Co-localization of the inositol 1,4,5-trisphosphate receptor and calreticulin in the equatorial segment and in membrane bounded vesicles in the cytoplasmic droplet of human spermatozoa

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Modulation of the intracellular calcium concentration within mammalian spermatozoa is important in several pre-fertilization events including hyperactivated motility and the acrosome reaction. To identify calcium binding proteins (CBP) potentially regulating these processes, a 45Ca overlay technique was employed on 2-D blots of human sperm extracts. Microsequencing by Edman degradation and CAD mass spectrometry identified a relatively abundant 60.5 kDa CBP with a pI of 4.2 as calreticulin (CRT). Immunofluorescent labelling with anti-CRT antibodies localized CRT to the acrosome, with highest fluorescence in the equatorial segment, and in the cytoplasmic droplets of 94 and 48% of human spermatozoa respectively. Double immunolabelling experiments demonstrated co-localization of CRT and the inositol 1,4,5-trisphosphate receptor (IP3R) in the acrosome, in the equatorial segment, and vesicular structures in the cytoplasmic droplets of the neck region. Electron microscopic immunogold labelling localized CRT to the equatorial segment of acrosome-reacted spermatozoa and to membrane-enclosed vesicles within the cytoplasmic droplet of both acrosome-intact and acrosome-reacted spermatozoa. Localization of the IP3 receptor to the CRT-containing vesicles, in the sperm neck and to the acrosome, suggests that capacitative calcium entry in human spermatozoa may be regulated from these putative calcium storage sites.

Key words: calcium regulation/calcium storage/calreticulin/human spermatozoa/inositol trisphosphate receptor

Introduction

Variations in the amplitude and frequency of intracellular calcium oscillations and a gradual increase in the mean cytosolic free calcium ([Ca2+]i) concentration during capacitation are essential for spermatozoa to attain hyperactivated motility and to undergo the acrosome reaction (AR) (Suarez et al., 1993; Yanagimachi, 1994; Suarez and Dai, 1995). These two pre-fertilization events occur independently (Yanagimachi, 1981; Suarez et al., 1986), suggesting either that they are driven by different Ca2+ regulatory pathways or that the pathways are activated at different times in distinct subcellular locations (Suarez et al., 1993). In the sperm tail, increased [Ca2+]i initiates hyperactivation (Yanagimachi, 1982) which comprises an increase in flagellar-bend amplitude and flagellar-beat asymmetry (Yanagimachi, 1994). Hyperactivated motility may be advantageous to spermatozoa for detaching from oviductal mucosa, for penetrating viscoelastic substances in the oviduct, and for penetrating the zona pellucida (Suarez et al., 1995).

In the sperm head, calcium is a key messenger in the AR. Calcium modulates, directly or via intermediary compounds, the activity of several enzymes including phospholipase C (PLC) (Roldan et al., 1994), protein kinase C (O’Toole et al., 1996), phospholipase A2 (PLA2) (Poldan and Fragio, 1993), adenylate cyclase (Gross et al., 1987), cyclic nucleotide phosphodiesterase (Wasco and Orr, 1984), and actin severing proteins (Spungin and Breitbart, 1996). Each of these molecules appears to participate in the signalling cascade, which, upon
activation by binding of spermatozoa to the zona pellucida, results in exocytosis of the acrosomal contents. Both G protein-coupled receptor and receptor tyrosine kinase have been suggested to initiate this event (Snell and White, 1996; Breitbart and Spungin, 1997). One of calcium’s initial roles in the AR is participation in the activation of PLC which yields effectors such as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) by hydrolysis of polyphosphoinositides stored in the plasma membrane (Thomas and Meizel, 1989).

