triguing that in the teleost retinas entirely separate horizontal cells have evolved for rods and cones (*11*), whereas in mammals one cell of the axon-bearing variety appears to perform both functions. A possible advantage in having single neurons with electrically isolated regions is that the number of independent integrating units within the brain (*12*) can be increased without adding more cells. It would therefore not be surprising if similar arrangements were present elsewhere in the central nervous system.

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8. The steady state attenuation of signals conducted passively through the axon from the cell body to the terminal arborization was calculated for the cell of Fig. 1, a and b, by using equation 3 of Rall [*Exp. Neurol.* **1**, 491 (1959)], setting B_1 of this equation equal to R_∞/R_t (see equation 13, *ibid.*); R_∞ is the input resistance of a semi-infinite axon (equation 10, *ibid.*) of the diameter of the horizontal cell axon, and R_t is the resistance of the terminal arborization [calculated from equation 5 of W. Rall, in *Excitatory Synaptic Mechanisms*, P. Andersen and J. K. S. Jansen, Eds. (Univ. Forlaget, Oslo, 1970), p. 175]. The following electrical parameters and dimensions enter into our calculations: R_m, 1500 ohm cm^2 [based on measurements of horizontal cells in *Necturus* retina, R. Nelson, *J. Neurophysiol.* **36**, 519 (1973)]; R_i, 100 ohm cm; axonal diameter, 0.5 μm; axonal length, 330 μm; membrane area of the terminal arborization, 17,800 μm^2; L for a typical axonal branch, 0.5 space constant (based on measurements of seven typical branches of Fig. 1b by using the above electrical parameters); membrane area of the cell body, 5100 μm^2. These values yield several derivative properties: axonal space constant, 137 μm; L_{axon}, 2.4 space constants; R_t, 9.1×10^6 ohms; $R_{cell\ body}$, 2.9×10^7 ohms, B_1, 77, signal transfer from cell body to terminal arborization, 0.23 percent; from terminal arborization to cell body, 0.73 percent. The cone signal seen in the terminal arborization of Fig. 1c is about 4 mv or no less than 10 percent of the largest cone signal seen in horizontal cell bodies. However, in order to obtain signal transfer as large as 10 percent either R_m would have to be 33,000 ohm cm^2 or the axon could be no longer than 16 μm. Much of the voltage attenuation is accounted for by the very low resistance of the terminal arborization as compared to the input resistance of the axon. Signals coming down the axon are effectively short-circuited by the terminal arborization. If the terminal arborization is cut off from the axon (mathematically) and the end of the axon sealed (corresponding to $B_1 = 0$), the signal transfer from the cell body to the sealed end jumps from 0.23 to 18 percent.
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Polyspermy Block of *Spisula* Eggs Is Prevented by Cytochalasin B

Abstract. *The eggs of the surf clam* Spisula solidissima *have a built-in mechanism that prevents polyspermy: the eggs show a 70 percent decrease in sperm receptivity 5 seconds after fertilization, and become completely resistant to sperm by 15 seconds. When the outer egg coat (vitelline layer) was removed, there was no change in fertilizability or the timing of the block to polyspermy. This suggests that the alteration occurs in or at the plasma membrane. Such changes in the egg surface were sensitive to low concentrations of cytochalasin B.*

A means for preventing more than one sperm nucleus from fusing with the egg nucleus is a prerequisite for preserving the diploid state of the genome in eukaryotic organisms. In eggs that do not contain yolks, this occurs by excluding all but one sperm and is referred to as the block to polyspermy. The mechanism of this block has been worked out for eggs of the sea urchin and the hamster. Earlier work on these eggs indicated that the fusion and exocytosis of the cortical granules, referred to as the "cortical reaction," was involved in the block to polyspermy (*1*). Later it was shown that macromolecules released from these granules at fertilization are the responsible factors (*2, 3*).

