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Voltage-dependent modulation of T-type calcium channels by protein tyrosine phosphorylation

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A T-type Ca^{2+} channel is expressed during differentiation of the male germ lineage in the mouse and is retained in sperm, where is it activated by contact with the the egg's extracellular matrix and controls sperm acrosomal exocytosis. Here, we examine the regulation of this Ca^{2+} channel in dissociated spermatogenic cells from the mouse using the whole-cell patch–clamp technique. T currents were enhanced, or facilitated, after strong depolarizations or high frequency stimulation. Voltage-dependent facilitation increased the Ca^{2+} current by an average of 50%. The same facilitation is produced by antagonists of protein tyrosine kinase activity. Conversely, antagonists of tyrosine phosphatase activity block voltage-dependent facilitation of the current. These data are consistent with the presence of a two-state model, in which T channels are maintained in a low (or zero) conductance state by tonic tyrosine phosphorylation and can be activated to a high conductance state by a tyrosine phosphatase activity. The positive and negative modulation of this channel by the tyrosine phosphorylation state provides a plausible mechanism for the control of sperm activity during the early stages of mammalian fertilization.

Keywords: fertilization/sperm/T-type calcium channel/tyrosine kinase/tyrosine phosphatase

Introduction

Calcium influx through voltage-sensitive channels controls a variety of cellular processes, including secretion and muscular contraction. Current flow through these channels is regulated primarily by membrane potential, but is also subject to facilitatory and inhibitory modulation that determine the resulting intracellular Ca^{2+} signal. Modulation has been attributed to alterations in the phosphorylation state of channel subunit proteins (Sculptoreanu *et al.*, 1993b), to the direct interaction of G protein subunits with ion channel constituents (Bean, 1989; Hille, 1994) and to voltage-dependent processes (Artalejo *et al.*, 1991). The mechanisms of modulation have been examined primarily in high voltage-activated Ca^{2+} channels. For example, L-type currents are positively modulated by protein kinases A and C, as well as by G_s, and are suppressed by G_o (reviewed by Hille, 1994). Similar mechanisms can regulate other high voltage-activated Ca^{2+} channels (Mintz and Bean, 1993; Hille, 1994).

The understanding of protein kinase-mediated channel regulation has progressed by the assignment of specific phosphorylation sites on recombinant channel subunits with selective aspects of modulation. In contrast, relatively little is known about the mechanism of voltage-dependent modulation. The potentiation, or facilitation, of Ca^{2+} currents by frequent or strong depolarizations was first described in chromaffin cells (Fenwick *et al.*, 1982) and subsequently found in cardiac myocytes, neurons and a number of other cellular systems (reviewed by Dolphin, 1996). Ca^{2+} channel facilitation increases catecholamine secretion during stress (Artalejo *et al.*, 1994), increases muscle contractile force (Sculptoreanu *et al.*, 1993b) and generally is believed to either maintain or enhance Ca^{2+} signals. Several mechanisms have been proposed to explain voltage-dependent facilitation, including a voltage-dependent alteration of channel protein conformation, with the resultant exposure of new phosphorylation sites (Artalejo *et al.*, 1992; Sculptoreanu *et al.*, 1993a,b; Bourinet *et al.*, 1994), the relief of a G protein-mediated inhibition of channel function (Grassi and Lux, 1989; Roche *et al.*, 1995) or an alteration in the voltage dependence of gating (Feldmeyer *et al.*, 1992). These studies focused on the high voltage-activated L- and N-type Ca^{2+} currents, whereas relatively little is known about the voltage-dependent facilitation of T-type channels (Alvarez *et al.*, 1996; Ganitkevich and Isenberg, 1996).

A functional low voltage-activated, T-type Ca^{2+} channel is expressed during the meiotic and post-meiotic stages of mammalian spermatogenesis (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996; Lievano *et al.*, 1996). Recently, we have shown that this channel is retained in mature mouse sperm and is activated during fertilization by sperm contact with the egg's extracellular matrix, or zona pellucida (Arnoult *et al.*, 1996). Ca^{2+} influx through the sperm T channel is required for the acrosome reaction, an exocytotic event that must be completed prior to sperm–egg fusion.

