Understanding the molecular basis of sperm capacitation through kinase design

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Thirty years ago, on July 25, Steptoe and Edwards reported the birth of Louise Joy Brown, the first successful "Test-Tube" baby (1). This achievement followed a lack of success of in vitro fertilization experiments for almost 80 years since the first attempts in 1878. These early failures were due mainly to a lack of comprehension of sperm physiology. In the early 1950s, Chang (2) and Austin (3) demonstrated independently that sperm had to be in the female reproductive tract for a finite period before acquiring fertilizing capacity. This phenomenon is known as sperm capacitation. What made this finding a necessary step for the consequent development of in vitro fertilization was the understanding that certain factors in the female were needed for the sperm to become fertile. A logical follow-up of the discovery of sperm capacitation occurred some years later when Chang demonstrated mammalian in vitro fertilization conclusively by showing that eggs from a black rabbit fertilized in vitro by capacitated sperm from a black male, and transferred to a white female, resulted in the birth of a litter of black offspring (4).

In vitro fertilization was made possible by the discovery of sperm capacitation and raised the interest to study the molecular basis of this process. Inherent to these studies, capacitation was defined as the physiological changes occurring in the female reproductive tract that render the sperm able to fertilize. These changes involved a series of sequential and parallel processes; some of them take place as soon as the sperm is ejaculated, whereas others arise over a longer period in the female tract or in a medium that supports in vitro capacitation. Interestingly, both early and late events are centrally regulated by protein kinase A (PKA). The work by Morgan *et al.* (5) in a recent issue of PNAS takes a chemical-genetic switch approach to understand the temporal action of this enzyme in sperm capacitation. Regarding this approach, to facilitate consideration of the complex cascade of molecular events that occur during capacitation, a discussion of this process may be divided into fast and slow capacitation events (Fig. 1).

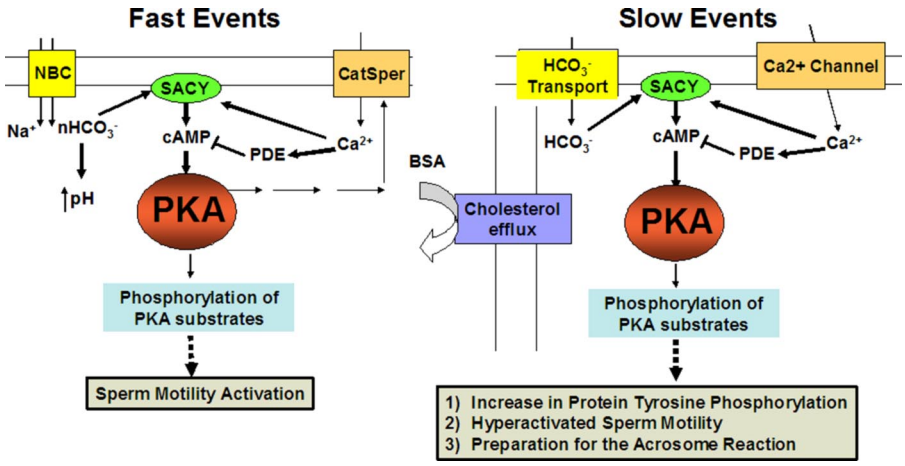

Fig. 1. Molecular basis of fast and slow events associated with sperm capacitation. (*Fast Events*) As soon as sperm are in contact with an isotonic solution containing HCO_3^- and Ca^{2+}, a vigorous flagellar movement is observed. At the molecular level, this process depends on the increase in PKA activity and is mediated by a Ca^{2+} and HCO_3^- coordinated stimulation of the atypical adenylyl cyclase SACY. At these instances, it is believed that HCO_3^- and Ca^{2+} are transported by a Na^+/HCO_3^- cotransporter (NBC) and a sperm-specific Ca^{2+} channel (CatSper). (*Slow Events*) After an extended period of incubation in vivo or in vitro, sperm acquire the ability to fertilize. The fertilization capacity is preceded by the preparation to undergo the exocytotic acrosome reaction and by changes in the motility pattern known as hyperactivation. At the molecular level, these changes are correlated with an increase in tyrosine phosphorylation. This increase is downstream of PKA stimulation; however, opposite to the fast processes, the increase in tyrosine phosphorylation also depends on the presence of cholesterol acceptors in the capacitation medium.

A very early event in sperm capacitation is the activation of sperm motility. Although sperm stored in the cauda epididymis consume oxygen at a high rate, they are immotile. The vigorous movement of the flagellum starts immediately after sperm are released from the epididymis and come into contact with high HCO_3^- and Ca^{2+} concentrations present in the seminal fluid. The transmembrane movement of HCO_3^- has been associated with the increase in intracellular pH (pHi) observed during capacitation. However, another likely target for HCO_3^- as well as Ca^{2+} actions in sperm is the regulation of cAMP metabolism through stimulation of a unique type of adenylyl cyclase (SACY). In contrast to transmembrane adenylyl cyclases, SACY does not respond to activators of Gs, the G protein stimulator of transmembrane cyclases, such as cholera toxin or nonhydrolyzable analogs of GTP (e.g., GTPγS). Furthermore, SACY is stimulated by HCO_3^- anions and multiple evidences indicate that this atypical cylase is the main HCO_3^- target during sperm capacitation. As a consequence of SACY activation, intracellular levels of cAMP increase and activate PKA. Once activated, PKA phosphorylates various target proteins that are presumed to initiate several signaling pathways. In sperm exposed to HCO_3^-, cAMP rises to a maximum within 60 sec, and the increase in PKA-dependent phosphorylation begins within 90 sec (for review, see ref. 6).

