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# Zona Pellucida and Progesterone-Induced $Ca^{2+}$ Signaling and Acrosome Reaction in Human Spermatozoa

Review

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## Acrosome Reaction

The mammalian acrosome reaction (AR) is the fusion, at multiple points, of the outer acrosomal membrane with the overlying plasma membrane. This fusion causes the release of acrosomal contents and the exposure of the inner acrosomal membrane. The time taken for this process to occur is not accurately known. Estimates for AR in mouse spermatozoa vary from ~2 minutes after stimulation with solubilized zona to 130 minutes with intact zona (Lee and Storey, 1989; Rockwell and Storey, 2000). In the human, latencies of 15–60 minutes have been observed with intact zona (Morales et al, 1994). Fusion of the plasma and outer acrosomal membranes in the human appears to progress in a different way from that of the rodent and other mammals, but the final result is the same (Nagae et al, 1986; Yanagimachi, 1994). Release of acrosomal enzymes (including hyaluronidase and acrosin), combined with vigorous sperm motility, has been postulated to enable sperm penetration through the zona, allowing binding of the sperm to the oolemma, the egg plasma membrane (Talbot, 1985). It should be noted that experiments with mouse genetic knockouts have shown that zona penetration and fertilization can still occur in the absence of acrosin (Baba et al, 1994). Receptors revealed on the surface of the spermatozoon (after the vesicle and outer acrosomal membrane have been shed) are important in oolemma binding. After binding, the sperm is able to fuse with the oocyte (Yanagimachi, 1994; Snell and White, 1996; Myles and Primakoff, 1997).

*Interaction of Sperm With Ovulated Oocytes and Induction of AR*—Before reaching the oocyte, human sperm may come into contact with periovulatory follicular fluid or oviductal fluid. Both of these have a demonstrated AR induction capability that is decreased by protein kinase A (PKA) and protein kinase C (PKC) inhibitors (De Jonge et al, 1993). A hyaluronic acid–rich layer of cumulus cells surrounds the ovulated oocyte. Although the cumulus is the first egg vestment to be penetrated by the spermatozoon, we know remarkably little about this process. The cumulus oophorus consists of 2 different structures, an outer cell layer/mass (cumulus cells) and an inner layer (cells of the corona radiata), both of which secrete steroids (Laufer et al, 1984; Osman et al, 1989) and proteins (Tesarik et al, 1988). The cells of the human corona radiata are contiguous with the zona pellucida (ZP) and pass through it. Corona cells have many gap junctions with each other and the oocyte, which allow molecules and calcium ion ($Ca^{2+}$) signals to propagate. These junctions are known to be important in signaling during oocyte growth (Motta et al, 1994, 1995; Mattioli and Barboni, 2000). At ovulation, the links between the cumulus and oocyte are thought to be reduced or completely lost (Gregory et al, 1994; Motta et al, 1994, 1995), but the cumulus cells still have projections running throughout the zona to near the oolemmal surface (Motta et al, 1994).

The effects of cumulus cells on spermatozoa in vivo are yet to be established. However, it appears likely that passage through the cumulus mediates or prepares the cells for AR (Tesarik et al, 1988). Frequency of AR in human spermatozoa is reported to increase significantly from control (spontaneous) levels of 14.5% plus or minus 1.5% to 24.5% plus or minus 1.9% when incubated with cumulus mass; a further increase to 49% plus 3.3% was reported for cells incubated with mature cumulus containing an oocyte (Carrell et al, 1993). These findings agree with earlier data (De Jonge et al, 1988) demonstrating an induction of AR in sperm incubated with vested human oocytes. A likely cause of the AR-inducing activity of the cumulus is progesterone (P), a hormone produced at levels of 5–10 μM, in the cumulus during ovulation (Osman et al, 1989). High levels of cumulus-secreted P have been directly correlated to significantly increased rates of fertilization and polyspermy in vitro

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(Hartshorne, 1989), possibly reflecting more successful penetration of the ZP. The effects of P on spermatozoa include rapid activation of $Ca^{2+}$ influx (see below) and appear to be mediated nongenomically. Sperm membrane receptor(s) for P are yet to be definitely identified (Bray et al, 1999), but 2 binding sites have been detected ($K_d$ = 0.06 and 26 μM; Luconi et al, 1998). Attempts to block P-induced signaling with guanosine triphosphatase binding protein (G protein) modulators have not been successful, suggesting that other downstream pathways may be involved (Tesarik et al, 1993). Studies of various mammals, including the human, suggest that, as well as P, prostaglandins, sterol sulfates, glycosaminoglycans, and estrogen are present in follicular fluid and cumulus cell secretions (Lenz et al, 1983; Hartshorne, 1989; Zaneveld et al, 1991). Prostaglandin E, in particular, has been demonstrated to mediate elevation of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and AR via a G protein–coupled receptor mechanism (Schaefer et al, 1998). An estrogen receptor has recently been discovered on mature human sperm (Luconi et al, 1999), and estrogen has been demonstrated to induce $Ca^{2+}$ influx and to modulate P-induced responses (Luconi et al, 1999; Baldi et al, 2000). These effects of estrogen may be due to direct modulation of voltage-operated calcium channel (VOCC) components, as demonstrated by Espinosa et al (2000).

A second function of the cumulus-corona may be removal or selection of spermatozoa. Leukocytes are present throughout the cumulus, and both they and corona cells appear to phagocytose abnormal and/or supernumerary sperm (Nottola et al, 1998). When in vivo fertilized eggs were examined, cumulus cells had phagocytosed 31 of 36 sperm found in the main cumulus; only 5 of 36 were wedged in the matrix between, and all of these were acrosome reacted with intact equatorial segments. Of a further 7 sperm found in the corona and ZP, 6 had lost their equatorial segments, and all were acrosome reacted (Pereda and Coppo, 1985). However, other authors have reported low numbers of acrosome-intact human sperm at the zona after cumulus penetration in vitro (Chen and Sathananthan, 1986). The cumulus oophorus and follicular fluid have also been demonstrated to influence and maintain the movement characteristics in subpopulations of human sperm in ways that may be relevant in cumulus penetration (Mendoza and Tesarik, 1990; Tesarik et al, 1990).

Having penetrated the cumulus mass, sperm bind to the ZP, a thick, highly glycosylated protein matrix secreted during oogenesis, which surrounds the oocyte. Functions of the ZP include prevention of polyspermy; protection of the embryo before implantation; regulation of endocrine profiles during folliculogenesis; and blockade of heterospecific fertilization (Yanagimachi, 1994). This last role reflects the species-specific nature of sperm-ZP binding. If the sperm, upon reaching the ZP, have yet to acrosome react, then the process of binding to the ZP may induce acrosomal exocytosis. Exactly how sperm bind to the zona is unknown. The murine ZP is thought to be composed of at least 3 glycoproteins, ZP1, ZP2, and ZP3. A revised model has recently been suggested for the human and, in all likelihood, other mammals (except rodents) with the discovery of a fourth zona gene, now giving a classification of ZP1, ZPB, ZP2/ZPA, and ZP3/ZPC (Hughes and Barratt, 1999). Mouse experiments suggest that carbohydrate moieties (O-linked oligosaccharides) of ZP3 are of particular importance in AR induction (Beebe et al, 1992). A number of sperm ZP receptors or binding proteins have been suggested, such as trypsinlike proteins, spermadhesins, β 1,4-galactosyltransferase, and a 56- and 95-kd protein (reviewed in Tulsiani et al, 1997). Incubation of human sperm with P (simulating passage through the cumulus) has been shown to enhance human zona binding (by hemizona assay) and penetration of hamster oocytes (Sueldo et al, 1993; Oehninger et al, 1994b). It also significantly enhances subsequent zona-induced AR (Roldan et al, 1994).

