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AN N-TERMINAL PARTIAL SEQUENCE OF THE 13 kDa *PYCNOPODIA HELIANTHOIDES* SPERM CHEMOATTRACTANT 'STARTRAK' POSSESSES SPERM-ATTRACTING ACTIVITY

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Summary

Freshwater extracts of starfish ovaries were used to purify the sperm-attracting peptide 'startrak' from *Pycnopodia helianthoides* using hydrophobic interaction chromatography and DEAE–high-pressure liquid chromatography. Partially purified attractant had a molecular mass of 13 kDa, estimated from gel filtration and polyacrylamide gel electrophoresis results. The purified attractant was subjected to amino acid analysis and direct sequencing, and was found to consist largely of a single peptide composed of an estimated 127 residues based on a molecular mass of 13 kDa. An N-terminal sequence of amino acids from positions 3 to 34 was obtained and synthesized as: NH_2-Ala-Glu-Leu-Gly-Leu-Cys-Ile-Ala-Arg-Val-Arg-Gln-Gln-Asn-Gln-Gly-Gln-Asp-Asp-Val-Ser-Ile-Tyr-Gln-Ala-Ile-Met-Ser-Gln-Cys-Gln-Ser-COOH. The synthetic peptide possessed sperm-attracting activity 130 times greater than the activity of partially purified startrak and showed a pattern of species-specificity of sperm chemotaxis similar to that of startrak. Antibody prepared against synthetic peptide removed the sperm-attracting activity from crude and partially purified preparations of startrak. The partial sequence of startrak was not homologous with that of any of the known echinoid sperm motility-activating peptides.

Key words: echinoderms, spermatozoa, chemoattractant, starfish, peptide, sequence, antibody, *Pycnopodia helianthoides*.

Introduction

Sperm chemotaxis has been described in numerous species from four invertebrate phyla: Cnidaria, Mollusca, Echinodermata and Urochordata (Miller, 1985*a*). Detailed plotting of sperm trails demonstrated that individual spermatozoa moved closer to an attractant source by altering their normal swimming behavior. Similar sperm trail shapes were observed in members of the four phyla, suggesting that a common mechanism was responsible for the sperm attraction behavior (Miller, 1985*a*,*b*). Species-specificity of sperm chemotaxis at the genus or order level was found in all the groups studied, and in some cases it existed between species in the same genus (Miller, 1979*a*, 1985*a*; R. L. Miller, unpublished data).

Knowledge of the chemistry of the more than 50 animal sperm attractants known to exist by 1985 consisted mainly of data on stability, crude molecular mass estimates and suggestions that most of the molecules might be peptides (Miller, 1979*b*, 1982; Carre and Sardet, 1981). The limited characterization of sperm attractants was largely the result of the very limited quantities of female gametes available during the short reproductive season of the species in which sperm chemotaxis occurred.

Recent work on coral and tunicate sperm attractants has revealed that some animal sperm attractants are not peptides. The sperm attractant of the scleractinian coral *Montipora digitata* has been identified using high-pressure liquid chromatography (HPLC), mass spectroscopy and organic synthesis as the most saturated of a group of three lipid alcohols with molecular masses of approximately 240 Da (Coll *et al.* 1994). It is apparently not species-specific (Coll and Miller, 1992; R. L. Miller, unpublished data). The sperm attractant of the tunicate *Ciona intestinalis* appears to be a small molecule resistant to proteases (Yoshida *et al.* 1993; Miller, 1982).

A 14 amino acid, sea urchin sperm-motility-activating egg-jelly peptide SAP-IIA (resact), purified from egg jelly of the sea urchin *Arbacia punctulata* using a sperm motility activation (oxygen uptake) assay (Suzuki, 1989), had sperm-attracting activity (Ward *et al.* 1985). The attraction behavior was genus- or order-specific and required external calcium. No

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evidence has been published that any of the other 70 sperm-motility-activating peptides (SAPs), purified so far from 18 species of sea urchins, possessed sperm-attracting activity (Suzuki and Yoshino, 1992). Molecular homologs of resact and the related egg-jelly peptide speract (Garbers *et al.* 1982; Garbers, 1989*a*,*b*; Suzuki *et al.* 1984) have been useful tools for dissecting the molecular pathways leading to sperm motility activation in sea urchin sperm (Smith and Garbers, 1983). It is not known how these pathways apply to sperm chemotactic behavior because no study of the relationship between chemotaxis and the molecular structure of the attractant has been published.

