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Cells: Functional Expression and Implication in Sperm Capacitation

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To fertilize, mammalian sperm must complete a maturational process called capacitation. It is thought that the membrane potential of sperm hyperpolarizes during capacitation, possibly due to the opening of K^+ channels, but electrophysiological evidence is lacking. In this report, using patch-clamp recordings obtained from isolated mouse spermatogenic cells we document the presence of a novel K^+-selective inwardly rectifying current. Macroscopic current activated at membrane potentials below the equilibrium potential for K^+ and its magnitude was dependent on the external K^+ concentration. The channels selected K^+ over other monovalent cations. Current was virtually absent when external K^+ was replaced with Na^+ or *N*-methyl-*D*-glucamine. Addition of Cs^+ or Ba^{2+} (IC_{50} of ~15 μM) to the external solution effectively blocked K^+ current. Dialyzing the cells with a Mg^{2+}-free solution did not affect channel activity. Cytosolic acidification reversibly inhibited the current. We verified that the resting membrane potential of mouse sperm changed from −52 ± 6 to −66 ± 9 mV during capacitation *in vitro*. Notably, application of 0.3–1 mM Ba^{2+} during capacitation prevented this hyperpolarization and decreased the subsequent exocytotic response to zona pellucida. A mechanism is proposed whereby opening of inwardly rectifying K^+ channels may produce hyperpolarization under physiological conditions and contribute to the cellular changes that give rise to the capacitated state in mature sperm.

Key Words: K^+ channel; inward-rectifier; spermatogenic cells; sperm capacitation; sperm acrosome reaction.

INTRODUCTION

Sperm interaction with the oocyte's zona pellucida (ZP) initiates the acrosome reaction (AR), an essential exocytotic event in fertilization. Before the AR, mammalian sperm must complete in the female genital tract a poorly understood maturational process called capacitation. Sperm capacitation can be also accomplished *in vitro* by incubating sperm in defined medium (Yanagimachi, 1994; Visconti *et al.*, 1995). *In vitro* studies have shown that capacitation is accompanied by a plasma membrane hyperpolarization that is thought to be mediated by an increase in K^+ permeability (Zeng *et al.*, 1995; Arnoult *et al.*, 1999). Capacitation takes from minutes to hours and during this process sperm membrane potential changes tens of millivolts, reaching values as negative as −80 mV (Arnoult *et al.*, 1999).

K^+ channels are a family of transmembrane proteins that allow the passive diffusion of K^+ down its electrochemical gradient (Hille, 1992; Coetzee *et al.*, 1999). Biochemical and molecular biology data suggest the presence of different K^+ channels in both mammalian testis and spermatozoa (Schreiber *et al.*, 1998; Salvatore *et al.*, 1999; Jacob *et al.*, 2000). However, electrophysiological investigation of ion channels in the plasma membrane of mature sperm has been precluded by the small size of these cells (Espinosa *et al.*, 1998). An alternative approach has been to study these channels in spermatogenic cells, developmental precursors that synthesize proteins for subsequent use in the translationally quiescent mature sperm. Initial studies revealed the presence of only one functional type of K^+ channel in rodent spermatogenic cells. This current was independent of external Ca^{2+} concentration and blocked by tetraethylammonium chloride (Hagiwara and Kawa, 1984). More recently, the combination of electrophysiology with molecular biology methods has been used to specifically study

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particular K^+ channel subtypes from sperm. Hence, a novel gene abundantly expressed in mammalian spermatocytes encoding a unique type of K^+ channel regulated by both intracellular pH and membrane voltage (called mSlo3) was cloned and expressed in *Xenopus* oocytes (Schreiber *et al.*, 1998). Its functional expression in spermatogenic cells and sperm is yet to be determined. Likewise, RT-PCR experiments revealed high levels of mRNA expression in rat testis of K_{ir} 5.1, a member of the inward rectifier K^+ channel superfamily. However, the function of this channel remains unknown since it does not form functional channels when expressed in *Xenopus* oocytes (Salvatore *et al.*, 1999). It has been also suggested that mammalian sperm may possess a Ca^{2+}-activated K^+ (MaxiK-type) channel, which has been implicated in the process of sperm activation and gamete interaction (Wu *et al.*, 1998). Considering this, we further characterized the functional whole-cell K^+ currents in mouse spermatogenic cells and examined their influence in the development of the capacitated state in mature sperm.

MATERIALS AND METHODS

Cell preparation. Spermatogenic cells were obtained following the procedure previously described by Espinosa *et al.* (1999) with minor modifications. Briefly, testes from adult CD1 mice were excised and suspended in ice-cold dissociation solution containing (in mM) NaCl 130; KCl 3; $MgCl_2$ 2; $NaHCO_3$ 1; NaH_2PO_4 0.5; Hepes 5; glucose 10 (pH 7.4) supplemented with DNase I (100 μg/ml). The tunica albuginea was removed and the seminiferous tubules were separated using tweezers under a stereoscopic microscope. Tubules were dispersed into individual cells or synplasts using Pasteur pipettes. The cells were collected by centrifugation (at 2500 rpm for 4 min), resuspended in external recording solution (see below), and stored on ice until assayed. Under these conditions, spermatogenic cells remained totally healthy for as long as 8 h. Although this cell suspension contains a heterogeneous cell population, individual spermatogenic cells or synplasts at three different stages of differentiation are preferentially present as determined by bright-field microscopy: pachytene spermatocytes, round spermatids, and condensing spermatids. These cells were used routinely in the electrophysiological recording. Inasmuch as similar results were obtained from these stages, data were pooled for presentation. Ten-microliter aliquots of cell suspension were dispensed into a recording chamber (~200-μl total volume) on the stage of an inverted microscope and subjected to electrophysiological recording as detailed below.

Caudal epididymal mouse mature sperm were mechanically collected from CD1 mice and placed in 1.5-ml microcentrifuge tubes containing 700 μl of medium 199 supplemented with BSA (0.4% wt/vol), sodium pyruvate (30 mg/liter), and $NaHCO_3$ (4.2 g/liter) at 37°C. After 5–10 min the fraction of motile sperm was determined by visual inspection and preparations with <75% motile cells were discarded. For *in vitro* capacitation, 300- to 400-μl aliquots of the sperm suspension (4–5 $\times$ 10^6 cells/ml) were incubated at 37°C as described previously (Visconti and Kopf, 1998; Visconti *et al.*, 1999). Following an incubation period of 30 min, sperm were collected and then assayed for the AR.

Electrophysiology. K^+ currents were recorded according to the whole-cell patch-clamp technique (Hamill *et al.*, 1981). All recordings were performed at room temperature using an Axopatch 200 A patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ micropipettes manufactured from borosilicate glass capillary tubes (KIMAX-51; Kimble Division, Owens-Illinois, Inc., Toledo, OH) on a horizontal puller (Sutter Instrument Co., Novato, CA). Unless otherwise stated, cells were clamped at a holding potential of 0 mV and capacity transients were electronically compensated. Currents were evoked by 200- to 1800-ms hyperpolarizing voltage steps (0.5 Hz) to test potentials ranging from −120 to +50 mV. Pulse protocols, data capture, and analysis of recordings were performed using pCLAMP software (Axon). Current records were captured on-line and digitized at a sampling rate of 5–10 kHz following filtering of the current record (2 kHz; internal 4-pole Bessel filter) using a personal computer attached to a DigiData 1200 interface (Axon). Linear leak-current component was subtracted off-line using the clampfit analysis routine of pCLAMP. To isolate inwardly rectifying K^+ currents, cells were bathed in different solutions as listed in Table 1. Bath solutions were adjusted to pH 7.3 with KOH and 300 ± 5 mosml $liter^{-1}$ with sucrose. The composition of internal (patch pipette) solutions used in this study is also given in Table 1. The control internal solution consisted of (mM): K^+-methanesulfonate ($MeSO_3$) 122; KF 20; $CaCl_2$ 2.5; $MgCl_2$ 1; EGTA 5; Hepes 10; (pH 7.3/KOH, 275 ± 5 mosml $liter^{-1}$ with sucrose).

Assay for acrosome reaction. As one end point of capacitation, we analyzed the ZP-induced AR in sperm, based on the premise that only capacitated sperm would undergo exocytosis in response to ZP. The percentage of AR was measured using Coomassie blue G-250 as described elsewhere (Visconti *et al.*, 1999). Briefly, sperm were incubated at 37°C for 30 min followed by the addition of 5 ZP eq/μl. After an additional 30 min incubation at 37°C, an equal volume of 2× fixative solution (10% formaldehyde in phosphate-buffered saline) was added to each tube. Following fixation, 10-μl aliquots of sperm suspension were spread onto glass slides and air-dried. The slides were then stained with 0.22% Coomassie blue G-250 in 50% methanol and 10% glacial acetic acid for 3–5 min, gently rinsed with deionized H_2O, air-dried, and mounted with 50% (v/v) glycerol in phosphate-buffered saline. To calculate the percentage of AR, at least 100 sperm were assayed per experimental condition for the presence or absence of the characteristic dark blue acrosomal crescent.

