CALCIUM- AND CADMIUM-BINDING PROTEINS OF SCALLOP (PLACOPECTEN MAGELLANICUS) SPERM FLAGELLA

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Calcium is well-established as an important "second messenger" that controls the motility of eukaryotic flagella and cilia. In general, an increase above basal levels in the intraflagellar (intraciliary) concentration of calcium (Ca^{2+}) brings about a transient "response state" during which the flagellar beat pattern is altered. After the brief physiological response, normal "steady-state" patterns of flagellar beat are restored (Sanderson, M.J., 1984, in Biology of the Integument, v.1, The Invertebrates, pp.36-38, Springer, Berlin). The particular type of stimulus (e.g., light, chemicals, mechanical stimuli) that elicits this response state and the nature of the Ca^{2+}-induced response itself (eg, arrest, reversal of beat direction, increased asymmetry of beat) are specific properties of the cell type in question. Many invertebrates including scallops reproduce by external fertilization, and a key component in this process is apparently chemotactic swimming of the sperm toward the egg (Brokaw, C.J., 1987, J. Cell. Biochem. 35:175-184). It is believed that the Ca^{2+}-dependent responses mentioned above underly this chemotactic behavior of sperm; however, the mechanism by which Ca^{2+} controls flagellar (or ciliary) beat is as yet unclear (Otter, T., 1989, in Cell Movement, v.1, The Dynein ATPases, pp.281-298, A.R. Liss, NY). As a first step in clarifying the Ca^{2+}-dependent mechanism(s) that control scallop sperm flagellar beat, I have sought to identify the flagellar calcium-binding proteins (CaBPs) that may be involved in this control. Thus, the studies described below are the start of a series of invesigations on the role of Ca^{2+} in scallop sperm motility and chemotaxis.

The sea scallop Placopecten magellanicus has rarely been the subject of study at MDIBL (at least in the last decade) and, in general, records of their whereabouts in the Frenchman's Bay area and their reproductive cycle (especially for male scallops) were difficult to obtain. In this report I have attempted to begin recording this information.

Scallops (P. magellanicus; n=21) were collected by bottom dragging (ca. 40 fathoms) from Frenchman's Bay or by SCUBA divers (1-2 fathoms) near Bartlett's Landing at Bartlett Narrows in Western Bay. The sex, shell diameter, total wet weight of visceral organs including the mantle, and gonad wet weight were measured and recorded (Table 1). The data were grouped according to sex and according to the month of collection, although several individuals were collected in June but not sacrificed until the end of July. During dissection of the gonad, care was taken to excise the gonad intact, extrude the gut contents, and trim away any adhering nephridial tissue before weighing the gonad. Thus gonad wet weight is probably accurate to within 5% or less. Females were so indicated by salmon-colored gonads that contained eggs. Males had cream-colored gonads that contained sperm. Sperm maturation was assessed by sperm morphology and motility of sperm extruded from testis tissue minced in filtered seawater containing 0.2mM EDTA (FSWE). In all males, vigorous motility was observed 2 - 30 minutes after mincing the testis (see below). This was true even for the male collected and examined on June 6; nevertheless, motility of sperm from the males collected in late June and throughout July appeared more vigorous and uniform than this early example (June 6). According to Langton et al. (1987, Mar. Ecol. Progr. Ser. 37:19-25; R.W. Langton, pers. comm.), development of Placopecten gonads begins in late spring and spawning takes place in September. Finally, the scallops collected at Bartlett's Landing were "hand-picked" at the site and

therefore probably represent some of the more mature individuals from that region. On the other hand, the sample of scallops from Frenchman's Bay (which included all of the females) is not biased in this way.

Table 1. Scallop size and gonad development.

	Shell Diameter (mm)	Gonad Wet Wgt. (g)	Gonad, % Wet Wgt.
FEMALES June 1989	100	nd	---
	104	nd	---
	86	imm	---
	94	10.29	19.2
	105	8.93	13.9
	110	12.36	15.7
	103	12.36	22.0
	90	6.99	16.2
		mean + S.D.	= 17.4 ± 3.2
MALES June 1989	73	nd	---
	87	6.21	nd
	101	8.57	15.3
	97	6.22	12.4
	125	20.21	18.7
	122	21.55	23.0
MALES July 1989	137	39.40	28.1
	123	25.11	24.5
	122	21.48	23.9
	111	24.30	27.6
	89	8.20	16.9
	95	6.39	12.9
	93	9.28	18.5
		mean + S.D.	= 20.2 ± 5.6

nd = not determined; imm = immature

To prepare sperm flagella, testis tissue was minced in two volumes of FSWE at 4°C and the suspension was stirred occasionally (on ice) for 30 minutes. During this period, sperm motility was assessed by phase contrast microscopy at 200x magnification (obj. n.a. = 0.4). In general, motility was poor immediately after mincing the tissue, but it improved rapidly over the next 5 minutes and remained vigorous for the next 25 minutes, until the end of the observation period. Percent motility approached 100% in the best preparations; the sperm swam in circles against the coverslip or slide surface.

