Chemotaxis of *Arbacia punctulata* Spermatozoa to Resact, a Peptide from the Egg Jelly Layer

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ABSTRACT Resact, a peptide of known sequence isolated from the jelly layer of *Arbacia punctulata* eggs, is a potent chemoattractant for *A. punctulata* spermatozoa. The chemotactic response is concentration dependent, is abolished by pretreatment of the spermatozoa with resact, and shows an absolute requirement for millimolar external calcium. *A. punctulata* spermatozoa do not respond to speract, a peptide isolated from the jelly layer of *Strongylocentrotus purpuratus* eggs. This is the first report of animal sperm chemotaxis in response to a defined egg-derived molecule.

The sea urchin egg is surrounded by an extracellular investment known as the jelly layer. The major macromolecular component of the jelly layer is a high molecular weight fucose-sulfate–rich glycoconjugate that induces the sperm acrosome reaction (1–4). Several small peptides also have been isolated from the jelly layer. The best characterized of these peptides is speract or sperm activating peptide H2, isolated from the jelly layer of *Strongylocentrotus purpuratus* and *Hemicentrotus pulcherrimus* eggs (5–7). The sequence of speract has been determined (Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly; references 7 and 8), analogues have been synthesized (8, 9), and a 77,000-D (77-kD) speract receptor on the sperm surface has been identified (10). Treatment of *S. purpuratus* spermatozoa with speract increases sperm respiration and motility when assayed at pH 6.6–6.8 (5–7, 11). Speract has only a minimal effect on respiration and motility at pH 7.8–8.0 (5, 11). Speract also causes a transient increase in sperm guanosine 3′,5′-cyclic monophosphate (cGMP)[1] levels (5, 6, 8).

A different peptide, resact, has been isolated from the jelly layer of *Arbacia punctulata* eggs (12, 13). Its sequence is Cys-Val-Thr-Gly-Ala-Pro-Gly-Cys-Val-Gly-Gly-Gly-Arg-Leu-NH_2 (13). Like speract, resact increases the cGMP levels, respiration, and motility of *A. punctulata* spermatozoa at pH 6.6, but has little effect on respiration or motility at pH 7.8–8.0 (13). Both resact and speract are species specific; neither peptide will induce changes in motility or respiration at pH 6.6 in the heterospecific spermatozoa (12, 13). Since neither peptide markedly stimulates sperm respiration or motility at pH 7.8 (the pH of normal seawater), the biological function of these peptides remains unknown (12, 14).

We have shown that exposing *A. punctulata* spermatozoa to resact at pH 7.9 results in a change in the electrophoretic mobility (from 160 kD to 150 kD) of an abundant sperm membrane protein (13, 15). Several lines of evidence suggest that the mobility shift is due to a receptor-mediated (15–17) dephosphorylation of the protein (15, 16). We have identified the sperm phosphoprotein as guanylate cyclase (18). Correlated with the change in electrophoretic mobility is a 38-fold decrease in the specific activity of the enzyme (18). It is not clear why the activity of the enzyme (measured in vitro) decreases under conditions in which cGMP levels transiently increase in the intact cell; various possibilities have been discussed (16). Regardless of how this question is resolved, these results show that the egg possesses a mechanism to alter the cGMP metabolism of the spermatozoon.

Here we demonstrate that resact is a potent chemoattractant for *A. punctulata* spermatozoa. This is the first demonstration of animal sperm chemotaxis in response to a precisely defined, egg-derived molecule.

MATERIALS AND METHODS

Reagents: Artificial seawater (ASW) was formulated as follows: 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 27.1 mM $MgSO_4$, 4.4 mM $NaHCO_3$,

[1] *Abbreviations used in this paper*: ASW, artificial seawater; OCaSW, calcium-free seawater; cGMP, guanosine 3′,5′-cyclic monophosphate.

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10 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 0.5% (wt/vol) polyvinylpyrrolidone, pH 7.9. Calcium-free seawater (OCaSW) was ASW from which the 10 mM $CaCl_2$ had been omitted. Acid-solubilized whole *A. punctulata* egg jelly was prepared as described (15). Resact and speract were synthesized as described (8, 12, 13). Sodium dodecyl sulfate (SDS, L-5750) was from Sigma Chemical Co. (St. Louis, MO), and 6-carboxyfluorescein was from Eastman Kodak Co. (Rochester, NY).