Measurement of membrane potential. Mature sperm (40 μl at 40 $\times$ 10^6 cells/ml) were capacitated *in vitro* as described above. After a 30-min incubation, the potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$; Molecular Probes, Eugene, OR) was added to the cell suspension at a final concentration of 1 μM. The fluorescence was then monitored with a Hansatech MkII fluorometer (Norfolk, UK) at 620/670-nm excitation/emission wavelength pair (Espinosa and Darszon, 1995). Cell hyperpolarization decreases the dye fluorescence. Recordings were initiated after dissipating mitochondrial membrane potential with 100 nM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma) and reaching a steady-state fluorescence (1–3 min). Fluorescence changes were converted to membrane potential (V_m) as follows. First, extracellular K^+ concentration was changed by adding KCl (1.25 to 10 mM final concentration) in the presence of 650 nM valinomycin (Sigma Chemical Co., St. Louis, MO), and the fluorescence was measured. V_m was then calculated according to the Nernst equation (Hille, 1992), assuming an intracellular K^+ concentration of 120 mM and that the membrane is a K^+ electrode under the influence of the K^+-selective ionophore valinomycin (Espinosa and Darszon, 1995). Finally, V_m in the absence and

TABLE 1
Composition of Solutions (mM)

	Bath solutions (BS)[a]							
BS	K-$MeSO_3$	NMDG-$MeSO_3$	Na-$MeSO_3$	RbOH	NaOH	KOH	$KOOCCH_3$	pH
I	150	—	—	—	—	—	—	7.4
II	2	—	126	—	—	—	—	7.4
III	2	126	—	—	—	—	—	7.4
IV	128	—	—	—	—	—	—	7.4
V	30	98	—	—	—	—	—	7.4
VI	60	68	—	—	—	—	—	7.4
VII	—	68	—	—	—	60	—	7.4
VIII	—	68	—	—	60	—	—	7.4
IX	—	68	—	60	—	—	—	7.4
X	75	—	—	—	—	—	75	8.1
XI	75	—	—	—	—	—	75	6.3
XII	150	—	—	—	—	—	—	6.3

	Pipette solution (PS)[b]						
PS	$MgCl_2$	Hepes	EGTA	EDTA	K-$MeSO_3$	KCl	pH
I	1	10	5	—	122	8	7.35
II	—	10	—	5	120	10	7.35
III	1	5	5	—	122	8	7.35

[a] All solutions contained 6.5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 3.3 glucose.
[b] All solutions contained 20 KF, 2.5 $CaCl_2$.

presence of 1 mM Ba^{2+} was calculated from the fluorescence intensity using this calibration curve.

RESULTS

A Hyperpolarization-Activated Inward Current

Previous patch-clamp studies have shown that the K^+ permeability of the plasma membrane in spermatogenic cells is controlled mainly by K^+-selective channels of the delayed rectifying type (Hagiwara and Kawa, 1984). Herein, we present evidence for an inwardly rectifying K^+ current in mammalian spermatogenic cells. The presence of inwardly rectifying K^+ channels in these cells was documented with a series of whole-cell patch-clamp experiments in which both the patch electrode and the cell bathing solution contained 150 mM K^+ (BSI, PSI; Table 1). Examples of the resulting current records are presented in Fig. 1A, which shows typical fast activating, noninactivating whole-cell inward currents of decreasing amplitude from an isolated mouse spermatogenic synplast clamped to 0 mV, in response to voltage steps of 200-ms duration applied from −110 to +50 mV in 10-mV increments. Immediately after the instantaneous jump in the inward direction, the currents underwent a slight reduction in amplitude most likely due to external Mg^{2+} and/or Ca^{2+} blockade (Jow and Numann, 1998). Even though negative applied potentials clearly activated macroscopic currents, no detectable outward current was observed at positive potential values, indicating an inwardly rectifying current–voltage (*I–V*) relationship (Hagiwara *et al.*, 1976). In this case, the *I–V* curve obtained in symmetrical high K^+ (not shown) pivots at 0 mV, indicating that K^+ ions carried the majority of the measured current (see below). Inasmuch as the presence of Cl^- channels has been reported in mammalian spermatogenic cells and sperm (Espinosa *et al.*, 1998), low symmetric Cl^- solutions (Table 1) were used to minimize the Cl^- background currents. Under these conditions the contribution of a Cl^- conductance to the inward current recorded in spermatogenic cells was negligible. Together these results are consistent with the expression of an inwardly rectifing K^+ current (K_{ir}) in spermatogenic cells. It should be noted that after the activating pulses used to evoke the inwardly rectifying K^+ current, a prominent time-dependent and rapidly inactivating inward current was observed when the voltage returned to holding potential (Fig. 1A). This most likely corresponds to the T-Ca^{2+} current previously reported and characterized in spermatogenic cells (Arnoult *et al.*, 1998; Santi *et al.*, 1996). When examined in detail, these currents showed voltage-dependent activation and inactivation with faster kinetics at more depolarizing voltages producing the criss-crossing pattern typical of T-type currents (not shown).

Dependence on Voltage and $[K^+]_o$

Additional experiments were carried out to confirm the identity of the inward current in spermatogenic cells. In the

