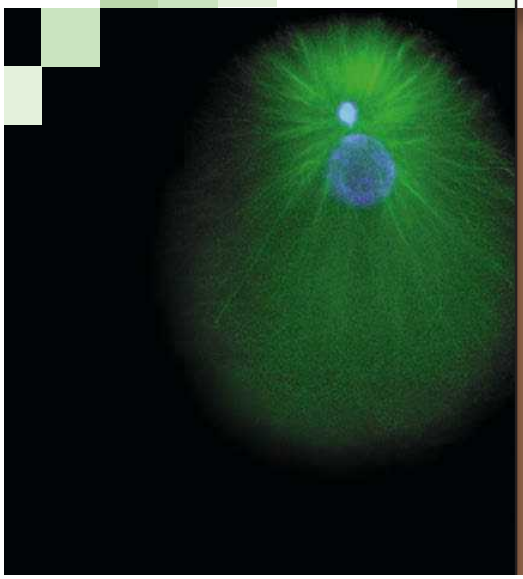
7

Fertilization

Beginning a New Organism

How do the sperm and egg nuclei find each other?



FERTILIZATION IS THE PROCESS WHEREBY THE GAMETES—sperm and egg—fuse together to begin the creation of a new organism. Fertilization accomplishes two separate ends: sex (the combining of genes derived from two parents) and reproduction (the generation of a new organism). Thus, the first function of fertilization is to transmit genes from parent to offspring, and the second is to initiate in the egg cytoplasm those reactions that permit development to proceed.

Although the details of fertilization vary from species to species, it generally consists of four major events:

1. *Contact and recognition* between sperm and egg. In most cases, this ensures that the sperm and egg are of the same species.
2. *Regulation* of sperm entry into the egg. Only one sperm nucleus can ultimately unite with the egg nucleus. This is usually accomplished by allowing only one sperm to enter the egg and actively inhibiting any others from entering.
3. *Fusion* of the genetic material of sperm and egg.
4. *Activation* of egg metabolism to start development.

This chapter will describe how these steps are accomplished in two groups of organisms: sea urchins (whose fertilization we know the best) and mammals.

The Punchline

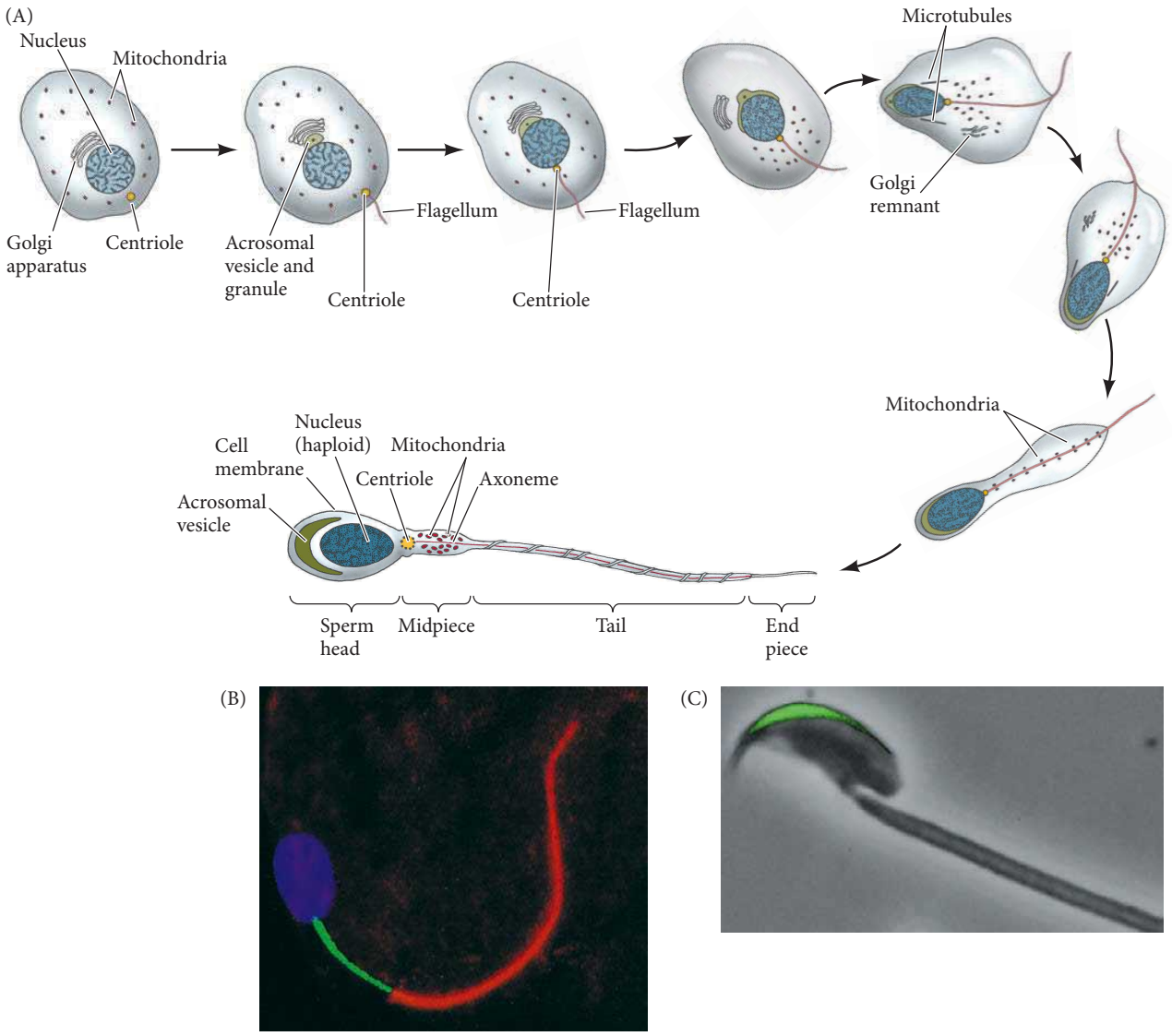
During fertilization, the egg and sperm must meet, the genetic material of the sperm must enter the egg, and the fertilized egg must initiate cell division and the other processes of development. Sperm and egg must travel toward each other, and chemicals from the eggs can attract the sperm. Gamete recognition occurs when proteins on the sperm cell membrane meet proteins on the extracellular coating of the egg. In preparation for this meeting, the sperm cell membrane is altered significantly by exocytotic events. The sperm activates development by releasing calcium ions (Ca^{2+}) from within the egg. These ions stimulate the enzymes needed for DNA synthesis, RNA synthesis, protein synthesis, and cell division. The sperm and egg pronuclei travel toward one another and the genetic material of the gametes combines to form the diploid chromosome content carrying the genetic information for the development of a new organism.

Structure of the Gametes

A complex dialogue exists between egg and sperm. The egg activates the sperm metabolism that is essential for fertilization, and the sperm reciprocates by activating the egg metabolism needed for the onset of development. But before we investigate these aspects of fertilization, we need to consider the structures of the sperm and egg—the two cell types specialized for fertilization.

Sperm

Sperm were discovered in the 1670s, but their role in fertilization was not discovered until the mid-1800s. It was only in the 1840s, after Albert von Kölliker described the formation of sperm from cells in the adult testes that fertilization research could really begin. Even so, von Kölliker denied that there was any physical contact between sperm and egg. He believed that the sperm excited the egg to develop in much the same way a magnet communicates its presence to iron. The first description of fertilization was



published in 1847 by Karl Ernst von Baer, who showed the union of sperm and egg in sea urchins and tunicates (Raineri and Tammiksaar 2013). He described the fertilization envelope, the migration of the sperm nucleus to the center of the egg, and the subsequent early cell divisions of development. In the 1870s, Oscar Hertwig and Herman Fol repeated this work and detailed the union of the two cells' nuclei.

WEB TOPIC 7.1 **THE ORIGINS OF FERTILIZATION RESEARCH** Our knowledge of fertilization is relatively recent. Although sperm was discovered in the 1670s, it didn't have a known job until 200 years later.

SPERM ANATOMY Each sperm cell consists of a haploid nucleus, a propulsion system to move the nucleus, and a sac of enzymes that enable the nucleus to enter the egg. In most species, almost all of the cell's cytoplasm is eliminated during sperm maturation, leaving only certain organelles that are modified for spermatic function (**FIGURE 7.1A,B**). During the course of maturation, the sperm's haploid nucleus becomes very streamlined and its DNA becomes tightly compressed. In front or to the side of this compressed haploid nucleus lies the **acrosomal vesicle**, or **acrosome** (**FIGURE 7.1C**). The acrosome is derived from the cell's Golgi apparatus and contains enzymes that digest proteins and complex sugars. Enzymes stored in the acrosome can digest a path through the outer coverings of the egg. In many species, a region of globular actin proteins lies between the sperm nucleus and the acrosomal vesicle. These proteins are used to extend a fingerlike **acrosomal process** from the sperm during the early stages of fertilization. In sea urchins and numerous other species, recognition between sperm and egg involves molecules on the acrosomal process. Together, the acrosome and nucleus constitute the **sperm head**.

The means by which sperm are propelled vary according to how the species has adapted to environmental conditions. In most species, an individual sperm is able to travel by whipping its **flagellum**. The major motor portion of the flagellum is the **axoneme**, a structure formed by microtubules emanating from the centriole at the base of the sperm nucleus. The core of the axoneme consists of two central microtubules surrounded by a row of nine doublet microtubules. These microtubules are made exclusively of the dimeric protein **tubulin**.

Although tubulin is the basis for the structure of the flagellum, other proteins are also critical for flagellar function. The force for sperm propulsion is provided by **dynein**, a protein attached to the microtubules. Dynein is an ATPase—an enzyme that hydrolyzes ATP, converting the released chemical energy into mechanical energy that propels the sperm.[1] This energy allows the active sliding of the outer doublet microtubules,

[1]The importance of dynein can be seen in individuals with a genetic syndrome known as the Kartagener triad. These individuals lack functional dynein in all their ciliated and flagellated cells, rendering these structures immotile (Afzelius 1976). Thus, males with Kartagener triad are sterile (immotile sperm). Both men and women affected by this syndrome are susceptible to bronchial infections (immotile respiratory cilia) and have a 50% chance of having the heart on the right side of the body (a condition known as *situs inversus*, the result of immotile cilia in the center of the embryo).

◀ **FIGURE 7.1** Modification of a germ cell to form a mammalian sperm. (A) The centriole produces a long flagellum at what will be the posterior end of the sperm. The Golgi apparatus forms the acrosomal vesicle at the future anterior end. Mitochondria collect around the flagellum near the base of the haploid nucleus and become incorporated into the midpiece ("neck") of the sperm. The remaining cytoplasm is jettisoned, and the nucleus condenses. The size of the mature sperm has been enlarged relative to the other stages. (B) Mature bull sperm. The DNA is stained blue, mitochondria are stained green, and the tubulin of the flagellum is stained red. (C) The acrosomal vesicle of this mouse sperm is stained green by the fusion of proacrosin with green fluorescent protein (GFP). (A after Clermont and Leblond 1955; B from Sutovsky et al. 1996, courtesy of G. Schatten; C courtesy of K.-S. Kim and G. L. Gerton.)

causing the flagellum to bend (Ogawa et al. 1977; Shinyoji et al. 1998). The ATP needed to move the flagellum and propel the sperm comes from rings of mitochondria located in the **midpiece** of the sperm (see Figure 7.1B). In many species (notably mammals), a layer of dense fibers has interposed itself between the mitochondrial sheath and the cell membrane. This fiber layer stiffens the sperm tail. Because the thickness of this layer decreases toward the tip, the fibers probably prevent the sperm head from being whipped around too suddenly. Thus, the sperm cell has undergone extensive modification for the transport of its nucleus to the egg.

In mammals, the differentiation of sperm is not completed in the testes. Although they are able to move, the sperm released during ejaculation do not yet have the capacity to bind to and fertilize an egg. The final stages of sperm maturation, cumulatively referred to as **capacitation**, do not occur in mammals until the sperm has been inside the female reproductive tract for a certain period of time.

The egg

CYTOPLASM AND NUCLEUS All the material necessary to begin growth and development must be stored in the egg, or **ovum**.[2] Whereas the sperm eliminates most of its cytoplasm as it matures, the developing egg (called the **oocyte** before it reaches the stage of meiosis at which it is fertilized) not only conserves the material it has, but actively accumulates more. The meiotic divisions that form the oocyte conserve its cytoplasm rather than giving half of it away; at the same time, the oocyte either synthesizes or absorbs proteins such as yolk that act as food reservoirs for the developing embryo. Birds' eggs are enormous single cells, swollen with accumulated yolk (see Figure 12.2). Even eggs with relatively sparse yolk are large compared to sperm. The volume of a sea urchin egg is about 200 picoliters (2×10^{-4} mm^3), more than 10,000 times the volume of sea urchin sperm (**FIGURE 7.2**). So even though sperm and egg have equal haploid

[2] Eggs over easy: the terminology used in describing the female gamete can be confusing. In general, an *egg*, or *ovum*, is a female gamete capable of binding sperm and being fertilized. An *oocyte* is a developing egg that cannot yet bind sperm or be fertilized (Wessel 2009). The problems in terminology come from the fact that the eggs of different species are in different stages of meiosis (see Figure 7.3). The human egg, for example, is in second meiotic metaphase when it binds sperm, whereas the sea urchin egg has completed all of its meiotic divisions when it binds sperm. The contents of the egg also vary greatly from species to species.

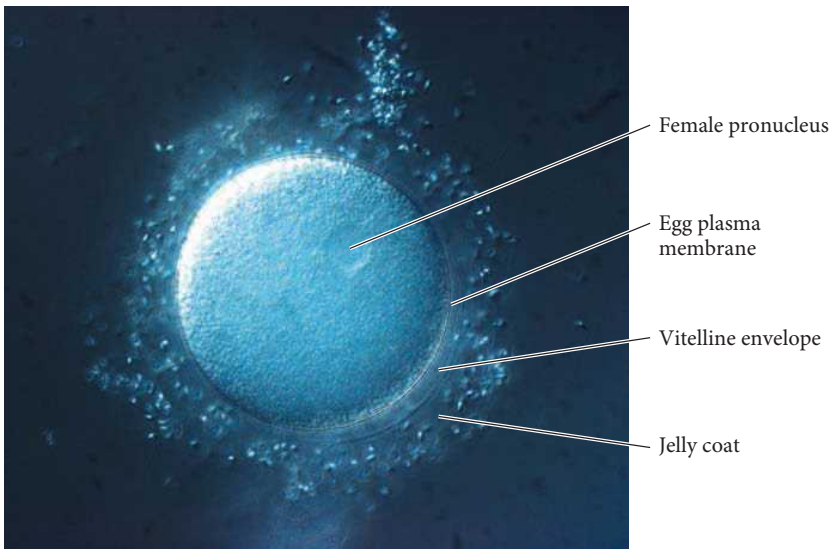


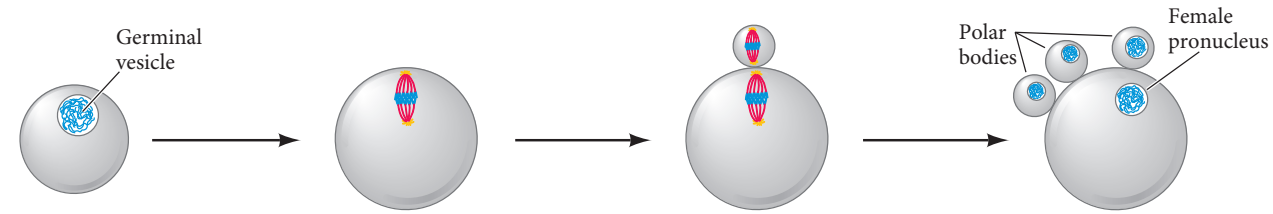
FIGURE 7.2 Structure of the sea urchin egg at fertilization. Sperm can be seen in the jelly coat and attached to the vitelline envelope. The female pronucleus is apparent within the egg cytoplasm. (Photograph by Kristina Yu © Exploratorium www.exploratorium.edu.)

nuclear components, the egg accumulates a remarkable cytoplasmic storehouse during its maturation. This cytoplasmic trove includes the following:

- **Nutritive proteins**. The early embryonic cells must have a supply of energy and amino acids. In many species, this is accomplished by accumulating yolk proteins in the egg. Many of these yolk proteins are made in other organs (e.g., liver, fat bodies) and travel through the maternal blood to the oocyte.
- **Ribosomes and tRNA**. The early embryo must make many of its own structural proteins and enzymes, and in some species there is a burst of protein synthesis soon after fertilization. Protein synthesis is accomplished by ribosomes and tRNA that exist in the egg. The developing egg has special mechanisms for synthesizing ribosomes; certain amphibian oocytes produce as many as 10^{12} ribosomes during their meiotic prophase.
- **Messenger RNAs**. The oocyte not only accumulates proteins, it also accumulates mRNAs that encode proteins for the early stages of development. It is estimated that sea urchin eggs contain thousands of different types of mRNA that remain repressed until after fertilization.
- **Morphogenetic factors**. Molecules that direct the differentiation of cells into certain cell types are present in the egg. These include transcription factors and paracrine factors. In many species, they are localized in different regions of the egg and become segregated into different cells during cleavage.
- **Protective chemicals**. The embryo cannot run away from predators or move to a safer environment, so it must be equipped to deal with threats. Many eggs contain ultraviolet filters and DNA repair enzymes that protect them from sunlight, and some eggs contain molecules that potential predators find distasteful. The yolk of bird eggs contains antibodies that protect the embryo against microbes.

Within the enormous volume of egg cytoplasm resides a large nucleus (see Figure 7.2). In a few species (such as sea urchins), this **female pronucleus** is already haploid at the time of fertilization. In other species (including many worms and most mammals), the egg nucleus is still diploid—the sperm enters before the egg's meiotic divisions are completed (**FIGURE 7.3**). In these species, the final stages of egg meiosis will take place after the sperm's nuclear material—the **male pronucleus**—is already inside the egg cytoplasm.

WEB TOPIC 7.2 **THE EGG AND ITS ENVIRONMENT** Most eggs are fertilized in the wild, not in the laboratory. In addition to the developmental processes in the egg, there are also factors in the egg that help the developing embryo cope with environmental stresses.



Primary oocyte

The roundworm *Ascaris*
The mesozoan *Dicyema*
The sponge *Grantia*
The polychaete worm *Myzostoma*
The clam worm *Nereis*
The clam *Spisula*
The echiuroid worm *Urechis*
Dogs and foxes

First metaphase

The nemertean worm *Cerebratulus*
The polychaete worm *Chaetopterus*
The mollusk *Dentalium*
The core worm *Pectinaria*
Many insects
Starfish

Second metaphase

The lancelet *Branchiostoma*
Amphibians
Most mammals
Fish

Meiosis complete

Cnidarians (e.g., anemones)
Sea urchins

FIGURE 7.3 Stages of egg maturation at the time of sperm entry in different animal species. Note that in most species, sperm entry occurs before the egg nucleus has completed meiosis. The germinal vesicle is the name given to the large diploid nucleus of the primary oocyte. The polar bodies are nonfunctional cells produced by meiosis (see Chapter 6). (After Austin 1965.)