T channel regulation can be observed readily in mouse spermatogenic cells, which lack detectable levels of other voltage-sensitive Ca^{2+} currents (Hagiwara and Kawa, 1984; Arnoult *et al.*, 1996; Lievano *et al.*, 1996). Here, we report the voltage-dependent facilitation of the spermatogenic cell T current. Pharmacological and biophysical analyses suggest that a protein tyrosine kinase activity is required for inhibitory modulation of these channels, whereas a tyrosine phosphatase activity reverses this effect during strong or high frequency depolarizations.

Results

Mouse spermatogenic cell Ca^{2+} currents were recorded from dissociated cells using the whole-cell configuration

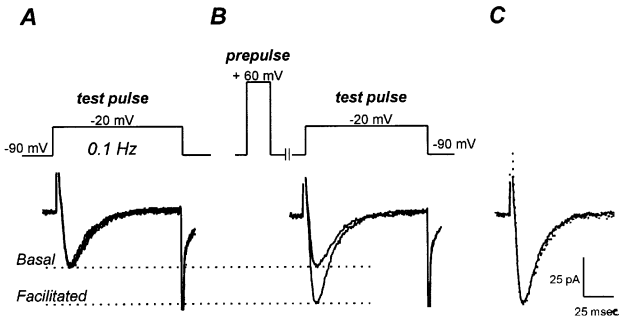

Fig. 1. The voltage-dependent facilitation of the mouse spermatogenic cell T current. Currents are obtained from a round spermatid stage spermatogenic cell in a bath solution that contained elevated (10 mM) Ca^{2+}. Similar results are obtained from meiotic (spermatocyte) and post-meiotic (spermatid) stages of germ cell differentiation. (**A**) The superimposed traces illustrating the basal current that is evoked by depolarization from a holding potential of –90 mV to a test potential of –20 mV at a frequency of 0.1 Hz. (**B**) Facilitated current is obtained by subjecting the cell to a depolarizing prepulse from holding potential (–90 mV) to +60 mV for 100 ms, repolarizing to the holding potential for 500 ms, and then applying a test depolarization to –20 mV. Current traces represent basal T current as well as the facilitated current during the final test depolarization to –20 mV. (**C**) Basal (solid line) and facilitated currents (dotted line) from (B) are normalized to the same peak current amplitude. Note that there is no significant difference in the activation or inactivation kinetics.

of the patch–clamp. The biophysical and pharmacological characteristics of this current indicate that it is mediated by T-type Ca^{2+} channels (Arnoult *et al.*, 1996). This T current is present during meiotic and post-meiotic stages of spermatogenesis, as are the regulatory processes described in these experiments.

Voltage-dependent facilitation of the T-type Ca^{2+} current

Maximal inward T current ('basal' current) is obtained when cells are depolarized from a holding potential of –90 mV to a test potential of –20 mV. Current amplitude is not affected by successive depolarizing steps to this potential at a frequency of <0.1 Hz (Figure 1A), but is enhanced by increasing the stimulation rate above 0.2 Hz (data not shown) or by providing a depolarizing prepulse to more positive membrane potential before the test depolarization (Figure 1B). The additional current recruited by such prepulses is referred to as 'facilitated' current. Voltage-dependent facilitation does not alter significantly the activation and inactivation kinetics of the current. This is demonstrated in Figure 1C, where basal and facilitated current traces superimpose after normalization to the same peak amplitude. The activation and inactivation time constants, as determined on 20 cells, are 3.6 ± 1.2 and 16.8 ± 3.6 ms respectively, for basal current, while comparable values for the facilitated current are 3.4 ± 1.2 and 14.8 ± 2.7 ms, respectively (mean ± SD). Similar results were obtained when Ba^{2+} replaced Ca^{2+} as a charge carrier (data not shown). These kinetic data suggest that faciltation reflects the regulation of a single population, rather than the activation of a different class of channels.

The voltage and time dependence of the facilitating prepulse were determined (Figure 2A). When cells are depolarized from a holding potential of –90 mV, the prepulse necessary for facilitation has the following characteristics: (i) a voltage threshold of –30 mV and a maximal response following depolarizations to +60 mV (Figure 2B); (ii) a temporal threshold of 5 ms producing a maximal effect following prepulses of 100 ms duration (Figure 2C); and (iii) a transitory effect, as a prepulse–test pulse interval of 0.5–1 s produced a maximal response and facilitation decreased as this interval was lengthened (loss of facilitation, $t_{1/2}$ ~4.8 s; Figure 2D). The degree of facilitation also decreased as the prepulse–test pulse interval was shortened, most likely due to incomplete recovery of T channels from inactivation during the prepulse. Optimal facilitation occurs when cells that are held at –90 mV are subjected to a 100 ms prepulse to +60 mV, returned to holding potential for 500 ms, and then subjected to test depolarizations.