Although P is an effective inducer of AR, it is generally accepted that this steroid functions as a secondary or coinducer (see above). The central role of ZP is based on the concept that it provides species specificity in fertilization via its role in induction of AR. However, we do not know that AR is the mechanism by which such specificity is achieved, nor do we know the site at which AR occurs during normal fertilization. It is yet to be established whether the occurrence of AR at the surface of the ZP is crucial to sperm penetration, a subject that is beyond the scope of this review. The possibility that ''cutting thrust'' rather than lytic events are the basis of eutherian ZP penetration has been thoroughly reviewed by Bedford (1998) and is consistent with the recent data on CatSper, a putative sperm cation channel that is localized in a principal piece of the mature mouse sperm tail (Ren et al, 2001). Mouse gene knockouts that are CatSper −/− have poorly motile sperm that completely fail to fertilize eggs unless the zona is removed (Ren et al, 2001), strongly indicating a requirement for vigorous motility in zona penetration. We are therefore not yet in a position to assess whether induction of AR during penetration of the cumulus should be regarded as ''premature.''

### *$Ca^{2+}$ and AR*

AR is mediated by a complex interaction of cellular responses, which includes kinase activation and consequent phosphorylation, gating of ion channels, and a plethora of other poorly defined processes (Ward and Kopf, 1993; Bielfeld et al, 1994a,b; Aitken, 1997; Breitbart and Spungin, 1997). However, as with virtually all other forms of stimulus-activated exocytosis, secretion of the acrosomal

contents, whether activated by P or ZP, is ultimately mediated by an elevation of $[Ca^{2+}]_i$. In both instances, this is composed of a transient $Ca^{2+}$ influx, followed by activation of a sustained influx pathway (Thomas and Meizel, 1989; Blackmore et al, 1990, 1991; Florman, 1994; Arnoult et al, 1996a,b; Kirkman-Brown et al, 2000). The P-induced $[Ca^{2+}]_i$ response is detectable in uncapacitated cells but is reportedly enhanced upon capacitation (Baldi et al, 1991; Mendoza and Tesarik, 1993; Garcia and Meizel, 1999). The evidence available at present is consistent with participation of both VOCCs and $Ca^{2+}$ store-regulated capacitative $Ca^{2+}$ entry (CCE) in the $Ca^{2+}$ signal leading to agonist-induced AR. Before reviewing this evidence and comparing the ZP- and P-induced $[Ca^{2+}]_i$ signals, the evidence for the existence of these influx mechanisms in spermatozoa is briefly summarized.

*VOCCs*—There is little doubt that VOCCs are present in mature spermatozoa and that they are of significance in $Ca^{2+}$ signaling. In patch-clamped mouse spermatogenic cells (spermatocytes and round spermatids), T-type (transient, low-voltage activated [LVA]) channels are the only detectable VOCC current (Arnoult et al, 1996a; Santi et al, 1996). T-type $\alpha_1$ subunits ($\alpha_1$ being the main pore-forming subunit of the VOCC) have been detected in rodent germ cells by RT-PCR (Serrano et al, 1999). T-channel $\alpha_1$ subunit transcripts are present in human testis and can be detected in germ cells using in situ hybridization (Jagannathan et al, 2000a,b). Despite the efforts of many laboratories, it has, to date, proved impossible to raise specific antibodies to this class of channels. However, small T-like VOCC currents have been detected in human spermatogenic cells held under a whole-cell clamp (Arnoult, personal communication). Several high-voltage–activated $\alpha_1$ subunits have also been detected in rodent spermatogenic cells and mature spermatozoa (Lievano et al, 1996; Serrano et al, 1999; Westenbroek and Babcock, 1999; Wennemuth et al, 2000). Novel splice variants of $\alpha 1_C$ (an L-type channel) have been reported by Benoff (1998) and Goodwin et al (1997, 1998, unpublished data) in germ cells of rodents and humans. We have observed modulation of $[Ca^{2+}]_i$ in human spermatozoa by specific agonists and antagonists of L-type VOCCs (Kirkman-Brown et al, unpublished data). We conclude that, apart from the T-type channels, which will provide only a transient $Ca^{2+}$ influx pathway, other functional VOCCs are present in these cells that could provide a route for a sustained $Ca^{2+}$ influx (Publicover and Barratt, 1999).

*$Ca^{2+}$ Stores and CCE*—There is evidence that at least 1 $Ca^{2+}$ store exists within mammalian spermatozoa, possibly located in the acrosome, specifically outlined as follows: 1) Inositol trisphosphate receptors ($IP_3Rs$) have been shown to be present in testes and male germ cells (Walensky and Snyder, 1995; Tovey et al, 1997; Dragileva et al, 1999; Kuroda et al, 1999); additionally, staining for $IP_3Rs$ in human spermatozoa has shown localization of type $IP_3Rs$ to the acrosomal cap, but this staining is lost after induction of AR (Kuroda et al, 1999). 2) Staining with thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ adenosine triphosphatases (ATPases) (SERCAs), localizes primarily to the acrosome and midpiece, and acrosomal staining with thapsigargin, as with staining of $IP_3Rs$, is lost after AR (Rossato et al, 2001). Finally, 3) digitonin-permeabilized mouse spermatozoa show adenosine triphosphate (ATP)-dependent uptake of $^{45}Ca^{2+}$, which is inhibited by $IP_3$ (Walensky and Snyder, 1995).

It should be noted, however, that isolated acrosomes from bovine spermatozoa were shown to accumulate $Ca^{2+}$ in an ATP-dependent manner but did not release $Ca^{2+}$ in response to 10 μM $IP_3$ (Spungin and Breitbart, 1996).

Thapsigargin, which selectively releases $Ca^{2+}$ from intracellular stores by inhibition of the SERCAs (Thastrup et al, 1990), elevates the $[Ca^{2+}]_i$ of capacitated or uncapacitated mammalian spermatozoa and initiates AR (human: Blackmore, 1993; Meizel and Turner, 1993; Spungin and Breitbart, 1996; bull and ram: Dragileva et al, 1999; mouse: O'Toole et al, 2000). Doses of thapsigargin required to exert this effect vary. Some authors report that the submicromolar (10–100 nM) doses will induce AR and $Ca^{2+}$ mobilization (Meizel and Turner, 1993; Spungin and Breitbart, 1996; Rossato et al, 2001), whereas others (including ourselves) only see significant effects at 1- to 10-μM doses (Blackmore, 1993; Walensky and Snyder, 1995; Dragileva et al, 1999; O'Toole et al, 2000). Effective doses for blockade of SERCAs of somatic cells are typically in the range of 1–100 nM, and micromolar doses may have nonspecific effects (Treiman et al, 1998). The actions of thapsigargin on spermatozoa when used in the micromolar range, though probably acceptably specific, should therefore be interpreted with some caution.

