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Review

Voltage-operated calcium channels in male germ cells

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The acrosome reaction is a key event in fertilization. Current models for induction of the acrosome reaction incorporate a necessary influx of Ca^{2+}, which is mediated by agonist-induced gating of ion channels in the sperm plasma membrane. The difficulty of applying electrophysiological techniques to spermatozoa has severely hampered studies on the expression of functional ion channels in these cells. However, during the last few years, a combination of molecular and physiological techniques (applied to immature spermatogenic cells) has elucidated both the expression of Ca^{2+} channels in male germ cells and their role in induction of the acrosome reaction. It now appears that a range of voltage-operated Ca^{2+} channels, similar to those that occur in somatic cells, is expressed in spermatozoa. Male rodent germ cells express a low-voltage activated (T-type) channel that is regulated by membrane potential and provides the primary Ca^{2+} influx mechanism in zona pellucida-stimulated spermatozoa. In human spermatozoa, similar channels are apparently expressed, but their function in induction of the acrosome reaction has yet to be established. A range of other, high voltage-activated channels also appear to be present in rodent and human spermatozoa, but their roles are not yet known. In this review, the structure and characteristics of voltage-operated Ca^{2+} channels are outlined and the evidence for their expression and function in male germ cells is assembled and discussed.

Sexual reproduction in mammals is achieved by the fusion of a spermatozoon with the oocyte. The spermatozoon must penetrate the egg vestments and bind to the oolemma before gamete fusion can occur. A pivotal event in this process is the acrosome reaction wherein the acrosome, a secretory vesicle in the apical region of the spermatozoon, fuses with the overlying plasma membrane. This fusion results in secretion of the acrosomal contents and also incorporation of the inner acrosomal membrane into the plasmalemma. These processes are believed to be important for penetration of the zona pellucida and sperm–oocyte fusion (Ward and Kopf, 1993).

Agonist-induced Ca^{2+} signalling in spermatozoa and male infertility

Acrosome reaction is a secretory event triggered as the spermatozoon approaches the egg (Yanagimachi, 1994). Although a number of intracellular messenger systems have been implicated and the biochemistry of the acrosome reaction is complex (Ward and Kopf, 1993; Breitbart and Spungin, 1997), it appears that gating of Ca^{2+} channels and consequent Ca^{2+} influx plays a central role. In several mammals, a sustained increase in $[Ca^{2+}]_i$, dependent upon influx of extracellular Ca^{2+}, is induced directly by solubilized zona pellucida. The initial phase of zona pellucida-induced Ca^{2+} influx appears to require activation of voltage-operated calcium channels (VOCCs). Organic and inorganic antagonists of VOCCs, including 1,4-dihydropyridines (DHPs), a class of drugs specific for these channels, inhibit both the zona pellucida-induced Ca^{2+} signal and the consequent acrosome reaction (Florman *et al.*, 1998; Darszon *et al.*, 1999; Publicover and Barratt, 1999). Progesterone is the only other well-characterized agonist of the acrosome reaction. In a similar manner to the zona pellucida, progesterone causes a rapid and transient increase in $[Ca^{2+}]_i$, accompanied by depolarization, followed by a sustained $[Ca^{2+}]_i$ response. Progesterone-induced acrosome reaction is also blocked by DHPs, but the role of VOCCs in this process is disputed (Publicover and Barratt, 1999).

Although sperm dysfunction is the single most common cause of human infertility (Hull *et al.*, 1985; Irvine, 1998), very little is known about the cellular, molecular or genetic causes of this pathology. Defective acrosome reaction and, by implication, $[Ca^{2+}]_i$ signalling is a common feature of male factor infertility (Barratt and Publicover, 2001). For example, Liu *et al.* (2001) calculated that approximately 25% of infertile men with normal semen parameters show disordered zona pellucida-induced acrosome reaction

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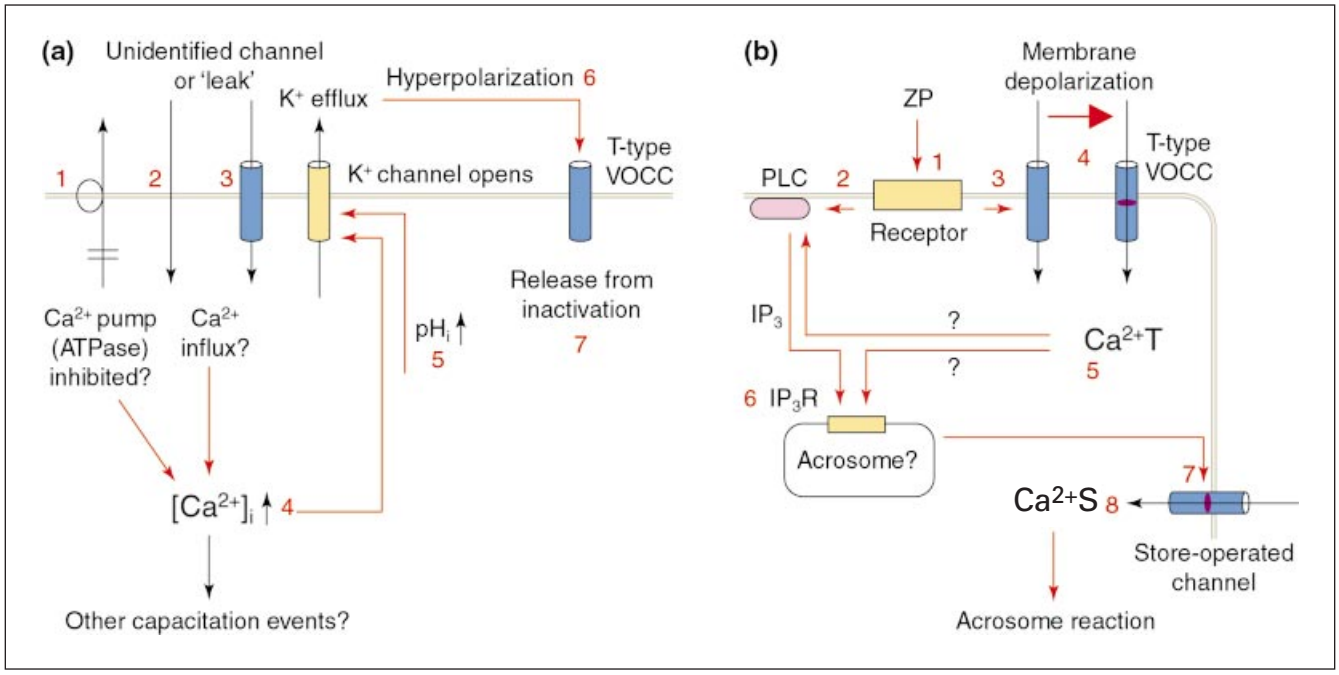