DAG activates PLA (Poldan and Fragio, 1993), inhibits lysophosphatidate acyltransferase (Harrison and Roldan, 1990; Momchilova et al., 1999) and may activate other phospholipases such as phosphatidylycerol-specific PLC (Roldan and Murase, 1994), resulting in an increase in fusogenic phospholipids in the acrosomal region and thus priming the cytoplasmic leaflets of the outer acrosome membrane and the overlying plasma membrane for fusion (Harrison and Roldan, 1990). DAG further stimulates Ca2+-dependent protein kinase C, which has been shown to be involved in the AR (de Jonge et al., 1991), possibly by direct activation of Ca2+ channels in the plasma membrane (Breitbart and Spungin, 1997). Finally, [Ca2+]i may promote fusion by direct interaction with the negatively charged headgroups of lipids, thus abolishing their bilayer stabilizing effects as well as the electrostatic repulsion between the membranes (Suarez et al., 1986).

The second effector generated by PLC is IP3, which modulates intracellular Ca2+ oscillations in a variety of cell types by activation of the inositol 1,4,5-trisphosphate receptor (IP3R), which spans the membrane of intracellular Ca2+ storage sites (Berridge, 1993). Binding of IP3 to the cytoplasmic N-terminus of the IP3R initiates conformational changes of the channel-forming C-terminus leading to channel opening (Berridge, 1993). Interactions between calreticulin (CRT) and the IP3R in regulation of IP3-induced calcium release from the endoplasmic reticulum (ER) have been proposed in Xenopus oocytes (Camacho and Lechleiter, 1995). Similarly IP3 appears to be the primary mechanism responsible for the sperm-induced release of Ca2+ from intracellular stores in oocytes of the sea urchin (Mohri et al., 1995).

CRT was first purified from sarcoplasmic reticulum of rabbit skeletal muscle and was subsequently shown to be the major Ca2+ binding/storage protein in the ER of a variety of non-muscle cell types (Krause and Michalak, 1997). CRT has been localized to the acrosome of rat spermatozoa (Nakamura et al., 1993). CRT consists of several structural and functional domains: a globular N-terminal domain, and a central proline rich P-domain with repeat sequences, separated from an acidic C-terminal stretch by a short hydrophobic sequence (Krause and Michalak, 1997). CRT contains an NH2-terminal signal sequence, and a KDEL ER retrieval signal at its C-terminus, and it has both high affinity/low capacity and low affinity/high capacity sites for calcium binding (Krause and Michalak, 1997). Like the closely related ER chaperone calnexin, CRT is a lectin, which interacts specifically with partially trimmed, monoglycosylated, N-linked oligosaccharides via its P-domain (Peterson et al., 1995).

In somatic cells, depletion of the ER Ca2+ stores activates Ca2+ channels in the plasma membrane resulting in Ca2+ being pumped into the cell (Putney, 1986). This process is called ‘store-operated calcium entry’ or ‘capacitative calcium entry’ (not to be confused with capacitation). The ‘conformational-coupling’ model, originally proposed by Irvine, suggests that store-operated Ca2+ channels interact directly with the ER IP3R during ‘capacitative calcium entry’ (Irvine, 1990). Recent data support the basic tenet of this coupling mechanism (Putney, 1999; Berridge et al., 2000).

The present paper is the first to identify, characterize and immunolocalize CRT in human spermatozoa. CRT was localized by immuno-electron microscopy in membrane-bound vesicles (CRT-containing vesicles; CCV) in the cytoplasmic droplet adjacent to the postacrosomal neck and anterior midpiece regions. CRT was additionally demonstrated in the acrosomal matrix and in the equatorial segment of acrosome-reacted spermatozoa. Immunofluorescence studies co-localized CRT and IP3R in the acrosome and neck of human spermatozoa, with highest concentrations of both antigens appearing in the equatorial segment and in the neck region. Based on their content of both CRT and IP3R, and their localization, these putative Ca2+ storage sites containing IP3R become prime candidates as regulators of ‘capacitative calcium entry’ during capacitation and the AR.