The extent of facilitation was also reciprocally dependent upon the basal current density. Spermatogenic cells were subjected to the optimal facilitation protocol described above. Cells with a basal current density of 2–5.5 mA/cm^2 exhibit a large facilitation (166 ± 43% of basal current, n = 27). In contrast, cells with a larger basal current density of 6–10 mA/cm^2 exhibit a much lower increase (108 ± 9% of initial current, n = 12). The mean degree of facilitation was 148 ± 45% increase (n = 39) and regression analysis of these pooled data indicate a maximal current of 9.2 mA/cm^2.

Role of protein phosphorylation in the voltage-dependent facilitation of T-type calcium current

As shown in Figure 2D, the potentiation of spermatogenic cell T currents by a facilitating prepulse decays slowly. Sustained facilitation was also reported for the L-type channels of chromaffin cells (Artalejo *et al.*, 1992) and skeletal muscle (Sculptoreanu *et al.*, 1993a,b), where the operation of a voltage-dependent protein kinase has been proposed. We therefore determined the role of the protein phosphorylation state in T channel facilitation using modulators of protein kinase and phosphatase activities.

Figure 3 illustrates the effects of tyrphostins A47 and A25, specific membrane-permeant inhibitors of protein tyrosine kinases, on the spermatogenic cell T current. The values of the basal and facilitated currents obtained after a depolarizing prepulse were relatively stable during sequential recording at a frequency of 0.1 Hz (Figure 3B–D). However, addition of A47 or A25 enhanced the initial current amplitude to a level equal to that of the prepulse-facilitated current in the absence of tyrphostin. This enhancement of basal currents occurs in the absence of any associated alterations in the activation and inactivation kinetics, thus producing a larger net conductance. This effect was produced by 2–50 μM tyrphostin A47, although higher concentrations acted more rapidly ($t_{1/2}$ at 2 and 10 μM of ~2.5 min and <0.5 min, respectively). These effects are readily reversible when tyrphostin A47 or A25 are removed by perfusion (Figure 3D). In contrast, no effects on T currents were observed when spermatogenic cells were treated with tyrphostin A1 (Figure 3D), which is not a potent inhibitor of protein tyrosine kinase activity, or with AG1478 (data not shown), which is a highly selective antagonist of the epidermal growth factor receptor tyrosine kinase (Levitzki and Gazit, 1995).

Several additional features of the tyrphostin-dependent enhancement of Ca^{2+} current are notable. (i) The increase

