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Identification of distinct K^+ channels in mouse spermatogenic cells and sperm

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Summary

Potassium (K^+) channels are believed to regulate mammalian sperm acquisition of fertilising capacity. However, the molecular identity of these proteins in sperm has not been elucidated. In this report, using immunoconfocal and electron microscopy we show that a minimum of four different classes of K^+ channels (Kv1.1, Kv1.2, Kv3.1 and GIRK1) are present and regionally distributed over the surface of mouse epididymal sperm. In addition, the use of reverse transcription polymerase chain reaction on RNA from mouse spermatogenic cells allowed the amplification of multiple transcripts corresponding to the channels identified by immunocytochemistry. Consistent with this, whole-cell patch-clamp recordings showed the expression of at least two different outwardly rectifying K^+ currents in spermatogenic cells.

Keywords: GIRK1, K^+ channel, Kv1.1, Kv1.2, Kv3.1, Sperm capacitation

Introduction

Mammalian sperm develop the ability to fertilise eggs after completing in the female tract a series of dynamic events known as capacitation. Cellular changes involved in sperm capacitation finally seem to permit the influx of calcium (Ca^{2+}) required for onset of the acrosome reaction (AR) (Yanagimachi, 1994). Although the theories on the mechanisms of capacitation are diverse (Baldi *et al*., 2000), there are some unifying aspects. Recent studies have established that capacitation is dependent on the extracellular environment, especially the presence of various ions. It has been proposed that during capacitation, there is a potassium (K^+)-mediated hyperpolarisation of sperm membrane that may act to prime Ca^{2+} channels for subsequent activation by ZP3 (Zeng *et al*., 1995; Arnoult *et al*., 1999), the physiological inductor of the AR. In spite of its importance it is not known how K^+ permeability is regulated during capacitation. Initial studies by Hagiwara & Kawa (1984) demonstrated the presence of voltage-gated K^+ channels in isolated rat male germ cells almost two decades ago, but the notion of their contribution to the K^+ efflux during mammalian sperm capacitation is recent (Arnoult *et al*., 1999; Muñoz-Garay *et al*., 2001). In addition, though K^+ channel transcript expression has been found in the reproductive system (Schreiber *et al*., 1998; Inanobe *et al*., 1999; Salvatore *et al*., 1999; Jacob *et al*., 2000) the molecular nature of sperm K^+ channels has not been elucidated. To address this issue, we began using a strategy that combines immunocytochemistry and molecular biology with electrophysiology to determine the expression of different types of K^+ channels in individual mouse spermatogenic cells and sperm.

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Materials and methods

Immunofluorescence

Epididymal sperm were obtained as described elsewhere (Serrano *et al.*, 1999). Cells were fixed with 4% formaldehyde and permeabilised with 1% Triton X-100. Samples were incubated for at least 1 h with specific primary antibodies (Table 1). After rinsing, samples were incubated with Alexa 594-conjugated (Molecular Probes) secondary antibodies (Table 1). Fluorescence images were acquired with a confocal microscope and analysed using Confocal Assistant software (BioRad).

Immunoelectron microscopy

Sperm were fixed with a mixture of 2% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M cacodylate buffer, and embedded in LR-White. Thin sections (90 nm) mounted in 300-mesh nickel grids were incubated with the different K^+ primary antibodies followed by incubation with the second antibody (Zymed Co.) conjugated to 20 nm gold particles (Table 1). Sections were contrasted with aqueous saturated uranyl acetate and lead citrate before electron microscope (Zeiss EM-10) observations.

Table 1 Specific K^+ channel antibodies used in immunocytochemistry experiments

Primary antibody[a]	Primary antibody dilution		Secondary antibody solution	
	IF	EM	IF[b]	EM[c]
APC-099 (anti-Kv1.1)	1/60	1/10	1/60	1/40
APC-010 (anti-Kv1.2)	1/200	1/10	1/200	1/40
APC-014 (anti-Kv3.1)	1/200	1/10	1/200	1/40
APC-005 (anti-GIRK1)	1/200	1/10	1/200	1/40

IF, immunofluorescence; EM, electron microscopy.
[a]Alomone Labs (Jerusalem, Israel); [b]Molecular Probes (Eugene, OR); [c]Zymed (San Francisco, CA).

