Effects of factors released from eggs and other agents on cyclic nucleotide concentrations of sea urchin spermatozoa

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Summary. Factors released from eggs (FRE) of the sea urchin, *Strongylocentrotus purpuratus*, caused up to 20-fold increases in sperm cyclic AMP levels after a 1-min incubation. Putative cyclic nucleotide phosphodiesterase inhibitors such as theophylline acted in a synergistic manner with FRE to cause even greater increases in sperm cyclic AMP levels. This effect appeared to be specific for egg factors since various hormones (triiodothyronine, norepinephrine, histamine), nucleosides (adenosine, guanosine), nucleophiles (azide), anaesthetics (procaine), ionophores (X537A, A23187), metals (Mn^{2+}) and neurotransmitters (acetylcholine) did not increase sperm cyclic AMP levels. Various mammalian tissue extracts (serum, uterus, adrenal, ovary, lung) also had no effect.

We suggest that the activity which elevates the cyclic AMP of sea urchin spermatozoa is specifically associated with sea urchin eggs.

Introduction

Sperm motility, respiration rate and fructolytic rate can be maintained or increased by cyclic nucleotide phosphodiesterase inhibitors and by exogenous adenosine 3':5'-monophosphate (cyclic AMP) or guanosine 3':5'-monophosphate (cyclic GMP) (Garbers, Lust, First & Lardy, 1971a; Garbers, First, Sullivan & Lardy, 1971b; Garbers, First & Lardy, 1973a; Garbers, First, Gorman & Lardy, 1973b; Hoskins, 1973; Frenkel, Peterson & Freund, 1973; Morton, Harrigan-Lum, Albagli & Jooss, 1974; Schoenfeld, Amelar & Dubin, 1975). These reports, and observations of high activities of cyclic AMP-dependent protein kinase (Hoskins, Casillas & Stephens, 1972; Garbers, First & Lardy, 1973c; Lee & Iverson, 1976), guanylate cyclase (Garbers, Hardman & Rudolph, 1974; Gray, Drummond, Luk, Hardman & Sutherland, 1976), cyclic nucleotide phosphodiesterase (Gray *et al.*, 1976; Tash, 1976; Wells & Garbers, 1976), phosphoprotein phosphatase (Tang & Hoskins, 1975) and adenylate cyclase (Casillas & Hoskins, 1971; Braun & Dods, 1975; Herman, Zahler, Doak & Campbell, 1976; Garbers, 1977) in the spermatozoa of various animal species, have led to speculation that the cyclic nucleotides may be involved in the modulation of various sperm functions (Garbers *et al.*, 1971a; Morton & Albagli, 1973; Rogers & Morton, 1973; Hoskins & Casillas, 1974; Toyoda & Chang, 1974; Rosado, Hicks, Reyes & Blanco, 1974). Potent natural effectors of sperm cyclic nucleotide metabolism, however, were discovered only recently (Garbers & Hardman, 1975, 1976). Factors released from sea urchin eggs (FRE) were shown to elevate sperm cyclic AMP levels by as much as 100-fold within 0.5 min. It has since been reported that the same factor which elevates sperm cyclic AMP levels also stimulates sperm respiratory rates (Kopf & Garbers, 1977). Although these observations lend strong support to the previous speculations, the specificity of the FRE-induced cyclic AMP elevation in sea urchin spermatozoa has not been studied.

We therefore investigated a number of substances known to increase cyclic nucleotide levels in other cells, and extracts from a number of mammalian tissues, for their effects on sperm cyclic AMP and cyclic GMP concentrations.

Materials and Methods

Sea urchins (*Strongylocentrotus purpuratus*) were obtained from Pacific Bio-Marine, Venice, California. Unlabelled nucleotides and other drugs were from Sigma, Schwartz/Mann, Fisher, Hoffmann-La Roche or Eli Lilly. Dowex-50 (AG 50W X8, 100–200 mesh) was from Bio-Rad.

Spermatozoa and eggs were collected and washed as described previously (Garbers & Hardman, 1976). Egg factors were obtained by allowing eggs (10–20 mg wet wt/ml) to set in seawater for 1–2 h and collecting the supernatant fluid above the eggs. This supernatant fluid is often termed 'egg water', and is known to elevate sperm cyclic AMP levels (Garbers & Hardman, 1975, 1976), to stimulate respiration and motility, and to induce the acrosome reaction (Rothschild, 1956; Hathaway, 1963).

