A low molecular weight factor from sea urchin eggs elevates sperm cyclic nucleotide concentrations and respiration rates

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Summary. A factor associated with sea urchin eggs that increases sperm cyclic nucleotide concentrations and respiration rates was identified as having a low molecular weight. The factor was more potent at elevating cyclic GMP concentrations than cyclic AMP concentrations, and represents the first demonstration of a factor associated with eggs that is capable of causing elevations of sperm cyclic GMP. Concentration–response curves of the crude mixture of egg factors to increase sperm cyclic AMP and cyclic GMP concentrations and respiratory rates were very similar, and comparable losses of these three activities were observed after extensive dialysis and heat treatment of the crude egg factors. The factor was partly purified by ethanol precipitation of a large molecular weight egg jelly component, and by charcoal adsorption and LH-20 chromatography of the resultant ethanol-soluble material. The factor was not extracted into a variety of organic solvents and had an apparent molecular weight of between 1000 and 2000, as estimated by gel filtration.

Introduction

It has been known for over 50 years that soluble components associated with the ova of certain species of sea urchins have dramatic metabolic and functional effects on sea urchin spermatozoa. Some effects of these components on spermatozoa include induction of isoagglutination (Lillie, 1913), changes in motility (Lillie, 1913; Hathaway, 1963), alterations in respiration rates (Gray, 1928; Rothschild, 1952; Hathaway, 1960, 1963), development of the acrosome reaction (Dan, 1952), and promotion of fertilization (Lybing & Hagström, 1957). Whether these effects are mediated by the same or by different components associated with the egg is not known.

Garbers & Hardman (1975, 1976) reported that factors (FRE) released from the eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* increased cyclic AMP concentrations in the spermatozoa of these sea urchins. A high molecular weight and low molecular weight factor, both capable of elevating sperm cyclic AMP concentrations, appeared to exist in the crude mixture of these egg factors. The high molecular weight egg factor demonstrated strict species specificity with respect to the induction of cyclic AMP elevations in spermatozoa (Tubb, Kopf & Garbers, 1979), but the low molecular weight factor was able to elevate cyclic AMP concentrations in a variety of sea urchin species. We have been investigating the effects of these egg factors on one sperm functional change, the stimulation of oxygen consumption. Since sperm cyclic AMP changes are known to be associated with altered sperm functions such as motility and metabolic rates (Garbers, Lust, First & Lardy, 1971b; Garbers, First & Lardy, 1973a; Garbers, First, Gorman & Lardy, 1973b; Hoskins, 1973; Frenkel, Peterson & Freund, 1973; Tash & Mann, 1973; Morton, Harrigan-Lum, Albagli & Jooss, 1974; Rosado, Hicks, Reyes & Blanco, 1974; Hoskins, Hall & Munsterman, 1975; Cascieri, Amann & Hammerstedt, 1976),

some or all of the previously studied effects of sea-urchin egg factors on sea-urchin sperm respiration could be explained by egg factor-induced alterations of sperm cyclic AMP concentrations, and we have examined this possibility.

The present report describes the partial purification and characterization of a low molecular weight egg-associated factor from sea urchins, and its effects on sea urchin sperm respiratory rates and cyclic nucleotide metabolism.

Materials and Methods

Lytechinus pictus and *Strongylocentrotus purpuratus* sea urchins were purchased from Pacific-Biomarine, Venice, California. Tritiated cyclic nucleotides were obtained from the Radiochemical Centre (Amersham, U.K.) and New England Nuclear (Boston, Massachusetts). Dowex-50 (AG 50W X8, 100–200 mesh), Bio Gel A 1.5 m and Bio Gel P-6 were from Bio-Rad (Richmond, California). Sephadex G-10 and G-50 (medium), ovalbumin, chymotrypsinogen and ribonuclease were from Pharmacia (Sweden). Cytochrome c was obtained from Nutritional Biochemical Corp. (Cleveland, Ohio), theophylline was from Merck and Co. (Rahway, New Jersey), and Tris (Trizma Base), pronase and trypsin were from Sigma (St Louis, Missouri). Activated coconut charcoal (50–200 mesh) was purchased from Fisher (U.S.A.).