Fig. 1. Regulation and activation of voltage-operated calcium channels (VOCCs) during capacitation and induction of the acrosome reaction by zona pellucida in mammalian spermatozoa. Red arrows show causative links between components of the pathways. Red numbers refer to specific components of the pathways explained below. Blue cylinders in the membrane represent Ca^{2+} channels or cation channels carrying inward ion flux. Yellow cylinders represent K^+ channels. Yellow boxes represent receptor molecules. The pink box represents phospholipase C (PLC). (a) Possible regulation of the T-type voltage-operated Ca^{2+} channels of mammalian spermatozoa by membrane potential. During capacitation, one or more of the following: (1) reduced Ca^{2+} efflux due to inhibition of the Ca^{2+} ATPase pump; (2) increased leakage of Ca^{2+} across the bilayer due to instability caused by removal of cholesterol; and (3) increased Ca^{2+} influx due to the opening of an unidentified channel, result in increased $[Ca^{2+}]_i$ (4). Cytoplasmic alkalinization (5) is also known to occur during capacitation. Either or both $[Ca^{2+}]_i$ and pH_i may regulate one or more types of K^+ channel in the sperm plasma membrane, causing hyperpolarization (6) of the membrane potential. This hyperpolarization releases the T-type voltage-operated Ca^{2+} channels from inactivation (7) such that they are competent to respond to a stimulus provided by zona pellucida. (b) Probable participation of T-type VOCCs and store-operated Ca^{2+} channels in induction of the acrosome reaction by zona pellucida. Binding of zona pellucida (ZP) to its receptor (1) activates both PLC (2) and an unidentified cation channel (3). Opening of the cation channel enables cation influx, causing depolarization (4) of the plasma membrane, resulting in activation of T-type voltage-operated Ca^{2+} channels. The resulting brief Ca^{2+} influx generates a transient increase in $[Ca^{2+}]_i$ (Ca^{2+}T; 5). Inositol trisphosphate (IP_3), generated by the activated phospholipase C, binds IP_3 receptors, probably on the acrosome (6), causing release of stored Ca^{2+}. Upon emptying of the store, store-operated channels in the plasma membrane open (7), allowing influx of Ca^{2+} and causing a sustained increase in $[Ca^{2+}]_i$ (Ca^{2+}S; 8), which leads to the acrosome reaction. Ca^{2+}S is never generated unless Ca^{2+}T occurs first, indicating that the Ca^{2+} that enters through the T-type VOCCs plays a part in events leading to opening of the store-operated channels. Possible sites of action are at the activation of PLC or at the activation of the IP_3 receptor (both labelled with a question mark).

(characterized by normal zona pellucida binding but failure to undergo acrosome reaction and penetrate the zona pellucida). Furthermore, several studies have shown that failure to generate a calcium influx in response to progesterone is strongly associated with sperm dysfunction (Oehninger *et al.*, 1994) and reduced fertilization success at *in vitro* fertilization (Krausz *et al.*, 1995). Currently, the specific defects in men who fail to generate a calcium signal are unknown. Failure to generate a calcium signal could be the result of several factors, including failure of agonist binding or defective Ca^{2+} signalling machinery. Impaired expression or function of VOCCs is a likely cause. The function and regulation of VOCCs in the working of the normal cell needs to be understood before their potential role in male factor infertility can be assessed. Elucidation of the nature of VOCCs in male germ cells is a vital first step in the development of effective rational therapy.

The difficulty of obtaining testicular biopsies and spermatogenic cells from fertile donors has proved an impediment to molecular and electrophysiological research on the VOCCs of human male germ cells, such that our knowledge is rudimentary. However, there has been considerable progress in characterizing the VOCCs in spermatozoa and male germ cells of other mammals, particularly in the activation of rodent spermatozoa by zona pellucida. The probable regulation and activation of VOCCs during capacitation and induction of the acrosome reaction by zona pellucida in mammals are summarized (Fig. 1). Most

of the relevant data, particularly on the response to zona pellucida, derive from studies in mice. This review discusses the status of VOCCs in spermatozoa (primarily those of humans and rodents) as determined from pharmacological, physiological and molecular studies, and attempts to place these channels in the context of the diversity of VOCCs that has been described in somatic cells. The role and regulation of VOCCs in animal and human spermatozoa are also discussed.

Voltage-operated Ca^{2+} channels

Physiology and pharmacology

On the basis of their biophysical and pharmacological properties, VOCCs have been classified into high voltage activated (HVA) and low voltage activated (LVA) channels.

HVA channels. HVA channels are so-called because of their requirement for a relatively large depolarization (positive shift of the membrane potential from its resting value, for example, from –80 to –30 mV) to induce channel opening. Typically, HVA channels open at voltages $\geqslant$ –30 mV. On the basis of biophysical and pharmacological characteristics, HVA channels have been divided into L, N, P, Q and R types. Consistent differences have been observed in rates and voltage-dependence of activation, inactivation and deactivation among these types of channel (see Box 1 for an explanation of these processes) such that each type has its own 'signature' (Randall, 1998). The genuine nature of these distinctions is confirmed by the discrete pharmacological sensitivity of each of the HVA channel subtypes (Randall, 1998; Tsien *et al.*, 1998). L-type channels show a particular sensitivity to DHPs, whereas N-type channels are uniquely sensitive to ω-conotoxin GVIA (from the cone snail *Conus geographus L.*). P, Q and R channels can be separated by their differential sensitivities to ω-conotoxins and ω-agatoxins (from the funnel web spider *Agelenopsis aptera*) (Mintz, 1994; Randall, 1998).

LVA channels. LVA channels activate in response to relatively modest depolarizations (typically $\geqslant$ –60 mV) and are characterized by the lowest single channel conductance of all the VOCCs and by the transient nature of their whole-cell current. Typically LVA channels inactivate within 50–100 ms in response to a step voltage change (Fig. 2b). Selective pharmacological modulation of T-type channels continues to present a problem. Nickel (Ni^{2+}), mibefradil and amiloride have been used to discriminate T-type channels from the HVA channels. Mibefradil exerts a potent inhibitory effect on T-type Ca^{2+} currents (Mishra and Hermsmeyer, 1994; Todorovic and Lingle, 1998) and has been used as a probe for the involvement of T-type channels in cellular responses, including those of spermatozoa. However, at higher concentrations, mibefradil can antagonize HVA Ca^{2+} currents (Martin *et al.*, 2000; Wu *et al.*, 2000) and in some cells studies show little selectivity between LVA and HVA channels. Similarly, low (μmol l^{-1}) concentrations of Ni^{2+} have been used to block T-type currents selectively in several types of cell, for example sino–atrial nodal cells and sensory neurones (Todorovic and Lingle, 1998), as well as spermatozoa. However, sensitivity to Ni^{2+} has been observed occasionally in HVA channels, as has low Ni^{2+} sensitivity of T-type currents in neuronal cells (Zamponi *et al.*, 1996). Therefore, the best that can be said is that there is a range of semi-selective compounds available, none of which offer selectivity such that they can be regarded as diagnostic of T-type channels.

Structure

The main structural constituent of all VOCCs is the single, pore-forming α_1 subunit. This subunit is the primary determinant of the biophysical and pharmacological variation among VOCCs. It is composed of four homologous domains (Fig. 2a), which are interconnected by cytoplasmic linker regions. Each domain is made up of six transmembrane helices, S1–S6. Between the S5 and S6 segments in each domain there is a non-helical P-loop. The four P-loops are believed to line the channel pore (Catterall, 1995).

