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OPINION

Voltage-operated Ca^{2+} channels and the acrosome reaction: which channels are present and what do they do?

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Evidence from pharmacological studies suggests that induction of the acrosome reaction of mammalian spermatozoa by solubilized zona pellucida, and possibly by progesterone, is dependent upon Ca^{2+} influx through voltage-operated Ca^{2+} channels. Studies on Ca^{2+} accumulation and membrane potential in ligand-stimulated or artificially depolarized spermatozoa support such a conclusion. Electrophysiological studies on rodent spermatogenic cells have revealed the presence of a 'T' type voltage-operated Ca^{2+} current. This current has pharmacological attributes consistent with those of the putative channel responsible for Ca^{2+} influx mediating the acrosome reaction. However, use of molecular techniques to study human and rodent testis and spermatogenic cells has detected the presence of three different voltage-operated Ca^{2+} channel subunits. One of these ($\alpha 1_E$) may generate T-currents, though this is currently disputed. Voltage-operated Ca^{2+} channel structure and the relationship between channel subunit expression and the characteristics of consequent Ca^{2+} currents is briefly reviewed. The nature and function of T-channel-mediated Ca^{2+} influx is examined in the context of the time-course of ligand- and depolarization-induced elevation of $[Ca^{2+}]_i$ in mammalian spermatozoa. It is likely that a secondary Ca^{2+} response (mobilization of stored Ca^{2+} or activation of a second Ca^{2+}-influx pathway) is required for the acrosome reaction. Evidence for the existence and participation of various candidates is discussed (including voltage-operated Ca^{2+} channels, which may be functionally expressed only in mature spermatozoa), the available evidence favouring a secondary Ca^{2+}-influx pathway. Immediate priorities for future research in this area are proposed.

Key words: acrosome reaction/calcium/ion channels/membrane potential/spermatozoa

The acrosome reaction and Ca^{2+} channels

The acrosome reaction (AR) of human spermatozoa, as in virtually all other forms of stimulus-activated exocytosis, is mediated by an elevation of $[Ca^{2+}]_i$. In various mammalian species, including man, this elevation of $[Ca^{2+}]_i$ and the consequent AR (induced by solubilized zona or progesterone) is greatly attenuated by reduction of $[Ca^{2+}]_o$ or by a non-specific blockade of Ca^{2+} channels by metal ions (Blackmore *et al.*, 1990; Florman *et al.*, 1992; Florman, 1994; Plant *et al.*, 1995; Aitken *et al.*, 1996). Furthermore, when mammalian spermatozoa are exposed to solubilized zona, both the $[Ca^{2+}]_i$ response and the AR are blocked by 1,4-dihydropyridines (DHPs), a class of drugs that are specific to voltage-operated Ca^{2+} channels (VOCCs; Florman *et al.*, 1992; Florman, 1994). DHPs can also block responses to progesterone and follicular fluid in both human and rodent spermatozoa (Blackmore *et al.*, 1990; McLaughlin and Ford, 1994; Shi and Roldan, 1995; O'Toole *et al.*, 1996), though the findings in this case are less consistent (see below). It has been reported that these drugs can cause reversible infertility of the human male *in vivo* (Hershlag *et al.*, 1995). The simplest interpretation of these data is that influx of Ca^{2+} through VOCCs, probably related to those of somatic cells, is necessary for elevation of $[Ca^{2+}]_i$ and, therefore, for successful AR (Benoff, 1998; Florman *et al.*, 1998).

Use of fluorescent dyes to monitor the membrane potential (E_m) of spermatozoa has confirmed that the mean E_m of bovine and rat spermatozoa depolarizes, from around –60mV to between –25 and –20 mV, upon exposure to solubilized zona (Arnoult *et al.*, 1996b). This depolarization is sufficient to activate VOCCs. It also appears that hyperpolarization of the spermatozoon E_m may be a necessary component of capacitation, releasing VOCCs from inactivation (Zeng *et al.*, 1995).