RT-PCR

RNA from spermatogenic cells, isolated as previously described (Serrano *et al.*, 1999), was extracted using RNAzol (Life Technologies). The Superscript system (Life Technologies) was used for reverse transcription polymerase chain reaction (RT-PCR). Total RNA was reverse transcribed using random primers (Table 2). The resulting cDNA was used for amplification of the K^+ channel genes. cDNA fragments amplified by PCR were analysed in agarose cells and sequenced.

Electrophysiology

K^+ currents were recorded using the patch-clamp technique as previously described (Muñoz-Garay *et al.*, 2001). Cells were clamped at a holding potential of –90 mV and capacity transients were electronically compensated. Current records were captured on-line and digitised (5 kHz) following filtering (2 kHz). Pulse protocols, data capture and analysis were performed using pCLAMP software (Axon). To isolate K^+ currents, cells were bathed in a solution containing (in mM): sodium methanesulphonate 118; NaCl 8; $CaCl_2$ 2.5; KSO_4 2; $MgCl_2$ 1; HEPES 10; glucose 3.3 (pH 7.4), with or without tetraethylammonium chloride (TEA^+) 50. The internal solution consisted of (mM): potassium methanesulphonate 122; KCl 8; KFl 20; $CaCl_2$ 2.5; $MgCl_2$ 1; EGTA 5; HEPES 10 (pH 7.35).

Results and discussion

A set of commercial antibodies against different K^+ channels (Alomone Labs; Table 1) were used to characterise the expression and cell distribution of these proteins in mouse epididymal sperm. We found that mature sperm's repertoire of K^+ channels includes at least four different proteins – Kv1.1, Kv1.2, Kv3.1 and GIRK1 – which are differentially distributed in the cells (Fig. 1). No specific signal was found for Kv1.3, Kv3.2, GIRK2 and ROMK1 K^+ channels (not shown). Confocal images from sperm stained with anti-Kv1.1 (Fig. 1*A*) and anti-Kv1.2 (Fig. 1*B*) antibodies showed that these proteins are localised to the principal piece of the

Table 2 Sets of primers designed to ampify K^+ channels from mouse spermatogenic cells and the predicted sizes of the PCR products

Gene	GenBank accession no.	Forward primer 5′→3′	Reverse primer 5′→3′	Temperature (°C)/ Size (bp)
Kv1.1	M30439	AGCAGGAGGGAAATCAGAAG	TCGGTGGTAGAAATAGTTGA	55/428
Kv1.2	M30440	ATGAAGGCTACATCAAGGAA	CAGGCAAAGAATCTAACCAG	55/346
Kv3.1	Y07521	ACAAGACCGAAATCGAGAA	TGGTGGCAAAGATGAGC	48/426
GIRK1	1582163	GTGTCTGCCGAGATTTGA	GCGTTGGAACCTCTTTAT	50/476