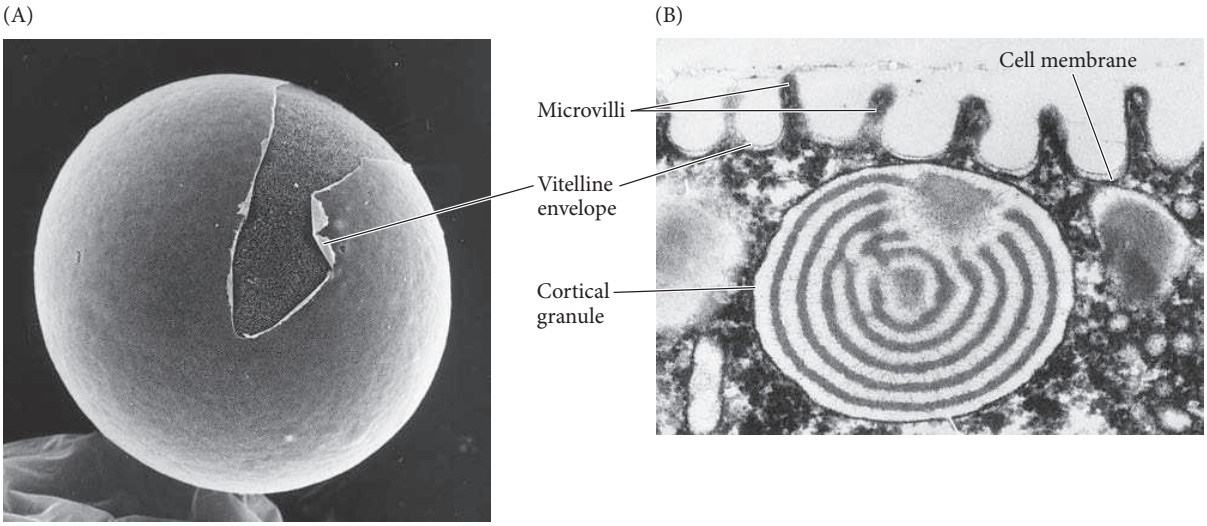


FIGURE 7.4 Sea urchin egg cell surfaces. (A) Scanning electron micrograph of an egg before fertilization. The cell membrane is exposed where the vitelline envelope has been torn. (B) Transmission electron micrograph of an unfertilized egg, showing microvilli and cell membrane, which are closely covered by the vitelline envelope. A cortical granule lies directly beneath the cell membrane. (From Schroeder 1979, courtesy of T. E. Schroeder.)

CELL MEMBRANE AND EXTRACELLULAR ENVELOPE The membrane enclosing the egg cytoplasm regulates the flow of specific ions during fertilization and must be capable of fusing with the sperm cell membrane. Outside this egg cell membrane is an extracellular matrix that forms a fibrous mat around the egg and is often involved in sperm-egg recognition (Wasserman and Litscher 2016). In invertebrates, this structure is usually called the **vitelline envelope** (**FIGURE 7.4A**). The vitelline envelope contains several different glycoproteins. It is supplemented by extensions of membrane glycoproteins from the cell membrane and by proteinaceous "posts" that adhere the vitelline envelope to the cell membrane (Mozingo and Chandler 1991). The vitelline envelope is essential for the species-specific binding of sperm. Many types of eggs also have a layer of **egg jelly** outside the vitelline envelope. This glycoprotein meshwork can have numerous functions, but most commonly it is used either to attract or to activate sperm. The egg, then, is a cell specialized for receiving sperm and initiating development.

Lying immediately beneath the cell membrane of most eggs is a thin layer (about 5 μm) of gel-like cytoplasm called the **cortex**. The cytoplasm in this region is stiffer than the internal cytoplasm and contains high concentrations of globular actin molecules. During fertilization, these actin molecules polymerize to form long cables of actin **microfilaments**. Microfilaments are necessary for cell division. They are also used to extend the egg surface into small projections called **microvilli**, which may aid sperm entry into the cell (**FIGURE 7.4B)**. Also within the cortex are the **cortical granules** (see Figures 7.4B). These membrane-bound, Golgi-derived structures contain proteolytic enzymes and are thus homologous to the acrosomal vesicle of the sperm. However, whereas a sea urchin sperm contains just one acrosomal vesicle, each sea urchin egg contains approximately 15,000 cortical granules. In addition to digestive enzymes, the cortical granules contain mucopolysaccharides, adhesive glycoproteins, and hyalin protein. As we will soon describe, the enzymes and mucopolysaccharides help prevent polyspermy—that is, they prevent additional sperm from entering the egg after the first sperm has entered—while hyalin and the adhesive glycoproteins surround the early embryo, providing support for cleavage-stage blastomeres.

In mammalian eggs, the extracellular envelope is a separate, thick matrix called the **zona pellucida**. The mammalian egg is also surrounded by a layer of cells called the **cumulus** (**FIGURE 7.5**), which is made up of the ovarian follicular cells that were nurturing the egg at the time of its release from the ovary. Mammalian sperm have to get past these cells to fertilize the egg. The innermost layer of cumulus cells, immediately adjacent to the zona pellucida, is called the **corona radiata**.

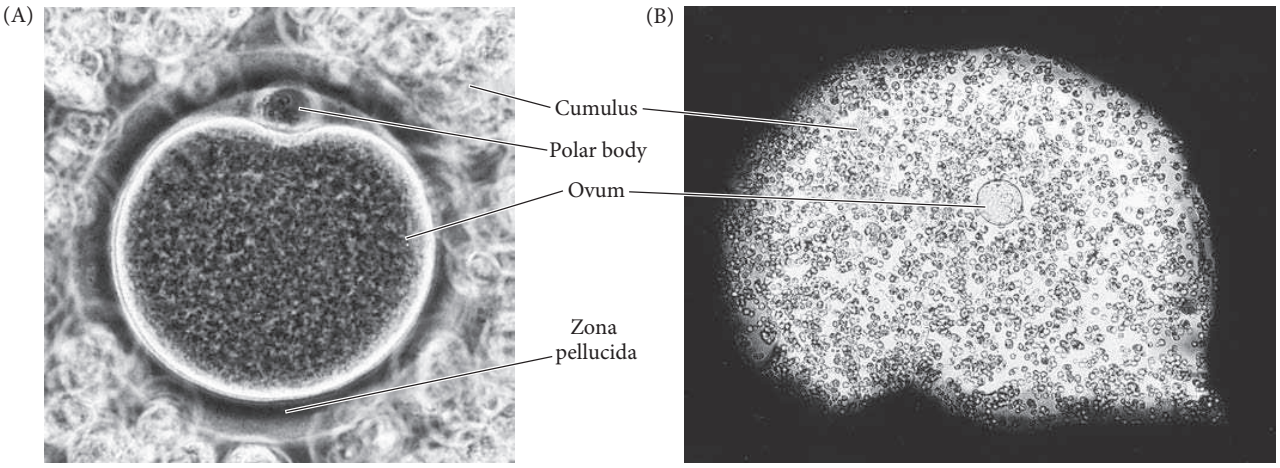


FIGURE 7.5 Mammalian eggs immediately before fertilization. (A) The hamster egg, or ovum, is encased in the zona pellucida, which in turn is surrounded by the cells of the cumulus. A polar body cell, produced during meiosis, is visible within the zona pellucida. (B) At lower magnification, a mouse oocyte is shown surrounded by the cumulus. Colloidal carbon particles (India ink, seen here as the black background) are excluded by the hyaluronidate matrix. (Courtesy of R. Yanagimachi.)

Recognition of egg and sperm

The interaction of sperm and egg generally proceeds according to five steps (**FIGURE 7.6**; Vacquier 1998):

1. *Chemoattraction* of the sperm to the egg by soluble molecules secreted by the egg
2. *Exocytosis* of the sperm acrosomal vesicle and release of its enzymes
3. *Binding of the sperm* to the extracellular matrix (vitelline envelope or zona pellucida) of the egg
4. *Passage of the sperm* through this extracellular matrix
5. *Fusion* of the egg and sperm cell membranes

After these steps are accomplished, the haploid sperm and egg nuclei can meet and the reactions that initiate development can begin. In this chapter, we will focus on these events in two well-studied organisms: sea urchins, which undergo external fertilization; and mice, which undergo internal fertilization. Some variations of fertilization events will be described in subsequent chapters as we study the development of particular organisms.

External Fertilization in Sea Urchins

Many marine organisms release their gametes into the environment. That environment may be as small as a tide pool or as large as an ocean and is shared with other species that may shed their gametes at the same time. Such organisms are faced with two problems: How can sperm and eggs meet in such a dilute concentration, and how can sperm be prevented from attempting to fertilize eggs of another species?

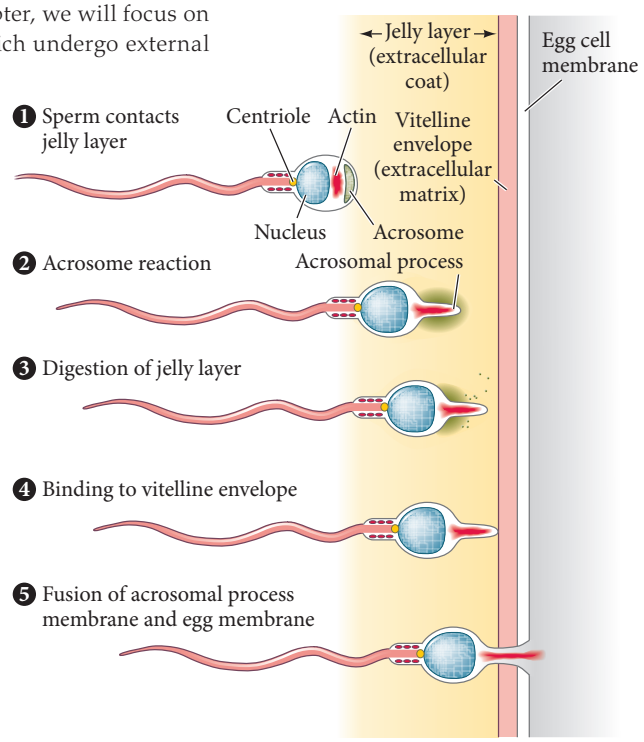


FIGURE 7.6 Summary of events leading to the fusion of egg and sperm cell membranes in sea urchin fertilization, which is external. (1) The sperm is chemotactically attracted to and activated by the egg. (2, 3) Contact with the egg jelly triggers the acrosome reaction, allowing the acrosomal process to form and release proteolytic enzymes. (4) The sperm adheres to the vitelline envelope and lyses a hole in it. (5) The sperm adheres to the egg cell membrane and fuses with it. The sperm pronucleus can now enter the egg cytoplasm.

VADE MECUM

The reactions that prevent polyspermy in a fertilized sea urchin egg can be seen in the raising of the fertilization envelope. See the segment on sea urchin fertilization to view these remarkable events.

In addition to simply producing enormous numbers of gametes, two major mechanisms have evolved to solve these problems: species-specific **sperm attraction** and species-specific **sperm activation**. Here we describe these events as they occur in sea urchins.

Sperm attraction: Action at a distance

Species-specific sperm attraction has been documented in numerous species, including cnidarians, mollusks, echinoderms, amphibians, and urochordates (Miller 1985; Yoshida et al. 1993; Burnett et al. 2008). In many species, sperm are attracted toward eggs of their species by **chemotaxis**—that is, by following a gradient of a chemical secreted by the egg. These oocytes control not only the type of sperm they attract, but also the time at which they attract them, releasing the chemotactic factor only after they reach maturation (Miller 1978).

The mechanisms of chemotaxis differ among species (see Metz 1978; Eisenbach 2004), and chemotactic molecules are different even in closely related species. In sea urchins, sperm motility is acquired only after the sperm are spawned. As long as sperm cells are in the testes, they cannot move because their internal pH is kept low (about pH 7.2) by the high concentrations of CO_2 in the gonad. However, once sperm are spawned into seawater, their pH is elevated to about 7.6, resulting in the activation of the dynein ATPase. The splitting of ATP provides the energy for the flagella to wave, and the sperm begin swimming vigorously (Christen et al. 1982).

But the ability to move does not provide the sperm with a direction. In echinoderms, direction is provided by small chemotactic peptides called **sperm-activating peptides** (**SAPs**). One such SAP is **resact**, a 14-amino acid peptide that has been isolated from the egg jelly of the sea urchin *Arbacia punctulata* (Ward et al. 1985). Resact diffuses readily from the egg jelly into seawater and has a profound effect at very low concentrations when added to a suspension of *Arbacia* sperm. When a drop of seawater containing *Arbacia* sperm is placed on a microscope slide, the sperm generally swim in circles about 50 μm in diameter. Within seconds after a small amount of resact is injected, sperm migrate into the region of the injection and congregate there (**FIGURE 7.7**). As resact diffuses from the area of injection, more sperm are recruited into the growing cluster.

Resact is specific for *A. punctulata* and does not attract sperm of other urchin species. (An analogous compound, speract, has been isolated from the purple sea urchin, *Strongylocentrotus purpuratus*.) *A. punctulata* sperm have receptors in their cell membranes that bind resact (Ramarao and Garbers 1985; Bentley et al. 1986). When the extracellular side of the receptor binds resact, it activates latent guanylyl cyclase in the cytoplasmic side of the receptor (**FIGURE 7.8**). Active guanylyl cyclase causes the sperm cell to produce more cyclic GMP (cGMP), a compound that activates a calcium channel in the cell membrane of the sperm tail, allowing the influx of calcium ions (Ca^{2+}) from the seawater into the tail (Nishigaki et al. 2000; Wood et al. 2005). These sperm-specific calcium channels are encoded by CatSper genes—the same genes that control the direction of sperm migration in mice and humans (Seifert et al. 2014). The increases in cGMP and Ca^{2+} activate both the mitochondrial ATP-generating apparatus and the dynein ATPase that

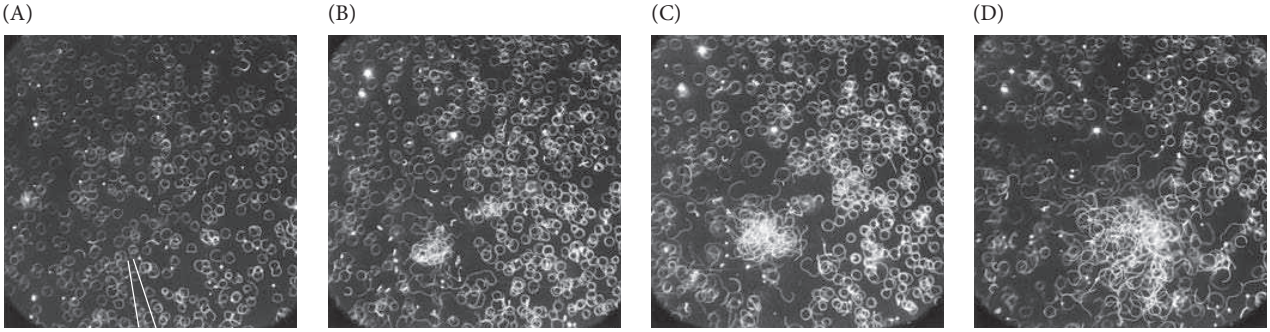


FIGURE 7.7 Sperm chemotaxis in the sea urchin *Arbacia punctulata*. One nanoliter of a 10-n*M* solution of resact is injected into a 20-microliter drop of sperm suspension. (A) A 1-second photographic exposure showing sperm swimming in tight circles before the addition of resact. The position of the injection pipette is shown by the white lines. (B–D) Similar 1-second exposures showing migration of sperm to the center of the resact gradient 20, 40, and 90 seconds after injection. (From Ward et al. 1985, courtesy of V. D. Vacquier.)

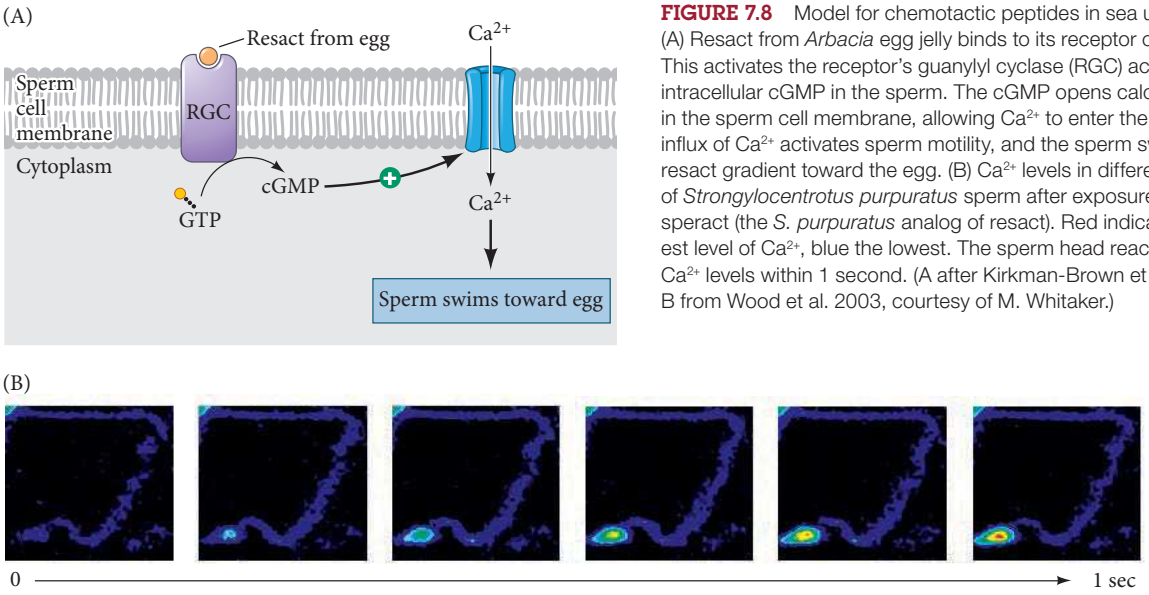


FIGURE 7.8 Model for chemotactic peptides in sea urchin sperm. (A) Resact from *Arbacia* egg jelly binds to its receptor on the sperm. This activates the receptor's guanylyl cyclase (RGC) activity, forming intracellular cGMP in the sperm. The cGMP opens calcium channels in the sperm cell membrane, allowing Ca^{2+} to enter the sperm. The influx of Ca^{2+} activates sperm motility, and the sperm swims up the resact gradient toward the egg. (B) Ca^{2+} levels in different regions of *Strongylocentrotus purpuratus* sperm after exposure to 125 n*M* speract (the *S. purpuratus* analog of resact). Red indicates the highest level of Ca^{2+}, blue the lowest. The sperm head reaches its peak Ca^{2+} levels within 1 second. (A after Kirkman-Brown et al. 2003; B from Wood et al. 2003, courtesy of M. Whitaker.)

stimulates flagellar movement in the sperm (Shimomura et al. 1986; Cook and Babcock 1993). In addition, the sperm sense the SAP gradient by curving their tails, interspersing straight swimming with a "turn" to sense the environment (Guerrero et al. 2010). The binding of a single resact molecule may be enough to provide direction for the sperm, which swim up a concentration gradient of this compound until they reach the egg (Kaupp et al. 2003; Kirkman-Brown et al. 2003). Thus, resact functions as a sperm-*attracting* peptide as well as a sperm-activating peptide. (In some organisms, the functions of sperm attraction and sperm activation are performed by different compounds.)

The acrosome reaction

A second interaction between sperm and egg jelly results in the **acrosome reaction**. In most marine invertebrates, the acrosome reaction has two components: the fusion of the acrosomal vesicle with the sperm cell membrane (an exocytosis that results in the release of the contents of the acrosomal vesicle), and the extension of the acrosomal process (Dan 1952; Colwin and Colwin 1963). The acrosome reaction in sea urchins is initiated by contact of the sperm with the egg jelly. Contact causes the exocytosis of the sperm's acrosomal vesicle. The proteolytic enzymes and proteasomes (protein-digesting complexes) thus released digest a path through the jelly coat to the egg cell surface. Once the sperm reaches the egg surface, the acrosomal process adheres to the vitelline envelope and tethers the sperm to the egg. It is possible that proteasomes from the acrosome coat the acrosomal process, allowing it to digest the vitelline envelope at the point of attachment and proceed toward the egg (Yokota and Sawada 2007).