A table-top oxygraph (Model K-1C, Gilson Medical Electronics, Inc., Wisconsin) equipped with a Clark-type oxygen electrode and a 1.8 ml capacity incubation chamber was used for the measurement of sperm oxygen consumption.

The heterologous system (*S. purpuratus* egg factors and *L. pictus* spermatozoa) employed in these studies was selected to avoid the sperm agglutination known to occur when using the egg jelly component of the same species (Lillie, 1913). Such isoagglutination can complicate interpretation of sperm respiratory measurements. All gamete preparations and incubations were carried out in sea water at pH 8.0.

Preparation of gametes and egg factors. Spermatozoa and eggs were collected and washed, and egg factors were prepared as previously described (Garbers & Hardman, 1976). Experiments were initiated within 15 min after resuspension of the spermatozoa.

Determination of cyclic nucleotide concentrations. Spermatozoa were resuspended in sea water to concentrations ranging from 39 to 97.5 mg (wet weight) cells/ml. The cells (0.2 ml) were then preincubated at 15–17°C in sea water alone, or in sea water containing theophylline, for 5 min. Incubations (1 min) were initiated by the addition of various quantities of sea water or egg factors. The final incubation volume was 0.65 ml. Incubations were terminated by the addition of 0.5 N-perchloric acid, containing tracer amounts of cyclic [³H]AMP or cyclic [³H]GMP for estimation of sperm cyclic nucleotide recoveries. Concentrations of cyclic nucleotides at zero time were estimated by adding the spermatozoa directly to sea water containing 0.5 N-perchloric acid. The acidified cell suspensions were then frozen and thawed 5 times before purification of the cyclic nucleotides on Dowex-50 columns (0.7 × 25.0 cm) as described by Schultz, Böhme & Hardman (1974). The column fractions containing cyclic AMP and 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 radioimmunoassay (Steiner, Kipnis, Utiger & Parker, 1969), as modified by Harper & Brooker (1975).

Determination of respiration. Spermatozoa were collected as described above and stored as a suspension (68.5–115 mg (wet weight) cells/ml) in a water bath (17°C) or ice bath (0°C). Various quantities of the sperm suspension were preincubated in sea water alone or in sea water containing theophylline in a temperature-controlled (17°C) chamber fitted with an oxygen electrode. The final preincubation volume was 1.8 ml. Within 2–3 min after addition of the spermatozoa, the respiratory rates had reached a constant basal rate. Various quantities of sea

water or egg factors were then added to the chamber and the new respiratory rates were calculated over a period of 5 min. Respiratory rates were expressed in terms of ng atoms O consumed/min/mg (wet weight) spermatozoa.

Different preparations of spermatozoa displayed considerable variation in the absolute rates of respiration, although consistency in qualitative patterns was always observed. The respiratory data presented therefore represent typical experiments.

Stability of the egg factors at 115°C. The crude mixture of egg factors collected in sea water (pH 8.0) was examined for stability of activity at neutral pH (by addition of 1.0 N-HCl to the sea water to bring the final pH to 7.0) and at acid pH (by addition of 11.6 N-HCl to give a final concentration of 1 N-HCl, pH 0.63). Egg factors treated in this manner were then placed in a Griffen-Worden pressure vessel, sealed *in vacuo*, and incubated at 115°C. At various times, an aliquot of the mixture was withdrawn and the samples incubated at acid pH were immediately titrated towards neutral pH with 11.0 N-NaOH. The sample was finally neutralized to pH 7.0 with Tris buffer. Tris buffer was also added in equal quantities to the samples incubated at neutral pH. The respective samples were then incubated with washed *L. pictus* spermatozoa, and respiration and cyclic nucleotide concentrations were determined as indicated above.

