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Molecular characterization of a voltage-gated potassium channel expressed in rat testis*

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Potassium (K^+) channels are present in both mammalian testis and spermatozoa. Immunofluorescent detection of sperm-bound biotinylated charybdotoxin, an inhibitor of Ca^{2+}-activated and of delayed rectifier K^+ channels, indicated that these ion channels are uniformly distributed over the surface of both heads and tails of unfixed rat epididymal spermatozoa. Reverse transcription–polymerase chain reaction (RT–PCR) analysis on rat testis RNA with PCR primers, based on known nucleotide sequences of different classes of K^+ channels, amplified sequences homologous to delayed rectifier K^+ channels. In-situ RT–PCR on rat testis sections showed that these K^+ channel transcripts are present in the cytoplasm of primary spermatocytes and post-meiotic elongating spermatids. Northern blot analysis of various rat tissues identified multiple K^+ channel transcripts, some of which were observed only in testis. An attempt to obtain a full length rat testis K^+ channel cDNA sequence gave an assembled sequence of 2693 base pairs with >90% homology to a delayed rectifier K^+ channel, Kv1.3. A method for rapid amplification of cDNA ends was employed to amplify the 5′ sequences of the rat testis cDNA but a unique sequence could not be obtained. DNA sequencer traces suggest that multiple related K^+ channels which differed at their 5′ ends were amplified in rat testis.

Key words: charybdotoxin labelling/potassium channel/sperm membrane/spermatozoa/testis

Introduction

The acrosome reaction is an exocytotic event induced by sperm binding to the zona pellucida and requires extracellular calcium ions (Ca^{2+}). The elevation of sperm intracellular Ca^{2+} triggers the fusion of plasma and outer acrosomal membranes and release of proteolytic enzymes. This Ca^{2+} enters spermatozoa as the end result of a complex of partially characterized signal transduction pathways activated both by sperm capacitation and by zona binding (review: Benoff, 1998a, 1999).

The acrosome reaction was first identified in echinoderms (Dan, 1956) and has been best studied in these invertebrates. Egg jelly-induced Ca^{2+} influx is accompanied by sodium (Na^+) uptake and potassium (K^+) and proton (H^+) efflux (Schackmann *et al*., 1978; Schackmann and Shapiro, 1981). Inhibitors of Ca^{2+} and K^+ channels in somatic cells inhibit induction of the acrosome reaction (Schackmann and Shapiro, 1981) and bind with high affinity to sperm membranes (Kazazoglou *et al*., 1985; Mikami-Takaei and Yasumasu, 1989), suggesting that sea urchin sperm plasma membrane permeability to Ca^{2+} and K^+ is regulated by ion channels. Electrophysiological studies of sperm membrane proteins transferred to planar lipid bilayers provided additional evidence for the existence of sperm Ca^{2+} channels (e.g. Guerrero and Darszon, 1989) and K^+ channels (e.g. Cook and Babcock, 1993).

While studies in mammalian spermatozoa have been limited, a body of data now indicates that ion currents similar to those observed in sea urchin are associated with the mammalian sperm acrosome reaction and that these ion movements are regulated by monovalent- and divalent-selective cation channels inserted in the plasma membrane (reviewed by Darszon *et al*., 1999). Recent electrophysiological characterization of the ion channels in human sperm plasma membranes provides evidence for Ca^+, K^+, Na^+ and chloride (Cl^-) channels (Chan *et al*., 1997; Shi and Ma, 1998). Monovalent ion movements precede Ca^{2+} influx (e.g. O'Toole *et al*., 1996). Sustained Ca^+ influx occurs via voltage-dependent Ca^{2+} channels (Florman, 1994; Benoff, 1998b). The exact nature of these Ca^{2+} channels remains controversial, i.e. whether they are low voltage- or high voltage-activated.

Rat and human spermatozoa express L-type voltage-dependent Ca^{2+} channels (Goodwin *et al*., 1997). Such channels require a large depolarization of membrane potential for full activation (Florman *et al*., 1992). As in sea urchin spermatozoa (Trimmer *et al*., 1986), the permeability of mammalian spermatozoa to Ca^{2+} is modulated by alkaline shifts in internal pH (Florman *et al*., 1989; Zeng *et al*., 1996). Monovalent cation channels participate in this pH shift (Arnoult *et al*., 1996). In sea urchin, K^+ channels are the first to be activated in the pathway leading to the rise in pH of the sperm cytosol

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(Schackmann and Shapiro, 1981; Schackmann *et al*., 1981; Lee and Garbers, 1986). Thus, K^+ channel activation could be the primary event in the pathway leading to acrosome reaction in mammalian spermatozoa (Benoff, 1999).

There are at least three general classes of K^+ channels: (i) cyclic nucleotide-gated channels, (ii) voltage-gated channels, including delayed (outward) rectifiers and Ca^{2+}-activated channels, and (iii) inward rectifiers, including G-protein-gated inward rectifiers, ATP-dependent channels and P_{2X} purinergic receptors (for review see Christie, 1995). The conductance of K^+ channels from sea urchin spermatozoa suggests that they are related to delayed rectifier or Ca^{2+}-activated K^+ channels (Lievano *et al*., 1985). Therefore, in this initial attempt to provide a molecular characterization of the K^+ channels in mammalian spermatozoa, we have examined the expression of Ca^{2+}-activated K^+ channels and of delayed rectifier K^+ channels in rat testis.

Materials and methods

Products and reagents

Modified Ham's F-10 medium (Formula No. 90-8050PG) was obtained from Gibco Laboratories (Grand Island, NY, USA). Rat liver cDNA was obtained from Clontech Laboratories (Palo Alto, CA, USA). Unless otherwise specified, all chemicals were reagent grade or higher. Except where noted, all reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Design of a probe to detect sperm K^+ channels

We proceeded on the assumption that voltage-gated K^+ channels, if expressed by rat spermatozoa, would be similar to those detected in sea urchin (Lievano *et al*., 1985). Charybdotoxin is a potent inhibitor of both Ca^{2+}-activated K^+ channels and delayed rectifier voltage-gated K^+ currents in somatic cells (Deutsch *et al*., 1991; Brugnara *et al*., 1993, 1995). Therefore we chose to use charybdotoxin as a probe to localize K^+ channels on the surface membranes of rat spermatoza. Charybdotoxin is a single chain polypeptide of 37 amino acid residues including one histidine (imidiazole), one tyrosine (phenol), four lysines (primary amines) and six cysteines (sulphydryl) (Gimenez-Gallego *et al*., 1988). A ^{1}H-NMR-based three-dimensional solution structure (Bontems *et al*., 1991) and site-specific mutagenesis (Stampe *et al*., 1994) ruled out cysteines and tyrosine as potential targets for derivatization. Three disulphide linkages involving the six cysteines maintained the rigid tertiary structure necessary for functionality. Replacing the one tyrosine with other amino acid residues drastically reduced the mutant charybdotoxin's affinity for K^+ channel. Histidine and three of the four lysines (excluding Lys-27) were classed as 'indifferent residues', at which chemically radical changes did not affect binding affinity (Stampe *et al*., 1994). Thus, these indifferent residues can be candidates for labelling (e.g. with biotin).