Thorough characterization of human sperm VOCCs is essential to a full understanding of the processes required for human fertilization and may also allow development of novel contraceptives. During the last few years, considerable progress has been made in elucidating the nature of the sperm VOCC(s). In particular, it has been shown that there is a T type channel (see below) present in the spermatogenic cells and probably in the spermatozoa of rodents (Florman *et al.*, 1998). However, the findings from different techniques, from different laboratories and from studies on different species are difficult to harmonize. It is arguable that our understanding of this field, particularly in humans, is far from complete. The purpose of this manuscript is to summarize and briefly review the available evidence and to suggest potentially fruitful areas for future work.

Voltage operated Ca^{2+} channels

Using both electrophysiological and molecular techniques, VOCCs have been described and characterized in a wide variety of somatic cells. These currents can be classified into at least six types (T, L, N, P, Q, R), based upon their biophysical characteristics and sensitivity to a range of drugs and toxins (Birnbaumer *et al.*, 1994; Dunlap *et al.*, 1995). All except one (T-type) channel require large depolarizations (to voltages ⩾–30 mV) to cause opening and are, therefore, referred to as high voltage activated (HVA) channels. The T-type typically activates at voltages ⩾–60 mV (Figure 1b) and is referred to as low voltage activated (LVA). Molecular studies have shown that all these VOCC types are members of the same protein family and are structurally similar, the main component being an α1 (pore-forming) protein subunit. This protein contains four homologous domains (repeats I–IV), each composed of six transmembrane alpha helical segments (S1–S6) interspersed by linkers (Figure 1a). Eight of these helices (S5, S6 and the 5–6 linker from each repeat) are believed to surround the channel pore (McClesky, 1994). Helices IIIS5, IIIS6 and IVS6 form the primary DHP binding region of L-type, HVA channels (Grabner *et al.*, 1996; Sinnegger *et al.*, 1997; Striessnig *et al.*, 1998). Expression studies suggest that the α_1 subunit may be able to form a functional channel on its own, but is usually associated with (and modulated by) β and α_2-δ auxiliary subunits. Ten different homologous α1 gene products ($\alpha1_{A/B/C/D/E/F/G/H/I/S}$) and a range of splice variants have been described. This molecular diversity of the α_1 subunit is believed to be the primary cause of the observed biophysical and pharmacological variation among voltage operated Ca^{2+} currents. However, an important role for the auxiliary subunits is also recognized, both in significantly modulating functional characteristics of the channels and also in regulating expression of α1 subunits in the plasmalemma (Birnbaumer *et al.*, 1998; Trimmer, 1998).