Column chromatography. Sephadex G-50, Bio Gel A 1.5 m, Sephadex G-10 and Bio Gel P-6 resins were suspended, equilibrated, and run in sea water (pH 8.0) at room temperature (24°C). All molecular weight calibration standards were suspended in sea water before chromatography.

Results

All respiratory and cyclic nucleotide studies, unless otherwise noted, were performed by preincubating the spermatozoa with 2.2 mM-theophylline and then incubating these cells with the egg factors. An example of the dramatic alteration of sperm respiration rates caused by the addition of a small amount of the crude mixture of egg factors is shown in Text-fig. 1. The respiratory rates were altered rapidly by, and continued at a linear rate after, addition of the egg factors. The respiratory stimulation by the factors was concentration-dependent (Text-fig. 2c). When

Text-fig. 1. The effect of *S. purpuratus* egg factors (FRE) on the respiratory rate of *L. pictus* spermatozoa at 17°C. The spermatozoa were preincubated with 2.2 mM-theophylline before addition of the egg factors. The figure represents a typical oxygraph tracing.

Text-fig. 2. Effects of dialysis of the *S. purpuratus* egg factors (FRE) on (a) the cyclic AMP-elevating activity, (b) the cyclic GMP-elevating activity, and (c) the respiratory stimulating activity when incubated with *L. pictus* spermatozoa. The crude egg factors, in sea water, were dialysed extensively at 4°C against sea water or distilled water (see 'Methods'). All values for cyclic AMP and cyclic GMP represent the mean $\pm$ s.e.m. ($n = 6$). The respiratory measurements represent a typical experiment. $\bullet$, $\blacksquare$ = non-dialysed FRE; O, Δ , $\square$ = dialysed FRE.

Text-fig. 3. The effects with time of *S. purpuratus* egg factors (FRE) on (a) the cyclic AMP and (b) cyclic GMP concentrations of *L. pictus* spermatozoa. Theophylline added alone to the sperm suspensions caused a 2-fold elevation of cyclic AMP concentrations above control values at all time points in (a) but had no significant effects of cyclic GMP concentrations (b). All values represent mean $\pm$ s.e.m. ($n = 4$). O, Spermatozoa only; $\bullet$, spermatozoa + 50 μl FRE + 1.54 mM-theophylline.

S. purpuratus egg factors were incubated with *L. pictus* spermatozoa, there was a concentration-dependent increase in both cyclic AMP and cyclic GMP concentrations (Text-fig. 2a, b). Egg factors and theophylline separately increased sperm cyclic AMP and cyclic GMP concentrations in a concentration-dependent manner (data not shown), but these changes were not as large as the synergistic responses observed when the egg factors and theophylline were combined (Text-fig. 2a, b). The egg factors appeared to be consistently more potent in elevating cyclic GMP concentrations since 1 μ l and 4 μ l crude egg factors were required for half-maximal elevations of sperm cyclic GMP and cyclic AMP concentrations, respectively (Text-fig. 2a, b).

The time course of the egg factor-induced changes in sperm cyclic AMP (Text-fig. 3a) and cyclic GMP (Text-fig. 3b) concentrations were also studied. Cyclic AMP and cyclic GMP concentrations were elevated maximally between 0.5 and 2 min, and then the levels declined gradually as a function of time.

Dialysis of the egg factors. As shown in Text-fig. 2, dialysis significantly reduced the cyclic AMP elevating activity (Text-fig. 2a) and the cyclic GMP elevating activity (Text-fig. 2b) of the crude egg factor mixture. A similar reduction in the respiratory stimulating activity was also observed (Text-fig. 2c).

Thermal and acid stability of the egg factors. Heating the egg factors at neutral pH for up to 8 h had no significant effects on any of the 3 activities measured (data not shown). However, heating at acid pH led to a complete loss of these activities within 2 h after the start of the incubation (Text-fig. 4).