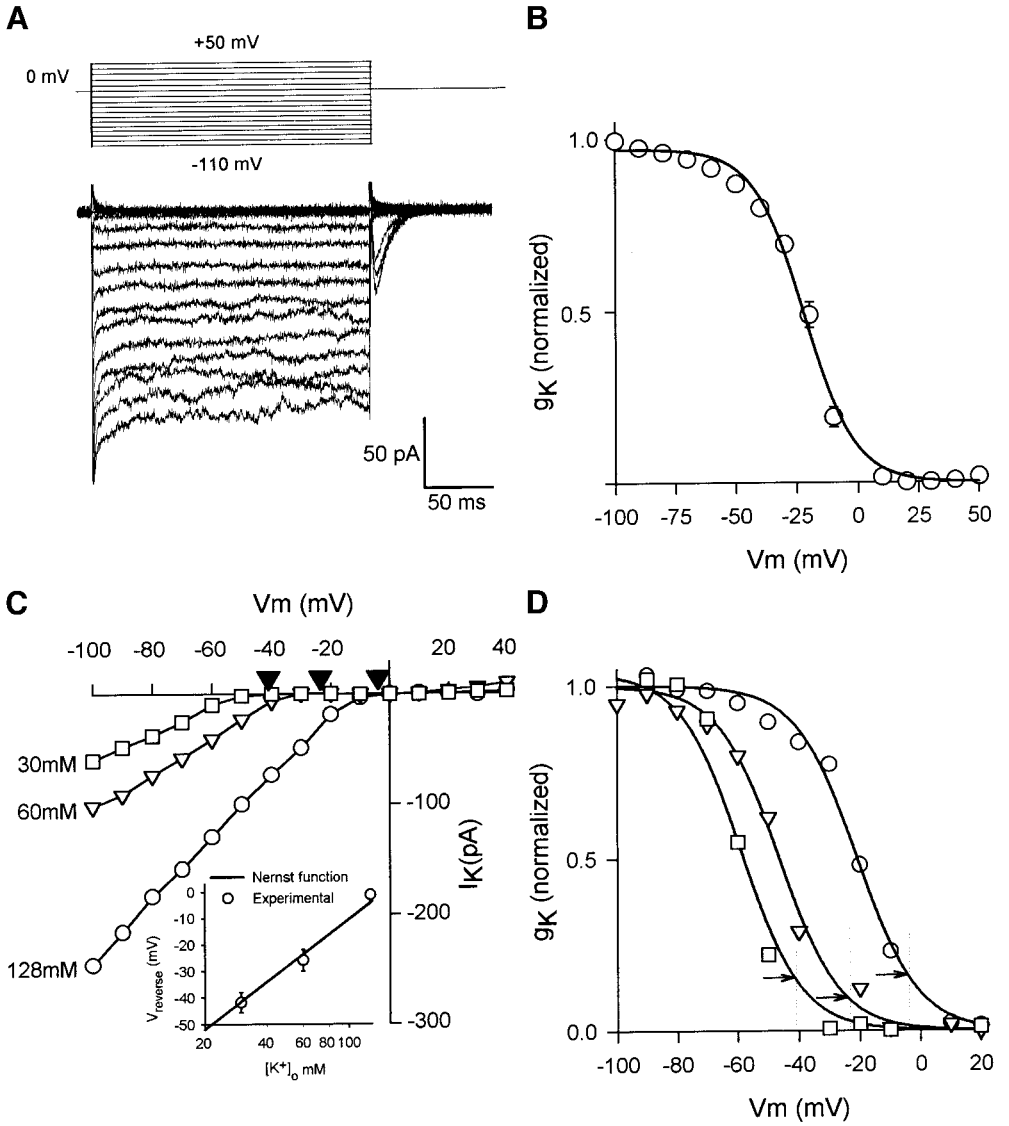

FIG. 1. Inwardly rectifying K^+ current in mouse spermatogenic cells. (A) Typical current responses recorded by using 10-mV voltage steps between −110 and +50 mV starting from a holding potential of 0 mV in symmetrical K^+ recording solutions (Table 1, BSI and PSI). The inward rectifier activates at potentials negative to V_K. Schematic representation of the voltage protocol is shown above the traces. All voltage-clamp recordings shown in this and subsequent figures are single traces and were obtained at room temperature. (B) Normalized conductance–voltage (*g–V*) curve at the steady state of currents obtained in a number of cells as illustrated in A. The voltage dependence of the measured conductance for K_{ir} channels followed a typical Boltzmann function with saturation. The conductance (g_K) at each membrane potential was estimated as $g_K = I_K/(V_m - V_K)$, where I_K is peak current amplitude and $V_m - V_K$ represents the driving force. Assuming that g_K was maximal when the command voltage step from 0 to −110 mV was applied, the conductance values were normalized as a function of membrane potential, and then the data were fitted to the Boltzmann equation, $I_K = [g(V_m - V_K)]/(1 + \exp[(V_m - V_{1/2})/s])$, where g is the conductance, V_m represents the test potential, V_K is the apparent reversal potential, and s is the range within the potential changes e-fold around $V_{1/2}$. Symbols represent means ± SEM of nine experiments; most of the SEM bars were smaller than symbols. (C) The reversal potential of the inward rectifier current in spermatogenic cells is displaced according to V_K. Currents were recorded from isolated cells in response to voltage steps in 10-mV increments from a holding potential of 0 mV (range −110 to +50 mV) in the presence of 30 (open squares), 60 (open triangles), and 128 mM (open circles) external K^+ (Table 1; BSV, BSVI, and BSIV, respectively). *I–V* curves at the steady state of currents are shown. Zero-current values (reversal potentials) of inward currents are in close agreement with theoretical V_K calculated by the Nernst equation (indicated by inverted filled triangles on the abscissas). The inset shows the relationship between reversal potential of the currents and extracellular K^+, measured by extrapolating the *I–V* curves. Each symbol represents the mean ± SD of at least five cells. (D) Normalized *g–V* curves of K_{ir} channels in a spermatogenic cell bathed with solutions containing 128, 60, and 30 mM potassium-$MeSO_3$, as in C. Conductance is plotted versus the membrane voltage and fitted by a Boltzmann function (smooth curves). The arrows denote V_K and the region to the right of the dotted line in each curve is the fraction of open channels at potentials positive to E_K.

presence of 128 mM K^+-$MeSO_3$ in the external recording solution current traces showed the typically noninactivating inward currents described previously. In contrast, replacement of extracellular K^+ by *N*-methyl-*D*-glucamine ($NMDG^+$) or Na^+ (Table 1; BSIII and BSIV, respectively) completely abolished the inward current, indicating that these two cations are quite impermeable (data not shown). Likewise, different concentrations of external K^+ were used to corroborate that the recorded current from spermatogenic cells was due to the activation of a K^+ permeability of the inward rectifier type (Hagiwara and Yoshii, 1979). Decreasing the K^+ concentration in the bath solution from 128 to 30 mM K^+-$MeSO_3$ resulted in a negative shift of the reversal potential value (Fig. 1C). The observed shift is close to −40 mV, the expected variation in zero current potential for a highly K^+-selective current. Hence, inward rectification was found to depend on the K^+ concentration, with no detectable outward currents at potentials positive to the K^+ reversal potential (V_K). The inset in Fig. 1C summarizes data on reversal potentials for at least five cells, showing good agreement between the measured reversal potential for the whole-cell currents as a function of external K^+ (open circles) and the potentials calculated using the Nernst equation (solid line). Along with these marked shifts in the reversal potential, the increased inward current amplitude was associated with an increased conductance. Figure 1D demonstrates that conductance, calculated as described above, was dependent on $[K^+]_o$, as determined from the *I–V* relationships obtained in cells bathed with K^+-containing solutions ranging from 30- to 128-mM. Interestingly, Fig. 1D also shows that at all $[K^+]_o$ tested there is a fraction of channels (about 9–16%) that are open at potentials near to the right of V_K (Fig. 1D, arrows).

Ionic Selectivity

The cation selectivity of the current was then investigated in synplasts with 150 mM $[K^+]_i$ and replacing $[K^+]_o$ with equimolar Rb^+ or Na^+ (Fig. 2A). The *I–V* plots obtained are shown in Fig. 2B. Selectivity was assessed from permeability ratios calculated from the reversal potentials. The profile of selectivity was $K^+ > Rb^+ > Na^+$. The permeability ratios of Rb^+ and of Na^+ over K^+ estimated using a modified Goldman-Hodgkin-Katz function were 0.21 and <0.021, respectively. Based on this empirical measure of ion selectivity, the channel exhibited the same ion-selective sequence as other reported inwardly rectifying K^+ channels (Hille, 1992).

Action of External Ba^{2+} and Cs^+

One of the main characteristic features of inward rectifiers is their susceptibility to blockade by external Ba^{2+} and Cs^+ (Hagiwara *et al.*, 1976, 1978). Figure 3 shows an example of whole-cell current recordings obtained with 300 μM Ba^{2+} (Fig. 3A) or Cs^+ (Fig. 3B) added to the 150 mM K^+-$MeSO_3$ bath solution. External Ba^{2+} completely abolished the macroscopic current at all voltages tested. A concentration-dependent current block was also observed with external Cs^+ (not shown). However, the block for Cs^+ was voltage dependent and largely confined to more hyperpolarizing potentials. Little or no observable block of membrane conductance was detected at potentials more depolarized than −60 mV. Figure 3C summarizes the results obtained from four different experiments where the inhibition in current amplitude during Ba^{2+} and Cs^+ block is plotted as a function of voltage. These results confirm the presence of an inwardly rectifying channel in spermatogenic cells characterized by a high sensitivity to both Ba^{2+} and Cs^+ ions. To quantitatively evaluate the degree of block, a dose–response curve was constructed using current values measured at the plateau (−90 mV) in the presence of different extracellular concentrations of Ba^{2+}. As illustrated in Fig. 3D, the effects of Ba^{2+} were dose-dependent and complete block required concentrations over 300 μM. The normalized currents were fitted with a sigmoid isotherm according to the following formalism: $f = a/(1 + \exp(-(x-x0)/b))$; a half-maximal inhibitory concentration (IC_{50}) of 15.4 μM was calculated.

Mechanisms of Rectification

Investigations in cloned inwardly rectifying K^+ channels have indicated that rectification can be fully explained considering channel block by intracellular Mg^{2+} and polyamines (Yang *et al.*, 1995; Taglialatela *et al.*, 1995). We examined the rectification of the native inwardly rectifying K^+ channels in spermatogenic cells using step depolarizations. Spermatogenic cells were dialyzed with one of two internal solutions (Table 1): (1) the control solution containing 1 mM Mg^{2+} in the form of $MgCl_2$ and (2) the nominally Mg^{2+}-free pipette solution containing a 5 mM concentration of the Ca^{2+} and Mg^{2+} chelator EDTA. Cells were bathed in external solution that contained 150 mM K^+-$MeSO_3$. The inwardly rectifying profile of the conductance was unchanged when Mg^{2+} was omitted in the intracellular solution (data not shown), thereby indicating that the rectification may not be attributable to a modulating effect of intracellular Mg^{2+}, as has been described for some kinds of inwardly rectifying K^+ channels (Hille, 1992). Hence, upon depolarizing voltage to 100 mV, outward currents decreased first rapidly after capacitative transients and then decayed more slowly describing the blockade of channels. The time course of these currents was well described by the sum of two exponential functions, and notably the time constants of blockade (τ_{block}) remained unaffected regardless of the absence of Mg^{2+}. According to this, τ_{fast} was 0.7 ± 0.2 and 0.7 ± 0.3 ms for the control ($n = 6$) and 0 Mg^{2+} ($n = 3$) solutions, whereas at the same voltage τ_{slow} was 7.5 ± 1.4 and 7.4 ± 1.7 ms, respectively. Since channel blocking by Mg^{2+} and channel blocking by polyamines are the two proposed mechanisms of rectification, it seems reasonable to propose that in the absence of Mg^{2+} ions the endogenous