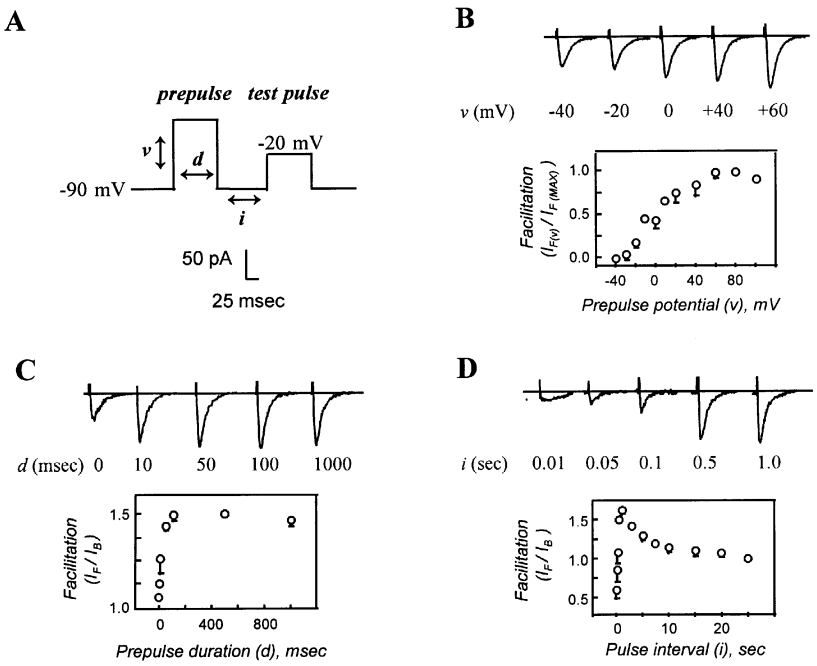

Fig. 2. Biophysical characterization of the voltage-dependent facilitation of mouse spermatogenic cell T channels. (**A**) Voltage protocols illustrate the relationship between prepulse and test pulse. The prepulse voltage (*v*) and duration (*d*), as well as the prepulse–test pulse interval (*i*), are varied systematically in subsequent panels. Voltage protocols are delivered at 15 s intervals. Time and current scaling factors for subsequent panels are also shown. (**B**) The relative magnitude of facilitation is determined by the size of the prepulse potential. Selective traces illustrate the Ca^{2+} current produced during test depolarizations to –20 mV following prepulses to the indicated membrane potentials (*v*). In these experiments, $d = 100$ ms and $i = 500$ ms. The normalized degree of facilitation following prepulses to membrane potential *v* is determined by subtracting the basal current and then expressing the ratio of the enhanced current [$I_{F(v)}$] to the maximal facilitated current ($I_{F(MAX)}$). No facilitation is noted for prepulses from a holding potential of –90 mV to a membrane potential of less than –30 mV, and maximal facilitation occurred following a prepulse of +60 mV. Data representing the mean current from 1–3 cells are presented below the traces. (**C**) The magnitude of facilitation is determined by the duration of the depolarizing prepulse. Selective current traces illustrate the effect of prepulse duration (*d*) on test facilitated current amplitude and data are summarized below these traces. Cells are depolarized from a holding potential of –90 mV to a prepulse potential of +60 mV for the indicated durations, returning to holding potential for 500 ms, and then depolarizing to a –20 mV test potential for 100 ms. Basal current is determined by depolarizing cells from the holding potential for 100 ms. Data is expressed as the ratio of the facilitated current (I_F) to the basal current (I_B), with each point representing the mean of observations on one to four cells. (**D**) The magnitude of facilitation is determined by the interval between the depolarizing prepulse and the test potential. Selective current traces illustrate the effect of prepulse–test pulse interval (*i*) on the facilitated current amplitude, and data are summarized below these traces. Facilitated current is determined by depolarizing cells from the holding potential (–90 mV) to a prepulse potential of +60 mV for 100 ms, by returning the membrane potential to the holding potential for the indicated intervals and by applying a test depolarization to –20 mV for 100 ms. Basal currents were determined by depolarization from holding potentials of –20 mV for 100 ms. Data are expressed as the ratio of the facilitated current (I_F) to the basal current (I_B), with each point representing the mean of observations on one to three cells. Note that T currents are reduced for brief prepulse–test pulse intervals due to incomplete recovery from steady-state inactivation.

in current produced by tyrphostin A47 and A25 is equal to, but never exceeds that produced by a strong depolarizing prepulse. (ii) The amplitude of the prepulse-facilitated current obtained after a depolarizing prepulse is not increased by tyrphostins A47 or A25 (Figure 3). (iii) Tyrphostins A47 and A25 had no stimulatory effect on those cells with large basal currents, in which depolarizing prepulses also failed to produce any enhancement.

These observations suggest a role for protein tyrosine kinase activity in the control of T channel activity. In order to explore this regulatory role further, we examined the effects of tyrosine phosphatase modulators on Ca^{2+} currents. As shown in Figure 4, current amplitude was relatively stable during repetitive depolarization at low frequency (<0.1 Hz) and voltage-dependent facilitation was observed following a depolarizing prepulse (point '1').

When 10 μM phenylarsine oxide, a specific and membrane-permeant protein tyrosine phosphatase inhibitor, was added to the external solution, there was a marked decline in the amplitude of both the basal current and the facilitated current produced by a depolarizing prepulse (Figure 4). However, the loss of facilitated current is always more rapid, and within 100–200 s there is no longer additional Ca^{2+} current as a result of a depolarizing prepulse (Figure 4, point '2').

Similar results were obtained when 1 mM Na^+-orthovanadate is added to the pipet solution. This compound is a membrane-impermeant inhibitor of a wide range of phosphatases, including protein tyrosine phosphatases. The additional current produced by a facilitating prepulse is reduced by 50% during 600 s of recording in the presence of Na^+-orthovanadate, whereas only a 15% reduction was observed in parallel experiments in the absence of this antagonist ($n = 5$). The requirement for intracellular diffusion of Na^+-orthovanadate from the pipet to its site of action most likely contributes to its relatively slow time course and low potency. These data support the hypothesis that the spermatogenic cell T current is regulated by its tyrosine phosphorylation state.