Gametes: *Arbacia punctulata* were spawned by intracoelomic injection of 0.2 ml of 0.5 M KCl. Spermatozoa were collected from the gonopores with a pipette, diluted with 2 vol of ASW (or OCaSW), and stored on ice. Immediately before observation, the spermatozoa were diluted to a final concentration of 1.5×10^6 cells/ml with ASW (or OCaSW).

Microscopy: Sperm swimming behavior was observed using a 2.5× objective lens under dark field conditions. Illumination was provided by a LX150F lamp (ILC Technology Inc., Sunnyvale, CA), a Chadwick-Helmuth model 136 flash power supply (Chadwick-Helmuth Co., Inc., El Monte, CA), and a General Radio model 1310B audio oscillator (GENRAD, INC., Concord, MA) generating trigger pulses at 70 Hz. The stage temperature was maintained at 18°C, and spermatozoa swimming at the upper and lower surfaces of an open drop were photographed (1-s exposures) on Kodak Plus-X film.

A microinjection apparatus (19) was used to introduce test solutions into the sperm suspension. Typically, 1 nl of test material was injected into a 20-μl drop of sperm suspension (using a micropipette of 50-μm bore diameter). In some experiments, a tracer dye (6-carboxyfluorescein) was co-injected with peptide. Immediately after injection (1 nl, 100 nM resact, 0.5 mM 6-carboxyfluorescein), visible fluorescence was confined to a circle of ~250 μm at the tip of the pipette. The diameter of the injected drop increased to 500 μm by 1 min postinjection. In all experiments shown the micropipette was withdrawn after injection, as reflections from the glass obscured the photographic image.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE): To prepare samples for SDS PAGE, spermatozoa were pelleted from a suspension of 1.5×10^6 cells/ml by 8-min centrifugation at 2,000 *g* (2°C), resuspended to a final dilution of 10^7 cells/ml in ASW (or OCaSW) from which the polyvinylpyrrolidone had been omitted, and extracted with 10% (wt/vol) trichloroacetic acid. Processing of trichloroacetic acid–insoluble material for SDS PAGE was as described (15). Gels were silver-stained by the method of Morrissey (20).

RESULTS

Response of Spermatozoa to Resact

The normal swimming behavior of *A. punctulata* spermatozoa in an open drop of ASW is shown in Fig. 1 *a*. Spermatozoa thigmotactically trapped on the upper and lower surfaces of the drop (21, 22) were found to swim in circles of 50–60-μm diam. The typical response of spermatozoa to 10 nM resact delivered into the drop by microinjection is shown in Fig. 1, *b–e*. Within seconds after injection of resact, many spermatozoa in the area of the injected material were swimming in straighter trajectories, and a slight accumulation of spermatozoa appeared to be forming (Fig. 1 *b*). By 40 s, the cluster had grown and a sperm-depleted zone surrounding the cluster was evident (Fig. 1 *c*). As resact diffused from the area of injection, both the number of spermatozoa recruited into the cluster and the size of the cluster continued to grow (Fig. 1, *d* and *e*). Eventually (90 s) the accumulated spermatozoa started to disperse, although the difference in track shape was still clearly evident (Fig. 1 *f*). At all times spermatozoa outside the zone of influence of the injected resact continued to swim,