Biotin is a target moiety that can initiate a number of alternative amplification and signal detection schemes. This flexibility allows selection of a scheme which optimizes detection sensitivity while minimizing non-specific signal in each particular application. Another factor influencing the choice of biotin was the commercial availability of reagents with biotin at one end of a 12–24 atom linker arm and moieties reactive with primary amines, sulphydryls, imidazoles, phenols and carboxylates at the other. In an accompanying model of a charybdotoxin–K^+ channel binding complex (Stampe *et al*., 1994), all four of these residues occupied the upper aqueous-accessible surface, so that attachment of biotin to any of these should not affect K^+ channel recognition. However, because the reagent for biotinylation of histidine, *p*-diazobenzyl biocytin, reacts with tyrosine under the same conditions, derivatization of charybdotoxin's histidine was ruled out. The usual protocol for biotinylation of protein lysines, which uses a large excess of biotinylating agent to maximize biotin incorporation, was obviously unsuitable as it would derivatize all four lysines, including the Lys-27 vital to K^+ channel binding. Two changes were made with the aim of limiting biotinylation to one or two residues: the molar ratio of biotinylating agent 'NHS–LC–biotin' (Pierce #21335, Rockford, IL, USA) to protein was markedly reduced, and a large excess of Tris buffer (containing tris[hydroxymethyl]aminomethane, a primary amine) was added to stop the reaction after an appropriate time by destroying the remaining NHS–LC–biotin.

Preparation of biotinylated charybdotoxin

Charybdotoxin (100 µg) was incubated with 1.5 µl of 10 mg/ml sulpho-NHS–LC–biotin in 100 µl for 2 h in an ice water bath. At the end of incubation, the biotinylation reaction was stopped by addition of 50 µl of a buffer containing 0.125 mol/l Tris base, 0.96 mol/l glycine and 10% methanol. Unreacted biotin and Tris–glycine were removed from the reaction mix by gel filtration chromatography over a 1.1 ml column of Bio Gel P polyacrylamide gel extra fine grade (Bio Rad Laboratories, Hercules, CA, USA) in phosphate-buffered saline (PBS). Ten µl of the 100 µl fractions collected from the Bio Gel P column was diluted with 49 µl PBS and blotted using a PR 648 Slot Blot Filtration Manifold (Hoefer Scientific Instruments, San Francisco, CA, USA) onto 100% methanol pre-washed Immobilon-PDVF membranes (Millipore Corp., Bedford, MA, USA). Biotinylated mannosylated bovine serum albumin (BSA; 1–4 µg) (Sigma No. 7924) was used as a positive control. Membranes were washed twice with PBS and blocked in a TTBS buffer (100 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% Tween-20, 1% BSA) for 1 h at room temperature. To evaluate biotin incorporation into charybdotoxin, membranes were incubated with avidin–horseradish peroxidase (HRP) and the HRP signal was developed using 3,3′,5,5′-tetramethylbenzidine substrate (TMB) kit according to protocols from the supplier (Vector Labs, Burlingame, CA, USA). Individual fractions that reacted with avidin–HRP were stored in aliquots with 0.01% sodium azide at –70°C.

Labelling rat spermatozoa with charybdotoxin

Spermatozoa collected from the cauda epididymides of 4 month old Sprague–Dawley male rats (>500 g) were divided into two aliquots. One aliquot was employed for immunohistochemistry with anti-Kv1.3 polyclonal antibodies (see below). The second aliquot was washed with Ham's F-10 medium and incubated with a 1:5 dilution of biotinylated charybdotoxin in PBS for 2 h at 37°C in 5% CO_2 in air. Free biotin replaced the biotinylated charybdotoxin in control reactions. After washing in PBS, spermatozoa were incubated with a 1:100 dilution of rabbit anti-biotin antibody (Catalog No. DS-861; Enzo Diagnostics, New York, NY, USA) for 16–18 h at 24°C. The sperm-bound biotinylated charybdotoxin was then detected with 1:160 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (Sigma No. F-0511) in PBS/0.05% BSA at 37°C for 45 min followed by 1:16 dilution of FITC-conjugated anti-goat immunoglobulin G (Sigma No. F-7367) in PBS-0.05% BSA at 37°C for 45 min. Additional control reactions were performed with unlabelled spermatozoa and each of the three antibodies alone or in all possible combinations. Labelled spermatozoa were then air-dried onto glass microscope slides and viewed at ×600 magnification with an Olympus BX50 microscope (Olympus Corp., Lake Success, NY, USA) equipped with epifluorescence optics. Labelled spermatozoa

were photographed at ×100 magnification on 35 mm/400 ASA black and white film (Eastman Kodak Co., Rochester, NY, USA).

Labelling of rat spermatozoa with anti-Kv1.3 polyclonal antibodies

Alternatively, freshly isolated epididymal spermatozoa were washed three times with PBS and smeared onto the well of a pre-cleaned heavy Teflon-coated (HTC) microscope slide (#10-1179; Cel-Line Associates, Inc., Newfield, NJ, USA) and allowed to air dry. Spermatozoa were sequentially reacted with anti-Kv1.3 polyclonal antibody prepared in rabbit (1:50 dilution; #APC-002; Alomone Labs, Jerusalem, Israel) and human spermatozoa pre-absorbed, fluorescein-conjugated sheep anti-rabbit IgG (1:160 dilution; Sigma No. F-0511). The labelling reactions employed buffers containing 0.5% Triton X-100 following protocols established in the laboratory (Benoff *et al*., 1996). Labelled spermatozoa were viewed as described above.

RNA isolation

Total cellular RNA was isolated from tissues from Sprague–Dawley male rats, 6 months of age with an approximate weight range of 300–400 g, using a modified protocol (Chomcznski and Sacchi, 1987) described previously (Goodwin *et al*., 1997). The RNA concentration was measured by ultraviolet absorbance at 260 nm and the RNA was stored at –70°C until analysis. For some experiments, total RNA (10 μg) was treated with 13 units of RNase-free DNase I (Gibco–BRL, Grand Island, NY, USA) at room temperature for 45 min. The DNase was then heat-inactivated for 15 min at 65°C. DNase-treated RNA preparations were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 3 mol/l sodium acetate, pH 5.2. The precipitate was stored at –70°C until use.

Design of K^+ channel PCR primers

Sequences for voltage-gated K^+ channels deposited in the National Center for Biotechnology Information (NCBI) database were identified using BLASTN 2.0 program (Altuschl *et al*., 1997) and accession numbers or key words for known Kv (delayed rectifier) channel sequences. The following sequences were identified: (i) mouse brain K^+ channel protein-1 (Accession No. Y00305); and (ii) rat mRNA for putative K^+ channel subunit proteins (RCK1 and RCK2, Accession No. X12589; RCK3, Accession No. X16001; RCK4, Accession No. X16002; RCK5, Accession No. X16003). The structure of the alpha subunit of these channels is shown in Figure 1. The amino acid sequences encoding transmembrane segments S1–S4 are highly conserved among all RCK channels and the amino acid sequences encoding transmembrane segments S5–S6 are identical (Stuhmer *et al*., 1989).