In contrast, modulators of serine/threonine phosphorylation, such as H-7 and H-9, do not increase the basal Ca^{2+} currents (200 mM; $n = 9$ for each). Furthermore,

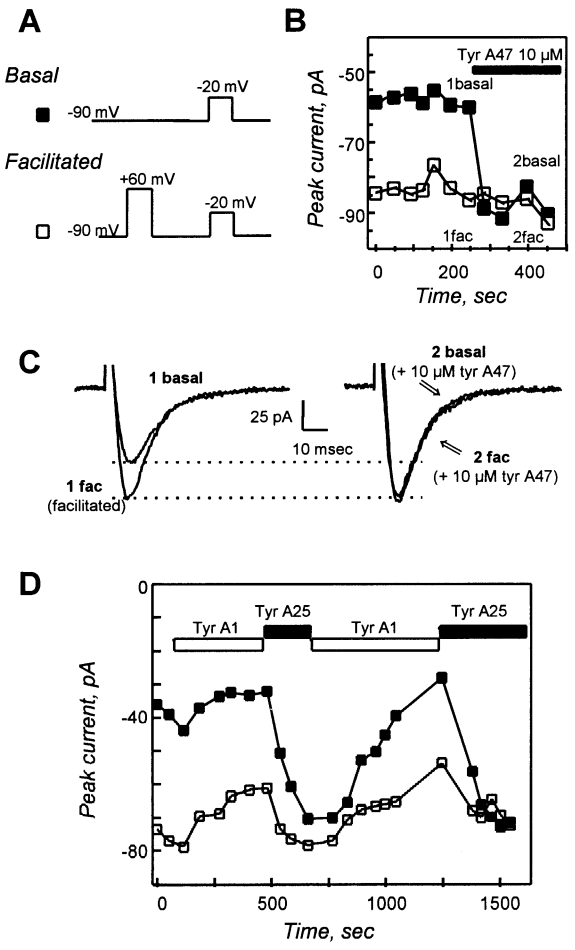

Fig. 3. The effects of protein tyrosine kinase inhibitors on the voltage-dependent facilitation of the mouse spermatogenic cell T-type Ca^{2+} current. Recordings were obtained from cells in the round spermatid stage of spermatogenesis and are representative of the responses of meiotic (spermatocyte) and post-meiotic (spermatid) stages of differentiation. (**A**) Voltage protocol for evoking basal (■) and facilitated (□) currents . The facilitated current is produced by a 100 ms depolarizing prepulse that is delivered 500 ms prior to test depolarization. Basal and facilitated currents at a single time point are obtained within 5 s and these observations are repeated at 50 s intervals. (**B**) Time course of the effects of tyrphostin A47 on voltage-dependent facilitation. Currents were evoked by the basal and facilitated voltage protocols (see A) for 250 s and then 10 μM tyrphostin A47 was introduced into the bath by perfusion (black bar). The basal current rapidly increases to the level of the facilitated current, whereas there is no effect on the peak current amplitude of the voltage-dependent facilitated current. (**C**) Current traces obtained at time points '1' and '2' from the round spermatid shown in (B). Note that at 'point 1', prior to the addition of tyrphostin A47, the voltage-dependent facilitation protocol enhances peak current amplitude by ~40% without affecting either the activation or inactivation kinetics. Following addition of tyrphostin A47 ('point 2'), the amplitude of the current evoked by a basal voltage protocol increases to the level of the facilitated current. (**D**) Time courses illustrating the effects of tyrphostins A1 and A25 on the voltage-dependent facilitation of T currents. Currents were obtained as described in (A). Tyrphostin A1 (50 μM) had no effect on the T current of this spermatid, whereas 50 μM tyrphostin A25 enhances the current evoked by a basal voltage protocol to the level of the facilitated current. Tyrphostin A25 is a potent inhibitor of some tyrosine kinases, whereas the A1 compound is relatively inactive. Note that the effects of tyrphostin A25 are reversible, as was also observed for tyrphostin A47 (not shown).