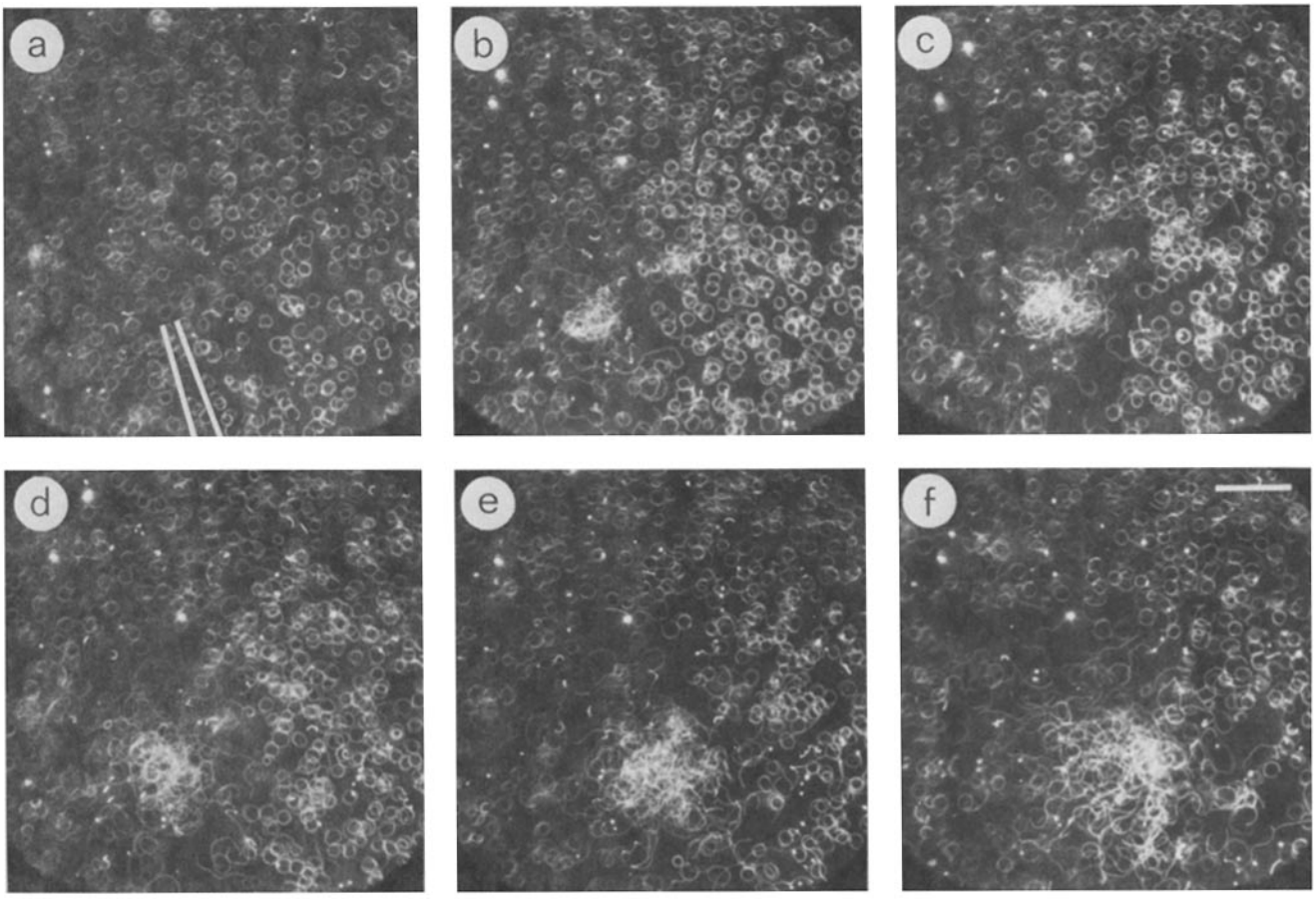

FIGURE 1 Effect of 10 nM resact on *A. punctulata* spermatozoa. A micropipette was inserted into a 20-μl drop of sperm suspension (position of the micropipette is indicated in *a*). 1 nl of 10 nM resact was injected into the drop and 5 s later the micropipette was removed. Photographs (1-s exposures) were taken (*a*) 5 s before insertion of the micropipette, and (*b*) 20 s, (*c*) 40 s, (*d*) 50 s, (*e*) 70 s, (*f*) 90 s postinjection. Bar, 200 μm. × 34.

