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K_{ATP} channels in mouse spermatogenic cells and sperm, and their role in capacitation

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Abstract

Mammalian sperm must undergo a series of physiological changes after leaving the testis to become competent for fertilization. These changes, collectively known as capacitation, occur in the female reproductive tract where the sperm plasma membrane is modified in terms of its components and ionic permeability. Among other events, mouse sperm capacitation leads to an increase in the intracellular Ca^{2+} and pH as well as to a hyperpolarization of the membrane potential. It is well known that ion channels play a crucial role in these events, though the molecular identity of the particular channels involved in capacitation is poorly defined. In the present work, we report the identification and potential functional role of K_{ATP} channels in mouse spermatogenic cells and sperm. By using whole-cell patch clamp recordings in mouse spermatogenic cells, we found K^+ inwardly rectifying (K_{ir}) currents that are sensitive to Ba^{2+}, glucose and the sulfonylureas (tolbutamide and glibenclamide) that block K_{ATP} channels. The presence of these channels was confirmed using inhibitors of the ATP synthesis and K_{ATP} channel activators. Furthermore, RT-PCR assays allowed us to detect transcripts for the K_{ATP} subunits SUR1, SUR2, $K_{ir}6.1$ and $K_{ir}6.2$ in total RNA from elongated spermatids. In addition, immunoconfocal microscopy revealed the presence of these K_{ATP} subunits in mouse spermatogenic cells and sperm. Notably, incubation of sperm with tolbutamide during capacitation abolished hyperpolarization and significantly decreased the percentage of AR in a dose-dependent fashion. Together, our results provide evidence for the presence of K_{ATP} channels in mouse spermatogenic cells and sperm and disclose the contribution of these channels to the capacitation-associated hyperpolarization.

Keywords: Sperm; Mammalian sperm; Spermatogenic cells; Ion channels; Sperm capacitation; Sperm acrosome reaction; K^+ channels; KATP channels; Inward-rectifying channels; SUR subunits

Introduction

Mammalian sperm are unable to fertilize eggs immediately after ejaculation. They acquire fertilization capacity after residing in the female tract for a finite period of time. The physiological changes occurring in the female reproductive tract rendering sperm able to fertilize constitute the process of "capacitation". Capacitation is associated with changes in tyrosine (tyr) phosphorylation of a subset of sperm proteins (Visconti et al., 1995a, 2002; Baker et al., 2004). Both capacitation and tyr-phosphorylation have been shown to be regulated by a cAMP-dependent pathway involving protein kinase A (PKA) (Visconti et al., 1995b; Galantino-Homer et al., 1997; Nolan et al., 2004). In addition, capacitation in mammalian sperm is accompanied by a hyperpolarization of the membrane potential (Em) (Zeng et al., 1995; Arnoult et al., 1999) and increases in intracellular pH (pHi) (Parrish et al., 1989; Zeng et al., 1996; Galantino-Homer et al., 2004) and Ca^{2+} ($[Ca^{2+}]i$) (Baldi et al., 1991; DasGupta et al., 1993). Mouse spermatogenic cells and mature sperm have voltage dependent Ca^{2+} (Ca_V) channels of the T-type (Ca_V3) that participate in the acrosome reaction (AR) (Arnoult et al.,

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1996; Lievano et al., 1996). A sperm hyperpolarization associated to capacitation could be important to remove inactivation from T-channels, driving them from an inactive state to a closed state from which they could be activated by zona pellucida (ZP) to trigger AR (Darszon et al., 2001; Florman et al., 1998).

The hyperpolarization that accompanies mouse sperm capacitation is influenced by external K^+ and K^+ channel blockers. It is thus thought that a K^+ permeability contributes to this process (Zeng et al., 1995; Arnoult et al., 1999; Muñoz-Garay et al., 2001). Molecular and functional studies of K^+ channels in mammalian male germ cells and mature sperm have indicated the presence of voltage-gated (Hagiwara and Kawa, 1984; Schreiber et al., 1998; Wu et al., 1998; Jacob et al., 2000; Felix et al., 2002) and inward rectifier (K_{ir}) channels (Salvatore et al., 1999; Muñoz-Garay et al., 2001; Felix et al., 2002). However, little is known about the regulation of the capacitation-associated hyperpolarization.

It was suggested that a pH-regulated K^+ channel with strong inward rectification contributes to the capacitation-associated hyperpolarization. The addition of Ba^{2+}, a K_{ir} channel blocker, eliminated inwardly rectifying K^+ currents in spermatogenic cells and prevented both the development of membrane hyperpolarization and partially the sperm AR (Muñoz-Garay et al., 2001). Therefore, an elevation in pHi, as it occurs during capacitation, could increase the open probability of these channels (Muñoz-Garay et al., 2001) driving Em towards the K^+ equilibrium potential and hyperpolarizing sperm. Considering that the K_{ir} family has 7 subgroups (1–7) at the present time (Coetzee et al., 1999; Bichet et al., 2003), it seemed important to identify the molecular identity of those present in spermatogenic cells and their possible influence on sperm physiology, particularly during capacitation.

Here, we report what is to our knowledge the first functional evidence for the presence of a weak inwardly rectifying K_{ATP} channel in spermatogenic cells. These channels are ubiquitously expressed in a variety of cell types, including pancreatic β cells, cardiac myocytes, skeletal muscle cells, neurons and pituitary cells (Ashcroft and Gribble, 1998). Molecular studies indicate that K_{ATP} channels are double tetramers formed from four K_{ir} channel (6.1 and/or 6.2) and four sulfonylurea receptor (SUR1, SUR2 A and B) subunits. When the ATP/ADP ratio rises in the cytoplasm, K_{ATP} channels close and the cell depolarizes. It is well known that in pancreatic β-cells this mechanism regulates insulin release (Nichols and Koster, 2002). In the current work we report K_{ir} currents from mouse spermatogenic cells that are sensitive to Ba^{2+}, glucose and sulfonylureas (tolbutamide and glibenclamide). Notably, these currents were augmented by K_{ATP} openers (pinacidil and diazoxide) as well as by agents that inhibit the production of ATP (2-deoxyglucose and 2,4-dinitrophenol). Furthermore, RNA messengers for SUR1, SUR2, K_{ir}6.1 and 6.2 were found in mouse spermatogenic cells, and specific antibodies to these K_{ATP} subunits detected all the proteins in this cells and mature sperm. The possible role of these channels during sperm capacitation is discussed.

Materials and methods

Chemicals

Tolbutamide, glibenclamide, 2-deoxyglucose, 2,4-dinitrophenol, diazoxide and pinacidil were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were analytical grade.