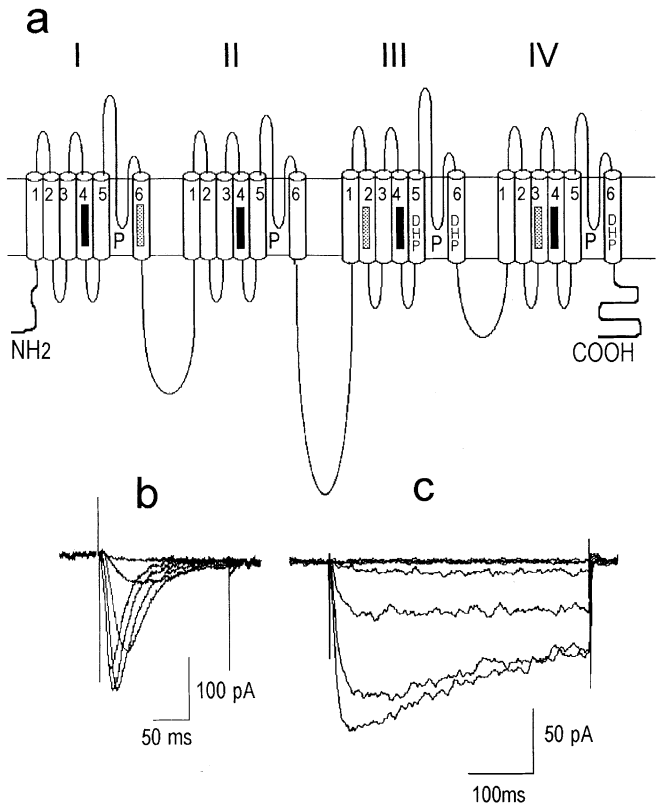

Figure 1. (**a**) Diagrammatic illustration of the structure of a generalized α1 subunit (modified from Grabner *et al.*, 1996) The structure is composed of four homologous domains (I–IV) each composed of six α helical segments (1–6) with linkers which project alternately outside and inside the membrane. The linker between segments 5 and 6 in each domain includes an intramembrane loop. These loops (P) are believed to line the aqueous pore. Segments which are believed to act as the primary voltage sensors are marked by black bars, those that form the primary 1,4 dihydropyridine binding site are marked DHP and those that at which splice variants are seen in testicular $\alpha1_C$ are marked by stippled bars. (**b**) Family of superimposed T currents activated by voltage pulses from –90 mV to –60, –50, –40, –30, –20, and –10 mV. Note the characteristic 'crossing' of the traces (see text) and the rapid inactivation of these currents. (**c**) Family of superimposed L currents activated by voltage steps from –90 mV to –50, –40, –30, –20, –10, 0 and 10 mV. Note the more positive activation voltages and the relatively slow inactivation compared to T currents. These currents were recorded from an osteoblast in primary culture and are probably carried by a mixture of $\alpha1_C$ and $\alpha1_D$, both of which are expressed in these cells (M.R.Gu, A.J.Preston, Y.El Haj, and S.J.Publicover, unpublished).

Characterization of voltage operated Ca^{2+} channels in male germ cells

Monitoring of $[Ca^{2+}]_i$ and use of drugs

Various methods have been employed for detection and characterization of sperm VOCCs. Use of fluorescence techniques for monitoring $[Ca^{2+}]_i$ in mammalian spermatozoa has shown that induction of AR by solubilized zona or by alkaline, depolarizing media is associated with elevation of $[Ca^{2+}]_i$ and that activation of VOCCs is a necessary step in this response (Florman *et al.*, 1992; Linares-Hernandez *et al.*, 1998). Imaging of individual, zona-stimulated cells shows that an initial focal influx through a non-specific cation channel precedes (and probably induces) a larger global elevation of $[Ca^{2+}]_i$ (Florman, 1994; Arnoult *et al.*, 1996a,b). Blockade of VOCCs causes failure of the global (but not the focal) $[Ca^{2+}]_i$ response to zona and consequent failure of the AR (Florman, 1994; Arnoult *et al.*, 1996a,b).

Similarly, activation of AR by progesterone may be VOCC dependent and it has been suggested that Cl^- efflux via a receptor/chloride channel, similar to the neuronal $GABA_A$ receptor, may provide the necessary depolarization (Turner and Meizel, 1995; Meizel *et al.*, 1997). However, the effects of DHPs and verapamil (a phenylalkylamine VOCC blocker) on responses to progesterone are not consistent. Different laboratories report that the $[Ca^{2+}]_i$ transient and/or AR which occurs upon application of progesterone or follicular fluid to mouse and human spermatozoa is sensitive (Blackmore *et al.*, 1990; McLaughlin and Ford, 1994; Shi and Roldan, 1995; O'Toole *et al.*, 1996) or insensitive (Thomas and Meizel, 1989; Foresta *et al.*, 1993; Aitken *et al.*, 1996) to blockade of VOCCs.

Linares-Hernandez *et al.* (1998) have recently undertaken a detailed study of the depolarization-induced elevation of $[Ca^{2+}]_i$ in non-capacitated human spermatozoa. Valinomycin

was used to increase membrane K^+ permeability, thus 'clamping' E_m at the K^+ equilibrium potential (E_K), which was then controlled by adjusting medium K^+ concentration. Differences in the responses of human spermatozoa, in comparison with rodent and bovine cells, included the ability of depolarization to induce Ca^{2+}-influx without alkalinization (though alkalinization enhanced the effect) and an insensitivity to the DHP, nifedipine. The T channel blocker Ni^{2+} inhibited the depolarization-induced elevation of $[Ca^{2+}]_i$, but required very high (non-selective) concentrations to achieve a significant effect (see below).