Materials and methods
Preparation of spermatozoa
Semen specimens were obtained from normal, healthy young men by masturbation. Individual semen samples were allowed to liquefy at room temperature and the mature spermatozoa were separated from seminal plasma, immature germ cells and non-sperm cells by Percoll (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation (Naaby-Hansen et al., 1997). In some experiments, motile, intact spermatozoa were obtained by the swim-up method, as previously described (Bronson and Fusi, 1990). All samples were obtained under informed consent using forms approved by the University of Virginia Human Investigation Committee.

Solubilization of sperm proteins for electrophoresis
Purified spermatozoa were solubilized in a lysis buffer containing: 2% (v/v) NP-40, 8.8 mol/l urea, 100 mmol/l dithiothreitol (DTT), 2% (v/v) ampholines pH 3.5–10, and the protease inhibitors 2 mmol/l PMSF, 5 mmol/l iodoacetamide, 5 mmol/l EDTA, 3 mg/ml TLCK, 1.46 mmol/l pepstatin A and 2.1 mmol/l leupeptin. 5×10⁸ cells per ml were solubilized by constant shaking at 4°C for 60 min. Insoluble material was removed by centrifugation at 10 000 g for 2 min, and the supernatant was applied to the first electrophoretic dimension. Protein concentrations were determined by using the bicinchoninic acid method (Pierce, Rockford, IL, USA), employing bovine serum albumin (BSA) as the standard.

Electrophoresis
Analytical 2-D gel electrophoresis was performed according to O’Farrell with minor modifications (Naaby-Hansen et al., 1997). Proteins in analytical gels were routinely visualized by silver staining as previously described (Naaby-Hansen et al., 1997). Preparative 2-D gel electrophoresis in large format gels (23×23 cm) was performed in the Investigator 2-D Electrophoresis System (Genomic Solutions, Huntingdon, Cambridgeshire, UK), employing the following ampholine (Pharmacia, Uppsala, Sweden) composition: 20%
pH 5–7, 20% pH 7–9 and 60% pH 3.5–10. Proteins separated by preparative 2-D gel electrophoresis were visualized by Coomassie staining. The spot of interest (migrating at a pH of 4.2 and an apparent mass of 60.5 kDa) was cut from the gel and microsequenced by CAD mass spectrometry, following tryptic degradation as described below.

Electrotransfer to nitrocellulose membranes was accomplished using an established method (Towbin et al., 1979). Electrotransfer to PVDF membranes (0.2 mm pore size; Pierce) was carried out as previously described (Henzel et al., 1993), using a published transfer buffer composition (Matsudaira, 1987) [10 mmol/l 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11]. Proteins were visualized by staining in a solution containing 0.1% Coomassie R250, 40% methanol and 0.1% acetic acid for 1 min, followed by destaining in a solution of 10% acetic acid and 50% methanol for 3×3 min. The centre of the Coomassie-stained spot was cut from the PVDF membrane and microsequenced by Edman degradation.

Detection of calcium binding proteins (CBP)
CBP were demonstrated using a 45Ca overlay assay (Maruyama and Ebashi, 1984). Briefly, proteins separated on 2-D gels were transferred to PVDF membranes (Jethmalani et al., 1994), and the membranes were washed 3×20 min in a washing buffer (10 mmol/l imidazole HCl, 60 mmol/l KCl and 5 mmol/l MgCl₂, pH 6.8) and incubated with 2 µCi/ml of 45CaCl₂ in washing buffer for 30 min at room temperature. The membranes were subsequently rinsed for 3 min in distilled H₂O followed by 2 min rinsing in 50% ethanol and were air-dried on filter paper for 15–20 min. The membranes were finally dried by hot air from a hair dryer and placed directly upon X-ray films (NEF-495; Du Pont, USA) for 18–24 days. Some of the PVDF membranes were subsequently stained with Coomassie to localize the CBPs in the total 2-D protein pattern, while other membranes were used for Western blot analysis as described below. Computerized pattern analysis and densitometry of the autoradiograms and the stained membranes were performed with 2-D Analyzer software (BioImage 2000), as noted in our previous publication (Naaby-Hansen et al., 1997).