Spermatozoa (in 0.2 ml seawater) were incubated immediately after being washed and were re-suspended in a final reaction mixture of 0.65 ml as described in the Tables. The incubations were initiated by the addition of spermatozoa to the assay tubes and were stopped by the addition of 1 ml 0.5 N-perchloric acid, containing tracer amounts of cyclic-[^3H]AMP or cyclic-[^3H]GMP for estimation of sperm cyclic nucleotide recoveries. The acidified cell suspensions were then frozen and thawed five times before purification of the cyclic nucleotides on Dowex-50 columns (0.7 cm $\times$ 25.0 cm) as described by Schultz, Böhme & Hardman (1974). The column fractions containing cyclic AMP or cyclic GMP were freeze dried and then resuspended in 0.5–1.0 ml distilled water. Cyclic AMP was assayed by the method of Gilman (1970), as modified by Brostrom & Kon (1974). Cyclic GMP was determined by the radioimmunoassay of Steiner, Kipnis, Utiger & Parker (1969), as modified by Harper & Brooker (1975).

Various tissue extracts were also prepared for incubation with the spermatozoa. Hamster serum was collected and heated at 56°C for 35 min to inactivate the toxic serum component described by Yanagimachi (1970). Hamster uteri were removed and flushed with 0.9% (w/v) NaCl solution. The flushings were centrifuged at 26 000 g for 20 min and the resulting supernatant fluid was used in subsequent experiments. Hamster adrenal glands were removed, trimmed of excess tissue, and homogenized (5–10 g/100 ml) in 0.9% (w/v) NaCl solution. The homogenate was centrifuged at 26 000 g for 20 min and the supernatant fluid was removed and heated at 56°C for 30 min. Hamster ovaries and rat lungs were removed, weighed and homogenized (5–10 g/100 ml) in 0.9% (w/v) NaCl solution. The homogenate was then centrifuged at 26 000 g for 20 min and the supernatant saved for incubation with the spermatozoa.

Results

The positive results are shown in detail in the Tables, but much of the negative data is not given.

Effects of various agents in the presence or absence of theophylline

As reported previously (Garbers & Hardman, 1975, 1976) there was a marked synergistic effect between theophylline and FRE to elevate sperm cyclic AMP levels (Table 1). However, agents known to elevate cyclic nucleotide levels in other tissues (Robison, Butcher & Sutherland, 1971; Kimura, Mittal & Murad, 1975a, b), and other compounds failed to elevate sperm cyclic AMP or cyclic GMP levels.

Some of the incubations with these agents were extended for more than 1 min. A23187, a divalent cation ionophore, was tested at 15 and 30 $\mu\text{g/ml}$ in normal seawater and did not affect cyclic AMP or cyclic GMP levels after 1, 2, 5 or 10 min of incubation. However, in Mg^{2+} -free seawater, the ionophore caused small but reproducible increases in sperm cyclic AMP concentrations in the presence of theophylline (Table 2). Thus, Ca^{2+} transport into the sperm cell may result in an elevation of cyclic AMP. A23187 significantly reduced the FRE-induced elevation of sperm cyclic AMP in the presence and absence of theophylline. Cyclic GMP concentrations were either decreased or not altered by the ionophore (Table 2).

Sodium azide, which possesses nucleophilic properties, has been reported to elevate cyclic GMP levels in various tissues (Kimura *et al.*, 1975b) and to stimulate guanylate cyclase activity in broken cell preparations (Kimura *et al.*, 1975a). This compound failed to elevate cyclic GMP levels at any concentration tested but depressed cyclic GMP levels at concentrations greater than 150 μM : at 1.5 mM-azide, cyclic GMP concentrations were depressed by 78%. Cyclic AMP levels were not affected by sodium azide at any concentration tested. Sodium nitroprusside (0.15 and 1.5 mM) did not elevate either cyclic nucleotide.

Table 1. The effect of various agents in the presence or absence of theophylline on sea urchin sperm cyclic AMP and cyclic GMP levels (nmol/g wet weight of spermatozoa)