Electrophysiological investigations

Ca^{2+}-sensitive fluorescent dyes monitor the concentration of Ca^{2+} in the cytosol, not transmembrane Ca^{2+} fluxes, and thus provide only indirect information on the nature of Ca^{2+} channel(s). However, direct electrophysiological recording from spermatozoa is currently impracticable due to the combination of shape and minute size. Some brief, cell-attached patch (single channel) records have been reported (e.g. Espinosa *et al.*, 1998), which have established that sperm membranes possess a range of anion and cation channels, similar to somatic cells, but these studies have provided little information on VOCCs of spermatozoa.

Reconstitution of purified sperm membrane proteins into artificial membranes has been used extensively and has occasionally revealed the presence of Ca^{2+} channels. A DHP-modulated channel has been described from boar spermatozoa, which shows very little voltage sensitivity of activation or inactivation (Tiwari-Woodruff and Cox, 1995). Purified membranes from mouse spermatozoa include a Ca^{2+} conducting channel of high, multistate conductance and low Ca^{2+} selectivity (Beltran *et al.*, 1994). These channels may play a role in the control of the AR but neither has the characteristics of the currents seen in spermatogenic cells (see below).

Electrophysiological studies of male germ cell VOCCs, employing the more informative 'whole cell' technique, have been restricted to 'spermatogenic cells'. Mature spermatozoa are not transcriptionally active so it is possible that spermatogenic cells express the same channels as mature spermatozoa (Arnoult *et al.*, 1996a; Lievano *et al.*, 1996), though insertion into the sperm membrane of previously transcribed proteins may occur. Records obtained from spermatogenic cells of rat and mouse show a short-lived (fast inactivating), LVA current similar to the T-type VOCC of somatic cells (Hagiwara *et al.*, 1984; Arnoult *et al.*, 1996a; Santi *et al.*, 1996). The channel shows a sensitivity to DHPs which is sufficient to account for the effect of these compounds on $[Ca^{2+}]_i$ responses and on AR. Furthermore, it has a pharmacology (relative potency of various blockers in inhibiting currents) similar to that of the putative AR-inducing channel (Arnoult *et al.*, 1996a; Santi *et al.*, 1996; Florman *et al.*, 1998). Arnoult *et al.* (1997) showed that the current facilitates in response to depolarising prepulses, the facilitated current being enlarged but having similar kinetics to control currents. This effect is tyrosine phosphatase dependent (Arnoult *et al.*, 1997), so activation of tyrosine kinase associated with capacitation and/or activation of the spermatozoa (Ward and Kopf, 1993; Visconti and Kopf, 1998) may suppress facilitation. No other types of VOCC current have been seen in these cells.

Molecular investigations

Use of reverse transcription–polymerase chain reaction (RT–PCR) to study expression of VOCC α1 subunits in male germ cells has produced evidence for the presence of three different VOCC α1 subunits. Studies by Darszon and colleagues on rat spermatogenic cells have detected primarily RNA coding for the $\alpha1_E$ subunit (Lievano *et al.*, 1996). $\alpha1_A$ was also detected, though at a lower level (Lievano *et al.*, 1996).