In contrast to the fast activation of sperm motility, other capacitation-associated processes require longer incubation periods. These slower processes can be accomplished in vitro by using sperm incubated in defined media. In all cases, in vitro capacitation media contain a protein source that usually is BSA; and an assortment of ions including HCO_3^- and Ca^{2+}. The mechanisms by which these media components are able to

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promote capacitation at the molecular level have been an active area of research in the past decade (ref. 6 and references therein). BSA is believed to function as a sink for the depletion of sperm plasma membrane cholesterol and can be replaced by other cholesterol-binding compounds such as β-cyclodextrins to induce capacitation (6). As in the early activation of sperm movement, HCO_3^- and Ca^{2+} are involved in the regulation of SACY and in the consequent increase in cAMP levels and PKA activation.

Sperm capacitation is also associated with an increase in tyrosine phosphorylation (6). This increase in tyrosine phosphorylation is a late event, it depends on the presence of BSA, Ca^{2+}, and HCO_3^- in the capacitation medium, and it is correlated with capacitation (6). Specifically, the absence of any one of these media constituents prevents both tyrosine phosphorylation and capacitation. It is also established that tyrosine phosphorylation is downstream of a PKA pathway. The involvement of PKA is illustrated by experiments demonstrating that cAMP-permeable analogs are able to induce the increase in tyrosine phosphorylation in the absence of BSA, HCO_3^-, or Ca^{2+}. The paradox in the regulation of fast and slow capacitation-associated events is that both are mediated by a HCO_3^-/SACY/cAMP/PKA pathway; however, although activation of this pathway occurs immediately and does not need cholesterol acceptors, the increase in tyrosine phosphorylation and other late events are not immediately stimulated and require the presence of cholesterol acceptors.

The report by Morgan *et al.* (5) provides the tools needed to elucidate this conundrum. Previously, this group generated mice that lack the unique sperm PKA catalytic subunit Cα2 (7). As predicted, these mice were infertile despite normal mating behavior, and their sperm presented defects in both early and late capacitation-associated events. This work, together with results from mice lacking the atypical HCO_3^--dependent adenylyl cyclase SACY (8, 9), has conclusively demonstrated that a HCO_3^--dependent modulation of a cAMP/PKA pathway is involved in the regulation of both fast and slow capacitation-associated processes. Despite these ground-breaking studies, little is known on the temporal pattern of PKA activation and how this activity mediates different aspects of sperm capacitation.

In vitro fertilization was made possible by the discovery of sperm capacitation.

Unlike gene deletions, inhibitors act quickly and reversibly, and do not allow the cell to compensate for the missing kinase activity during development. Taking this into account, if inhibitors were keys that fit into a lock, could it be possible to modify the lock to accept a predesigned key rather than designing a new key for the lock? This approach was first described by Bishop *et al.* (10). This group showed that it is possible to introduce a point mutation deeply into the ATP binding pocket of any protein kinase, in a way that a "gate-keeper" side chain is eliminated, making room for the bulky group of a kinase inhibitor that competes with ATP for binding. The large substituted inhibitor would otherwise not fit into the pocket of wild-type kinases and therefore would not affect other cellular processes.

By using this rationale, Morgan *et al.* (5) investigated the temporal requirement and regulation of PKA in sperm. For this analysis, the authors engineered a targeting vector that contained the NEO gene, a Cα minigene, and a mutant form of exon 5 with a point mutation in the ATP binding pocket of the PKA catalytic subunit. This mutation modified the kinase in a way that a bulky kinase inhibitor such as 1NM-PP1 would inhibit only the engineered kinase without affecting its specificity. Homozygous mice carrying the mutant PKA were then used to investigate sperm capacitation. As expected, 1NM-PP1 had no effect in wild-type sperm; however, this bulky inhibitor blocked the HCO_3^--dependent increase in flagellar beat frequency. Moreover, this inhibitor blocked phosphorylation of PKA substrates occurring within 90 sec of addition of HCO_3^- to sperm as well as the increase in tyrosine phosphorylation. Most interestingly, the inhibition in tyrosine phosphorylation was only observed when mutant sperm were incubated for an extended period with 1NM-PP1. This experiment allowed the authors to conclude that PKA played at least two independent roles in the regulation of sperm motility. A "fast" action that is required for the activation of flagellar beat; and a "slow" action such as the change in the flagellum waveform symmetry, that needs PKA to be active for an extended time period.

In summary, this manuscript will open new avenues of investigation in sperm signal transduction. First, it produces a model that would allow dissection of PKA regulated events in sperm. Second, it serves as a proof of principle to study other protein kinases in sperm. Finally, although in this case the authors introduced the mutant PKA in the whole mice, the design of the targeting vector will allow for specific introduction of the mutant PKA in any tissue for which specific expression of cre is available.

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