One RCK nucleotide sequence, RCK3 [the rat neural (cerebral) cortex voltage-dependent K^+ channel], was chosen at random for use in design of PCR primers. Note that RATKV3 (Accession No. M31744) is essentially homologous to RCK3 [differing only at three nucleotides encoding amino acid number 106, resulting in a substitution of phenylalanine in RATKV3 for leucine in RCK3 (Swanson *et al*.., 1990)] and that these sequences have been renamed Kv1.3 (Chandy and Gutman, 1995).

Initially, a single set of PCR primers complementary to the S4 and S6 regions of Kv1.3 (respectively, S4F and S6R) were synthesized on an Applied Biosystems Model 393 DNA synthesizer (Foster City, CA, USA). These primers were used in the original reverse transcription–polymerase chain reaction (RT–PCR), and in the Northern analysis and in-situ RT–PCR hybridizations. To amplify the full length cDNA, additional sets of paired primers were then synthesized. These primers were defined as rat voltage-gated K^+ channel (RVGKC) forward (F) and reverse (R) primers and are listed as follows: S4F, 5′ GTAAGGGTCTTCCGCATCTTCAA; S6R, 5′ AACAATCACAGGAACCGGCAATGC; RVGKC1F, 5′ AGTTCCCTGAGACGCTGCTAG; RVGKC1R, 5′ CATGATATTTCTGGAGAAGGTG; RVGKC2F, 5′ AGGACGTGTTTGAGGCTGCCAA; RVGKC2R, 5′ AACAATCACAGGAACCGGCAATGC; RVGKC3F, 5′ CATTGCCGGTTCCTGTGAT; RVGKC3R, 5′ AAGAGCTGAGAGAATGCAG; RVGKC4F, 5′ CTGCATTCTCTCAGCTCTT; RVGKC4R, 5′ ATCTTCCTGTGATACTGCA; RVGKC5F, 5′ TGCAGTATCACAGGAAGAT; RVGKC5R, 5′ ATTGAGGAAATCTGAAGGTA; RVGKC6F, 5′ ACCACCTGCTGGAGCCAGAAGC; RVGKC6R, 5′ TGTTGGCAGCCTCAAACACGTC.

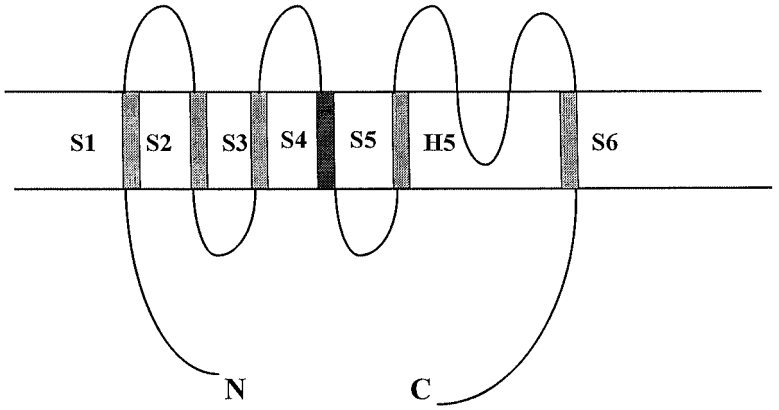

Figure 1. Schematic representation of alpha subunit of a voltage-gated K^+ channel. The alpha subunit of a typical voltage-gated K^+ channel consists of a single domain similar to the repetitive domains found in the alpha subunits of Na^+ and Ca^{2+} voltage-gated ion channels (Catterall, 1995; Benoff, 1998b), which are divided into six putative transmembrane domains designated as S1–S6 (shaded regions). Transmembrane segment S4 is the voltage sensor. The region between transmembrane segments S5 and S6, which protrudes into the membrane and is designated as H5, is the pore region. Amino and carboxy terminals, represented as N and C respectively, extend towards the inside of the cell.

Based on an earlier study (Kohler *et al*., 1996), *Rattus norvegicus* Ca^{2+}-activated K^+ channel sequences were retrieved from the NCBI database: (i) rSk1 mRNA (Accession No. U69885); (ii) rSk2 mRNA (Accession No. U69882); and (iii) rSk3 mRNA (Accession No. U69884). As above, one sequence (rSk1) was chosen at random for use in design of PCR primers. Two sets of paired primers were synthesized and are listed as follows: RCAKC1F, 5′ TGCTGTTGGTCTTCAGCAT; RCAKC1R, 5′ ACATCCAGGCGGCTCTCCA; RCAKC2F, 5′ TAAACGACTCAGTGACTAT; RCAKC2R, 5′ AGCCAGCAGATAGAGGCGCT.

Generation of PCR products from rat tissue RNA

To prepare first strand cDNA, total RNA (2 μg) was reverse transcribed in a total volume of 20 μl containing 5 mmol/l $MgCl_2$, 1× reverse transcription buffer (10 mmol/l Tris–HCl, 50 mmol/l KCl, and 0.1% Triton X-100), 1 mmol/l each dNTP, 20 IU of rRNasin ribonuclease inhibitor and 20 units of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of oligo $(dT)_{12}$ primer using a Reverse Transcription System Kit (Promega, Madison, WI, USA). The reaction was incubated at 42°C for 1 h. The resulting cDNA was precipitated with 3.5 mol/l ammonium acetate and 3 volumes of ethanol at –70°C overnight. The precipitate was centrifuged at 17 000 *g* for 30 min and the dried pellet was resuspended in 40 μl of H_2O. For PCR amplification of K^+ channel sequences, 3 μl cDNA, 1× PCR buffer (50 mmol/l KCl, 10 mmol/l Tris–HCl, pH 8.3), 1.5 mmol/l $MgCl_2$, 250 mmol/l dNTP, 50 pmol of each primer and 2.5 units of Taq

polymerase (Boehringer–Mannheim, Indianapolis, IN, USA) in a final volume of 100 µl were subjected to 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 4 min in a DNA Thermal cycler (Perkin–Elmer, Foster City, CA, USA). The size of the PCR products was estimated by co-electrophoresis of 20 µl of the completed PCR reaction and molecular weight size standards (Bio Rad Laboratories, Hercules, CA, USA) on a 1.2% agarose gel. Size-separated nucleic acids were visualized following ethidium bromide (EB) staining and photographed using a Gel Doc 1000 (Bio Rad).

5′ rapid amplification of cDNA ends (RACE)

To amplify the 5′ region of the rat testis Kv channels, additional sets of primers were synthesized from Kv1.3 sequences: RVGKCE1R, 5′ CCATAGCCTCCTCACCCAGTTGGT; RVGKCE2R, 5′ TCAAACACGTCCTGCGACGGAGAGG; RVGKCE3R, 5′ CCTCGGAGAAGATGTCGATGGGCAC; RVGKCE4R, 5′ CCAGCACTGACACGATGGCAATGCC.

