phosphoenolpyruvate (A grade, Calbiochem), 2 units pyruvate kinase (600 I.U./ml, A grade, Calbiochem) and 10 mM caffeine (Sigma), in a total volume of 100 µl. Reaction mixtures were incubated at 37°C for 20-30 min in an oscillating water bath (100 rpm, Paton Industries Pty. Ltd., South Australia). The reaction was stopped by the addition of 100 µl of stopping solution, which contained 40 mM ATP, 1.4 mM cAMP, 2% sodium dodecyl sulfate at pH 7.5. ^{3}H-cAMP (25,000 cpm) (30 Ci/mmole from Radiochemical Centre, Amersham) in 50 µl was added, to determine the efficiency of recovery of cAMP. The total 250 µl volume was placed in a boiling water bath for 1.5 min. After cooling, 0.8 ml of distilled water was added to each tube and the sample was then applied to the columns for isolation of [α^{32}P]-cAMP. It was found that if the lysed or homogenized cells were sedimented (centrifuged at 20,000 g for 10 min), and only the supernatant, where more than 95% of [α^{32}P]-cAMP was present, was allowed to pass through the columns, the flow rate was better. Eluates from the Dowex cation-exchange resin and alumina columns were then collected into scintillation vials, each containing 10 ml Insta-gel, and were counted in a Packard Tri-Carb (Model 3255) Scintillation Counter.

Experiment B. 1 ml of washed human spermatozoa were sonicated, as stated above, and 50 µl aliquots were added to separate glass test-tubes and incubated at 37°C. The standard assay mixture, as used above, and 10-100 µM Gpp(NH)p (test) or the same amount of distilled water (control) was added to each tube in a final reaction volume of 100 µl. The tubes were then incubated at 37°C in an oscillating water bath (100 rpm) for between 1 and 30 min. The subsequent procedures for the measurement of [α^{32}P]-cAMP formed from [α^{32}P]-ATP by the adenylate cyclase were the same as described above.

RESULTS

In the absence of cholera toxin and Gpp(NH)p, the specific activities found for adenylate cyclase in washed human spermatozoa which had been sonicated, or freezed-thawed, ranged between 102 and 114 pmoles/10^8 sperm/20 min in 20 determinations from 8 different experiments. If 10 mM caffeine (a phosphodiesterase inhibitor) was excluded from the assay mixture, the rate of cAMP production was between 24.8 and 37.05 pmoles/10^8 sperm/20 min. It was found that adenylate cyclase activity decayed rapidly when spermatozoa were incubated at 37°C (Fig. 1). The recovery of cAMP from the Dowex and alumina columns was between 48% and 58.8%.

Where cholera toxin was incubated with intact human spermatozoa for between 5 min and 5 h at 20 µg/ml, the toxin was then removed and the spermatozoa sonicated, and the rate of cAMP formation assayed, no demonstrable effect on adenylate cyclase activity was observed (Fig. 1). In some experiments,

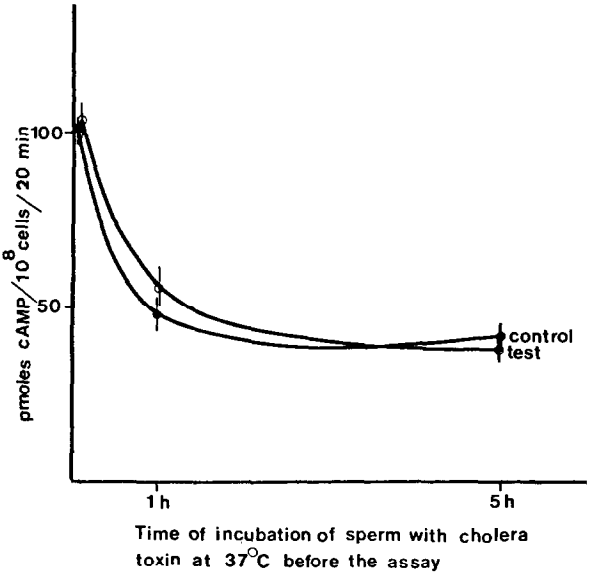

Fig. 1. Adenylate cyclase activity of human spermatozoa (1 x 10^8 cells/ml) incubated with cholera toxin (20 µg/ml). Washed human spermatozoa were incubated either with cholera toxin, 5 mM ATP, 1 mM DTT and 1 mM NAD (○—○) or buffer without toxin, 5 mM ATP, 1 mM DTT and 1 mM NAD (●—●) at 37°C for between 5 min and 5 h. After each incubation period, 1.5 ml ice-cold KRP was added to each assay tube, the cells were sedimented and were subjected to sonication. Adenylate cyclase activity was then assayed by the addition of standard assay mixture for 20 min at 37°C. Note the decrease of adenylate cyclase activity at 37°C. All points are Mean±SEM of at least 6 determinations from 3 different experiments, where pooled semen samples from different donors were used.