Edman degradation
The protein spots were sequenced in an Applied Biosystems 470A protein sequencer operated according to the manufacturers specifications, using a cartridge and cycles for PVDF membranes.

Mass spectrometry analysis
Protein spots were cut from gels, as accurately as possible to minimize extra polyacrylamide, and were divided into several pieces. Gel pieces were washed and destained in 500 µl 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 50 µl of 10 mmol/l DTT in 0.1 mol/l ammonium bicarbonate and reduced at room temperature for 1 h. The DTT solution was removed and the sample was alkylated in 50 µl 50 mmol/l iodoacetamide/0.1 mol/l ammonium bicarbonate at room temperature for 1 h in the dark. This reagent was removed and the gel pieces were washed with 100 µl 0.1 mol/l ammonium bicarbonate and dehydrated in 100 µl acetonitrile for 5 min. The acetonitrile was removed and the gel pieces were rehydrated in 100 µl 0.1 mol/l ammonium bicarbonate. Gel pieces were then dehydrated in 100 µl acetonitrile, the acetonitrile was removed, and the material was completely dried by vacuum centrifugation. Gel pieces were next rehydrated in 20 ng/µl trypsin in 50 mmol/l ammonium bicarbonate and incubated on ice for 10 min. Excess trypsin solution was removed, 20 µl 50 mmol/l ammonium bicarbonate was added, samples were digested overnight at 37°C and peptides were extracted from the polyacrylamide in 70 µl 50% acetic acid/5% formic acid. Extracts from two gels were combined and evaporated to <20 µl for mass spectrometry (LC-MS) analysis.

The LC-MS system consisted of a Finnigan TSQ7000 system with an electrospray ion source interfaced to a 10 cm×75 µm (i.d.) Poros 10 R2 reversed phase capillary column. 1 µl of the extract was injected and the peptides were eluted from the column by an acetonitrile/0.1 mol/l acetic acid gradient at a flow rate of 0.6 µl/min. The electrospray ion source was operated at 4.5 kV with a 1.2 µl/min coaxial sheath liquid flow of 70% methanol/30% water/0.125% acetic acid and a coaxial nitrogen flow adjusted as needed for optimum sensitivity. The digest was analysed by capillary LC-electrospray mass spectrometry to measure the mol. wt of the peptides present in the digest. Peptide sequences for the peptides detected were determined by collisionally activated dissociation using LC-electrospray-tandem mass spectrometry with argon as the collision gas.

Oligonucleotides, reverse transcription–polymerase chain reaction (RT-PCR) and Northern analysis
The oligonucleotide probes, 5’-GC(T/C)-GC(G/C)-TAC-TTC-AAGGAG-CAG-TTC-CT-3’, corresponding to protein sequence #1 (EPA-VYFKEQFLDGD), and 5’-CTT-GTA-GTT-GAA-GAT-CAC-(A/G) TG-CAC-CT-3’, corresponding to protein sequence #2 (KVHV(I/L)JNYK), were prepared by the Biomedical Research Facility of the University of Virginia. PCR cloning was performed by denaturing 0.05 µg testicular poly-(A)+ RNA (Clontech, Palo Alto, CA, USA) at 65°C for 10 min prior to assembly of a 20 µl reverse transcription reaction consisting of 2 µl 10×RT buffer, 0.5 µl plasmid ribonuclease inhibitor (36 U/µl; Promega), 1 µl 10 mmol/l 4dNTP and 1 µl AMV reverse transcriptase (23 U/µl; Stratagene, La Jolla, CA, USA). After incubation for 60 min at 42°C, 80 µl of diethylpyrocarbonate-treated H₂O was added and 1 µl aliquots of the cDNA solution were amplified for 40 cycles (94°C for 45 s, 38/44/50/56°C for 45 s, 72°C for 2 min) as specified by the Taq polymerase manufacturer (Promega). Separation and isolation of the PCR products was achieved by electrophoresis of reaction aliquots in 2.4% agarose gels made 1× in TAE (40 mmol/l Tris–acetate, 1 mmol/l EDTA) buffer. After precipitation and quantification PCR fragments were ligated into PCR-Script vectors according to manufacturer’s instructions (Stratagene) and sequenced.