Recently, the sperm attractant of the forcipulate starfish *Pycnopodia helianthoides* was partially purified, by adsorption and gel filtration chromatography and reverse-phase and hydrophobic interaction HPLC, and identified using a sperm chemotaxis assay (Miller *et al.* 1992; Punnett *et al.* 1992). The molecule was found to be a heat- and freeze–thaw-stable peptide with an apparent molecular mass of 13 kDa. Its biological activity could be abolished by the proteases trypsin, chymotrypsin and pronase. The attractant showed only weak staining with silver using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). Its relatively large size suggested that it might be a suitable candidate to examine the relationship between attractant structure and sperm chemotactic behavior.

We report here the complete purification of the *Pycnopodia helianthoides* sperm attractant and the sequencing and synthesis of a 32 amino acid fragment with sperm-attracting activity from the amino-terminal portion of the molecule. No sequence homology was found between the starfish attractant and sea urchin SAPs. The chemoattractant activity of both the natural and synthetic attractant peptides are blocked by an antibody raised against the synthetic peptide. These data suggest that we have identified a new class of sperm attractant peptide, which we have named 'startrak' in view of its isolation from starfish.

Materials and methods

Animals

The large, multi-armed starfish *Pycnopodia helianthoides* (Brandt) was used because a single female yielded up to 500 g (wet mass) of ovaries, the males can be maintained in ripe condition out of season, the 'dry' sperm retain viability in the cold for several days and the sperm attraction behavior is easy to observe during bioassay. Sexually ripe specimens, collected by SCUBA diving and trawling in the waters surrounding the San Juan Islands, WA, USA, from January to June, were sorted by sex into shaded pens hanging under the boat docks of the Friday Harbor Laboratories of the University of Washington. The animals were fed salmon cannery waste or dogfish flesh weekly. Dissected testes were placed in a dry dish and held at 4 °C for up to a week. Small samples of the 'dry' sperm were removed as required for bioassay of the sperm attractant. Ovaries were removed from ripe females just after they had been sexed, and the damp gonads from each animal were weighed and frozen at −40 °C.

Assay and purification of sperm-attracting activity

Less than 0.5 μl of sperm was added to a broad, flat drop of sea water on a microscope slide in an area coated by a solution of 1 % polyethylene glycol (10 kDa) to keep the sperm from sticking to the slide. The sperm rapidly dispersed. Cells that came into contact with the slide surface exhibited sperm thigmotactic behavior by swimming in circles against it (Miller, 1985*a*). Attractant samples of 10 or 25 μl were serially diluted 1:1 until they produced a just-visible sperm turning response: sperm suddenly altered their thigmotactic circular pathways in a manner that rapidly brought them closer to the pipette tip and then reinitiated circling concentric with the pipette tip. As a result, the sperm concentration close to the pipette tip rapidly increased (Miller, 1985*a*).

The number of dilutions required to attain complete loss of attracting activity is defined as the titer number. A change in titer equivalent to one dilution equals a 50 % drop in biological activity with a defined error of ± one serial half-dilution (±50 %). The titer number is converted to units of activity by taking it as the power of two (a titer of $10=2^{10}=1024$ units) (Miller, 1966, 1985*a*,*b*).

Seawater (SW) extracts of the ovaries were filtered, eluted from columns of the Amberlite hydrophobic resin XAD-7 and subjected to Bio-gel P60 gel filtration (see Punnett *et al.* 1992, for details of the methods used). The fractions showing the highest sperm-attracting activity were then subjected to reverse-phase (RP) HPLC (Vydac C_4 RP300 column) followed by hydrophobic interaction (HIC) HPLC (Vydac diphenyl column). The fractions with highest activity were combined, lyophilized and injected into a TSK–DEAE 5PW column (0.75 cm×7.5 cm) (Supelco Inc.) using a gradient of 0 mol l^{-1} to 0.05 mol l^{-1} sodium chloride in 0.003 mol l^{-1} sodium phosphate, pH 7.5, with absorbance monitored at 220 nm. Flow rate was 1.0 ml min^{-1} for 30 min with the column held at room temperature. Phosphate and sodium salts were removed by passage of fractions with the highest activity through a Sep-Pak column, which was then flushed with water. The purified biological activity was eluted in a few milliliters of methanol, which was removed by flash evaporation. The active material was lyophilized, bioassayed and subsequently analyzed for amino acid composition and sequence.