Benoff and colleagues report detection of message for $\alpha1_C$ in rat and human testis (Goodwin *et al.*, 1997; Benoff, 1998; Goodwin *et al.*, 1998a,b,c). In-situ RT–PCR for $\alpha1_C$ was carried out on frozen sections from rat testis and PCR product was present at all stages of germ cell differentiation (Goodwin *et al.*, 1998b). The laboratory has recently reported that they have successfully carried out RT–PCR on RNA from motile, mature human spermatozoa and were able to detect mRNA for $\alpha1_C$ (Goodwin *et al.*, 1998a). The testicular $\alpha1_C$ is truncated at the 5′ end and sequencing has revealed splice variants in regions IS6, IIIS2 and IVS3 of the molecule (Goodwin *et al.*, 1997; Benoff, 1998; Goodwin *et al.*, 1998a,b,c; Figure 1a), which may be functionally significant (Benoff, 1998). Region IS6 is known to contribute significantly to determination of the kinetics of inactivation in channels expressed in *Xenopus* oocytes (Zhang *et al.*, 1994), though other features of the channel (particularly the type of β subunit co-expressed) are very important in this respect (Birnbaumer *et al.*, 1998). Alternative splicing in segment IIIS2 of $\alpha1_C$ may affect the voltage-dependence of channel block by DHPs. Human $\alpha1_C$ has two splice variants in this region (Soldatov *et al.*, 1995). The variant which is expressed in the testis has been shown to have higher sensitivity to DHPs when cells are held at –90 mV, though at –40 mV the two splice variants showed similar sensitivity (Soldatov *et al.*, 1995). The main DHP-biding regions (IIIS5, IIIS6 and IVS6; Grabner *et al.*, 1996; Sinnegger *et al.*, 1997; Striessnig *et al.*, 1998) and the voltage sensors (S4 in each repeat; McClesky, 1994) do not seem to be subject to alternative splicing in testicular $\alpha1_C$ (Figure 1a).

$\alpha1_G$, a subunit believed to generate T-currents in neurons, is not expressed at detectable levels in rat testis (Perez-Reyes *et al.*, 1998a). Another possible T channel, $\alpha1_H$, has also been described and is expressed primarily in heart (Cribbs *et al.*, 1998; Perez-Reyes *et al.*, 1998b), but information on its presence/absence in male germ cells is not yet available.

Which α1 subunit generates the T-current of spermatogenic cells?

The data from electrophysiological and molecular studies on the VOCCs of male germ cells are not easily harmonized. It is generally accepted that $\alpha1_A$ codes for the P/Q channel normally found in brain (Birnbaumer *et al.*, 1994), an HVA current. It is most unlikely that this subunit is responsible for the LVA current of germ cells. The situation with regard to α_{1E} is more complex. This subunit was initially considered to be an LVA channel (Soong *et al.*, 1993), but subsequent investigations cast doubt upon this classification. The consensus

is now that it probably codes for the R type HVA channel, but the identity of $\alpha1_E$ is still open to question since different conclusions have been reached depending on the nature of the characteristics assessed (Williams *et al.*, 1994; Bourinet *et al.*, 1996; Randall and Tsien, 1997). A feature of T currents which has been proposed as diagnostic is that current families (currents induced by a series of incremented voltage steps), when superimposed, cross over (Randall and Tsien; 1997; Figure 1b). Currents carried by expressed recombinant $\alpha1_E$ do not show crossing over (Randall and Tsien, 1997) but currents seen in rodent spermatogenic cells do (Arnoult *et al.*, 1996a; Santi *et al.*, 1996).

Expression of $\alpha1_C$ subunit normally produces HVA currents, of the L-type, which inactivate relatively slowly (Figure 1c). Such currents were not detected in spermatogenic cells of rodents (see above). It has been argued that alternative splicing in the testis specific form of $\alpha1_C$ could cause changes in both voltage dependence and kinetics of the channel such that, though the currents in spermatogenic cells are generated by testicular $\alpha1_C$, they have been wrongly identified as T type (Benoff, 1998). There is no precedent for such a fundamental transformation of all biophysical characteristics in a splice variant. However, it is impossible to assess the impact of the alternative splicing in the testis-specific channel until it is expressed, with an appropriate β subunit, in oocytes or a cell line, allowing the application of electrophysiological techniques.

A further complication in interpretation of the molecular data has been introduced by the recent findings of Meir and Dolphin (1998) that several α1 subunits ($\alpha1_B$, $\alpha1_C$ and $\alpha1_E$) can generate T-like single channel currents upon expression in a VOCC-null (COS-7) cell line. The HVA channels observed in previous studies on expression of these subunits were also present, though when $\alpha1_B$ was expressed without any auxiliary subunits only the T-like channels were observed (Meir and Dolphin, 1998). There are, as yet, no reports of significant whole cell currents being generated in this way.