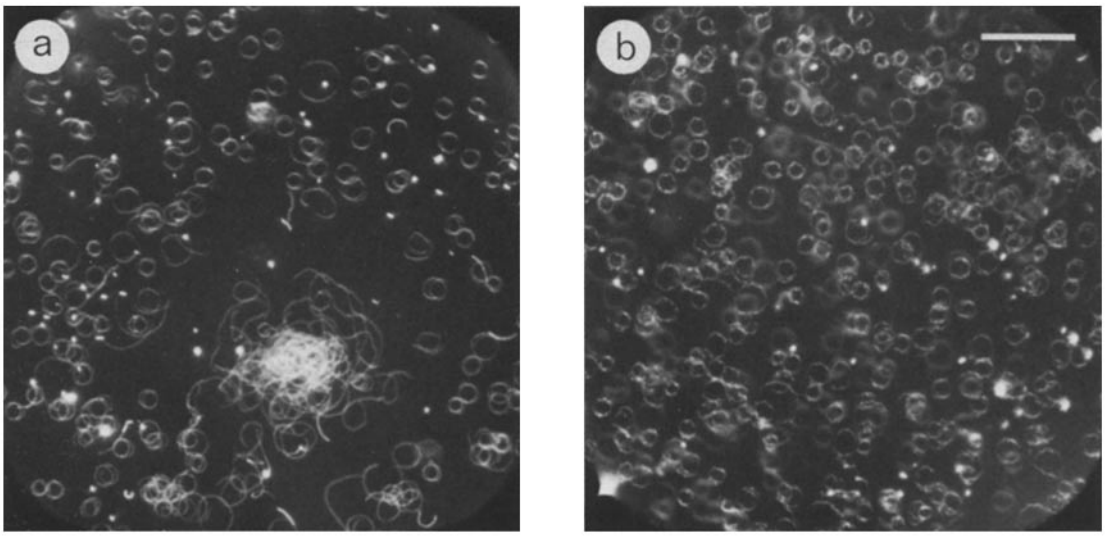

FIGURE 2 Comparison of the effects of (*a*) 100 nM resact and (*b*) 10 μm speract on *A. punctulata* spermatozoa. Sperm tracks were photographed 50 s after peptide injection. In both cases, peptide was injected just below the center of the field. Bar, 300 μm. × 41.

in place, in 50–60-μm circles. Injection of whole acid-solubilized *A. punctulata* egg jelly had qualitatively the same effect as resact (not shown). However, injection of an equivalent volume of speract, at concentrations as high as 10 μM, had no effect on either the swimming behavior or the distribution of the spermatozoa (Fig. 2*b*).

The effects of resact on sperm swimming behavior were dose-dependent. Injection of 100 pM resact had no effect (Fig. 3*a*). A response was first detectable at 1 nM resact, which elicited a definite change in swimming behavior, but no accumulation (Fig. 3*b*). At 3.3 nM resact both a change in swimming pattern and an accumulation of spermatozoa were evident (Fig. 3*c*). At increasing concentrations of resact, the number of spermatozoa recruited into the cluster and the tightness of the cluster increased (Fig. 3, *d–f*), as did the length of time for which the cluster persisted (not shown). When spermatozoa at the concentration used for microscopic observation (1.5×10^6 cells/ml) were exposed to solutions of resact, the electrophoretic mobility shift of the sperm guanylate cyclase (15) also showed a concentration-dependent response to resact (Fig. 3, lower panel): no effect at 10 pM, slight mobility shift at 100 pM, a nearly complete shift at 1 nM, and complete shift from 3.3 nM–1 μM. Since resact microinjected into a drop of sperm suspension will diffuse away from the area of injection, the actual concentration of resact that elicited a threshold chemotactic response (Fig. 3*b*, upper panel) was probably slightly less than 1 nM. This correlates well with the minimum amount of resact required to induce the mobility shift of the guanylate cyclase (100 pM–1 nM; Fig. 3, *b* and *c*, lower panel).

Pretreatment of the Spermatozoa with Resact

Prior exposure of spermatozoa to concentrated solutions of resact abolished their response to resact from the micropipette. A small volume of concentrated sperm suspension (2.5×10^9 cells/ml) was incubated in either 1 μM resact or ASW (control). After 2 min, the spermatozoa were diluted 1,000-fold, and resact was added to the control sample to a final concentration of 1 nM (both sperm suspensions then contained 1 nM resact). The control spermatozoa showed a good chemotactic response to 1 μM resact (Fig. 4*a*). In contrast, the spermatozoa pretreated with resact showed no chemotaxis to 1 μM resact (Fig. 4*b*).