Marathon Ready rat testis cDNA (Clontech, Palo Alto, CA, USA) was amplified with 5′ adaptor primer (AP1; component of the kit) and a 3′ gene-specific primer derived from the Kv1.3 nucleotide sequence (RVGKCE1R or RVGKCE2R) using the Advantage cDNA PCR kit (Clontech) according to manufacturer's protocols. A 1 µl aliquot of this PCR product was used as template in a second PCR reaction using 5′ nested adaptor primer (AP2; component of the kit) and nested 3′ gene-specific primers (RVGKCE3R or RVGKCE4R) according to methods described in the manufacturer's manual for 'touchdown PCR'. Touchdown PCR (Don *et al*., 1991; Roux, 1995) involves using an annealing temperature during the initial PCR cycles that is several degrees higher than the T_m of the adaptor primer (T_m >70°C). This allows the gene-specific product to accumulate. Then the annealing temperature is reduced to the adaptor primer T_m (68°C).

DNA sequence analysis

Amplified products were directly purified with Wizard PCR kit (Promega). The purified fragments were then sequenced directly using the original primers in an automated Model 370 DNA Sequencing System (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocol for fluorescence-based DNA sequencing with Taq polymerase. The sequences obtained were assembled to construct a full length cDNA sequence. Compiled sequences were translated by PC/Gene Release 7.1 (IntelliGenetics, Mountain View, CA, USA) and the longest open reading frame was identified. Derived amino acid sequences were then aligned with the Kv1.3 amino acid sequence and with the sequences deposited in the NCBI database using BLASTN 2.0 program.

Northern blot analysis

Total RNA (10 µg) from various rat tissues was size-fractionated on denaturing agarose gels, transferred to nylon membranes (Micron Separation Inc., Westboro, MA, USA) and hybridized according to published protocols (Maniatis *et al*., 1987) with ^{32}P-random-primed probe derived from a 2169 nucleotide clone encoding a portion of the rat testis-specific L-type voltage-dependent Ca^{2+} channel alpha-1C subunit (Goodwin *et al*., 1997) or actin cDNA from exons 4 and 5 (Goodwin *et al*., 1999). The membranes were then exposed to X-ray film (Kodak, New Haven, CT, USA) and the resultant autoradiogram photographed on GS-700 imaging densitometer (Bio Rad). The blot was stripped and reprobed with a ^{32}P-labelled PCR product generated using rat testis cDNA and S4F/S6R primers as per the standard laboratory protocol.

In-situ RT–PCR hybridization

We chose to use cryosections of rat testis rather than sections from paraffin-embedded tissues due to ease of preparation (Ausubil *et al*., 1991). Rat testis frozen sections (5 µm) were collected on in-situ PCR glass slides (Perkin–Elmer) and fixed in formalin as described earlier (Nuovo, 1994). The S4F/S6R primer pair in conjunction with an EZ rTth RNA PCR kit (Perkin–Elmer) and digoxigenin dUTP (Boehringer–Mannheim) was used to amplify Kv1.3 RNA sequences in these testis sections in a Gene Amp in-situ PCR System 1000 (Perkin–Elmer) following protocols established in the laboratory (Goodwin *et al*., 1998). Actin primers (forward and reverse, to exon 5) and primers derived against *E.coli* DNA origin of replication (forward primer 5′ CGAGATTACAAAAGTTACCTG and reverse primer 5′ CGTTAGCCCACCCAGCAAAA) were used in parallel control reactions. Prior to PCR amplification, testis sections were treated with 2 mg/ml pepsin for either 15 min or 30 min and subsequently DNase-treated as described previously (Goodwin *et al*., 1998). Testis sections which were protease-treated for 30 min but not DNase-treated were also included in this experiment. PCR products were detected using alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer–Mannheim) as previously described (Goodwin *et al*., 1998). Slides were mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA), examined at ×600 magnification with an Olympus BX50 microscope and photographed.

Results

Labelling rat spermatozoa with charybdotoxin

Before embarking upon an extensive molecular search, it was imperative to determine if the surface of mature rat spermatozoa contained K^+ channels related to Ca^{2+}-activated or delayed rectifier K^+ channels, and, if present, simultaneously determine their distribution within the various compartments of the sperm surface membrane. K^+ channel-specific antibodies would have been ideal probes for these purposes. However, at the time this study was initiated, such antibodies were not commercially available. Labelling of charybdotoxin with biotin was the chosen alternative. Charybdotoxin, one of a class of scorpion peptide neurotoxins, blocks voltage-gated K^+ channels by binding specifically to the external surface of K^+ channels at low concentrations (1 pmol/l to 10 nmol/l) (Deutsch *et al*., 1991; Brugnara *et al*., 1993, 1995; Stampe *et al*., 1994). It is implausible that a simple peptide such charybdotoxin would exhibit non-specific binding with such a high affinity to a random site on the sperm surface. Dissociation constants for non-specific interactions are typically in the micromolar range or higher, even at lower ionic strengths. Inhibition constants in the picomolar range, on the other hand, imply a specific binding site, as has been documented for this ion channel inhibitor.

Charybdotoxin was minimally biotinylated to preserve its K^+ channel binding activity (see Materials and methods) and unfixed rat epididymal spermatozoa were incubated with this preparation. Sperm-bound probe was localized following triple antibody staining employing anti-biotin antibodies. Spermatozoa incubated with free biotin served as a control. The pattern of non-specific staining observed with free biotin clearly differed from the staining pattern obtained with biotinylated charybdotoxin. While free biotin weakly reacted with the post-acrosomal segment of the sperm head and with the midpiece region (Figure 2C and D), a strong fluorescence signal was detected over the entire sperm head and sperm tail in aliquots labelled with the biotinylated charybdotoxin (Figure 2A and B).

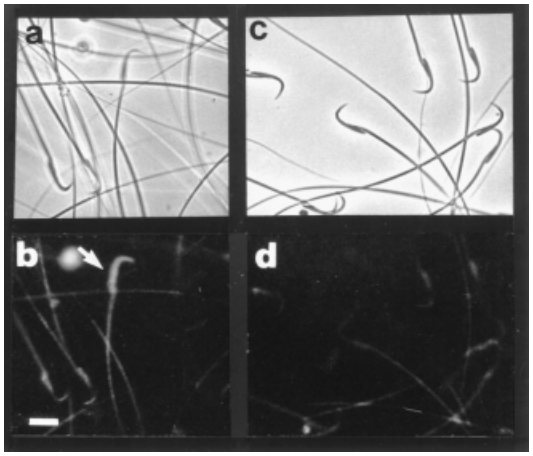

Figure 2. Localization of rat sperm K^+ channels using biotinylated charybdotoxin. Paired phase and epifluorescence photomicrographs are shown. Sperm-bound charybdotoxin (**a**, **b**) or free biotin (**c**, **d**) was detected by indirect immunocytochemistry (see Materials and methods). Note that free biotin binds only weakly to unfixed spermatozoa and this binding is observed primarily in the post-acrosomal segment of the sperm head and the midpiece (**d**). In contrast, charybdotoxin exhibits strong binding over the entire sperm head (**b**, arrow) and sperm tail. Scale bar = 15 μm.