Amino acid analysis, sequencing and peptide synthesis

Amino acid analysis, sequencing of the intact native protein and synthesis of the N-terminal peptide sequence was performed by the Protein and Nucleic Acid Chemistry Facility of the Yale University School of Medicine. A 1.6 μg sample of purified peptide was subjected to amino acid analysis using a Beckman model 7300 amino acid analyzer. N-terminal sequence analysis was performed using an Applied Biosystems model 477 pulse liquid-phase microsequencer equipped with an ABI model 120A on-line phenylthiohydantoin detector.

A 32 amino acid peptide was synthesized by the Biotechnology Resource Laboratory of the W. M. Keck Foundation, Yale University, on the basis of the amino acid sequence with certain substitutions. Terminal modifications were made to counteract what we suspected might be poor solubility of the synthetic compound compared with the original purified product. The biological activity and species-specificity of the synthetic peptide were tested in the same way as the natural sperm-attracting activity (Miller, 1985*a*,*b*).

Antibody experiments

Antisera were prepared from 10 mg of pure, unconjugated, synthetic peptide by Pocono Rabbit Farms. Immune and pre-immune sera were initially tested for activity against the synthetic peptide using an enzyme-linked immune serum assay (ELISA). The sera were then tested for sperm-attractant binding activity by mixing diluted samples with a small volume of sea water containing a known concentration of sperm attractant at 4 °C. After 30 min, protein-A–sepharose (PAS) (untreated or preblocked with 10 % by mass of non-fat dried milk) was added at a concentration of 1.0 mg per 20 μl and the mixture was incubated for 10 min at 4 °C with continuous agitation. The suspension was centrifuged at 12 000 ***g*** for 5 min to remove PAS and any bound activity, and the supernatant was assayed for residual sperm-attracting activity. During these experiments, we discovered that some sperm-attracting activity bound to the Eppendorf tubes, but this binding was eliminated in the presence of serum or of the non-fat dried milk preblocker at the concentrations used in the binding studies.

Sequence comparisons

A computer-based amino acid sequence comparison search was carried out using the National Center for Biotechnology Information (NCBI) BLAST network server and by direct comparison of SAP-I and SAP-II sequences using the DNASIS program (Hitachi).

Results

Sperm attractant purification

A typical DEAE–HPLC run is shown in Fig. 1. The biological activity eluted from the DEAE column in fractions 17–19 and there was a peak of acivity in fraction 18 which contained 20 times the biological activity added in the original sample. This suggested that some inhibitory substance(s) had been removed. The DEAE–HPLC chromatography step yielded 2.1 mg of white powder with a total activity of 5.4×10^7 units (or 420 % of the original starting material) at a specific activity of 2.6×10^7 units mg^{-1} and a purification factor of 10^6. The recoveries of the previous steps in the purification procedure up to the HIC–HPLC step are shown in Table 1 (from Punnett *et al.* 1992). The material recovered contained 0.06 % protein by mass (the rest was residual gradient salts), and amino acid analysis and sequencing indicated that it was