In sea urchins, the acrosome reaction is initiated by sulfate-containing polysaccharides in the egg jelly that bind to specific receptors located directly above the acrosomal vesicle on the sperm cell membrane. These polysaccharides are often highly species-specific, and egg jelly factors from one species of sea urchin generally fail to activate the acrosome reaction even in closely related species (**FIGURE 7.9**; Hirohashi and Vacquier 2002; Hirohashi et al. 2002; Vilela-Silva et al. 2008). Thus, activation of the acrosome reaction serves as a barrier to interspecies (and thus unviable) fertilizations. This is important when numerous species inhabit the same habitat and when their spawning seasons overlap.

In *Strongylocentrotus purpuratus*, the acrosome reaction is initiated by a repeating polymer of fucose sulfate. When this sulfated polysaccharide binds to its receptor on the

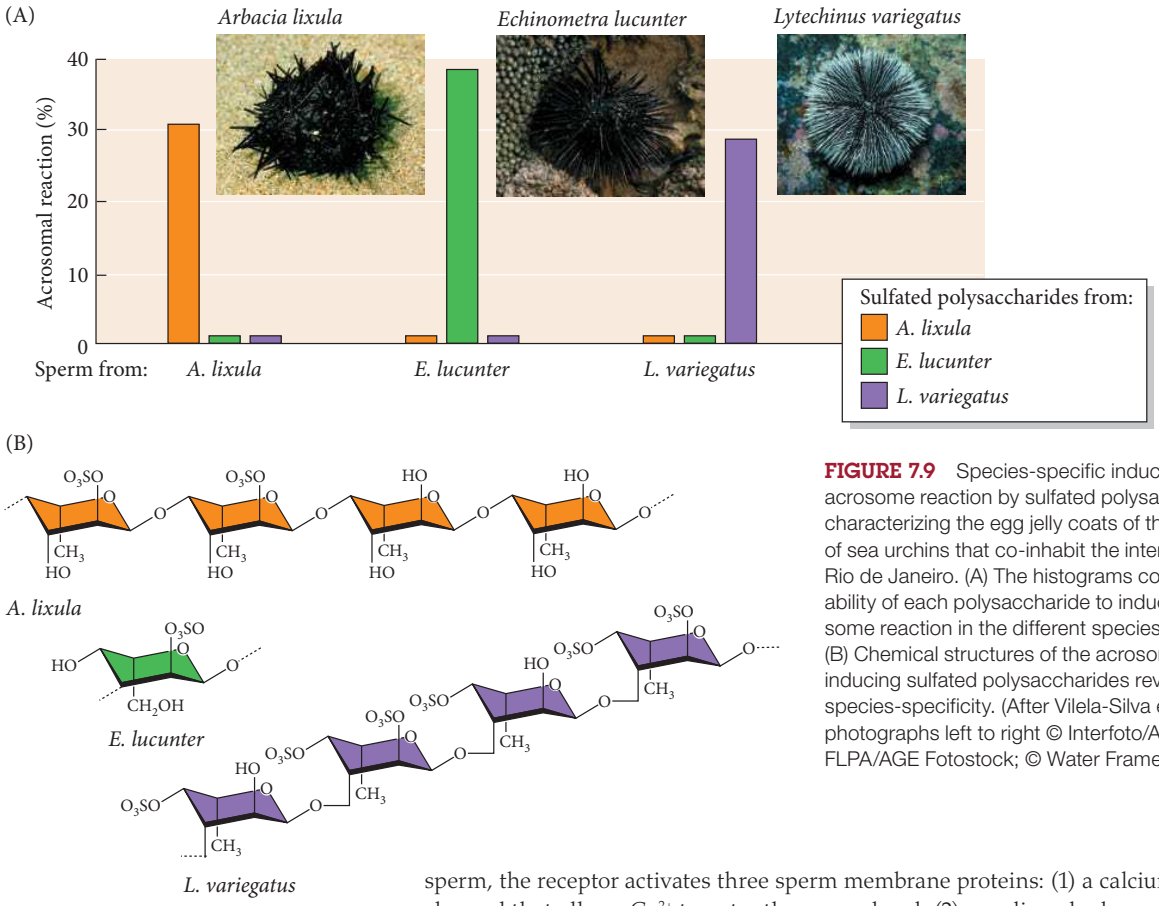


FIGURE 7.9 Species-specific induction of the acrosome reaction by sulfated polysaccharides characterizing the egg jelly coats of three species of sea urchins that co-inhabit the intertidal around Rio de Janeiro. (A) The histograms compare the ability of each polysaccharide to induce the acrosome reaction in the different species of sperm. (B) Chemical structures of the acrosome reaction-inducing sulfated polysaccharides reveal their species-specificity. (After Vilela-Silva et al. 2008; photographs left to right © Interfoto/Alamy; © FLPA/AGE Fotostock; © Water Frame/Alamy.)

sperm, the receptor activates three sperm membrane proteins: (1) a calcium transport channel that allows Ca^{2+} to enter the sperm head; (2) a sodium-hydrogen exchanger that pumps sodium ions (Na^+) into the sperm as it pumps hydrogen ions (H^+) out; and (3) a phospholipase enzyme that makes another second messenger, the phospholipid **inositol 1,4,5-trisphosphate** (**IP_3**, of which we will hear much more later in the chapter). IP_3 is able to release Ca^{2+} from *inside* the sperm, probably from within the acrosome itself (Domino and Garbers 1988; Domino et al. 1989; Hirohashi and Vacquier 2003). The elevated Ca^{2+} level in a relatively basic cytoplasm triggers the fusion of the acrosomal membrane with the adjacent sperm cell membrane (**FIGURE 7.10A–C**), releasing enzymes that can lyse a path through the egg jelly to the vitelline envelope.

The second part of the acrosome reaction involves the extension of the acrosomal process by the polymerization of globular actin molecules into actin filaments (**FIGURE 7.10D**; Tilney et al. 1978). The influx of Ca^{2+} is thought to activate the protein RhoB in the acrosomal region and midpiece of the sperm (Castellano et al. 1997; de la Sancha et al. 2007). This GTP-binding protein helps organize the actin cytoskeleton in many types of cells and is thought to be active in polymerizing actin to make the acrosomal process.

Recognition of the egg's extracellular coat

The sea urchin sperm's contact with an egg's jelly coat provides the first set of species-specific recognition events (i.e., sperm attraction, activation, and acrosome reaction). Another critical species-specific binding event must occur once the sperm has penetrated the egg jelly and its acrosomal process contacts the surface of the egg (**FIGURE 7.11A**). The acrosomal protein mediating this recognition in sea urchins is an insoluble, 30,500-Da protein called **bindin**. In 1977, Vacquier and co-workers isolated bindin from the acrosome of *Strongylocentrotus purpuratus* and found it to be capable of binding to dejellied eggs of the same species. Further, sperm bindin, like egg jelly polysaccharides,

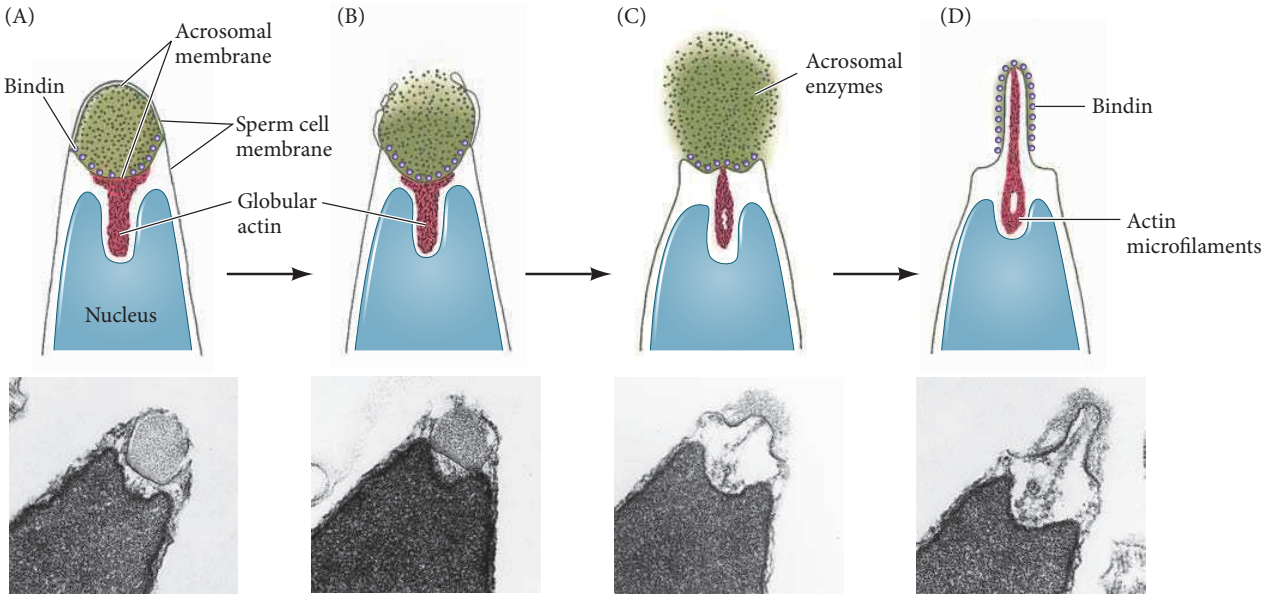


FIGURE 7.10 Acrosome reaction in sea urchin sperm. (A–C) The portion of the acrosomal membrane lying directly beneath the sperm cell membrane fuses with the cell membrane to release the contents of the acrosomal vesicle. (D) The actin molecules assemble to produce microfilaments, extending the acrosomal process outward. Actual photographs of the acrosome reaction in sea urchin sperm are shown below the diagrams. (After Summers and Hylander 1974; photographs courtesy of G. L. Decker and W. J. Lennarz.)

is usually species-specific: bindin isolated from the acrosomes of *S. purpuratus* binds to its own dejellied eggs but not to those of *S. franciscanus* (**FIGURE 7.11B**; Glabe and Vacquier 1977; Glabe and Lennarz 1979).

Biochemical studies have confirmed that the bindins of closely related sea urchin species have different protein sequences. This finding implies the existence of species-specific **bindin receptors** on the egg vitelline envelope (**FIGURE 7.12A**). Indeed, a 350-kDa glycoprotein that displays the properties expected of a bindin receptor, has been isolated

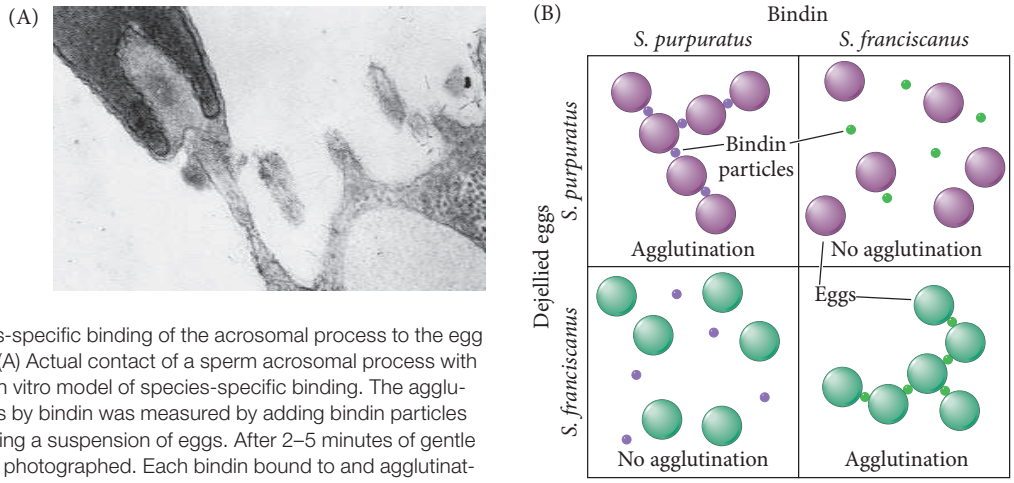


FIGURE 7.11 Species-specific binding of the acrosomal process to the egg surface in sea urchins. (A) Actual contact of a sperm acrosomal process with an egg microvillus. (B) In vitro model of species-specific binding. The agglutination of dejellied eggs by bindin was measured by adding bindin particles to a plastic well containing a suspension of eggs. After 2–5 minutes of gentle shaking, the wells were photographed. Each bindin bound to and agglutinated only eggs from its own species. (A from Epel 1977, courtesy of F. D. Collins and D. Epel; B based on photographs in Glabe and Vacquier 1978.)

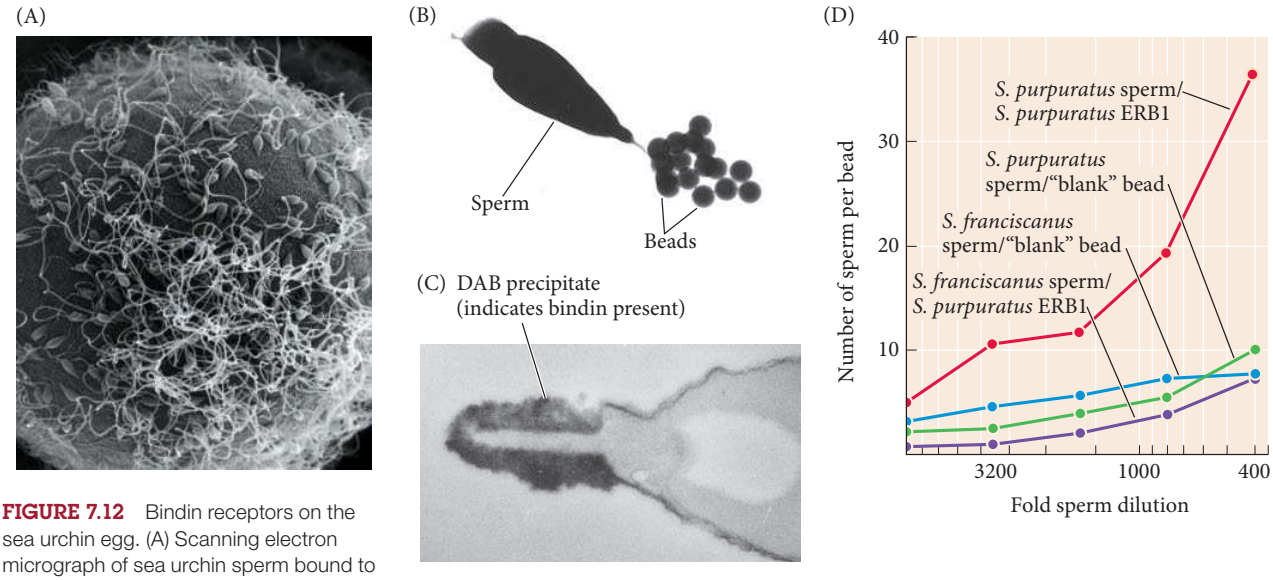


FIGURE 7.12 Bindin receptors on the sea urchin egg. (A) Scanning electron micrograph of sea urchin sperm bound to the vitelline envelope of an egg. Although this egg is saturated with sperm, there appears to be room on the surface for more sperm, implying the existence of a limited number of bindin receptors. (B) *Strongylocentrotus purpuratus* sperm bind to polystyrene beads that have been coated with purified bindin receptor protein. (C) Immunochemically labeled bindin (the label manifests as a dark precipitate of diaminobenzidine, DAB) is seen to be localized to the acrosomal process after the acrosome reaction. (D) Species-specific binding of sea urchin sperm to ERB1. *S. purpuratus* sperm bound to beads coated with ERB1 bindin receptor purified from *S. purpuratus* eggs, but *S. franciscanus* sperm did not. Neither sperm bound to uncoated "blank" beads. (A © Mia Tegner/SPL/Science Source; B from Foltz et al. 1993; C from Moy and Vacquier 1979, courtesy of V. Vacquier; D after Kamei and Glabe 2003.)

from sea urchin eggs (**FIGURE 7.12B**; Kamei and Glabe 2003). These bindin receptors are thought to be aggregated into complexes on the vitelline envelope, and hundreds of such complexes may be needed to tether the sperm to the egg. The receptor for sperm bindin on the egg vitelline envelope appears to recognize the protein portion of bindin on the acrosome (**FIGURE 7.12C**) in a species-specific manner. Closely related species of sea urchins (i.e., different species in the same genus) have divergent bindin receptors, and eggs will adhere only to the bindin of their own species (**FIGURE 7.12D**). Thus, species-specific recognition of sea urchin gametes can occur at the levels of sperm attraction, sperm activation, the acrosome reaction, and sperm adhesion to the egg surface.

Bindin and other gamete recognition proteins are among the fastest evolving proteins known (Metz and Palumbi 1996; Swanson and Vacquier 2002). Even when closely related urchin species have near-identity of every other protein, their bindins and bindin receptors may have diverged significantly.

Fusion of the egg and sperm cell membranes

Once the sperm has traveled to the egg and undergone the acrosome reaction, the fusion of the sperm cell membrane with the egg cell membrane can begin (**FIGURE 7.13**). Sperm-egg fusion appears to cause the polymerization of actin in the egg to form a **fertilization cone** (Summers et al. 1975). Homology between the egg and the sperm is again demonstrated, since the sperm's acrosomal process also appears to be formed by the polymerization of actin. Actin from the gametes forms a connection that widens the cytoplasmic bridge between the egg and sperm. The sperm nucleus and tail pass through this bridge.

Fusion is an active process, often mediated by specific "fusogenic" proteins. In sea urchins, bindin plays a second role as a fusogenic protein. In addition to recognizing the egg, bindin contains a long stretch of hydrophobic amino acids near its amino terminus, and this region is able to fuse phospholipid vesicles in vitro (Ulrich et al. 1999; Gage et al. 2004). Under the ionic conditions present in the mature unfertilized egg, bindin can cause the sperm and egg membranes to fuse.

One egg, one sperm

As soon as one sperm enters the egg, the fusibility of the egg membrane—which was necessary to get the sperm inside the egg—becomes a dangerous liability. In the normal case—**monospermy**—only one sperm enters the egg, and the haploid sperm nucleus

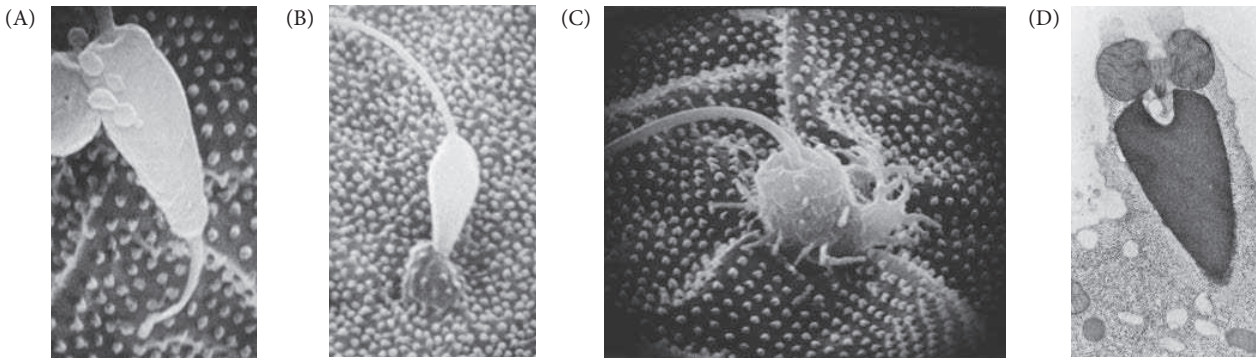
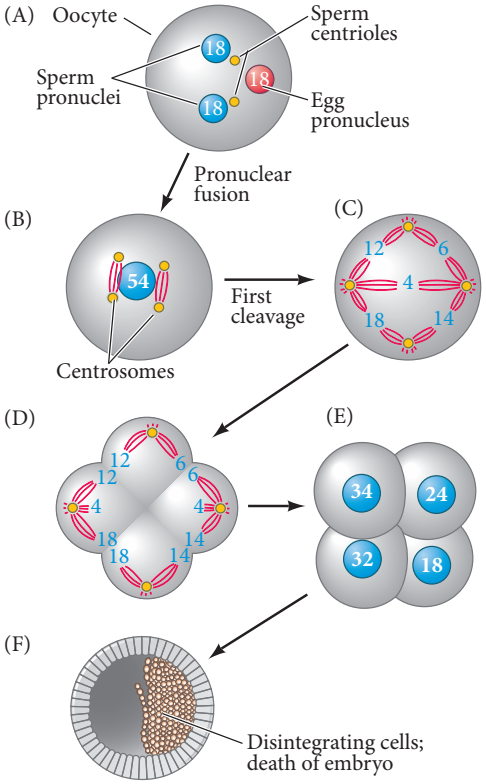


FIGURE 7.13 Scanning electron micrographs of the entry of sperm into sea urchin eggs. (A) Contact of sperm head with egg microvillus through the acrosomal process. (B) Formation of fertilization cone. (C) Internalization of sperm within the egg. (D) Transmission electron micrograph of sperm internalization through the fertilization cone. (A–C from Schatten and Mazia 1976, courtesy of G. Schatten; D courtesy of F. J. Longo.)

combines with the haploid egg nucleus to form the diploid nucleus of the fertilized egg (zygote), thus restoring the chromosome number appropriate for the species. During cleavage, the centriole provided by the sperm divides to form the two poles of the mitotic spindle while the egg-derived centriole is degraded.

