Effects of epinephrine and hypotaurine on in-vitro fertilization in the golden hamster

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Summary. Using an experimental design in which the addition of hypotaurine or epinephrine was staggered through time, evidence was found that suggests these two compounds are working independently and sequentially to stimulate the fertilizing capacity of hamster spermatozoa *in vitro*. Prior exposure of spermatozoa to hypotaurine is a prerequisite for the action of epinephrine in causing activation and penetration of hamster ova. A definite role for hypotaurine in inducing capacitation of hamster spermatozoa is also demonstrated.

The α-adrenergic antagonist, phentolamine, was more effective in blocking fertilization of hamster ova *in vitro* than was propranolol, a β-antagonist. This indicates that catecholamines may be working by way of α-adrenergic receptors in causing capacitation of hamster spermatozoa. The failure to block fertilization with phentolamine after epinephrine has exerted its effect implies that epinephrine acts in a hormone-like fashion.

Introduction

For mammalian fertilization to occur spermatozoa must undergo a series of physiological and morphological alterations defined as capacitation and the acrosome reaction. In the golden hamster 3 factors controlling these events *in vitro* have been identified. A β-amino acid (taurine or hypotaurine) maintains hamster sperm motility and viability (Mrsny, Waxman & Meizel, 1979; Meizel, Lui, Working & Mrsny, 1980) but may also play a role in sperm capacitation (Meizel *et al.*, 1980; Leibfried & Bavister, 1981). Catecholamines may cause activation of sperm motility (Bavister, Chen & Fu, 1979), a process closely identified with capacitation (Yanagimachi, 1970). They may also cause induction of the acrosome reaction (Cornett & Meizel, 1978; Meizel & Working, 1980). Serum albumin allows the occurrence of the acrosome reaction (Lui, Cornett & Meizel, 1977; Bavister, 1981a) but may also aid capacitation (Meizel, 1978; Bavister, 1981b) and maintain sperm motility and viability (Meizel, 1978).

The presence of a β-amino acid, a catecholamine and serum albumin in an appropriate physiological saline with energy sources (Bavister & Yanagimachi, 1977) yields an almost completely defined system for the capacitation and fertilization of hamster gametes *in vitro* (Leibfried & Bavister, 1981). Beta-amino acids (Hamner, 1971; Meizel *et al.*, 1980) and serum albumin (Hamner, 1971; Stanke, Sikes, DeYoung & Tumbleson, 1974) are present in the oviducal fluid of several mammals and oviducal tissue contains catecholamines (Bodkhe & Harper, 1972). These factors may also be endogenous promoters of fertilization since they are found at the site of fertilization *in vivo*, the oviduct.

How these 3 agents interact in enabling hamster spermatozoa to penetrate ova is unclear. Analysis of the role of serum albumin in fertilization is not easily amenable to experimental

manipulation. Although a synthetic macromolecule, polyvinyl alcohol, can replace albumin in maintaining sperm viability and motility, serum albumin is still essential for the occurrence of acrosome reactions *in vitro* (Bavister, 1981b). The β-amino acids and catecholamines together stimulate the fertilizing ability of spermatozoa in the presence of serum albumin (Leibfried & Bavister, 1981), but individually are less effective in maintaining motility (Bavister *et al.*, 1979; Leibfried & Bavister, 1981), or causing activation and acrosome reactions (Cornett & Meizel, 1978; Mrsny *et al.*, 1979; Meizel *et al.*, 1980) and fertilization (Cornett, Bavister & Meizel, 1979; Leibfried & Bavister, 1981). The purpose of the present study was to examine further the interactions between hypotaurine and epinephrine in stimulating the fertilizing ability of hamster spermatozoa.