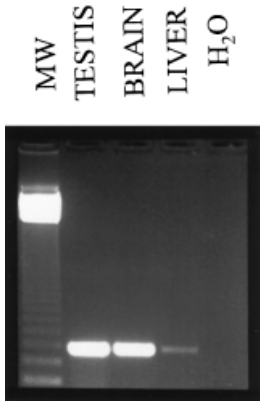

Figure 3. Expression of voltage-gated, delayed rectifier K^+ channels in rat tissues. Total RNA isolated and reverse transcribed to cDNA from rat testis (TESTIS), rat brain (BRAIN) and cDNA from rat liver (LIVER) was amplified using S4F/S6R primers. A 316 bp fragment was amplified from RNA from all tissues tested. Fragment size was determined by extrapolation from the 123 bp DNA ladder molecular weight size standard (MW; Cat No. 15613-011; Gibco–BRL, Grand Island, NY, USA). No EB staining is detectable in the lane containing the H_2O control (H_2O).

These studies were subsequently repeated using a polyclonal antibody directed against a recombinant fusion protein containing the carboxy terminus of the human homologue of the rat delayed rectifier K^+ channel Kv1.3. As observed for biotinylated charybdotoxin, this antibody specifically bound over the entire rat sperm head (not shown). Sperm-bound antibody was also detected on tails.

These data provide evidence for the presence of proteins related to voltage-gated K^+ channels in mature rat spermatozoa.

A delayed rectifier K^+ channel is expressed in rat testis

The initial attempt to amplify K^+ channel sequences in RNA isolated from rat testis employed primer pairs RCAKC1F/1R and RCAKC2F/2R, which were designed from a small-conductance, Ca^{2+}-activated K^+ channel expressed in brain (Kohler *et al*., 1996). PCR products of the expected size, 618 and 420 bp, respectively, were obtained with brain cDNA template. In contrast, PCR reaction performed with rat testis cDNA and the RCAKC1F/1R primer pair yielded a series of fragments unrelated in size to the gene-specific products (not shown). PCR reactions performed with rat testis cDNA and the RCAKC2F/2R primer pair resulted in amplification of 320 and 148 bp DNA fragments (not shown). Direct sequencing of the 320 bp product revealed 64–65% identity to *H.sapiens* mRNA for nuclear proteins SA-1 (EMBL 275331) and SA-2 (EMBL 275330). Direct sequencing of the 148 bp fragment yielded a sequence without significant homology to any sequence deposited in the NCBI database.

To search for the expression of delayed rectifier K^+ channels in rat testis, PCR was performed with primers to the S4–S6 region of Kv1.3. This region contains the ion conducting pore. A 316 bp PCR product was amplified from cDNA generated from testis (Figure 3). A similar-sized product was obtained from rat brain and liver templates (Figure 3). The nucleotide sequences obtained from direct sequencing of these PCR products were aligned with the sequence of the S4–S6 region of Kv1.3 sequence (not shown). This analysis demonstrated that the PCR products exhibited 96.8% homology with Kv1.3 cDNA. A further sequence homology search using BLASTN 2.0 non-redundant DNA sequence alignment from NCBI (Altschul *et al*., 1997) demonstrated that the PCR products from rat tissues were also related to rat voltage-gated K^+ channel protein, RATRGK5 (Accession No. M30312), with similar homology in the corresponding region of S4–S6 (not shown).

These data suggest that mRNA for small-conductance, Ca^{2+}-activated K^+ channels are not abundant in rat testis. In addition, these observations provide the first molecular evidence that a gene encoding a voltage-gated, delayed rectifier K^+ channel is expressed in rat testis.

Localization of voltage-gated K^+ channel RNA transcripts in rat testis

In-situ RT–PCR was performed using S4F/S6R primers and digoxigenin dUTP as a tagged nucleotide in order to examine the expression pattern of voltage-gated K^+ channels in rat testis (Figure 4A). PCR products were identified by a positive colour reaction (i.e. blue precipitate) with the alkaline phosphatase-conjugated anti-digoxigenin antibody.

Control testis sections were pepsin-digested for 30 min and not treated with DNase. Following in-situ RT–PCR with the S4F/S6R primer pair, such sections exhibited a relatively uniform, high intensity blue precipitate, suggesting that amplification of genomic K^+ channel sequences had occurred (not shown). In contrast, in sections that were both protease- and DNase-treated, the PCR signal was markedly reduced and limited primarily to the cytoplasm of primary spermatocytes in stages VII through IX/X of rat spermatogenesis. In addition, there was a dramatic decrease in the ability to detect K^+

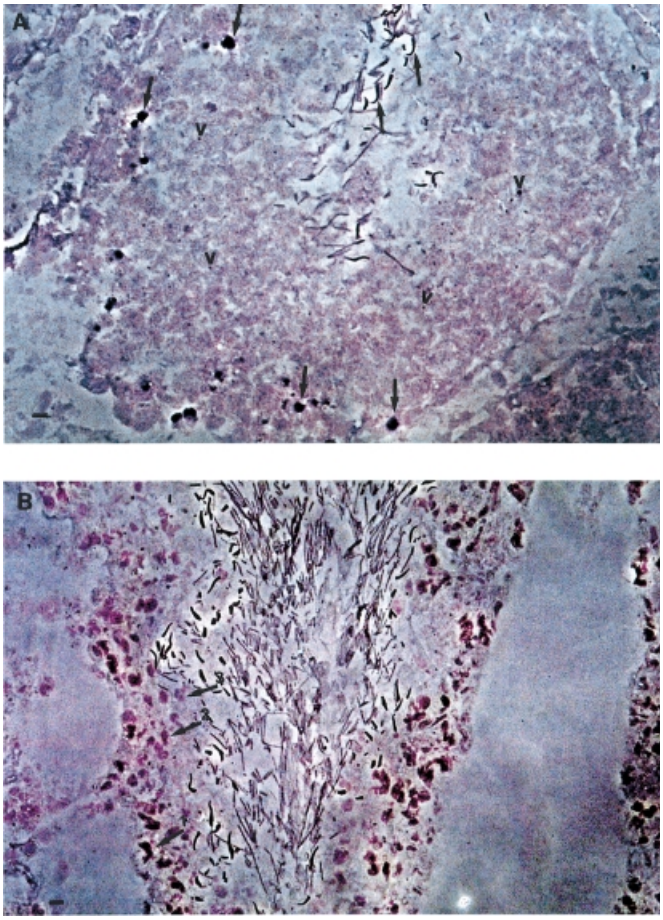

Figure 4. K^+ channel transcripts are observed in post-meiotic male germ cells. Typical results are shown. Scale bars represent 10 μm. (**A**) Following in-situ reverse transcription–polymerase chain reaction (RT–PCR) using the S4F/S6R primer pair, a blue precipitate, indicative of interaction of alkaline phosphatase-conjugated antidigoxigenin antibody with digoxigenin-conjugated nucleotides in the PCR product, is observed over the heads of post-meiotic elongating spermatids and mature spermatozoa. Arrows indicate products amplified in mature spermatozoa present in the lumen of seminiferous tubules as well as in primary spermatocytes located in the adluminal compartment and basal cells (which could include spermatogonia and muscle cells). Open arrow heads point to smaller precipitates occurring throughout the seminiferous epithelium, which may represent decaying K^+ channel transcripts or low levels of K^+ channel gene transcription. Further studies are required to distinguish between these two possibilities. (**B**) In contrast to K^+ channel transcripts, actin transcripts are present throughout the seminiferous tubules. A gradient of precipitate densities is observed from the cells in the basement membrane (i.e. darker; arrow no. 1) to the cells closer to the lumen (i.e. lighter; arrow nos. 2 and 3). Amplified product is also detectable in mature spermatozoa.

channel-specific transcripts when the sections were exposed to shorter protease digestion time (data not shown).

