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Minireview

A Perspective on the Control of Mammalian Fertilization by Egg-Activated Ion Channels in Sperm: A Tale of Two Channels[1]

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PERSPECTIVE

Mammalian sperm contain a single large secretory vesicle, or acrosome. Exocytosis of this vesicle (the acrosome reaction) occurs as an early step in the gamete interaction process and is an essential prerequisite for late events, including sperm-egg fusion [1]. The control of fertilization is, from this perspective, a question of the regulation of a secretory event.

It may be useful to compare sperm to other secretory systems. Typical somatic secretory cells such as mast cells, the neuronal presynaptic terminal, and exocrine cells contain large numbers of secretory vesicles whereas sperm possess a single acrosome. Those familiar somatic secretory models tolerate a low level of basal vesicle release that is augmented upon the arrival of an external stimulus. For example, miniature synaptic potentials are due to the stochastic release of individual vesicles, and stimuli, in the form of membrane depolarization, increase the number of released vesicles [2, 3].

In contrast, it can be argued that spontaneous exocytosis must be strictly controlled in sperm and may require a specialized regulatory mechanism. Key elements in this argument include the observations that 1) sperm that complete the acrosome reaction at a distance from eggs have decreased capacity to penetrate through the cumulus oophorus [4] and to adhere to the egg's extracellular matrix, or zona pellucida [5, 6], and 2) sperm that fail to initiate acrosome reactions cannot penetrate the zona pellucida and hence are denied access to the egg plasma membrane [1]. Sperm consequently face an ''acrosome reaction problem,'' in which secretion must be coordinated with egg contact and yet spontaneous secretory rates must be suppressed. It is now understood that the initiation of acrosome reactions during egg contact is accomplished by the presence in the zona pellucida of an acrosome reaction-inducing agonist, the glycoprotein ZP3 [7]. However, individual sperm must still prevent spontaneous secretion until the time of sperm-egg contact.

One common theme in the regulation of exocytotic processes is the general role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) as a mediator of stimulus-secretion coupling. In both sperm and somatic cells, elevations of $[Ca^{2+}]_i$ are necessary and sufficient to initiate secretion [8–11]. In this review we describe the mechanisms by which mammalian sperm elevate $[Ca^{2+}]_i$ during stimulation by ZP3 and the manner in which this signal-transducing mechanism is in turn modulated during capacitation. In this minireview we will suggest that the control of sperm ion channel function accounts, at least in part, for the stringent control of acrosome reactions and, particularly, for minimizing rates of spontaneous acrosome reactions.

VOLTAGE-SENSITIVE CALCIUM CHANNELS ARE ACTIVATED DURING THE ACROSOME REACTION

Two classes of voltage-sensitive Ca^{2+} channels have been described in somatic cells on the basis of their biophysical characteristics. 1) High voltage-activated channels require depolarizations to > -20 mV for activation, conduct maximal currents at $> +10$ mV, and may account for the L-, N-, P-, Q-, and R-type currents. These channels have a heteromeric protein composition consisting of a pore-forming $\alpha 1$ subunit and auxiliary regulatory $\alpha 2/\delta$ and β subunits [12, 13]. Many of the high voltage-activated channels can be differentiated from each other as well as from the T-type low voltage-activated T channels by pharmacological criteria [14]. 2) A low voltage-activated channel that has a voltage threshold of approximately -60 mV conducts maximal current at -30 to -20 mV and accounts for the T-type current. There is presently no consensus regarding the molecular components of T channels and, in fact, pharmacological studies suggest that there may be a diversity of T channels.

Sperm are transcriptionally and translationally inert. Ion channels that are utilized in sperm must therefore be synthesized during spermatogenesis. A T-type low voltage-activated current is the only Ca^{2+} current that can be detected in mouse spermatogenic cells by whole cell-patch clamp methods [15–19]. In addition, $\alpha 1A$ and $\alpha 1E$ genes may be expressed during mouse spermatogenesis, as indicated in reverse transcription-polymerase chain reaction experiments using spermatogenic cell RNA [17]. The Ca^{2+} currents that are carried by these gene products have not yet been assigned unequivocally. It has been suggested that the $\alpha 1A$ gene product mediates both P- and Q-type high voltage-activated currents [14, 20], possibly in association with distinct auxiliary subunits [21]. Similarly, recombinant $\alpha 1E$ protein produces a current in cellular expression systems that was initially described as a low voltage-activated Ca^{2+} current [22, 23]. Further analysis has shown that the $\alpha 1E$ gene product produces a high voltage-activated class and may be the R-type current [13, 24]. While transcription of