Ten α_1 subunit genes, ($\alpha_{1A\text{-}I}$ and $\alpha 1_S$) have been identified thus far by molecular cloning, mostly in neuronal and cardiac tissues. Of these, α_{1A}, α_B, α_C, α_D, α_E, α_F and α_{1S} all appear to form HVA channels. Some of these subunits, when expressed as recombinant channels, closely resemble one of the biophysical–pharmacological subtypes described above. α_{1B} is clearly N-type and α_{1C}, α_{1D} and α_{1S} are all types of L-channel. α_{1A} and α_{1E} probably form P/Q- and R-type channels, respectively, although this has yet to be firmly established. α_{1G}, α_{1H} and α_{1I} have been discovered relatively recently (Perez-Reyes *et al.*, 1998; Williams *et al.*, 1999; Monteil *et al.*, 2000a) and constitute the LVA channel family. These subunits vary in their biophysical properties (activation, inactivation, deactivation; Box 1) and in their pharmacological characteristics. For instance, α_{1H} is considerably more Ni^{2+}-sensitive than α_{1G} or α_{1I} (Lacinová *et al.*, 2000). Members of the LVA family of α_1 gene products are particularly subject to alternative splicing, which leads to a considerable increase in functional diversity beyond that derived from the three genes.

In HVA channels, the α_1 subunit is associated with β, α_2–δ, and possibly γ subunits (Birnbaumer *et al.*, 1998; Walker and De Waard, 1998). These auxiliary subunits regulate expression and biophysical characteristics of the α_1 subunit with which they associate. However, the domain within HVA α_1 subunits that interacts with the β subunit (Fig. 2) is not present in LVA channels. Although modest effects of auxiliary subunits on T-type channels have been reported (Dolphin *et al.*, 1999; Gao *et al.*, 2000), most data on expression of recombinant channels indicate that LVA channels can function independently, without associating with any of the auxiliary subunits (Perez-Reyes *et al.*, 1998; Lacinová *et al.*, 2000).

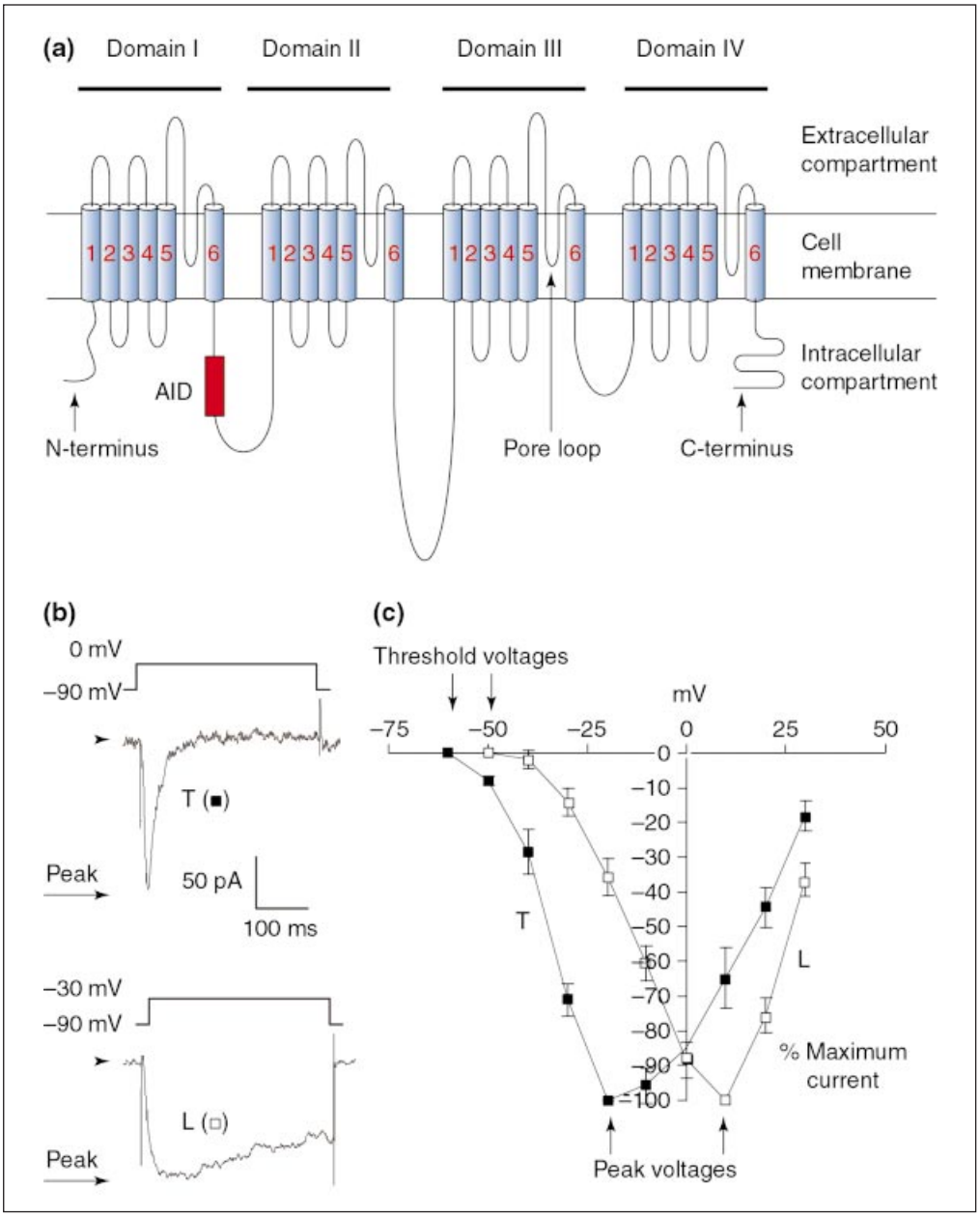

Fig. 2. Structure and functional characteristics of voltage-operated Ca^{2+} channels (VOCCs). (a) Putative structure of a VOCC α_1 subunit. All VOCCs are made up of four repeating domains numbered I–IV from the N-terminus. Each domain is composed of six membrane-spanning alpha-helical regions (blue cylinders) numbered 1–6 (labelled in red). The membrane-spanning regions are interspersed by linkers that extend alternately outside and inside of the membrane. The four 5–6 linkers each include an intramembrane stretch called the pore loop. The four pore-loops are believed to extend into the pore (which is lined by helices 5 and 6 in each domain) and to contribute to selectivity. Transmembrane helix 4 in each domain includes a high proportion of charged residues and is believed to act as a voltage sensor. The I–II (intracellular) linker includes sites at which the α_1 subunit interacts with the β subunit (alpha interaction domain (AID) shown as a red box) and sites at which channel activity can be regulated by phosphorylation (Dolphin, 1998; Dunlap and Ikeda, 1998; Zamponi and Snutch, 1998). The sequence for interaction with the β subunit is not present on the I–II linker of low voltage activated (LVA) α1 subunits (Lacinová *et al.*, 2000). (b) T- and L-type currents recorded by the whole-cell variant of the patch clamp technique, in which the record shows the summed activity of a population of channels (see Box 1). The upper trace shows a fast-inactivating T-type (LVA) current. Upon application of a depolarizing pulse to the cell (from –90 to 0 mV; marker above the trace) the inward Ca^{2+} current