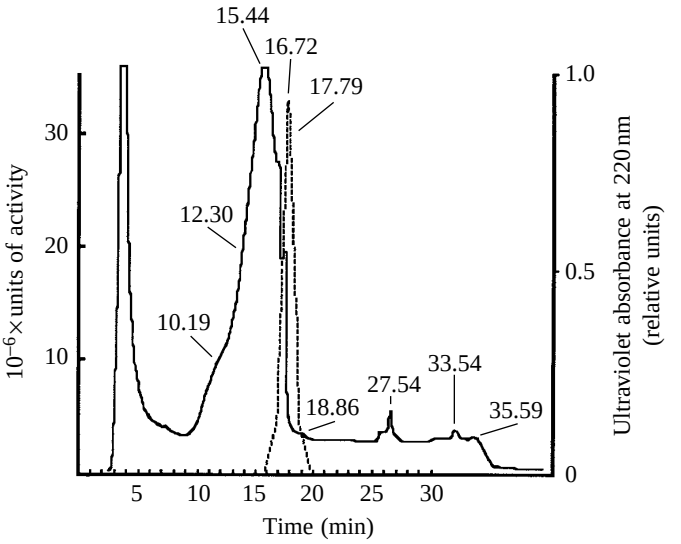

Fig. 1. DEAE–HPLC run of HIC–HPLC-purified startrak. The attractant (dashed line) elutes as a single peak of biological activity at 17.79 min associated with a very small peak on the downslope of the major ultraviolet peak (solid line) at 16.72 min. See Materials and methods section for details. Elution times in minutes are given beside the absorbance peaks.

substantially pure. We named the natural sperm attractant 'startrak' based on the words *star*fish at*trac*tant.

Amino acid analysis

The amino acid composition of a sample of the DEAE–HPLC-purified startrak is shown in Table 2. The total number of residues is based on our estimates of molecular mass (13 kDa) from gel fitration and polyacrylamide gel electrophoretic studies (Miller *et al.* 1992; Punnett *et al.* 1992). The amino acid analysis indicated 0.289 μg of peptide in the original sample. This suggests an estimated 412 pmol of a 127 residue protein, assuming a molecular mass of 13 kDa. Since this material yielded an unambiguous amino acid sequence that encoded strongly biologically active material, the sperm attractant peptide probably comprised the major component of the protein in the sample.

Purified startrak yielded a partial N-terminal amino acid sequence

Of the first 34 amino acids at the amino terminal end of startrak, 32 were sequentially identified (Table 3). Yields for all amino acids in the sequence were 10 % or higher. No other sequence was obtained with 10 % or more yield at any position. Ser′ (Table 3) represents a dithiothreitol (DTT) adduct of dihydroserine and probably indicates Cys at positions 8 and 32. Ser is present at positions 23 and 30. Ambiguity was observed at positions 7 (Leu and Phe) and 29 (Met and Val), indicating possible allelic heterogeneity at the two locations. Some heterogeneity is likely, since the initial ovarian material was pooled from several starfish (Punnett *et al.* 1992). The

Table 1. *Purification of* Pycnopodia helianthoides *sperm attractant**

Step	Mass (mg)	Total number of units	Recovery[a] (%)	Specific activity[a] (units mg^{-1})	Relative purification[a]
1. SW extract	500 000[b]	1.28×10^7	100[c]	25.6	1.0
2. XAD-7[d]	11 120	5.87×10^7	460	5.30×10^3	2.1×10^2
3. P-60	135	1.41×10^7	110	1.05×10^5	4.1×10^3
4. HPLC–HIC run[e]	6.3	3.20×10^7	250	5.10×10^6	2.0×10^5
5. DEAE–HPLC	2.1	5.40×10^7	420	2.60×10^7	10^6

*Steps 1–4 are previously published data (Punnett *et al.* 1992).

[a]Data shown in the recovery, specific activity and relative purification columns have an uncertainty of ±50 % owing to the use of the end-point assay (see text). This error is calculated as 0.35 (Margenau and Murphy, 1943).

[b]Wet mass of ovaries prior to extraction.

[c]We use the seawater extract as 100 %. The high recovery in the next step suggests that an attractant inhibitor in the seawater extract has been removed.

[d]Methanol wash. In view of the large increase in recovered biological activity, we can use 5.87×10^7 units as the base for purification. Percentage recoveries for the last three steps are then 24, 54 and 91. The large increase in biological activity between step 3 and step 5 suggests that another inhibitor may have been removed.

[e]Data for the HPLC–RP run are not included because of the problem of accounting for the morpholine residue.

calculated molecular mass of the sequenced fragment was 3.7 kDa, representing about 28 % of the native peptide.