Although thapsigargin has been reported to cause a marked elevation of $[Ca^{2+}]_i$ in human spermatozoa in the absence of extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$) (Rossato et al, 2001), most laboratories observe no effects of the drug on $[Ca^{2+}]_i$ or AR under such conditions. This $[Ca^{2+}]_o$ dependence indicates that the primary effect of the drug in elevating $[Ca^{2+}]_i$ is to induce an influx of $Ca^{2+}$ across the plasma membrane. The simplest interpretation of these findings is that the $Ca^{2+}$ store of spermatozoa does not normally contain sufficient $Ca^{2+}$ to significantly elevate $[Ca^{2+}]_i$ but that, upon the emptying of this store, CCE is activated. CCE is an as-yet poorly defined process by which the emptying of an intracellular $Ca^{2+}$ store results in activation of a ''store-operated channel (SOC)'' in the cell membrane, permitting $Ca^{2+}$ influx (Berridge, 1995; Putney and McKay, 1999). On the basis of the available evidence, 2 models have been proposed for the mechanism by which the state of the $Ca^{2+}$ store is communicated

to the SOC to activate CCE. The first involves direct coupling between the $Ca^{2+}$ release channels on the store and the $Ca^{2+}$ entry (store operated) channels on the plasma membrane, and the second requires the synthesis/release of a diffusible ''calcium influx factor'' from the depleted store, which activates the SOC (Bootman et al, 2001). It appears that there are a number of membrane channels responsible for CCE (Putney and McKay, 1999; Bootman et al, 2001). Though these are yet to be fully characterized, the strongest candidates are the transient receptor potential (Trp) gene family, encoding $Ca^{2+}$ channel proteins. Expressed, recombinant Trp channels vary in their characteristics, but a number are known to form functional CCE channels. Human Trp3 (hTrp3) has been shown to interact functionally with $IP_3$Rs, apparently as part of direct $Ca^{2+}$-store-plasmalemma coupling for CCE (Kiselyov et al, 1998; Putney, 1999; see above). In the context of the sustained $Ca^{2+}$ influx of spermatozoa, it is noteworthy that the acrosome (the putative $Ca^{2+}$ store) is located close to the plasmalemma and is thus ideally suited for direct coupling. In human testis, transcripts for hTrp1 (which, when expressed, acts as an SOC; Zitt et al, 1996) and hTrp6 are strongly expressed (Wes et al, 1995; Hofmann et al, 1999). In the mouse, Trp2 is strongly expressed in testis (Vannier et al, 1999).

### *Mechanisms of Agonist-Induced $Ca^{2+}$ Influx*

*ZP-Induced $Ca^{2+}$ Influx in Mouse Spermatozoa*—The ZP-induced $[Ca^{2+}]_i$ signaling pathway is best characterized in the mouse, where there is an initial depolarizing cation influx, followed by a 2-stage influx of $Ca^{2+}$ (Florman et al, 1998). The first stage of $Ca^{2+}$ influx is brief (200–300 ms; Arnoult et al, 1999) and occurs through a dihydropyridine-sensitive VOCC. The pharmacology of ZP-induced $Ca^{2+}$ influx closely resembles that of the LVA channel in mouse spermatogenic cells (Arnoult et al, 1996a,b; Santi et al, 1996; Florman et al, 1998). This initial signal is then followed by a sustained elevation of $[Ca^{2+}]_i$, which is dependent on the initial $[Ca^{2+}]_i$ transient but occurs by a separate influx pathway (Florman et al, 1998; Darszon et al, 1999). Various mechanisms have been proposed, but recent data strongly support the idea that this second phase is mediated by CCE through SOCs (O'Toole et al, 2000; Jungnickel et al, 2001).

In the simplest model based on these data, the initial $[Ca^{2+}]_i$ transient may activate phospholipase C (PLC), which in turn generates $IP_3$ and diacylglycerol (DAG). This would ultimately result in the emptying of intracellular $Ca^{2+}$ stores and consequent CCE (O'Toole et al, 2000). Though this model awaits firm verification, the evidence available at present suggests that it is essentially correct. For example, Fukami et al (2001) have reported recently that disruption of the PLCδ4 gene in mice results in few, small litters or complete sterility in the male. The sperm were also unable to initiate AR in response to ZP. Jungnickel et al (2001) have studied the role of Trp proteins in the sustained calcium entry pathway induced by ZP3 in mouse sperm. Using an antibody raised to mouse Trp2, they have shown the presence of a 123-kd protein in mouse sperm, localized primarily to the anterior of the sperm head. Further experiments with this antibody have shown that it blocks thapsigargin-activated CCE and decreases the ZP-evoked $Ca^{2+}$ influx rate. The location of Trp2 to the anterior head of mouse sperm is ideal for interaction with the adjacent $IP_3$R that is present in the acrosomal membrane (see above). Interestingly, in the hamster, there may be a contribution of VOCCs to the late component of $Ca^{2+}$ influx since this part of the signal is selectively blocked by nifedipine (Shirakawa and Miyazaki, 1999).

A neuronal glycine receptor/$Cl^-$ channel (GlyR) has been identified on mammalian sperm plasma membranes, and there is evidence for the role of a GlyR in the initial ZP-evoked response (reviewed in Llanos et al, 2001). Experiments on human, rodent, and porcine sperm suggest involvement of GlyR channels in zona-induced AR, GlyR agonists promoting AR in a dose-dependent manner (Melendrez and Meizel, 1995; Turner et al, 1997; Llanos et al, 2001). In addition, bicuculline, strychnine, and other GlyR antagonists severely impaired or abolished ZP-mediated AR (Llanos et al, 2001). Mice with mutations in the GlyR α and β subunits (termed ''spasmodic'' and ''spastic,'' respectively) have been shown to lack the ability to undergo either ZP- or glycine-mediated AR (Sato et al, 2000). In addition, Sato and colleagues observed that a GlyR antibody abolished ZP-induced AR in wild-type mouse sperm. The relationship of the GlyR to the model outlined above is uncertain, but one possibility is that the channel provides a mechanism of chloride efflux, and consequent depolarization, for activation of VOCCs (Garcia and Meizel, 1999).

*P-Induced $Ca^{2+}$ Influx in Human Spermatozoa*—The response to P is best characterized in the human. When spermatozoa are treated with P, there is a rapid (within seconds), transient elevation of $[Ca^{2+}]_i$ lasting 1–2 minutes, which is observed both by fluorimetry and single-cell imaging (Blackmore et al, 1990; Foresta et al, 1993; Plant et al, 1995; Aitken et al, 1996; Tesarik et al, 1996; Meizel et al, 1997; Kirkman-Brown et al, 2000). This $[Ca^{2+}]_i$ transient is abolished in a low-$Ca^{2+}$ medium, indicating that it reflects an influx of extracellular $Ca^{2+}$ (Blackmore et al, 1990; Foresta et al, 1993; Plant et al, 1995; Aitken et al, 1996), and is accompanied by depolarization of the membrane potential (Foresta et al, 1993). There has been considerable debate about the involvement of VOCCs in this response. P-induced AR is sensitive to modulators of VOCCs (Shi and Roldan, 1995; O'Toole et al, 1996a; Kirkman-Brown et al, 2000, un-

published data), but most authors report little, if any, effect of VOCC blockers on the amplitude of the $Ca^{2+}$ transient (Thomas and Meizel, 1989; Foresta et al, 1993; Aitken et al, 1996). More recently, this question has been reexamined in light of the detection of T channels in mouse germ cells (see above). Garcia and Meizel (1999), using fluorimetric measurement of $[Ca^{2+}]_i$ in human spermatozoa, observed inhibition of the P-induced $[Ca^{2+}]_i$ transient by pimozide and mibefradil, drugs that affect the mouse spermatogenic cell T current (Arnoult et al, 1998) and are known to show some selectivity for T channels over other VOCCs. However, the effects were difficult to interpret, the dose-dependency of these effects being inconsistent both with the action of these drugs on mouse spermatogenic cell T currents (Arnoult et al, 1998) and with their effects on P-induced AR. Blackmore and Eisoldt (1999), using a similar approach, concluded that T channels were not involved in the $[Ca^{2+}]_i$ response to P. In contrast, Morales et al (2000) and Patrat et al (2000a,b) both reported strong inhibitory effects of T-channel blocking drugs on the P-induced $[Ca^{2+}]_i$ response. However, inconsistencies between laboratories and the lack of specificity of the compounds used are such that it is difficult to draw conclusions from these studies. Using single-cell imaging, we have detected an effect of VOCC blockers on the duration of the transient. Our data indicate that the transient $Ca^{2+}$ influx includes a ''late'' VOCC-mediated component as well as an ''early'' non-VOCC component (Kirkman-Brown et al, 2000, unpublished data). It is not yet possible to determine whether the VOCC involved is a T channel or one of the high-voltage–activated VOCCs that are also present in spermatozoa (see above). However, progress in identifying the molecular nature of sperm T channels may soon permit a comparison of the pharmacologies of the ''late'' P-induced transient and recombinant human sperm T channels. The nature of the early component of the early P transient still remains elusive, the only proven antagonist being lanthanum $(La)^{3+}$ (Blackmore et al, 1990; Plant et al, 1995; Aitken et al, 1996). An intriguing possibility is that reported differences in susceptibility of the P-induced $[Ca^{2+}]_i$ transient to VOCC antagonists reflect differences in the contributions of the 2 components.