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these Ca^{2+} channel genes occurs during spermatogenesis, the associated high voltage-activated currents are not observed [15–19]. Thus, functional expression of one type of voltage-sensitive Ca^{2+} channel can be detected during spermatogenesis. However, other channel types may be present either at low copy number or in an inactive form.

In contrast to the situation with spermatogenic cells, it is relatively difficult to form the high-resistance (>1GΩ) seals required for patch clamp studies on sperm membranes. However, the presence and functional role of voltage-sensitive Ca^{2+}channels in sperm can be determined using ion-selective fluorescent probes, either in cell populations using spectrofluorometric approaches or in single cells using digital image processing-enhanced fluorescence microscopy. Such studies indicate that sperm maintain a free internal Ca^{2+} concentration ($[Ca^{2+}]_i$) of 50–100 nM; that, during capacitation, this increases to 125–175 nM with a monotonic time course (~0.5 nM/min in bovine sperm); and that these values then stabilize. Increases in $[Ca^{2+}]_i$ during capacitation occur uniformly throughout both the head and flagellar regions of the sperm [25]. The mechanisms that account for this physiological alteration in the sperm $[Ca^{2+}]_i$ set point have not been determined, and the role of Ca^{2+} transport systems, including channels, remains uncertain. What is clear is that these minor increases in $[Ca^{2+}]_i$ do not initiate acrosome reactions.

Addition of ZP3 produces an additional increase to 300–500 nM within minutes, at which point $[Ca^{2+}]_i$ values either are stabilized or slowly decline. Peak rates of $[Ca^{2+}]_i$ elevation during this response are ~150 nM/min and are associated with the initiation of acrosome reactions. The evidence that T channel activation is an essential component of the ZP3-activated signaling pathway in sperm may be summarized as follows. T currents in spermatogenic cells are inhibited by several drugs and ions in the following order of potency (IC_{50}): PN200-110 (4×10^{-8} M) > nifedipine (4×10^{-7} M) > pimozide (4.6×10^{-7} M) > Ni^{2+} > (3.4×10^{-5} M) > verapamil (7×10^{-5} M) > amiloride (2.4×10^{-4} M) > Cd^{2+} (2.8×10^{-4} M) [16]. In sperm, both the elevations of $[Ca^{2+}]_i$ and the acrosome reaction induced by ZP3 are inhibited by these same compounds with similar potencies [16, 19, 26]. These observations suggest that ZP3 stimulation of sperm evokes a T-type Ca^{2+} current that is an essential component of the signal transduction mechanism regulating the acrosome reaction.

Utilization of a T current to mediate Ca^{2+} influx during acrosome reactions can be understood in light of the biophysical characteristics of this class of channels. T channels open following weak depolarizations, when there is a large electrochemical driving force for Ca^{2+} entry [27]. This provides a mechanism through which nonexcitable cells, which do not produce the strong depolarizations that are associated with action potentials and that are required for the opening of high voltage-activated channels, are nevertheless able to transduce external signals into a $[Ca^{2+}]_i$ response.

In summary, T-type low voltage-activated Ca^{2+} channel genes are synthesized during mouse spermatogenesis, as revealed by direct examination using patch clamp methods. No other functional channels can be detected during spermatogenesis, although transcription of other channel genes has been reported. T channels are retained in sperm following terminal differentiation and play an essential role in the initiation of the acrosome reaction during zona pellucida contact. Since conductance through T channels is regulated by membrane potential, it is then pertinent to ask how eggs depolarize sperm membrane potential.