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Molecular studies

As spermatozoa are believed to be transcriptionally inactive, studies of gene expression in these cells have been carried out primarily on RNA isolated from progenitor spermatogenic cells (see Table 1). Transcripts for a number of HVA channels encoding $\alpha_{1A,}$ $\alpha_{1B,}$ $\alpha_{1C,}$ α_{1D} and α_{1E} subunits have been detected by PCR using total RNA from mouse seminiferous tubules (Liévano *et al.*, 1996). RT–PCR on RNA from purified mouse spermatogenic cells detected primarily $\alpha 1_E$ transcript; α_{1A} and α_{1C} transcripts were present in small amounts (Lievano *et al.*, 1996; Espinosa *et al.*, 1999). Benoff and colleagues have consistently detected transcripts for α_{1C} in rat and human testis (Goodwin *et al.*, 1997, 1998). *In situ* RT–PCR of rat testis sections indicated that testis-specific α_{1C} transcripts were present at all stages of the germ cell lineage (Goodwin *et al.*, 1998). In addition, Goodwin *et al.* (2000) reported the detection of message for α_{1C} in RNA from mature ejaculated human spermatozoa.

Detection of LVA channels has proved problematic. Using a series of primers against various regions of the LVA α_{1G} subunit, Jacob and Benoff (2000) detected only transcripts encoding for domain IV and the C-terminus in rat testis RNA. No PCR products were generated with cDNA from human spermatozoa (Jacob and Benoff, 2000). Espinosa *et al.* (1999), using primers directed against the C-termini of α_{1G} and α_{1H}, obtained PCR products from mouse spermatogenic cell cDNA. Son *et al.* (2000) used degenerate primers on cDNA from human testicular biopsies to obtain a 489 bp fragment of α_{1H}, but neither α_{1G} nor α_{1I} was detected. Recently, the full-length sequences of both α_{1G} and α_{1H} LVA channels from human testicular cDNA were obtained by amplifying a series of overlapping PCR products. Both channels are present in multiple isoforms. These transcripts have also been observed in human male germ cells (Jagannathan *et al.*, 2000a,b, in press).

Immunocytochemical studies using anti-peptide antibodies raised to cytosolic domains of rat brain VOCCs have shown the presence and regional localization of $\alpha_{1A,}$ $\alpha_{1B,}$ α_{1C} and α_{1E} subunit proteins on mouse spermatozoa (Westenbroek and Babcock, 1999; Wennemuth *et al.*, 2000). All of the four channel subunits generate a punctate staining pattern. Intriguingly, all four of these channel proteins have distinct localization patterns, indicating specialization of function. Serrano *et al.* (1999) have also detected α_{1A} and α_{1C} in mouse spermatogenic cells, the protein being detectable in the cytoplasm as well as at the cell surface. No specific binding, in mouse spermatogenic cells or spermatozoa, was detected with antibodies to α_{1B} or α_{1D} (Serrano *et al.*, 1999). Goodwin *et al.* (1997) showed that an antibody directed against rabbit α_{1S}, probably detecting the α_{1C} protein, labelled the postacrosomal region of human spermatozoa. Immunolocalization of $\alpha_{1A,}$ $\alpha_{1B,}$ and α_{1E} proteins have also been shown in rat Sertoli cells and within the seminiferous epithelium, peritubular and interstitial tissues, indicating a role at the blood–testis barrier (Fragale *et al.*, 2000).

The only study to date on expression of VOCC auxiliary subunits was carried out by Serrano *et al.* (1999), who reported detection of all four types of VOCC β subunit by RT–PCR of mouse germ cell mRNA. Use of specific antibodies also identified all β subunits except b4 in spermatogenic cells and spermatozoa, and staining in the spermatozoon showed regional localization.

$[Ca^{2+}]_i$ responses to depolarization

In most cells, the resting membrane potential (E_m) is determined primarily by the equilibrium potential for K^+ (E_K; see Box 2). Therefore, an increase in $[K^+]_o$ and the consequent positive shift of E_K can be used to activate VOCCs and processes mediated by VOCCs. Several studies have addressed the response of $[Ca^{2+}]_i$ in mature spermatozoa to K^+-induced depolarization. Spermatozoa of the sea urchin *Lytechinus pictus* show an increase in $[Ca^{2+}]_i$ in response to K^+ depolarization, provided that they have already undergone a valinomycin-induced hyperpolarization (see Box 2).

(downward deflection of the trace) reaches a peak (arrow) and then rapidly decays as the channels inactivate, returning to resting levels (arrowhead at the start of the trace) within 100 ms. The lower trace shows a more slowly inactivating L-type current, induced by a voltage step from –90 to –30 mV (marker above the trace). The inward Ca^{2+} current reaches a peak (arrow) and decays less than halfway back towards resting levels (arrowhead at the start of the trace) during the duration of the depolarizing pulse (400 ms). Scale bars show 50 pA and 100 ms and refer to both traces. Both currents were recorded from osteoblasts. Peak (arrows) indicates the maximum current used for constructing current voltage relationships (see Box 1). (c) Current–voltage relationships for whole-cell T-currents (■) and L-currents (□) of the type shown in (b). These relationships were constructed by applying a series of voltage steps from a resting membrane voltage (–90 mV) to various values of membrane potential, to obtain a 'family' of currents. The maximum current evoked by each step (see arrows in (b)) was then measured and plotted against the voltage to which the membrane potential was stepped to evoke that current. For each current type, data from five cells have been normalized (by expressing as percentages of the maximum current) and plotted as mean ± SEM. The inward Ca^{2+} current is plotted below the horizontal axis, consistent with the downward deflection of the current trace for an inward current (see (b) and Box 1). Plots of this type are characteristically biphasic for VOCCs, increasing from a 'threshold' voltage, at which the channels are just activated (see Box 1), to a peak value and then decreasing in amplitude at more positive values. The increase in current amplitude that occurs in the voltage range between the threshold and the peak reflects recruitment of an increasing proportion of the population of channels as the depolarizing steps become larger (see Box 1). Peak current occurs close to the value at which all the channels open. The reduced current amplitude that is seen upon steps to more positive voltages reflects the reduced driving force for ion influx as the voltage begins to approach the equilibrium potential for Ca^{2+} (see Box 2). Osteoblasts, from which these recordings were made, express $\alpha_{1C,}$ α_{1D} and α_{1G} subunits (Gu *et al.*, 1999) but not α_{1H} (Y. Gu, S. Jagannathan, T. Snow and S. J. Publicover, unpublished).