Effect of External Calcium

The clustering of spermatozoa in response to resact required millimolar concentrations of calcium in the surrounding seawater. The extent of accumulation and tightness of the clusters decreased markedly from 10 mM Ca^{2+} (the concentration in normal seawater) to 3.5 mM Ca^{2+} (Fig. 5, *a* and *b*). The response was even less evident at 1.15 mM Ca^{2+} (Fig. 5*c*), and a cluster was just barely detectable at 0.5 mM Ca^{2+} (Fig. 5*d*). At calcium concentrations of 0.15 mM or lower, no accumulation was seen (Fig. 5*e*). Although the clustering response was blocked at these lower calcium concentrations, resact still induced a change in swimming behavior, manifest as an increase in track diameter (from 50–60 μm to 80–100 μm; Fig. 5, *d* and *e*).

DISCUSSION

The chemotaxis of animal spermatozoa to eggs or secretions from the female reproductive system is a widespread phenomenon. Sperm chemotaxis has been demonstrated in four major phyla (Cnidaria, Mollusca, Echinodermata, and Urochordata), and suggestive evidence exists in the Bryozoa, Annelida, Chaetognatha, Nematoda, and the lower vertebrates (see reference 22 for review). Very little is known in any animal species about the mechanism by which the attractants influence the direction of sperm motility or the chemical nature of the attractants themselves.

Previous attempts to characterize animal sperm chemoattractants have been limited to descriptions of the chemical stability, electrophoretic behavior, and protease sensitivity of crude or partially purified attractant preparations. Using this approach it has been suggested that the attractants of certain echinoderms (22) and hydroids (23) might be peptides. A siphonophore sperm attractant has been partially characterized electrophoretically, but the chemical nature of the sub-