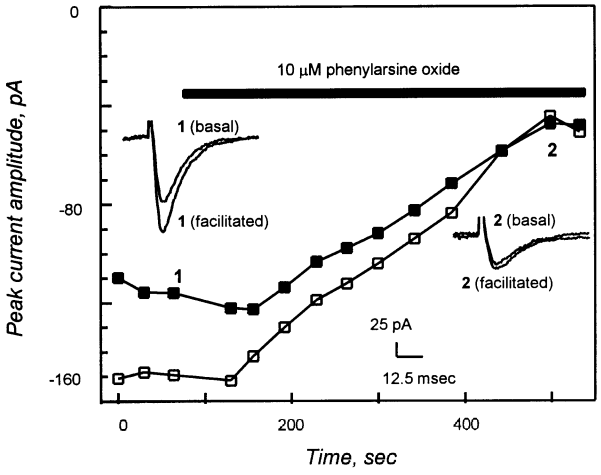

Fig. 4. Time course of the effects of the protein tyrosine phosphatase inhibitor, phenylarsine oxide, on the voltage-dependent facilitation of the mouse spermatogenic cell T current. Recordings were obtained from cells in the pachytene spermatocyte stage and are representative of the responses of meiotic (spermatocyte) and post-meiotic (spermatid) stages of differentiation. The voltage protocols used to evoke basal (■) and facilitated currents (□) are identical to those illustrated in Figure 3A. The peak current amplitude was obtained before and after introduction of 10 μM phenylarsine oxide by bath perfusion (black bar). Note that phenylarsine oxide inhibits both the basal and facilitated current amplitudes, yet the rate of inhibition of the facilitated current is greater. Insets: current traces obtained before the addition of phenylarsine oxide ('point 1') and in the presence of inhibitor ('point 2') are shown. The current and time scales for both traces are 25 pA and 12.5 ms.

H-7 does not affect the relative amplitude of the facilitated current obtained following depolarizing prepulses (data not shown). Okadaic acid (1 μM), an inhibitor of the protein phosphatase 2A, has no effect on the amplitude of the basal or the facilitated currents (data not shown). Finally, neither the basal nor the facilitated Ca^{2+} currents of spermatogenic cell are affected by the addition of 500 μM guanine nucleotides (GDP, GTP) or of their thiophosphate derivatives (GDPβS, GTPγS) to the pipet solution (data not shown).

Discussion

We have demonstrated that the T-type Ca^{2+} current is enhanced when mouse spermatogenic cells are subjected to either a depolarizing prepulse or to inhibition of protein tyrosine phosphorylation. Three types of evidence indicate that these two treatment protocols produce the same facilitated state. (i) The extent of the effects of these treatments are similar. For example, the basal current is enhanced selectively by tyrphostin A25 and A47 to the level of the facilitated current, but these inhibitors of protein tyrosine kinase activity never produce currents that exceed the facilitated level. Similarly, protein tyrosine phosphatase inhibitors selectively suppress the facilitated current to the level of the standard current, but never produce currents that are smaller than the affected basal current. (ii) The effects of these protocols are not additive. For example, tyrphostin treatment precludes further enhancement of current by a depolarizing prepulse. (iii) The effects of a depolarizing prepulse and of an altered protein tyrosine phosphorylation state on the biophysical characteristics of the T current are similar: both treatments

alter the current amplitude without affecting either the activation or the inactivation kinetics.

The indistinguishable biophysical characteristics of the basal and facilitated currents suggest that facilitation is due to an increase in the T current rather than the recruitment of a class of different cryptic channels, such as may occur in chromaffin cells (Artalejo *et al.*, 1992). The enhancement of spermatogenic cell T currents can be due either to an increase in the number of functional channels or to alteration in the conductance characteristics of individual T channels. Taken together with the conclusion that a single facilitated state is produced by either experimental treatment, these observations suggest that spermatogenic cell T-type Ca^{2+} channels are present in two functional states: a low conductance, or even silent, basal state and a higher conductance facilitated state. Transitions between these functional states are promoted either by voltage stimulation or by modulation of protein tyrosine phosphorylation. Single channel recordings will be required to assess this model.

Voltage-dependent facilitation has been reported previously for Ca^{2+} channels, as well as a number of other voltage-sensitive ion channels (Fenwick *et al.*, 1982). This stimulation has been attributed to several mechanisms, including phosphorylation of channel subunits through either Ca^{2+}- or voltage-dependent protein kinases as well as the voltage-dependent relief of a G protein-mediated inhibition (Hille, 1994; Dolphin, 1996). However, two observations indicate that a Ca^{2+}-regulated process is unlikely to mediate the voltage-dependent facilitation of the spermatogenic cell T channel: (i) the effects of a depolarizing prepulse are observed when Ba^{2+} replaces Ca^{2+} as a charge carrier; and (ii) internal Ca^{2+} is buffered in all recordings by EGTA or by BAPTA.