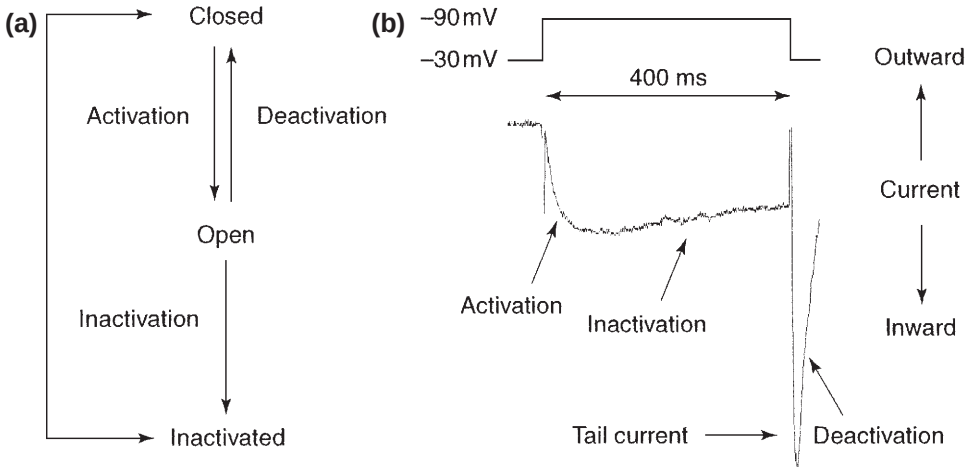
Box 1. Biophysical characteristics of voltage-operated Ca^{2+} channels

Classification of voltage-operated calcium channels (VOCCs) is based upon pharmacological sensitivity, relative permeability to Ca^{2+}/Ba^{2+} and biophysical characteristics. Diagnostic biophysical features include characteristics of activation, characteristics of inactivation and rate of deactivation (Randall, 1998).

Activation is the voltage-activated transition of a channel from the closed to the open state that occurs in response to depolarization of membrane potential (E_m). The probability of opening for any individual channel is a function of E_m. In a population of channels, there is a sigmoid relationship between membrane voltage and the proportion of open channels, with a threshold value below which the channels remain closed and a saturation value above which virtually all channels will open. The voltage range between threshold and saturation is characteristic of the type of channel. Low voltage-activated (LVA) and high voltage-activated (HVA) channels are clearly separable in this way and there are also small differences between channels within the two families. The latency of channel activation in response to a depolarizing step is also variable. In whole-cell clamp records, this results in a 'ramp' at the start of the whole-cell current as individual channels are recruited into the open population (see figure).

Inactivation is the process by which channels in the open configuration spontaneously adopt a non-permeable state despite maintenance of E_m at a value sufficient to permit channel opening. In whole-cell records, this adoption of a non-permeable state appears as a decay of current amplitude during the depolarizing pulse that is used to activate the channels. There appear to be three types of VOCC inactivation, including a Ca^{2+}–calmodulin mediated effect, a voltage-mediated effect and a poorly understood slow form of inactivation (Stotz and Zamponi, 2001). The rapid inactivation of LVA channels is voltage-induced, depolarization itself causing a structural rearrangement of the channel. Both the rate of inactivation of open channels and the extent of inactivation (proportion of channels that adopt the inactivated state) are voltage-dependent. The 'inactivated' state is different from the normal closed state, and inactivated channels do not re-open without first returning to the closed state, which requires a return of E_m to a more negative voltage (see figure).

Deactivation is the process by which channels that have opened in response to depolarization (but have not yet inactivated) return to the closed state upon repolarization of E_m. When a voltage pulse terminates and E_m returns to more negative values, there is a brief increase in current amplitude before deactivation (called the **tail current;** see figure) as a result of the increase in inward driving force on the charge carrier. In whole-cell records, the time course of decay of the tail current reflects the distribution of latencies for deactivation of the individual channels. This time course is particularly slow in T channels and is one of the diagnostic features of this type of channel.



(a) Simple model for transition of a voltage-operated channel between states, based upon models for inactivation of voltage-operated Na^+ channels, in which there may be several closed states, only one of which can undergo the transition to the open state. When the membrane is at resting potential, the channels are closed. Upon depolarization, a proportion of channels undergo activation to adopt the open state (see above) and may then 'flicker' between open and closed states. While the membrane is depolarized, open channels can become inactivated. From the inactivated state, the channels do not re-open but they can return to the closed state upon repolarization of E_m to sufficiently negative values. It is not clear whether channels can pass directly between closed and inactivated states (shown by the long, double-headed arrow that bypasses the open state) but this seems likely. Those channels that are still open at the end of a period of depolarization return to the closed state by deactivation. (b) Whole-cell record from a cell expressing L-type VOCCs. This type of record shows the summed currents from a large number of channels (see Fig. 2b). Although transitions between the various channel states are rapid and appear as steps on single channel records, in whole-cell records, the kinetics of the transition of the population of channels between one state and another can be visualized and measured. Upon application of a 400 ms depolarization from –90 to –30 mV (shown by the marker above the trace), a population of L channels undergoes activation, which, in this case, takes approximately 100 ms to reach completion. A proportion of these open channels (approximately 25%) then inactivate during the pulse (duration = 400 ms), which appears as a slow decay in the current amplitude. Upon repolarization of E_m a large tail current is seen that returns to zero as the channels deactivate. This recording was made in the presence of the VOCC agonist FPL 64716, the use of which results in very slow deactivation.

The initial hyperpolarization causes an increase in intracellular pH, pH_i, which is apparently necessary for subsequent activation of Ca^{2+} influx (Gonzalez-Martinez *et al.*, 1992). The increase in $[K^+]_o$ causes an increase in $[Ca^{2+}]_i$ in capacitated bovine spermatozoa that is sensitive to blockers of VOCCs (Babcock and Pfeiffer, 1987; Florman *et al.*, 1992; Arnoult *et al.*, 1996a). This VOCC-mediated Ca^{2+} influx, in a similar manner to that in sea urchin spermatozoa, requires a coincident increase in pH_i. Arnoult *et al.* (1996a) simultaneously measured pH_i and $[Ca^{2+}]_i$ in individual bovine spermatozoa and showed that an increase in $[K^+]_o$ to 80 mmol l^{-1} induces Ca^{2+} influx only in conjunction with a measurable increase in pH_i (induced by extracellular application of NH_4^+). Both depolarization and alkalinization occur upon zona pellucida binding, and the increase in pH_i is mediated by a pertussis toxin-sensitive mechanism (Florman *et al.*, 1992; Arnoult *et al.*, 1996a).