Protease pretreatment can distort the morphology of cryosections (Nuovo, 1994). Therefore sperm cell associations in sectioned rat seminiferous tubules were identified using a staging scheme (Leblond and Clermont, 1952). In these frozen sections, no signal was seen in the regions of the seminiferous tubule where immature spermatozoa were present near the lumen of the tubule. Signal was only seen above the germinal layer in cells presumed to be primary spermatocytes by their association with mature sperm heads and spermatozoa undergoing release (spermiation) near the tubule lumen (Figure 4A). K^+ channel transcripts were also observed in mature spermatozoa released into the lumen of the seminiferous tubules (Figure 4A). No blue precipitate was observed in negative controls, i.e. testis sections which were not protease digested or testis sections amplified with *E.coli* primers (data not shown), confirming that the signal present in the post-meiotic elongating spermatids and mature spermatozoa was specific for K^+ channel mRNA.

In-situ RT–PCR with rat testis sections and actin primers was employed as positive control for this study. Actin transcripts were detected throughout the testis, including cells in interstitial regions. The density of the actin hybridization signal was more abundant in cells closer to the basement membrane of the seminiferous tubules as compared to cells closer to the lumen, i.e. with differentiation of cells of the male germ line (Figure 4B). This is consistent with reports that actin mRNA levels are higher in pre-meiotic cells than in post-meiotic cells (Hecht and Penshow, 1987; Caldwell and Handel, 1991).

These data illustrate the differing patterns of gene expression which occur during spermatogenesis in the rat.

Northern blot analysis

To provide an estimate of the number of structurally distinct delayed rectifier K^+ channel sequences expressed in different rat tissues, total RNA from various rat tissues were size-fractionated by electrophoresis through denaturing agarose gels, blotted onto nylon membranes and hybridized with ^{32}P-labelled 316 bp PCR product generated using rat testis RNA as template and the S4F/S6R primer pair (Figure 5). A 5 kb RNA transcript was detected in rat heart, thymus, kidney, spleen, skeletal muscle, liver, lung and testis which hybridized to the Kv1.3 cDNA probe sequence. Two less abundant transcripts of 4.2 and 2.4 kb were observed only in the testis.

It is important to note that a hybridization signal was detected only after a relatively long exposure of the membrane to X-ray film, i.e. 10 days, as compared to the 2 day exposures required to detect L-type voltage-dependent Ca^{2+} channel alpha-1C subunit transcripts and actin transcripts (Goodwin *et al.*, 1999). These data suggest that transcripts encoding K^+ channels in testis are less abundant than those encoding Ca^{2+} channels or a housekeeping gene.

Attempts to amplify full length rat testis K^+ channel cDNA sequences

To further characterize the K^+ channel expressed in rat testis, six sets of paired primers were synthesized. These primers were designed so that they spanned the entire cDNA sequence of the rat neural cortex voltage-gated K^+ channel Kv1.3 (see Materials and methods and Figure 6). These primers were employed in PCR reactions using testis cDNA as template.

With the one exception of the RAKC6F/RAKC6R primer pair, the primer sets amplified robust products of the expected size (not shown). However, a series of additional minor products was observed in each case. Such products could have resulted from amplification of related sequences or non-

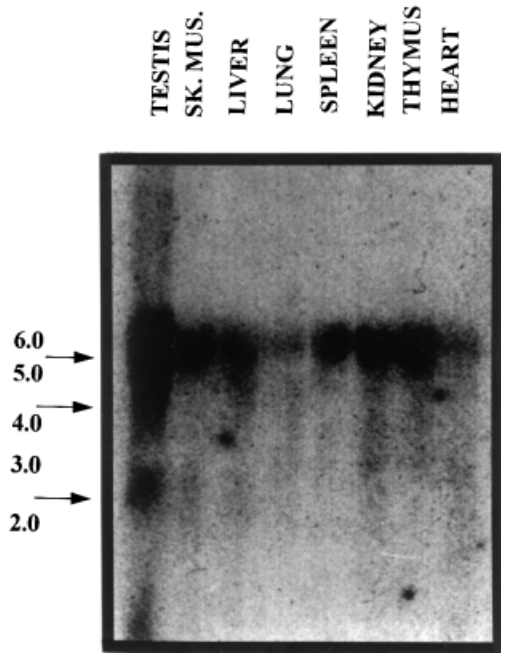

Figure 5. Northern hybridization of total RNA from different rat tissues with ^{32}P-labelled polymerase chain reaction product prepared with rat testis RNA template and the S4F/S6R primer pair. The numbers to the left of the blot represent sizes (in kb) extrapolated from the 1 kb marker RNA ladder (no. 15620-016; Gibco–BRL). Arrows identify transcripts which hybridized with the probe. A 5 kb transcript was detected in all tissues analysed. Additional 4.2 and 2.4 kb transcripts were detected only in testis. SK. MUS. = skeletal muscle.