After the initial P-induced $[Ca^{2+}]_i$ transient, a second, sustained elevation of $[Ca^{2+}]_i$ occurs. This sustained $[Ca^{2+}]_i$ elevation is primarily, but not exclusively, observed in cells that show the initial transient (Kirkman-Brown et al, 2000). We have recently found that this sustained response to P is partially occluded by pretreatment with thapsigargin (an activator of CCE) and is reduced in amplitude in the presence of 2-aminophenyl borate (an inhibitor of $Ca^{2+}$ store depletion and consequent downstream events; Punt et al, 2001, in preparation). Since it is already known that the treatment of human or mouse sperm with P stimulates turnover of phosphoinositides and generation of $IP_3$ and DAG (Thomas and Meizel, 1989; Roldan et al, 1994; O'Toole et al, 1996b), it appears likely that the sustained phase of the P-induced $[Ca^{2+}]_i$ response is supported by CCE.

Similar to the putative role of the GlyR/$Cl^-$ channel in ZP-induced AR, the P-mediated calcium influx and AR may involve a γ-aminobutyric acid (GABA) receptor/$Cl^-$ channel. GABA induces AR in human spermatozoa in a dose-dependent manner, this response being inhibited by $GABA_A$ receptor antagonists such as bicuculline and, to a lesser extent, by $GABA_B$ receptor blockers such as baclofen (Wistrom and Meizel, 1993; Calogero et al, 1999). Inhibition of P-induced AR by bicuculline is also observed in the mouse (Roldan et al, 1994). Sperm incubated with P, and then challenged with GABA, show a significant increase in AR compared to sperm incubated with P alone (mouse: Roldan et al, 1994; human: Calogero et al, 1999). GABA and P may act through the same receptor or converge on a similar signaling pathway, as their AR-inducing abilities are not simply additive (Calogero et al, 1999). Blockade of $Cl^-$ channels with picrotoxin is reported to inhibit both the GABA- and P-induced AR (Calogero et al, 1999; Kuroda et al, 1999). In addition, $Cl^-$ efflux has been shown to take place during the P-induced AR (Turner and Meizel, 1995). As with the ZP-GlyR system, the subsequent membrane depolarization could activate a VOCC. There is also evidence to suggest that the activation of the GABA receptor may increase the activity of a sodium/bicarbonate ion cotransporter, leading to increased bicarbonate ion concentrations within the sperm cytosol (Turner and Meizel, 1995). However, it should be noted that not all laboratories detect effects of GABA receptor manipulation on $[Ca^{2+}]_i$ or AR (eg, Baldi et al, 1991; Blackmore et al, 1994). Blackmore et al (1994) showed no correlation between the ability of a number of modified steroids to initiate $Ca^{2+}$ influx in human spermatozoa and to modify $Cl^-$ uptake or $Cl^-$ currents in human embryonic kidney (HEK)-293 cells transfected with $GABA_A$ subunits. In addition, P-mediated $Ca^{2+}$ influx was unaffected by a series of $GABA_A$ receptor/chloride channel modulators (Blackmore et al, 1994). This conflicts with the data of Meizel et al (1997), who observed a strong inhibition of P-induced $Ca^{2+}$ influx by picrotoxin. Finally, it must be noted that in the 2 studies that have investigated the actions of GABA itself and GABA agonists on $[Ca^{2+}]_i$ in human spermatozoa, no calcium-mobilizing effect was observed (Blackmore et al, 1994; Aitken et al, 1996).

### *ZP- and P-Induced $[Ca^{2+}]_i$ Responses—Untangling Species and Agonist Differences*

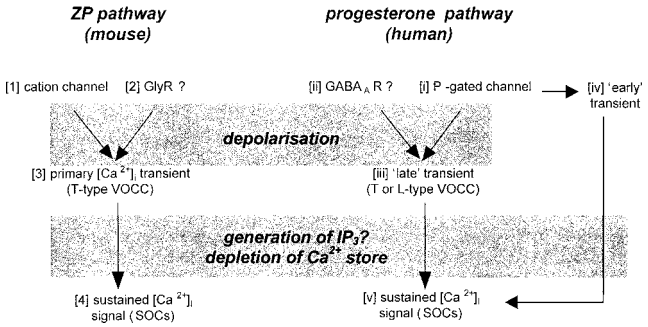
Comparisons between ZP- and P-induced $Ca^{2+}$ signaling in spermatozoa are complicated by the nature of the data.

Our understanding of ZP-induced $[Ca^{2+}]_i$ signaling is primarily derived from studies on the mouse. It has been known for some time that solubilized zona induces AR in the human (eg, Cross et al, 1988; De Jonge, 1996; Liu and Baker, 1996; Henkel et al, 1998; Sabeur et al, 1998), and it is clear that, as in the mouse system, this is associated with a rise in $[Ca^{2+}]_i$ (Patrat et al, 2000b). Studies using recombinant human ZP3 have also detected an elevation of $[Ca^{2+}]_i$ and induction of AR in human spermatozoa (Brewis et al, 1996). AR in human spermatozoa can even be induced by solubilized mouse ZP (Lee et al, 1987). However, it is not yet known whether the $[Ca^{2+}]_i$ signals in mouse and human spermatozoa are generated in the same way. Fluorimetric records of ZP-induced $[Ca^{2+}]_i$ signaling in populations of human spermatozoa show a biphasic response with a small initial transient and a rather larger sustained phase, the kinetics being similar to those of the response to P (Patrat et al, 2000b). Preliminary studies suggest that both components of the response are inhibited (but not abolished) by the VOCC blocker pimozide (10 μM) (Patrat et al, unpublished data). The AR and $[Ca^{2+}]_i$ responses of human spermatozoa induced by neoglycoproteins, which may activate the same signaling pathway as ZP (Brandelli et al, 1995, 1996; Blackmore and Eisoldt, 1999), are sensitive to blockers of T-type VOCCs, consistent with a mechanism not unlike that characterized in the mouse (Brandelli et al, 1996; Blackmore and Eisoldt, 1999; Son et al, 2000). However, the sensitivity to some of these blockers is strikingly different from that of the T channel in mouse spermatogenic cells (Arnoult et al, 1998; Son et al, 2000). With regard to the likely participation of SOCs downstream of the initial $Ca^{2+}$ influx, the human and murine responses must differ somewhat, since Trp2, which is believed to support CCE in ZP-stimulated mouse spermatozoa, is a pseudogene in the human (Vannier et al, 1999). Since other Trp channels (hTrp1 and hTrp6) are believed to be expressed in human testis (see above), one possibility is that, in the human, the functional role of Trp2 is taken by another member of the family. It thus appears that ZP-induced $Ca^{2+}$ signaling in the human may be similar to that in the mouse but is most unlikely to be identical.