Cell preparation

Spermatogenic cells for electrophysiological recording were obtained as previously described (Muñoz-Garay et al., 2001). Briefly, testes from adult CD1 mice were excised and suspended in ice-cold dissociation solution containing (in mM) 130 NaCl; 3 KCl; 10 $CaCl_2$, 2 $MgCl_2$; 1 $NaHCO_3$; 0.5 NaH_2PO_4; 5 HEPES; 10 glucose (pH 7.4/NaOH). The tunica albuginea was removed and the seminiferous tubules separated. Tubules were dispersed into individual cells or symplasts using Pasteur pipettes. The cells were stored at 8°C until assayed. Subsequently, 100 μl aliquots of cell suspension were dispensed into a recording chamber (500-μl total volume) and subjected to electrophysiological recording.

Electrophysiology

K^+ currents were recorded according to the whole-cell patch-clamp technique (Marty and Neher, 1995). All recordings were performed at room temperature using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and 2- to 4-MΩ micropipettes. Cells were clamped at a holding potential of 0 mV and currents were evoked by 200 ms voltage steps (0.5 Hz) to test potentials ranging from −100 to +40 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 currents (2 kHz; internal 4-pole Bessel filter) using a personal computer attached to a DigiData 1200 interface (Axon). To record inwardly rectifying K^+ currents, cells were bathed in a solution containing (in mM): 150 K-methanesulfonate ($MeSO_3$); 6.5 $CaCl_2$; 1 $MgCl_2$; 10 HEPES; 10 Glucose (pH 7.4/KOH). The internal solution consisted of (mM): 122 K-$MeSO_3$; 20 KF; 8 KCl; 2.5 $CaCl_2$; 1 $MgCl_2$; 5 EGTA; 10 HEPES; (pH 7.3/KOH). Tolbutamide, glibenclamide and pinacidil were prepared as 100 mM stock solutions in DMSO. Ba^{2+}, 2-deoxyglucose and 2,4-dinitrophenol were made at 100 mM in external solution and diazoxide 50 mM in 0.1 N NaOH. These compounds were diluted in external solution to the indicated concentration and perfused by gravity at ~1 ml/min. Controls with the highest DMSO volume used in the experiments with inhibitors or activators had no effect on the recorded currents.

Assay for capacitation and acrosome reaction

Caudal epididymal mouse sperm were collected from CD1 mice and placed in capped 1.5-ml microcentrifuge tubes containing medium 199 supplemented with BSA (0.4% wt/vol), Na^+ pyruvate (30 mg/l) and $NaHCO_3$ (2.2 g/l) at 37°C ($4–5 \times 10^6$ cells/ml). The swim-up method (Henkel and Schill, 2003) was used to separate sperm with >90% motility. The sperm suspension was incubated for 10 min and the top ~1 ml separated and capacitated incubating it 30 min at 37°C (Visconti et al., 1999). AR was induced after capacitation in a 30 μl aliquot by adding 5 eq/μl *zona pellucida*. The percentage of AR, which also measures capacitation indirectly, was determined 30 min later, after adding an equal volume of fixative (10% formaldehyde in phosphate-buffered saline). Following fixation, 10 μl aliquots of the sperm suspension were spread onto glass slides and air-dried. The slides were stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% glacial acetic acid for ~5 min, rinsed and mounted with 50% (v/v) glycerol in phosphate-buffered saline (Muñoz-Garay et al., 2001). At least 100 sperm were assayed per experimental condition to calculate the percentage of AR.

Measurement of membrane potential

Mature sperm were capacitated in vitro as mentioned above. After a 30-min incubation, the potential sensitive dye 3,3′-dipropylthiocarbocyanine iodide

Table 1
K_{ATP} channel subunit specific primers

Primer name	Primer sequence	Product size (bp)	GenBank Acc. no.
K_{ir}6.1	F: CGCATCCAGGTGGTCAAG R: TGACTGAGGAGGAGGGCGT	325	NM_008428
K_{ir}6.2	F: CACCCTGCGCCATGGCCGCC R: TCGCACCCCACCACTCTACA	685	BC057006
SUR1	F: GGTCAGCGTCAGCGAATC R: CAAGCAGGGACAAGAGCAG	552	NM_011510
SUR2	F: TGGTCAGATTCGCAGTCA R: AGAAGGCTCAGATTCATTTAC	SUR2A 340 SUR2B 445	MMU97066

($DiSC_3(5)$; Molecular Probes, Eugene, OR) was added to the cell suspension at a final concentration of 1 μM. Fluorescence was then monitored with a Hansatech MkII fluorometer (Norfolk, UK) at a 620/670-nm excitation/emission wavelength pair (Espinosa and Darszon, 1995). Mitochondrial membrane potential was dissipated with 100 nM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma). Cell hyperpolarization decreases the dye fluorescence. Recordings were initiated after reaching steady-state fluorescence (1–3 min) and were converted to Em as described previously (Muñoz-Garay et al., 2001).

RNA isolation and RT-PCR experiments

Total RNA was prepared from isolated mouse elongated spermatids (Bellve, 1993) using TRIzol Reagent (Sigma) as previously described (Serrano et al., 1999). cDNA was synthesized from total RNA samples by random hexamer-primed reverse transcription (Superscript II RNase H-Reverse Transcriptase, Invitrogen). cDNA was then subjected to PCR amplification using Taq DNA Polymerase (Invitrogen). The primers used to amplify PCR fragments for the four K_{ATP} subunits are summarized in Table 1. The absence of genomic contamination in the RNA samples was confirmed with reverse transcription-negative controls (no RT) for each experiment. Amplified products were analyzed by DNA sequencing in order to confirm their identity.

Immunolocalization

Mouse epididymal sperm were obtained as described elsewhere (Espinosa et al., 1998). For immunodetection, the cells were smeared on slides and allowed to air-dry, fixed with formalin (Merck, 5% final concentration), permeabilized with 0.1% Triton X-100 (Sigma) for 10 min, washed three times with PBS 1× and blocked with 2% BSA-PBS. Then, they were treated with anti-K_{ir}6.1, anti-SUR1, anti-SUR2B (SantaCruz Biotechnologies) and anti-K_{ir}6.2 (Alomone Labs) antibodies at a 1:50 dilution in 2% BSA in PBS overnight at 4°C. Immunofluorescence labeling for confocal microscopy was performed treating the cells with Alexa Fluor 594 (goat anti-rabbit) or Alexa Fluor 647 (donkey anti-goat) antibodies (Molecular probes) using a 1:100 dilution. Control experiments performed by pre-incubation of the primary antibody with the respective antigenic peptide (1:5 K_{ir}6.2, SUR1 and SUR2B) or with the secondary antibody alone (K_{ir}6.1), did not show positive staining under the same experimental conditions utilized (see Supplementary Figs. 1–4). Identical settings were used for all the specimens. Similarly, aliquots of mouse spermatogenic cells were obtained and processed for immunolocalization as described above (Serrano et al., 1999), with the exception of using 4% paraformaldehyde to fix, no permeabilization and 1:100 dilutions of the K_{ATP} subunit antibodies. Control experiments were processed likewise except by the replacement of the primary antibody with peptide-blocked antibody prepared by incubation with its corresponding peptide antigen (1:5 ratio), and no significant staining was observed (not shown).