For Northern blot analysis, 50 ng of either the purified RT–PCR-generated CRT fragment or β-actin cDNA were radiolabelled by the random primer method (Feinberg and Vogelstein, 1984), isolated by Elutip-D column (Schleicher and Schuell) and hybridized to a Human Multiple Tissue Northern Blot (Clontech) in ExpressHyb (Clontech). Washing of the blot was performed according to manufacturer’s instructions.

Immunoblotting analysis
Immunoblotting from 2-D gels was performed according to published methods (Naaby-Hansen et al., 1997). Blots containing human sperm proteins were incubated for 1 h at 22°C with a 1:3000 dilution of the monospecific monoclonal rabbit anti-human CRT antiserum PA3-900 (Affinity Bioreagents, Golden, CO, USA), under constant gentle rocking. Polyclonal goat antiserum raised against recombinant rabbit CRT was employed at a 1:2000 dilution, while monospecific

Calreticulin and IP3 receptor in human spermatozoa

925
rabbit antiserum to the ER retrieval signal (QA)KDEL was diluted 1:5000 (gifts from Dr Marek Michalak, University of Alberta, Canada). This antibody is specific for CRT and only faintly recognizes other KDEL proteins such as glucose-regulated protein 78 (BiP), ERP72 and PDI (Dr M.Michalak, personal communication). Antibody binding was visualized employing diaminobenzidine (DAB) as a substrate, following incubation with HRP-conjugated secondary antibodies (HRP-conjugated goat anti-rabbit-Ig antibodies and HRP-conjugated rabbit anti-goat-Ig antibodies from Jackson ImmunoResearch Lab, West Grove, PA, USA). In some experiments secondary antibodies were employed alone as a control.

Indirect immunofluorescence of human spermatozoa

For the indirect immunofluorescent studies, fresh human spermatozoa were harvested over a discontinuous 55/80% Percoll gradient and subsequently washed three times with Ham’s F-10 medium. The spermatozoa were counted using a haemocytometer and diluted with medium to a concentration of 1×10⁶ spermatozoa/ml. Twenty µl of the sperm solution was added per well (2×10⁵ spermatozoa) onto poly-l-lysine-coated slides. Cells were allowed to bind to the slide and the excess was removed. The spermatozoa were air-dried onto the slides at 40°C, and then some of the slides were fixed with methanol for 10 min. After washing 3×5 min in phosphate-buffered saline (PBS), the slides were either used fresh or were frozen at −70°C for no longer than a week. All subsequent incubations were performed in a humid chamber. The slides were blocked in 10% normal donkey serum (NDS) in PBS with 0.05% Tween-20 (PBS-tw) for 30 min. For the dual labelling experiment, the goat anti-CRT (a gift from Dr M.Michalak) and rabbit anti-IPR (PA3-901; Affinity BioReagents, Golden, CO, USA) primary antibodies were each diluted 200-fold with 1% NDS in PBS-tw and the slides were incubated overnight at 4°C. The slides were then washed 3×5 min in PBS-tw and the secondary antibodies, a donkey anti-goat IgG rhodamine conjugate and a goat anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate (both from Jackson ImmunoResearch, West Grove, PA, USA), were applied at a 1:200 dilution in 1% NDS in PBS-tw for 1 h at 37°C. The slides were washed 3×5 min in PBS-tw, and Slow Fade–Light Antifade Kit (Molecular Probes, Inc., Eugene, OR, USA) was used to reduce the fading rate of the fluorescein. Each primary antibody was tested against the other secondary antibody to ensure no cross-reactivity occurred. The rabbit anti-CRT antibody (PA3-900) was also used individually at a 1:200 dilution followed by the goat anti-rabbit FITC conjugate as a secondary antibody to immunostain the spermatozoa.