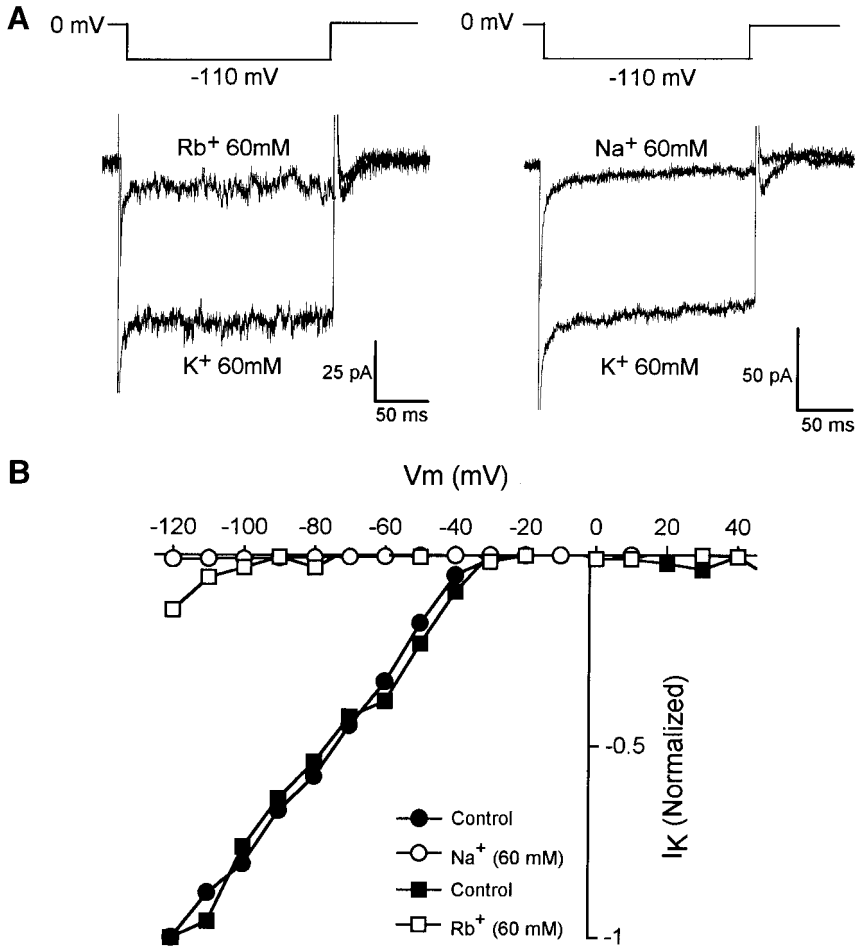

FIG. 2. Cation selectivity of inwardly rectifying K^+ currents in spermatogenic cells. (A) Inward currents recorded in mouse synplasts in response to 200-ms hyperpolarizing pulses applied from a holding potential of 0 mV to −110 mV. Cells were bathed in external solutions containing 68 mM $NMDG^+$ and 60 mM XOH, where X was K^+, Na^+, or Rb^+ (Table 1; BSVII, BSVIII, and BSIX, respectively). (B) *I–V* relationships for whole-cell inward currents in the same cells shown in A obtained during exposure to control (filled symbols) and to Na^+- and Rb^+-containing solutions (open symbols).

polyamines are responsible for inward rectification of the K_{ir} channels recorded in spermatogenic cells.

Modulation by Internal pH

We next investigated whether inwardly rectifying K^+ macroscopic whole-cell currents from spermatogenic cells were subjected, like other types of inward rectifiers, to pH regulation (Hagiwara *et al.*, 1978). A complete family of currents illustrating the effect of lowering pH in a synplast is given in Fig. 4A. The cell was maintained at 0 mV throughout the recording. In this experiment, the pH of the extracellular recording solution (150 mM K^+-$MeSO_3$) was changed from 7.4 (upper left panel) to 6.3 by titrating with $MeSO_3$ (upper right panel) and the current was measured. Under these recording conditions, in no case did a reduction in external pH (pH_o) cause significant changes in current amplitude ($n = 4$). To examine the effects of internal pH (pH_i) on macroscopic conductance we used a bath solution containing (in mM) 75 methanesulfonate and 75 K^+ acetate (pH 6.3). As previously described (Choe *et al.*, 1997), pH_i can be modified with membrane-permeable acetate solutions. Although pH_i was not directly measured in this experiment, in contrast to what was observed when pH_o was lowered in the solution containing the membrane-impermeable K^+ methanesulfonate, equimolar replacement of $[K^+]_o$ with the membrane-permeable K^+ acetate solution completely inhibited the current (Fig. 4, lower left panel). This inhibition could be partially reversed by returning to the 150 mM K^+-$MeSO_3$ control bath solution (lower right panel). Figure 4B compares current values at different voltages recorded during a decrease in the bath pH from 7.4 to 6.3 in several spermatogenic cells. Inward currents were normalized to the maximum value in the control. Filled

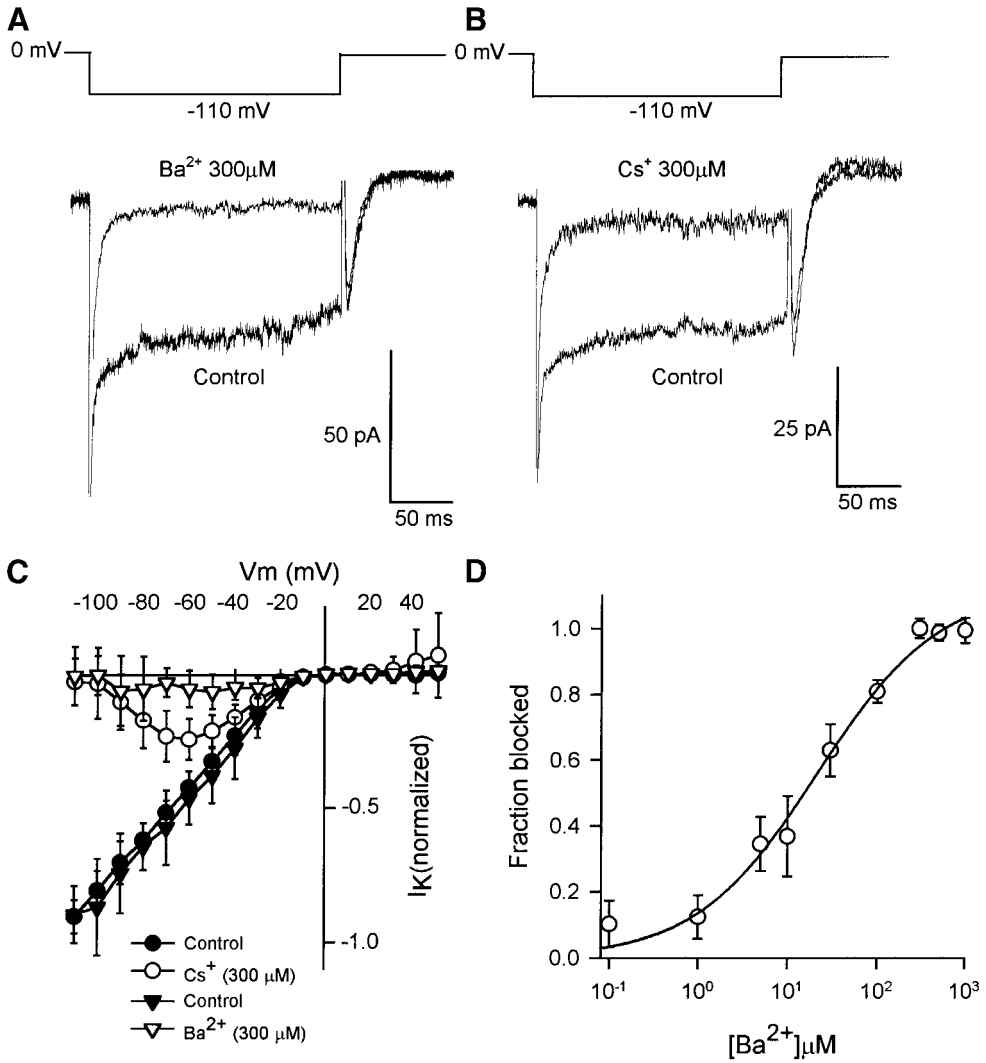

FIG. 3. Ba^{2+} and Cs^{+} block the inwardly rectifying current. (A and B) Current responses to voltage steps to −90 mV from a holding potential of 0 mV before and after superfusion of BSI supplemented with 300 μM Ba^{2+} or Cs^{+}, respectively. (C) Steady-state *I–V* relationships for a number of cells as shown in A and B. Note that the *I–V* curve in the presence of Cs^{+} shows a region of negative slope conductance at potentials in excess of −60 mV revealing a voltage-dependent blocking by this cation. Symbols represent means ± SD of four independent experiments. (D) Dose-dependent block of the steady-state inward currents at −90 mV by Ba^{2+}. Currents were obtained in response to 200-ms hyperpolarizations to −90 mV from a holding potential of 0 mV, measured at steady state and normalized to the control. Symbols represent mean values of the fraction of current blocked at various Ba^{2+} concentrations, and vertical lines indicate means ± SD (n = 3–7). The smooth curve is the least-squares fit of mean values to a sigmoid function (see Results) with an IC_{50} value of 15.4 μM.

triangles depict control inward currents where the bath recording solution contained 150 mM K^{+}-$MeSO_3$; the *I–V* relation was linear below V_K. Open circles are normalized current values after changing pH_o (with no change in pH_i) to 6.3. Filled circles denote inhibited inward currents measured 5 min after the change in the composition of the bath solution as described above. At this time the currents and presumably pH_i should both be in a steady state. Open triangles represent the normalized currents after the K^{+} acetate solution was washed out. Together, these results strongly suggest that the K_{ir} channels in spermatogenic cells are pH_i sensitive.