Results

Previously, our group showed that strong K_{ir} channels are expressed in mouse spermatogenic cells and proposed that they participate in the capacitation-associated hyperpolarization (Muñoz-Garay et al., 2001). Since several types of K_{ir} channels exist (Coetzee et al., 1999), it is necessary to identify which of them are present in the later stages of spermatogenic cells and in sperm, to understand how they are regulated and if they influence sperm capacitation.

As pointed out earlier, K_{ATP} channels are octamers formed from four SUR subunits and four K_{ir} subunits. Sulfonylureas such as tolbutamide and glibenclamide, inhibit K_{ATP} channels by interacting with their SUR subunits. Hence, we initially used these compounds at >3 fold their IC_{50}s (Seino, 1999) to investigate the expression of K_{ATP} channels in mouse spermatogenic cells. As shown in Fig. 1, repetitive voltage steps from −100 mV to +40 mV, from a holding potential of 0 mV, revealed weak inward rectification K^+ currents (I_K). These experiments were performed using symmetrical K^+ concentrations (see Methods). Tolbutamide (300 μM) reversibly decreased the K^+ whole-cell current at the voltages tested (Figs. 1A and C). At −100 mV this drug reduced membrane current to 45% ± 12% (n = 13) of the control. Glibenclamide (20 μM) also decreased the K^+ whole-cell current at the voltages tested (38% ± 13%; n = 16) but in contrast to tolbutamide, the inhibition was not reversible (Figs. 1B and D). Fig. 2 summarizes the time course of I_K decay when perfusing 300 μM tolbutamide (A) or 20 μM glibenclamide (B) and their degree of reversibility. The ratio of K_{ATP} currents in the presence and absence of the drug ($I_{sulfonylurea}/I_{control}$) did not vary considerably from cell to cell and its average was only mildly dependent on Em between −100 and +40 mV (not shown). The irreversible nature of the glibenclamide inhibition of K_{ATP} currents has been reported in other systems (Allen and Brown, 2004; Lim et al., 2004).

A notable feature of K_{ATP} channels is precisely their inhibition by increased intracellular ATP levels (Aguilar-Bryan and Bryan, 1999). Hence, in order to decrease the cellular ATP content, spermatogenic cells were superfused several minutes with a glucose-free solution. This maneuver resulted in a significant increase in the amplitude of the whole-cell currents recorded (Figs. 3A and B). As expected, the addition of glibenclamide attenuated the response evoked by the glucose-free solution (Figs. 3C and D). The involvement of ATP in the regulation of the K^+ currents was corroborated in experiments where the patch pipette contained 1 mM ATP. Under this condition, glucose removal was unable to stimulate the current (Figs. 3C and D).

K_{ir} channels are known to be susceptible to blockade by μM concentrations of external Ba^{2+} (Hagiwara et al., 1978). In particular, K_{ATP} channels are blocked by 100–200 μM Ba^{2+} (Bonev and Nelson, 1993; Takano and Ashcroft, 1996). Therefore, we determined what percentage of the K^+ current was sensitive to this cation. Fig. 4A shows that Ba^{2+} blocked ~49% ± 5% (n = 13) of the current in spermatogenic cells in a dose-dependent fashion, revealing the possible presence of two classes of K_{ir} channels with different sensibility. The magnitude of the Ba^{2+} blockade was partially reversible (Fig. 4B), only mildly voltage dependent (not shown) and similar in magnitude to that found with tolbutamide and glibenclamide.

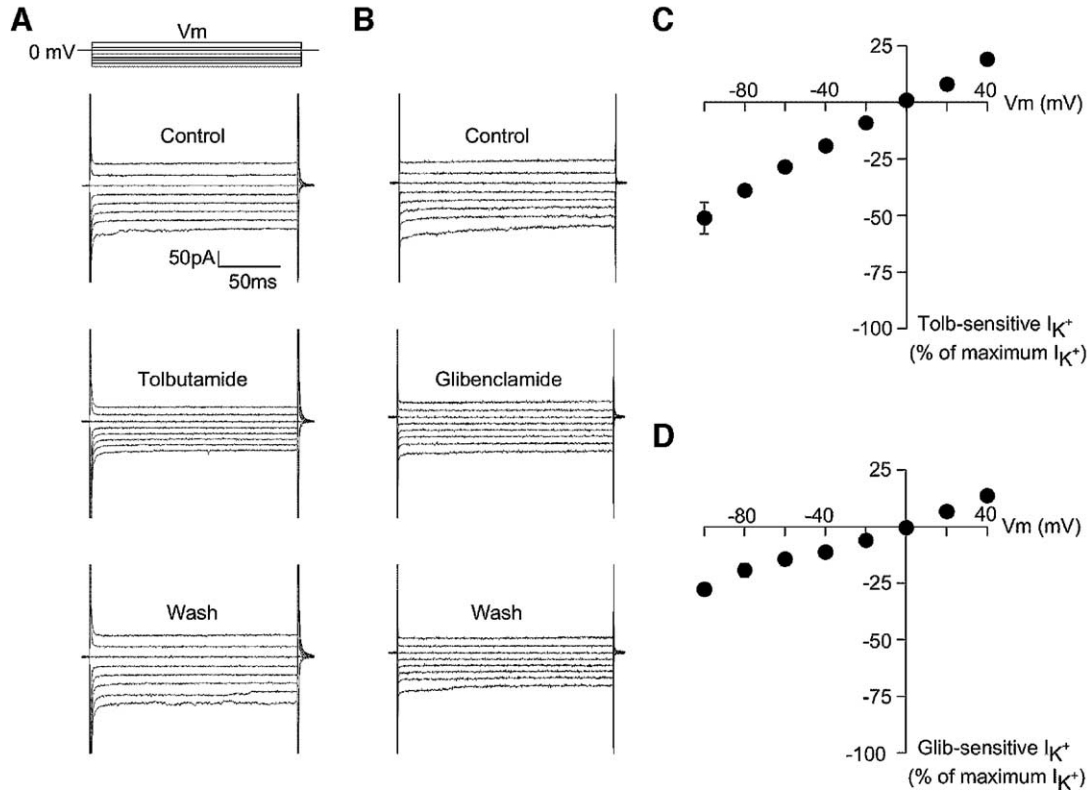

Fig. 1. Sensitivity to sulfonylureas of the inwardly rectifying K^+ (K_{ir}) currents recorded in mouse spermatogenic cells. (A) Top, representative whole-cell patch clamp currents recorded by applying voltage steps (20 mV) from a holding potential of 0 mV to test potentials ranging from −100 to +40 mV in symmetrical K^+ recording solutions. The protocol used for eliciting K_{ir} currents is shown above traces. Center and bottom, after establishing the whole-cell configuration, the K^+ current developed within some minutes; thereafter, tolbutamide was applied to the bath and washed out. Inhibition by 300 μM tolbutamide was fully reversible. (B) Whole cell K_{ir} currents recorded at different test potentials in the same conditions as in A. Top, Control K^+ current traces recorded in spermatogenic cells at 8 to 12 min after establishing the whole-cell configuration. Center and bottom, Glibenclamide at 20 μM produced an important inhibition of the K_{ir} current which was not reversible after washout for about 20 min. (C–D) Averaged current–voltage (I–V) plots of the sulfonylurea-sensitive K^+ currents measured at the end of the voltage steps. Steady-state currents were recorded in the presence of the sulfonylureas as well as after washout ($n \geq 13$). Subtraction of the currents recorded as control from the corresponding values recorded for sulfonylurea addition yields the currents through putative K_{ATP} channels. The currents were normalized with respect to the maximum I_K of each experiment and expressed as the percentage of maximum I_K.