there was no attempt to remove the cholera toxin from spermatozoa after it had been incubated with the sperm, and so it could be in contact with the adenylate cyclase of the disrupted spermatozoa during the 20 min cAMP formation assay. But, the adenylate cyclase activity still showed no response to cholera toxin (Table 1a). However, adenylate cyclase activity of pigeon erythrocytes was susceptible to cholera toxin (Table 1b). With both 5 min and 1 h preincubation of erythrocytes with cholera toxin, the basal activity of adenylate cyclase increased by about 400%, but similar treatment in human spermatozoa did not elicit any change in adenylate cyclase activity (Table 1a,b). The basal activity of adenylate cyclase in pigeon erythrocytes was found between 1.24 and 1.56 pmoles of cAMP formed/ µl of cells/h, comparable with the results of 1-3 pmoles of cAMP formed/µl of cells/h reported by Gill and King (20).

Table 1. The activity of adenylate cyclase in human spermatozoa (a) and pigeon erythrocytes (b) after incubation at 37°C with cholera toxin (5 µg/ml).*

Time of incubation before cell lysis and adenylate cyclase assay	Adenylate cyclase activity	
a.	pmoles of cAMP formed/10^9 cells/20 min	
	Without Toxin (Control)	With Cholera Toxin (Test)
5 min	109.90±6.22	101.44±7.11
1 h	43.45±3.11	42.28±2.41
b.	pmoles of cAMP formed/50 µl of cells/20 min	
	Without Toxin (Control)	With Cholera Toxin (Test)
5 min	26.00±2.11	85.16±2.17
1 h	20.72±2.62	86.95±3.41

*After incubation, the cells were lysed by incubating at -70°C for 3 min and thawing at 37°C and the rate of [α^{32}P]-cAMP synthesized was assayed. During the 20 min assay period, cholera toxin, presumably, could contact the adenylate cyclase. All data are Mean±SEM of 8 determinations.

Gpp(NH)p at a concentration of 10 µM caused stimulation of adenylate cyclase activity in human spermatozoa. This stimulation was found to be time-dependent. The adenylate cyclase activity of sperm after incubation with Gpp(NH)p for 1 min was found not be any greater than that where no Gpp(NH)p was added but, incubation for 5 min or more was found to produce increasing adenylate cyclase activity (Table 2). After sperm were incubated with Gpp(NH)p for 20 min at 37°C in the standard assay mixture, the basal activity of adenylate cyclase was increased by 25%, but after a period of 30 min at 37°C, the increase in basal activity was about 150% (Fig. 2). Where Gpp(NH)p was used at a concent-

Table 2. Adenylate cyclase activity of human spermatozoa after incubation with Gpp(NH)p for 20 min at 37°C.*

Additions	Adenylate cyclase activity (pmoles of cAMP formed/20 min/10^9 sperm)
None	106.66±8.71
Gpp(NH)p, 10 µM	133.12±5.44
Gpp(NH)p, 100 µM	152.43±6.11

*Gpp(NH)p was included in the standard assay mixture and incubated with human spermatozoa for 20 min at 37°C. All data are Mean±SEM of 6 determinations.