ZP3-DEPENDENT CATION CHANNEL: MECHANISMS OF MEMBRANE DEPOLARIZATION

The discussion to this point has focused on the identification of a T-type voltage-sensitive Ca^{2+} channel and the demonstration of its essential role in the ZP3-activated signal transduction mechanism. This suggests that a depolarization of sperm membrane potential may be an upstream element of this pathway. To address this question we incubated sperm with the cationic carbocyanine, 3,3′-dipropylthiadicarbocyanine iodide [5], and with the anionic oxonol, *bis*-(1,3-diethylthiobarbituric acid) trimethine oxonol [3]. These compounds are redistribution-type fluorescent probes of membrane potential and have been used previously in a variety of cellular systems [28–30], including sperm [31–36].

Capacitated sperm populations from mice and cattle have resting membrane potentials of approximately −60 mV that is due to permeability of several ions, including K^+ [37] and possibly Ca^{2+} [36]. This population-averaged membrane potential hyperpolarizes from ~−30 mV to ~−60 mV during capacitation in vitro [37]. Approximately 50% of sperm within a population capacitate in vitro, as determined from chlortetracycline fluorescence [38] and as estimated from the secretory response to ZP3 [38, 39]. The membrane potential of capacitated sperm cells has not yet been determined on the single-cell level. However, it is likely that the reported membrane potential of capacitated populations reflects contributions of the relatively depolarized uncapacitated fraction of sperm and that the potential of capacitated fraction may be more hyperpolarized than the population average.

ZP3 stimulation depolarizes sperm membrane potential to ~−25 mV [40]. This is a specific response to homologous ZP3 when tested in mouse and bovine sperm: it is not observed when sperm are treated with other zona pellucida glycoproteins, with unrelated control glycoproteins, or with ZP3 from another species.

Depolarization is due to the activation of a voltage-insensitive ion channel. The characteristics of this channel were determined with potentiometric fluorescent probes [40] and also in a series of fura 2-fluorescence quenching studies [25]. This channel is permeable to Na^+ and to many divalent cations (Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}), but it is impermeable to larger organic cations such as *N*-methyl-D-glucamine$^+$ and to trivalent cations such as La^{3+}. In addition, the channel appears to be completely impermeable to organic and inorganic anions. Thus, this ZP3-activated pathway has the characteristics of a poorly selective cation channel.

Cation channels gated by cyclic nucleotides [41], by progesterone [42], and by extracellular ATP [43] may be present in mammalian sperm. Moreover, both the progestin- and the ATP-gated channels depolarize sperm membrane potential. Comparisons between these channels are difficult because of the incomplete nature of the data set, the physiological state of the sperm cells, and the use of different detector systems. Ion selectivity studies suggest that the ZP3-dependent cation channel may conduct certain divalent metal cations [25, 40] whereas other sperm cation channels may be more selective for monovalent ions. Thus, there may be several cation-selective channels in mammalian sperm.