However, the cortical reaction may not constitute the sole block to polyspermy. In the sea urchin, numerous sperm attach to an egg within the first second after insemination and hundreds or even thousands of sperm are attached to the egg surface by the time the cortical reactions are initiated 25 seconds later (*4*). Many workers, therefore, have suggested that some earlier change in the egg results in the block to polyspermy, this change presumably occurring at the plasma membrane. Kinetic analyses of sea urchin fertilization indicate that two types of blocks occur. The first is rapid and only partially effective, and could correspond to a rapid change in the plasma membrane. The other block is later-acting and completely effective, and its time sequence corresponds to that of the cortical reactions (*5*). Whether the fast block occurs at all, or indeed whether it occurs in sea urchin eggs, is still a matter of debate (*4*).

There are eggs of other organisms that do not exhibit any sort of cortical reaction but must have a block to polyspermy. The best-studied examples of these are the pelecypod mollusk *Spisula solidissima* (*6, 7*) and the echiuroid worm *Urechis caupo* (*8*). These eggs are useful for studying blocks to polyspermy in the absence of cortical granule exocytosis. The experiments described here demonstrate a fast block to polyspermy in *Spisula* and may provide a model for the proposed fast incomplete block in eggs that do have cortical reactions, such as those of the sea urchin and hamster.

Ripe ovaries from *S. solidissima* females were minced with scissors, and the eggs were strained through several layers of cheesecloth into seawater. The eggs were immediately sedimented by a hand centrifuge, then suspended in filtered seawater; this washing procedure was repeated three to four times. Semen was obtained as the exudate from excised testes that had been stored at 4°C for 15 minutes; the exudate was filtered through cheesecloth and stored at 4°C until it was diluted just before use (*9*). All experiments were

Table 1. Effect of cytochalasin B (CB) on the block to polyspermy in *Spisula*.

Conditions	Percentage of polyspermy at CB concentrations (μg/ml)			
	0	0.5	1.0	5.0
Initial light insemination of eggs pretreated 10 minutes with CB	5	3	3	20
Light insemination of eggs first treated 10 minutes with CB, followed by heavy insemination at 2 minutes	6	67	69	68
Initial light insemination in the presence of CB	0	0	0	0
Light insemination in the presence of CB, followed by heavy insemination at 2 minutes	0	20	20	89

performed at room temperature on the day the gametes were collected.

For monospermic fertilization, 0.2 percent (by volume) egg suspensions were inseminated with a 0.01 percent sperm suspension (*10*). For polyspermic fertilization, a 0.2 percent egg suspension was inseminated with a 1.0 percent sperm suspension. To determine polyspermy, eggs were fixed 50 minutes after insemination, and the decondensed sperm nuclei in each egg were counted (*11*). Eggs were prepared for electron microscopy by the procedure of Longo (*7*), except that bovine serum albumin was omitted from the fixative.

The cytology of fertilization has been well described in *Spisula* (*6, 7*). Briefly, the egg is surrounded by a fibrous vitelline layer into which are interdigitated numerous microvilli emanating from the egg. Numerous sperm attach to the egg, presumably to the microvilli (*7*). The sperm remain very tightly attached after fertilization and cannot be removed by simple mechanical agitation. They detach after fertilization, but the rate and extent of detachment is much less rapid than in the sea urchin egg where sperm detachment from the vitelline layer is complete within 30 seconds after the cortical reactions begin (*4*). In *Spisula*, detachment is first evident about 5 minutes after fertilization and is not complete until about 60 minutes have elapsed.

We also looked for similarities between *Spisula* and sea urchin fertilization in terms of release of substances from the eggs after fertilization which affect the fertilizability of test eggs. In the sea urchin, components of the cortical granules effect the block to polyspermy by altering the sperm receptor sites on the vitelline layer. If sea urchin eggs that have had their vitelline layer removed are fertilized in concentrated suspensions and the soluble exudate from these eggs is applied to unfertilized eggs, a protease present in the exudate renders the other eggs nonfertilizable (*2*). Although *Spisula* eggs do not have cortical granule exocytosis, we examined the possibility that soluble products which alter the egg or sperm are released at fertilization. The vitelline layers were removed with β-alanine (described below), and a 20 percent suspension of eggs was lightly inseminated. The eggs were allowed to stand for 5 minutes; they were then removed by hand centrifugation, and the sperm were removed by centrifugation at 10,000*g* for 20 minutes. Unfertilized eggs with and without vitelline layers were then incubated in the supernatant for 15 and 30 minutes. There was no apparent loss in fertilizability in any case. Thus, unlike the sea urchin egg, *Spisula* eggs upon fertilization release no substances that affect subsequent fertilization of test eggs.