Although it is known that P will induce AR in nonhuman mammals (and even activate a $Ca^{2+}$-dependent AR-like response in octopus spermatozoa) (Tosti et al, 2001), the characteristics of the P-induced $[Ca^{2+}]_i$ response described above were derived entirely from human studies. Kobori et al (2000) recently investigated the P-induced $[Ca^{2+}]_i$ signal in mouse spermatozoa. They showed that the treatment of mouse spermatozoa with P, as in the human, results in an elevation of $[Ca^{2+}]_i$. However, there were clear species differences. Transient responses (similar to those observed in human spermatozoa) and more prolonged, plateaulike responses were seen. Biphasic responses, as described in the human (Kirkman-Brown et al, 2000), were not reported. Sensitivity to P was low, less than 50% of cells responding at a dose of 40 μM and less than 25% at 4 μM, a dose that typically activates 80%–90% of human spermatozoa. Prolonged $[Ca^{2+}]_i$ responses were only seen at the higher doses. Use of VOCC blockers showed differential sensitivity of the 2 components. Prolonged responses were abolished by 1 μM pimozide (a dose that effectively blocks the T-type current of mouse spermatogenic cells; Arnoult et al, 1998), but the transient responses were much less sensitive. Neither of the response types was affected by 5 μM verapamil, a blocker of L-type VOCCs. It appears likely that, as with the response to ZP, the $Ca^{2+}$-mobilizing action of P is not identical in the 2 species but clearly has similarities.

The mouse ZP- and human P-induced $[Ca^{2+}]_i$ signaling responses, although clearly different, do possess some striking parallels. Both are multiphasic, with an initial, transient phase that activates the later component(s). It is likely that the transient phase in both instances includes depolarization (which may involve $Cl^-$ efflux) and activation of VOCCs (though in the case of P, the response is not totally dependent on VOCC activation). Downstream of the initial transient, there is, in both responses, a sustained influx of $Ca^{2+}$, probably due to activation of SOCs. It is likely that key components of the 2 responses (VOCCs, store emptying, and CCE) are at least similar. The simplest model, on the basis of these observations, would be that the initial receptors/responses for ZP and P are separate but that the signals subsequently employ common components (Figure). In this context, it is of interest that Roldan et al (1994) observed that larger numbers of mouse sperm underwent AR when challenged with solubilized ZP subsequent to P exposure than when the mouse sperm were challenged by the agonists in the inverse order or simultaneously. The authors suggested that P primes the sperm to respond to ZP, a suggestion that is consistent with convergence of the signal transduction pathways.

An intriguing observation is that of Krausz et al (1995, 1996), who showed that the P-induced $[Ca^{2+}]_i$ signal in human spermatozoa is highly predictive of fertilization success in vitro. Furthermore, Oehninger et al (1994a) showed significant impairment of the $[Ca^{2+}]_i$ response to P in cases of teratozoospermia (normally associated with subfertility). Two simple interpretations of these data are: 1) the $Ca^{2+}$ influx mechanisms activated by P and ZP in human spermatozoa are largely similar such that, in cases of impaired ZP-induced $Ca^{2+}$ influx, P acts as a good predictor of the response to ZP, or 2) responsiveness to P, during penetration of the cumulus, is a key factor in the ability to respond to and/or penetrate the ZP. An impaired




Proposed parallels between intracellular calcium ion concentration ($[Ca^{2+}]_i$) signals induced by zona pellucida (ZP) in the mouse and progesterone (P) in the human. Shaded areas indicate key events occurring in both pathways. Left-hand pathway represents ZP-induced signaling in murine spermatozoa: an unidentified cation channel **(1)** and possibly efflux of $Cl^-$ through a glycine receptor (GlyR) **(2)** provide initial depolarization (shading) leading to activation of a T channel **(3)**. Downstream generation of inositol trisphosphate ($IP_3$) and depletion of calcium ion ($Ca^{2+}$) stores (shading) cause store-operated channel (SOC) activation **(4)**, which provides the sustained component of the signal. Right-hand pathway represents P-induced signaling in human spermatozoa: activation of an unidentified P-gated channel **(i)** and possibly efflux of $Cl^-$ through a γ-aminobutyric acid ($GABA_A$) receptor ($GABA_A$ R) **(ii)** provide initial depolarization (shading) leading to activation of a voltage-operated calcium channel (VOCC) **(iii)**. Additionally, the P-gated channel **(i)** carries the "early" non-VOCC $Ca^{2+}$ influx **(iv)**. Downstream of **(iii)** or **(iv)**, generation of $IP_3$ and depletion of $Ca^{2+}$ stores (shading) cause SOC activation **(v)**, which provides the sustained component of the signal.

response to P is thus itself a cause of male-factor infertility.

It is not possible, from the data available at present, to discount either of these suggestions.

### *Final Thoughts and Future Directions*

In this review, we have tried to make clear that there is still much that is poorly understood about the actions of ZP and P in inducing AR, particularly in the human. Our model for induction of AR in the human is very dependent on lessons learned from studies on the mouse. We have almost no direct evidence regarding the mechanism of action of ZP in the human, and the large body of data on the action of P is remarkably inconsistent. Furthermore, we know almost nothing of the induction of AR in vivo or the role of the cumulus and P in this process.

An immediate aim for work on human spermatozoa must be to investigate ZP- and P-induced $[Ca^{2+}]_i$ signaling to permit a detailed understanding of the events underlying $Ca^{2+}$ mobilization equivalent to that which has been achieved (for ZP) in the mouse. A secondary aim must be for workers to start to employ experimental protocols more comparable to the in vivo situation. If either 1) or 2) outlined in the previous section is correct, the role of the cumulus (and P in particular) in induction of AR and fertilization deserves considerably more attention.

Recommended reviews include those by Tanghe et al (2002) on functions of the cumulus; Bray et al (1999) on progesterone receptors; Patrat et al (2000b) on AR/ZP receptors; Darszon et al (1999) and Publicover and Barratt (1999) on VOCCs and sperm; and Ward and Kopf (1993), Bielfeld et al (1994a,b), Aitken (1997), and Breitbart and Spungin (1997) on other signals and AR.

## *References*


Aitken RJ. Molecular mechanisms regulating human sperm function. *Mol Hum Reprod.* 1997;3:169–173.

Aitken RJ, Buckingham DW, Irvine DS. The extragenomic action of progesterone on human spermatozoa: evidence for a ubiquitous response that is rapidly down-regulated. *Endocrinology.* 1996;137:3999–4009.

Arnoult C, Cardullo RA, Lemos JR, Florman HM. Activation of mouse sperm T-type $Ca^{2+}$ channels by adhesion to the egg zona pellucida. *Proc Natl Acad Sci USA.* 1996a;93:13004–13009.

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M, Florman HM. Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proc Natl Acad Sci USA.* 1999;96:6757–6762.

Arnoult C, Villaz M, Florman HM. Pharmacological properties of the T-type $Ca^{2+}$ current of mouse spermatogenic cells. *Mol Pharmacol.* 1998;53:1104–1111.

Arnoult C, Zeng Y, Florman HM. ZP3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. *J Cell Biol.* 1996b;134:637–645.

Baba T, Azuma S, Kashiwabara S, Toyoda Y. Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J Biol Chem.* 1994;269:31845–31849.

Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J Androl.* 1991;12:323–330.

Baldi E, Luconi M, Muratori M, Forti G. A novel functional estrogen receptor on human sperm membrane interferes with progesterone effects. *Mol Cell Endocrinol.* 2000;161:31–35.

Bedford JM. Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biol Reprod.* 1998;59:1275–1287.

Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, Dean J, Saling P. Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. *Dev Biol.* 1992;151:48–54.