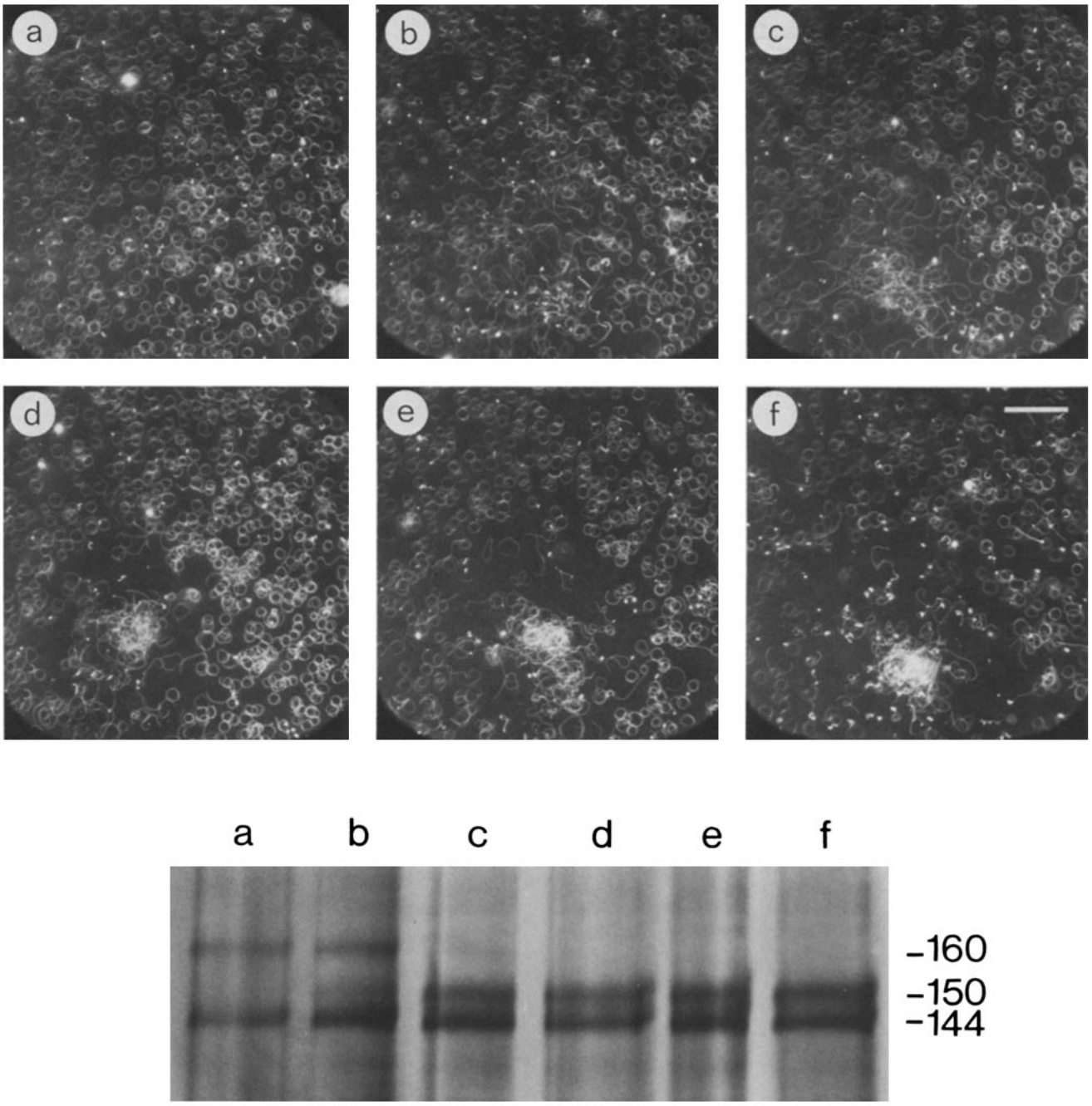

FIGURE 3 Effects of various concentrations of resact on sperm swimming behavior and on the electrophoretic mobility of guanylate cyclase. Upper panels, photographs were taken 40 s after the injection of 1 nl of various concentrations of resact into a 20-μl drop of sperm suspension (1.5×10^6 cells/ml). Injection in each case was just below the center of the field. Bar, 300 μm. $\times$ 34. Lower panel, resact was added to 50 ml of sperm suspension (1.5×10^6 cells/ml) to various final concentrations. After 2 min at 18°C the spermatozoa were processed for SDS PAGE as described in Materials and Methods. A portion of the silver-stained gel is shown, and the positions of the 144-kD membrane protein (15) and the 160- and 150-kD forms of the guanylate cyclase are indicated. In previous experiments (13, 15), it was shown that the 160- to 150-kD mobility shift is induced within 5 s after the spermatozoa are exposed to resact. (*a*) 100 pM, (*b*) 1 nM, (*c*) 3.3 nM, (*d*) 10 nM, (*e*) 100 nM, (*f*) 1 μM resact.

stance is unknown (24). Our results are the first description of animal sperm chemoattraction in response to a defined molecule of egg origin.

This work also represents the first demonstration of sperm chemotaxis in the sea urchin (see also references 22 and 25a). The chemotactic response to resact is distinctly different from the swarming response of sea urchin spermatozoa to solubilized egg jelly (25). The chemotactic response to resact requires millimolar external calcium (Fig. 5), whereas *A. punctulata* spermatozoa will swarm in response to egg jelly in OCaSW containing 1 mM EGTA (Ward, G., unpublished observations). Resact induces the mobility shift of the guanylate cyclase under calcium-free conditions (Ward, G., unpublished observations), demonstrating that the spermatozoa are still able to bind the peptide in the absence of external calcium. Speract binds to *S. purpuratus* spermatozoa in the absence of