The timing of the block to polyspermy was determined as follows. Eggs were inseminated with an initial low sperm dose (light insemination) sufficient to give 100 percent monospermy. The occurrence of a block to polyspermy was then tested at intervals after this initial light insemination by the addition of a second dose of sperm at 100 times the concentration of the light insemination (heavy insemination) which, if given initially, would have resulted in more than 80 percent polyspermy. The time at which there was no appreciable polyspermy upon addition of the heavy sperm dose was taken as the completion time for the block to polyspermy (*5*).

In our first experiments, the heavy insemination was added to separate portions at 15-second intervals for 2 minutes. The results revealed that a complete block to polyspermy had occurred by 15 seconds. The exact timing of this change was determined in a second experiment in which heavy sperm concentrations were added at 5-second intervals after the initial light insemination. The initial light insemination yielded about 4 percent polyspermy, whereas the initial heavy insemination resulted in 83 percent polyspermy (Fig. 1A). By 5 seconds, the susceptibility to further insemination was reduced 70 percent, and by 15 seconds the eggs were completely refractory to further insemination. Thus, the block to polyspermy develops in less than 5 seconds after the initial contact with sperm.

We next undertook to determine whether the block to polyspermy involved a change in the vitelline layer. We tried numerous procedures to remove the vitelline layer from unfertilized eggs (*12*). At the suggestion of Professor L. Lorand we tried compounds that increase the dielectric constant of the medium and found that a 1- to 2-minute exposure to isotonic (1*M*) glycine or β-alanine would remove the vitelline layer from unfertilized eggs (*13*). Phase-contrast microscopy of eggs immersed in 1*M* β-alanine revealed that the vitelline layer of these eggs hydrated or swelled and sloughed off into the medium. Electron microscopy of treated and control eggs showed that the fibrous material between the microvilli was almost completely removed by this treatment. Such eggs lost their rigidity, were flaccid, and became unable to cleave. Similar observations were reported by Rebhun on eggs whose vitelline layers were removed with isotonic NaCl after fertilization, a procedure that does not work with unfertilized eggs (*6*).

Nevertheless, eggs treated with 1*M* β-

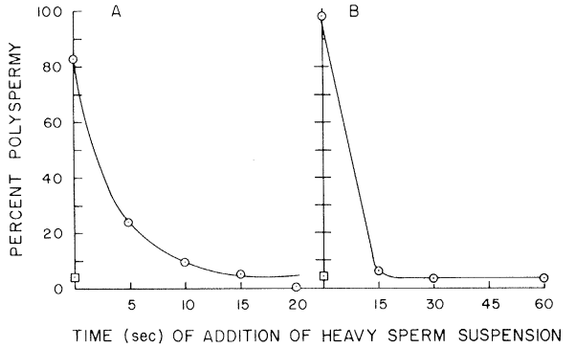

Fig. 1. (A) Polyspermy as a function of time of heavy insemination after an initial light insemination of *Spisula* eggs. (B) Polyspermy as a function of the time of heavy insemination after an initial light insemination of vitelline layer–free *Spisula* eggs. ⊡, Initial light insemination.

alanine and lacking vitelline layers are normal in their ability to be fertilized and to undergo maturation divisions. This treatment has no effect on the fertilizability of these eggs compared to control untreated eggs (as tested by sperm dilution assay on treated and control eggs). Eggs without vitelline layers also develop a rapid and complete block to polyspermy 15 seconds after the initial insemination (Fig. 1B).