Collectively, the above presented results are consistent with the functional expression of K_{ATP} channels in mouse male germ cells. To verify this further, different agents that affect K_{ATP} channel activity were directly assessed by whole-cell patch-clamp studies on these cells (Fig. 5). Panels A and B depict averaged I–V relationships of K_{ir} currents under control conditions and after application of two ATP synthesis inhibitors. As can be seen, peak K_{ir} currents increased after a ~5 min exposure; 25.1 ± 13.7% to 5 mM 2-deoxyglucose, 12.4 ± 3.2% to 1 mM 2,4-dinitrophenol and 99.1 ± 39.5% to

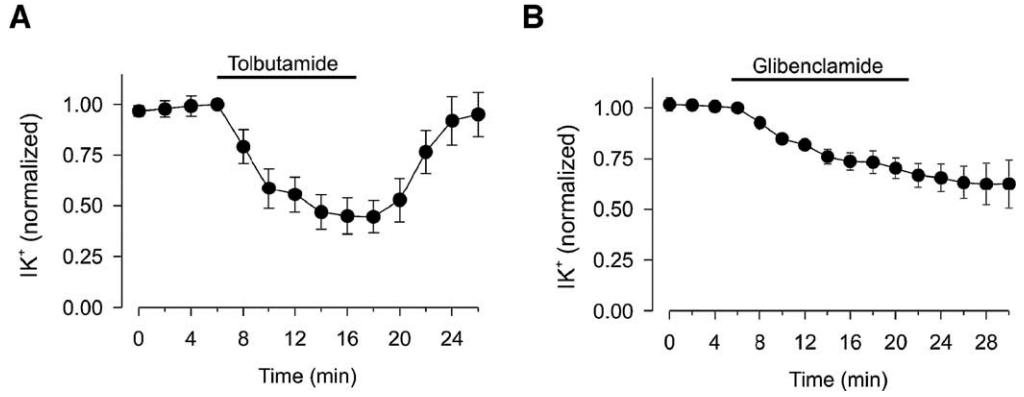

Fig. 2. Time course of the sulfonylurea-induced inhibition of K_{ir} currents in spermatogenic cells. (A) Normalized whole-cell currents (mean ± SEM) in 13 cells elicited by hyperpolarizing voltage steps to −100 mV from a holding potential of 0 mV, plotted as a function of the time after tolbutamide (300 μM) application to the bath and washed out. (B) Time course of inhibition by glibenclamide (20 μM) of the K_{ir} currents recorded as described for A in spermatogenic cells. Sulfonylurea application is indicated by the solid bar.

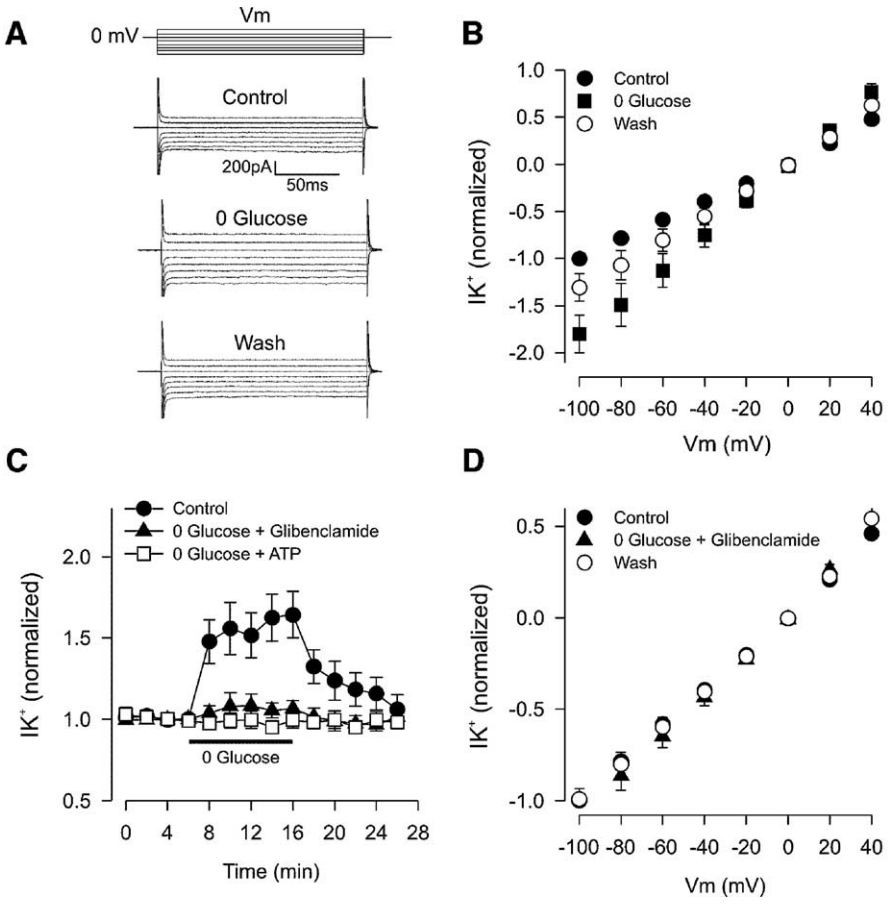

Fig. 3. Superfusion with a glucose-free solution increases K_{ir} currents in spermatogenic cells. (A) Three sets of current records in response to voltage steps between −100 and +40 mV (in 20 mV increments) from a holding potential of 0 mV are shown. Recordings were made at about 10 min (control), 20 min (0 glucose) and 30 min (washout) after the whole-cell mode was established. (B) $I-V$ relationships constructed from current records evoked by the voltage step protocol described in panel A. The curves show almost a linear current–voltage relationship and reverse at 0 mV (n = 15). (C) Effects of glucose depletion on the currents elicited by hyperpolarizing voltage step in the presence of ATP and glibenclamide. The normalized current amplitude in response to voltage-clamp steps between −100 and +40 mV from a holding potential of 0 mV is plotted as a function of time. Glibenclamide and ATP strongly antagonize the increases in membrane conductance produced by extracellular depletion of glucose. (D) Normalized $I-V$ relationships for control, glucose depletion (0 glucose) plus 20 μM glibenclamide and washout (n = 10).