Synthetic peptide sperm-attracting activity

A peptide corresponding to the partial N-terminal sequence was synthesized with Cys at positions 8 and 32 (indicated by bold type in Table 3), Leu at position 7, and Met at position 29 (indicated by bold type). Position 3 (Ala) was blocked by acetylation and position 34 (Ser) by amidation in an effort to aid in future labeling procedures and to counteract anticipated solubility problems. The predicted molecular mass of 3595 Da was confirmed by mass spectroscopy (data not shown). The synthetic sequence is shown in Table 3.

The sperm-attracting activity and species-specificity of the synthetic peptide were confirmed using sperm from *Pycnopodia helianthoides* and four additional echinoderm species. The sperm from the two asteriid starfishes *Orthasterias koehleri* and *Stylasterias forerri* were used because these sperm are known to respond to the *P. helianthoides* native attractant (Miller, 1985*b*), and sperm of the velatid starfish *Pteraster tesselatus* and the sea cucumber *Stichopus californicus* (Class Holothuroidea) were used as negative controls, because these sperm show no response to the *P. helianthoides* native attractant (Miller, 1985*b*). The sperm-attracting activity of the synthetic peptide was entirely consistent with that of the native attractant, inducing chemotaxis of *P. helianthoides*, *O. koehleri* and *S. forerri* sperm but eliciting no response from *P. tesselatus* or *S. californicus* sperm (Table 4).

Table 2. *Amino acid analysis of purified starfish sperm-attracting protein*

Amino acid*	Number of residues	Mole percent
Asx	16.5	12.093
Thr	3.9	2.847
Ser†	18.5	13.533
Glx	33.6	24.635
Gly	19.7	14.460
Ala	12.1	8.826
Val	6.7	4.931
Ileu	9.7	7.114
Leu	9.7	7.112
Arg	6.1	4.448

Total number of residues = 132 (based on a molecular mass of 13 kDa as estimated by SDS–PAGE).

*Sequence data show one tyrosine which would normally be degraded during the amino acid analysis.

†Two of the serines are probably cysteine.

Table 3. *Amino acid sequence of the startrak molecule*

1 *X-X*†-Ala-Glu*-Leu-Gly-Leu-Ser′‡-Ile-Ala-Arg-Val-Arg-Gln-Gln-
16 Asn-Gln-Gly-Gln-Asp-Asp-Val-Ser-Ile-Tyr-Gln-Ala-Ile-Val-Ser-
31 Gln-Ser′-Gln-Ser-

*Underlined residues constitute a Q-linker sequence (see Discussion).

†The identity of the first two residues could not be determined.

‡Ser′ represents a dithiothreitol adduct of dihydroserine and probably indicates Cys at positions 8 and 32.

Ambiguity was observed at positions 7 (Leu and Phe) and 29 (Met and Val), suggesting possible allelic heterogeneity at the two locations.

Synthetic startrak fragment modified sequence

3* NH_2-Ala-Glu-Leu-Gly-Leu-**Cys**§-Ile-Ala-Arg-Val-Arg-Gln-Gln-
16 Asn-Gln-Gly-Gln-Asp-Asp-Val-Ser-Ile-Tyr-Gln-Ala-Ile-**Met**-Ser-
31 Gln-**Cys**-Gln-Ser-COOH

*Numbering based on original sequence.

§Bold residues represent substitutions (see Results).

Table 4. *Species-specificity testing of synthetic chemotactic peptide*

Compound tested	Sperm tested	Titer*
HPLC-purified natural attractant (1.0 mg ml^{-1})	PYC	17
Synthetic peptide attractant (1.0 mg ml^{-1})	PYC	18
Synthetic peptide attractant (1.0 mg ml^{-1})	ORT	21
Synthetic peptide attractant (1.0 mg ml^{-1})	PTER	–
Synthetic peptide attractant (1.0 mg ml^{-1})	STYL	17
Synthetic peptide attractant (1.0 mg ml^{-1})	STICH	–
STICH attractant (crude at 12*)	STICH	12
Speract (0.2 mg ml^{-1})	ORT	–
Resact (0.1 mg ml^{-1})	ORT	–

*Number of serial half-dilutions for loss of biological activity. The assay is described in the Materials and methods section.