Materials and Methods

General

The methods used for obtaining fertilization *in vitro* were as previously described (Leibfried & Bavister, 1981). The only departure from these techniques was the use of (−)-epinephrine for the catecholamine. A stock solution containing (−)-epinephrine (25 μM), hypotaurine (250 μM), D-penicillamine (500 μM), sodium metabisulphite (500 μM) and sodium lactate (3 mM) dissolved in saline (9·21 g NaCl/l of ×3 distilled water) was prepared, divided into aliquants and frozen until just before use. When epinephrine or hypotaurine were to be administered separately, (−)-epinephrine stock solutions were made as described above with the elimination of hypotaurine. Stock solutions of hypotaurine (250 μM) were prepared in saline alone, divided into portions and frozen until required. Stocks of the catecholamine antagonists (250 μM), phentolamine and propranolol, dissolved in saline, were made shortly before use. All reagents mentioned in this paragraph and succeeding portions of 'Materials and Methods' were obtained from Sigma Chemical Co., St Louis, Missouri, except for phentolamine (CIBA Pharmaceutical Co., Summit, New Jersey).

Droplets of culture medium under paraffin oil in Petri dishes (Falcon Plastics, #1007) were used for sperm and egg incubations. The final volume of all droplets was 50 μl. To droplets of the appropriate size were added 2 μl of the hypotaurine and/or (−)-epinephrine stocks for final concentrations of 10 and 1 μM, respectively, and 1 to 4 μl of the antagonists to yield concentrations of 5, 10, 15 or 20 μM. Whenever the volume of additions varied between treatments in an experiment, droplets with fewer additions were supplemented with appropriate volumes of saline.

Hamster spermatozoa were recovered from each cauda epididymidis of adult males and inseminated into droplets at concentrations of approximately $1–4 \times 10^5$ spermatozoa/ml (Leibfried & Bavister, 1981). Ova were recovered from female golden hamsters that had received 25 i.u. PMSG on the day of the post-oestrous discharge followed 48–54 h later by 25 i.u. hCG. Ova surrounded by the cumulus oophorus were removed from oviducts 15–17 h after the hCG and placed into medium containing 1 mg hyaluronidase/3 ml and 0·01 mg soybean trypsin inhibitor/ml. After cumulus removal was complete (a period of 10–15 min), ova were recovered, pooled from all animals, rinsed 3 times in medium and added to treatment droplets containing spermatozoa. The medium used for recovery and rinsing of ova was similar to the modified Tyrode's used for culture except that the concentration of $NaHCO_3$ was reduced to 2 mM and Hepes buffer (10 mM) was added. The pH of this medium was 7·4 in air at 20–25°C.

After sperm/ova cultures had incubated for 1½–2 h, 25 μl 10% neutral buffered formalin were added to the droplets to halt further development. Ova were removed from the culture and agitated in a pipette to dislodge excess spermatozoa. They were then transferred to microscope slides and compressed slightly with coverslips ringed with petroleum jelly for microscopic examination. Ova were classified as having: (a) spermatozoa in the perivitelline space; (b)

decondensing sperm heads in the vitellus; and (c) male pronucleus, female pronucleus, two polar bodies and a sperm tail in the vitellus. Proportions of ova found in the 3 stages of fertilization never differed between treatments within an experiment or between experiments. For the purpose of this paper ova were scored as penetrated if observed in any of the 3 stages of fertilization. The majority of penetrated ova had decondensing or pronuclear spermatozoa in the vitellus (see footnotes, Tables 2, 3 and 4).

Experimental designs

Experiments 1 and 2. Experiment 1 was designed to determine the time course of effects exerted by epinephrine and hypotaurine and whether they have sequential or synergistic effects. Seven treatment groups were utilized. One contained both epinephrine and hypotaurine throughout the incubation period, 3 groups contained hypotaurine throughout and 3 groups contained epinephrine throughout the period of culture. At 2 and 4 h after the start of sperm incubation, epinephrine or hypotaurine was added to groups lacking these compounds at the start of sperm incubation. Two groups received only epinephrine or hypotaurine during the period of culture. At 2, 4 and 6 h of incubation, the percentage of motile spermatozoa was estimated. Activation (spermatozoa demonstrating the whiplash movement characteristic of capacitated hamster spermatozoa) was scored as 0 (no spermatozoa), 1 ($<50\%$ motile spermatozoa activated) or 2 (most motile spermatozoa activated). Triplicate observations were made for each treatment within a replicate. Replication was over days using a new male each time ($r = 6$).