In most animals, any sperm that enters the egg can provide a haploid nucleus and a centriole. The entrance of multiple sperm—**polyspermy**—leads to disastrous consequences in most organisms. In sea urchins, fertilization by two sperm results in a triploid nucleus, in which each chromosome is represented three times rather than twice. Worse, each sperm's centriole divides to form the two poles of a mitotic apparatus, so instead of a bipolar mitotic spindle separating the chromosomes into two cells, the triploid chromosomes may be divided into as many as four cells, with some cells receiving extra copies of certain chromosomes while other cells lack them (**FIGURE 7.14**). Theodor Boveri demonstrated in 1902 that such cells either die or develop abnormally.



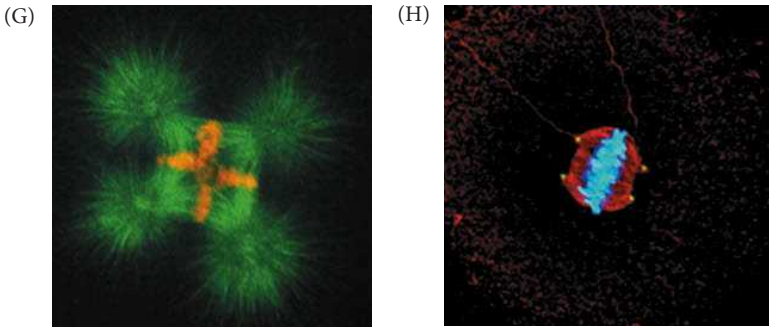
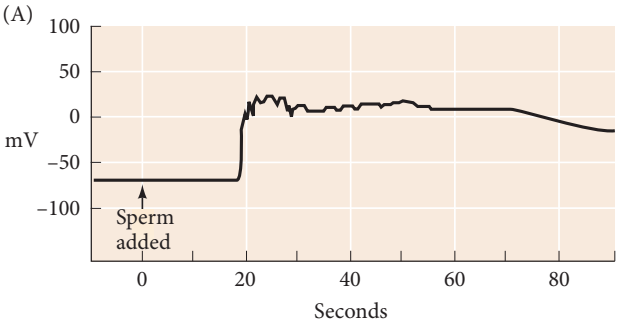


FIGURE 7.14 Aberrant development in a dispermic sea urchin egg. (A) Fusion of three haploid nuclei, each containing 18 chromosomes, and the division of the two sperm centrioles to form four centrosomes (mitotic poles). (B,C) The 54 chromosomes randomly assort on the four spindles. (D) At anaphase of the first division, the duplicated chromosomes are pulled to the four poles. (E) Four cells containing different numbers and types of chromosomes are formed, thereby causing (F) the early death of the embryo. (G) First metaphase of a dispermic sea urchin egg akin to (D). The microtubules are stained green; the DNA stain appears orange. The triploid DNA is being split into four chromosomally unbalanced cells instead of the normal two cells with equal chromosome complements. (H) Human dispermic egg at first mitosis. The four centrioles are stained yellow, while the microtubules of the spindle apparatus (and of the two sperm tails) are stained red. The three sets of chromosomes divided by these four poles are stained blue. (A–F after Boveri 1907; G courtesy of J. Holy; H from Simerly et al. 1999, courtesy of G. Schatten.)



(B)

Na^+	Polyspermic eggs (%)
490	22
360	26
120	97
50	100

FIGURE 7.15 Membrane potential of sea urchin eggs before and after fertilization. (A) Before the addition of sperm, the potential difference across the egg cell membrane is about –70 mV. Within 1–3 seconds after the fertilizing sperm contacts the egg, the potential shifts in a positive direction. (B,C) *Lytechinus* eggs photographed during first cleavage. (B) Control eggs developing in 490 m*M* Na^+. (C) Polyspermy in eggs fertilized in similarly high concentrations of sperm in 120 m*M* Na^+ (choline was substituted for sodium). (D) Table showing the rise of polyspermy with decreasing Na^+ concentration. Salt water is about 600 m*M* Na^+. (After Jaffe 1980; B,C courtesy of L. A. Jaffe.)

Developing Questions

Sodium ions can readily orchestrate the fast block to polyspermy in salty seawater. But amphibians spawning in freshwater ponds also use ion channels to achieve a fast block to polyspermy. How is this achieved in an environment that lacks the ocean's high concentrations of Na^+?

The fast block to polyspermy

The most straightforward way to prevent the union of more than two haploid nuclei is to prevent more than one sperm from entering the egg. Different mechanisms to prevent polyspermy have evolved, two of which are seen in the sea urchin egg. An initial, fast reaction, accomplished by an electric change in the egg cell membrane, is followed by a slower reaction caused by the exocytosis of the cortical granules (Just 1919).

The **fast block to polyspermy** is achieved by a change in the electric potential of the egg cell membrane that occurs immediately upon the entry of a sperm. Once one sperm has fused with the egg, soluble material from that sperm (probably nicotinic acid adenine dinucleotide phosphate, NAADP) acts to change the egg cell membrane (McCulloh and Chambers 1992; Wong and Wessel 2013). Sodium channels are closed, thereby preventing the entry of sodium ions (Na^+) into the egg, and the egg cell membrane maintains an electrical voltage gap between the interior of the egg and its environment. This **resting membrane potential** is generally about 70 mV, which is expressed as –70 mV because *the inside of the cell is negatively charged with respect to the exterior.* Within 1–3 seconds after the binding of the first sperm, the membrane potential shifts to a *positive* level—about +20 mV—with respect to the exterior (**FIGURE 7.15A**; Jaffe 1980; Longo et al. 1986). The shift from negative to positive is the result of a small influx of Na^+ into the egg through newly opened sodium channels. Sperm cannot fuse with egg cell membranes that have a positive resting potential, so the shift means that no more sperm can fuse to the egg.

The importance of Na^+ and the change in resting potential from negative to positive was demonstrated by Laurinda Jaffe and colleagues. They found that polyspermy can be induced if an electric current is applied to artificially keep the sea urchin egg membrane potential negative. Conversely, fertilization can be prevented entirely by artificially keeping the membrane potential of eggs positive (Jaffe 1976). The fast block to polyspermy can also be circumvented by lowering the concentration of Na^+ in the surrounding water (**FIGURE 7.15B**). If the supply of sodium ions is not sufficient to cause the positive shift in membrane potential, polyspermy occurs (Gould-Somero et al. 1979; Jaffe 1980). An electric block to polyspermy also occurs in frogs (Cross and Elinson 1980; Iwao et al. 2014), but probably not in most mammals (Jaffe and Cross 1983).

WEB TOPIC 7.3 **BLOCKS TO POLYSPERMY** The work of Theodor Boveri and E. E. Just were critical in elucidating the blocks against multiple sperm entry.

The slow block to polyspermy

The fast block to polyspermy is transient, since the membrane potential of the sea urchin egg remains positive for only about a minute. This brief potential shift is not sufficient to prevent polyspermy permanently, and polyspermy can still occur if the sperm bound to the vitelline envelope are not somehow removed (Carroll and Epel 1975). This sperm removal is accomplished by the **cortical granule reaction**, also known as the **slow block to polyspermy.** This slower, mechanical block to polyspermy

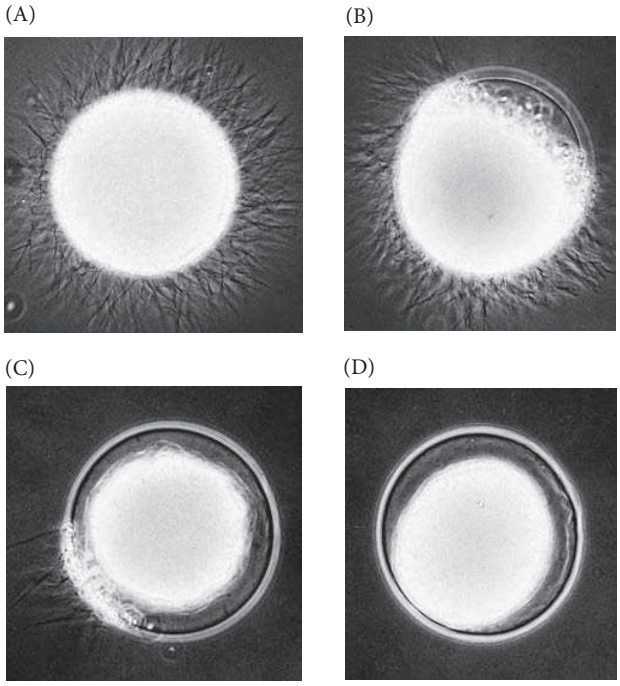


FIGURE 7.16 Formation of the fertilization envelope and removal of excess sperm. To create these photographs, sperm were added to sea urchin eggs, and the suspension was then fixed in formaldehyde to prevent further reactions. (A) At 10 seconds after sperm addition, sperm surround the egg. (B,C) At 25 and 35 seconds after insemination, respectively, a fertilization envelope is forming around the egg, starting at the point of sperm entry. (D) The fertilization envelope is complete, and excess sperm have been removed. (From Vacquier and Payne 1973, courtesy of V. D. Vacquier.)

becomes active about a minute after the first successful sperm-egg fusion (Just 1919). This reaction is found in many animal species, including sea urchins and most mammals.

Directly beneath the sea urchin egg cell membrane are about 15,000 cortical granules, each about 1 μm in diameter (see Figure 7.4B). Upon sperm entry, cortical granules fuse with the egg cell membrane and release their contents into the space between the cell membrane and the fibrous mat of vitelline envelope proteins. Several proteins are released by cortical granule exocytosis. One of these, the enzyme cortical granule serine protease, cleaves the protein posts that connect the vitelline envelope proteins to the egg cell membrane; it also clips off the bindin receptors and any sperm attached to them (Vacquier et al. 1973; Glabe and Vacquier 1978; Haley and Wessel 1999, 2004).

The components of the cortical granules bind to the vitelline envelope to form a **fertilization envelope**. The fertilization envelope starts to form at the site of sperm entry and continues its expansion around the egg. This process starts about 20 seconds after sperm attachment and is complete by the end of the first minute of fertilization (**FIGURE 7.16**; Wong and Wessel 2004, 2008).

WATCH DEVELOPMENT 7.1 See the fertilization envelope rise from the egg surface.

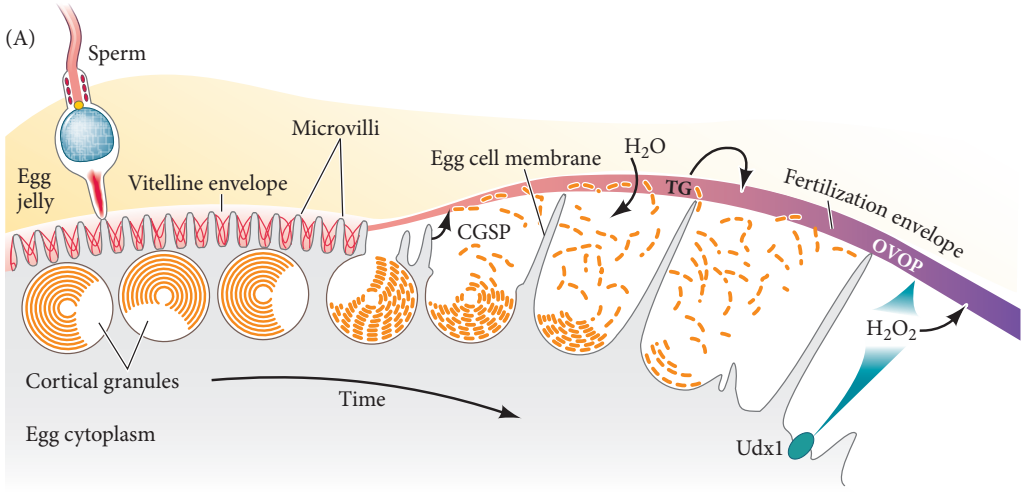
The fertilization envelope is elevated from the cell membrane by mucopolysaccharides released by the cortical granules. These viscous compounds absorb water to expand the space between the cell membrane and the fertilization envelope, so that the envelope moves radially away from the egg. The fertilization envelope is then stabilized by crosslinking adjacent proteins through egg-specific peroxidase enzymes and a transglutaminase released from the cortical granules (**FIGURE 7.17**; Foerder and Shapiro 1977; Wong et al. 2004; Wong and Wessel 2009). This crosslinking allows the egg and early embryo to resist the shear forces of the ocean's intertidal waves. As this is happening, a fourth set of cortical granule proteins, including hyalin, forms a coating around the egg (Hylander and Summers 1982). The egg extends elongated microvilli whose tips attach to this **hyaline layer**, which provides support for the blastomeres during cleavage.

VADE MECUM

The two blocks to polyspermy were discovered in the early 1900s by the African-American embryologist Ernest Everett Just. The sea urchin segment contains videos of Just's work on these embryos.

Calcium as the initiator of the cortical granule reaction

The mechanism of cortical granule exocytosis is similar to that of the exocytosis of the acrosome, and it may involve many of the same molecules. Upon fertilization, the concentration of free Ca^{2+} in the egg cytoplasm increases greatly. In this high-calcium environment, the cortical granule membranes fuse with the egg cell membrane, releasing their contents (see Figure 7.17A). Once the fusion of the cortical granules begins near the point of sperm entry, a wave of cortical granule exocytosis propagates around the cortex to the opposite side of the egg.



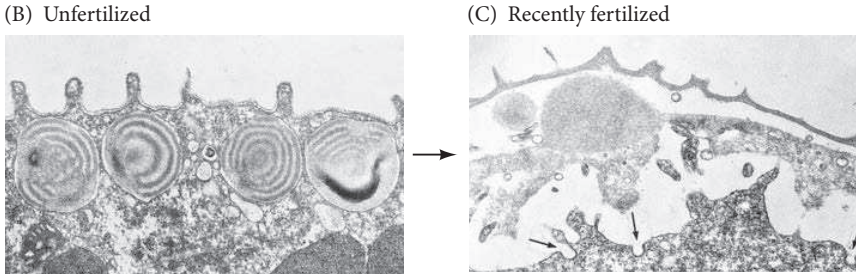


FIGURE 7.17 Cortical granule exocytosis and formation of the sea urchin fertilization envelope. (A) Schematic diagram of events leading to the formation of the fertilization envelope. As cortical granules undergo exocytosis, they release cortical granule serine protease (CGSP), an enzyme that cleaves the proteins linking the vitelline envelope to the cell membrane. Mucopolysaccharides released by the cortical granules form an osmotic gradient, causing water to enter and swell the space between the vitelline envelope and the cell membrane. The enzyme Udx1 in the former cortical granule membrane catalyzes the formation of hydrogen peroxide (H_2O_2), the substrate for soluble ovoperoxidase (OVOP). OVOP and transglutaminases (TG) harden the vitelline envelope, now called the fertilization envelope. (B,C) Transmission electron micrographs of the cortex of an unfertilized sea urchin egg and the same region of a recently fertilized egg. The raised fertilization envelope and the points at which the cortical granules have fused with the egg cell membrane of the egg (arrows) are visible in (C). (A after Wong et al. 2008; B,C from Chandler and Heuser 1979, courtesy of D. E. Chandler.)

In sea urchins and mammals, the rise in Ca^{2+} concentration responsible for the cortical granule reaction is not due to an influx of calcium into the egg, but comes from within the egg itself. The release of calcium from intracellular storage can be monitored visually using calcium-activated luminescent dyes such as aequorin (a protein that, like GFP, is isolated from luminescent jellyfish) or fluorescent dyes such as fura-2. These dyes emit light when they bind free Ca^{2+}. When a sea urchin egg is injected with dye and then fertilized, a striking wave of calcium release propagates across the egg and is visualized as a band of light that starts at the point of sperm entry and proceeds actively to the other end of the cell (**FIGURE 7.18**; Steinhardt et al. 1977; Hafner et al. 1988). The entire release of Ca^{2+} is complete within roughly 30 seconds, and free Ca^{2+} is re-sequestered shortly after being released.

WATCH DEVELOPMENT 7.2 This video of sea urchin fertilization shows waves of calcium ions starting at the point of sperm attachment and traversing the sea urchin egg.

Several experiments have demonstrated that Ca^{2+} is directly responsible for propagating the cortical granule reaction, and that these ions are stored within the egg itself. The drug A23187 is a calcium ionophore—a compound that allows the diffusion of ions such as Ca^{2+} across lipid membranes, permitting them to travel across otherwise impermeable barriers. Placing unfertilized sea urchin eggs into seawater containing A23187 initiates the cortical granule reaction and the elevation of the fertilization envelope. Moreover, this reaction occurs in the absence of any Ca^{2+} in the surrounding water; thus the A23187 must be stimulating the release of Ca^{2+} that is already sequestered in organelles within the egg (Chambers et al. 1974; Steinhardt and Epel 1974).

In sea urchins and vertebrates (but not snails and worms), the Ca^{2+} responsible for the cortical granule reaction is stored in the endoplasmic reticulum of the egg (Eisen and Reynolds 1985; Terasaki and Sardet 1991). In sea urchins and frogs, this reticulum

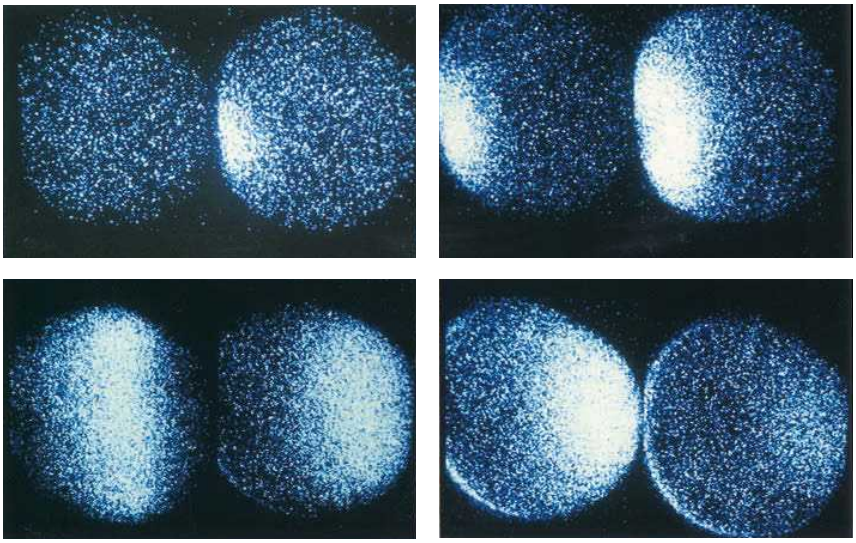


FIGURE 7.18 Calcium release across a sea urchin egg during fertilization. The egg is pre-loaded with a dye that fluoresces when it binds Ca^{2+}. When a sperm fuses with the egg, a wave of calcium release is seen, beginning at the site of sperm entry and propagating across the egg. The wave does not simply diffuse but travels actively, taking about 30 seconds to traverse the egg. (Courtesy of G. Schatten.)

is pronounced in the cortex and surrounds the cortical granules (**FIGURE 7.19**; Gardiner and Grey 1983; Luttmer and Longo 1985). The cortical granules are themselves tethered to the cell membrane by a series of integral membrane proteins that facilitate calcium-mediated exocytosis (Conner et al. 1997; Conner and Wessel 1998). Thus, as soon as Ca^{2+} is released from the endoplasmic reticulum, the cortical granules fuse with the cell membrane above them. Once initiated, the release of calcium is self-propagating. Free calcium is able to release sequestered calcium from its storage sites, thus causing a wave of Ca^{2+} release and cortical granule exocytosis.