The above results indicate that a rapid change in sperm receptivity, evidenced as a block to polyspermy, does not involve the vitelline layer and may occur at the plasma membrane. In considering possible mechanisms for such a fast change, we recalled that cortical contractions in response to fertilization and wound healing have been described in several types of eggs (*14, 15*). In amphibian eggs, this wound healing contraction is prevented by cytochalasin B (CB) (*15*). We therefore designed an experiment to determine whether the block to polyspermy could also be prevented by this drug. Eggs were first treated for 10 minutes with CB at 0.5, 1.0, and 5.0 μg/ml in dimethyl sulfoxide or in seawater containing dimethyl sulfoxide. They were then washed and inseminated with a light sperm dose; 2 minutes later they were given a heavy polyspermy-producing dose. As shown in the upper part of Table 1, prior treatment with the lower concentrations of CB did not result in increased polyspermy after the initial light insemination. There was an increase at 5 μg/ml. All concentrations, however, prevented the development of the block, and the eggs remained receptive to sperm. As seen, approximately 70 percent of the eggs became polyspermic after the second heavy insemination. This situation is different when the CB is added directly with the sperm (lower part of Table 1). Under these conditions, CB prevents the block best at the higher concentrations, suggesting that the drug did not have sufficient time to act to prevent the establishment of the block.

We then examined the question of whether CB could reverse an already established block. For this experiment, eggs were lightly inseminated and at 2 minutes, when the block was fully established, the eggs were exposed to CB (5 μg/ml) for 5 minutes. They were then washed twice and heavily inseminated at 12 minutes. There was no increase in polyspermy in these eggs.

These results show that fertilization of *S. solidissima* eggs result in a rapid loss in receptivity of the egg to sperm, evidenced as a block to polyspermy. The mechanism of this block is radically different from that in the intensively studied sea urchin and hamster eggs. In *Spisula*, as compared to these eggs, a complete block to polyspermy is established rapidly; and there is no cortical granule exocytosis, no evidence of substances released from the egg which affect fertilizability, and no rapid detachment of sperm. The block occurs in the absence of a vitelline layer, strongly suggesting that the alteration in sperm receptivity occurs at the level of the plasma membrane.

The change resulting in the block to polyspermy in *Spisula* is sensitive to CB. Efforts to directly demonstrate such a surface contraction in *Spisula* eggs were unsuccessful. These included placing particles of carmine or kaolin directly on eggs whose vitelline layers had been removed with β-alanine. The eggs were immobilized on slides coated with protamine sulfate (*16*) and we looked for particle movement after fertilization or artificial activation with ionophore A23187 (*17*).

The mechanism of CB action is unknown in this system. Operationally the egg remains "fertilizable"; that is, it continues to accept sperm, in the presence of the drug. The simplest interpretation is that normally some rapid CB-sensitive conformational change is triggered in the egg plasma membrane by the first successful sperm. This change renders the egg nonreceptive to other sperm. Similar concentrations (1 to 10 μg/ml) of CB inhibit polar body formation and cleavage in *Spisula* (*7*), suggesting that the action of the drug is on a microfilament system. The block to polyspermy could involve a microfilament-mediated alteration of the surface. This could simply make the cortex more rigid so that additional sperm cannot enter. Alternatively, a microfilament-sperm receptor complex could undergo a conformational change after fertilization; sperm could remain attached but cannot enter. Finally, the drug sensitivity may be simply through general interference with membrane function because CB binds to plasma membrane components (*18*).

Our experiments also show that the polyspermy block is not reversed by CB. This could indicate that the CB-sensitive function is not continuously required to maintain the block; a more permanent alteration in sperm receptivity may subsequently be superimposed on the CB-sensitive one.

It would be of great interest to know whether a similar type of change occurs in eggs in which release of cortical granule contents are involved in the block to polyspermy. The sea urchin and hamster are the classic models for this type of block, but here too there is kinetic evidence for an earlier fast block to polyspermy. In these eggs it is conceivable that a rapid alteration of the egg surface, such as may occur in *Spisula* eggs, results in the fast block. The slower, cortical-granule-mediated change permanently stabilizes the egg against further fertilization.

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12. Among the procedures tried were incubation in 0.01*M* dithiothreitol, 1*M* glycerol, 0.05*M* sodium bromide, 0.9*M* sucrose, and, at 1 mg/ml, Pronase, trypsin, amylase, hyaluronidase, pepsin, neuraminidase, and various combinations of enzymes. Pronase, trypsin, amylase, and glycerol were found to activate the eggs.
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