Linares-Hernandes *et al.* (1998) carried out similar experiments on uncapacitated human spermatozoa, labelled with Fura-2 and $DiSC_3$ to allow simultaneous monitoring of $[Ca^{2+}]_i$ and E_m. Cells responded to K^+ with an increase in $[Ca^{2+}]_i$ that included an initial transient component (approximately 30 s) and a smaller sustained response. Both the transient and sustained components of the K^+-induced $[Ca^{2+}]_i$ increase were dependent upon influx of Ca^{2+}. Alkalinization was not necessary to permit Ca^{2+} influx, but simultaneous addition of NH_4^+ with K^+ increased the $[Ca^{2+}]_i$ response by approximately 90%. Fifty micromoles per litre of Ni^{2+} inhibited the transient component, but the sustained component was blocked only partially by 600 μmol Ni^{2+} l^{-1}. Fifty micromoles per litre of nifedipine had no effect. The response to the increase in $[K^+]_o$ was enhanced by prior hyperpolarization of E_m (with valinomycin). The estimated value of E_m before application of valinomycin was –40 mV, so this hyperpolarization may have been necessary to release VOCCs in uncapacitated cells from inactivation, as has been proposed for the hyperpolarizing effect of capacitation (Zeng *et al.*, 1995; Florman *et al.*, 1998; Arnoult *et al.*, 1999; see below). Silvestroni *et al.* (1997) observed an increase in $[Ca^{2+}]_i$ in human spermatozoa treated with the insecticide lindane. This effect was believed to reflect alteration of the membrane dipole potential, resulting in activation of VOCCs. The effect was reduced by 45% in the presence of the DHP nicardipine, and was also inhibited by Cd^{2+}.

Electrophysiological studies

Although there is a range of techniques that can shed light on the expression and activity of ion channels, detailed knowledge of functional expression and biophysical and pharmacological characteristics can be obtained only by direct application of electrophysiological techniques. Despite considerable efforts, it has proved impossible to obtain records of voltage-operated Ca^{2+} currents from mature spermatozoa. Darszon and colleagues have used insertion of sperm channels into artificial bilayers and have also successfully applied the cell-attached patch clamp technique to osmotically swollen sea urchin and mouse spermatozoa. These technically demanding studies have provided valuable data on expression of various channels, including a Ca^{2+} channel in sea urchin and mouse spermatozoa that resembles the ryanodine receptor (Darszon *et al.*, 1999). Unfortunately, this approach has provided little information as yet on the expression of VOCCs in these cells. Tiwari-Woodruff and Cox (1995) inserted boar sperm proteins into artificial bilayers and observed a Ca^{2+} channel that displayed sensitivity to DHP drugs but showed no voltage sensitivity. In humans, there has been only one report of patching (Weyand *et al.*, 1994), in which a cyclic nucleotide gated Ca^{2+} channel was observed. Shi and colleagues have inserted human sperm proteins in artificial bilayers and observed various channels, including a Ca^{2+}-permeable channel that shows voltage sensitivity (Chan *et al.*, 1997; Ma and Shi, 1999).

An alternative approach has been to apply the patch clamp technique to immature germ cells. As mature spermatozoa are considered to be transcriptionally inactive, all manufacture of proteins by the germ cell must occur before maturity. Therefore, ion channels of the mature cell may be functionally expressed at the immature stage. The first study on mammalian spermatogenic cells was carried out by Hagiwara and Kawa (1984) on cells dissociated from rat seminiferous tubules. These authors observed a tetraethyl ammonium-sensitive K^+ current and a transient, LVA, T-like VOCC current that increased in density in more mature cells. More recently, this technique has been applied to spermatogenic cells of mice. Similar to the situation in rats, the only VOCCs observed in these studies were LVA, T-like currents (Arnoult *et al.*, 1996b; Santi *et al.*, 1996). Characterization showed that these currents possessed an unusually high sensitivity to DHPs (IC_{50} for nifedipine = 0.4 μmol l^{-1} (Arnoult *et al.*, 1998) or 10 μmol l^{-1} (Santi *et al.*, 1996)) and were blocked by intermediate concentrations of Ni^{2+} (IC_{50} = 34 μmol l^{-1} (Arnoult *et al.*, 1998) or 100–200 μmol l^{-1} (Santi *et al.*, 1996)). The sensitivity of the mouse spermatogenic cell current to channel-specific toxins has been investigated in elongating spermatids (Wennemuth *et al.*, 2000). These authors observed a 38% blockade of the current after 5 min exposure to ω-conotoxin GVIA, a toxin considered to be diagnostic for N-type HVA channels (see above). The remaining portion of the current was almost completely blocked by 100 mmol Ni^{2+} l^{-1}, whereas this dose gave only 50% block of non-toxin-pretreated currents. These findings indicate that the LVA current of mouse spermatogenic cells is made up of two components that have similar kinetics but different pharmacology, one of which is Ni^{2+}-sensitive, whereas the other shows a sensitivity to ω-conotoxin GVIA that is unlike the T channel. Small, T-like currents have recently been detected in human spermatogenic cells (Jagannathan *et al.*, in press).

In neuronal cells, HVA currents are regulated by a number of mechanisms, many of which are downstream

Table 1. Molecular studies on the expression of voltage-operated calcium channels in testis and germ cells

Tissue	Method	α_1-Subunit type(s) investigated and result	Reference
Mouse			
Testis	RT–PCR	A, B, C, D, E detected (836–240 bp)	Liévano *et al.*, 1996
Germ cells	RT–PCR	Primarily E detected (240 bp); A (753 bp) C, G (520 bp) and H (351 bp) also present	Liévano *et al.*, 1996; Espinosa *et al*, 1999
Germ cells	Northern blot	C detected (data not shown)	Espinosa *et al.*, 1999
Germ cells	Immunostaining	A, C detected; B,D not detected	Serrano *et al.*, 1999
Spermatozoa	Immunostaining	A, C detected; B,D not detected	Serrano *et al.*, 1999
Spermatozoa	Immunostaining–immunoblot	A, C, E – detection/regional localization, B – detection/regional localization	Westenbroek and Babcock, 1999; Wennemuth *et al.*, 2000
Rat			
Testis	RT–PCR	C detected (full sequence)	Goodwin *et al.*, 1997
Spermatozoa	Immunostaining	L-type detected (antibody raised against α_{1S})	Goodwin *et al.*, 1997
Spermatozoa	*In situ* RT–PCR	C detected	Goodwin *et al.*, 1997
Testis/spermatozoa	Western blot	L-type detected (antibody raised against α_{1S})	Goodwin *et al.*, 1998
Testis sections	Immunostaining	L-type detected (antibody raised against α_{1S})	Goodwin *et al.*, 2000
Testis sections	*In situ* RT–PCR	C detected	Goodwin *et al.*, 2000
Testis	RT–PCR	G (domain IV and C-terminus, domains I–III not detected)	Jacob and Benoff, 2000
Multi-tissue northern blot panel	Northern blot	G not detected	Perez-Reyes *et al.*, 1998
Testis sections	Immunostaining, *in situ* hybridization	A and B detected, E not detected (data not shown)	Fragale *et al.*, 2000
Human			
Ejaculated spermatozoa	*In situ* RT–PCR	C detected	Goodwin *et al.*, 2000
Sperm RNA	RT–PCR	G not detected	Jacob and Benoff, 2000
Testis/male germ cells	PCR	G and H full sequence	Jagannathan *et al.*, 2000a,b, in press
Testis	*In situ* hybridization	G and H in germ cells and somatic cells	Jagannathan *et al.*, in press
Multi-tissue northern blot panel	Northern blot	G detected (small amounts), I not detected	Monteil *et al.*, 2000a,b
Testis	RT–PCR	H detected (489 bp); G and I not detected	Son *et al.*, 2000

effects of neurotransmitters. There have been relatively few reports of regulation of LVA channel currents, although it is clear that such regulation can occur, both in excitable and non-excitable cells (Barrett *et al.*, 1991; Chesnoy-Marchais and Fritsch, 1994; Pemberton *et al.*, 2000). The T-like current of mouse male germ cells is subject to control by tyrosine phosphorylation–dephosphorylation. Dephosphorylation in response to depolarizing prepulses causes an increase in current amplitude (Arnoult *et al.*, 1997). Espinosa *et al*. (2000) reported that oestradiol exerts a rapid (non-genomic), inhibitory effect on current amplitude, as has been observed in somatic cells (Zhang *et al.*, 1994; Ogata *et al.*, 1996).