Text-fig. 4. The effects of incubation of the *S. purpuratus* egg factors (FRE) at 115°C in 1 N-HCl on: (a) cyclic AMP-elevating activity, (b) cyclic GMP-elevating activity and (c) respiration-stimulating activity, when incubated with *L. pictus* spermatozoa. Cyclic nucleotide values represent mean $\pm$ s.e.m. ($n = 6$). Respiratory measurements represent a typical experiment.

Gel filtration of the egg factors. When the crude mixture of egg factors was applied to Bio-Gel A 1.5 m and Sephadex G-50 columns equilibrated with sea water, respiratory-stimulating activity, cyclic AMP-elevating activity, and cyclic GMP-elevating activity elution profiles were very similar (data not shown). Gel filtration of the egg factors on Sephadex G-10 and Bio-Gel P-6 columns, calibrated with dextran blue T-2000, poly-L-lysine, polyethylene glycol 4000, and polyethylene glycol 900, demonstrated that the three activities migrated coincidentally in a molecular weight range of between 1000 and 2000 (data not shown).

Partial purification of the low molecular weight egg factor. When 1 litre sea water containing the egg factors was added to 3 litres 95% ethanol, with constant stirring at 0–2°C, a large molecular weight egg-associated component was precipitated. The small molecular weight respiratory stimulatory factor was not precipitated by this step and remained in the supernatant fluid. Factor activity throughout this and subsequent purification steps was monitored by measuring sperm respiratory stimulation. The suspension containing both factors was then centrifuged at 3500 *g* for 15 min and the supernatant fluid collected and flash evaporated to a final volume of 800–1000 ml. The concentrate was then acidified to pH 5.0 with HCl and applied to a 2.6 × 30 cm charcoal column which had been equilibrated with 5 l distilled water.

Under these conditions, all of the factor activity was adsorbed to the column. The column was then washed successively with 5 l H₂O, 5 l 50% ethanol and then 5 l H₂O. The factor remained completely adsorbed to the column throughout this washing procedure. Elution of the factor from the column was accomplished by the addition of 3 l of a solution containing 70% ethanol and 0.2% (NH₄)OH. The eluate was flash evaporated and the remaining aqueous phase was freeze-dried. This freeze-dried material was then resuspended in 10–15 ml 70% ethanol and 5 ml of the solution was applied to a 2.6 × 80 cm Sephadex LH-20 column, equilibrated with 70% ethanol and 0.1 M-NaCl. The fractions from the column containing the factor were then pooled and flash evaporated. The remaining aqueous solution containing the factor was used in the subsequent studies. The recovery of factor throughout these procedures, as determined by the amount of factor required for half-maximal stimulation of respiration, was about 30%.

Effects of pronase on the activity of the low molecular weight egg factor. When the partly purified factor was incubated with 20 µg pronase/ml, respiratory-stimulating activity disappeared within 2 h (data not shown). Although these results suggest that the factor is a peptide, it is still possible that the factor is adsorbed by pronase at a rate similar to that expected for enzymic degradation. Trypsin and a variety of other hydrolytic enzymes failed to inactivate the factor.

Organic solvent extraction of the low molecular weight factor. An aliquot (1 ml) of a solution containing the factor was mixed with 5 ml chloroform, ether or benzene. The aqueous phase was then tested for activity, and it was observed that the factor remained quantitatively in this phase.

Molecular weight. The respiratory-stimulating factor migrated at the front of Sephadex G-10 columns (exclusion limit approximately 1000), but migrated between cytochrome *c* (mol. wt 13 000) and ³H₂O (inclusion volume) on Sephadex G-50 columns. Subsequently, the factor was found to migrate with a molecular weight of 1000–2000 as estimated on Bio-Gel P-6 columns by using [³H]polyethylene glycol (mol. wt 4000 and 900) and poly-L-lysine (mol. wt 3000) as standards.