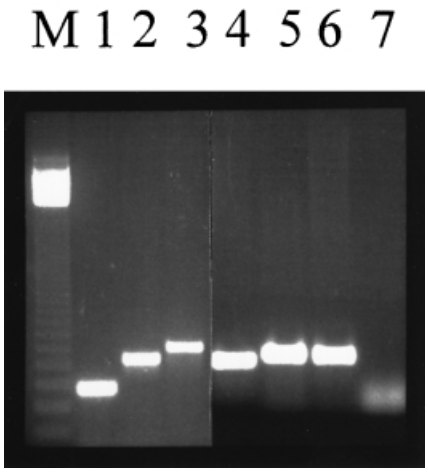

Figure 6. Robust polymerase chain reaction (PCR) products were obtained when total RNA isolated from rat testis was reverse transcribed into cDNA and then amplified using primers designed from internal sequences of the rat cerebral cortex voltage-dependent potassium channel Kv1.3. The region amplified by a specific primer pair is as indicated: (1) S4F/S6R (nucleotides 962 to 1274), (2) RVGKC3F/3R (nucleotides 1276 to 1793, (3) RVGKC4F/4R (nucleotides 1775 to 2392, (4) RVGKC5F/5R (nucleotides 2374 to 2940), (5) RVGKC2F/2R (nucleotides 669 to 1298), and (6) RVGKC1F/1R (nucleotides 228 to 841). In contrast, primers designed to amplify the 5′ end of the transcript (7), RVGKC6F/6R (nucleotides 27 to 693), failed to elicit a gene-specific product; only primer dimers were detected. The sizes of PCR-amplified products were calculated based on their mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp molecular weight marker ladder (M).

```
RSKCP   M-------------------------------------------------   1
KV1.3   MTVVPGDHLLEPEAAGGGGGDPPQGGCVSGGGCDRYEPLPPALPAAGEQD  50
        *

RSKCP   -------------------------------------RYFDPLRNEYFFD  14
KV1.3   CCGERVVINISGLRFETQLKTLCQFPETLLGDPKRRMRYFDPLRNEYFFD 100
                                              ************

RSKCP   RNRPSFDAILYYYQSGGRIRRPVNVPIDIFSEEIRFYQLGEEAMEKFRED  64
KV1.3   RNRPSLDAILYYYQSGGRIRRPVNVPIDIFSEEIRFYQLGEEAMEKFRED 150
        *****.*******************************************

RSKCP   EGFLREEERPLPRRDFQRQVWLLFEYPESSGPARGIAIVSVLVILISIVI 114
KV1.3   EGFLREEERPLPRRDFQRQVWLLFEYPESSGPARGIAIVSVLVILISIVI 200
        **************************************************

RSKCP   FCLETLPEFRXEKDYPASPSQDVFEAANNSTSGASSGASSFSDPFFVVET 164
KV1.3   FCLETLPEFRDEKDYPASPSQDVFEAANNSTSGASSGASSFSDPFFVVET 250
        ********** ***************************************

RSKCP   LCIIWFSFELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTELAER 214
KV1.3   LCIIWFSFELLVRFFACPSKATFSRNIMNLIDIVAIIPYFITLGTELAER 300
        **************************************************

RSKCP   QGNGQQAMSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGL 264
KV1.3   QGNGQQAMSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGL 350
        ************************************************** 
                                          b
                                            c
RSKCP   LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDM 314
KV1.3   LIFFLFIGVILFSSAVYFAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDM 400
        **************************************************

RSKCP   HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEGEEQAQYM 364
KV1.3   HPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETEGEEQAQYM 450
        ************************************************** 
                                                      a
RSKCP   HVGSCQHLSSSAEELRKARSNSTLSKSEYMVIEEGGMNHSAFPQTPFKTG 414
KV1.3   HVGSCQHLSSSAEELRKARSNSTLSKSEYMVIEEGGMNHSAFPQTPFKTG 500
        ************************************************** 
          d
RSKCP   NSTATCTTNNNPNSCVNIKKIFTDV  439
KV1.3   NSTATCTTNNNPNSCVNIKKIFTDV  525
        *************************
```

Figure 7. Alignment of the deduced amino acid sequence of the rat testis voltage-gated K^+ channel (RSKCP) with Kv1.3 sequences. Perfect sequence conservation is denoted by an asterisk below the sequence and conservative amino acid changes are indicated by a full point. Lines in the figure denote sites for modification of channel activity: (a) a potential site for tyrosine phosphorylation (Holmes *et al*., 1996), (b, c) two potential protein kinase C sites, and (d) a potential protein kinase A site (Stuhmer *et al*., 1989).

functional shortened transcripts or from amplification of contaminating genomic DNA. To eliminate the latter possibility, total RNA from testis was digested with RNase-free, DNase I prior to RT into cDNA. After DNase treatment, only a single PCR product of the predicted size was obtained (Figure 6).

Each PCR product was purified and subjected to direct DNA sequencing. The sequence of each PCR fragment of the testis K^+ channel sequence exhibited a 90–95% homology with its corresponding sequence within Kv1.3. The sequences from the individual PCR products were assembled into a 2693 base pair sequence. The longest open reading frame in this sequence is 439 amino acids in length. This deduced amino acid sequence was aligned with the amino acid sequences of Kv1.3, demonstrating an overall 99% identity in the corresponding region (Figure 7). Note that a phenylalanine is present at position 106 of the partial amino acid sequence of the rat testis K^+ channel. The sites for phosphorylation by protein kinase C (PKC) and by tyrosine kinase (TK) which are present in Kv1.3 were also identified in the testis K^+ channel.

These data demonstrate that a K^+ channel transcript expressed in rat testis encodes a delayed rectifier K^+ channel.

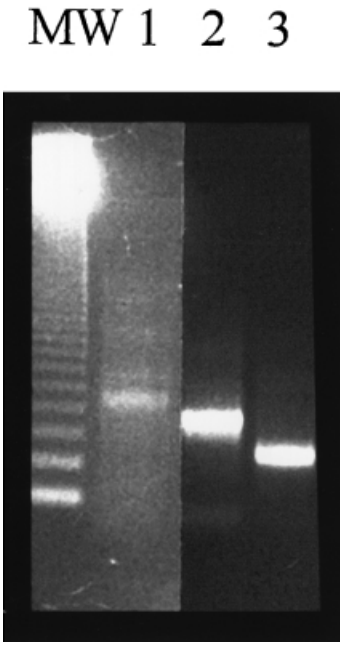

Figure 8. Results from 5′ rapid amplification of cDNA ends. Typical results are shown. The sizes of polymerase chain reaction (PCR)-amplified products were calculated based on their mobility in a 1.2% agarose gel containing ethidium bromide relative to the DNA fragments of the 123 bp marker ladder (M). Rat testis cDNA was amplified using 5′ adaptor primer (AP1) and 3′ gene-specific primer (RVGKCE1) (lane 1). A 1 µl aliquot of the PCR reaction from lane 1 was reamplified with 5′ nested adaptor primer (AP2) and 3′ gene-specific primers RVGKCE3 (lane 2) and RVGKCE4 (lane 3).

5′ RACE

The failure to obtain a PCR product with extreme 5′ primer pair RAKC6F/RAKC6R suggests that the 5′ end of the K^+ channel transcript in rat testis is structurally different from Kv1.3. Therefore, the method for rapid amplification of cDNA ends (RACE) was employed in an attempt to amplify the 5′ sequences of the rat testis cDNA. The gene-specific primers used in these reactions were RVGKCE1 or RVGKCE2. A smear of EB staining was observed when the products of these 5′ RACE reactions were analysed by electrophoresis through an agarose gel (e.g. Figure 8, lane 1). According to the supplier of the reagents used for RACE, a smear is most often seen when amplifying cDNA from a multigene family in which the gene-specific primer may simultaneously amplify several highly homologous cDNA.

To obtain a single product, an aliquot of this RACE reaction was used in a second PCR using a nested 5′ primer (AP2) and two different nested 3′ gene-specific primers (RVGKCE3 and RVGKCE4) (Figure 8, lanes 2 and 3). The PCR reactions generated single robust products of 300 or 400 bp with the 3′ gene specific primers. Both products were purified and subjected to direct DNA sequencing. A unique sequence could not be obtained with either product. The inability to obtain a single sequence was not due to lower amounts of PCR products amplified from the RACE reaction (see Figure 8, lanes 2 and 3). Rather, the chromatogram from the automated DNA sequencer suggested that multiple cDNA sequences were imposed on each other.

These data suggest that multiple related K^+ channel sequences were present in rat testis.