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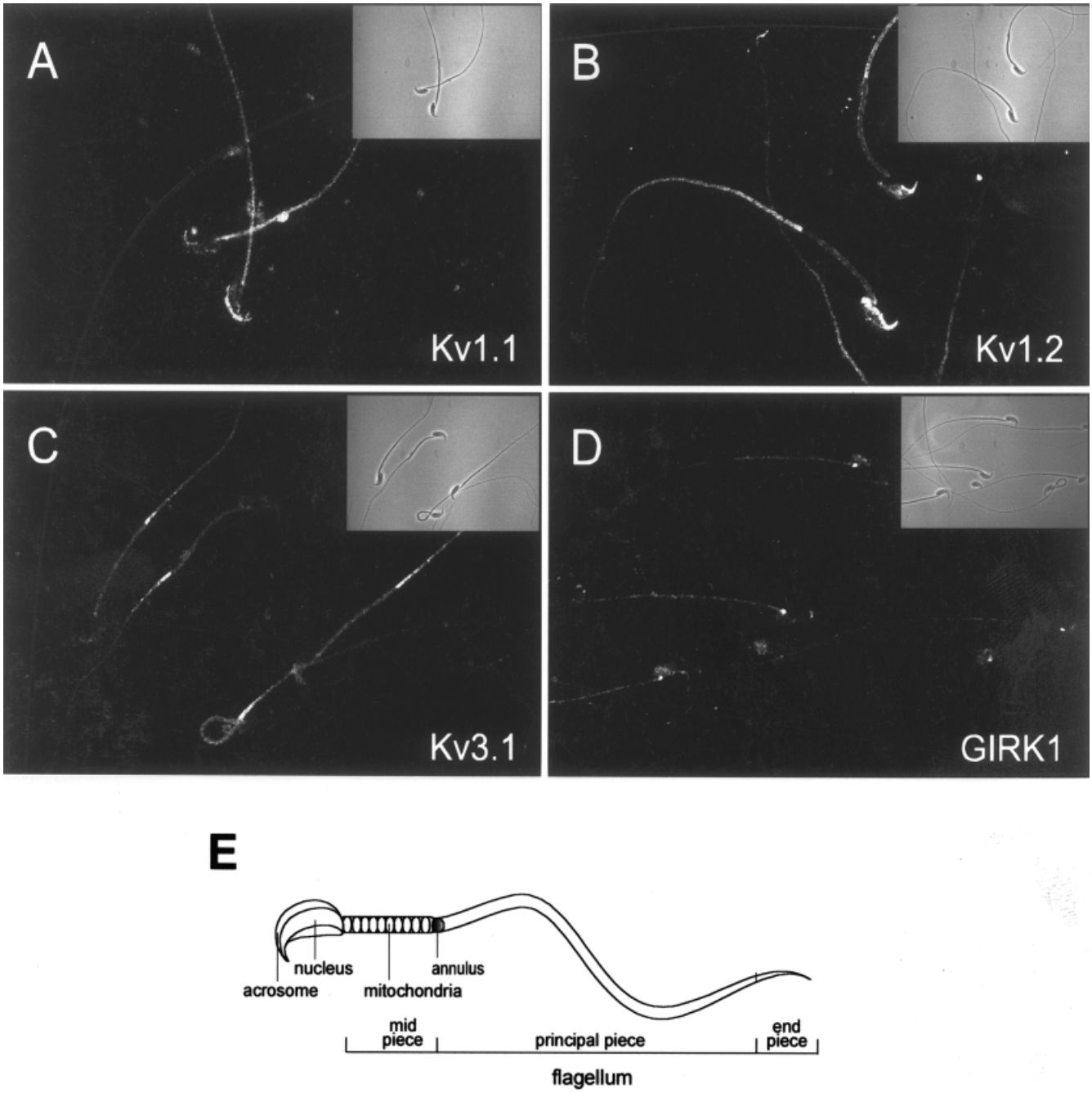

Figure 1 Immunolocalisation of distinct K⁺ channels in sperm. (*A*)–(*D*) Confocal immunofluorescence images of mouse mature sperm stained with Kv1.1, Kv1.2, Kv3.1 and GIRK1 antibodies, respectively, illustrating the immunoreactivity observed in different regions of the cells. Insets represent the corresponding phase-contrast images. (*E*) Schematic representation of the anatomy of a sperm.

flagellum and in the sperm head including the apical tip (see Fig. 1*E*). KV3.1 immunoreactivity was intense and regular in the annulus, and no signal was observed in the head (Fig. 1*C*), while GIRK-1 specific signal was confined mostly to the connecting piece between the tail and the head (Fig. 1*D*). Control experiments using antibodies blocked by exposure to the corresponding peptide antigen exhibited very low residual staining (not shown).