It is also unlikely that the voltage-dependent facilitation of the spermatogenic T-type current is due to the reversal of G protein-dependent inhibition. In a number of other cellular systems, external signals suppress Ca^{2+} currents through a G protein-mediated mechanism, and voltage-dependent facilitation is due to the reversal of this inhibition (Doupnik and Pun, 1994; Roche *et al.*, 1995; Dolphin, 1996). The voltage-dependent reversal of G protein-mediated inhibition has been studied extensively in the high voltage-activated class of Ca^{2+} channels.

However, several observations are inconsistent with the presence of a similar G protein-mediated pathway in the facilitation of spermatogenic cell T currents. First, facilitation by either a depolarizing prepulse or by the application of tyrosine kinase inhibitors is not affected by the intracellular addition of G protein inhibitors (GDPβS or absence of GTP) or activators (GTPγS or GTP). For example, while GDPβS inhibits either the voltage-dependent facilitation or the receptor-mediated inhibition of Ca^{2+} currents in several other preparations (Grassi and Lux, 1989; Buisson *et al.*, 1992, 1995; Doupnik and Pun, 1994; Roche *et al.*, 1995; Alvarez *et al.*, 1996), we were able to record stable facilitation for long periods (10–15 min) in the presence of this G protein blocker. Second, the duration of the voltage-dependent facilitation of the spermatogenic cell T current is not consistent with the presence of a G protein-mediated pathway. We observed that a depolarizing prepulse produces a relatively long-lived facilitation that decays with a time constant of 4.8 s. In contrast, facilitation is transitory in those examples in which it is due to removal of G protein-mediated inhibition and decays with a time constant of ~30 ms (Tsunoo *et al.*, 1986; Golard *et al.*, 1993). Finally, a characteristic feature of voltage-dependent relief of G protein inhibition is an increased rate of current inactivation (Hille, 1994; Roche *et al.*, 1995; Alvarez *et al.*, 1996), whereas the facilitated current of spermatogenic cells does not exhibit any change in kinetics (Figure 1C).

The most plausible mechanism for the control of this T current is by the regulation of tyrosine phosphorylation. The pharmacological experiments presented here suggest a model in which dephosphorylation of key but unidentified tyrosine residues accounts for the transition of the channel to the facilitated state, whereas the phosphorylation of these residues produces the lower conductance basal state. It is now well accepted that tyrosine phosphorylation alters the function of the nicotinic acetylcholine receptor (reviewed by Huganir, 1991) and of a number of voltage-gated ion channels (reviewed by Siegelbaum, 1994; Jonas and Kaczmarek, 1996). In addition, it has been suggested that tyrosine phosphorylation also modulates Ca^{2+} channel function, including a possible role in the angiotensin II-dependent inhibition of the T-type current of NG108-15 cells (Buisson *et al.*, 1992, 1995). The consensus of these previous studies is that Ca^{2+} currents are increased by tyrosine phosphorylation. In contrast, the T channel of mouse spermatogenic cells represents, to the best of our knowledge, the first example of a Ca^{2+} current that is potentiated by a voltage-dependent dephosphorylation process.

The inhibitory and facilitatory modulation of T currents by protein tyrosine kinases and tyrosine phosphatases, respectively, may control sperm function during mammalian fertilization. We have shown recently that T channels are retained on the mature sperm cell following the completion of spermatogenesis (Arnoult *et al.*, 1996). Prior to penetrating the egg's zona pellucida and fusing with the egg plasma membrane, sperm must complete the acrosome reaction, a Ca^{2+}-dependent exocytotic event. Zona pellucida contact activates sperm T channels, and the resultant Ca^{2+} entry is essential for the acrosome reaction (Arnoult *et al.*, 1996). Sperm have only a single secretory vesicle, and available evidence indicates that cells that complete the acrosome reaction prior to contact with eggs are infertile (Ward and Kopf, 1993; Yanagimachi, 1994). Thus, a key feature of sperm physiology is the suppression of spontaneous exocytosis until the activation of specific signal transducing pathways by the zona pellucida glycoprotein, ZP3 (Ward and Storey, 1984; Florman and First, 1988; Florman and Babcock, 1990).