Benoff S. Voltage dependent calcium channels in mammalian spermatozoa. *Front Biosci.* 1998;3:D1220–D1240.

Berridge MJ. Capacitative calcium entry. *Biochem J.* 1995;312:1–11.

Bielfeld P, Anderson RA, Mack SR, De Jonge CJ, Zaneveld LJ. Are capacitation or calcium ion influx required for the human sperm acrosome reaction? *Fertil Steril.* 1994a;62:1255–1261.

Bielfeld P, Faridi A, Zaneveld LJ, De Jonge CJ. The zona pellucida-induced acrosome reaction of human spermatozoa is mediated by protein kinases. *Fertil Steril.* 1994b;61:536–541.

Blackmore PF. Thapsigargin elevates and potentiates the ability of progesterone to increase intracellular free calcium in human sperm: possible role of perinuclear calcium. *Cell Calcium.* 1993;14:53–60.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. Progesterone and 17-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J Biol Chem.* 1990;265:1376–1380.

Blackmore PF, Eisoldt S. The neoglycoprotein mannose-bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa. *Mol Hum Reprod.* 1999;5:498–506.

Blackmore PF, Im WB, Bleasdale JE. The cell surface progesterone receptor which stimulates calcium influx in human sperm is unlike the

A-ring reduced steroid site on the $GABA_A$ receptor/chloride channel. *Mol Cell Endocrinol.* 1994;104:237–243.

Blackmore PF, Neulen J, Lattanzio FA, Beebe SJ. Cell surface receptors for progesterone mediate calcium uptake in human sperm. *J Biol Chem.* 1991;266:18655–18659.

Bootman MD, Collins TJ, Peppiatt CM, et al. Calcium signalling—an overview. *Semin Cell Dev Biol.* 2001;12:3–10.

Brandelli A, Miranda PV, Anon-Vazquez MG, Marin-Briggiler CI, Sanjurjo C, Gonzalez-Echeverria F, Blaquier JA, Tezon JG. A new predictive test for in-vitro fertilization based on the induction of sperm acrosome reaction by N-acetylglucosamine-neoglycoprotein. *Hum Reprod.* 1995;10:1751–1756.

Brandelli A, Miranda PV, Tezon JG. Voltage-dependent calcium channels and Gi regulatory protein mediate the human sperm acrosomal exocytosis induced by N-acetylglucosaminyl/mannosyl neoglycoproteins. *J Androl.* 1996;17:522–529.

Bray C, Brown JCK, Publicover SJ, Barratt CL. Progesterone interaction with sperm plasma membrane, calcium influx and induction of the acrosome reaction. *Rep Med Rev.* 1999;7:81–93.

Breitbart H, Spungin B. The biochemistry of the acrosome reaction. *Mol Hum Reprod.* 1997;3:195–202.

Brewis IA, Clayton R, Barratt CL, Hornby DP, Moore HD. Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. *Mol Hum Reprod.* 1996;2: 583–589.

Calogero AE, Burrello N, Ferrara E, Hall J, Fishel S, D'Agata R. Gamma-aminobutyric acid (GABA) A and B receptors mediate the stimulatory effects of GABA on the human sperm acrosome reaction: interaction with progesterone. *Fertil Steril.* 1999;71:930–936.

Carrell DT, Middleton RG, Peterson CM, Jones KP, Urry RL. Role of the cumulus in the selection of morphologically normal sperm and induction of the acrosome reaction during human in vitro fertilization. *Arch Androl.* 1993;31:133–137.

Chen C, Sathananthan AH. Early penetration of human sperm through the vestments of human eggs in vitro. *Arch Androl.* 1986;16:183–197.

Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod.* 1988;38:235–244.

Darszon A, Labarca P, Nishigaki T, Espinosa F. Ion channels in sperm physiology. *Phys Rev.* 1999;79:481–510.

De Jonge C. The cAMP-dependent kinase pathway and human sperm acrosomal exocytosis. *Front Biosci.* 1996;1:d234–d240.

De Jonge CJ, Barratt CLR, Radwanska E, Cooke ID. The acrosome reaction-inducing effect of human follicular and oviductal fluid. *J Androl.* 1993;14:359–365.

De Jonge CJ, Rawlins RG, Zaneveld LJ. Induction of the human sperm acrosome reaction by human oocytes. *Fertil Steril.* 1988;50:949–953.

Dragileva E, Rubinstein S, Breitbart H. Intracellular $Ca^{2+}$-$Mg^{2+}$-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. *Biol Reprod.* 1999;61:1226–1234.

Espinosa F, Lopez-Gonzalez I, Munoz-Garay C, Felix R, De la Vega-Beltran JL, Kopf GS, Visconti PE, Darszon A. Dual regulation of the T-type $Ca^{2+}$ current by serum albumin and beta-estradiol in mammalian spermatogenic cells. *FEBS Lett.* 2000;475:251–256.

Florman HM. Sequential focal and global elevations of sperm intracellular calcium are initiated by the zona pellucida during acrosomal exocytosis. *Dev Biol.* 1994;165:152–164.

Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CMB. A perspective on the control of mammalian fertilisation by egg-activated ion channels in sperm: a tale of two channels. *Biol Reprod.* 1998;59:12–16.

Foresta C, Rossato M, Di Virgilio F. Ion fluxes through the progesterone-activated channel of the sperm plasma membrane. *Biochem J.* 1993; 294:279–283.

Fukami K, Nakao K, Inoue T, et al. Requirement of phospholipase Cdelta4 for the zona pellucida-induced acrosome reaction. *Science.* 2001;292:920–923.

Garcia MA, Meizel S. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod.* 1999;60:102–109.

Goodwin LO, Leeds NB, Hurley I, Cooper GW, Pergolizzi RG, Benoff S. Alternative splicing of exons in the alpha1 subunit of the rat testis L-type voltage-dependent calcium channel generates line-specific dihydropyridine binding sites. *Mol Hum Reprod.* 1998;4:215–226.

Goodwin LO, Leeds NB, Hurley I, Mandel FS, Pergolizzi RG, Benoff S. Isolation and characterization of the primary structure of testis-specific L-type calcium channel: implications for contraception. *Mol Hum Reprod.* 1997;3:255–268.

Gregory L, Booth AD, Wells C, Walker SM. A study of the cumulus–corona cell complex in in-vitro fertilization and embryo transfer; a prognostic indicator of the failure of implantation. *Hum Reprod.* 1994; 9:1308–1317.

Hartshorne GM. Steroid production by the cumulus: relationship to fertilization in vitro. *Hum Reprod.* 1989;4:742–745.

Henkel R, Franken DR, Habenicht UF. Zona pellucida as physiological trigger for the induction of acrosome reaction. *Andrologia.* 1998;30: 275–280.

Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. *Nature.* 1999;397:259–263.

Hughes DC, Barratt CL. Identification of the true human orthologue of the mouse Zp1 gene: evidence for greater complexity in the mammalian zona pellucida? *Biochim Biophys Acta.* 1999;1447:303–306.

Jagannathan S, Barratt CLR, Publicover SJ. Characterisation of alpha 1H, a T-type calcium ion channel from human germ cells. *Eur J Neurosci.* 2000a;12(suppl):383.

Jagannathan S, Punt EL, Ivic A, Zamponi GW, Hamid J, Barratt CLR, Publicover SJ. Evidence for the expression of alpha 1G (T-type) voltage operated $Ca^{2+}$ channels in human male germ cells. *J Androl.* 2000b;108(suppl):61.

Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM. Trp2 regulates entry of $Ca^{2+}$ into mouse sperm triggered by egg ZP3. *Nat Cell Biol.* 2001;3:499–502.