Activation of Egg Metabolism in Sea Urchins

Although fertilization is often depicted as nothing more than the means to merge two haploid nuclei, it has an equally important role in initiating the processes that begin development. These events happen in the cytoplasm and occur without the involvement of the parental nuclei.[3] In addition to initiating the slow block to polyspermy (through cortical granule exocytosis), the release of Ca^{2+} that occurs when the sperm enters the egg is critical for activating the egg's metabolism and initiating development. Calcium ions release the inhibitors from maternally stored messages, allowing these mRNAs to be translated; they also release the inhibition of nuclear division, thereby allowing

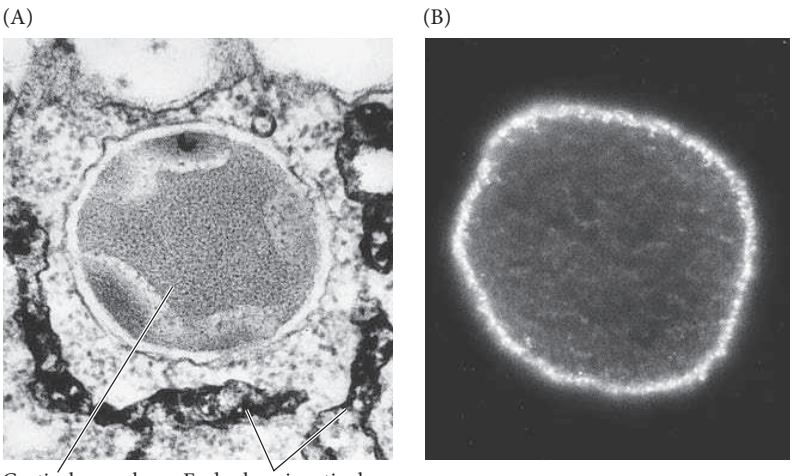


FIGURE 7.19 Endoplasmic reticulum surrounding cortical granules in sea urchin eggs. (A) The endoplasmic reticulum has been stained to allow visualization by transmission electron microscopy. The cortical granule is seen to be surrounded by dark-stained endoplasmic reticulum. (B) An entire egg stained with fluorescent antibodies to calcium-dependent calcium release channels. The antibodies show these channels in the cortical endoplasmic reticulum. (A from Luttmer and Longo 1985, courtesy of S. Luttmer; B from McPherson et al. 1992, courtesy of F. J. Longo.)

[3] In certain salamanders, this function of fertilization (i.e., initiating development of the embryo) has been totally divorced from the genetic function. The silver salamander *Ambystoma platineum* is a hybrid subspecies consisting solely of females. Each female produces an egg with an unreduced chromosome number. This egg, however, cannot develop on its own, so the silver salamander mates with a male Jefferson salamander (*A. jeffersonianum*). The sperm from the Jefferson salamander merely stimulates the egg's development; it does not contribute genetic material (Uzzell 1964). For details of this complex mechanism of procreation, see Bogart et al. 1989, 2009.

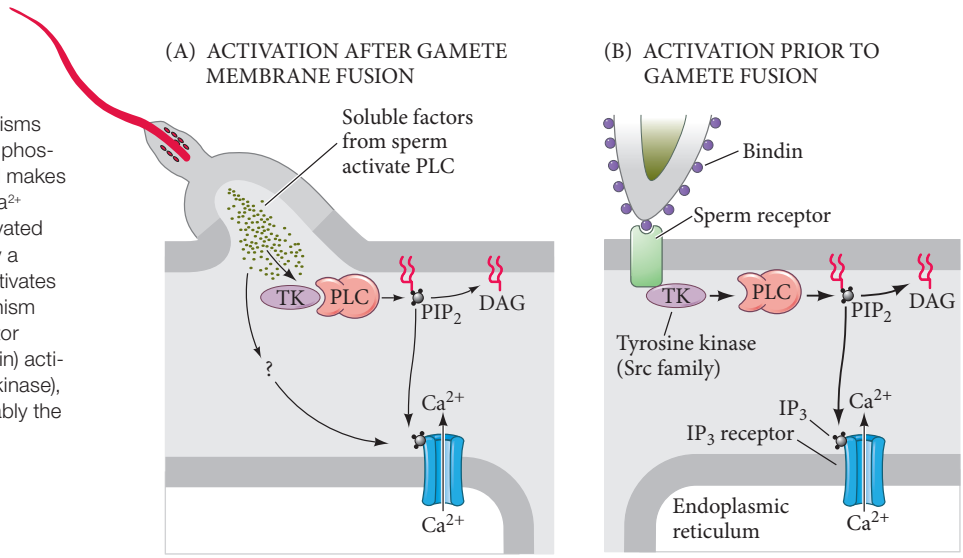


FIGURE 7.20 Probable mechanisms of egg activation. In both cases, a phospholipase C (PLC) is activated and makes IP_3 and diacylglycerol (DAG). (A) Ca^{2+} release and egg activation by activated PLC directly from the sperm, or by a substance from the sperm that activates egg PLC. This may be the mechanism in mammals. (B) The bindin receptor (perhaps acting through a G protein) activates tyrosine kinase (TK, an Src kinase), which activates PLC. This is probably the mechanism used by sea urchins.

cleavage to occur. Indeed, throughout the animal kingdom, calcium ions are used to activate development during fertilization.

Release of intracellular calcium ions

The way Ca^{2+} is released varies from species to species (see Parrington et al. 2007). One way, first proposed by Jacques Loeb (1899, 1902), is that a soluble factor from the sperm is introduced into the egg at the time of cell fusion, and this substance activates the egg by changing the ionic composition of the cytoplasm (**FIGURE 7.20A**). This mechanism, as we will see later, probably works in mammals. The other mechanism, proposed by Loeb's rival Frank Lillie (1913), is that the sperm binds to receptors on the egg cell surface and changes their conformation, thus initiating reactions within the cytoplasm that activate the egg (**FIGURE 7.20B**). This is probably what happens in sea urchins.

IP_3: THE RELEASER OF CA^{2+} If Ca^{2+} from the egg's endoplasmic reticulum is responsible for the cortical granule reaction and the reactivation of development, what releases Ca^{2+}? Throughout the animal kingdom, it has been found that **inositol 1,4,5-trisphosphate (IP_3)** is the primary agent for releasing Ca^{2+} from intracellular storage.

The IP_3 pathway is shown in **FIGURE 7.21**. The membrane phospholipid **phosphatidylinositol 4,5-bisphosphate (PIP_2)** is split by the enzyme **phospholipase C (PLC)** to yield two active compounds: IP_3 and **diacylglycerol (DAG)**. IP_3 is able to release Ca^{2+} into the cytoplasm by opening the calcium channels of the endoplasmic reticulum. DAG activates protein kinase C, which in turn activates a protein that exchanges sodium ions for hydrogen ions, raising the pH of the egg (Nishizuka 1986; Swann and Whitaker 1986). This Na^+-H^+ exchange pump also requires Ca^{2+}. The result of PLC activation is therefore the liberation of Ca^{2+} and the alkalinization of the egg, and both of the compounds this activation creates—IP_3 and DAG—are involved in the initiation of development.

WATCH DEVELOPMENT 7.3 See a movie of fertilization with and without PLC activation.

In sea urchin eggs, IP_3 is formed initially at the site of sperm entry and can be detected within seconds of sperm-egg attachment. Inhibiting IP_3 synthesis prevents Ca^{2+} release (Lee and Shen 1998; Carroll et al. 2000), whereas injected IP_3 can release sequestered Ca^{2+} and lead to cortical granule exocytosis (Whitaker and Irvine 1984; Busa et al. 1985). Moreover, these IP_3-mediated effects can be thwarted by pre-injecting the egg with calcium-chelating agents (Turner et al. 1986).

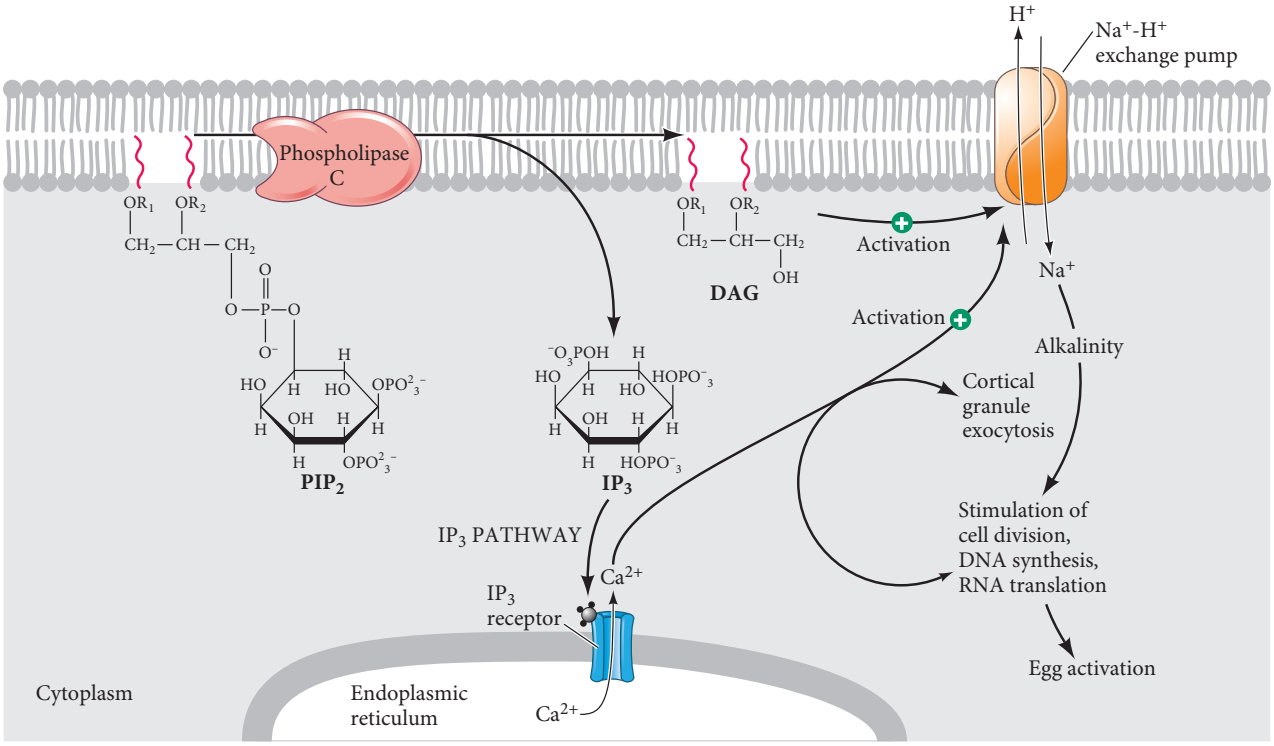


FIGURE 7.21 Roles of inositol phosphates in releasing calcium from the endoplasmic reticulum and the initiation of development. Phospholipase C splits PIP_2 into IP_3 and DAG. IP_3 releases calcium from the endoplasmic reticulum, and DAG, with assistance from the released Ca^{2+}, activates the sodium-hydrogen exchange pump in the membrane.

IP_3-responsive calcium channels have been found in the egg endoplasmic reticulum. The IP_3 formed at the site of sperm entry is thought to bind to IP_3 receptors in these calcium channels, effecting a local release of Ca^{2+} (Ferris et al. 1989; Furuichi et al. 1989). Once released, Ca^{2+} can diffuse directly, or it can facilitate the release of more Ca^{2+} by binding to *calcium-triggered calcium-release receptors*, also located in the cortical endoplasmic reticulum (McPherson et al. 1992). These receptors release stored Ca^{2+} when they bind Ca^{2+}, so binding Ca^{2+} releases more Ca^{2+}, which binds to more receptors, and so on. The resulting wave of calcium release is propagated throughout the cell, starting at the point of sperm entry (see Figure 7.18). The cortical granules, which fuse with the cell membrane in the presence of high Ca^{2+} concentrations, respond with a wave of exocytosis that follows the calcium wave. Mohri and colleagues (1995) have shown that IP_3-released Ca^{2+} is both necessary and sufficient for initiating the wave of calcium release.

PHOSPHOLIPASE C: THE GENERATOR OF IP_3 If IP_3 is necessary for Ca^{2+} release and phospholipase C is required in order to generate IP_3, the question then becomes, What activates PLC? This question has not been easy to address since (1) there are numerous types of PLC that (2) can be activated through different pathways, and (3) different species use different mechanisms to activate PLC. Results from studies of sea urchin eggs suggest that the active PLC in echinoderms is a member of the γ (gamma) family of PLCs (Carroll et al. 1997, 1999; Shearer et al. 1999). Inhibitors that specifically block PLCγ inhibit IP_3 production as well as Ca^{2+} release. Moreover, these inhibitors can be circumvented by microinjecting IP_3 into the egg. How PLCγ is activated by sperm is still a matter of controversy, although inhibitor studies have shown that membrane-bound kinases (Src kinases) and GTP-binding proteins play critical roles (**FIGURE 7.22**; Kinsey and Shen 2000; Giusti et al. 2003; Townley et al. 2009; Voronina and Wessel 2003, 2004). One possibility is that NAADP brought in by the sperm to initiate electrical depolarization also activates the enzyme cascade leading to IP_3 production and calcium release (Churchill et al. 2003; Morgan and Galione 2007).

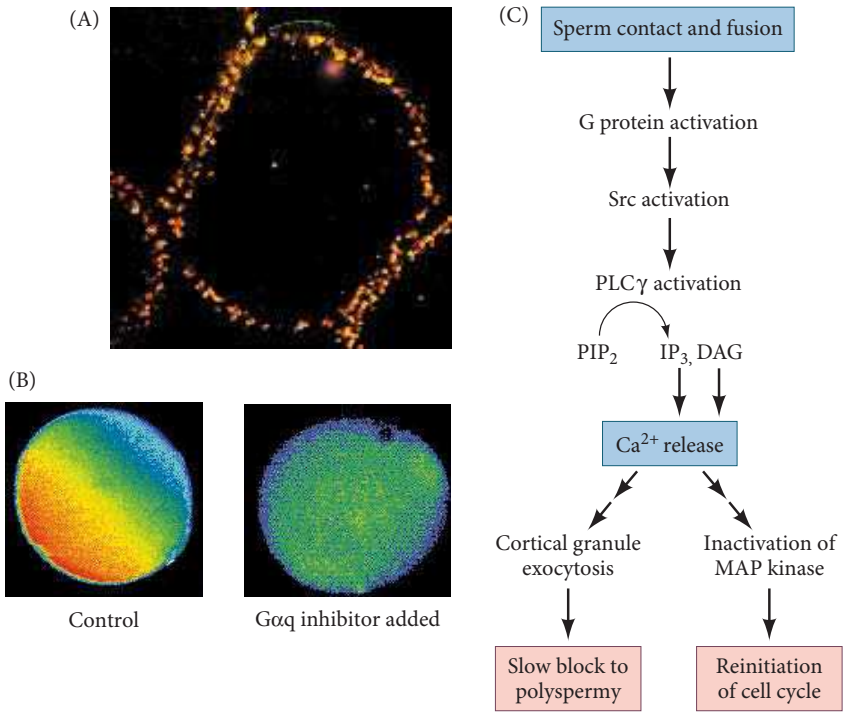


FIGURE 7.22 G protein involvement in Ca^{2+} entry into sea urchin eggs. (A) Mature sea urchin egg immunologically labeled for the cortical granule protein hyaline (red) and the G protein Gαq (green). The overlap of signals produces the yellow color. Gαq is localized to the cortex. (B) A wave of Ca^{2+} appears in the control egg (computer-enhanced to show relative intensities, with red being the highest), but not in the egg injected with an inhibitor of the Gαq protein. (C) Possible model for egg activation by the influx of Ca^{2+}. (After Voronina and Wessel 2003; photographs courtesy of G. M. Wessel.)

Effects of calcium release

The flux of calcium across the egg activates a preprogrammed set of metabolic events. The responses of the sea urchin egg to the sperm can be divided into "early" responses, which occur within seconds of the cortical granule reaction, and "late" responses, which take place several minutes after fertilization begins (**TABLE 7.1**).

EARLY RESPONSES As we have seen, contact or fusion of a sea urchin sperm and egg activates two major blocks to polyspermy: the fast block, mediated by sodium influx into the cell; and the cortical granule reaction, or slow block, mediated by the intracellular release of Ca^{2+}. The same release of Ca^{2+} responsible for the cortical granule reaction is also responsible for the re-entry of the egg into the cell cycle and the reactivation of egg protein synthesis. Ca^{2+} levels in the egg increase from 0.05 to between 1 and 5 μ*M*, and in almost all species this occurs as a wave or succession of waves that sweep across the egg beginning at the site of sperm-egg fusion (see Figure 7.18; Jaffe 1983; Terasaki and Sardet 1991; Stricker 1999).

Calcium release activates a series of metabolic reactions that initiate embryonic development (**FIGURE 7.23**). One of these is the activation of the enzyme NAD^+ kinase, which converts NAD^+ to $NADP^+$ (Epel et al. 1981). Since $NADP^+$ (but not NAD^+) can be used as a coenzyme for lipid biosynthesis, such a conversion has important consequences for lipid metabolism and thus may be important in the construction of the many new cell membranes required during cleavage. Udx1, the enzyme responsible for the reduction of oxygen to crosslink the fertilization envelope, is also NADPH-dependent (Heinecke and Shapiro 1989; Wong et al. 2004). Lastly, NADPH helps regenerate glutathione and ovothiols, molecules that may be crucial scavengers of free radicals that could otherwise damage the DNA of the egg and early embryo (Mead and Epel 1995).

DEV TUTORIAL ***Find it/lose it/move it*** **The basic pattern of biological evidence—find it/lose it/move it—can be followed in the discoveries involving gamete adhesion and calcium activation of the egg.**

TABLE 7.1 Events of sea urchin fertilization

Event	Approximate time postinsemination[a]
EARLY RESPONSES	
Sperm-egg binding	0 sec
Fertilization potential rise (fast block to polyspermy)	within 1 sec
Sperm–egg membrane fusion	within 1 sec
Calcium increase first detected	10 sec
Cortical granule exocytosis (slow block to polyspermy)	15–60 sec
LATE RESPONSES	
Activation of NAD kinase	starts at 1 min
Increase in $NADP^+$ and NADPH	starts at 1 min
Increase in O_2 consumption	starts at 1 min
Sperm entry	1–2 min
Acid efflux	1–5 min
Increase in pH (remains high)	1–5 min
Sperm chromatin decondensation	2–12 min
Sperm nucleus migration to egg center	2–12 min
Egg nucleus migration to sperm nucleus	5–10 min
Activation of protein synthesis	starts at 5–10 min
Activation of amino acid transport	starts at 5–10 min
Initiation of DNA synthesis	20–40 min
Mitosis	60–80 min
First cleavage	85–95 min

Main sources: Whitaker and Steinhardt 1985; Mohri et al. 1995.