The design of Exp. 2 was similar to that of Exp. 1 except that the percentage of ova fertilized *in vitro* by spermatozoa was the endpoint observed. Ova were added to incubations of spermatozoa 6 h after the beginning of culture and removed $1\frac{1}{2}$-2 h after exposure to spermatozoa. Again replication was over days ($r = 6$).

Experiment 3. The dose–response of spermatozoa to phentolamine and propranolol was examined. Four concentrations (5, 10, 15 and 20 μM) were tested for both antagonists. Ova were added to incubations of spermatozoa 4 h after the beginning of culture and examined for stage of fertilization $1\frac{1}{2}$-2 h later. All treatments contained epinephrine and hypotaurine. The endpoint used for statistical analysis was the percentage of ova penetrated. Replication was done over days ($r = 6$).

Experiment 4. This experiment was designed to determine when epinephrine becomes necessary in causing sperm activation and capacitation and if it is acting as a hormone-like stimulus. Epinephrine and hypotaurine were present in all treatments. Phentolamine (15 μM) was added to different groups at 0, 1, 2, 3 and 4 h of sperm incubation. Ova were placed with spermatozoa after 5 h of culture and removed $1\frac{1}{2}$-2 h later and examined for penetration. Replication was over days ($r = 7$).

Statistical analysis

An analysis of variance for 1-way classifications was used to analyse all experimental data. In Exp. 1, individual analyses were performed for each of the 3 observation times. Treatments with no variation were not included in the analyses. When treatment effects were significant, Duncan's multiple range test was used to determine which treatment means were different. Both the 1-way ANOVAs and Duncan's tests were done using the SAS computing programme (Barr, Goodnight, Sall & Helwig, 1976).

Results

Experiments 1 and 2

Hypotaurine and epinephrine had sequential effects on capacitation of hamster spermatozoa. When only epinephrine was present at the beginning of sperm incubation, motility decreased to

Table 1. Effect of adding hypotaurine and epinephrine after the beginning of incubation on motility and activation of hamster spermatozoa

| | 2 hours | 4 hours | | 6 hours | |
| | | | | | |
Treatment*	% Motility†	% Motility†	Activation‡	% Motility†	Activation‡
Ht + Epi	^{a}85·2 ± 2·5	^{a}64·0 ± 3·0	^{a}1·08 ± 0·08	^{a}50·5 ± 4·7	^{a}1·34 ± 0·22
Ht + Epi at 2 h	^{b}71·8 ± 4·3	^{a}65·0 ± 4·4	^{a}1·29 ± 0·17	^{a,b}43·3 ± 5·4	^{a}1·19 ± 0·13
Ht + Epi at 4 h	^{b}69·3 ± 5·2	^{b}43·0 ± 10·0	^{b}0·29 ± 0·19	^{b}35·2 ± 8·0	^{a}0·93 ± 0·16
Ht only	^{b}68·0 ± 5·7	^{b}36·8 ± 11·1	^{b}0·33 ± 0·12	^{c}11·2 ± 3·4	^{b}0·04 ± 0·04
Epi + Ht at 2 h	^{c}4·0 ± 2·0	^{c}11·3 ± 3·7	^{b}0·13 ± 0·13	^{c}6·5 ± 1·2	^{b}0·13 ± 0·13
Epi + Ht at 4 h	^{c}3·8 ± 2·2	0	—	0	—
Epi only	^{c}4·0 ± 2·0	0	—	0	—

All means (±s.e.m.) are based on 6 replicates, each replicate containing triplicate observations per treatment.

* Ht = hypotaurine (10 μM). Epi = (−)-epinephrine (1 μM). These agents were present throughout the period of sperm incubation unless stated otherwise.

† Mean percentage of motile spermatozoa present at the time of observation. Treatment means with different superscripts are different ($P < 0.05$) within each time.