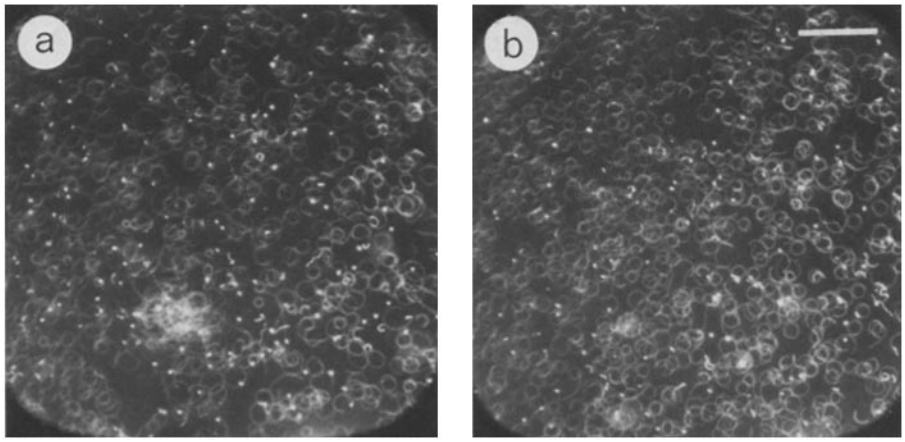

FIGURE 4 Pretreatment of spermatozoa with 1 μM resact abolishes the ability of the spermatozoa to respond chemotactically to resact from the micropipette. Spermatozoa were diluted to 2.5×10^9 cells/ml in either ASW (*a*) or 1 μM resact (*b*). After 2 min at 18°C, 50 μl of each suspension was removed and diluted 1,000-fold with ASW. 50 μl of 1 μM resact was then added to the ASW sample, and a 20-μl drop of each suspension was put on a slide. 1 nl of 1 μM resact was injected into each drop near the center of the field. Sperm tracks were photographed 40 s after injection. Bar, 300 μm. × 34.