[a]Approximate times based on data from *S. purpuratus* (15–17°C), *L. pictus* (16–18°C), *A. punctulata* (18–20°C), and *L. variegatus* (22–24°C). The timing of events within the first minute is best known for *L. variegatus*, so times are listed for that species.

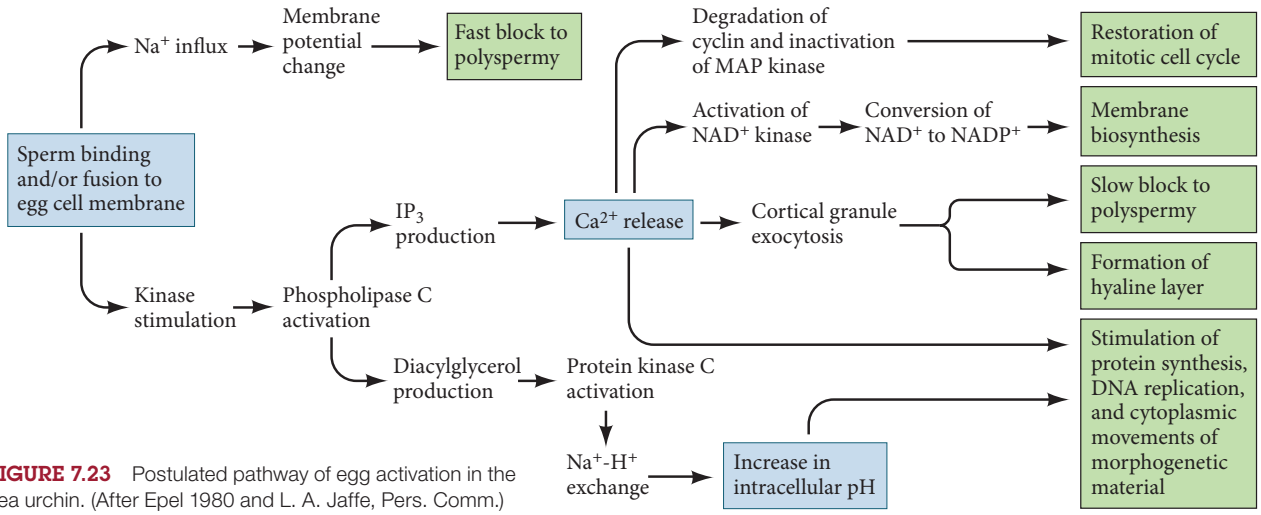


FIGURE 7.23 Postulated pathway of egg activation in the sea urchin. (After Epel 1980 and L. A. Jaffe, Pers. Comm.)

WEB TOPIC 7.4 **RULES OF EVIDENCE** The "find it/lose it/move it" pattern for experimentation fits into a larger system of scientific evidence, as shown by these examples from fertilization research.

LATE RESPONSES: RESUMPTION OF PROTEIN AND DNA SYNTHESIS The late responses of fertilization include the activation of a new burst of DNA and protein synthesis. In sea urchins, the fusion of egg and sperm causes the intracellular pH to increase. This rise in intracellular pH begins with a second influx of Na^+ from seawater, which results in a 1:1 exchange between these sodium ions and hydrogen ions (H^+) from inside the egg. This loss of H^+ causes the pH within the egg to rise (Shen and Steinhardt 1978; Michael and Walt 1999).

It is thought that pH increase and Ca^{2+} elevation act together to stimulate new DNA and protein synthesis (Winkler et al. 1980; Whitaker and Steinhardt 1982; Rees et al. 1995). If one experimentally elevates the pH of an unfertilized egg to a level similar to that of a fertilized egg, DNA synthesis and nuclear envelope breakdown ensue, just as if the egg were fertilized (Miller and Epel 1999). Calcium ions are also critical to new DNA synthesis. The wave of free Ca^{2+} inactivates the enzyme MAP kinase, converting it from a phosphorylated (active) to an unphosphorylated (inactive) form, thus removing an inhibition on DNA synthesis (Carroll et al. 2000). DNA synthesis can then resume.

In sea urchins, a burst of protein synthesis usually occurs within several minutes after sperm entry. This protein synthesis does not depend on the synthesis of new messenger RNA, but uses mRNAs already present in the oocyte cytoplasm. These mRNAs encode proteins such as histones, tubulins, actins, and morphogenetic factors that are used during early development. Such a burst of protein synthesis can be induced by artificially raising the pH of the cytoplasm using ammonium ions (Winkler et al. 1980).

One mechanism for this global rise in the translation of messages stored in the oocyte appears to be the release of inhibitors from the mRNA. In Chapter 2 we discussed maskin, an inhibitor of translation in the unfertilized amphibian oocyte. In sea urchins, a similar inhibitor binds translation initiation factor eIF4E at the 5′ end of several mRNAs and prevents these mRNAs from being translated. Upon fertilization, however, this inhibitor—the eIF4E-binding protein—becomes phosphorylated and is degraded, thus allowing eIF4E to complex with other translation factors and permit protein synthesis from the stored sea urchin mRNAs (Cormier et al. 2001; Oulhen et al. 2007). One of the mRNAs "freed" by the degradation of eIF4E-binding protein is the message encoding cyclin B protein (Salaun et al. 2003, 2004). Cyclin B combines with Cdk1 to create **mitosis-promoting factor** (**MPF**), which is required to initiate cell division.

Fusion of Genetic Material in Sea Urchins

After the sperm and egg cell membranes fuse, the sperm nucleus and its centriole separate from the mitochondria and flagellum. The mitochondria and the flagellum disintegrate inside the egg, so very few, if any, sperm-derived mitochondria are found in developing or adult organisms. Thus, although each gamete contributes a haploid genome to the zygote, the *mitochondrial* genome is transmitted primarily by the maternal parent. Conversely, in almost all animals studied (the mouse being the major exception), the centrosome needed to produce the mitotic spindle of the subsequent divisions is derived from the sperm centriole (see Figure 7.14; Sluder et al. 1989, 1993).

Fertilization in sea urchin eggs occurs after the second meiotic division, so there is already a haploid female pronucleus present when the sperm enters the egg cytoplasm. Once inside the egg, the sperm nucleus undergoes a dramatic transformation as it decondenses to form the haploid male pronucleus. First, the nuclear envelope degenerates, exposing the compact sperm chromatin to the egg cytoplasm (Longo and Kunkle 1978; Poccia and Collas 1997). Kinases from the egg cytoplasm phosphorylate the sperm-specific histone proteins, allowing them to decondense. The decondensed histones are then replaced by egg-derived, cleavage-stage histones (Stephens et al.

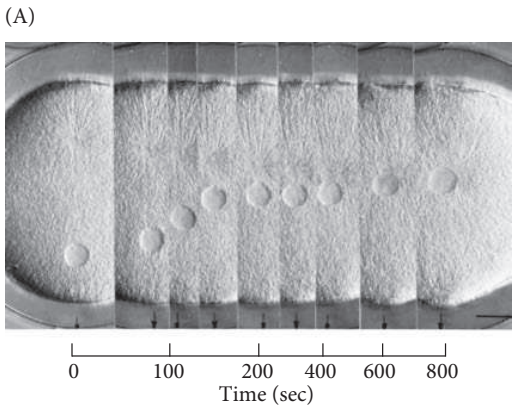


FIGURE 7.24 Nuclear events in the fertilization of the sea urchin. (A) Sequential photographs showing the migration of the egg pronucleus and the sperm pronucleus toward each other in an egg of *Clypeaster japonicus*. The sperm pronucleus is surrounded by its aster of microtubules. (B) The two pronuclei migrate toward each other on these microtubular processes. (The pronuclear DNA is stained blue by Hoechst dye.) The microtubules (stained green with fluorescent antibodies to tubulin) radiate from the centrosome associated with the (smaller) male pronucleus and reach toward the female pronucleus. (C) Fusion of pronuclei in the sea urchin egg. (A from Hamaguchi and Hiramoto 1980, courtesy of the authors; B from Holy and Schatten 1991, courtesy of J. Holy; C courtesy of F. J. Longo.)

2002; Morin et al. 2012). This exchange permits the decondensation of the sperm chromatin. Once decondensed, the DNA adheres to the nuclear envelope, where DNA polymerase can initiate replication (Infante et al. 1973).

But how do the sperm and egg pronuclei find each other? After the sea urchin sperm enters the egg cytoplasm, the sperm nucleus separates from the tail and rotates 180° so that the sperm centriole is between the developing male pronucleus and the egg pronucleus. The sperm centriole then acts as a microtubule organizing center, extending its own microtubules and integrating them with egg microtubules to form an aster. Microtubules extend throughout the egg and contact the female pronucleus, at which point the two pronuclei migrate toward each other. Their fusion forms the diploid zygote nucleus (**FIGURE 7.24**). DNA synthesis can begin either in the pronuclear stage or after the formation of the zygote nucleus, and depends on the level of Ca^{2+} released earlier in fertilization (Jaffe et al. 2001).

At this point, the diploid nucleus has formed. DNA synthesis and protein synthesis have commenced, and the inhibitions to cell division have been removed. The sea urchin can now begin to form a multicellular organism. We will describe the means by which sea urchins achieve multicellularity in Chapter 10.

WATCH DEVELOPMENT 7.4 Two movies show the sperm pronucleus and egg pronucleus travelling toward each other and fusing.

Internal Fertilization in Mammals

It is very difficult to study any interactions between the mammalian sperm and egg that take place prior to these gametes making contact. One obvious reason for this is that mammalian fertilization occurs inside the oviducts of the female. Although it is relatively easy to mimic the conditions surrounding sea urchin fertilization using natural or artificial seawater, we do not yet know the components of the various natural environments that mammalian sperm encounter as they travel to the egg.

A second reason why it is difficult to study mammalian fertilization is that the sperm population ejaculated into the female is probably heterogeneous, containing spermatozoa at different stages of maturation. Out of the 280×10^6 human sperm normally ejaculated during coitus, only about 200 reach the vicinity of the egg (Ralt et al. 1991). Thus, since fewer than 1 in 10,000 sperm even gets close to the egg, it is difficult to assay those molecules that might enable the sperm to swim toward the egg and become activated.

A third reason why it has been difficult to elucidate the details of mammalian fertilization is the recent discovery that there may be multiple mechanisms (discussed later in the chapter) by which mammalian sperm can undergo the acrosome reaction and bind to the zona pellucida (see Clark 2011).

WATCH DEVELOPMENT 7.5 A video from the laboratory of Dr. Yasayuki Mio shows the events of human fertilization and early development in vitro.

Getting the gametes into the oviduct: Translocation and capacitation

The female reproductive tract is not a passive conduit through which sperm race, but a highly specialized set of tissues that actively regulate the transport and maturity of both gametes. Both the male and female gametes use a combination of small-scale biochemical interactions and large-scale physical propulsion to get to the **ampulla**, the region of the oviduct where fertilization takes place.

TRANSLOCATION The meeting of sperm and egg must be facilitated by the female reproductive tract. Different mechanisms are used to position the gametes at the right place at the right time. A mammalian oocyte just released from the ovary is surrounded by a matrix containing cumulus cells. (Cumulus cells are the cells of the ovarian follicle to which the developing oocyte was attached; see Figure 7.5.) If this matrix is experimentally removed or significantly altered, the fimbriae of the oviduct will not "pick up" the oocyte-cumulus complex (see Figure 12.11), nor will the complex be able to enter the oviduct (Talbot et al. 1999). Once it is picked up, a combination of ciliary beating and muscle contractions transport the oocyte-cumulus complex to the appropriate position for its fertilization in the oviduct.

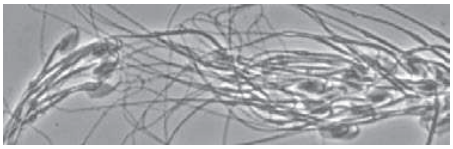
The sperm must travel a longer path. In humans, about 300 million sperm are ejaculated into the vagina, but only one in a million enters the Fallopian tubes (Harper 1982; Cerezales et al. 2015). The translocation of sperm from the vagina to the oviduct involves several processes that work at different times and places.

- *Sperm motility.* Motility (flagellar action) is probably important in getting sperm through the cervical mucus and into the uterus. Interestingly, in those mammals where the female is promiscuous (mating with several males in rapid succession), sperm from the same male will often form "trains" or aggregates where the combined propulsion of the flagella make the sperm faster (**FIGURE 7.25**). This strategy probably evolved for competition between males. In those species without such female promiscuity, the sperm usually remain individual (Fisher and Hoeckstra 2010; Foster and Pizzari 2010; Fisher et al. 2014).

- *Uterine muscle contractions.* Sperm are found in the oviducts of mice, hamsters, guinea pigs, cows, and humans within 30 minutes of sperm deposition in the vagina—a time "too short to have been attained by even the most Olympian sperm relying on their own flagellar power" (Storey 1995). Rather, sperm appear to be transported to the oviduct by the muscular activity of the uterus.

- *Sperm rheotaxis.* Sperm also receive long-distance directional cues from the flow of liquid from the oviduct to the uterus. Sperm display rheotaxis—that is, they will migrate against the direction of the flow—using CatSper calcium channels (like sea urchin sperm) to sense calcium influx and monitor the direction of the current (Miki and Clapham 2013). Such sperm rheotaxis has been observed in mice and in humans.

(A)



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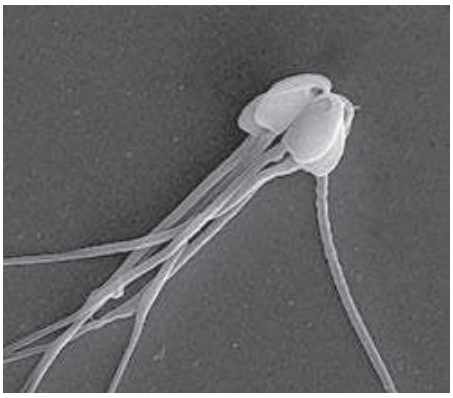

FIGURE 7.25 Sperm associations can occur in species where females mate with several males in a brief timespan. (A) The "sperm train" of the wood mouse *Apodemus sylvaticus*. Sperm are joined by their acrosomal caps. (B) Close-up of the sperm heads of the field mouse *Peromyscus maniculatus*, showing hook-to-hook attachment. (A from Foster and Pizzari 2010, courtesy of T. Pizzari and H. Moore; B from Fischer et al. 2014, courtesy of H. S. Fischer and H. Hoekstra.)

CAPACITATION During the trek from the vagina to the ampullary region of the oviduct, the sperm matures such that it has the capacity to fertilize the egg when the two finally meet. Unlike the sperm of frogs or sea urchins, newly ejaculated mammalian sperm are immature and cannot fertilize the egg; they are unable to undergo the acrosome reaction or to sense the cues that will eventually guide them to the egg. To achieve such competence, the sperm must undergo a suite of sequential physiological changes called **capacitation**. These changes are accomplished only after a sperm has resided for some time in the female reproductive tract (Chang 1951; Austin 1952). Sperm that are not capacitated are "held up" in the cumulus matrix and are unable to reach the egg (Austin 1960; Corselli and Talbot 1987).

DEV TUTORIAL ***Capacitation*** **The knowledge that recently ejaculated mammalian sperm could not fertilize an egg was a critical breakthrough in the development of successful in vitro fertilization techniques.**

Contrary to popular belief, the race is not always to the swift. A study by Wilcox and colleagues (1995) found that nearly all human pregnancies result from sexual intercourse during a 6-day period ending on the day of ovulation. This means that the fertilizing sperm could have taken as long as 6 days to make the journey to the oviduct. Although some human sperm reach the ampulla of the oviduct within half an hour of intercourse, "speedy" sperm may have little chance of fertilizing the egg because they have not undergone capacitation. Eisenbach (1995) proposed a hypothesis wherein capacitation is a transient event, and sperm are given a relatively brief window of competence during which they can successfully fertilize the egg. As the sperm reach the ampulla, they acquire competence—but they lose it if they stay around too long.

The molecular processes of capacitation prepare the sperm for the acrosome reaction and enable the sperm to become hyperactive (**FIGURE 7.26**). Although the details of these processes still await description (they are notoriously difficult to study), two sets of molecular changes are considered to be important:

1. *Lipid changes.* The sperm cell membrane is altered by the removal of cholesterol by albumin proteins in the female reproductive tract (Cross 1998). The cholesterol efflux from the sperm cell membrane is thought to change the location of its "lipid rafts," isolated regions that often contain receptor proteins that can bind the zona pellucida and participate in the acrosome reaction (Bou Khalil et al. 2006; Gadella et al. 2008). Originally located throughout the sperm cell membrane, after cholesterol efflux lipid rafts are clustered over the anterior sperm head. The outer acrosomal membrane changes and comes into contact with the sperm cell membrane in a way that prepares it for the acrosome reaction (Tulsiani and Abou-Haila 2004).
2. *Protein changes.* Particular proteins or carbohydrates on the sperm surface

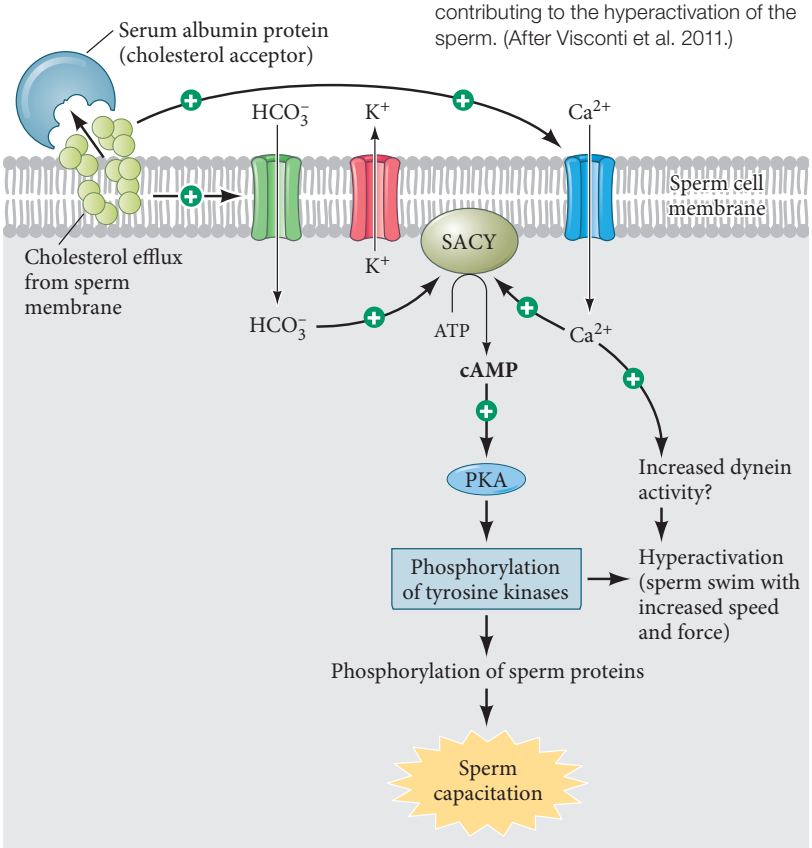

FIGURE 7.26 Hypothetical model for mammalian sperm capacitation. The pathway is modulated by the removal of cholesterol from the sperm cell membrane, which allows the influx of bicarbonate ions (HCO_3^-) and calcium ions (Ca^{2+}). These ions activate adenylate kinase (SACY), thereby elevating cAMP concentrations. The high cAMP levels then activate protein kinase A (PKA). Active PKA phosphorylates several tyrosine kinases, which in turn phosphorylate several sperm proteins, leading to capacitation. Increased intracellular Ca^{2+} also activates the phosphorylation of these proteins, as well as contributing to the hyperactivation of the sperm. (After Visconti et al. 2011.)