5 mM of this later compound, respect to controls measured at −100 mV). In a similar manner, panels C and D show the effects of ~5 min exposure to 100 μM pinacidil or diazoxide, two K_{ATP} channel activators, on the K_{ir} currents recorded from spermatogenic cells. The mean K_{ir} current amplitudes were increased by 34.6 ± 6.2% and 24.7 ± 7.1% of the control measured at −100 mV, respectively.

Having determined the presence of K_{ATP} channels in functional assays, we next looked for the corresponding RNA messengers for the K_{ir} and SUR subunits known to constitute

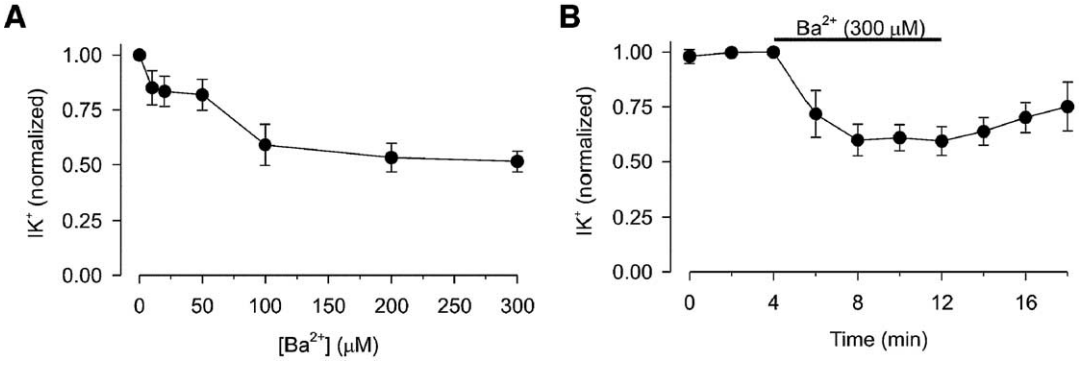

Fig. 4. Effects of Ba^{2+} on the K_{ir} currents expressed in spermatogenic cells. (A) Relative amplitude of inwardly rectifying K^+ currents evoked by hyperpolarizing voltage steps to −100 mV from a holding potential of 0 mV at various concentrations of $BaCl_2$ as listed. K_{ir} current amplitudes were normalized to the current amplitudes just before Ba^{2+} application. (B) Averaged time course of K_{ir} current block by Ba^{2+} (300 μM). Ba^{2+} application is indicated by the solid bar.

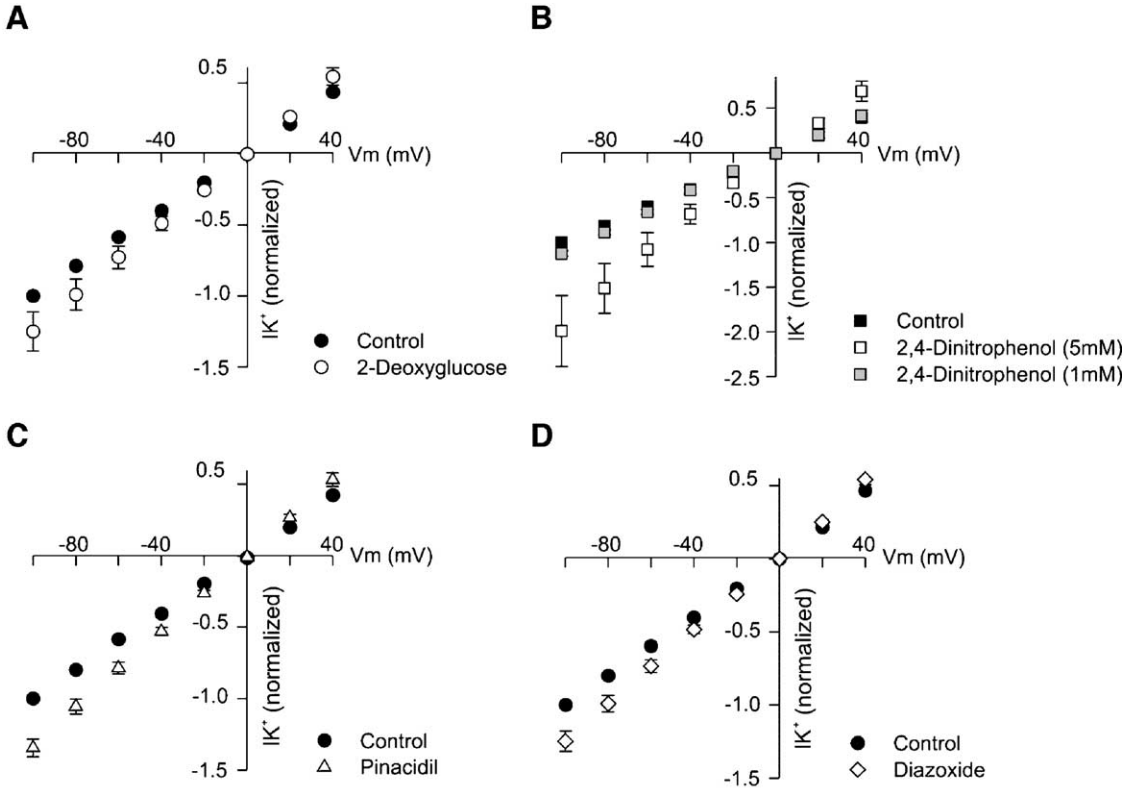

Fig. 5. Inhibitors of the ATP synthesis and K_{ATP} channel activators increase K_{ir} currents in spermatogenic cells. Averaged $I-V$ relationships of K_{ir} currents measured at the end of the voltage steps. Currents were elicited from a holding potential of 0 mV by voltage steps (in 20 mV increments) to potentials ranging from −100 to +40 mV. Current amplitudes were normalized with respect to the maximum I_K of each experiment and expressed as the fraction of maximum I_K. Data are shown for at least 8 representative cells in each condition, and were collected before (control) and after exposure to 5 mM 2-deoxyglucose (panel A), 1 and 5 mM 2,4-dinitrophenol (panel B) and 100 μM pinacidil or diazoxide (panels C and D, respectively).

these channels. RT-PCR analysis was performed with total RNA isolated from elongated spermatids of adult mice using the primers shown in Table 1. This analysis revealed the expected fragment sizes for $K_{ir}6.1$ (325 bp), $K_{ir}6.2$ (685 bp), SUR1 (552 bp) and SUR2 (445 bp) (Fig. 6A). Each of these products was confirmed by DNA sequencing. In addition, to further characterize the expression of K_{ATP} channels, we lastly performed immunolabeling experiments on mouse spermatogenic cells using specific anti-K_{ir} and anti-SUR antibodies to assess for the presence of the channel proteins. A typical result is shown in Fig. 6B. K_{ATP} channel subunits appeared to be broadly expressed in all spermatogenic cells examined. Immunofluorescence appears associated with the plasma membrane, though important intracellular reactivity excluding the nuclear region was also observed in the cells. Antibodies against $K_{ir}6.1$ and $K_{ir}6.2$ labeled spermatogenic cells with a punctuate pattern while SUR1 and SUR2 displayed a more diffuse staining. The reason for the heterogeneity in the K_{ATP} channel subunits distribution and the significance of their intracellular localization is not presently clear and needs further examination. Lastly, as mentioned in the Methods section, the specificity of the antibodies was evaluated by preadsorbing them with the corresponding synthesized antigen peptides. No significant staining was seen under these conditions (data are not shown).