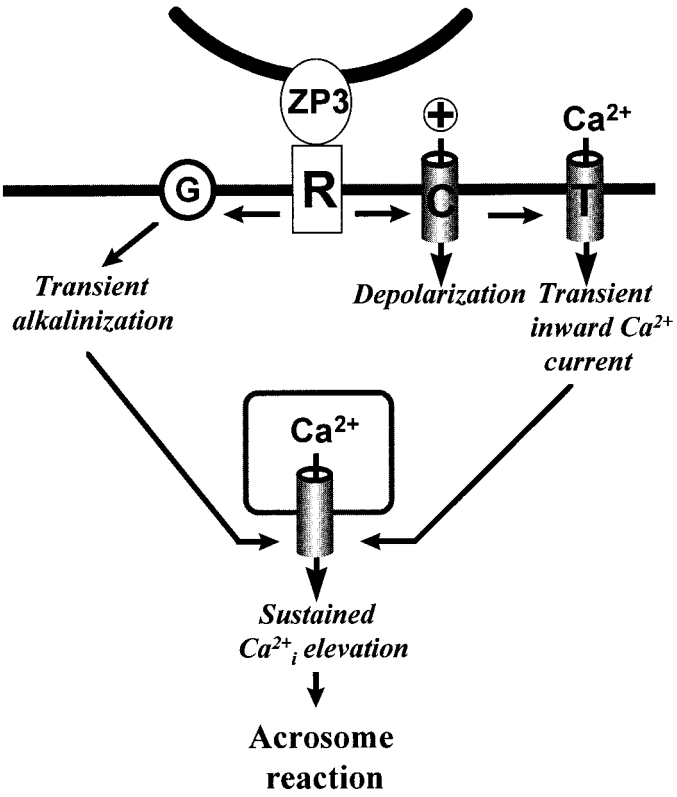

FIG. 1. Model of the mechanism of sperm ion channel activation by the zona pellucida. During gamete adhesion, ZP3 in the zona pellucida associates with a sperm surface receptor (R) and produces two separate intracellular signals. One pathway consists of the activation of a cation channel (C) through a pertussis toxin-insensitive and voltage-insensitive mechanism. Sperm maintain an inwardly negative membrane potential, and conductance through cation channels produces a depolarizing current. The result of membrane depolarization is the activation of a low voltage-activated T-type Ca^{2+} channel (T). The sperm T channel may additionally be modulated by its tyrosine phosphorylation state during capacitation and ZP3 stimulation. The second signaling pathway results from ZP3 activation of a pH regulator, resulting in a transient alkalinization of internal pH. ZP3 activates this pH regulator through a pertussis toxin-sensitive mechanism that likely reflects mediation of the G_i-class of G proteins (G). Transient Ca^{2+} and pH elevations act in synergy to promote a sustained Ca^{2+} elevation, most likely through release from an IP3-sensitive intracellular store. This sustained Ca^{2+} leads to acrosome reactions. This pathway depends on T channel activation, and sustained Ca^{2+} elevations are inhibited by T channel antagonists.

In summary, ZP3 activates a cation channel in sperm during gamete adhesion. This channel conducts monovalent and divalent cations but is not permeable to anions. The roles of this channel and of the T-type Ca^{2+} channel are considered in the next section.

A MODEL OF THE IONIC EVENTS IN ZP3 SIGNAL TRANSDUCTION

As discussed earlier, elevation of intracellular Ca^{2+} is a central event in the control of acrosome reactions. Figure 1 presents a model for the mechanisms by which zona pellucida adhesion regulates sperm Ca^{2+} levels.

The initial event in this pathway is the activation of a sperm surface receptor (R) by ZP3 binding. According to this model, receptor activation initiates two signaling sequences. First, a cation channel (C) is activated through a pertussis toxin-insensitive mechanism. Given the polarized membrane potential maintained by sperm, activation of a cation channel is expected to produce an inward, depolarizing current. Under physiological conditions this cation channel produces a small, transient influx of Ca^{2+} into the sperm head [25]. However, Ca^{2+} entry by this mechanism does not initiate acrosome reactions. The cation channel should also mediate an inward Na^+ current. We have suggested that the major function of these inward currents is to depolarize sperm membrane potential and thereby open voltage-sensitive T-type Ca^{2+} channels (T on Fig. 1) [25, 40].

The calculated membrane potential of capacitated sperm is consistent with an essential role of a T-type channel. T-type channels, including that in male germ cells [16], exhibit voltage-dependent inactivation at membrane potentials above ~ -80 mV [27, 44, 45]. As discussed previously, it is as though capacitated sperm within a population have a membrane potential considerably more negative than the -60 mV reported for the heterogeneous population [37]. Thus, sperm membrane potential is sufficiently polarized to permit activation of low voltage-activated T channels. ZP3 depolarizes membrane potential from resting values to ~ -25 mV [40], a value that produces a peak T current in germ cells [16–18] and somatic cells [27, 45]. In contrast, the zona pellucida-dependent depolarization would not be expected to activate high voltage-activated Ca^{2+} currents.

Voltage-dependent inactivation of T currents occurs within 50–100 msec during depolarization [16–18], thereby terminating the ZP3-induced Ca^{2+}influx. In contrast, the sustained Ca^{2+} elevations that are required for acrosome reactions are not detected for 1–2 min after zona stimulation [16, 25, 40, 46, 47]. These protracted Ca^{2+} responses require T channel activation and are inhibited by T channel antagonists [16]; however, they are not a direct measure of T channel function. In this regard, inositol 1,4,5-trisphosphate (IP3) receptors are present in sperm acrosomes [48], and release of sequestered Ca^{2+}produces acrosome reactions [48, 49]. A plausible mechanism of action is that T channel activation produces a transient Ca^{2+} influx that in turn initiates a downstream process of Ca^{2+}-induced Ca^{2+} release leading to acrosome reactions [16, 48]. Other intracellular effectors of ZP3 action, such as elevated pH_i [26, 40, 46, 50] and IP3 [51], may also contribute to Ca^{2+} pool mobilization. In fact, internal alkalinization is initiated by ZP3 through a pertussis toxin-sensitive pathway that likely signifies the mediation of sperm G proteins (G on Fig. 1) in this process. Alkalinization is required for robust Ca^{2+} elevations in response to ZP3 [40] or membrane depolarization [34, 40].