Kirkman-Brown JC, Bray C, Stewart PM, Barratt CL, Publicover SJ. Biphasic elevation of $[Ca^{2+}]_i$ in individual human spermatozoa exposed to progesterone. *Dev Biol.* 2000;222:326–335.

Kiselyov K, Xu X, Mozhayeva G, Kuo T, Pessah I, Mignery G, Zhu X, Birnbaumer L, Muallem S. Functional interaction between $InsP_3$ receptors and store-operated hTrp3 channels. *Nature.* 1998;396:478–482.

Kobori H, Miyazaki S, Kuwabara Y. Characterization of intracellular $Ca^{2+}$ increase in response to progesterone and cyclic nucleotides in mouse spermatozoa. *Biol Reprod.* 2000;63:113–120.

Krausz C, Bonaccorsi L, Luconi M, Fuzzi B, Criscuoli L, Pellegrini S, Forti G, Baldi E. Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with in-vitro fertilization. *Hum Reprod.* 1995;10:120–124.

Krausz C, Bonaccorsi L, Maggio P, et al. Two functional assays of sperm responsiveness to progesterone and their predictive values in in vitro fertilisation. *Hum Reprod.* 1996;11:1661–1667.

Kuroda Y, Kaneko S, Yoshimura Y, Nozawa S, Mikoshiba K. Are there inositol 1,4,5-triphosphate (IP3) receptors in human sperm? *Life Sci.* 1999;65:135–143.

Laufer N, DeCherney AH, Haseltine FP, Behrman HR. Steroid secretion by the human egg–corona–cumulus complex in culture. *J Clin Endocrinol Metab.* 1984;58:1153–1157.

Lee MA, Storey BT. Endpoint of first stage of zona pellucida-induced acrosome reaction in mouse spermatozoa characterized by acrosomal

$H^+$ and $Ca^{2+}$ permeability: population and single cell kinetics. *Gamete Res.* 1989;24:303–326.

Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. *Fertil Steril.* 1987;48:649–658.

Lenz RW, Ball GD, Lohse JK, First NL, Ax RL. Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and in vitro fertilization. *Biol Reprod.* 1983;28:683–690.

Lievano A, Santi CM, Serrano CJ, Trevino CL, Bellve AR, Hernandez-Cruz A, Darszon A. T-type channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Lett.* 1996;388:150–154.

Liu DY, Baker HW. Relationship between the zona pellucida (ZP) and ionophore A23187-induced acrosome reaction and the ability of sperm to penetrate the ZP in men with normal sperm-ZP binding. *Fertil Steril.* 1996;66:312–315.

Llanos MN, Ronco AM, Aguirre MC, Meizel S. Hamster sperm glycine receptor: evidence for its presence and involvement in the acrosome reaction. *Mol Reprod Dev.* 2001;58:205–215.

Luconi M, Bonaccorsi L, Maggi M, Pecchioli P, Krausz C, Forti G, Baldi E. Identification and characterisation of functional nongenomic progesterone receptors on human sperm membrane. *J Clin Endocrinol Metab.* 1998;83:877–855.

Luconi M, Muratori M, Forti G, Baldi E. Identification and characterization of a novel functional estrogen receptor on human sperm membrane that interferes with progesterone effects. *J Clin Endocrinol Metab.* 1999;84:1670–1678.

Mattioli M, Barboni B. Signal transduction mechanism for LH in the cumulus–oocyte complex. *Mol Cell Endocrinol.* 2000;161:19–23.

Meizel S, Turner KO. Initiation of the human sperm acrosome reaction by thapsigargin. *J Exp Zool.* 1993;267:350–355.

Meizel S, Turner KO, Nuccitelli R. Progesterone triggers a wave of increased free calcium during the human sperm acrosome reaction. *Dev Biol.* 1997;182:67–75.

Melendrez CS, Meizel S. Studies of porcine and human sperm suggesting a role for a sperm glycine receptor/$Cl^-$ channel in the zona pellucida-initiated acrosome reaction. *Biol Reprod.* 1995;53:676–683.

Mendoza C, Tesarik J. Effect of follicular fluid on sperm movement characteristics. *Fertil Steril.* 1990;54:1135–1139.

Mendoza C, Tesarik J. A plasma-membrane progesterone receptor in human sperm is switched on by increasing intracellular free calcium. *FEBS Lett.* 1993;330:57–60.

Morales P, Pizarro E, Kong M, Kerr B, Ceric F, Vigil P. Gonadotropin-releasing hormone-stimulated sperm binding to the human zona is mediated by a calcium influx. *Biol Reprod.* 2000;63:635–642.

Morales P, Vigil P, Franken DR, Kaskar K, Coetzee K, Kruger TF. Sperm–oocyte interaction: studies on the kinetics of zona pellucida binding and acrosome reaction of human spermatozoa. *Andrologia.* 1994;26: 131–137.

Motta PM, Makabe S, Naguro T, Correr S. Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy. *Arch Histol Cytol.* 1994;57:369–394.

Motta PM, Nottola SA, Pereda J, Croxatto HB, Familiari G. Ultrastructure of human cumulus oophorus: a transmission electron microscopic study on oviductal oocytes and fertilized eggs. *Hum Reprod.* 1995; 10:2361–2367.

Myles DG, Primakoff P. Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg. *Biol Reprod.* 1997;56: 320–327.

Nagae T, Yanagimachi R, Srivastava PN, Yanagimachi H. Acrosome reaction in human spermatozoa. *Fertil Steril.* 1986;45:701–707.

Nottola SA, Macchiarelli G, Familiari G, Stallone T, Sathananthan AH, Motta PM. Egg–sperm interactions in humans: ultrastructural aspects. *Ital J Anat Embryol.* 1998;103(4 suppl 1):85–101.

Oehninger S, Blackmore P, Morshedi M, Sueldo C, Acosta AA, Alexander NJ. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. *Fertil Steril.* 1994a;61:349–354.

Oehninger S, Sueldo C, Lanzendorf S, Mahony M, Burkman LJ, Alexander NJ, Hodgen GD. A sequential analysis of the effect of progesterone on specific sperm functions crucial to fertilization in vitro in infertile patients. *Hum Reprod.* 1994b;9:1322–1327.

Osman RA, Andria ML, Jones AD, Meizel S. Steroid induced exocytosis: the human sperm acrosome reaction. *Biochem Biophys Res Commun.* 1989;160:828–833.

O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. $Ca^{2+}$ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol Biol Cell.* 2000;11: 1571–1584.

O'Toole CMB, Roldan ERS, Fraser LR. Role for $Ca^{2+}$ channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. *Mol Reprod Dev.* 1996a;45:204–211.

O'Toole CMB, Roldan ERS, Hampton P, Fraser LR. A role for diacylglycerol in human sperm acrosomal exocytosis. *Mol Hum Reprod.* 1996b;2:317–326.

Patrat C, Serres C, Jouannet P. Induction of a sodium ion influx by progesterone in human spermatozoa. *Biol Reprod.* 2000a;62:1380–1386.

Patrat, C, Serres S, Jouannet P. The acrosome reaction in human spermatozoa. *Biol Cell.* 2000b;92:255–266.

Pereda J, Coppo M. An electron microscopic study of sperm penetration into the human egg investments. *Anat Embryol (Berl).* 1985;173:247–252.

Plant A, McLaughlin EA, Ford WCL. Intracellular calcium measurements in individual human sperm demonstrate that the majority can respond to progesterone. *Fertil Steril.* 1995;64:1213–1215.

Publicover SJ, Barratt CLR. Voltage operated $Ca^{2+}$ channels and the acrosome reaction: which channels are present and what do they do? *Hum Reprod.* 1999;14:873–879.

