0014-4754/93/490593-03$1.50 + 0.20

Two-step acquisition of motility by insect spermatozoa

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Received 14 December 1992; accepted 16 March 1993

Abstract. High motility of eupyrene sperm of a grasshopper (*Omocestus ventralis*) was induced by cAMP. Both trypsin and cAMP were necessary for high motility of eupyrene sperm of three other species of grasshoppers. The same was found for apyrene sperm of the silkmoth, *Bombyx mori*, when they had been washed free from seminal plasma. On the other hand, in locusts (*Locusta migratoria*), in which the yellow gland has high Arg-C endopeptidase activity, sperm motility was induced by trypsin, like that of apyrene sperm of Lepidoptera. Thus in these two orders of Insecta, sperm motility appears to be induced by the same two-step process: the first step by Arg-C endopeptidase, and the second by cAMP.
Key words. Motility acquisition of spermatozoa; insects; Arg-C endopeptidase; cAMP; initiatorin.

In the silkmoth *Bombyx mori*, a factor dissociating eupyrene (nucleated) sperm bundles, and also a factor activating apyrene sperm, are known to be localized in the glandula (g.) prostatica[7,8,11,14]. The addition of bovine trypsin to seminal fluid from the v. seminalis of this insect led to both the dissociation of eupyrene bundles, and the acquisition of motility by apyrene sperm[15,17]. This indicated that the two factors involved in sperm maturation may be identical.

Another protease, initiatorin, an Arg-C endopeptidase that cleaves proteins on the C-side of arginine residues, also induces both dissociation of eupyrene bundles and apyrene motility. In *Bombyx*, this enzyme is localized only in the g. prostatica[1], the most distal exocrine gland of the male reproductive tract[14,16]. However, when the apyrene spermatozoa were washed free of seminal plasma, both cAMP (cyclic adenosine 2′:3′-monophosephate) and an Arg-C endopeptidase were necessary for acquisition of motility[18].

In this paper, a study of the mechanism of induction of motility of spermatozoa of five species of orthopterans is described, and compared with the process in the silkmoth *Bombyx*. Unlike Lepidoptera, but like other animals, Orthoptera have a single type of spermatozoa, the eupyrene type. The mode of acquisition of motility could differ in insects of these two orders, and also in apyrene and eupyrene spermatozoa.

Materials and methods

Freshly emerged males of *Locusta migratoria* were kept in an incubator at 20 °C. Males of four species of short-horn grasshoppers, *Euchorthippus declivus*, *Chorthippus longcornis*, *Ch. dorsatus* and *Ommocestus ventralis*, were collected in a field in the suburbs of Siena, Italy. Unmated but fully mature male grasshoppers were selected for use.

Induction of sperm motility was assessed microscopically, as described previously[15,17,18]. The seminal fluid containing immotile eupyrene spermatozoa from the yellow gland of unmated grasshoppers was diluted with Ringer solution to 20 placed on a glass slide and mixed with 10 µl of trypsin (Type I Sigma, USA: final concentration, 2–5 µM) and/or cAMP (Sigma, USA: final concentration, 20–100 µM) in Ringer solution. Motility was examined under the 5, 10, 15 and 20 min after mixing. A control sperm suspension was mixed with Ringer solution.

For the assay of endopeptidase activity, pooled specimens of the yellow gland, the white gland and the transparent gland from five 14 day-old unmated adult male locusts were homogenized in 50 µl of cold 50 mM Tris-HCl buffer (pH 7.8) containing 0.1% cetyltrimethyl-ammonium bromide (CTAB, Wako Pure Chemicals, Osaka, Japan). The homogenates were centrifuged at 4500 × g for 20 min at 4 °C and the supernatants were used for assay of Arg-C endopeptidase using BAEE (N_α-benzoyl-L-arginine ethylester, Sigma Chemical Co., St. Louis, U.S.A.).

One unit (U) of BAEEase activity was defined as the activity hydrolyzing 1 µmole of BAEE per min[1,22,25]. The reaction mixture contained Tris-HCl buffer (pH 9.0), 1.0 mM BAEE and 100 µl of enzyme preparation in a final volume of 500 µl. Assays were carried out in 10 mm quartz cuvettes at 25 °C. The reaction was started by adding the enzyme preparation and the change in absorbancy was monitored with an MPS-200 spectrophotometer (Shimadzu, Kyoto, Japan) at 253 nm. The amount of BAEE hydrolyzed was calculated, taking the molar absorbancy coefficient as 1150 $cm^{-1} \cdot M^{-1}$. Activity was determined in four replicate samples. The protein concentration in the enzyme preparation was determined colorimetrically with Folin-Ciocalteau's phenol reagent[4].