REGULATION OF ZP3 SIGNAL TRANSDUCTION DURING CAPACITATION

Mammalian sperm require a period of capacitation, or functional reprogramming, in order to exhibit fertilizing ability [1]. Capacitation is also required for the induction of the acrosome reaction by ZP3 [38, 39]. This observation has led to the suggestion that the ZP3 signal-transducing mechanisms are regulated during capacitation.

In fact, sperm T channels may modulated in several ways during capacitation. First, the conductance state is controlled by membrane potential. As discussed previously, voltage-dependent inactivation of T channels occurs with a low voltage threshold. Inactivation of the germ cell channel is first observed at ~ -70 mV and is complete by -55 mV [16]. Importantly, the probability of T channel opening directly from the inactivated state is low [27].

The membrane potential of uncapacitated sperm popu-

lations is relatively depolarized, with values of ~−30 mV calculated for both mouse and bull sperm in vitro [37]. Under these conditions there is a maximal voltage-dependent inactivation of T channels [16], and Ca^{2+} current through this pathway is minimal. Thus, conditions that may further depolarize membrane potential of uncapacitated sperm will not enhance T currents and are unlikely to initiate acrosomal secretion. During capacitation, sperm membrane potential hyperpolarizes due to an enhanced contribution of K^+ permeability [37]. Hyperpolarization relieve steady-state inactivation of T channels. As a result, channels tend to dwell in the closed state, from which opening occurs with high probability after depolarization [27]. Thus, hyperpolarization may act to prime T channels for subsequent activation by ZP3.

T channel activation is regulated in a second way during capacitation. As discussed previously, a ZP3-activated cation channel produces an inward current carried by Ca^{2+} and probably also by Na^+. This depolarizing current provides the coupling between gamete contact and T channel activation. Cation channel opening in response to zona pellucida interaction has been assessed in single sperm using Ca^{2+}-selective fluorescent dyes. The sensitivity of this cation channel to evoked opening in response to zona pellucida stimuli is low in incapacitated sperm and increases during capacitation in vitro [25]. Thus, the ability of ZP3 to depolarize sperm membrane potential, and hence to activate the voltage-sensitive T channel, is modulated at the level of an upstream cation channel during capacitation.

Finally, and moving into the realm of speculation, T channels may also be modulated during capacitation by tyrosine phosphorylation. Recently, it has been shown that tyrosine phosphorylation of either the T channel or of a channel regulator decreases current through the channel. Conversely, tyrosine phosphatase activity enhances T current [19]. This type of modulation has not yet been observed in somatic cell T channels and may reflect a novel regulatory mechanism. Alternatively, tyrosine phosphorylation-dependent modulation may be more readily detectable in the relatively simple germ cell model system, where other Ca^{2+} currents are not present.

In this regard, capacitation is associated with a wave of protein tyrosine phosphorylation [52, 53]. It is tempting to speculate that the T channel, or a channel regulator, is one substrate for a capacitation-dependent tyrosine kinase. The resultant negative modulation of T currents would reduce Ca^{2+} currents and could minimize spontaneous acrosome reactions. This suggestion implies that ZP3 activates a tyrosine phosphatase activity during the initiation of acrosome reactions. Further studies are required to test this suggested mechanism.

The mechanisms by which K^+ permeability and ZP3-regulated cation channel conductance are regulated during capacitation are not understood. Plausible and intriguing mechanisms may be proposed on the basis of altered protein kinase activity during capacitation [1, 52, 53] and on the known effect of posttranslational modifications on somatic cell K^+ channel function [54, 55]. Future efforts will focus on testing such proposed mechanisms in sperm. Thus, sperm T channels represent both an essential step in the ZP3 signal transduction pathway and a site of regulation during capacitation.

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