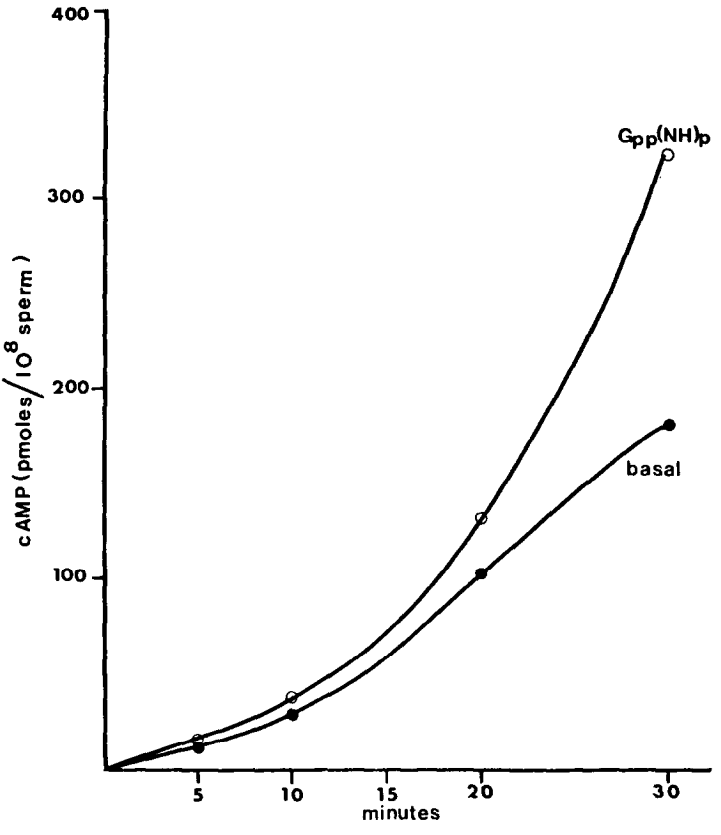

Fig. 2. Adenylate cyclase activity of human spermatozoa (1 x 10^8 cells/ml) incubated with Gpp(NH)p (10 μM). Washed human spermatozoa were sonicated, 50 μl was then incubated with standard assay mixture either including 10 μM Gpp(NH)p (O—O) or not (●—●), for between 1 and 30 min, during which [α^{32}P]-cAMP was synthesized from [α^{32}P]-ATP. All data reported here are results of 3 different experiments where pooled semen samples from different donors were used. Each experiment had at least 2 replicates.

ration of 100 μM, it was more effective in activating the basal activity of adenylate cyclase than it was at 10 μM during the 20 min assay period (Table 2).

DISCUSSION

It has been demonstrated that the adenylate cyclase activity of human spermatozoa is insensitive to a number of hormones including epinephrine, norepinephrine, isoproterenol, follicle-stimulating hormone, human chorionic gonadotrophin, luteinizing hormone, prostaglandin E_1, and insulin (1). The search for a specific hormonal activator of adenylate cyclase in other mammalian spermatozoa can be judged to be marginally successful (6-8). Therefore,

cholera toxin and Gpp(NH)p were used here to study their effects on adenylate cyclase activity in human spermatozoa.

Cholera toxin at the range of concentrations tested was unable to elicit any significant response of adenylate cyclase in human spermatozoa. This lack of response may indicate that spermatozoa lack surface receptors for cholera toxin, of which there are about 30 per pigeon erythrocyte, and to which the toxin attaches by its B components before it can activate the underlying adenylate cyclase system (13, 20). It has also been reported that treatments that decrease the ability of toxin to bind to mouse thymus cells cause a concomitant reduction in the toxin's ability to activate cAMP production rate (21). However, in some experiments (Table 1a) where the spermatozoa were lysed and cholera toxin could presumably have direct access to the adenylate cyclase, it still did not produce any observable effect on the rate of cAMP production, though NAD, ATP and cytosol,which are considered to be essential for the action of cholera toxin (13), were present. The reason for the lack of response of human spermatozoal adenylate cyclase to cholera toxin remains unknown, though it seems likely to be due to the lack of a specific cytosol factor(s) in human spermatozoa since relatively little cytosol is present in spermatozoa.

However, adenylate cyclase of pigeon erythrocytes was found to be activated by the cholera toxin that was inactive with spermatozoa (Table 1a,b). It was also observed that the adenylate cyclase activity of the erythrocytes incubated with cholera toxin for 5 min before the cells were lysed and cAMP formation assayed, was not significantly different from where an incubation time of 1 h was used (Table 1b). However, in other studies it has been found that the amount of cAMP produced in erythrocytes increases with increasing time of incubation with cholera toxin (13) but, in those studies the cholera toxin was removed before the cells were lysed and cAMP was assayed. In the studies undertaken here the cholera toxin was not removed, and would have been able to contact the adenylate cyclase during the 20 min assay period. It has been reported (13) that the response of pigeon erythrocytes to cholera toxin is more rapid in conditions where the toxin can contact the adenylate cyclase.

The results reported here indicated that adenylate cyclase activity of human spermatozoa can be increased by Gpp(NH)p in the range of concentrations tested. It has also been reported recently (22) that the adenylate cyclase activity of bovine spermatozoa recovered from the caput epididymis was activated by Gpp(NH)p. The underlying mechanism for this activation is unknown, but it has been reported that Gpp(NH)p stimulates adenylate cyclase activity by a mechanism independent of receptor occupation, and that the response of adenylate cyclase to the nucleotide occurs by means of a time-dependent process and is dose-dependent (15), as has been found here.

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