Electron microscopic localization

Spermatozoa from four donors were pooled and washed twice by centrifugation at 550 g in wash buffer [Ham’s F-10 Nutrient Mixture (Gibco/BRL, Gaithersberg, MD, USA) with 3% sucrose]. The washed spermatoza were resuspended in fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in wash buffer for 15 min at 22°C. After removing fixative by centrifugation and washing three times with wash buffer, the spermatozoa were dehydrated through a graded series of ethanol washes from 40 to 100%. The cells were infiltrated with and embedded in Lowicryl K4M. The blocks were polymerized with UV light for 72 h at −20°C and ultrathin sections of 100 µm thickness were cut. Non-specific sperm–antibody interactions were blocked by incubating the sections in undiluted NGS for 15 min at 22°C, and the specimens were washed once with wash buffer. Rabbit anti-CRT polyclonal antibody (No. PA3-900; ABR Affinity BioReagents Inc.) was diluted 1:25 in wash buffer with 1% NGS, 1% BSA and 0.05% Tween 20. Lowicryl sections were incubated with diluted anti-CRT or wash buffer alone at 4°C for 16 h. After washing four times in wash buffer, sections were incubated for 1.5 h at 22°C with 5 nm gold-conjugated secondary antibody, goat anti-rabbit IgG (Goldmark Biologicals, Phillipsburg, NJ, USA) diluted 1:35 in wash buffer. The sections were then washed with distilled water and stained with uranyl acetate before examination with a Jeol 100CX electron microscope.

In some experiments, pre-embedding immunogold labelling was performed on fresh swim-up spermatozoa or acrosome-reacted spermatozoa. Swim-up spermatozoa were first capacitated in human tubal fluid medium (HTF; Irvine Scientific) containing 30 mg/ml human serum albumin (Quinn et al., 1985; Byrd et al., 1989) and 2 µmol/l progesterone (Garcia and Meizel, 1999) at 37°C for 2 h in 5% CO₂. To induce the AR in capacitated spermatozoa, Ca²⁺ ionophore A23187 (Byrd and Wolf, 1989) was added to a final concentration of 10 µmol/l in the presence of 5 µmol/l CaCl₂ for 1 h at 37°C. Cells were incubated in pre-embedding blocking solution containing 3% sucrose and 10% normal goat serum (NGS) in Ham’s F-10 (Walensky and Snyder, 1995), incubated in the anti-CRT antibody (1:25) in F-10 buffer containing 3% sucrose, 1% BSA and 1% NGS, washed, incubated in 5 nmol/l gold-labelled goat anti-rabbit IgG (1:50; Goldmark) and washed. Cells were fixed in Ham’s F-10 containing 2% glutaraldehyde, 4% tannic acid, and 2 mmol/l MgSO₄, pH 7.2, serially dehydrated through ethanol and embedded in Araldite resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Sections were cut at 100 µm, mounted onto nickel grids (Electron Microscopy Sciences), counterstained with 7% uranyl acetate and lead citrate, and viewed by transmission electron microscopy.

Results

Sperm CBP were identified by incubating 2-D PVDF blots of sperm extracts with ⁴⁵Ca (Figure 1). In the mol. wt and pl ranges from 5 to 200 kDa and 3 to 8.5 respectively, five major CBP were resolved migrating at the following mol. wt/pl: 86/3.8–4.0, 60/4.2, 55/4.9–5.25, 26.5/5.2, and 16.5/3.9. These proteins showed ⁴⁵Ca binding capacity in four replications of the overlay experiment. Densitometric scanning of the autoradiographs indicated that >80% of the human sperm ⁴⁵Ca binding capacity in the acidic and neutral pH range (pl 3–8.5) is accounted for by these five acidic proteins. The protein with the highest ⁴⁵Ca binding capacity was identified as calmodulin, a known constituent of mammalian spermatozoa, based on its electrophoretic migration at mol. wt of 16.5 and pl of 3.9 (Figure 1). In addition, a small amount of ⁴⁵Ca bound to a 78 kDa protein, which was identified as BiP (Figure 1) by mass spectrometry analysis (data not shown).