PYC, *Pycnopodia helianthoides*; ORT, *Orthasterias koehleri*; STYL, *Stylasterias forerri* (Asteroidea: Forcipulatida); PTER, *Pteraster tesselatus* (Asteroidea: Velatida); STICH, *Stichopus californicus* (Holothuroidea).

As controls, sperm responses were also tested against the native attractants of both *P. helianthoides* and *Stichopus californicus* and against the two sea urchin sperm-motility-activating peptides speract (SAP-I) and resact (SAP-II) (Table 4). *Pycnopodia helianthoides* sperm responded strongly to the native *P. helianthoides* sperm attractant, but were not responsive to the *Stichopus californicus* sperm attractant or to speract and resact at concentrations from 0.1 to 1 mg ml^{-1} even though resact attracts echinoid sperm at much lower concentrations (Ward *et al.* 1985).

Anti-synthetic-peptide blocks sperm-attracting activity

Antibody was raised against the synthetic peptide and tested for the ability to recognize and block the activity of the native attractant. Immune (positive) and pre-immune (negative) sera were tested for antigenic activity against the synthetic peptide by ELISA at dilutions to 1:500. Only immune serum precipitated native or synthetic attractant. Attractant activity in samples of (1) crude attractant purified by XAD-7 chromatography (Punnet *et al.* 1992), (2) DEAE–HPLC-purified startrak, and (3) synthetic peptide was removed from solution by treatment with immune serum but not by treatment with pre-immune serum (Fig. 2; see Materials and methods for details). These findings confirm that the partial peptide sequence obtained from the purified native attractant represents the *bona fide* sperm attractant peptide.

Sequence comparisons

Sequence comparisons revealed that the known biologically active startrak sequence is not homologous with the published sequences of echinoid SAPs. However, the sequence was found to resemble a Q-linker, a stretch of amino acids (enriched in Gln, Arg, Asn, Glu, Ser and Pro) that possesses a high degree of structural flexibility (see Discussion). The amino acids which indicate Q-linker structure are underlined in Table 3.

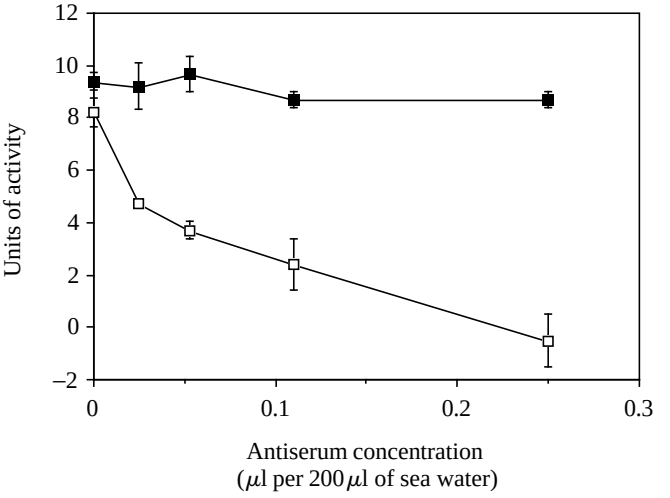

Fig. 2. Effects of antiserum concentration on loss of sperm-attracting activity. The crude antiserum was prepared against the 3.6 kDa synthetic peptide fragment. The graph shows the levels of sperm-attracting activity remaining in the supernatant (mean ± S.E.M. of three experiments) after a 20 min incubation of 1 μl containing 2^{18} units of synthetic peptide sperm attractant in 202 μl of sea water with increasing concentrations of the immune serum (open squares) or pre-immune serum (filled squares). Nearly identical results were obtained using natural peptide sperm-attracting activity.