The presence of these four classes of sperm K⁺ channels was corroborated using immunogold analysis. Longitudinal sections and cross-sections showed the presence of the channels closely associated with the cell surface in both sperm head and tail. As shown in Fig. 2, the distribution pattern of Kv1.1 (Fig. 2*A*, *B*), Kv1.2 (Fig. 2*C*, *D*) and Kv3.1 (Fig. 2*E*, *F*) was essentially in accordance with that of immunofluorescence. Semiquantitative analysis confirmed that Kv1.1 and

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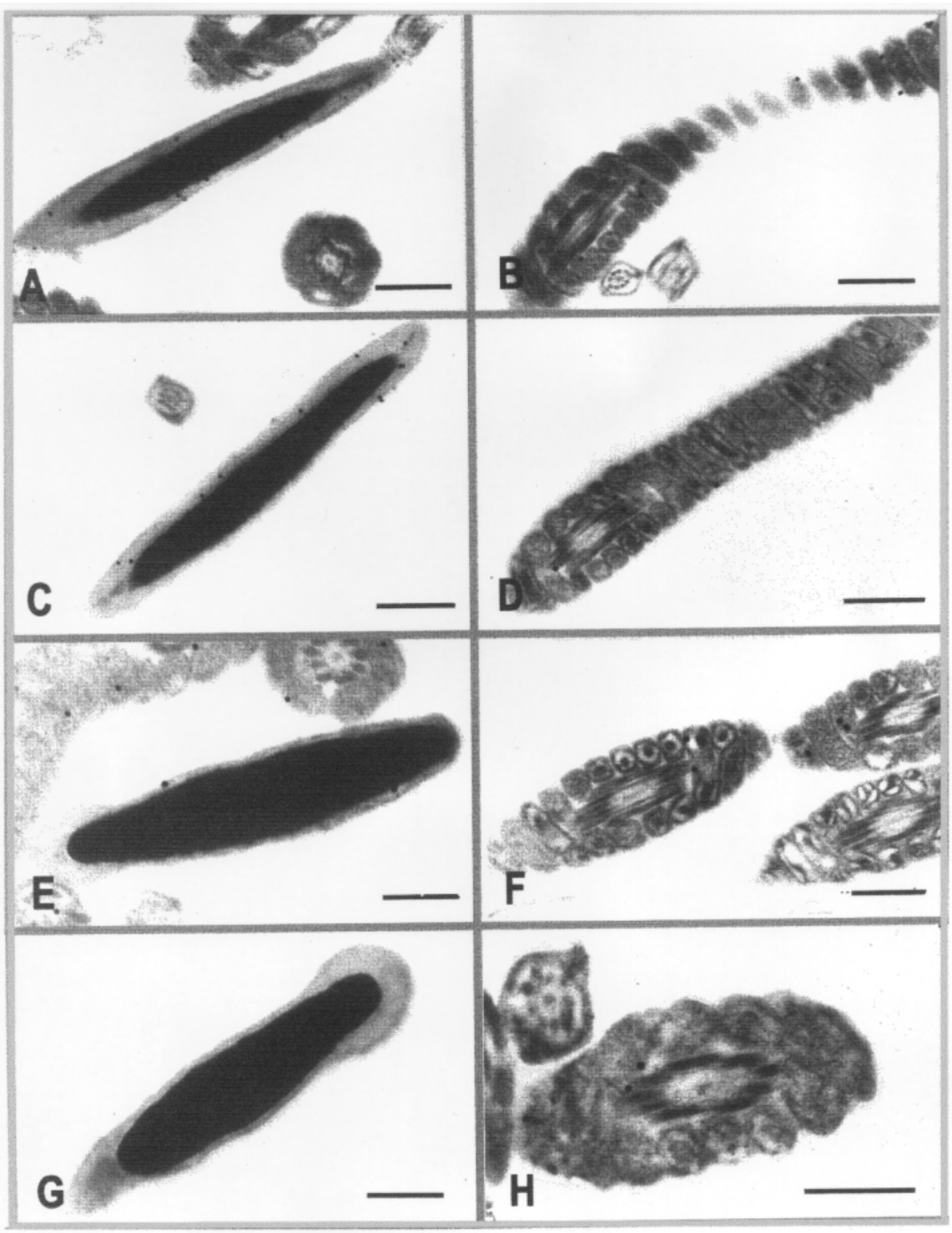