The relatively abundant 60.5 kDa CBP in an isolated position at pl 4.2 was chosen for microsequencing (horizontal arrows in Figures 1 and 2). Edman degradation of the protein spot, retrieved from a Coomassie-stained PVDF membrane, yielded 15 N-terminal amino acids. To obtain internal amino acid sequences, the protein was isolated by preparative 2-D gel electrophoresis and visualized by Coomassie staining, and the core of the 60.5 kDa spot was excised and digested with trypsin. The mol. wts of the resultant tryptic peptides were determined by LC-MS analyses and the amino acid sequences determined by LC-tandem MS. Masses for nine peptides and sequence information on five of these were obtained (Table 1). Database searches using CAD spectrum information (MSTag)
Calreticulin and IP3 receptor in human spermatozoa

Figure 1. Identification of calcium binding proteins (CBP) in human spermatozoa by $^{45}$Ca overlay. (A) The acidic region of $23\times 23$ cm SDS/PAGE of human sperm proteins visualized by silver staining. (B) Identification of sperm CBP on a PVDF membrane by the $^{45}$Ca overlay technique. The six CBP indicated in (B) were identified within the total, stainable protein pattern by a combination of gold/immunostaining of the PVDF membranes following autoradiography and computer-aided comparison of the images. The isolated CBP spot indicated by a star and a horizontal arrowhead was microsequenced and identified as calreticulin. Calmodulin, which gave the strongest $^{45}$Ca signal, migrated at its expected mol. wt (16.5 kDa) and pI (3.9) and was weakly stained by silver (A). The high mol. wt CBP migrating at pl 5.1 was identified as glucose-regulated protein 78 (BiP), which we have previously microsequenced and catalogued. Three unidentified CBP are indicated by vertical arrows. The 26.5 kDa CBP (B, arrow) did not stain with silver (A).

Table I. Peptide sequences obtained by mass spectrometry from protein spot of mass 60.5 kDa, pl 4.2

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Measured mol. wt. (M+H+, Da)</th>
<th>Peptide sequence by CAD1 (calculated mol. wt, M+H+, Da)</th>
<th>Peptide sequence from Swissprotein accession no. P27797</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1411.4</td>
<td>EQFXDGDGWTSR (1411.5)</td>
<td>EQFLDGDGWTSR</td>
</tr>
<tr>
<td>5</td>
<td>1220.0</td>
<td>GQTXVVQFTVK (1220.5)</td>
<td>GQTLVVQFTVK</td>
</tr>
<tr>
<td>4</td>
<td>1148.0</td>
<td>KVHVXFNYK (1148.4)</td>
<td>KVHVIFNYK</td>
</tr>
<tr>
<td>1</td>
<td>852.8</td>
<td>VAEPAVYFK (853.0)</td>
<td>VAEPAVYFK</td>
</tr>
<tr>
<td>2</td>
<td>1019.6</td>
<td>VHVXFNKY (1020.2)</td>
<td>VHVIFNYK</td>
</tr>
</tbody>
</table>

X designates I or L which cannot be distinguished by low energy CAD.

and partial peptide sequences (BLAST) determined that these five peptides (Table I), as well as the amino terminal sequence EAPAVYFKEQFLDGDG obtained by Edman degradation, corresponded to peptides in the sequence of human calreticulin. The observed N-terminal sequence starts at position 18 in the predicted amino acid sequence of human CRT (Swissprotein accession no. P27797), immediately following the leader sequence (amino acids 1–17), which is processed co-translationally (Krause and Michalak, 1997).