Implication in Sperm Capacitation

It is well known that during *in vitro* capacitation, the membrane potential of mature mammalian sperm hyperpolarizes from approximately −50 mV to −80 mV (Arnoult *et al.*, 1999). To establish whether this hyperpolarization is correlated with the activation of inwardly rectifying K^{+}

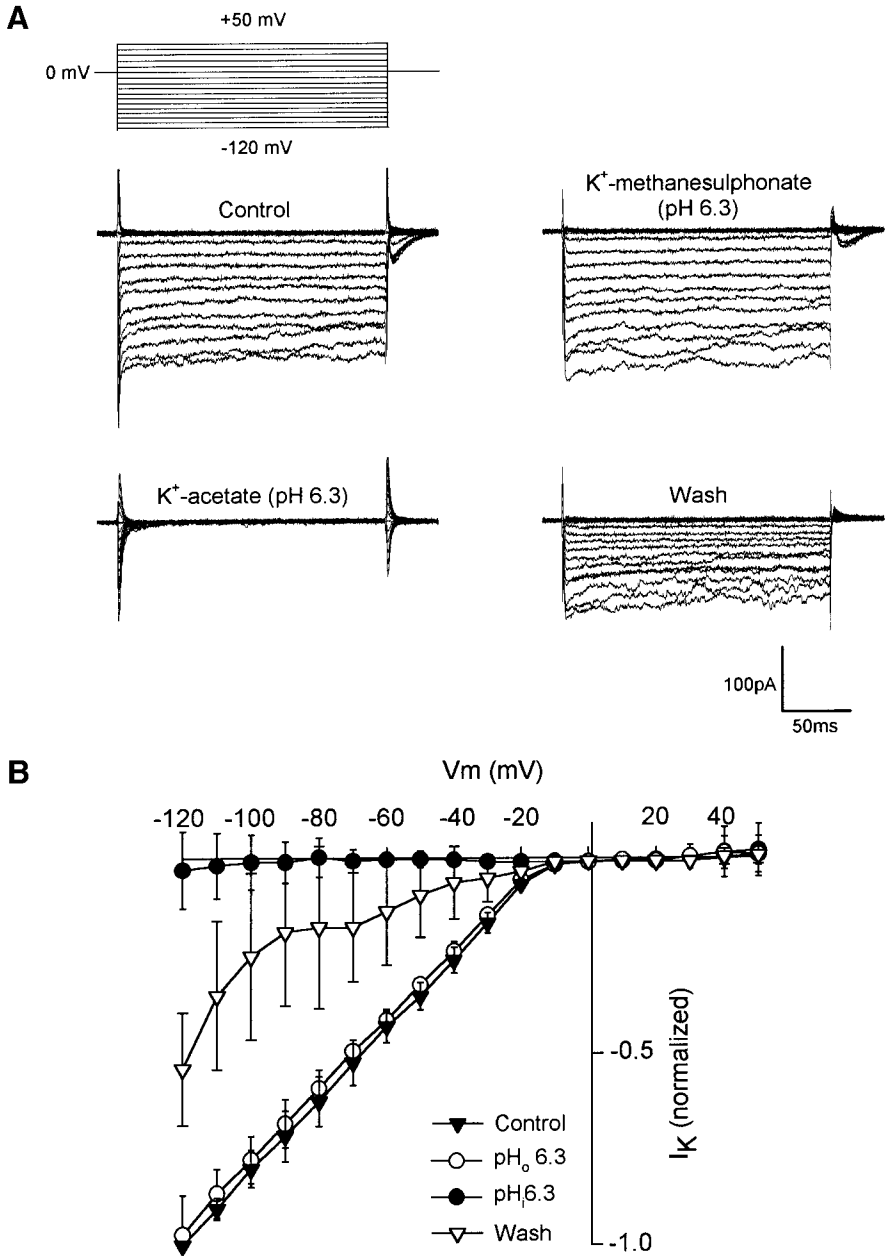

FIG. 4. Inhibition of K_{ir} currents in spermatogenic cells by pH_i. Whole-cell currents were recorded with symmetric K^+ concentration on both sides of the membrane before, during, and after lowering pH_o from a control value of 7.4 to 6.3. (A) Representative current families in response to pulse command potentials from −120 mV to +50 mV applied with an increment of 10 mV from a holding potential of 0 mV (upper left panel). Exposure of the cells to K^+-methanesulfonate, pH 6.3, did not affect inwardly rectifying currents (upper right panel). In contrast, the currents were completely abolished during a 5-min exposure to a K^+-acetate bath solution (pH 6.3; lower left panel) and returned near to the control level (lower right panel) 20 min after the exposure to K^+ acetate ended. (B) *I–V* plots for the steady-state component of the currents normalized to the current at −120 mV. Cells were superfused with high K^+ external solutions at various pH_o levels as listed. Solutions as in Table 1 (PSIII, BSI, and BSX to BSXII). The points in the *I–V* curves represent means ± SD of four to nine experiments.

channels we examined the effects of Ba^{2+} on the capacitation process, as measured by the percentage of ZP-induced sperm AR, based on the premise that only capacitated sperm would undergo AR in response to the physiological inductor (ZP). Figure 5A shows that only a small fraction of mature sperm incubated in medium devoid of ZP under-