Results and discussion

Acquisition of sperm motility in Orthoptera

The modes of acquisition of motility of eupyrene spermatozoa of different species of Orthoptera varied. It was induced by trypsin or cAMP alone, or trypsin plus cAMP (table 1). The spermatozoa of *Locusta migratoria* acquired motility on treatment with trypsin, like the apyrene spermatozoa of Lepidoptera[17,21]. But the spermatozoa of three of the species of small Italian grasshoppers, *Euchorthippus declivus*, *Chorthippus longicornis* and *Ch. dorstatus*, acquired motility only slowly on treatment with trypsin alone. Addition of cAMP was needed for them to swim actively, but cyclic AMP alone did not activate these spermatozoa. Thus, their activation mechanism seems to be similar to that of washed *Bombyx* apyrene spermatozoa[18]. In *Omocestus ventralis*, high motility of eupyrene spermatozoa was induced by cAMP alone, but not by trypsin alone, or by trypsin plus cAMP.

These findings show that two steps are involved in the process of acquisition of motility of orthopteran spermatozoa: the first induced by a trypsin-like endopeptidase, and the second by cAMP. This is a similar two-step process to that which occurs in Lepidoptera.

In *Omocestus ventralis*, the first step had apparently been completed by an endopeptidase in vivo, so the motility of isolated spermatozoa could be induced by the addition of cAMP only. Indeed, added trypsin seemed to impair the process induced by the endogeneous endopeptidase. (It has been found in *Bombyx* that there is an optimal concentration range of trypsin for inducing motility of spermatozoa, and that the spermatozoa were not activated when the trypsin concentration was above or below this level[17].) In *Locusta migratoria*, as in *B. mori*, addition of trypsin alone induced high sperm motility, presumably because sufficient cAMP was present in the seminal plasma to induce active movement[13].

Acquisition of sperm motility in other insect species. There have been few studies on the mechanism of acquisition of motility of insect sperm. In some species of saturniid moths, various compounds were tested for induction of motility of apyrene sperm[21]. With sperm of *Hyalophora cecropia* and *Antheraea polyphenmus*, high concentrations of trypsin and chymotrypsin induced motility, but cAMP and ATP did not. From these findings a peptide, but not the protease, was thought at that time to be the natural inducer of sperm motility[22].

Motility of apyrene spermatozoa and the flagellar membrane. Large ultrastructural differences can be observed between the cell membranes of motile and immotile apyrene spermatozoa of *B. mori* under natural conditions[13]. Immotile apyrene spermatozoa have a thick, compact cell membrane, whereas motile spermatozoa have a thin cell membrane with numerous microslits. Motile eupyrene spermatozoa of some lepidopterans were found to have a similar thin cell membrane with microslits[20]. Moreover, the same ultrastructural changes in the cell membrane were observed on treatment of immotile spermatozoa with Arg-C endopeptidase to induce motility[13,19].

Polysaccharide is present on the surface of the cell membrane of *Bombyx* sperm, as in other insects[2], as an extracellular matrix, and forms glycoprotein by binding with the surface protein. Sperm motility probably results from selective degradation of this glycoprotein by an Arg-C endopeptidase, and then deposition of cAMP on the surface of the cell membrane or in the microslits[19].

Arg-C endopeptidase activity in the yellow gland of locusts. If the hypothesis that a two-step process is necessary for the complete induction of sperm motility in insects in general is correct, an Arg-C endopeptidase should be present in the 'g. prostatica' of all acridids, as it is in *Bombyx*. The structure in acridids which probably corresponds to the 'g. prostatica'[6] of *Bombyx*, is the male reproductive tract as a complex. This gland complex is distinguishable morphologically into three portions, a white gland, a large transparent gland and a small yellow gland (figure 1). The two former seem to consist of many blind tubes, but the third may be an open tube, because it contains many eupyrene sperma-

Table 1. Acquisition of motility of insect spermatozoa on treatment with trypsin and cAMP