‡ Subjective estimate of the number of spermatozoa demonstrating the whiplash flagellar movement of capacitated hamster spermatozoa (see text). Treatment means with different superscripts are different ($P < 0.05$) within each time.

low levels after 2 h and could not be restored by adding hypotaurine at 2 or 4 h of culture (Table 1). When hypotaurine was added after 2 h of culture, activation was sometimes seen in the small percentage of spermatozoa that remained motile (Table 1) but penetration of ova did not occur (Table 2). Hypotaurine did not merely maintain sperm motility and viability. After 2 or 4 h of incubation, if epinephrine was added to sperm cultures containing hypotaurine, by the next 2 h observation period activation was observed and was comparable to treatments receiving epinephrine 2 h earlier (Table 1). This is shorter than the normal 3–4 h needed for the capacitation of hamster spermatozoa *in vitro* (Yanagimachi, 1969). The ability of hamster spermatozoa to penetrate ova was equal when epinephrine was added to sperm cultures containing hypotaurine at 0, 2 or 4 h of incubation (Table 2). Although not presented as separate data, the stage of fertilization reached during the time allotted for sperm/ova incubation did not

Table 2. Effect of adding hypotaurine or epinephrine after the start of sperm incubation on fertilization *in vitro*

Treatment*	Total no. of ova†	Means ± s.e.m. penetration (%)‡
Ht + Epi	165	^{a}71·7 ± 9·8
Ht + Epi at 2 h	153	^{a}69·9 ± 9·0
Ht + Epi at 4 h	150	^{a}52·8 ± 12·3
Ht only	168	^{b}6·6 ± 4·6
Epi + Ht at 2 h	156	0

* Ht = hypotaurine (10 μM). Epi = (−)-epinephrine (1 μM). Agents were present throughout the period of sperm incubation unless stated otherwise.

† Incubated with spermatozoa over 6 replicates. Ova added 6 h after the start of sperm incubation.

‡ An ovum was considered penetrated if spermatozoa were found in the perivitelline space or at any subsequent stage of fertilization. Vitelline spermatozoa were found in 75% of penetrated ova. Treatment means with different superscripts are different ($P < 0.05$).

Table 3. Effect of dose of phentolamine or propranolol on hamster fertilization *in vitro*

Catecholamine antagonist	Dose (μM)	Total no. of ova*	Mean ± s.e.m. penetration (%)†
None	—	101	[a]93·2 ± 2·8
Propranolol	5	103	[a]84·4 ± 10·6
	10	105	[a]83·5 ± 6·1
	15	106	[a,b]70·8 ± 8·3
	20	99	[b]57·1 ± 14·5
Phentolamine	5	107	[b,c]50·3 ± 14·0
	10	103	[c,d]31·8 ± 15·4
	15	103	[d]13·0 ± 13·0
	20	103	0

* Incubated with spermatozoa over 6 replicates. Ova added 4 h after the start of sperm incubation.

† An ovum was considered penetrated if spermatozoa were found in the perivitelline space or at any subsequent stage of fertilization. Vitelline spermatozoa were found in 86%, of penetrated ova. Treatment means with different superscripts are different ($P < 0.05$).

differ between treatments in this experiment or subsequent experiments. This implies that penetration of ova by spermatozoa began at essentially the same time across treatments.

Experiment 3

At all 4 concentrations phentolamine was more effective in blocking penetration of ova by hamster spermatozoa than was propranolol (Table 3). At 5 μM, phentolamine impaired the fertilizing ability of spermatozoa whereas propranolol had no effect until a concentration of 20 μM was reached. Phentolamine or propranolol at a concentration of 20 μM decreased the percentage of motile spermatozoa present by 3–4 h of incubation. A dose of 15 μM phentolamine was chosen for the next experiment since it effectively blocked fertilization (Table 3) without impairing sperm motility.

Experiment 4

Phentolamine was most effective in preventing fertilization *in vitro* when added within the first 3 h of sperm incubation (Table 4). Addition at later times (3 or 4 h of incubation) gave progressively less impairment of fertilization.

Discussion

Sequential roles for hypotaurine and epinephrine in stimulating the fertilizing ability of hamster spermatozoa were demonstrated in Exps 1 and 2. Prior exposure of spermatozoa to hypotaurine was a prerequisite for the action of epinephrine in causing activation of spermatozoa and penetration of ova. A definite role for hypotaurine in stimulating capacitation of spermatozoa was also demonstrated. If the addition of epinephrine to cultures of sperm containing hypotaurine was delayed for 2 or 4 h, activation was observed after incubation for a further 2 h. This is a shorter interval than the normal 3–4 h of incubation needed for activation of hamster spermatozoa *in vitro* (Yanagimachi, 1969).