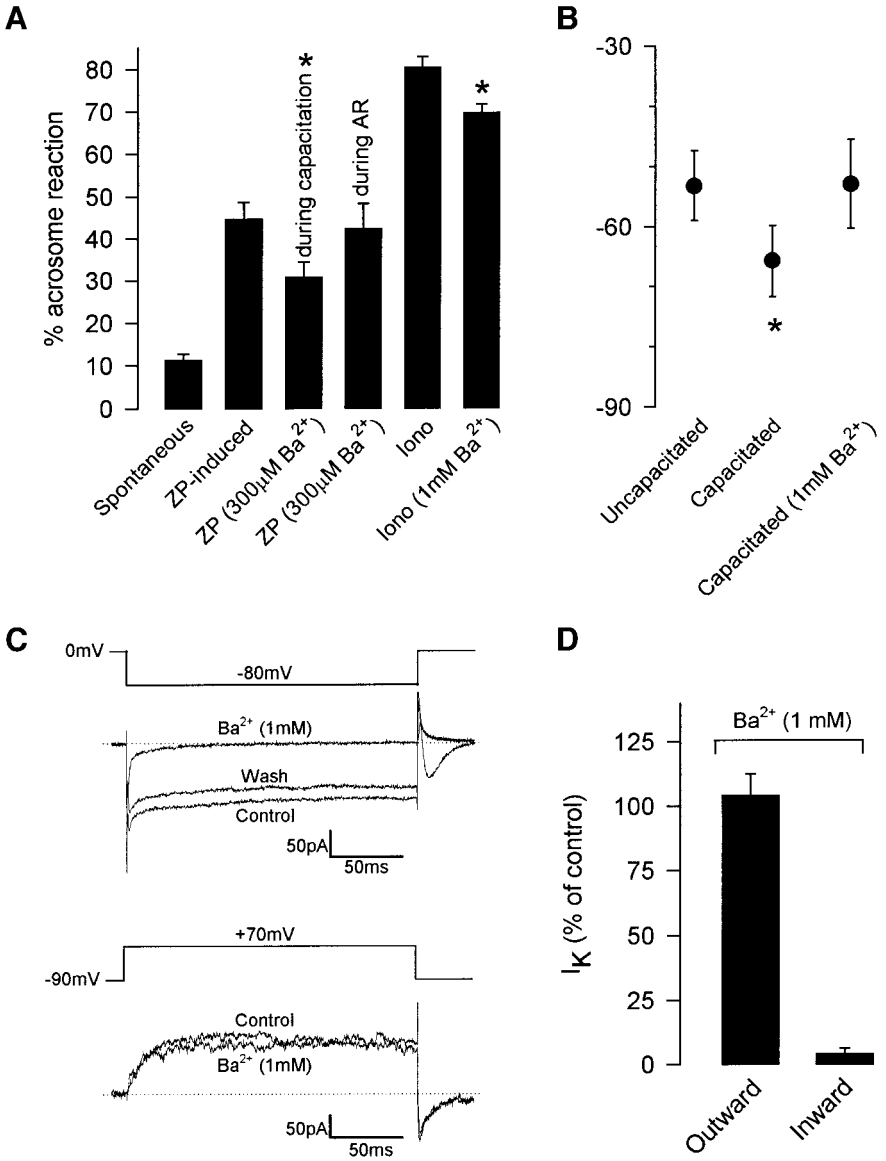

FIG. 5. Effects of extracellular Ba^{2+} on the ZP-induced sperm AR and membrane hyperpolarization during capacitation. (A) External Ba^{2+} inhibits the ZP-induced sperm AR by acting on capacitation. Capacitated mouse sperm were incubated for 30 min in the presence of ZP and AR was measured as described under Materials and Methods. Bars represent spontaneous AR that occurs in the absence of the physiological inductor and the percentage of acrosome-reacted sperm after ZP-induction in the absence or presence of Ba^{2+} (300 μM) during capacitation or the AR (5 s before ZP addition), as indicated. Two of the samples were incubated for 30 min in the presence (15 μM) of the cation ionophore ionomycin (Iono) alone or in combination with 1 mM Ba^{2+} as positive controls (n = 5 and 10, respectively). One hundred sperm were evaluated per assay. Data represent means ± SEM of at least four independent experiments. (B) Sperm membrane hyperpolarization associated with capacitation. Sperm membrane potential was measured using the fluorescence dye $DiSC_3(5)$ as indicated under Materials and Methods. Symbols represent the mean with standard deviation bars of membrane potential in uncapacitated as well as in capacitated sperm when 1 mM Ba^{2+} was added to the capacitating medium, as listed. The hyperpolarization that occurs with sperm capacitation is statistically significant (t value = 4.796; $P < 0.001$; n = 10 separate determinations obtained from four animals). (C) External Ba^{2+} blocks the inward but not the outward K^+ currents. Superimposed records of inward (top) and outward (bottom) K^+ currents obtained from representative spermatogenic cells before and after incubation in the presence of Ba^{2+} (1 mM). Dotted horizontal lines mark zero-current level. Recordings were obtained using the voltage protocol illustrated above the traces. (D) Statistical summary of steady-state K^+ current percentage in spermatogenic cells subjected to Ba^{2+} blockade as in C (n = 4).

went spontaneous AR. In contrast, a significant percentage of AR was observed when sperm were exposed to ZP, indicating that they were capacitated. The ZP-induced increase in the percentage of AR was significantly reduced when the cells were preincubated with 300 μM Ba^{2+} during capacitation, but remained unaffected when Ba^{2+} was added during AR. Furthermore, the reduction of the AR induced by Ba^{2+} seemed to be specifically related to an effect on K^+ channels, since this divalent did not affect the ability of sperm to undergo AR when incubated with the Ca^{2+} ionophore ionomycin, an agent capable of inducing acrosomal exocytosis in a nonregulated fashion (Brucker and Lipford, 1995).

A second series of experiments provided an alternative method to examine the consequences of manipulating the inwardly rectifying K^+ current during mature sperm *in vitro* capacitation, by monitoring the resting membrane potential using a voltage-sensitive dye. The mean resting membrane potential of mature sperm determined by this method was -52 ± 6 mV ($n = 10$ separate determinations). After completing *in vitro* capacitation sperm underwent an ~14-mV hyperpolarization (-66 ± 6 mV, $n = 10$; Fig. 5B). Notably, blockade of the inward-rectifying K^+ current by 1 mM Ba^{2+} prevented hyperpolarization (Fig. 5B) and V_m values were comparable with those of the resting membrane potential (-53 ± 7 mV). Since high concentrations of Ba^{2+} can block other K^+ channels (Nichols and Lopatin, 1997), the blocking effect of 1 mM Ba^{2+} was compared (Fig. 5C) in isolated synplasts expressing either inwardly rectifying or the outwardly noninactivating K^+ channels previously described (Hagiwara and Kawa, 1984). It can be seen that Ba^{2+} addition to the bath solution markedly reduced inward currents (upper panel) with no effect on the outward currents (lower panel). A comparison of normalized current amplitude in the presence and absence of Ba^{2+} is shown in Fig. 5D. Data were obtained from the currents recorded in response to the voltage steps indicated above the traces in Fig. 5C.

DISCUSSION

Here we describe the identification and characterization of a novel inwardly rectifying K^+ current expressed in mammalian spermatogenic cells. This current is carried through a new and unique type of K^+ channel that may participate in the preliminary phase of mammalian sperm activation, designated "capacitation," which regulates the efficiency of the AR during the early stages of fertilization.

The small size, complex geometry, and highly differentiated and motile nature of mature sperm complicate their electrophysiological characterization. To overcome these difficulties, an alternative approach has been to examine the ion channels of testicular spermatogenic cells using the patch-clamp technique (Liévano *et al.*, 1996; Arnoult *et al.*, 1998). Here we have found that most of the mouse synplasts of round and condensing spermatids display a rapidly activating inward current whose average amplitude was sustained throughout the duration of the voltage pulse and showed rectification around the K^+ equilibrium potential. The magnitude of the currents was strongly dependent on external K^+ concentration and the channels were highly selective for K^+ over other monovalent cations (Figs. 1 and 2). Addition of micromolar concentrations of Cs^+ and Ba^{2+} in the external solution resulted in a drastic inhibition of the whole-cell inward currents in spermatogenic cells. The degree of Cs^+ block was increased substantially by hyperpolarization, while a voltage-dependent block of macroscopic currents by Ba^{2+} is less evident (Fig. 3). In addition, because channel blockade by Mg^{2+} and channel blockade by polyamines are the two major mechanisms of channel rectification, it is reasonable to propose that in the absence of Mg^{2+} ions the endogenous polyamines are responsible for inward rectification of the K_{ir} channels recorded in spermatogenic cells.

Inhibition of strong inwardly rectifying K^+ channels may be mediated by decreases in intra- and extracellular pH (Zhu *et al.*, 2000). To investigate whether inwardly rectifying K^+ channels in spermatogenic cells have pH sensing mechanisms, whole-cell currents were measured in pH 6.3 bath solutions containing either 150 mM K^+ methanesulfonate or 75 mM K^+ methanesulfonate and 75 mM K^+ acetate. Current inhibition was observed only in cells bathed in the membrane-permeable acetate solution, indicating that K_{ir} is inhibited by intracellular acidification.

It is well established that in excitable cells, the hyperpolarized resting membrane potential (RP) is maintained by inward rectifier K^+ channels (Reimann and Ashcroft, 1999). In addition to stabilizing the RP, this current also plays an important role in modulating cellular excitability and its blockade leads to membrane depolarization (Nichols *et al.*, 1996). In addition, it is known that to achieve successful fertilization *in vivo,* mammalian spermatozoa must first undergo capacitation and then the AR, the exocytotic event that allows sperm to penetrate the zona pellucida and fuse with the oocyte.

Several physiological events lead to capacitation. These include an increase in $[Ca^{2+}]_i$, an increase in pH_i, activation of adenylyl cyclase by bicarbonate and hydrogen peroxide, production of cAMP, stimulation of PKA, and phosphorylation of certain proteins (Baldi *et al.*, 1996; Visconti and Kopf, 1998). Based on the effects of external Ba^{2+}, our results suggest that the inwardly rectifying K^+ current could be involved in sperm capacitation. To test this possibility, membrane potential (V_m) was investigated in mature sperm populations using a membrane potential-sensitive fluorescent dye ($DiSC_3(5)$) as described previously (Zeng *et al.*, 1995; Espinosa and Darszon, 1995). The calculated V_m value of uncapacitated sperm was -52 ± 6 mV. In contrast, *in vitro* capacitated populations of sperm have a V_m value of -66 ± 6 mV ($n = 10$). It has been suggested that V_m differences may regulate the activation of sperm, thereby suppressing premature AR in uncapacitated sperm and permitting capacitated sperm to respond to the physiologi-