Thus, it is conceivable that the testicular $\alpha1_E$ and/or $\alpha1_C$ are responsible for the spermatocyte T type current. However, the weight of available evidence does not support such a conclusion and it appears probable that at least the testicular $\alpha1_C$ (and possibly the $\alpha1_E$) is separate from the subunit responsible for the observed T currents. If so then (i) male germ cells also express α1 subunits for LVA channels, such as $\alpha1_H$ or $\alpha1_I$ (Cribbs *et al.*, 1998; Perez-Reyes *et al.*, 1998b) and (ii) currents carried by the subunits $\alpha1_A$, $_C$ and $_E$ are not detected by electrophysiological study of spermatogenic cells, possibly being available for activation only in mature spermatozoa (see below)

Is the T channel the primary Ca^{2+} influx pathway for AR?

Electrophysiological data (from rodent spermatogenic cells) and data from experiments on the effects of VOCC blockers on AR strongly suggest that spermatozoa posses a DHP-sensitive T channel that must be activated for successful induction of AR. Ca^{2+} flux through T channels can perform a second messenger function, such as in induction of steroidogenesis in adrenal cortex (Enyeart *et al.*, 1993; Rossier *et al.*, 1996), but this does not appear to be typical. Rather than providing a major pathway for Ca^{2+} influx, T currents frequently act to modulate E_m, supporting pacemaking, rhythmic or oscillatory activity (Carbonne and Swandulla, 1990; Huguenard, 1996) and may provide a depolarizing current sufficient to recruit Ca^{2+} entry through HVA channels. Analysis of the currents in spermatogenic cells has shown some overlap between the voltage dependencies of activation and inactivation (i.e. some activation can occur at voltages at which steady-state inactivation is not complete; Santi *et al.*, 1996) and it is, therefore, theoretically possible for these channels to support sustained Ca^{2+} influx at suitable membrane potentials (–65 to –45 mV). However, even at the centre of this range (–55 mV) the current is likely to be only ~2.5% of the possible maximum. Furthermore, upon exposure to solubilized zona, E_m is not held in this range but depolarizes to more positive values within a minute (Arnoult *et al.*, 1996b). T channel currents inactivate rapidly at voltages positive to –60 mV (Carbonne and Swandulla, 1990) and the currents carried by these channels, therefore, normally last $<$100 ms when induced by step depolarizations (Figure 1). The ramp depolarization of E_m that occurs in response to solubilized zona (Arnoult *et al.*, 1996b) may permit T channels to remain open for longer, but the consequent current would still be (probably) small and of no more than a few seconds in duration. In contrast, the AR-linked $[Ca^{2+}]_i$ response in bovine spermatozoa persists for several minutes before AR (Florman, 1994; Arnoult *et al.*, 1996b).

To support such a substantial and sustained $[Ca^{2+}]_i$ response either repetitive activation of T currents (repetitive Ca^{2+} spiking) or a secondary event (mobilization of intracellular stores or activation of a second Ca^{2+} influx pathway), subsequent to T-current activation, must occur.

Repetitive Ca^{2+} spiking?

T currents of neurons can support Ca^{2+} action potentials, (known as low threshold spikes; LTSs) which are of lower amplitude but longer duration than normal action potentials (amplitude of 15–25 mV and duration of 20–150 ms). LTSs are normally induced by 'rebound' following transient hyperpolarization of E_m and consequent release of T channels from inactivation (Huguenard, 1996). When expressed in combination with other conductances which can cause a post-LTS hyperpolarization the cycle can repeat causing oscillation of E_m and consequent repeated activation of T currents (Huguenard, 1996). Though such activity could conceivably generate a prolonged (possibly oscillating) Ca^{2+} signal in spermatozoa induced to undergo AR, there is currently no evidence to this effect.

Is there a Ca^{2+} store in spermatozoa?