Discussion

Our findings demonstrate that a new sperm attractant peptide, startrak, has been purified and partially identified; the first sperm attractant purified using a behavioral bioassay for sperm chemotaxis. Other sperm attractants have been purified using quite different bioassays. A 14-carbon lipid alcohol, known to attract sperm of the coral *Montipora digitata*, was recently purified using a surface binding assay (Coll *et al.* 1994). A number of sperm-motility-activating peptides (SAPs) of low molecular mass have been purified from sea urchin egg jellies (Suzuki, 1989; Suzuki *et al.* 1981). Bioassays were based on sperm motility activation and oxygen uptake (Suzuki and Garbers, 1984; Suzuki *et al.* 1984). Pure SAP-I (speract) (Garbers *et al.* 1982) had no sperm-attracting activity at concentrations as high as 10 μmol l^{-1} and apparently is not an attractant for the sperm of its own species (Ward *et al.* 1985). However, SAP-II (resact) induced chemotactic behavior in *Arbacia punctulata* sperm at concentrations as low as 1 nmol l^{-1} (Ward *et al.* 1985). These two SAPs showed complete specificity of sperm respiration activation at the order level and of sperm chemotaxis at the genus level (although speract does not attract sperm of the species that produces it). The many other effects of these and other similar peptides on sea urchin sperm motility and physiology have recently been reviewed (Ward and Kopf, 1993).

Regulation of sperm-attracting activity

The sperm-attracting activities of *Arbacia punctulata* (SAP-II), the hydrozoan *Tubularia crocea*, the ascidian *Ciona*

intestinalis and the starfish *Pycnopodia helianthoides* (startrak) all require external Ca^{2+} for normal activity (Miller, 1975*a*,*b*, 1985*a*; Ward *et al.* 1985), suggesting that they may have similar regulatory mechanisms (Miller, 1985*a*). Sea urchin SAPs at nanomolar concentrations elevate cyclic nucleotide content, internal pH (pHi) and internal calcium concentration ($[Ca^{2+}]_i$) of sperm with rapid associated fluxes of Na^+ and Ca^{2+} (inward) and K^+ and H^+ (outward) (Repaske and Garbers, 1983; Schackmann and Chock, 1986; Lee and Garbers, 1986). Chemoattractant-stimulated alterations in $[Ca^{2+}]_i$ appear to be largely responsible for the chemotactic turning response seen in sea urchin sperm (Cook *et al.* 1994).

Picomolar concentrations of the peptide speract produced long-lasting K^+-selective channel activation in hypotonically swollen sperm (Babcock *et al.* 1992). At nanomolar concentrations, both native and synthetic startrak affect a variety of cellular processes in starfish sperm, including increasing O_2 uptake and K^+-activated Ca^{2+} transport and changing pHi and levels of cyclic AMP and cyclic GMP. A comparison of ion flux and second messenger responses produced by SAPs in sea urchin sperm with the actions of startrak or its synthetic peptide in starfish sperm indicated at least 10 significant differences, particularly in the regulation of $[Ca^{2+}]_i$ (D. F. Babcock and R. L. Miller, unpublished results). The physiological response to a chemoattractant signal may not be identical in sea urchin and starfish sperm.

A comparison of the structure of startrak and SAPs

In excess of 70 SAPs have been characterized and most of these are decapeptides or tetradecapeptides showing little homology between orders (Suzuki *et al.* 1982; Suzuki and Yoshino, 1992; Yoshino and Suzuki, 1992). The known sequence of startrak shares no similarity with any of the SAPs or SAP propeptides. Asx-rich 12 kDa peptides that enhance the acrosome reaction have been isolated from the egg jelly of the western Pacific forcipulate starfish *Asterias amiurensis*. Their sequences have not yet been compared with that of startrak and they have yet to be tested for sperm-attracting activity (Hoshi *et al.* 1994; M. Hoshi, personal communication). A crude ovarian extract of *Asterias amiurensis* attracts the sperm of the forcipulate starfishes *Asterias forbesi* and *Pycnopodia helianthoides* (R. Miller, unpublished data).

Other sequence comparisons

The startrak N-terminal fragment sequence resembles a Q-linker, a stretch of amino acids that possesses a high degree of structural flexibility that is typically found at the ‘boundaries of structurally and functionally distinct but otherwise interacting domains of regulatory or sensory transducing proteins’ (Sutrina *et al.* 1990). Their sequences are not conserved in otherwise homologous proteins (Vogler and Lengeler, 1991; Wootton and Drummond, 1989).