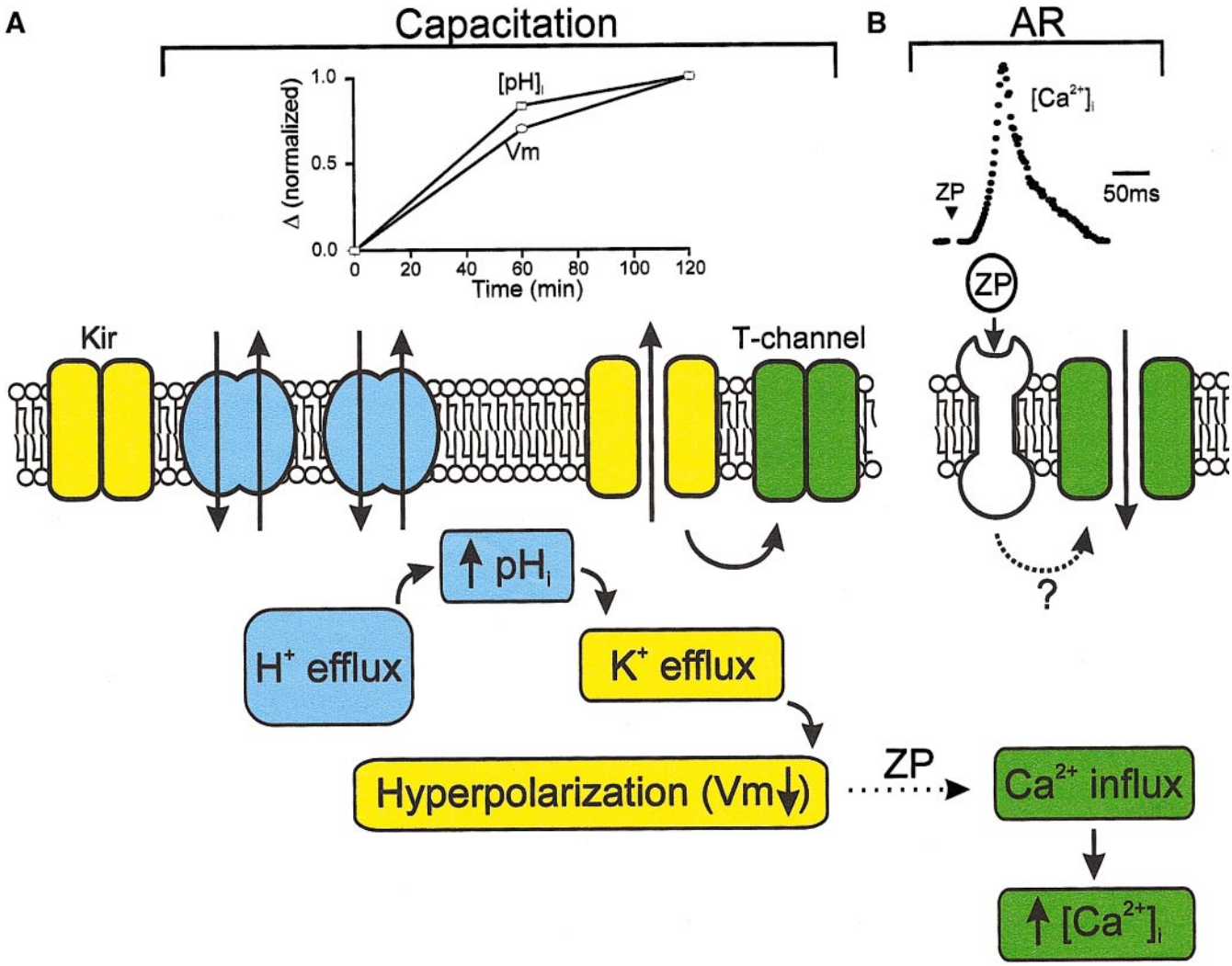

FIG. 6. Model of the functional consequences of the activation of inwardly rectifying K^+ channels of mouse sperm by pH_i during capacitation. (A) *In vitro* capacitation is accompanied by a pH_i increase due to the activation of two acid efflux mechanisms. This cytosolic alkalization results in the activation of inwardly rectifying K^+ channels in sperm plasma membrane leading to membrane hyperpolarization. Sperm transmembrane potential (V_m) can become as negative as −80 mV, a value sufficient to relieve steady-state, voltage-dependent inactivation of the voltage-gated Ca^{2+} currents. The upper panel illustrates the negative change in V_m of immobilized sperm normalized to the resting potential and the increase in pH_i normalized to its initial value calculated using fluorescent dyes during a 120-min incubation under conditions that support capacitation *in vitro* (Zeng *et al.*, 1996; Arnoult *et al.*, 1999). (B) Ca^{2+} influx through these T channels activated by ZP (arrow) via an unknown mechanism (question mark) during fertilization may drive AR. The upper panel depicts a typical $[Ca^{2+}]_i$ response from a representative sperm stimulated with the physiological inductor of the AR (Arnoult *et al.*, 1999).

cal agonist ZP. Notably, the addition of 1 mM Ba^{2+} to the capacitating medium prevented the development of membrane hyperpolarization. Since this Ba^{2+} concentration completely blocked inward K^+ current in spermatogenic cells, but had no effect on the outward K^+ current, it is suggested that membrane hyperpolarization during capacitation is due, at least in part, to an enhanced K^+ permeability through K_{ir} channels.

As discussed above, our findings are the first indication that a K^+ channel in spermatogenic cells may be modulated by pH_i. This has important physiological implications. According to our data, inwardly rectifying K^+ channels may contribute to determination of the resting potential of the spermatogenic cells. The question arises as to whether the pH_i changes that occur during capacitation activate K_{ir} channels and thus influence this process under physiological conditions. Interestingly, the resting pH_i of mammalian sperm is relatively acidic compared to values routinely

observed in somatic cells, as determined by indicator dyes (Zeng *et al.*, 1996), and may impose a functionally quiescent state. Intracellular pH regulation results from the activation of two acid efflux pathways: one mechanism dependent upon extracellular Na^+, Cl^-, and HCO_3^-, and a second pathway selectively inhibited by arylaminobenzoates (Zeng *et al.*, 1996). During storage in the epididymis sperm must maintain viability and suppress spontaneous AR. An acidic pH_i contributes to the maintenance of an uncapacitated state (Parrish *et al.*, 1989) and this is associated with prolonged viability (Yanagimachi, 1994). In addition, an acidic pH_i may act as a negative regulator of sperm K_{ir} channels, thereby maintaining depolarized membrane potential values and indirectly preventing unregulated Ca^{2+} entry and thus AR. On the other hand, in mature sperm pH_i may increase during capacitation by more than 0.2 pH units (Zeng *et al.*, 1996), a change sufficient to induce a 0.5- to 3-fold increase in the open probability of some K_{ir} channels (Choe *et al.*, 1997; Qu *et al.*, 1999). Hence, under physiological conditions, an increase in pH_i would activate K_{ir} channels, permitting K^+ ions to flow out of the cell, driving the potential toward V_K (Johns *et al.*, 1999), and hyperpolarizing sperm (Fig. 6). Consistent with this, the *g–V* curve in Figs. 1B and 1D shows that there is a small but significant fraction (9–16%) of open channels at potentials more positive to V_K. Inasmuch as K^+ ions are at higher concentrations intracellularly than extracellularly, the opening of K^+ channels causes these cations to leave the cell and hyperpolarize the membrane. Hyperpolarization during sperm capacitation is sufficient to relieve steady-state voltage-dependent inactivation of the T-type Ca^{2+} currents (Santi *et al.*, 1996; Arnoult *et al.*, 1998), recruiting them from an inactivated state to a closed state that can be subsequently activated to initiate AR. Indeed, previous studies have suggested that ZP-evoked Ca^{2+} entry into sperm through T-type Ca^{2+} channels is an essential step in the AR (Arnoult *et al.*, 1999; Darszon *et al.*, 1999). Moreover, the presence of a novel pH_i-sensitive K_{ir} channel may also help to explain early observations in which the increase of $[Ca^{2+}]_i$ in mammalian sperm induced by membrane depolarization with elevated K^+ is dependent on an increase in pH_i that results from treatment in alkaline medium (Babcock *et al.*, 1983; Babcock and Pfeiffer, 1987).

Finally, it should be noted that the native inwardly rectifying K^+ channels of spermatogenic cells share biophysical and pharmacological properties with the cloned members of the $K_{ir}2$ and $K_{ir}4$ superfamilies previously expressed in heterologous systems (Coetzee *et al.*, 1999). There is a strong correlation between structure and function within these subfamilies. $K_{ir}2$ channels are inward rectifiers and are subject to modulation by various effectors including pH_o, intracellular ATP, PKC activity, G-protein, and Mg^{2+} (Reimann and Ashcroft, 1999), while $K_{ir}4$ channel activity is subject to modulation by pH_i (Tsai *et al.* 1995). Interestingly enough, $K_{ir}4.1$ has additionally been shown to form novel functional heteromultimeric channels with $K_{ir}5.1$ (Pessia *et al.*, 1996; Salvatore *et al.*, 1999), a channel protein highly expressed in spermatozoa and spermatogenic cells that does not form functional homomeric channels when expressed in heterologous systems. Although the functional relevance of $K_{ir}5.1$ is not yet clear, it has been proposed recently that it may play an important role in conferring pH sensitivity to $K_{ir}4.1$ recombinant channels (Tucker *et al.*, 2000). Further studies will be needed to determine whether K_{ir} channels form heteromultimers in native tissue and whether the inwardly rectifying currents expressed in spermatogenic cells are homo- or heterotetramers and to determine their molecular identity.

Taken as a whole, our findings about a novel inwardly rectifying current of mouse spermatogenic cells contribute to establish the repertoire of K^+ channels present in these cells. Our results suggest that this inwardly rectifying channel plays an important role during capacitation and may participate in other physiological events during spermatogenesis.

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REFERENCES

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

Arnoult, C., Kaza, I. G., Visconti, P. E., Kopf, G. S., Villaz, M., and Florman, H. M. (1999). Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proc. Natl. Acad. Sci. USA* **96,** 6757–6762.

Babcock, D. F., Rufo, G. A. Jr., and Lardy, H. A. (1983). Potassium-dependent increases in cytosolic pH stimulate metabolism and motility of mammalian sperm. *Proc. Natl. Acad. Sci. USA* **80,** 1327–1331.