Punt EL, Barratt CLR, Publicover SJ. Involvement of store operated channels in the sustained component of the progesterone-induced $[Ca^{2+}]_i$ signal in human spermatozoa. *J Androl Suppl.* May/June 2001:90.

Putney JW Jr. TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc Natl Acad Sci USA.* 1999;96:14669–14671.

Putney JW Jr, McKay RR. Capacitative calcium entry channels. *Bioessays.* 1999;21:38–46.

Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, Tilly JL, Clapham D. A sperm ion channel required for sperm motility and male fertility. *Nature.* 2001;413:603–609.

Rockwell PL, Storey BT. Kinetics of onset of mouse sperm acrosome reaction induced by solubilized zona pellucida: fluorimetric determination of loss of pH gradient between acrosomal lumen and medium monitored by dapoxyl (2-aminoethyl) sulfonamide and of intracellular $Ca^{2+}$ changes monitored by fluo-3. *Mol Reprod Dev.* 2000;55:335–349.

Roldan ER, Murase T, Shi QT. Exocytosis in spermatozoa in response to progesterone and zona-pellucida. *Science.* 1994;266:1578–1581.

Rossato M, Di Virgilio F, Rizzuto R, Galeazzi C, Foresta C. Intracellular calcium store depletion and acrosome reaction in human spermatozoa: role of calcium and plasma membrane potential. *Mol Hum Reprod.* 2001;7:119–128.

Sabeur K, Cherr GN, Yudin AI, Overstreet JW. Hyaluronic acid enhances induction of the acrosome reaction of human sperm through interaction with the PH-20 protein. *Zygote.* 1998;6:103–111.

Santi CM, Darszon A, Hernandez-Cruz A. A dihydropyridine-sensitive T-type $Ca^{2+}$ current is the man $Ca^{2+}$ current carrier in mouse primary spermatocytes. *Am J Physiol.* 1996;271:C1583–C1593.

Sato Y, Son JH, Tucker RP, Meizel S. The zona pellucida-initiated acrosome reaction: defect due to mutations in the sperm glycine receptor/$Cl^-$ channel. *Dev Biol.* 2000;227:211–218.

Schaefer M, Hofmann T, Schultz G, Gudermann T. A new prostaglandin E receptor mediates calcium influx and acrosome reaction in human spermatozoa. *Proc Natl Acad Sci USA.* 1998;95:3008–3013.

Serrano CJ, Trevino CL, Felix R, Darszon A. Voltage-dependent $Ca^{2+}$ channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. *FEBS Lett.* 1999;462:171–176.

Shi QX, Roldan ERS. Evidence that a $GABA_A$ like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. *Biol Reprod.* 1995;52:373–381.

Shirakawa H, Miyazaki S. Spatiotemporal characterization of intracellular $Ca^{2+}$ rise during the acrosome reaction of mammalian spermatozoa induced by zona pellucida. *Dev Biol.* 1999;208:70–78.

Snell WJ, White JM. The molecules of mammalian fertilisation. *Cell.* 1996;85:629–637.

Son WY, Lee JH, Lee JH, Han CT. Acrosome reaction of human spermatozoa is mainly mediated by 1H T-type calcium channels. *Mol Hum Reprod.* 2000;6:893–897.

Spungin B, Breitbart H. Calcium mobilization and influx during sperm exocytosis. *J Cell Sci.* 1996;109:1947–1955.

Sueldo CE, Oehninger S, Subias E, Mahony M, Alexander NJ, Burkman LJ, Acosta AA. Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. *Fertil Steril.* 1993;60: 137–140.

Talbot P. Sperm penetration through oocyte investments in mammals. *Am J Anat.* 1985;174:331–346.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev.* 2002;61:414–424.

Tesarik J, Carreras A, Mendoza C. Single cell analysis of tyrosine kinase dependent and independent $Ca^{2+}$ fluxes in progesterone induced acrosome reaction. *Mol Hum Reprod.* 1996;2:225–232.

Tesarik J, Mendoza Oltras C, Testart J. Effect of the human cumulus oophorus on movement characteristics of human capacitated spermatozoa. *J Reprod Fertil.* 1990;88:665–675.

Tesarik J, Moos J, Mendoza C. Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm. *Endocrinology.* 1993;133:328–335.

Tesarik J, Pilka L, Drahorad J, Cechova D, Veselsky L. The role of cumulus cell-secreted proteins in the development of human sperm fertilizing ability: implication in IVF. *Hum Reprod.* 1988;3:129–132.

Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase. *Proc Natl Acad Sci USA.* 1990;87:2466–2470.

Thomas P, Meizel S. Phosphatidylinositol-4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent on $Ca^{2+}$ influx. *Biochem J.* 1989;264:539–546.

Tosti E, Di Cosmo A, Cuomo A, Di Cristo C, Gragnaniello G. Progesterone induces activation in Octopus vulgaris spermatozoa. *Mol Reprod Dev.* 2001;59:97–105.

Tovey SC, Godfrey RE, Hughes PJ, Mezna M, Minchin SD, Mikoshiba K, Michelangeli F. Identification and characterization of inositol 1,4,5-trisphosphate receptors in rat testis. *Cell Calcium.* 1997;21:311–319.

Treiman M, Caspersen C, Christensen SB. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum $Ca^{2+}$-ATPases. *Tips.* 1998;19:131–135.

Tulsiani DR, Yoshida-Komiya H, Araki Y. Mammalian fertilization: a carbohydrate-mediated event. *Biol Reprod.* 1997;57:487–494.

Turner K, Meizel S. Progesterone-mediated efflux of cytosolic chloride during the human sperm acrosome reaction. *Biochem Biophys Res Commun.* 1995;212:774–780.

Turner KO, Syvanen M, Meizel S. The human acrosome reaction is highly sensitive to inhibition by cyclodiene insecticides. *J Androl.* 1997; 18:571–575.

Vannier B, Peyton M, Boulay G, Brown D, Qin N, Jiang M, Zhu X, Birnbaumer L. Mouse trp2, the homologue of the human trpc2 pseudogene, encodes mTrp2, a store depletion-activated capacitative $Ca^{2+}$ entry channel. *Proc Natl Acad Sci USA.* 1999;96:2060–2064.

Walensky LD, Snyder SH. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *J Cell Biol.* 1995;130:857–869.

Ward CR, Kopf GS. Molecular events mediating sperm activation. *Dev Biol.* 1993;158:9–34.

Wennemuth G, Westenbroek RE, Xu T, Hille B, Babcock DF. CaV2.2 and CaV2.3 (N- and R-type) $Ca^{2+}$ channels in depolarization-evoked entry of $Ca^{2+}$ into mouse sperm. *J Biol Chem.* 2000;275:21210–21217.

Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C. TRPC1, a human homolog of a Drosophila store-operated channel. *Proc Natl Acad Sci USA.* 1995;92:9652–9656.

Westenbroek RE, Babcock DF. Discrete regional distributions suggest diverse functional roles of calcium channel alpha1 subunits in sperm. *Dev Biol.* 1999;207:457–469.

Wistrom CA, Meizel S. Evidence suggesting involvement of a unique human sperm steroid receptor/$Cl^-$ channel complex in the progesterone-initiated acrosome reaction. *Dev Biol.* 1993;159:679–690.

Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. *Zygote.* 1994;2:371–372.

Zaneveld LJ, De Jonge CJ, Anderson RA, Mack SR. Human sperm capacitation and the acrosome reaction. *Hum Reprod.* 1991;6:1265–1274.

Zitt C, Zobel A, Obukhov AG, Harteneck C, Kalkbrenner F, Luckhoff A, Schultz G. Cloning and functional expression of a human $Ca^{2+}$-permeable cation channel activated by calcium store depletion. *Neuron.* 1996;16:1189–1196.