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The Presence of the GABA-synthesizing Enzyme, Glutamate Decarboxylase, in *Spisula* Sperm

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Glutamate decarboxylase (GAD) is an enzyme that produces γ-aminobutyric acid (GABA), an inhibitory neurotransmitter. In many mammals, both GAD and GABA appear to be co-localized in various parts of the brain, but they are also found in pancreatic β-cells and testis. Two cDNAs coding for GAD were recently identified in rat (1). The cDNAs are products of two different genes on chromosomes 2 and 10, which encode for GAD-67 and GAD-65, respectively. In human pancreatic β-cells, GAD-65 appears to be the predominant form and was recently identified as an antigen for an autoantibody produced by insulin-dependent diabetes patients (2). The presence of GAD-like immunoreactivity has also been detected in the midpiece of ejaculated human spermatozoa (3), but no further information on the significance of this observation is available.

In this communication, we report evidence for the presence of GAD-active materials in homogenates of *Spisula* sperm. Homogenates of motile sperm contain significant GAD activity (0.5 nmol CO_2/min/mg protein). In addition, GAD-immunoreactive material can be identified in *Spisula* sperm homogenates. Two antibodies, AB108 and GAD-6, were used as probes for the Western blots. The former, a polyclonal antibody, recognizes GAD-67, and the latter, a monoclonal antibody, recognizes GAD-65. Each antibody is highly specific to its target GAD when mammalian brain tissues are examined. We have found that AB108 strongly stains protein bands with molecular masses estimated to be 79, 77, 58, 46, 44, and 28 kDa, and weakly stains 67 kDa band. On the other hand, GAD-6 stains bands corresponding to 76 and 28 kDa. Those bands at 77–79 and 76 kDa are somewhat larger than 67 and 65 kDa, which are observed for the mammalian GADs. The results suggest, although they are speculative, that the *Spisula* sperm GAD can be larger in size than the mammalian GADs.

We have previously found that a gossypolone-sepharose affinity column specifically absorbs surface macromolecules of *Spisula* sperm, with masses of 46, 44, and 28 kDa. This column does not, however, absorb rat GAD-65 expressed in yeast (Ueno, Kanai, Atomi, Ueda, and Tanaka, in prep.). When *Spisula* sperm proteins are passed through the gossypolone-sepharose affinity column, proteins of 79, 77, 76, and 58 kDa are not absorbed, but proteins of 46, 44, and 28 kDa are absorbed. The nonabsorbed cluster of macromolecules may be derived from sperm GAD, but further study is needed to determine whether these bands are related to GAD. Multiple bands are often observed in brain GADs from various species. For example, monkey and rat brain homogenates showed such multiple bands.

The present findings indicate that *Spisula* sperm exhibits GAD activity and contains immunoreactive GAD-like proteins. The results suggest that there may be GAD in *Spisula* sperm; thus, it raises an interesting question about the role of GABA in sperm function.

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Cytoplasmic Microtubule Arrays in *Oryzias latipes* (Medaka) Eggs During Ooplasmic Segregation

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Ooplasmic segregation in the fertilized medaka egg consists of the streaming of ooplasm toward the animal pole to form a blastodisc, the movement of oil droplets toward the vegetal pole, and the saltatory motion of small parcels toward both polar regions (1). In earlier studies we found that microtubule poisons and 5,5′-dibromo-BAPTA, a weak calcium buffer that dissipates the polar gradients of cytosolic free [Ca^{2+}] present during segregation (4), inhibit segregation in similar ways (1, 3). In the present study, using a protocol for indirect immunofluorescence developed by Gard (5), we characterized the microtubule arrays in medaka eggs during segregation and examined microtubule arrays in eggs injected with dibromo-BAPTA.

At regular intervals after fertilization eggs were fixed for 2–4 h at room temperature in a microtubule-stabilizing medium (3.7% formaldehyde; 0.05% glutaraldehyde; 5 m*M* EGTA; 1 m*M* $MgCl_2$; 80 m*M* PIPES, pH 6.8); fixed overnight in methanol at −20°C; permeabilized with 0.5% Triton X-100; treated with 100 m*M* $NaHB_4$; and incubated first with a monoclonal anti-alpha tubulin antibody (mouse, clone #DM-1A, ICN Immunobiologicals) and then with a rhodamine-labeled secondary

antibody. The eggs were examined by conventional fluorescence microscopy and also by laser scanning confocal microscopy. To correct for differences in the rate of development at different temperatures, we used a scale of normalized time (t_n) in which cytokinesis begins at $t_n = 1.0$ units.