Given that sperm membrane potential can be influenced by changes in K^+ channel activity, notably that of K_{ir} channels (Muñoz-Garay et al., 2001), experiments were carried out to determine whether the K_{ATP} channels may play a role during sperm capacitation. When tolbutamide was added during sperm incubation in capacitating media, the percentage of AR decreased significantly in a dose-dependent fashion (Fig. 7A). However, the percentage of sperm undergoing AR was not affected when the drug was applied after capacitation. These findings suggest that tolbutamide prevented in vitro sperm capacitation.

In order to test whether tolbutamide affected sperm capacitation by preventing hyperpolarization, sperm Em was then determined using a potential sensitive fluorescence dye, $DiSC_3(5)$. The population-averaged Em reported by this probe was −43 ± 1 mV in uncapacitated sperm and −63 ± 2 mV after 30 min of incubation in capacitating medium (Fig. 7B). These estimates agree with previous data (Espinosa and Darszon, 1995; Arnoult et al., 1999; Muñoz-Garay et al., 2001). As expected, sperm hyperpolarization during capacitation was inhibited by tolbutamide in a concentration dependent manner similar to the one found for capacitation itself (Fig. 7B).

Lastly, the presence and distribution pattern of K_{ATP} channel subunits was examined by immunocytochemistry on adult mouse sperm. As shown in Fig. 8, the $K_{ir}6.1$ subunit

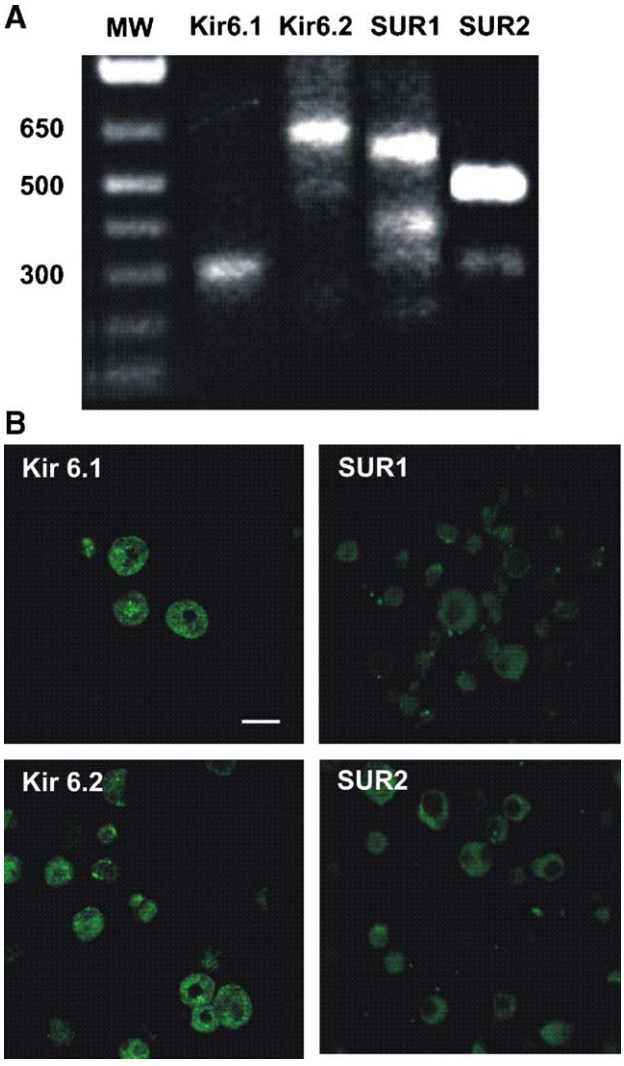

Fig. 6. Expression of SURx and K_{ir}6.x subunit isoforms in mouse spermatogenic cells. (A) RT-PCR was used to amplify K_{ir}6.1, K_{ir}6.2, as well as SUR1 and SUR2 mRNAs from mouse male germ cells. Representative agarose gel showing the amplification of cDNA fragments of the anticipated molecular weights (see Table 1). (B) Immunolocalization of KATP channel subunits in male germ cells. Representative fluorescence micrographs of permeabilized spermatogenic cells incubated with primary specific antibodies against K_{ir}6. 1, K_{ir}6.2, SUR1, and SUR2 recognized with a secondary anti-rabbit antibody conjugated to Alexa Fluor (green). Peptide controls (see Materials and methods) are not shown but gave no significant staining. Scale bar: 10 μm.

was detected in the flagellum midpiece while the K_{ir}6.2 subunit was localized in both the flagellum midpiece and in the postacrosomal region of the sperm head. Interestingly, the SUR1 subunit displays the same distribution pattern as K_{ir}6.2 in the flagellum midpiece and postacrosomal region of the sperm head. The SUR2 subunit was strongly immunolabeled in the flagellum principal piece and to a lesser extent in the flagellum midpiece. Also, a clear dot signal could be observed in the connecting sperm piece. These results suggest that K_{ir}6.2 and SUR1 subunits may co-localize, considering their very similar distribution pattern. These observations indicate that K_{ATP} channel subunits are expressed in mature mouse sperm.

Discussion

Membrane potential is a fundamental variable that cells use to translate and signal cues from the outside and inside. It provides the basis for cell bioenergetics and for fast or long-distance communication in excitable cells and tissues such as nerve and muscle. In sperm, there is evidence suggesting that motility, capacitation and the AR are influenced by Em (Florman et al., 1998; Darszon et al., 1999). In particular, capacitation is associated to changes in ion permeability (Baldi et al., 2000; Darszon et al., 2005) and biochemical parameters (Aitken, 1997; Visconti et al., 2002), which lead, among other physiological alterations, to a hyperpolarization in mouse sperm (Zeng et al., 1995; Arnoult et al., 1999; Muñoz-Garay et al., 2001).