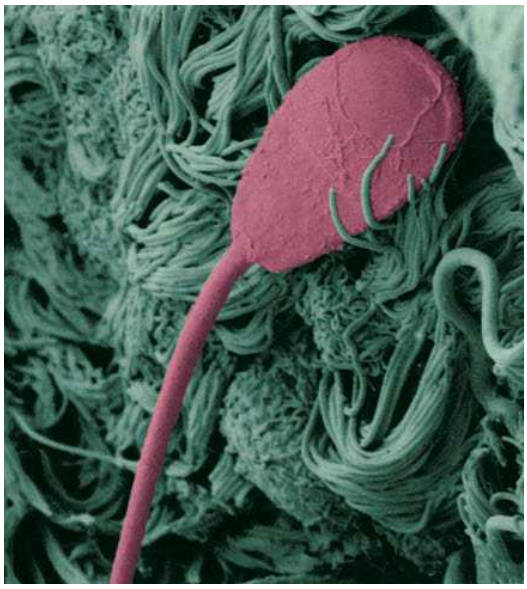

FIGURE 7.27 Scanning electron micrograph (artificially colored) showing bull sperm as it adheres to the membranes of epithelial cells in the oviduct of a cow prior to entering the ampulla. (From Lefebvre et al. 1995, courtesy of S. Suarez.)

are lost during capacitation (Lopez et al. 1985; Wilson and Oliphant 1987). It is possible that these compounds block the recognition sites for the sperm proteins that bind to the zona pellucida. It has been suggested that the unmasking of these sites might be one of the effects of cholesterol depletion (Benoff 1993). The membrane potential of the sperm cell becomes more negative as potassium ions leave the sperm. This change in membrane potential may allow calcium channels to be opened and permit calcium to enter the sperm. Calcium and bicarbonate ions are critical in activating cAMP production and in facilitating the membrane fusion events of the acrosome reaction (Visconti et al. 1995; Arnoult et al. 1999). The influx of bicarbonate ions (and possibly other ions) alkalinizes the sperm, raising its pH. This will be critical in the subsequent activation of calcium channels (Navarro et al. 2007). As a result of cAMP formation, protein phosphorylation occurs (Galantino-Homer et al. 1997; Arcelay et al. 2008). Once they are phosphorylated, some proteins migrate to the surface of the sperm head. One of these proteins is Izumo, which is critical in sperm-egg fusion (see Figure 7.30; Baker et al. 2010).

There may be an important connection between sperm translocation and capacitation. Smith (1998) and Suarez (1998) have documented that before entering the ampulla of the oviduct, the uncapacitated sperm bind actively to the membranes of the oviduct cells in the narrow passage (the isthmus) preceding it (**FIGURE 7.27**; see also Figure 12.11). This binding is temporary and appears to be broken when the sperm become capacitated. Moreover, the life span of the sperm is significantly lengthened by this binding. This restriction of sperm entry into the ampulla during capacitation, and the expansion of sperm life span may have important consequences (Töpfer-Petersen et al. 2002; Gwathmey et al. 2003). The binding action may function as a block to polyspermy by preventing many sperm from reaching the egg at the same time (if the oviduct isthmus is excised in cows, a much higher rate of polyspermy results). In addition, slowing the rate of sperm capacitation and extending the active life of sperm may maximize the probability that sperm will still be available to meet the egg in the ampulla.

In the vicinity of the oocyte: Hyperactivation, thermotaxis, and chemotaxis

Toward the end of capacitation, sperm become hyperactivated—they swim at higher velocities and generate greater force. Hyperactivation appears to be mediated by the opening of a sperm-specific calcium channel in the sperm tail (see Figure 7.26; Ren et al. 2001; Quill et al. 2003). The symmetric beating of the flagellum is changed into a rapid asynchronous beat with a higher degree of bending. The power of the beat and the direction of sperm head movement are thought to release the sperm from their binding with the oviduct epithelial cells. Indeed, only hyperactivated sperm are seen to detach and continue their journey to the egg (Suarez 2008a,b). Hyperactivation may enable sperm to respond differently to the fluid current. Uncapacitated sperm move in a planar direction, allowing more time for the sperm head to attach to the oviduct epithelial cells. Capacitated sperm rotate around their long axis, probably enhancing the detachment of the sperm from the epithelia (Miki and Clapham 2013). Hyperactivation, along with a hyaluronidase enzyme on the outside of the sperm cell membrane, enables the sperm to digest a path through the extracellular matrix of the cumulus cells (Lin et al. 1994; Kimura et al. 2009).

An old joke claims that the reason a man has to release so many sperm at each ejaculation is that no male gamete is willing to ask for directions. So what *does* provide

the sperm with directions? Heat is one cue: there is a thermal gradient of 2°C between the isthmus of the oviduct and the warmer ampullary region (Bahat et al. 2003, 2006). Capacitated mammalian sperm can sense thermal differences as small as 0.014°C over a millimeter and tend to migrate toward the higher temperature (Bahat et al. 2012). This ability to sense temperature difference and preferentially swim from cooler to warmer sites (**thermotaxis**) is found only in capacitated sperm.

By the time the sperm are in the ampullary region, most of them have undergone the acrosome reaction (La Spina et al. 2016; Muro et al. 2016). Now, a second sensing mechanism, **chemotaxis**, may come into play. It appears that the oocyte and its accompanying cumulus cells secrete molecules that attract capacitated (and only capacitated) sperm toward the egg during the last stages of sperm migration (Ralt et al. 1991; Cohen-Dayag et al. 1995; Eisenbach and Tur-Kaspa 1999; Wang et al. 2001). The identity of these chemotactic compounds is being investigated, but one of them appears to be the hormone **progesterone**, which is made by the cumulus cells. Guidobaldi and colleagues (2008) have shown that rabbit sperm binds progesterone secreted from the cumulus cells surrounding the oocyte and uses the hormone as a directional cue. In humans, progesterone has been shown to bind to a receptor that activates Ca^{2+} channels in the cell membrane of the sperm tail, leading to sperm hyperactivity (Lishko et al. 2011; Strünker et al. 2011). Mouse cumulus cells also secrete a substance, CRISP1, that attracts sperm and hyperactivates them through CatSper channels (Ernesto et al. 2015). The human cumulus also appears to make a substance (or substances) that attracts sperm, and it appears to form a gradient permitting the sperm to move through the cumulus toward the egg (Sun et al. 2005; Williams et al. 2015). Whether these are the same chemoattractants or different ones has yet to be resolved. This activation takes place only after the sperm's intracellular pH has increased, which may help explain why capacitation is needed in order for sperm to reach and fertilize the egg (Navarro et al. 2007).

Thus it appears that, just as in the case of sperm-activating peptides in sea urchins, progesterone both provides direction and activates sperm motility. Moreover, as in certain invertebrate eggs, it appears that the human egg secretes a chemotactic factor only when it is capable of being fertilized, and that sperm are attracted to such a compound only when they are capable of fertilizing the egg.

In summary, three calcium-mediated sensing processes get the mammalian sperm to the egg: rheotaxis (long-range), thermotaxis (moderate range), and finally chemotaxis, which works within millimeters of the egg.

Developing Questions

Sometimes the egg and sperm fail to meet and conception does not take place. What are the leading causes of infertility in humans, and what procedures are being used to circumvent these blocks?

The acrosome reaction and recognition at the zona pellucida

Before the mammalian sperm can bind to the oocyte, it must first bind to and penetrate the egg's zona pellucida. The zona pellucida in mammals plays a role analogous to that of the vitelline envelope in invertebrates; the zona, however, is a far thicker and denser structure than the vitelline envelope. The mouse zona pellucida is made of three major glycoproteins—**ZP1**, **ZP2**, and **ZP3** (**zona proteins 1, 2,** and **3**)—along with accessory proteins that bind to the zona's integral structure. The human zona pellucida has four major glycoproteins—ZP1, ZP2, ZP3, and ZP4.

The binding of sperm to the zona is relatively, but not absolutely, species-specific, and a species may use multiple mechanisms to achieve this binding. Early evidence from rabbits and hamsters (Huang et al. 1981; Yanagimachi and Phillips 1984) suggested that the sperm arriving at the egg had already undergone the acrosome reaction. More recently, Jin and colleagues (2011) showed that the mouse acrosome reaction occurs prior to the sperm binding to the zona (**FIGURE 7.28A**). They found that "successful" sperm—i.e., those that actually fertilized an egg—had already undergone the acrosome reaction by the time they were first seen in the cumulus. Sperm that underwent the acrosome reaction on the zona were almost always unsuccessful.

Thus it appears that most sperm undergo the acrosome reaction in or around the cumulus. Moreover they probably bind to the egg through ZP2 on the zona pellucida. In a gain-of-function experiment, ZP2 was shown to be critical for human sperm-egg

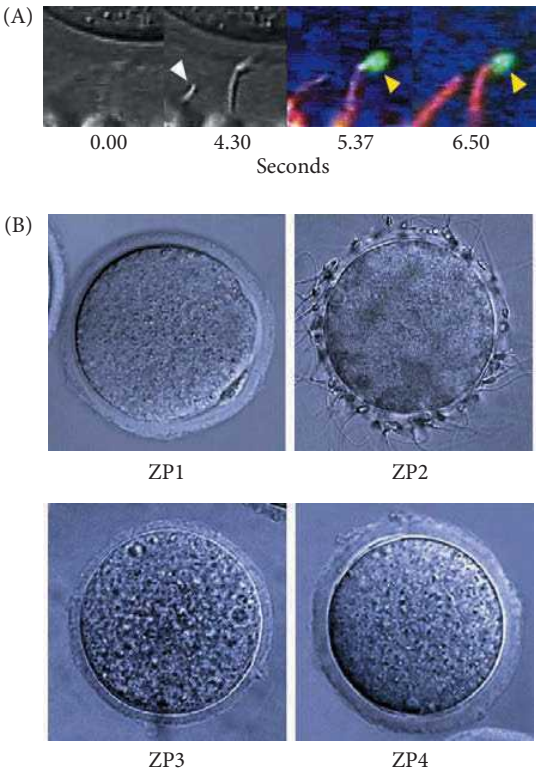

FIGURE 7.28 Acrosome-reacted mouse sperm bind to the zona and are successful at fertilizing the egg. (A) The acrosomes of mouse sperm were labeled with GFP such that intact acrosomes fluoresced green; sperm tails were labeled with red fluorescent markers. When the labeled sperm were allowed to interact with a mouse egg and cumulus, the resulting video revealed that the fertilizing sperm (arrowhead at 4.30 seconds) showed no green fluorescence when it reached the surface of the zona pellucida at 6.20 seconds—indicating that it had undergone the acrosome reaction before that time. An adjacent sperm did fluoresce green, meaning its acrosome remained intact. Such acrosome-intact sperm remain bound to the zona without undergoing the acrosome reaction or progressing to the egg cell membrane. (B) Gain-of-function experiment demonstrating that human sperm bind to ZP2. Of the four human zona pellucida proteins, only ZP4 is *not* found in mouse zona. Transgenic mouse oocytes were constructed that expressed the three normal mouse zona proteins and also one of the four human zona proteins. When human sperm were added to the mouse oocytes, they bound only to those transgenic oocytes that expressed human ZP2. Human sperm did not bind to cells expressing human ZP1, ZP3, or ZP4. (A from Jin et al. 2011, courtesy of N. Hirohashi; B from Baibakov et al. 2012.)

binding. Human sperm does not bind to the zona of mouse eggs, so Baibakov and colleagues (2012) added the different human zona proteins separately to the zona of mouse eggs. Only those mouse eggs with *human* ZP2 bound human sperm (**FIGURE 7.28B**). Using mutant forms of ZP2, Avella and colleagues (2014) demonstrated that there is a particular region of the mouse ZP2 protein (between amino acids 51 and 149) that bound the sperm. This region is seen in human ZP2 and may be responsible for sperm-zona binding in humans as well. ZP3 was the other candidate for binding sperm; however, Gahlay and colleagues (2010) provided evidence that mouse eggs with mutations in ZP3 were still fertilized.

In mice, there is also evidence that acrosome-intact sperm can bind to ZP3, and that ZP3 can cause the acrosome reaction directly on the zona (Bleil and Wassarman 1980, 1983). And in humans, there is evidence that the reaction can also be induced by the zona proteins, perhaps by all of them acting in concert (Gupta 2015). Indeed, there may be several means to initiate the acrosome reaction and to bind to and penetrate the zona pellucida. These mechanisms may act simultaneously, or perhaps one mechanism is used for acrosome-intact sperm and another for acrosome-reacted sperm. Given that the zona's biochemical composition differs in different species, the mechanisms that predominate in one species need not be the same in another. The sperm receptor that binds to the zona proteins has not yet been identified. It is probably a complex containing several proteins that bind to both the protein and carbohydrate portions of the zona glycoproteins (Chiu et al. 2014).

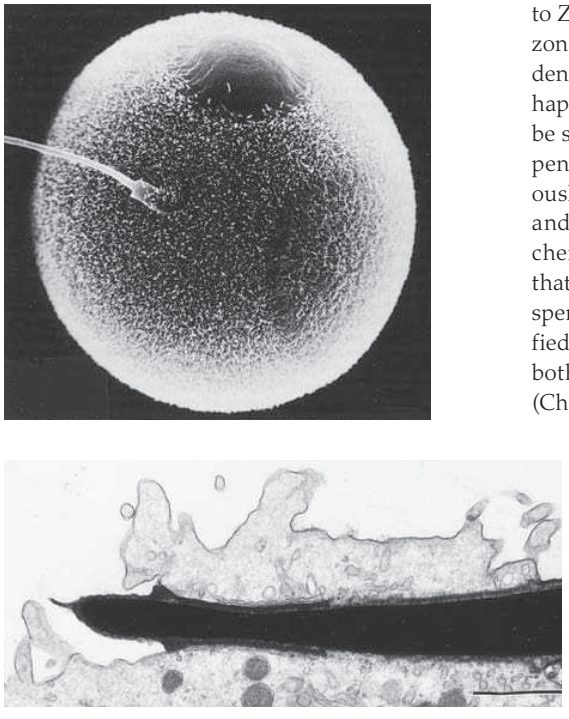

FIGURE 7.29 Entry of sperm into a golden hamster egg. (A) Scanning electron micrograph of sperm fusing with egg. The "bald" spot (without microvilli) is where the polar body has budded off. Sperm do not bind there. (B) Transmission electron micrograph of the sperm fusing parallel to the egg cell membrane. (From Yanagimachi and Noda 1970 and Yanagimachi 1994, courtesy of R. Yanagimachi.)

Gamete fusion and the prevention of polyspermy

In mammals, it is not the tip of the sperm head that makes contact with the egg (as happens in the perpendicular entry of sea urchin sperm) but the side of the sperm head (**FIGURE 7.29**). The acrosome reaction, in addition to expelling the enzymatic contents of the acrosome, also exposes the inner acrosomal membrane to the outside. The junction between this inner acrosomal membrane and the sperm cell membrane is called the **equatorial region**, and this is where membrane fusion between sperm and egg begins (**FIGURE 7.30A**). As in sea urchin gamete fusion, the sperm is bound to regions of the egg where actin polymerizes to extend microvilli to the sperm (Yanagimachi and Noda 1970).

The mechanism of mammalian gamete fusion is still controversial (see Lefèvre et al. 2010; Chalbi et al. 2014). On the sperm side of the mammalian gamete fusion process, Inoue and colleagues (2005) have implicated an immunoglobulin-like protein, named Izumo after a Japanese shrine dedicated to marriage. This protein is originally found in the membrane of the acrosomal granule (**FIGURE 7.30B**). However, after the acrosome reaction, Izumo redistributes along on the surface of acrosome-reacted sperm, where it is found primarily in the equatorial section, where mammalian sperm-egg binding takes place (see Figure 7.30A; Satouh et al. 2012). Sperm from mice carrying loss-of-function mutations in the *Izumo* gene are able to bind and penetrate the zona pellucida, but they are not able to fuse with the egg cell membrane. Human sperm also contain Izumo protein, and antibodies directed against Izumo prevent sperm-egg fusion in humans as well. There are other candidates for sperm fusion proteins, and there may be several sperm-egg binding systems operating, each of which may be necessary but not sufficient to ensure proper gamete binding and fusion.

Izumo binds to an oocyte protein called Juno (after the Roman goddess of marriage and fertility), and eggs deficient in Juno cannot bind or fuse with acrosome-reacted sperm (Bianchi et al. 2014). The interaction of Izumo and Juno recruits the egg membrane protein CD9 to the area of sperm-egg adhesion (Chalbi et al. 2014.) This protein appears to be involved with sperm-egg fusion, since female mice with the *CD9* gene knocked out are infertile due to fusion defects (Kaji et al. 2002; Runge et al. 2006). It is not known exactly how these proteins facilitate membrane fusion, but CD9 protein is also known to be critical for the fusion of myocytes (muscle cell precursors) to form striated muscle (Tachibana and Hemler 1999).

Polyspermy is a problem for mammals just as it is for sea urchins. In mammals, no electrical fast block to polyspermy has yet been detected; it may not be needed, given the limited number of sperm that reach the ovulated egg (Gardner and Evans 2006). However, a *slow* block to polyspermy occurs when enzymes released by the cortical granules modify the zona pellucida sperm receptor proteins such that they can no longer bind sperm (Bleil and Wassarman 1980). ZP2 is clipped by the protease ovastacin and loses its ability to bind sperm (**FIGURE 7.31**; Moller and Wassarman 1989). Ovastacin is found in the cortical granules of unfertilized eggs and is released during cortical granule fusion. Indeed, polyspermy occurs more frequently in mouse eggs bearing mutant ZP2 that cannot be cleaved by ovastacin (Gahlay et al. 2010; Burkart et al. 2012).

Another slow block to polyspermy involves the Juno protein (Bianchi and Wright 2014). As the sperm and egg membranes fuse, Juno protein appears to be released from the plasma membrane. Thus, the docking site for sperm would be removed. Moreover, this soluble Juno protein can bind sperm in the perivitelline space between the zona pellucida and oocyte,

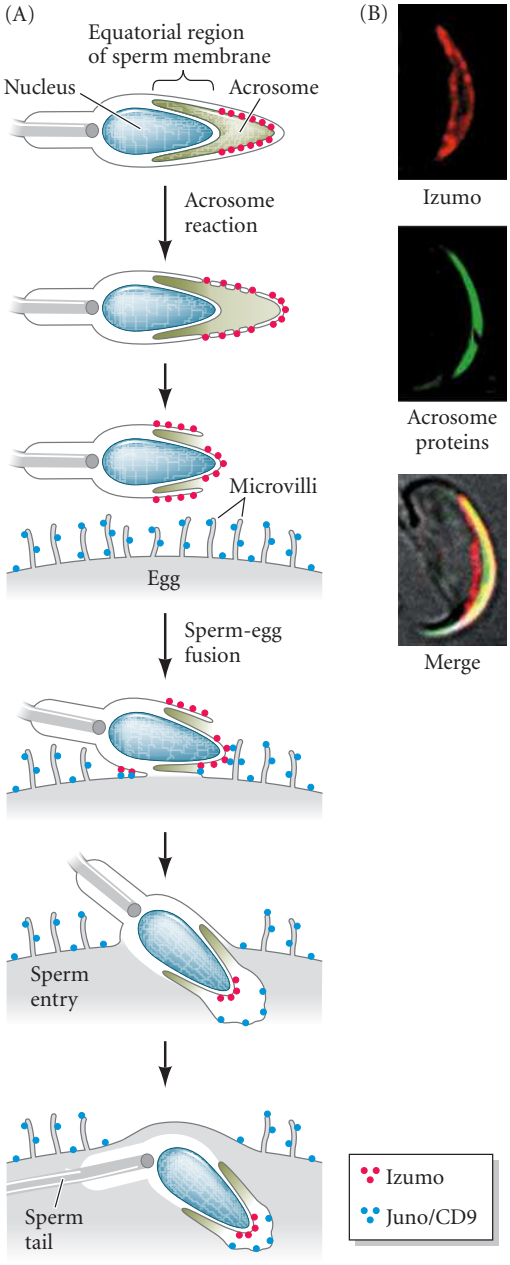

FIGURE 7.30 Izumo protein and membrane fusion in mouse fertilization. (A) Localization of Izumo to the inner and outer acrosomal membrane. Izumo is stained red, acrosomal proteins green. (B) Diagram of sperm-egg cell membrane fusion. During the acrosome reaction, Izumo localized on the acrosomal becomes translocated to the sperm cell membrane. There it meets the complex of Juno and CD9 proteins on the egg microvilli, initiating membrane fusion and the entry of the sperm into the egg. (After Satouh et al. 2012; photographs courtesy of M. Okabe.)