The minced testis/ sperm suspension was filtered through four layers of cheesecloth that had previously been soaked and thoroughly rinsed 3x in ice-cold FSWE, and the filtrate was spun at 5000xg for 5 min. (4°C) to collect the sperm. The pellet was resuspended in FSWE and the remaining steps for separating heads from tails (Kontes Dounce tight pestle "B", 6-8 strokes), collecting tails (flagella) and demembranating the flagella with 1% Nonidet P-40 to yield "9+2" axonemes were essentially as described by Stephens (1986, Meth. Cell. Biol. 27:217-227). Final pellets of flagella or axonemes were resuspended in a small volume of 30 mM Tris, 3 mM $MgSO_4$, 0.1 mM EDTA, 1 mM PMSF, pH 8.0 to a protein concentration of ca. 20 mg/ml. This suspension was mixed 1:1 with "Laemmli 2X" sample buffer, heated in boiling water (90 sec), and then frozen and stored.

SDS polyacrylamide gel electrophoresis of sperm flagellar and axonemal polypeptides was performed according to Otter and Galgoci (1988, Cell Motility and the Cytoskeleton 11: 215-216) and Otter et al. (1987, Anal. Biochem. 162: 370-377) using either: 12% acrylamide, 4 M urea or 15% acrylamide, 4 M urea, or

12%-16% acrylamide, 1-8 M urea gradient minislabs. Gels were stained with Coomassie brilliant blue R-250. Stained gels were directly contact printed on Kodak EDP paper using an amber filter to enhance contrast.

Gel profiles of low M_r polypeptides in the flagella and axonemes are shown in Figure 1. Lanes 5- 8 are heavily loaded to reveal minor components and to indicate typical loadings for diagonal gels. In addition, this comparison reveals the consistency of protein composition among preparations and some of the reproducible differences between sperm axonemes (lanes 1-5, 7) and whole flagella (lanes 6, 8). Although tubulin (M_r50,000) and proteins migrating more slowly remain essentially unresolved on these high percentage gel slabs, the low M_r polypeptides (45,000 >M_r> 8,000) migrate as distinct bands (Fig 1A). Thus, this gel composition is well-suited for the analysis of flagellar calmodulin and related low M_r CaBPs. As described below, CaBPs were identified by a two-dimensional "diagonal" electrophoresis technique.

Diagonal electrophoresis is a two-dimensional analytical technique that I have devised to identify CaBPs in a complex mixture of proteins (Otter and Galgoci, 1988, ibid.). The first dimension of electrophoresis is carried out in the presence of EDTA to chelate Ca^{2+}. The gel lane of interest is cut out of the slab and mounted on top of a second gel containing Ca^{2+}. With the axis of the second dimension perpendicular to the first, the proteins form a diagonal pattern, except for those polypeptides whose migration is Ca^{2+}-dependent. These