Ion channels usually contribute significantly to determine Em in cells (Hille, 2001). However, the small size, complex geometry, as well as the highly differentiated and motile nature of mature sperm, complicates the electrophysiological characterization of their ion channels. Though new strategies are emerging (Gorelik et al., 2002; Gu et al., 2004), an alternative to surmount these difficulties has been to examine the ion channels of spermatogenic cells that are larger and actively synthesize these proteins, some of which will end up in mature sperm (Darszon et al., 1999, 2001; Jagannathan et al., 2002). Using the patch-clamp technique, we characterized a strong inwardly rectifying, pH-sensitive, K^+ channel from mouse pachytene spermatocytes and spermatids (Muñoz-Garay et al., 2001). Notably, 0.3–1 mM Ba^{2+}, that blocks most K_{ir} channels (von Beckerath et al., 1996; Leung et al., 2000), inhibited the capacitation-associated hyperpolarization and partially decreased the subsequent exocytotic response to *zona pellucida* in mature sperm (Muñoz-Garay et al., 2001). These findings point out the importance of determining the molecular identity of the K_{ir} channels present in spermatogenic cells and sperm, and their contribution to the hyperpolarization associated to capacitation.

Here, we report the presence of K_{ATP} channels in mouse spermatogenic cells and sperm. As indicated earlier, K_{ATP} channels are heteromeric (4:4) complexes of two types of protein subunits: the K_{ir}6 subfamily and the sulfonylurea receptors (SUR; a member of the ATP-binding cassette (ABC) family). The resulting K^+ channels are sensitive to intracellular ATP, phospholipids, several therapeutic agents including sulfonylureas and K^+-channel openers (Seino, 1999; Mannhold, 2004). Different types of SUR subunits endow the channel with differential sensitivity to sulfonylureas: SUR1 possesses a high-affinity binding site for tolbutamide and glibenclamide, whereas SUR2A binds glibenclamide but not tolbutamide with high affinity (Dorschner et al., 1999). The physiological importance of the K_{ATP} channel is illustrated by the severe alterations in glucose- and sulfonylurea-induced insulin secretion caused by inactivation of genes encoding these proteins (Miki et al., 1998; Shiota et al., 2002). Though none of the K_{ATP} subunit knock-out mice has been reported infertile, unfortunately their detailed fertilization status has not been assessed (Minami et al., 2004). Furthermore, since the

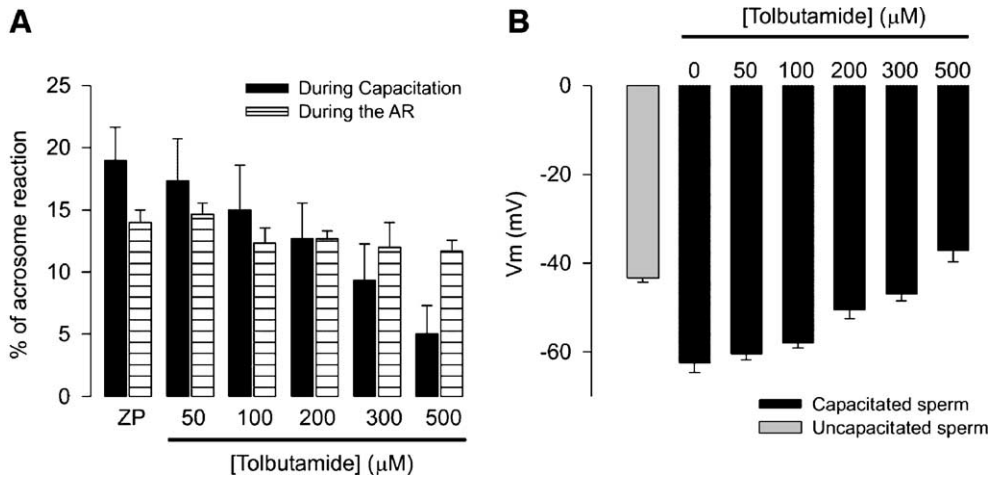

Fig. 7. Effects of tolbutamide on the *zona pellucida* (ZP)-induced sperm acrosome reaction (AR) and membrane hyperpolarization during capacitation. (A) Tolbutamide inhibits the ZP-induced AR by acting on capacitation. Capacitated mouse sperm were incubated 30 min in the presence of ZP and AR was measured. Bars represent the percentage of acrosome-reacted sperm after ZP-induction in the presence of the drug during capacitation or the AR (5 s before ZP addition). The ZP-induced AR values were normalized with respect to the control corrected for spontaneous AR which included the maximum DMSO volume used in the studies with the inhibitor. Tolbutamide did not significantly affect spontaneous AR: average values after capacitation were 16.6 ± 2.4 and 12 ± 3.7% after drug application. Data represent mean ± SEM of at least four independent experiments. One hundred sperm were evaluated per assay. (B) Sperm membrane hyperpolarization associated to capacitation. Bars represent mean ± SEM membrane potentials in uncapacitated as well as in capacitated sperm when increasing concentrations of tolbutamide are added to the capacitating medium. Sperm membrane potential was measured using the fluorescence dye $DiSC_3(5)$ (n = 4).

four K_{ATP} channel subunits are found in sperm, establishing the physiological role of K_{ATP} channels in these cells would require double knock-outs of $K_{ir}6.1$ and $K_{ir}6.2$ or SUR1 and SUR2 to rule out possible compensation.

We used three strategies to determine the presence and possible function of K_{ATP} channels in mouse spermatogenic cells and sperm. (1) Patch-clamp studies of K_{ir} currents in spermatogenic cells were carried out to explore their sensitivity to K_{ATP} channel inhibitors, to ATP and ATP synthesis inhibitors as well as to channel openers. (2) Membrane potential and AR measurements were determined in capacitated sperm populations in the absence and presence of tolbutamide. (3) K_{ATP} channel subunit mRNAs and proteins were identified using RT-PCR and immunolocalization.

(1) Under whole-cell patch-clamp conditions, we recorded a weak inwardly rectifying K^+ current that was sensitive to the sulfonylureas tolbutamide and glibenclamide (~40% inhibition at −100 mV; Figs. 1 and 2). The ability of these agents to significantly inhibit the current at micromolar concentrations, together with their partial reversibility, resembles their effects on both the native and cloned pancreatic β cell K_{ATP} channels (Aguilar-Bryan et al., 1995, 1998; Inagaki et al., 1995, 1996). As anticipated, these currents in spermatogenic cells increased (64 ± 14%) when glucose was removed from the external