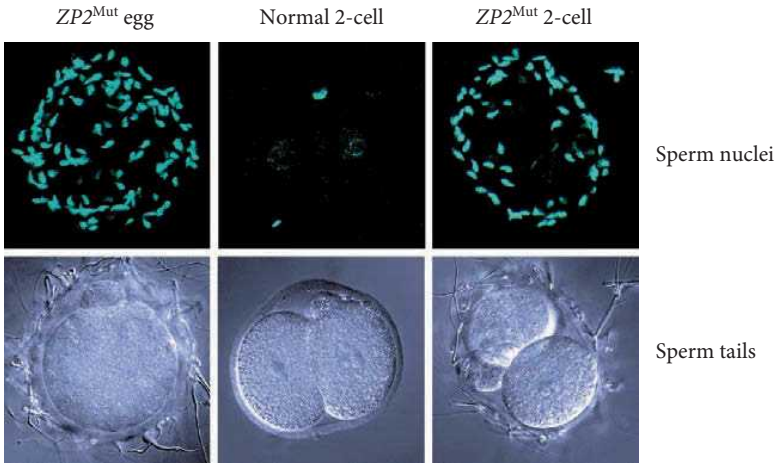

FIGURE 7.31 Cleaved ZP2 is necessary for the block to polyspermy in mammals. Eggs and embryos were visualized by fluorescence microscopy (to see sperm nuclei; top row) and brightfield microscopy (differential interference contrast, to see sperm tails; bottom row). Sperm bound normally to eggs containing a mutant ZP2 that could not be cleaved. However, the egg with normal (i.e., cleavable) ZP2 got rid of sperm by the 2-cell stage, whereas the egg with the mutant (uncleavable) ZP2 retained sperm. (From Gahlay et al. 2010, photograph courtesy of J. Dean.)

Developing Questions

One of the goals of modern pharmacology is to create a male contraceptive. Reviewing the steps of fertilization, what steps do you think it might be possible to block pharmacologically in order to produce such a contraceptive for males?

thereby preventing the sperm from seeing any Juno protein that may still reside on the oocyte membrane.

Fusion of genetic material

As in sea urchins, the single mammalian sperm that finally enters the egg carries its genetic contribution in a haploid pronucleus. In mammals, however, the process of pronuclear migration takes about 12 hours, compared with less than 1 hour in the sea urchin. The DNA of the sperm pronucleus is bound by protamines—basic proteins that are tightly compacted through disulfide bonds. Glutathione in the egg cytoplasm reduces these disulfide bonds and allows the sperm chromatin to uncoil (Calvin and Bedford 1971; Kvist et al. 1980; Sutovsky and Schatten 1997).

The mammalian sperm enters the oocyte while the oocyte nucleus is "arrested" in metaphase of its second meiotic division (**FIGURE 7.32A,B**; see also Figure 7.3). As described for the sea urchin, the calcium oscillations brought about by sperm entry inactivate MAP kinase and allow DNA synthesis. But unlike the sea urchin egg, which is already in a haploid state, the chromosomes of the mammalian oocyte are still in the middle of meiotic metaphase. Oscillations in the level of Ca^{2+} activate another kinase that leads to the proteolysis of cyclin (thus allowing the cell cycle to continue) and securin (the protein that holds the metaphase chromosomes together), eventually resulting in a haploid female pronucleus (Watanabe et al. 1991; Johnson et al. 1998).

DNA synthesis occurs separately in the male and female pronuclei. The centrosome produced by the male pronucleus generates asters (largely from microtubule proteins stored in the oocyte). The microtubules join the two pronuclei and enable them to migrate toward one another. Upon meeting, the two nuclear envelopes break down. However, instead of producing a common zygote nucleus (as in sea urchins), the chromatin condenses into chromosomes that orient themselves on a common mitotic spindle (**FIGURE 7.32C,D**). Thus, in mammals a true diploid nucleus is seen for the first time not in the zygote, but at the 2-cell stage.

Each sperm brings into the egg not only its nucleus but also its mitochondria, its centriole, and a tiny amount of cytoplasm. The sperm mitochondria and their DNA are degraded in the egg cytoplasm, so all of the new individual's mitochondria are derived from its mother. The egg and embryo appear to get rid of the paternal mitochondria both by dilution and by actively targeting them for destruction (Cummins et al. 1998; Shitara et al. 1998; Schwartz and Vissing 2002). In most mammals, however, the sperm centriole not only survives but appears to serve as the organizing agent for making the new mitotic spindle. Moreover, the sperm cytoplasm has recently been found to contain enzymes that activate egg metabolism, as well as RNA fragments that may alter gene expression (Sharma et al. 2016).

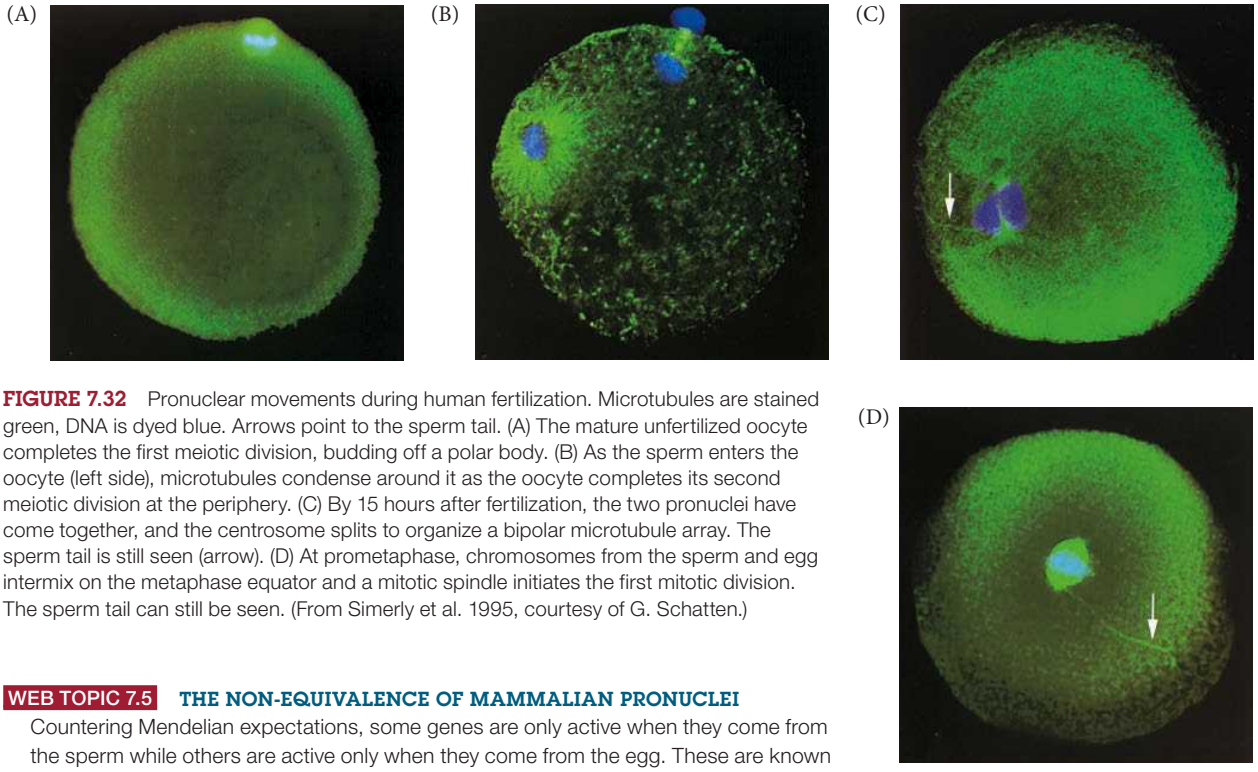

FIGURE 7.32 Pronuclear movements during human fertilization. Microtubules are stained green, DNA is dyed blue. Arrows point to the sperm tail. (A) The mature unfertilized oocyte completes the first meiotic division, budding off a polar body. (B) As the sperm enters the oocyte (left side), microtubules condense around it as the oocyte completes its second meiotic division at the periphery. (C) By 15 hours after fertilization, the two pronuclei have come together, and the centrosome splits to organize a bipolar microtubule array. The sperm tail is still seen (arrow). (D) At prometaphase, chromosomes from the sperm and egg intermix on the metaphase equator and a mitotic spindle initiates the first mitotic division. The sperm tail can still be seen. (From Simerly et al. 1995, courtesy of G. Schatten.)

WEB TOPIC 7.5 **THE NON-EQUIVALENCE OF MAMMALIAN PRONUCLEI**

Countering Mendelian expectations, some genes are only active when they come from the sperm while others are active only when they come from the egg. These are known as "imprinted" genes.

Activation of the mammalian egg

As in every other animal group studied, a transient rise in cytoplasmic Ca^{2+} is necessary for egg activation in mammals. The sperm induces a series of Ca^{2+} waves that can last for hours, terminating in egg activation (i.e., resumption of meiosis, cortical granule exocytosis, and release of the inhibition on maternal mRNAs) and the formation of the male and female pronuclei. And, again as in sea urchins, fertilization triggers intracellular Ca^{2+} release through the production of IP_3 by the enzyme phospholipase C (Swann et al. 2006; Igarashi et al. 2007).

However, the mammalian PLC responsible for egg activation and pronucleus formation may in fact come from the sperm rather than from the egg. Some of the first observations for a sperm-derived PLC came from studies of intracytoplasmic sperm injection (ICSI), an experimental treatment for curing infertility. Here, sperm are directly injected into oocyte cytoplasm, bypassing any interaction with the egg cell membrane. To the surprise of many biologists (who had assumed that sperm *binding* to an egg receptor protein was critical for egg activation), this treatment worked. The human egg was activated and pronuclei formed. Injecting mouse sperm into mouse eggs will also induce fertilization-like Ca^{2+} oscillations in the egg and lead to complete development (Kimura and Yanagimachi 1995).

It appeared that an activator of Ca^{2+} release was stored in the sperm head (see Figure 7.20A). This activator turned out to be a soluble sperm PLC enzyme, **PLCζ** (zeta), that is delivered to the egg by gamete fusion. In mice, expression of PLCζ mRNA in the egg produces Ca^{2+} oscillations, and removing PLCζ from mouse sperm (by antibodies or RNAi) abolishes the sperm's calcium-inducing activity (Saunders et al. 2002; Yoda et al. 2004; Knott et al. 2005). Human sperm that are unsuccessful in ICSI have been shown to have little or no functional PLCζ. In fact, normal human sperm can activate Ca^{2+} oscillations when injected into mouse eggs, but sperm lacking PLCζ do not (Yoon et al. 2008).

Whereas sea urchin eggs usually are activated as a single wave of Ca^{2+} crosses from the point of sperm entry, the mammalian egg is traversed by numerous waves of calcium ions (Miyazaki et al. 1992; Ajduk et al. 2008; Ducibella and Fissore 2008). The extent (amplitude, duration, and number) of these Ca^{2+} oscillations appears to regulate the timing of mammalian egg activation events (Ducibella et al. 2002; Ozil et al. 2005; Toth et al. 2006). In this way, cortical granule exocytosis occurs just before resumes meiosis and much before the translation of maternal mRNAs.

In mammals, the Ca^{2+} released by IP_3 binds to a series of proteins including calmodulin-activated protein kinase (which will be important in eliminating the inhibitors of mRNA translation), MAP kinase (which allows the resumption of meiosis), and synaptotagmin (which helps initiate cortical granule fusion). Unused Ca^{2+} is pumped back into the endoplasmic reticulum, and additional Ca^{2+} is acquired from outside the cell. This recruitment of extracellular Ca^{2+} appears to be necessary for the egg to complete meiosis. If Ca^{2+} influx is blocked, the second polar body does not form; instead, the result is two nonviable (triploid) egg pronuclei (Maio et al. 2012; Wakai et al. 2013).

DEV TUTORIAL ***Legends of the sperm*** **The stories people tell about fertilization are often at odds with the actual data of biology.**

WEB TOPIC 7.6 **A SOCIAL CRITIQUE OF FERTILIZATION RESEARCH** How we envision fertilization says a lot about us as well as about the science.

Coda

Fertilization is not a moment or an event, but a process of carefully orchestrated and coordinated events including the contact and fusion of gametes, the fusion of nuclei, and the activation of development. It is a process whereby two cells, each at the verge of death, unite to create a new organism that will have numerous cell types and organs. It is just the beginning of a series of cell-cell interactions that characterize animal development.

Next Step Investigation

Fertilization is a field ripe with important questions to be answered. Some of the most important involve the physiological changes that render the gametes "fertilization-competent." The mechanisms by which sperm become hyperactive and sense the egg are just beginning to become known, as are the mechanisms of sperm capacitation. Meiosis is resumed in mammalian oocytes, but the physiological mechanisms for this resumption remain largely unexplored. How is the polar body formed in a way that the oocyte retains most of the cytoplasm? And how do the gamete recognition proteins interact with cell fusion proteins to allow the sperm to enter the egg? Even the ways by which sperm activate the internal calcium ion channels is an open question. About 6% of American men and women between 15 and 44 years of age are infertile, and this makes the answering these questions extremely important.

Closing Thoughts on the Opening Photo

When Oscar Hertwig (1877) discovered fertilization in sea urchins, he delighted in seeing what he called "the sun in the egg." This was evidence that the fertilization was going to be successful. This glorious projection turns out to be the microtubular array generated by the sperm centrosome. This set of microtubules reaches out and finds the female pronucleus, and the two pronuclei migrate toward one another on these microtubular tracks. In this micrograph, the DNA of the pronuculei is stained blue, and the female pronucleus is much larger than that derived from the sperm. The microtubules are stained green. (Photograph courtesy of J. Holy and G. Schatten.)

7 Snapshot Summary
Fertilization

1. Fertilization accomplishes two separate activities: sex (the combining of genes derived from two parents) and reproduction (the creation of a new organism).
2. The events of fertilization usually include (1) contact and recognition between sperm and egg; (2) regulation of sperm entry into the egg; (3) fusion of genetic material from the two gametes; and (4) activation of egg metabolism to start development.
3. The sperm head consists of a haploid nucleus and an acrosome. The acrosome is derived from the Golgi apparatus and contains enzymes needed to digest extracellular coats surrounding the egg. The midpiece of the sperm contain mitochondria and the centriole that generates the microtubules of the flagellum. Energy for flagellar motion comes from mitochondrial ATP and a dynein ATPase in the flagellum.
4. The female gamete can be an egg (with a haploid nucleus, as in sea urchins) or an oocyte (in an earlier stage of development, as in mammals). The egg (or oocyte) has a large mass of cytoplasm storing ribosomes and nutritive proteins. Some mRNAs and proteins that will be used as morphogenetic factors are also stored in the egg. Many eggs also contain protective agents needed for survival in their particular environment.
5. Surrounding the egg cell membrane is an extracellular layer often used in sperm recognition. In most animals, this extracellular layer is the vitelline envelope. In mammals, it is the much thicker zona pellucida. Cortical granules lie beneath the egg's cell membrane.
6. Neither the egg nor the sperm is the "active" or "passive" partner; the sperm is activated by the egg, and the egg is activated by the sperm. Both activations involve calcium ions and membrane fusions.
7. In many organisms, eggs secrete diffusible molecules that attract and activate the sperm.
8. Species-specific chemotactic molecules secreted by the egg can attract sperm that are capable of fertilizing it. In sea urchins, the chemotactic peptides resact and speract have been shown to increase sperm motility and provide direction toward an egg of the correct species.
9. The acrosome reaction releases enzymes exocytotically. These proteolytic enzymes digest the egg's protective coating, allowing the sperm to reach and fuse with the egg cell membrane. In sea urchins, this reaction in the sperm is initiated by compounds in the egg jelly. Globular actin polymerizes to extend the acrosomal process. Bindin on the acrosomal process is recognized by a protein complex on the sea urchin egg surface.
10. Fusion between sperm and egg is probably mediated by protein molecules whose hydrophobic groups can merge the sperm and egg cell membranes. In sea urchins, bindin may mediate gamete recognition and fusion.
11. Polyspermy results when two or more sperm fertilize an egg. It is usually lethal, since it results in blastomeres with different numbers and types of chromosomes.
12. Many species have two blocks to polyspermy. The fast block is immediate and causes the egg membrane resting potential to rise. Sperm can no longer fuse with the egg. In sea urchins this is mediated by the influx of sodium ions. The slow block, or cortical granule reaction, is physical and is mediated by calcium ions. A wave of Ca^{2+} propagates from the point of sperm entry, causing the cortical granules to fuse with the egg cell membrane. The released contents of these granules cause the vitelline envelope to rise and harden into the fertilization envelope.
13. The fusion of sperm and egg results in the activation of crucial metabolic reactions in the egg. These reactions include re-initiation of the egg's cell cycle and subsequent mitotic division, and the resumption of DNA and protein synthesis.
14. In all species studied, free Ca^{2+}, supported by the alkalinization of the egg, activates egg metabolism, protein synthesis, and DNA synthesis. Inositol trisphosphate (IP_3) is responsible for releasing Ca^{2+} from storage in the endoplasmic reticulum. DAG (diacylglycerol) is thought to initiate the rise in egg pH.
15. IP_3 is generated by phospholipases. Different species may use different mechanisms to activate the phospholipases.
16. Genetic material is carried in a male and a female pronucleus, which migrate toward each other. In sea urchins, the male and female pronuclei merge and a diploid zygote nucleus is formed. DNA replication occurs after pronuclear fusion.
17. Mammalian fertilization takes place internally, within the female reproductive tract. The cells and tissues of the female reproductive tract actively regulate the transport and maturity of both the male and female gametes.
18. The translocation of sperm from the vagina to the egg is regulated by the muscular activity of the uterus, by the binding of sperm in the isthmus of the oviduct, and by directional cues from the oocyte and/or the cumulus cells surrounding it.
19. Mammalian sperm must be capacitated in the female reproductive tract before they are capable of fertilizing the egg. Capacitation is the result of biochemical changes in the sperm cell membrane and the alkalinization of its cytoplasm. Capacitated mammalian sperm can penetrate the cumulus and bind the zona pellucida.

20. In one model of sperm-zona binding, the acrosome-intact sperm bind to ZP3 on the zona, and ZP3 induces the sperm to undergo the acrosome reaction on the zona pellucida. In a more recent model, the acrosome reaction is induced in the cumulus, and the acrosome-reacted sperm bind to ZP2.
21. In mammals, blocks to polyspermy include modification of the zona proteins by the contents of the cortical granules so that sperm can no longer bind to the zona.
22. The rise in intracellular free Ca^{2+} at fertilization in amphibians and mammals causes the degradation of cyclin and the inactivation of MAP kinase, allowing the second meiotic metaphase to be completed and the formation of the haploid female pronucleus.
23. In mammals, DNA replication takes place as the pronuclei are traveling toward each other. The pronuclear membranes disintegrate as the pronuclei approach each other, and their chromosomes gather around a common metaphase plate.

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