Discussion

Our interest in mammalian sperm K^+ channels stems from four sets of observations. First, it was reported that exposure of human spermatozoa to progesterone results in the activation of a monovalent cation channel (Foresta *et al*., 1993). Second, it was found that progesterone-regulated ion movements in human spermatozoa are involved in cytosolic alkalinization (Garcia and Meizel, 1996). Third, altered sperm membrane potential in many infertile men is associated with defective K^+ transport (Calzada and Tellez, 1997). Fourth, we observed that: (i) progesterone binding to the human sperm surface non-nuclear progesterone receptor activates K^+ channels; (ii) K^+ channel inhibitors block the acrosome reaction induced by model zona ligands containing mannose; and (iii) inhibition of progesterone-stimulated acrosome loss is a biomarker for the effects on sperm function of environmental exposure to heavy metals such as lead (Benoff, 1999; Benoff *et al*., 1999; A.Jacob, I.R.Hurley, G.M.Centola, G.W.Cooper and S.Benoff, unpublished observations).

Molecular characterization of K^+ channels in spermatozoa or testis has been limited. To our knowledge only four such channels have been reported. First, sea urchin spermatozoa express K^+ channels on their head (Guerrero *et al*., 1987) and flagellar (Lievano *et al*., 1985; Labarca *et al*., 1996) plasma membranes. Their electrophysiological characterization indicated that sea urchin sperm K^+ channels exhibit a relatively large unitary conductance, suggesting that they are related to voltage-gated Ca^{2+}-activated (Latorre and Miller, 1983) or delayed rectifier (Conti and Neher, 1980) K^+ channels (for discussion, see Lievano *et al*., 1985). Second, a hyperpolarization-activated K^+ channel (SPIH) regulated by voltage and adenosine 3′,5′-cyclic monophosphate was recently cloned in sea urchin spermatozoa (Gauss *et al*., 1998). This channel activity is confined to the sperm flagellum, suggesting that SPIH channels are involved in flagellar beating. Third, a cyclic nucleotide-gated (CNG) channel has been cloned from bovine testis (Weyand *et al*., 1994). CNG K^+ channels have also been shown in sea urchin spermatozoa (Cook and Babcock, 1993; Labarca *et al*., 1996). Fourth, a pH-sensitive K^+ channel (Slo3) has been identified in mammalian spermatocytes (Schreiber *et al*.., 1998) that is highly homologous to the large conductance Ca^{2+}-activated (BK) K^+ channel, Slo1. BK K^+ channels are expressed in spermatogenic cells from rat testis (Chan *et al*., 1998). Thus, this report is the first to document the expression of delayed rectifier K^+ channels in mammalian testis and spermatozoa.

Multiple mammalian K^+ channel gene families have been identified using a *Drosophila Shaker* K^+ channel cDNA probe (Baumann *et al*., 1988; Stuhmer *et al*., 1989). The Kv1 family is the best characterized and its members primarily encode delayed rectifiers when expressed *in vitro* (Pongs, 1992; Salkoff *et al*., 1992). The Kv1 gene family is comprised of at least eight members, Kv1.1–Kv1.8 (Lock *et al*., 1994). The rat testis K^+ channel sequence described herein showed 96–98%

nucleotide sequence identity to the corresponding regions of Kv1.3 cDNA and a 99% amino acid sequence identity to Kv1.3 protein.

Our in-situ RT–PCR localization of K^+ channel transcripts indicated that the channel mRNA is observed in the cytoplasm of primary spermatocytes in stages VII through IX/X of spermatogenesis, when actin interacts with the developing acrosome granule to regulate shaping of the acrosome around the sperm nucleus and establish the mature acrosome and head shape (Leblond and Clermont, 1952; Vogl, 1989). These stage- and cell type-specific findings on K^+ channel gene expression are in contrast to similar studies performed to detect the mRNA encoding a component of another ion channel participating in the human sperm acrosome reaction, the alpha-1C subunit of L-type voltage-dependent Ca^{2+} channels (Goodwin *et al*., 1998, 2000). L-type alpha-1C transcripts were amplified in all cell types found within the rat seminiferous tubules but not in the interstitial spaces. Further, accumulation of L-type alpha-1C transcripts was not limited to a particular stage of the cycle of the rat seminiferous epithelium.

Nevertheless, as observed for Ca^{2+} channel transcripts (Goodwin *et al*., 1998, 2000), K^+ channel transcripts are also present in post-meiotic elongating spermatids and mature spermatozoa. Hybridization signal was not detected in intervening stages (e.g. round spermatids) of sperm development. This observation is somewhat perplexing. Since the S4F/S6R primer pair used to amplify K^+ channel in rat testis is derived from a highly conserved region of different classes of K^+ channels, one possible explanation for this discrepancy is that the transcript observed in the cytoplasm of primary spermatocytes is distinct from that present in the elongating spermatids. In this regard, we note that a unique form of actin mRNA is expressed solely in the post-meiotic stages (Gu *et al*., 1996).

Our Northern blot analysis provides evidence for expression of at least three Kv1-related transcripts in rat testis, i.e. 5, 4.2 and 2.4 kb. Multiple related mRNA for K^+ channel proteins have also been observed in somatic tissues in the rat (Baumann *et al*., 1988). These differentially expressed transcripts are either product of alternate splicing of the primary transcript or transcripts from independent genes. Kv channels in mammalian systems arise primarily from independent genes, and in most cases a single exon specifies the entire K^+ channel coding region (Salkoff *et al*., 1992; Christie, 1995). However, diversity of *Drosophila Shaker* channels is generated through alternate splicing to produce different mRNA from a single large transcription unit (Kamb *et al*., 1987; Pongs *et al*., 1988; Schwarz *et al*., 1988). Although less prominent, K^+ channel genes are alternatively spliced to produce different subunits [e.g. Kv4.3 (Ohya *et al*., 1997), Kv3.1 (Perney *et al*., 1992)]. At present, it is not clear whether the differentially expressed transcripts are products of multiple independent genes or of alternate splicing.

Mammalian somatic Kv channels, such as Kv1.3, are modulated by phosphorylation, i.e. K^+ currents are upregulated by PKC (Cai *et al*., 1992; Payet and Dupuis, 1992; Chung and Schlichter, 1997) and down-regulated by TK activity (Holmes *et al*., 1996; Bowlby *et al*., 1997). The Kv1.3 protein expressed in rat testis, based on the deduced amino acid sequence, potentially can be modulated in a similar manner. It is therefore important to note that, in human sperm, the K^+ channel proteins co-localize with surface plasma membrane non-nuclear progesterone receptors (Benoff *et al*., 1995, 1999; Jacob *et al*., 1998a). Progesterone-stimulated Ca^{2+} influx is mediated by PKC (Foresta *et al*., 1995) and TK activity prevents voltage-induced current facilitation (Arnoult *et al*., 1997). These findings, together with the fact that inhibitors of delayed rectifier K^+ channels block progesterone-stimulated acrosome loss, suggest a role for these channels in the mammalian acrosome reaction (Benoff, 1999).

Preliminary findings, based on inter-male variations in inhibitor sensitivities of progesterone-stimulated acrosome loss, suggest that multiple isoforms of the human homologue of rat Kv1.3 exist and that each man expresses only one isoform in his ejaculated spermatozoa (Jacob *et al*., 1998b; Benoff, 1999; S.Benoff and G.M.Centola, unpublished data). We believe that such diversity is of clinical importance. We postulate that differential expression of these K^+ channel isoforms could underlie inter-male differences in acrosome response to progesterone exposure as well as susceptibility to environmental lead exposures. Experiments are in progress to test this hypothesis.

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