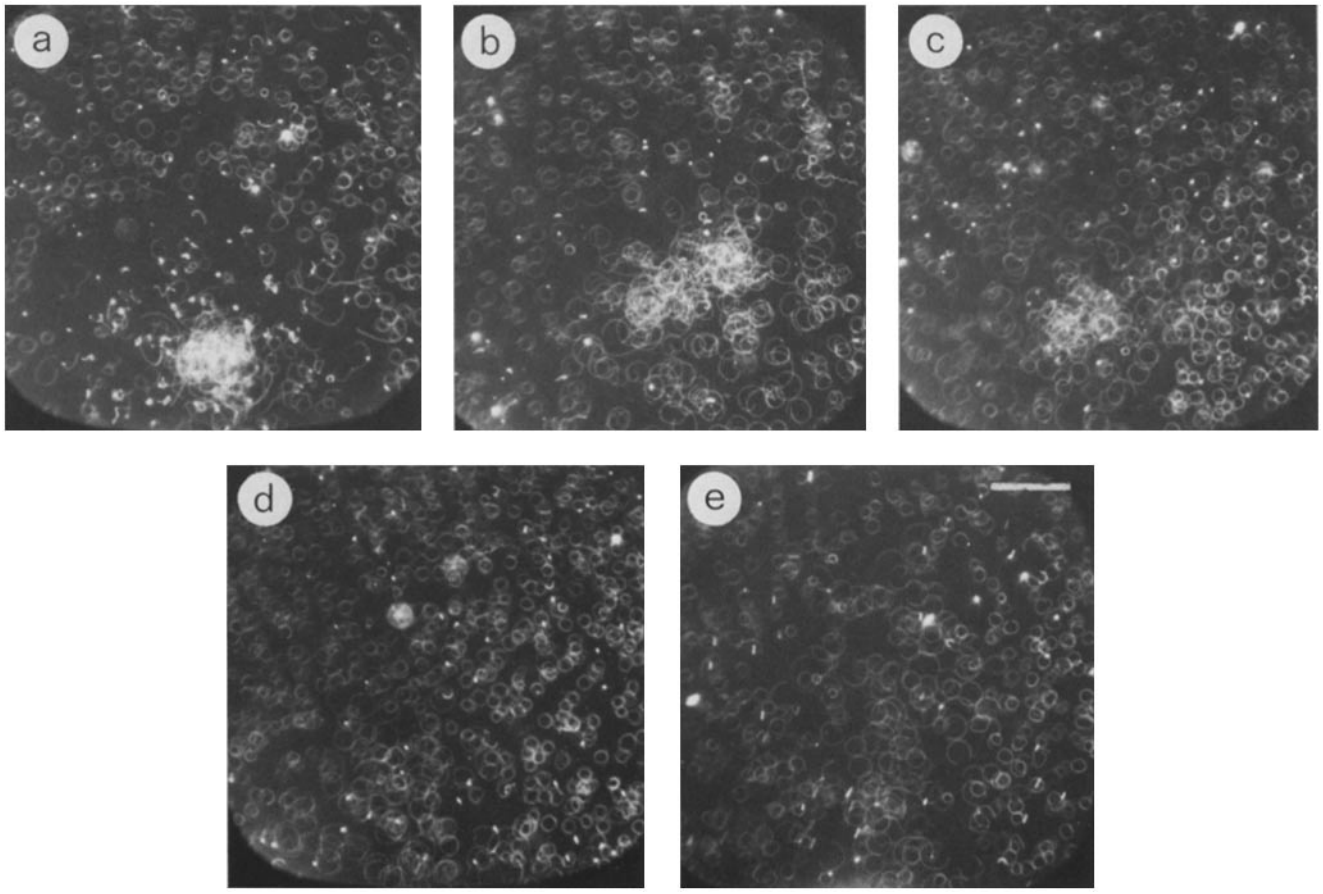

FIGURE 5 Resact acts as a chemoattractant only in the presence of millimolar external calcium. ASW and OCaSW were mixed to give final Ca^{2+} concentrations of (*a*) 10 mM, (*b*) 3.5 mM, (c) 1.15 mM, (*d*) 0.5 mM, (e) 0.15 mM. Solutions of resact (1 μM) and suspensions of sperm (1.5×10^7 cells/ml) were prepared at each calcium concentration. A 20-μl drop of sperm suspension was put on a slide, and photographs taken 30 s after injecting 1 nl resact. In all cases, injection was in the middle lower half of the field. Bar, 300 μm. × 34.

external calcium (26).

Spermatozoa pretreated with concentrated solutions of resact do not respond to resact from the micropipette (Fig. 4). If the dissociation of resact from its surface receptor (15–17) is relatively slow (as appears to be the case with speract and the speract receptor [26]), it may be that the pretreated spermatozoa cannot respond chemotactically to resact because their resact receptors are already occupied. Studies of receptor occupancy await the synthesis of radioactive resact.

The observation that the chemotactic response shows an absolute dependence on external calcium may be significant in terms of understanding the basic mechanism underlying sperm chemotaxis. Calcium has a profound effect on the asymmetry of the flagellar waveform in detergent-demembranated, reactivated sea urchin spermatozoa (27–29). These effects have been reproduced with *A. punctulata* spermatozoa (Brokaw, C. J., unpublished observations). Since directed turning of a spermatozoon towards a chemoattractant probably involves changes in the symmetry of the flagellar beat (30, 31), levels of calcium in the axoneme may modulate the

chemotactic response. The dependence of sperm chemotaxis on external calcium has been observed in other organisms (24, 30, 32–34).

Sea urchin gametes may represent a useful model system for studying the basic mechanisms underlying sperm chemotaxis and animal cell chemoreception. We have previously shown that resact induces a change in the phosphorylation state and enzymatic activity of sperm guanylate cyclase (13, 15, 16, 18). Guanylate cyclase is heavily enriched in the sperm flagellum (15, 35, 36). The results presented here demonstrate a correlation between the effects of resact on guanylate cyclase (as reflected by the enzyme's electrophoretic mobility) and the ability of the peptide to act as a chemoattractant (Fig. 3). These results raise the possibility that cGMP and changes in cGMP metabolism may be involved in some aspect of the chemotactic response. While the evidence at this point is strictly correlative, the fact that invertebrate spermatozoa in general (and sea urchin spermatozoa in particular) contain extremely high levels of guanylate cyclase activity (37, 38) makes the possibility intriguing.

We thank Dr. Hiromi Shimomura for his generous gift of synthetic resact and Sandee Nagayama for technical assistance.

This work was supported by National Institutes of Health grants HD-12896 to V. D. Vacquier, GM-18711 to C. J. Brokaw, and HD-10254 to D. L. Garbers.

Received for publication 12 June 1985, and in revised form 22 August 1985.

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