Table 4. Effect of adding phentolamine at intervals after the start of sperm incubation on hamster fertilization *in vitro*

Time of addition* (h)	Total no. of ova†	Mean ± s.e.m. penetration (%)‡
No additions	179	[a]77·2 ± 4·6
0	162	[c]7·2 ± 6·3
1	163	[c]14·2 ± 11·9
2	176	[c]23·0 ± 13·0
3	177	[b]45·5 ± 10·0
4	173	[a]65·5 ± 12·0

* Time from beginning of incubation at which phentolamine (15 μM) was added.

† Incubated with spermatozoa over 7 replicates. Ova were added 5 h after the start of sperm incubation.

‡ An ovum was considered penetrated if spermatozoa were found in the perivitelline space or at any subsequent stage of fertilization. Vitelline spermatozoa were found in 80% of penetrated ova. Treatment means with different superscripts are different ($P < 0.05$).

In the presence of hypotaurine only, no significant level of activated motility was found, a result that is similar to previous work from our laboratory (Leibfried & Bavister, 1981) and that is compatible with earlier work using adrenal extracts of the sperm motility factor (Bavister *et al.*, 1979), now known to be one of the β-amino acids. Cornett & Meizel (1978) also found that adrenal extracts of sperm motility factor were ineffective in inducing activation of hamster spermatozoa unless catecholamines were present. Later work from the same laboratory, and using hypotaurine rather than adrenal extracts as source of sperm motility factor, showed high levels of activated motility even without catecholamines (Meizel *et al.*, 1980). There are major differences in incubation methods between the work reported here and the results obtained by Meizel *et al.* (1980) including their use of 10-fold higher sperm concentration, 50-fold increase in hypotaurine and 3-fold greater bovine serum albumin. The most striking difference between the work of Meizel *et al.* (1980) and the results reported here is the use of a selected suspension of hamster spermatozoa produced by elution through a glass bead column (Lui, Mrsny & Meizel, 1979). Earlier work (Cornett & Meizel, 1978) in which non-selected sperm populations were used yielded results that are compatible with ours. Whether or not the use of a pre-selected population of spermatozoa accounts for the disparity, the work reported here provides no evidence for suggesting that hypotaurine alone is capable of inducing significant activation of motility or fertility in hamster spermatozoa.

The effects of hypotaurine on hamster spermatozoa *in vitro*, leading to capacitation, occur within the first 2 h of sperm incubation. This was indicated by the motility and activation (Table 1) and fertility (Table 2) of spermatozoa when epinephrine was added at 2 h versus 0 h incubation and the inability to block fertilization completely with phentolamine when added after 2 h of culture (Table 4). After sensitization of spermatozoa by hypotaurine, addition of epinephrine may be delayed until at least 4 h after the start of incubation and still cause significant levels of activation (Table 1) and fertility (Table 2). Epinephrine, therefore, can be the direct stimulus for activation and possibly the acrosome reaction since its effect is more closely linked in time to these events compared to hypotaurine.

Failure to block fertilization *in vitro* by the addition of a catecholamine antagonist after 2 h of sperm incubation suggests that epinephrine acts by way of a hormone-like stimulus. Identification of adrenergic receptors on spermatozoa would provide definitive evidence for this. Phentolamine, an α-adrenergic antagonist was more effective in blocking fertilization than was propranolol, a β-adrenergic antagonist, yet both α- and β-agonists have been shown to stimulate

motility, activation and acrosome reactions in hamster spermatozoa (Cornett & Meizel, 1978; Bavister *et al.*, 1979; Meizel & Working, 1980). If epinephrine is truly interacting with adrenergic receptors on spermatozoa, it is not clear whether α- or β-receptors or both are involved.

In summary, evidence is presented that suggests hypotaurine and epinephrine are working independently and sequentially to stimulate the fertilizing capacity of hamster spermatozoa. The cellular mechanisms being affected by these agents must now be elucidated.

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