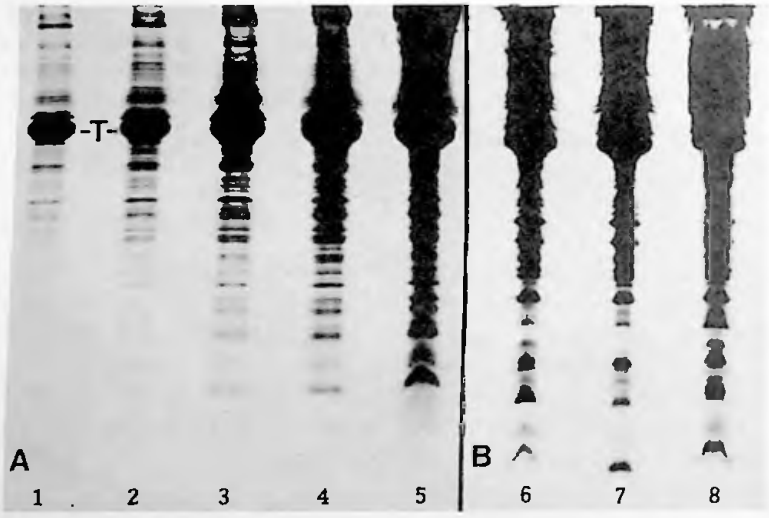

Figure 1. Scallop flagellar proteins separated on a 12-16% acrylamide, 1-8M urea gradient gel slab (A) or a 12% acrylamide, 4M urea slab (B). Dilution series of axonemal proteins (A) reveals resolution of low M_r bands; (B) shows reproducible differences between axonemes and whole flagella and indicates typical loadings for diagonal gels (see Fig. 2). T = tubulin, M_r50,000.

lie conspicuously off the diagonal (see Figure 2). By systematically varying the Ca^{2+}-concentration in the second dimension, one can estimate the affinity of the protein for Ca^{2+}. The basis for this assay is a difference in the electrophoretic mobility of a protein in the presence of a ligand (e.g., Ca^{2+}) and in its absence. Diagonal electrophoresis is therefore a type of affinity electrophoresis, and the type of ligand can be modified to identify proteins with other specific binding properties. For example, by substituting Cd^{2+} for Ca^{2+} in the second dimension I have identified three Cd^{2+}-BPs in sperm flagella.

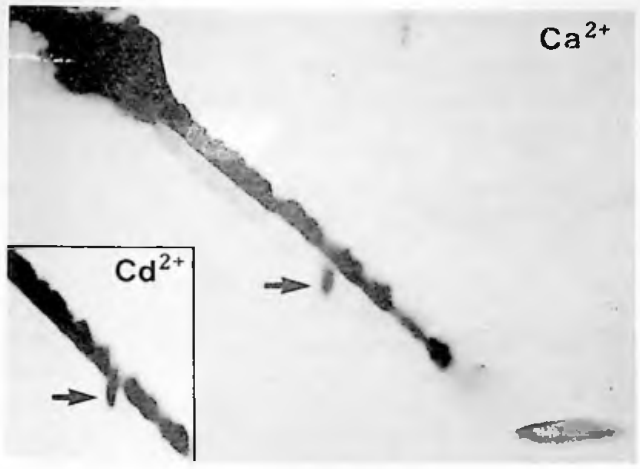

Figure 2. Diagonal electrophoresis of scallop sperm flagellar proteins reveals a major CaBP (arrow). Inset: a flagellar cadmium-binding protein, probably the same polypeptide as the CaBP, is detected. Second dimension of electrophoresis contained 200μM calcium or 200μM cadmium (inset).

Analysis of scallop flagellar proteins by diagonal electrophoresis is shown in Figure 2. In this example, one major CaBP was detected at M_r 18,000 (determined by comparison with low M_r standards). This protein is probably calmodulin, which has been identified in sperm flagella from many other species and is a bona fide component of gill cilia isolated from the bay scallop Aequipecten irradians (Otter, T., 1989, ibid.; Stommel et al., 1982, J. Cell Biol. 92:622-628). When scallop axonemal proteins were analyzed on diagonal gels, two CaBPs were detected (not shown). One of them, at M_r18,000, appeared to comigrate with calmodulin; the second CaBP was smaller, M_r15,000. The origin and biochemical characteristics of this smaller CaBP are not known.

Cadmium (Cd^{2+}) is a well-known heavy metal toxin that affects smooth muscle, vascular walls, and some nervous tissue. In spite of this widespread toxicity, its cellular site(s) of action has not been clearly identified. As an approach to identify the cellular targets of Cd^{2+} toxicity, I set out to determine whether diagonal electrophoresis could be modified to reveal CdBPs in a complex sample. When $CdCl_2$ (200μM) was substituted for $CaCl_2$ in the second dimension of electrophoresis, three low M_r CdBPs were identified in scallop flagella. One of these was flagellar calmodulin (Fig. 2, inset). The other two CdBPs were identified at M_r±10,000, barely resolved from the diagonal; these may correspond to metallothioneins, which are known to bind Cd^{2+} in cells other than sperm. These two polypeptides were never identified as CaBPs, indicating that they bind metals selectively. On the other hand, both Cd^{2+} and Ca^{2+} bind tightly to calmodulin (Fig. 2; Suzuki, Y. et al., 1985, Archiv. Toxicol. 57:205-211). Thus, diagonal electrophoresis appears to be an appropriate technique for identifying cellular targets of cadmium toxicity.

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