Addition	Concentration	Theophylline (1.5 mM)	Cyclic AMP	Cyclic GMP
None	—	—	2.4 ± 0.4	0.78 ± 0.06
		+	4.6 ± 0.4	1.23 ± 0.04
FRE	—	—	34.1 ± 2.4	0.49 ± 0.03
		+	117.0 ± 2.4	1.19 ± 0.05
Triiodothyronine	15 µg/ml	—	3.2 ± 0.5	0.85 ± 0.04
		+	4.7 ± 0.3	1.51 ± 0.16
X537A	15 µg/ml	—	2.5 ± 0.5	0.89 ± 0.06
		+	4.2 ± 0.2	1.25 ± 0.16
A23187	15 µg/ml	—	2.9 ± 0.2	0.75 ± 0.05
		+	5.1 ± 0.2	1.59 ± 0.11
Adenosine	77 µM	—	2.4 ± 0.2	0.75 ± 0.05
		+	5.0 ± 0.2	1.22 ± 0.11
Guanosine	77 µM	—	2.9 ± 0.3	0.96 ± 0.14
		+	5.7 ± 0.2	1.25 ± 0.17
Norepinephrine	77 µM	—	2.9 ± 0.3	0.80 ± 0.04
		+	5.0 ± 0.2	1.21 ± 0.06
Histamine	77 µM	—	2.2 ± 0.2	0.87 ± 0.05
		+	3.7 ± 0.1	0.95 ± 0.13
Acetylcholine	77 µM	—	2.1 ± 0.3	0.80 ± 0.05
		+	4.1 ± 0.2	1.48 ± 0.10
Phenylephrine	77 µM	—	1.9 ± 0.3	0.99 ± 0.04
		+	4.0 ± 0.3	1.19 ± 0.12
Procaine	77 µM	—	1.8 ± 0.2	0.98 ± 0.05
		+	3.6 ± 0.2	1.40 ± 0.09
Propranolol	77 µM	—	2.2 ± 0.4	0.84 ± 0.06
		+	4.8 ± 0.3	1.23 ± 0.11
MnCl ₂	770 µM	—	3.0 ± 0.4	0.86 ± 0.04
		+	4.3 ± 0.3	1.21 ± 0.11
Azide	77 µM	—	2.3 ± 0.4	0.72 ± 0.05
		+	3.9 ± 0.6	1.09 ± 0.12
Dinitrophenol	77 µM	—	2.1 ± 0.4	0.92 ± 0.04
		+	4.7 ± 1.0	1.21 ± 0.10

All values represent the mean ± s.e.m. of 4 observations. All incubations were for 1 min at 15–17°C, and each contained 20–25 mg (wet weight) spermatozoa.

Concentrations of acetylcholine (77 µM) and physostigmine (7.7 µM) which are known to stimulate sperm motility (Nelson, 1972; McGrady & Nelson, 1976) were tested in the presence and absence of 4% dimethylsulphoxide but there was no alteration of the cyclic nucleotide levels in sea urchin spermatozoa at 1, 3 or 6 min of incubation under these conditions.

Various fatty acids (7.7 µM) were also tested for effects on sperm cyclic nucleotide levels after incubation for 1 min, but of those studied (arachidonic, myristic, oleic and palmitic) none affected sperm cyclic AMP or cyclic GMP levels.

Effects of various agents on the FRE cyclic AMP-elevating activity

FRE elevated sperm cyclic AMP levels approximately 10-fold (Table 3), but only the putative phosphodiesterase inhibitors, theophylline and 1-methyl-3-isobutylxanthine, acted in a synergistic manner with FRE to elevate sperm cyclic AMP levels. However, sodium azide and dinitrophenol appreciably inhibited the cyclic AMP elevation caused by FRE. FRE slightly depressed sperm cyclic GMP levels, and this decrease was not alleviated by a number of agents tested except for theophylline and 1-methyl-3-isobutylxanthine (Table 3). In Mg²⁺-free seawater, A23187 caused a significant decrease in the FRE-induced elevation of cyclic AMP (Table 2).

Table 2. The effect of A23187 on cyclic nucleotide levels (nmol/g wet weight of spermatozoa) of sea urchin spermatozoa in Mg^{2+} -free seawater

Addition	Theophylline (1.5 mM)	Cyclic AMP	Cyclic GMP
None	—	2.7 ± 0.3	0.54 ± 0.07
	+	3.2 ± 0.2	0.89 ± 0.14
A23187 (15.4 µg/ml)	—	2.3 ± 0.1	0.30 ± 0.01*
	+	3.4 ± 0.2	0.45 ± 0.02
A23187 (31 µg/ml)	—	3.9 ± 0.6	0.31 ± 0.03
	+	6.2 ± 1.1*	0.55 ± 0.04
FRE	—	59.2 ± 2.9	0.45 ± 0.09
	+	176 ± 10.1	0.83 ± 0.11
FRE + A23187 (31 µg/ml)	—	10.6 ± 3.5*	0.17 ± 0.03
	+	45.9 ± 4.8*	0.44 ± 0.03

All incubations were for 1 min at 15–17°C. A23187 was added in a solution containing dimethylformamide; equal concentrations of this solvent were added to the incubation mixtures from which A23187 was excluded.

* Values (mean ± s.e.m. of 4 observations) significantly different from those of respective control without A23187 ($P < 0.05$).