The sperm attractant of the hydrozoan leptomedusan *Orthopyxis compressa* (Freeman and Miller, 1982) is released suddenly during emission of the second polar body (Miller, 1978), a time when the animal pole fertilization site becomes receptive to sperm. Preliminary observations suggest that the *Orthopyxis compressa* attractant is associated with the egg vitelline envelope (R. L. Miller, unpublished observations). It is tempting to suggest that startrak is a diffusable, Q-linker-like fragment released by protease processing of membrane-bound receptor proteins at egg maturation. Confirmation of this hypothesis requires the preparation of a labeled antibody to determine the precise location of the sperm attractant in the egg.

The potent biological activity of the synthetic peptide suggests that the chemotactic activity of startrak depends on the sequence at the amino terminus of the native peptide, assuming that the molecular mass of 13 kDa is accurate. In contrast, the sperm respiratory stimulating activity of SAP-II (resact) is located at the C terminus of the molecule (Shimomura and Garbers, 1986). This type of comparison is hazardous, however, since different assays (increased guanylate cyclase activity for resact and speract; sperm attraction for startrak) were employed in these studies and the state of processing of the purified startrak molecule has yet to be determined. The function of the remaining two-thirds of the molecule is unknown, though a tandem repeat of the N-terminal active sequence, such as that in the propeptide of speract (Ramarao *et al.* 1990), is a possibility.

Do SAPs and startrak utilize the same receptor mechanism?

The speract receptor is an M_r 77 000 protein resembling the macrophage scavenger receptor that binds acetylated low-density lipoprotein (Doolittle, 1985; Guo *et al.* 1994). The resact receptor is a single transmembrane domain receptor (M_r 160 000) with a ligand-binding site oriented outside and guanylyl cyclase activity oriented inside the cell membrane. Both receptors have been sequenced, and cDNA clones have been prepared (Dangott *et al.* 1989; Singh *et al.* 1988). Activation of the receptor by resact leads directly to a transitory increase in cyclic GMP levels followed by an increase in metabolic activity, rapid dephosphorylation of the receptor and a decrease in the rate of cyclic GMP production (Ward and Vacquier, 1983; Ward and Kopf, 1993). The resact receptor is homologous with a family of membrane-bound guanylyl cyclases that are widely distributed in vertebrates and invertebrates (Schulz, 1992). There appears to be little homology between the resact and speract receptors; an important finding given the similarity of the responses induced (Suzuki *et al.* 1982).

The speract receptor may function in conjunction with another protein with guanylyl cyclase activity (Thorpe and Garbers, 1989), or cooperativity of multiple receptors for SAPs may occur (Yoshino and Suzuki, 1992). The failure of speract to induce a chemotactic response is puzzling (Hardy *et al.* 1994), but may be a consequence of the differences in receptor composition and function mentioned above.

Almost nothing is known about the nature of the startrak receptor. The SAP-I and SAP-II receptors may serve as models for understanding the underlying mechanisms of startrak activity. Like the SAP-I and SAP-II receptors (Harumi *et al.*

1991; Suzuki *et al.* 1987), the startrak receptor is confined to the sperm tail membrane (Miller and Crawford, 1994). The receptor is heat-sensitive in the absence of bound ligand and is not denatured in the presence of weak formalin. Its sequence, secondary structure and other properties remain to be elucidated.

The significance of sperm chemotaxis in nature remains unclear. It is probably not effective at distances of more than one or two egg diameters (0.2–0.5 mm) (Miller and King, 1983; Denny and Shibata, 1989; Levitan, 1993). Yet, for broadcast-spawning animals, any process that enhances the rate and specificity of sperm and egg interactions is likely to be selected for. Human sperm may be capable of chemotaxis (Ralt *et al.* 1991), suggesting that sperm attraction to eggs may occur during internal fertilization in vertebrates when the gametes are already in close proximity. Its widespread distribution and position in the hierarchical events of fertilization argue for its importance. Marine invertebrates represent outstanding model systems for investigating the molecular mechanisms underlying sperm attraction as well as its significance in the ecology of fertilization success.

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