There is evidence that at least one Ca^{2+} store exists within mature rodent and bovine spermatozoa, probably in the acrosome (Walensky and Snyder, 1995; Spungin and Breitbart, 1996). Whether this store is mobilized by inositol trisphosphate (IP_3) is disputed (Walensky and Snyder, 1995; Spungin and Breitbart, 1996). Thapsigargin, which selectively releases Ca^{2+}

from intracellular stores of somatic cells (Thastrup *et al.*, 1990), elevates $[Ca^{2+}]_i$ of capacitated or uncapacitated human and non-human spermatozoa and initiates AR. However, these effects are observed only in the presence of extracellular Ca^{2+}, responses being suppressed in 'Ca^{2+}-free media' (Blackmore, 1993; Meizel and Turner, 1993; Spungin and Breitbart, 1996). Similar results are reported with 2,5-di(*tert*-butyl)hydroquinone (Perry *et al.*, 1997). The significance of a Ca^{2+} store in the events which follow zona binding and result in AR should, therefore, remain open to question.

Is there a second Ca^{2+} influx pathway?

Since three different α1 subunits have already been detected in spermatogenic cells (see above), it is likely that at least one HVA channel is present in these cells and could provide a route for a secondary, prolonged Ca^{2+} influx, or allow a major influx leading to mobilization of other sources of Ca^{2+}. The novel splice variants of $\alpha1_C$ detected by Benoff and colleagues (Goodwin *et al.*, 1997; Benoff, 1998; Goodwin *et al.*, 1998a,b,c) are possible candidates, though it is important to know the activation and inactivation characteristics of expressed channels before their potential contribution to sustained Ca^{2+} influx can be assessed. The $\alpha1_A$ detected at low levels by Lievano *et al.* (1996) may also be significant, particularly since the P/Q channels which are believed to reflect expression of this subunit do not show complete inactivation even at 0 mV and could, therefore, support sustained influx of Ca^{2+} over a wide voltage range (Regan, 1991).

Since electrophysiological studies of spermatogenic cells have detected no HVA currents, it is likely that these channels, if present, are non-functional at this stage (see above). However, such channels could subsequently become inserted into the plasma membrane, or become functional through the activity of second messengers or addition of necessary auxiliary subunits. Plasma membrane vesicles from bovine spermatozoa possess a Ca^{2+} channel that is sensitive to DHPs and becomes functional only after stimulation of protein kinase C (Spungin and Breitbart, 1996). Dolphin and colleagues have recently demonstrated that auxiliary subunits may be able to reveal hidden currents. Undifferentiated NG108–15 neuroblastoma/glioma hybrid cells express $\alpha1_{A,\ B,\ C,\ D}$ and $_E$ proteins ($\alpha1_G$ and $_H$ were not tested) and low levels of auxiliary (α2-δ, and β) subunits, but electrophysiological recording detects almost exclusively LVA currents. However, after over-expression of auxiliary subunits by transfection of cDNAs an additional sustained, HVA component to the currents is seen (Wyatt *et al.*, 1998).

The data of Linares-Hernandez *et al.* (1998) on K^+ (depolarization)-induced $[Ca^{2+}]_i$ responses in non-capacitated human spermatozoa are consistent with the existence of both a T channel-like and a slowly-inactivating, voltage activated Ca^{2+} influx pathway in these cells. An early component of the response to depolarization was blocked by 50 μM Ni^{2+} but very high doses (600 μM) were required to achieve a lasting inhibitory effect. Doses for 50% block of T-channel currents, including those of mouse spermatogenic cells, are typically around 10–50 μM, HVA currents being significantly blocked at 200–300 μM Ni^{2+} (Fox *et al.*, 1987; Narahashi *et al.*, 1987; Gu and Spitzer, 1993; Arnoult *et al.*, 1998). The results of experiments on the persistence of the voltage-activated Ca^{2+} influx pathway are particularly striking. When cells were depolarized to –17 mV (mean for population) in a low Ca^{2+} medium (no added Ca^{2+}, 0.5 mM EGTA) for up to 90 s, no elevation of $[Ca^{2+}]_i$ occurred. However, subsequent addition of extracellular Ca^{2+} caused an immediate rise in $[Ca^{2+}]_i$, indicating that an influx pathway was available (Linares-Hernandez *et al.*, 1998). T channels should inactivate completely within 1 s at this value of E_m and a separate voltage activated influx pathway must, therefore, exist in human spermatozoa. The $[Ca^{2+}]_i$ response induced by depolarization of these cells was not inhibited by the DHP nifedipine.