Table 3. The effect of various agents, in the presence or absence of factors released from sea urchin eggs (FRE), on cyclic nucleotide levels (nmol/g wet weight of spermatozoa) in sea urchin spermatozoa

Addition	Concentration (µM)	FRE	Cyclic AMP	Cyclic GMP
None	—	—	2.3 ± 0.2	0.45 ± 0.03
		+	23.9 ± 0.9	0.30 ± 0.02
T ₃	77	—	2.2 ± 0.05	0.56 ± 0.03
		+	21.3 ± 4.2	0.42 ± 0.02
A23187	15 (µg/ml)	—	2.4 ± 0.2	0.58 ± 0.04
		+	20.6 ± 1.0	0.36 ± 0.02
Adenosine	77	—	2.3 ± 0.2	0.47 ± 0.02
		+	16.0 ± 0.6	0.36 ± 0.01
Guanosine	77	—	2.0 ± 0.1	0.51 ± 0.03
		+	16.4 ± 0.7	0.36 ± 0.02
Phenylephrine	77	—	2.0 ± 0.2	0.46 ± 0.02
		+	16.5 ± 1.5	0.36 ± 0.03
Acetylcholine	77	—	2.1 ± 0.1	0.49 ± 0.03
		+	17.2 ± 0.8	0.33 ± 0.02
Azide	385	—	2.2 ± 0.1	0.34 ± 0.02
		+	10.3 ± 0.6	0.33 ± 0.04
Dinitrophenol	77	—	2.2 ± 0.1	0.52 ± 0.02
		+	11.3 ± 0.4	0.36 ± 0.02
Theophylline	1500	—	4.1 ± 0.3	0.68 ± 0.04
		+	79.0 ± 12.0	0.74 ± 0.08
1-Methyl-3-isobutylxanthine	150	—	4.5 ± 0.2	0.75 ± 0.04
		+	82.0 ± 6.0	0.82 ± 0.04

All values represent the mean ± s.e.m. of 4 observations. Incubations, each containing 20–30 mg spermatozoa, were for 1 min at 15–17°C.

Effects of tissue extracts on sperm cyclic nucleotide levels

Hamster serum and adrenal extracts have been reported to contain a substance of low molecular weight which stimulates hamster sperm motility (Bavister, Yanagimachi & Teichman, 1976). In the present study only sea urchin FRE caused an elevation in sperm cyclic AMP levels (Table 4). Likewise, sperm cyclic GMP levels were not affected by any of the added tissue extracts (Table 4).

Table 4. The effect of various tissue fluids on the cyclic nucleotide levels (nmol/g wet weight spermatozoa) in sea urchin spermatozoa

Species	Tissue	Theophylline (1.5 mM)	Cyclic AMP	Cyclic GMP
None	—	—	3.1 ± 0.4	0.37 ± 0.05
		+	4.9 ± 0.5	0.52 ± 0.03
Sea urchin	FRE	—	44.1 ± 2.4	0.33 ± 0.03
		+	127 ± 6.7	0.78 ± 0.05
Ox	Serum albumin	—	3.0 ± 0.4	0.31 ± 0.02
		+	4.6 ± 0.9	0.48 ± 0.04
Hamster	Serum	—	2.4 ± 0.4	0.38 ± 0.03
		+	3.3 ± 0.4	0.94 ± 0.27
	Uterine fluid	—	2.7 ± 0.3	0.31 ± 0.02
		+	3.8 ± 0.7	0.61 ± 0.28
	Adrenal	—	3.8 ± 1.0	0.36 ± 0.02
		+	3.9 ± 0.6	0.52 ± 0.04
	Ovary	—	2.5 ± 0.2	0.43 ± 0.07
		+	3.3 ± 0.2	0.62 ± 0.06
Rat	Lung	—	2.9 ± 0.4	0.41 ± 0.04
		+	2.8 ± 0.4	0.66 ± 0.06

All values represent the mean ± s.e.m. of 4 observations. All incubations (0.65 ml) were for 1 min at 15–17°C and each contained 15–25 mg (wet weight) of spermatozoa and 50 µl of each tissue fluid or extract.

Discussion

The chemical nature of the substance(s) released by sea urchin eggs remains to be determined, but they apparently do not resemble a number of agents (e.g. triiodothyronine, adenosine, norepinephrine, histamine, acetylcholine and phenylephrine) that are known to alter cyclic AMP levels in other tissues. The divalent cation ionophores (X537A and A23187) also failed to alter cyclic nucleotide levels in normal seawater, but A23187 caused elevations in sperm cyclic AMP levels in Mg²⁺-free seawater, suggesting that A23187-mediated Ca²⁺ transport might be responsible for the elevation of cyclic AMP. The cyclic AMP elevations are slight, however, and A23187 actually inhibits the FRE-induced elevation of sperm cyclic AMP. Therefore, FRE probably do not elevate sperm cyclic AMP by a similar mediation of Ca²⁺-transport.

The experiments with mammalian tissues demonstrate that cyclic AMP levels of sea urchin spermatozoa are elevated specifically by factor(s) associated with the sea urchin egg. The origin of the FRE remains to be clarified. Sea urchin eggs are surrounded by a jelly coat but it is not yet known whether the cyclic AMP-elevating activity is associated with the jelly coat or diffuses from the egg itself.

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