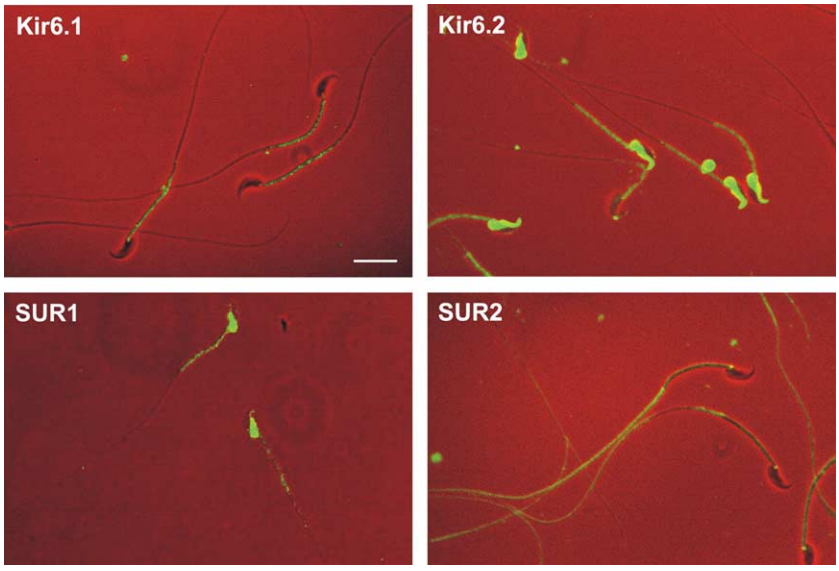

Fig. 8. Immunolocalization of K_{ATP} channel subunits in mature mouse sperm. Permeabilized cells were treated with $K_{ir}6.1$, $K_{ir}6.2$, SUR1, and SUR2B primary specific antibodies and were recognized with a secondary anti-rabbit antibody conjugated to Alexa Fluor (green). Peptide controls (see Materials and methods) are not shown but gave no significant staining. Scale bar: 10 μm.

media (Fig. 3), a maneuver to reduce internal ATP. This increase was prevented by glibenclamide. The presence of these K_{ATP} channels was confirmed using blockers of ATP synthesis and channel activators. Furthermore, these currents were also inhibited by $BaCl_2$ (Fig. 4) in a mildly voltage-dependent manner. The Ba^{2+} concentration dependence of the blockade suggests that more than one K_{ir} is present in spermatogenic cells, consistent with our findings that these cells display strongly rectifying K_{ir} (Muñoz-Garay et al., 2001) and K_{ATP} channels (this work). It is worth noting that in our earlier work studying the strongly rectifying K_{ir} current, spermatogenic symplasts displaying significant rectification were selected and the initial linear positive outward currents were subtracted (Muñoz-Garay et al., 2001). In the present work to characterize the weakly rectifying K_{ir} currents, all symplasts were recorded and no subtraction was carried out. This is the reason why under the present conditions Ba^{2+} blocks only 49% of the current. The remaining current probably includes non-inactivating, nonselective cation channels for instance of the TRP family (Darszon et al., 2005) and/or leak K^+ channels of the two pore family (Buckingham et al., 2005). Interestingly, the sulfonylureas inhibited ~38% of the current, indicating that the majority of the K_{ir} current flows through K_{ATP} channels.

(2) As reported previously (Arnoult et al., 1996, 1999; Espinosa and Darszon, 1995; Muþoz-Garay et al., 2001) and confirmed here (Fig. 5B), the resting Em in sperm is around −45 mV and after capacitation it hyperpolarizes to about −65 mV. The sperm resting Em, as in other somatic cells, is considerably less negative than the equilibrium potential for K^+ ions (E_K) (~−90 to −110 mV depending on the cell type). This indicates that K^+ channels do not completely dominate the permeability of the cell membrane at rest. As E_K is negative to the resting Em, opening K^+ channels will displace Em towards E_K resulting in a hyperpolarization; while closure of K^+ channels will cause a depolarization. Tolbutamide inhibited the sperm hyperpolarization that accompanies capacitation and capacitation itself (Fig. 5B), suggesting the involvement of K_{ATP} channels in this process. Sulfonylureas can also block other proteins of the ABC superfamily, such as the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel (Chang, 2003). Though CFTR mRNA has been detected in testis (Sheppard and Welsh, 1999) and specifically in spermatogenic cells, Western blot analysis performed with a CFTR specific antibody revealed immunoreactivity in membranes of round and elongated spermatids, but not in the fully developed sperm (Gong et al., 2001). Considering these findings and the blockade that Ba^{2+} causes on the capacitation associated hyperpolarization, the inhibition of sperm capacitation by tolbutamide suggests that indeed K_{ATP} channels participate in this important maturing process. However, our results do not rule out the involvement of other tolbutamide sensitive hyperpolarizing transporters in capacitation.

Recently, it has been shown that during capacitation the ATP content of sperm decreases (Baker et al., 2004). This would lead to an increase in the open time of K_{ATP} channels and would cause sperm hyperpolarization, which is consistent with the observed behavior of Em during capacitation. In addition, it is worth noting that K_{ir} channels and specifically K_{ATP} channels are regulated by CO_2/pH. The maximal activation occurs at pH 6.5 to 6.8, and the current through these channels seems to be inhibited at pH 6.2 to 5.9 (Wang et al., 2003). Since pHi increases during sperm capacitation from ~6.5 to ~6.7 (Vredenburgh-Wilberg and Parrish, 1995; Zeng et al., 1996), K_{ATP} channels would also be activated by this change.

(3) As a first step in the molecular identification of the different subunits that compose the sperm K_{ATP} channel, we searched for the presence of transcripts that correspond to the most likely composition. We found that transcripts for K_{ir}6.1 and K_{ir}6.2 and SUR1 and SUR2 are expressed in mouse spermatogenic cells (Fig. 6A). In addition, our immunocytochemical data show that K_{ir}6.1 and K_{ir}6.2 as well as SUR1 and SUR2B subunits are expressed in both spermatogenic cells and mature sperm (Figs. 6B and 8). Interestingly, the analysis of the regional expression of these subunits may give clues on the molecular entity responsible for K_{ATP} currents in these cells. A careful examination of the overall expression pattern and cellular localization of K_{ATP} channel subunits in mature sperm suggested that K_{ir}6.2 may co-localize and therefore associate predominantly with the SUR1 subunit. This points out to the presence of sulfonylurea-sensitive channels with molecular similarities to those found in the pancreatic β cells. K_{ATP} channels in these cells comprise K_{ir}6.2 and SUR1 subunits, while those of cardiac and skeletal muscle appear to be composed of K_{ir}6.2 and a splice variant of SUR2, SUR2A (Aguilar-Bryan and Bryan, 1999). Nevertheless, our results show that K_{ir}6.1 and SUR2 are also present in mature sperm, therefore other combinations of K_{ir} and SUR subunits are possible and may contribute to the hyperpolarization associated to capacitation or to other sperm functions such as hyperactivation and/or the AR. The possibility that K_{ATP} could be formed from heterotetramers has not been discarded (Cui et al., 2001). Furthermore, recent findings indicate SUR subunits may associate to other K_{ir} channels such as K_{ir}1 (ROMK) (Dong et al., 2001). Thus, a more detailed characterization of these channels both in spermatogenic cells and in sperm is required to fully define their molecular identity and function.

In summary, this work provides the fist evidence for the presence of K_{ATP} channels in mouse spermatogenic cells and sperm. In addition, evidence is presented for their contribution to the hyperpolarization that is associated to sperm capacitation. Evidently, these channels could participate also in other important functions such as spermatogenesis, sperm hyperactivation and the AR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.11.002.

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