Another possible (non-VOCC) Ca^{2+} influx route is capacitative Ca^{2+} entry. This influx pathway is not voltage activated, but is induced upon emptying of intracellular stores, possibly due to release from the depleted store of a 'Ca^{2+} influx factor', which stimulates a Ca^{2+} release activated current (I_{CRAC}; Fasolato *et al.*, 1994). I_{CRAC} is an important route for prolonged Ca^{2+} influx in somatic cells. Activation of such a pathway as a secondary response after initial VOCC activation is a possibility. It is also consistent with the ability of thapsigargin to elevate $[Ca^{2+}]_i$ only in the presence of extracellular Ca^{2+} (see above), assuming that the Ca^{2+} store in spermatozoa is too small to cause significant elevation of $[Ca^{2+}]_i$. There is currently no other evidence regarding capacitative Ca^{2+} entry in spermatozoa.

Could the T channel have a function in spermatogenesis?

Since it has only been possible to obtain direct records of T currents from differentiating (spermatogenic) cells, it is pertinent to ask whether these currents could have a role in spermatogenesis (Santi *et al.*, 1996). There is evidence that T currents are involved in generating periodic Ca^{2+} transients in immature neuronal cells, which are necessary for differentiation (Gu and Spitzer, 1993; Gu *et al.*, 1994). In both excitable and non-excitable cells, T currents tend to be most strongly expressed early during the process of differentiation (HVA currents subsequently becoming prevalent) and their expression is enhanced upon artificial induction of differentiation (Gottmann *et al.*, 1988; Bickmeyer *et al.*, 1993; Gu and Spitzer, 1993; Publicover *et al.*, 1994; Preston *et al.*, 1996). Both observations are consistent with a role for T currents in this process. The T-current of rodent spermatogenic cells shows voltage dependent facilitation, a process that is tyrosine phosphatase dependent (Arnoult *et al.*, 1997; see above). A similar voltage-induced enhancement of T currents has been observed in rat bone marrow stromal cells (Publicover *et al.*, 1995). This facilitation is greatly reduced in mature osteoblasts (M.R. Preston *et al.*, unpublished data) again consistent with a function in differentiation. It is, therefore, conceivable that the T currents which have been observed in spermatogenic cells play a role in the differentiation of male germ cells, though this does not preclude a further role in signalling in the mature spermatozoon.

Conclusions

It is clear that, despite considerable progress, a number of questions regarding the VOCCs of human spermatozoa and their role in the AR are still to be answered. Though evidence for the necessary participation of a T current in activation of the AR is strong, it appears likely that at least one further Ca^{2+} influx mechanism is involved. Three different VOCC α1 subunits, which probably form non-T-type channels, have been detected in male germ cells and these must be candidates for secondary influx pathways. The characteristics of these VOCCs should be established. Intracellular Ca^{2+} stores and I_{CRAC} are possible contributors to Ca^{2+} signalling during AR and merit further investigation. It is also crucial to establish whether the observations made on animal (particularly rodent) spermatozoa and the models based upon them hold good for the human. Studies that could be undertaken in the near future include: (i) recording of Ca^{2+} currents from human spermatogenic cells; (ii) cloning and expression of animal and human testis/sperm VOCCs. Expression of the human channel(s) in a system in which they can be analysed will allow both characterization and assessment of the importance of co-expression of auxiliary subunits; and (iii) use of preparations of permeabilized spermatozoa to determine the nature, capacity and regulation of Ca^{2+} stores.

Though not yet possible, the application of the whole cell patch technique to spermatozoa (allowing thorough assessment of ionic currents and their regulation in individual cells stimulated to undergo AR) may ultimately be required in order to answer many of the questions which have been raised here.

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