Figure 2 K^+ channel immunogold labelling of mouse sperm. Representative transmission electron micrograph sections of mature sperm stained with Kv1.1 (*A*, *B*), Kv1.2 (*C*, *D*), Kv3.1 (*E*, *F*) and GIRK1 (*G*, *H*) antibodies. Gold labels are shown as small black dots in the sperm head and flagellum. Scale bar represents 0.5 μm.

Kv1.2 were distributed to the head and tail of sperm, while Kv3.1 was localised mainly to the midpiece of the flagellum (not shown). GIRK1 immunogold signal was too faint for semiquantitative analysis and detection of any differential labelling between the head and the flagellum. As in immunofluorescence control experiments, the use of primary antibodies blocked with the peptide antigen completely abolished immunogold staining (not shown).

Having shown that different K^+ channels are pre-

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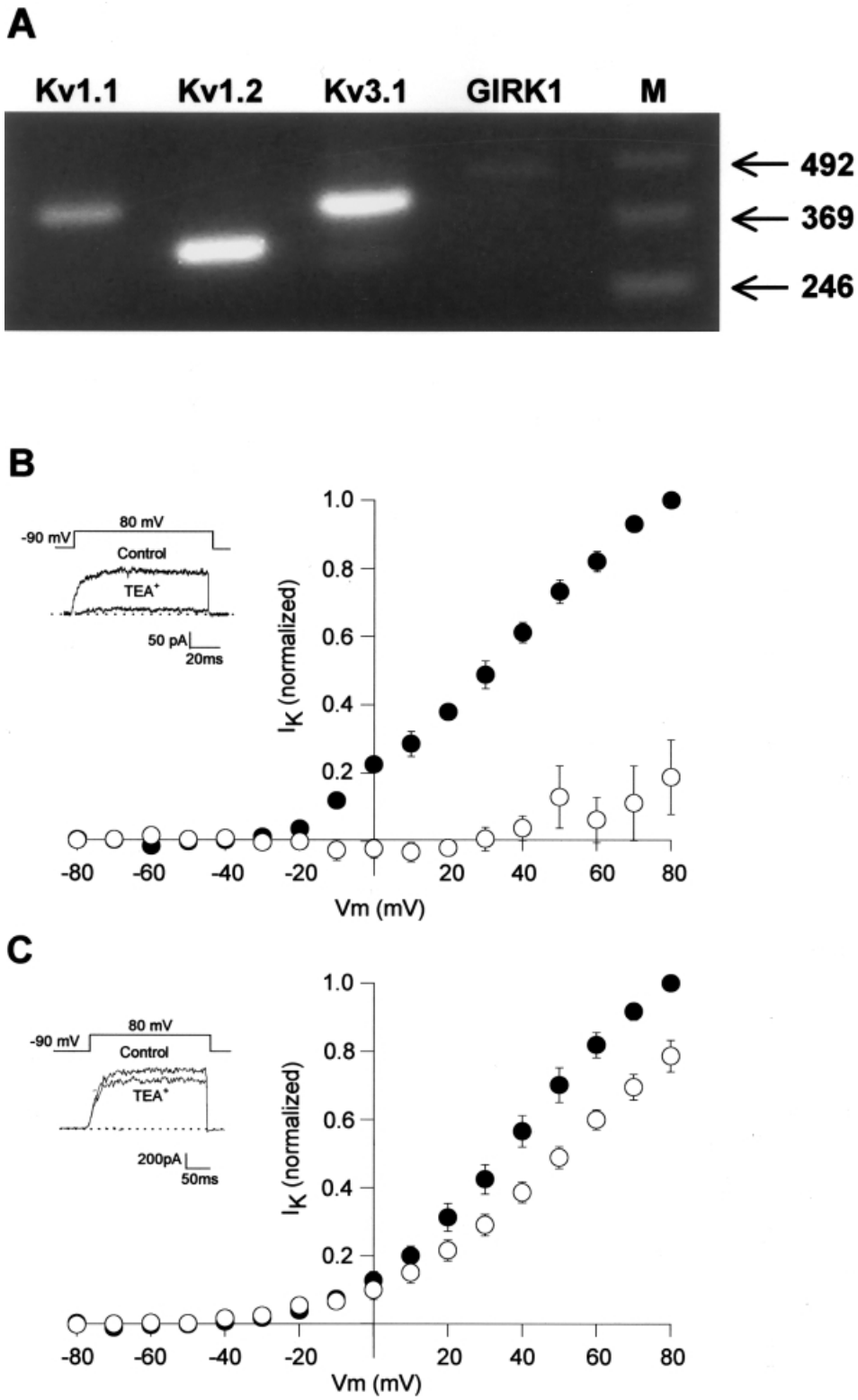