In eggs fixed soon after fertilization ($t_n = 0.02$, Fig. 1A), we saw punctate sources of fluorescence. A loose network of microtubules, lacking any preferred orientation, was present in the interpolar regions of the egg by $t_n = 0.16$ (Fig. 1B); and by $t_n = 0.24$, the density of this network had increased (Fig. 1C). A microtubule-organizing center (MTOC) was present at the animal pole, as evidenced by the convergence of microtubules into this region. At the vegetal pole of the egg, a mat consisting of mostly parallel microtubules was present during ooplasmic segregation (Fig. 1D). The parallel organization of this mat was lost by $t_n = 1.0$. We saw no linear elements in eggs treated with microtubule poisons or in eggs that were not incubated with the primary antibody.

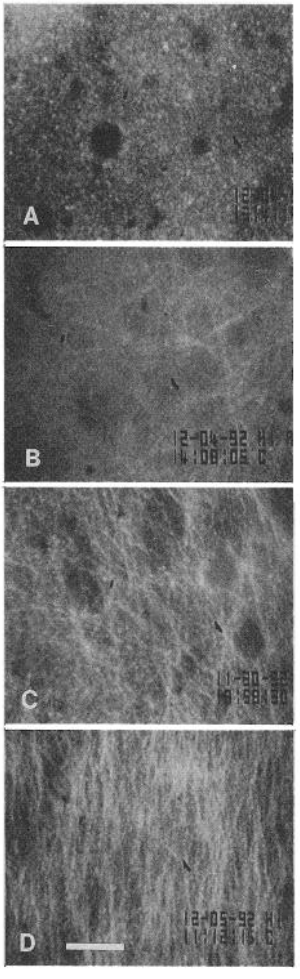

Figure 1. *Microtubule arrays in developing medaka eggs. (A) $t_n = 0.02$, equatorial region. Punctate foci of fluorescence can be seen, but no linear elements are apparent. (B) $t_n = 0.16$, equatorial region. A loose network of microtubules, lacking any preferred orientation, can be seen. (C) $t_n = 0.24$, equatorial region. The number of microtubules in the interpolar ooplasm has increased. (D) $t_n = 0.40$, vegetal pole. A mat of parallel microtubules can be seen. Scale bar, 10 μm.*

Fertilized eggs were injected with dibromo-BAPTA (50 m*M* dibromo-BAPTA, tetra potassium salt; 5 m*M* HEPES, pH 7.3) within 8 min after fertilization. Typically, the oil droplets in the hemisphere into which the buffer was injected were dislodged from their positions and floated to the top of the egg, but the oil droplets in the hemisphere opposite the injection site appeared to be frozen in place; that is, they moved neither toward the top of the egg nor to the vegetal pole (3). We also examined control eggs into which we had injected 150 m*M* KCl and 5 m*M* HEPES, pH 7.3. All eggs were fixed at $t_n = 0.76$.

When we injected sufficient dibromo-BAPTA to raise the cytosolic concentration to 2.7 m*M*, microtubules near the animal pole no longer converged into this region as they did in control eggs, suggesting a disruption of the MTOC. However, the mat of parallel microtubules at the vegetal pole was apparently unaffected by dibromo-BAPTA. Interpolar ooplasm near the injection site contained a very sparse network of microtubules, whereas ooplasm on the opposite side of the egg, where oil droplets were frozen in place, contained a microtubule network having a density comparable to that seen in control eggs. In both regions, the proportion of very long microtubules was greater in eggs receiving dibromo-BAPTA. Eggs into which we injected 150 m*M* KCl were indistinguishable from untreated eggs.

In summary, we have demonstrated (a) the development of a complex pattern of cytoplasmic microtubules during ooplasmic segregation in the medaka egg and (b) the alteration of this pattern by injection of the dibromo-BAPTA. Two features of this pattern—the apparent presence of a MTOC near the animal pole and the development of an array of microtubules between fertilization and the first cleavage—have been seen in developing sea urchin (6) and *Xenopus* eggs (2, 5), and the mat of parallel microtubules at the vegetal pole of the medaka egg is very similar to the one present in *Xenopus* eggs (2). The results of the present study are consistent with the suggestion that microtubules have a role in the propulsion of oil droplets toward the vegetal pole of the medaka egg during ooplasmic segregation (1) and that cytosolic calcium gradients are important for the development of the pattern of microtubules in the medaka egg.

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