Orthoptera Acrididae	Motility trypsin	cAMP	trypsin + cAMP
Locusta migratoria	++	−	
Euchorthippus declivus	+	−	++
Chorthippus longicornis	+	−	++
Ch. dorsatus	+	−	++
Omocestus ventralis	−	++	−

− immotility; + slightly motile; ++ highly motile or swimming

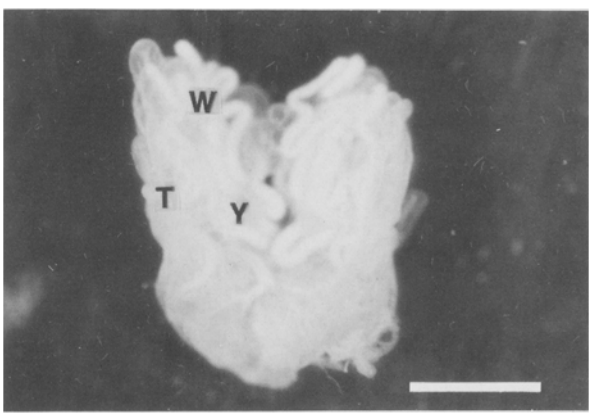

Figure. 'G. prostatica' of *Locusta migratoria*. This morphology and structure are very similar in other grasshopper species, but *Locusta* is much larger. Y, yellow gland; W, white gland; and T, transparent gland. Scale bar, 2.5 mm.

Table 2. Weights and BAEEase activities of three glands of the 'g. prostatica' complex of *Locusta migratoria* (n = 4)

	Weight of gland (mg/insect)	BAEEase activity (mU/mg tissue)	(mU/mg protein)
Yellow gland (Vesicula seminalis)	5.9 + 1.4	14.21 + 0.65	179.7 + 8.2
White gland	14.7 + 4.5	7.15 + 0.97	68.1 + 9.2
Transparent gland	16.5 + 3.1	4.58 + 0.64	62.8 + 8.8

tozoa derived from the testis. Therefore, this gland can be regarded as the true v. seminalis, being the storage organ for spermatozoa until their ejaculation. It is called the yellow gland, because it has a yellow substance, probably carotenoid, on its surface. The other two glands contain white and transparent secretions, respectively.

The Italian acridid grasshoppers used here were too small to allow examination of Arg-C endopeptidase activity in the glands. However, in the bigger grasshopper *Locusta migratoria*, BAEEase activity was found in all three glands of the g. prostatica complex, its activity being especially high in the yellow gland (table 2). The spermatozoa accumulated in the v. seminalis are immotile, but after their ejaculation, Arg-C endopeptidase secreted from cells of this gland presumably plays a part in their activation.

In conclusion, is it imprudent to generalize about the mechanism for acquisition of motility of spermatozoa in the class Insecta as a whole? Lepidoptera have two types of spermatozoa, apyrene and eupyrene. Orthoptera, which are taxonomically and phylogenetically very different, have only eupyrenes. Moreover, during mating both Lepidoptera and Orthoptera form spermatophores[9], but the modes of formation and transfer to the female are different. Nevertheless, in species of these two orders of Insecta, in both Holometabola and Hemimetabola, sperm motility has been found to be induced by the same two-step process.

In the spermatophore of *Bombyx*, a system for energy supply for protein, the arginine degradation cascade[14], which is initiated by the prostatic endopeptidase initiatorin, is involved in sperm maturation. A similar metabolic system has been found in the uterus of the fruit fly, *Drosophila melanogaster* (Diptera), which has only eupyrene spermatozoa and forms no spermatophore[12].

Thus there is evidence that in Insecta, although there are many different morphological and behavioral types of reproduction, certain fundamental physiological features are very similar. The process of activation of spermatozoa may also be similar in other animals; in this connection, it is interesting that cAMP also induces motility of spermatozoa of fish and mammals that have been demembranated with Triton-X[5, 10], and that motile mammalian spermatoza are inactivated by serine-protease inhibitors, although no natural endopeptidase for induction of their motility has yet been found[3]. A cAMP-dependent protein kinase is required for flagellar activation of sperm in the dog[24].

Acknowledgments. We thank Dr K. Yamazaki, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Hachiohji, Tokyo, Japan for supplying locusts. We also thank Professor P. S. Chen, Department of Biology, Zürich University, Zürich, Switzerland for revising this manuscript. This work was supported in part by research grants (No. 02304020 and 03455026) from the Ministry of Education, Science and Culture of Japan.

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