Participation of voltage-operated Ca^{2+} channels in agonist-induced signalling in mammalian spermatozoa and their regulation during capacitation

Zona pellucida-induced Ca^{2+} influx

Initial studies in which it was shown that DHPs inhibit the acrosome reaction and the zona pellucida-induced $[Ca^{2+}]_i$ response were interpreted as evidence for involvement of L-type VOCCs in transduction of zona pellucida binding (Florman, 1994). Therefore, it was somewhat surprising when the only functional VOCC in mouse spermatogenic cells (even at the elongating spermatid stage) was shown to be an LVA-T-type channel (see above). The $[Ca^{2+}]_i$ response to zona pellucida is prolonged and the acrosome reaction occurs some minutes after the start of this response. In contrast, the influx of Ca^{2+} through T-channels is transient (usually < 500 ms). However, comparison of the pharmacological sensitivities of the LVA current of spermatogenic cells and the zona pellucida-induced $[Ca^{2+}]_i$ increase in mouse spermatozoa shows great similarity (Arnoult *et al.*, 1996b, 1998). Furthermore, the initial response to zona pellucida is a large, brief $[Ca^{2+}]_i$ 'spike' with kinetics comparable to those of T-currents (Arnoult *et al.*, 1999). Therefore, it appears that a transient Ca^{2+} influx through the T-type channel seen in patch-clamped spermatogenic cells is an essential, early event in the response to zona pellucida binding. This initial $[Ca^{2+}]_i$ spike induces a second, sustained Ca^{2+} influx. Recent evidence indicates that the sustained component of Ca^{2+} influx is mediated primarily by a store-operated channel (Putney and McKay, 1999),

Box 2. Equilibrium potentials and the use of K^+ as a depolarizing stimulus

For any species of ion, movement across a biological membrane (through open ion channels) is determined by: (i) the concentration gradient (the difference in concentrations of the ion on the two sides of the membrane); and (ii) the electrostatic force (a force exerted on the ion by any difference in electrical potential across the membrane). Hence, the size and sign of the charge on the ion are important.

There will be a value of membrane potential (E_m) at which these two forces are equal in amplitude but opposite in direction, such that there is no net flux across the membrane. This value of E_m is called the **equilibrium potential**. Since the ionic species at a membrane vary in both their concentration gradient and their valency, each ion will have its own equilibrium potential (E_x, where X is the species of ion). Using the intracellular and extracellular concentrations, the equilibrium potential for any ion can be calculated using the Nernst equation:

$$E_X = RT/ZF * \ln([X]_o / [X]_i)$$

where E_X is equilibrium potential for ion X; R is the gas constant; T is temperature in degrees absolute; Z is valency of ion X; F is Faraday's constant; and $[X]_o$ and $[X]_i$ are the extracellular and intracellular concentrations of the ion X.

If a membrane is permeable to only one ion, then E_m will settle at the equilibrium potential for that ion. Resting cells are normally most permeable to K^+ and therefore resting potential of the cell is determined primarily by E_K, which is typically around –80 mV. However, resting cell membranes also show some permeability to other ions (such as Na^+) that have more positive equilibrium potentials. Therefore, resting potential is less negative than E_K, the actual value depending on relative permeabilities to K^+ and to these other ions. Cells can control E_m by opening and closing channels selective for K^+ or other ions, thus changing the relative contribution of the various equilibrium potentials to determination of E_m. In studies in spermatozoa, the K^+ ionophore valinomycin has been used to render membranes more K^+-permeable, thus moving E_m towards E_K (and hyperpolarizing resting cells). A common technique for investigating voltage-operated calcium channels is to depolarize cells by increasing $[K^+]_o$. The increased $[K^+]_o$ results in a positive shift in E_K (see Nernst equation above), thus acting as a depolarizing stimulus.

activated after depletion of a small Ca^{2+} store, probably in the acrosome (O'Toole *et al.*, 2000; Jungnickel *et al.*, 2001). Treatment of mouse spermatozoa with an antibody directed against an extracellular region of the store-operated channel Trp2 not only inhibits the sustained component of the zona pellucida-induced $[Ca^{2+}]_i$ signal but also reduces zona pellucida-induced acrosome reaction by > 75% (Jungnickel *et al.*, 2001). This finding not only identifies Trp2 as an important component in the Ca^{2+} influx pathway in the response of mouse spermatozoa to zona pellucida but also demonstrates the pivotal importance of the sustained component of Ca^{2+} influx for the acrosome reaction. The mechanism of activation of Trp2 is yet to be established. In somatic cells, these channels open in response to depletion of the Ca^{2+} store in the endoplasmic reticulum (Putney and MacKay, 1999). The signal transduction components required for mobilization of stored Ca^{2+} (IP_3 receptors, sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPases (SERCAs), a putative Ca^{2+} store) are present in rodent and human spermatozoa (Walensky and Snyder, 1995; Dragileva *et al.*, 1999; Kuroda *et al.*, 1999; Rosato *et al.*, 2001) but the emptying of a store upon agonist stimulation has yet to be demonstrated. The mature spermatozoon also possesses a number of HVA VOCCs and so some participation of one or more of these channels in sustained Ca^{2+} influx cannot be discounted. In hamsters, 10 μmol nifedipine l^{-1}, a blocker of L-channels (but also highly effective against the mouse spermatogenic cell T-channel; see above) inhibits the later part of the sustained component of the $[Ca^{2+}]_i$ signal and strongly inhibits the acrosome reaction (Shirakawa and Miyazaki, 1999). It has not yet been determined whether the mouse model holds good in humans or other mammals. However, solubilized zona pellucida induces a prolonged $[Ca^{2+}]_i$ response in populations of human spermatozoa, monitored by fluorimetry (Patrat *et al.*, 2000), and the magnitude of this response is strongly inhibited by pimozide, indicating the involvement of VOCCs.