A second characteristic of mammalian sperm is that fertility is only expressed during the final phase of functional maturation, or capacitation, that occurs within the female reproductive tract prior to sperm–egg contact (Yanagimachi, 1994). Recently, it was shown that one component of capacitation is the enhanced tyrosine phosphorylation of an array of sperm proteins (Visconti *et al.*, 1995a,b). Tyrosine phosphorylation of the sperm T channel or associated proteins during capacitation would minimize Ca^{2+} influx through this pathway, thereby suppressing spontaneous exocytosis as sperm prepare for fertilization. A corollary of this model, which has not yet

been demonstrated, is that a sperm tyrosine phosphatase is activated by ZP3 during sperm–zona pellucida contact, thus relieving T channel inhibition and permiting egg-induced acrosome reactions.

Sperm receive a variety of chemical signals in addition to that provided by ZP3 as they approach and contact eggs. Progesterone (Foresta *et al.*, 1993) and extracellular ATP (Foresta *et al.*, 1992, 1996) depolarize sperm membrane potential *in vitro* and may be present at the site of fertilization *in vivo*. It is plausible that these and other compounds act either through voltage- or receptor-dependent mechanisms to switch the sperm T channel from the low conductance state to a facilitated state, thereby enhancing the Ca^{2+} current triggered by ZP3.

In sum, the present study demonstrates that the T channel of the mouse male germ cell is modulated by its tyrosine phosphorylation state. In particular, the potentiation of this current by tyrosine dephosphorylation provides a new model for Ca^{2+} channel regulation. This type of modulation may regulate the development of fertility in mature sperm.

Materials and methods

Biological preparations

Seminiferous tubules were isolated from the testis of CD1 mice (16 weeks old; Charles River Laboratories) and incubated at 37°C for 30 min in 3 ml of a solution containing (mM): NaCl (150), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), NaH_2PO_4 (1), $NaHCO_3$ (12), glucose (11), pH 7.3 and collagenase type IA (1 mg/ml, Sigma). Tubules were rinsed twice in collagenase-free medium and cut into 2 mm sections. Spermatogenic cells were obtained by manual trituration and were attached to culture dishes coated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA). The pachytene spermatocyte and the round spermatid are the prominent cell types obtained from the diploid meiotic and the haploid post-meiotic stages of spermatogenesis, respectively. These cells are readily distinguished based on cellular and nuclear size and morphology, as described previously (Romrell *et al.*, 1976; Arnoult *et al.*, 1996). These stages were used routinely for electrophysiological recordings.

Electrophysiological recordings

Ca^{2+} currents were recorded in the whole-cell configuration of the patch–clamp technique (Hamill *et al.*, 1981) and analyzed using Biopatch (BioLogic, FR). Pipets were pulled from 7052 thin glass (Gardner Glass Co., CA), coated with Sylgard 184 (Dow Corning, MI), and fire-polished. Pipet resistance was 2–10 MΩ. Currents were obtained with an Axopatch 1-D amplifier (Axon Inst., Burlingame, CA). All traces were corrected for leakage and capacitance currents, filtered at 3 kHz, and digitized every 250 msec. Other details of the voltage protocols used here are provided in the Results section.

The pipet solution, which was designed to eliminate K^+ currents, contains (in mM): Cs-glutamate (120), TEA-Cl (20), $MgCl_2$ (5), D-glucose (5), MgATP (3), EGTA (10), HEPES (10), pH 7.0 (adjusted with CsOH). Spermatogenic cells were attached to culture dishes during 5 min incubations, following which the bath solution was changed to a standard recording solution, containing (mM): NaCl (100), KCl (5), $CaCl_2$ (10), $MgCl_2$ (1), TEA-Cl (26), Na-lactate (6), HEPES (10), D-glucose (3.3), pH 7.4 (adjusted with 1 M NaOH). The cells were isolated in a 1 ml chamber and perfused at a rate of 4–8 ml/min. Tyrphostin A1, A25, A47, AG1478 (Calbiochem, CA) and phenylarsine oxide (Sigma) were prepared as dimethylsulfoxide (DMSO) stocks. The DMSO concentration during recording was $<0.01\%$ (v/v). H-7 and H-9 (RBI, Natick, MA) were prepared in the standard recording solution. All experiments were preformed at room temperature (~25°C).

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