Exogenous cyclic nucleotides. Incubation of 5 mM-cyclic AMP, cyclic GMP, 8-Br-cyclic GMP or of dibutyryl cyclic AMP with sea urchin spermatozoa did not stimulate sperm respiration at pH 8.0 in the presence or absence of theophylline (data not shown).

Discussion

A number of workers have demonstrated species-specific effects of invertebrate egg factors on sperm respiration, ranging from positive effects (Gray, 1928; Carter, 1930; Hathaway, 1963), to no effects (Tyler, 1948) or negative effects (Rothschild 1952). The chemical nature of the sperm respiratory-stimulating factor remains unclear, and it may be different from species to species (Woodward, 1918; Clowes & Bachman, 1921; Carter, 1930; Hartmann & Schartau, 1939; Tyler, 1939; Cornman, 1941; Lybing & Hagström, 1957; Hathaway, 1960, 1963; Ohtake, 1976a, b). In general, however, the factor appears to be of low molecular weight and to be heat stable.

Studies in mammalian systems have provided evidence that factors which can stimulate sperm motility and respiration exist in the male and female reproductive tract fluids (Iritani, Gomes & VanDemark, 1969; Morton & Chang, 1973; Lindholmer, 1974; Hoskins *et al.*, 1975; Acott & Hoskins, 1976; Bavister & Yanagimachi, 1977; Brandt, Acott, Johnson & Hoskins, 1977), certain organ extracts (Bavister, Yanagimachi & Teichman, 1976), and in the sera of a variety of animals (Morton & Bavister, 1974; Bavister, 1975). The factor studied by Bavister (1975) and Bavister *et al.* (1976) is known to be of low molecular weight. Although the biochemical basis for the stimulatory properties of these mammalian factors has not been thoroughly investigated, it is known that mammalian sperm motility, respiration rates, and fructolytic rates can be maintained or increased by cyclic nucleotide phosphodiesterase inhibitors and by exogenous cyclic AMP or cyclic GMP (Garbers, First, Sullivan & Lardy, 1971a; Garbers *et al.*, 1971b, 1973a, b; Hoskins, 1973; Frenkel *et al.*, 1973; Schoenfeld, Amelar & Dubin, 1975). In addition, positive correlations between endogenous sperm cyclic AMP levels and sperm motility and respiration rates have been demonstrated (Tash & Mann, 1973; Morton *et al.*, 1974; Hoskins, Stephens & Hall, 1974; Cascieri *et al.*, 1976; Gray, Drummond, Luk, Hardman & Sutherland, 1976; Kopf & Garbers, 1978). It is, therefore, possible that the cyclic nucleotides serve as intracellular mediators for some or all of the sperm functional changes that occur in response to these mammalian factors. The observations of Berger & Clegg (1977) on the activation of pig sperm adenylate cyclase by sow uterine fluids support this suggestion.

The present report demonstrates that there is a low molecular weight factor released from the eggs of *S. purpuratus* sea urchins that will elevate the respiratory rates and the cyclic AMP and cyclic GMP concentrations of *L. pictus* spermatozoa. This report is the first to demonstrate an egg factor-induced elevation of sperm cyclic GMP concentrations. However, a purified large molecular weight factor that causes large elevations of sperm cyclic AMP concentrations failed to elevate sperm respiration rates in the homologous species (G. S. Kopf & D. L. Garbers, unpublished data), and elevation of sperm cyclic AMP concentrations only may not result in increased respiration rates. Whether or not cyclic GMP may mediate some of these respiratory changes remains to be studied, although exogenously added cyclic nucleotides did not appear to mimic the respiratory stimulation of the egg factors in *L. pictus* spermatozoa in the presence or absence of theophylline. Such studies are difficult to interpret, however, due to possible permeability problems.

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