Figure 3 Expression of K^+ channels in spermatogenic cells. (*A*) RT-PCR from mouse spermatogenic cells for Kv1.1, Kv1.2, Kv3.1 and GIRK1 as listed. Products were sequenced to verify specific amplification and corresponded to the indicated channels. *M* denotes molecular weight standards. (*B*), (*C*) Plots of the normalized peak current–voltage relationship in a group of cells exhibiting sensitivity to TEA^+, and in a subset of cells insensitive to the blocking cation, respectively. Inset to figures show representative patch-clamp recordings of the whole-cell outward K^+ currents recorded in mouse spermatogenic cells in response to depolarising pulses from a holding potential of –90 to +80 mV in the presence and absence of TEA^+ in the two subgroups of cells (n = 5). Note the change in scaling.

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sent in sperm, we next wanted to explore their functional expression. However, sperm are very small and difficult to study electrophysiolgically; therefore K^+ currents must be examined in the larger and spherical progenitor spermatogenic cells. We first confirmed the presence of the corresponding mRNAs by using RT-PCR. Specific oligonucleotides (Table 2) were used to amplify the genes encoding the K^+ channels (Fig. 3*A*). Next, by using patch-clamp recordings we documented the presence of two K^+-selective currents. The first activated at potentials above –20 mV whose magnitude did not decay during the voltage step (Fig. 3*B*) and was effectively blocked by the addition of 50 mM of TEA^+ to the bath recording solution. This type of current was recorded in nearly all cells and had similar properties to that reported previously by Hagiwara & Kawa (1984) in rat spermatogenic cells. The activity of non-inactivating TEA^+-sensitive Kv3.1 channels may mediate most of this current. However, these results do not exclude the presence of Kv1.1, Kv1.2 and/or GIRK1 functional channels; their low level of expression and the lack of specific blockers impeded their detection. Interestingly, a subset of spermatogenic cells (it should be noted that at least three different stages of differentiation can be found in the cell suspension used in the electrophysiological recordings) displayed a fast activating, long-lasting outward K^+ current insensitive to TEA^+ (Fig. 3*C*). We noted that the size of this current was much larger than the current sensitive to the blocking cation. Some of the properties of this current (e.g. voltage dependence, kinetics, selectivity, and insensitivity to TEA^+) resembled the mSlo3 current through recombinant channels expressed in *Xenopus* oocytes (Schreiber *et al*., 1998) obtained from mouse. Corroboration of this speculation is an interesting topic for future studies.

Learning how K^+ channels operate and are regulated in sperm is essential to understand fertilisation. Correlating the presence and cellular distribution of K^+ channels with their functional status in spermatogenic cells and sperm will allow a better understanding of their participation in differentiation, capacitation and the sperm AR.

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