Progesterone

The $[Ca^{2+}]_i$ response to progesterone has been studied primarily in humans and the participation of VOCCs in this process is disputed. Although several laboratories, including our own, have observed strong inhibition of progesterone-induced acrosome reaction in human spermatozoa by blockers of VOCCs (Shi and Roldan, 1995; O'Toole *et al.*, 1996), there are no unequivocal reports that these drugs can inhibit the progesterone-induced $[Ca^{2+}]_i$ signal. Similar to the situation in zona pellucida, the response to progesterone involves at least two phases of Ca^{2+} influx. An initial transient phase (lasting > 1 min and clearly different from that activated by zona pellucida) is followed by a sustained phase (Kirkman-Brown *et al.*, 2000). Significant effects of VOCC blockers on the amplitude of the $[Ca^{2+}]_i$ transient phase occur only with very high, unselective doses (Blackmore *et al.*, 1990; McLaughlin and Ford, 1994). However, a recent study on mouse spermatozoa by Kobori *et al.* (2000) reported effects of 1 μmol pimozide l^{-1}, a dose appropriate for blockade of the mouse spermatogenic cell T-type current (Arnoult *et al.*, 1998), on prolonged responses to progesterone. Fluorimetric studies on the interaction between the effects of progesterone and thapsigargin (commonly used as an activator of store-operated channels) showed that the response to

thapsigargin and the progesterone-induced transient were essentially additive (Blackmore, 1993). Effects on the sustained response have proved more difficult to assess.

Regulation of VOCCs during capacitation

An important aspect of the induction of the acrosome reaction via VOCC activation is that the channels should remain closed until required, to avoid premature Ca^{2+} influx and the acrosome reaction. Since the ability of agonists to induce Ca^{2+} influx and the acrosome reaction is poor in uncapacitated cells (Florman *et al.*, 1998; Visconti and Kopf, 1998; Baldi, 2000), it is likely that release of VOCCs from tonic inhibition occurs during capacitation. Evidence from studies in mice indicates that, for LVA VOCCs, this release may be achieved by regulation of the E_m. Estimates of E_m in uncapacitated mouse and bovine spermatozoa lie between –10 and –50 mV (Espinosa and Darszon, 1995; Zeng *et al.*, 1995; Arnoult *et al.*, 1999). LVA channels (believed to be crucial in the response to zona pellucida; see above) will be inactivated at these membrane potentials and therefore will be unable to respond to a depolarizing stimulus (Box 1). The membrane potential in these cells is sensitive to changes in $[K^+]_o$, indicating that K^+ permeability contributes to its determination. However, the effect is less than half of that predicted by the Nernst equation (see Box 2), indicating that other conductances, probably unselective cation channels, also contribute to resting potential (Zeng *et al.*, 1995). The membrane potential hyperpolarizes during capacitation of mouse, bovine and human spermatozoa (Zeng *et al.*, 1995; Arnoult *et al.*, 1999; Brewis *et al.*, 2000). In mouse spermatozoa, this hyperpolarization is associated with an increase in the $[K^+]_o$ sensitivity of E_m, indicating that it is the result largely of increased K^+ permeability (Zeng *et al.*, 1995) and a consequent shift of E_m towards E_K (Box 2). Single cell analysis of E_m in capacitating mouse spermatozoa shows that only those cells that undergo strong hyperpolarization are able to generate the zona pellucida-induced Ca^{2+} spike (believed to be Ca^{2+} influx through the LVA VOCC; see above) and carry out the acrosome reaction upon exposure to the zona pellucida (Arnoult *et al.*, 1999). Thus, it appears that LVA VOCCs are maintained in an inactivated state in uncapacitated cells and that membrane hyperpolarization during capacitation releases these channels from inactivation such that they can respond to a depolarizing stimulus provided by an agonist (Fig. 1a).

Darszon and colleagues have described a pH-regulated, inward rectifier K^+ channel in mouse spermatogenic cells (Gonzalez-Martinez *et al.*, 2001). The increase in pH_i that occurs during capacitation (Visconti and Kopf, 1998; Baldi, 2000) may activate this channel, contributing at least a part of the enhanced K^+ permeability that leads to hyperpolarization (Gonzalez-Martinez *et al.*, 2001; Fig. 1a). A second K^+ channel (slo3) that is sensitive to increased pH is present in mouse and (probably) human spermatocytes (Schreiber *et al.*, 1998). However, this channel is also strongly voltage-sensitive, and negligible activity is detected at negative membrane voltages (Schreiber *et al.*, 1998). A third putative mechanism for hyperpolarization of capacitating cells is activation of a Ca^{2+}-activated K^+ channel. Injection of rat testis mRNA into *Xenopus* oocytes results in expression of currents that show strong similarity to maxi-K, Ca^{2+}-activated K^+ channels of somatic cells. Immunolocalization and RT–PCR showed these channels to be present in spermatogenic cells including spermatozoa (Wu *et al.*, 1998). Such channels may be activated by the increase in $[Ca^{2+}]_i$ upon capacitation (see below), contributing to hyperpolarization (Fig. 1a).

Regulation of HVA VOCCs during capacitation may also occur, but it seems unlikely that this is achieved entirely, if at all, by membrane potential. Membrane potentials of uncapacitated spermatozoa (–10 to –50 mV; see above) are sufficient to release a large proportion of L-type (α_{1C}) and P-type (probably α_{1A}) channels from inactivation (Randall, 1998). Both of these channels are present in mouse spermatozoa and possibly germ cells (Table 1). However, HVA channels of somatic cells are known to be regulated by a number of other mechanisms, including G-protein interaction and phosphorylation (Dolphin, 1998; Walker and De Waard, 1998; Zamponi and Snutch, 1998). Potential mechanisms for regulation of VOCCs during capacitation include tyrosine phosphorylation and changes in membrane lipid composition. In mice, capacitation is correlated with increased tyrosine phosphorylation of a subset of sperm proteins. The T-currents of mouse spermatogenic cells can be enhanced by tyrosine dephosphorylation (Arnoult *et al.*, 1997; see above) but there is, as yet, no evidence for regulation of T-channel tyrosine phosphorylation during capacitation. Induction of capacitation *in vitro* requires inclusion in the medium of serum albumin, which appears to function primarily as a cholesterol acceptor, removing cholesterol from the membrane and increasing membrane fluidity (Visconti and Kopf, 1998; Baldi, 2000). Changes in phospholipid complement and distribution also occur during capacitation (Flesch and Gadella, 2000). Cholesterol can both inhibit (Jennings *et al.*, 1999) and increase (Sen *et al.*, 1992) VOCC currents. However, the increase in $[Ca^{2+}]_i$ that accompanies capacitation (Visconti and Kopf, 1998; Baldi, 2000) may reflect leakiness of the fluid or disordered cell membrane. Espinosa *et al.* (2000) observed an increase in the amplitude of mouse spermatogenic cell T-type currents in response to application of serum albumin, but the mechanism by which this effect is achieved does not seem to involve removal of cholesterol from the plasmalemma.

Future directions

The evidence summarized above indicates that male germ cells express a diverse range of VOCCs. Most of these channels appear to be non-functional in immature cells and therefore it appears that, if the channels possess functional roles, these are likely to be in the mature spermatozoon. The only one of these channels for which a function has been elucidated is the T-type channel, and even here, there

is strong evidence only in mice. Potential roles for other channels include processes occurring during capacitation, control of hyperactivation and the transduction of signals during interactions with cells of the female tract. Areas for future study should include all of these, as well as elucidation of the mechanisms underlying the acrosome reaction. Progress in the characterization of the various VOCCs in spermatozoa and in the elucidation of the control of E_m will be integral to success in this endeavour.

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