Babcock, D. F., and Pfeiffer, D. R. (1987). Independent elevation of cytosolic $[Ca^{2+}]$ and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J. Biol. Chem.* **262,** 15041–15047.

Baldi, E., Luconi, M., Bonaccorsi, L., Krausz, C., and Forti, G. (1996). Human sperm activation during capacitation and acrosome reaction: Role of calcium, protein phosphorylation and lipid remodelling pathways. *Front. Biosci.* **1,** d189–d205.

Brucker, C., and Lipford, G. B. (1995). The human sperm acrosome reaction: Physiology and regulatory mechanisms. An update. *Hum. Reprod. Update* **1,** 51–62

Choe, H., Zhou, H., Palmer, L. G., and Sackin, H. (1997). A conserved cytoplasmic region of ROMK modulates pH sensitivity, conductance, and gating. *Am. J. Physiol.* **273,** F516–F529.

Coetzee, W. A., Amarillo, Y., Chiu, J., Chow, A., Lau, D., McCormack, T., Moreno, H., Nadal, M. S., Ozaita, A., Pountney, D., Saganich, M., Vega-Saenz de Miera, E., and Rudy, B. (1999). Molecular diversity of K^+ channels. *Ann. NY Acad. Sci.* **868,** 233–285.

Darszon, A., Labarca, P., Nishigaki, T., and Espinosa, F. (1999). Ion channels in sperm physiology. *Physiol. Rev.* **79,** 481–510.

Espinosa, F., and Darszon, A. (1995). Mouse sperm membrane potential: Changes induced by Ca^{2+}. *FEBS Lett.* **372,** 119–125.

Espinosa, F., De la Vega-Beltrán, J. L., López-González, I., Delgado, R., Labarca, P., and Darszon, A. (1998). Mouse sperm patch-clamp recordings reveal single Cl^- channels sensitive to niflumic acid, a blocker of the sperm acrosome reaction. *FEBS Lett.* **426,** 47–51.

Espinosa, F., López-González I., Serrano, C. J., Gasque, G., de la Vega-Beltrán, J. L., Treviño, C. L., and Darszon, A. (1999). Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, α_{1E} currents expressed in *Xenopus* oocytes and the sperm acrosome reaction. *Dev. Genet.* **25,** 103–114.

Hagiwara, S., and Kawa, K. (1984). Calcium and potassium currents in spermatogenic cells dissociated from rat seminiferous tubules. *J. Physiol.* **356,** 135–149.

Hagiwara, S., Miyazaki, S., Moody, W., and Patlak, J. (1978). Blocking effects of barium and hydrogen ions on the potassium current during anomalous rectification in the starfish egg. *J. Physiol.* **279,** 167–185.

Hagiwara, S., Miyazaki, S., and Rosenthal, N. P. (1976). Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *J. Gen. Physiol.* **67,** 621–638.

Hagiwara, S., and Yoshii, M. (1979). Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *J. Physiol.* **292,** 251–265.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391,** 85–100.

Hille, B. (1992). "Ionic Channels in Excitable Membranes," 2nd ed., Sinauer, Sunderland, MA.

Jacob, A., Hurley, I. R., Goodwin, L. O., Cooper, G. W., and Benoff, S. (2000). Molecular characterization of a voltage-gated potassium channel expressed in rat testis. *Mol. Hum. Reprod.* **6,** 303–313.

Johns, D. C., Marx, R., Mains, R. E., O'Rourke, B., and Marban, E. (1999). Inducible genetic suppression of neuronal excitability. *J. Neurosci.* **19,** 1691–1697.

Jow, F., and Numann, R. (1998). Divalent ion block of inward rectifier current in human capillary endothelial cells and effects on resting membrane potential. *J. Physiol.* **512,** 119–128.

Liévano, A., Santi, C. M., Serrano, C. J., Treviño, C., Bellvé, A. R., Hernández-Cruz, A., and Darszon, A. (1996). T-type Ca^{2+} channels and α_{1E} expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Lett.* **388,** 150–154.

Nichols, C. G., and Lopatin, A. N. (1997). Inward rectifier potassium channels. *Annu. Rev. Physiol.* **59,** 171–191.

Nichols, C. G., Makhina, E. N., Pearson, W. L., Sha, Q., and Lopatin, A. N. (1996). Inward rectification and implications for cardiac excitability. *Circ. Res.* **78,** 1–7.

Parrish, J. J., Susko-Parrish, J. L., and First, N. L. (1989). Capacitation of bovine sperm by heparin: Inhibitory effect of glucose and role of intracellular pH. *Biol. Reprod.* **41,** 683–699.

Pessia, M., Tucker, S. J., Lee, K., Bond, C. T., and Adelman, J. P. (1996). Subunit positional effects revealed by novel heteromeric inwardly rectifying K^+ channels. *EMBO J.* **15,** 2980–2987.

Qu, Z., Zhu, G., Yang, Z., Cui, N., Li, Y., Chanchevalap, S., Sulaiman, S., Haynie, H., and Jiang, C. (1999). Identification of a critical motif responsible for gating of Kir2.3 channel by intracellular protons. *J. Biol. Chem.* **274,** 13783–13789.

Reimann, F., and Ashcroft, F. M. (1999). Inwardly rectifying potassium channels. *Curr. Opin. Cell. Biol.* **11,** 503–508.

Salvatore, L., D'Adamo, M. C., Polishchuk, R., Salmona, M., and Pessia, M. (1999). Localization and age-dependent expression of the inward rectifier K^+ channel subunit Kir 5.1 in a mammalian reproductive system. *FEBS Lett.* **449,** 146–152.

Santi, C. M., Darszon, A., and Hernández-Cruz, A. (1996). A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. *Am. J. Physiol.* **271,** C1583–C1593.

Schreiber, M., Wei, A., Yuan, A., Gaut, J., Saito, M., and Salkoff, L. (1998). Slo3, a novel pH-sensitive K^+ channel from mammalian spermatocytes. *J. Biol. Chem.* **273,** 3509–3516.

Taglialatela, M., Ficker, E., Wible, B. A., and Brown, A. M. (1995). C-terminus determinants for Mg^{2+} and polyamine block of the inward rectifier K^+ channel IRK1. *EMBO J.* **14,** 5532–5541.

Tsai, T. D., Shuck, M. E., Thompson, D. P., Bienkowski, M. J., and Lee, K. S. (1995). Intracellular H^+ inhibits a cloned rat kidney outer medulla K^+ channel expressed in *Xenopus* oocytes. *Am. J. Physiol.* **268,** C1173–C1178.

Tucker, S. J., Imbrici, P., Salvatore, L., D'Adamo, M. C., and Pessia, M. (2000). pH dependence of the inwardly rectifying potassium channel, kir5.1, and localization in renal tubular epithelia. *J. Biol. Chem.* **275,** 16404–16407.

Visconti, P. E., Bailey, J. L., Moore, G. D., Pan, D., Olds-Clarke, P., and Kopf, G. S. (1995). Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121,** 1129–1137.

Visconti, P. E., Galantino-Homer, H., Ning. X., Moore, G. D., Valenzuela, J. P., Jorgez, C. J., Alvarez, J. G., and Kopf, G. S. (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm. β-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J. Biol. Chem.* **274,** 3235–3242.

Visconti, P. E., and Kopf, G. S. (1998). Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod.* **59,** 1–6.

Wu, W. L., So, S. C., Sun, Y. P., Zhou, T. S., Yu, Y., Chung, Y. W., Wang, X. F., Bao, Y. D., Yan, Y. C., and Chan, H. C. (1998). Functional expression of a Ca^{2+}-activated K^+ channel in *Xenopus* oocytes injected with RNAs from the rat testis. *Biochim. Biophys. Acta* **1373,** 360–365.

Yanagimachi, R. (1994). Mammalian fertilization. *In* "The Physiology of Reproduction" (E. Knobil and J. D. Neil, Eds.), pp. 189–317. Raven Press, New York.

Yang, J., Jan, Y. N., and Jan, L. Y. (1995). Control of rectification and permeation by residues in two distinct domains in an inward rectifier K^+ channel. *Neuron* **14,** 1047–1054.

Zeng, Y., Clark, E. N., and Florman, H. M. (1995). Sperm membrane potential: Hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Dev. Biol.* **171,** 554–563.

Zeng, Y., Oberdorf, J. A., and Florman, H. M. (1996). pH regulation in mouse sperm: Identification of Na^+, Cl^-, and HCO_3^--dependent and arylaminobenzoate-dependent regulatory mechanisms and characterization of their roles in sperm capacitation. *Dev. Biol.* **173,** 510–520.

Zhu, G., Liu, C., Qu, Z., Chanchevalap, S., Xu, H., and Jiang, C. (2000). CO_2 inhibits specific inward rectifier K^+ channels by decreases in intra- and extracellular pH. *J. Cell Physiol.* **183,** 53–64.

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