When the monospecific antiserum to CRT (PA3-900) was employed in 2-D immunoblotting, a major immunoreactive form of CRT was observed at 60.5 kDa, accompanied by a slightly higher mol. wt form and at least two slightly lower mol. wt forms (58.8 and 57.4 kDa respectively, Figure 2, insert D). Thus, four forms of human sperm CRT, each varying slightly in mass, were resolved. The major 60.5 kDa form showed charge heterogeneity in both immunoblotting analysis (Figure 2, vertical arrows in inserts D and F) and on silver stained gels (Figure 2, vertical arrow in insert B). Immunoblotting analysis with antiserum to (G)KDEL demonstrated this C-terminal ER retrieval sequence in both the high mol. wt and the lower mol. wt CRT forms (Figure 2, horizontal arrows in insert F). This immunoblot may be compared to a gold stain of the identical membrane prior to incubation with antisera (Figure 2E). The lectins Concanavalin A (Figure 2, insert C), Pisum sativum agglutinin, Ricinus communis agglutinin 1, and Ulex europeus agglutinin 1 were employed in 2-D affinity blotting analysis. Although each lectin was reactive with different subsets of sperm glycoproteins on individual blots, no lectin recognized any of the four CRT spots, indicating that human sperm CRT is probably not glycosylated.

To determine whether the different mol. wt forms of sperm CRT represented multiple transcripts, we isolated a 396 bp testicular CRT cDNA fragment by RT–PCR for Northern blot analysis. Optimized degenerate oligonucleotides, based on the obtained CRT peptide sequences, were employed in PCR reactions with testicular cDNA manufactured by reverse transcription of human testicular poly-(A)$^+$ mRNA. Analysis of the PCR reaction products on agarose gels (Figure 3) demonstrated a single prominent PCR product migrating at 396 bp from the reactions at the 44° and 50°C annealing
S.Naaby-Hansen et al.

**Figure 2.** Two-dimensional gel electrophoretic purification and characterization of human sperm calreticulin (CRT). Sperm proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to PVDF membranes, and stained with R-250 Coomassie (A). Black horizontal arrowheads point to CRT. The centre of the major spot indicated by the top horizontal arrowhead was cut from the membrane for protein sequencing by Edman degradation. Fifteen amino acids identical to the N-terminus of human CRT were obtained. The major 60.5 kDa form of CRT migrated slightly above two lightly stained protein spots (lower horizontal arrows). The positions of α- and β-tubulins are indicated by an oblique white arrow. (B) One spot above the major 60.5 kDa form could be demonstrated by silver staining (top horizontal arrow in insert B). (C) None of these four proteins bound any of the lectins tested, including Concanavalin A. The black arrowhead indicates the position of the four unstained proteins (D). Immunoblotting with the monospecific antiserum PA3-900 identified all four protein spots as immunoreactive forms of CRT. Charge heterogeneity of the major 60.5 kDa form was evident on immunoblots (vertical arrow in inserts D and F) and horizontal streaking of the 60.5 kDa form in the basic direction was observed following silver staining (vertical arrow in insert B). Nitrocellulose-membrane immobilized sperm proteins were visualized by gold staining (E), and subsequent incubation with monospecific rabbit antiserum raised against the C-terminal GKDEL peptide (F). Note that all four forms of sperm CRT, including the high and the lower mol. wt forms, contained the retrieval signal (horizontal arrows in F).

Electroelution of the 396 bp band and cloning into the pCR-Script vector, followed by sequencing and computer analysis revealed that the band was identical to the N-terminus (minus the leader sequence) of human CRT (Swissprotein accession no. P27797).

To ascertain if there were any tissue-specific variants of the CRT message, a multiple tissue Northern blot containing mRNA from spleen, thymus, prostate, testis, ovary, small intestine, colon and leukocyte was probed with the PCR-derived cDNA. Under both short and long exposure conditions, hybridization with the CRT cDNA probe revealed a single positive mRNA band at ~2.0 kb in all mRNA samples (Figure 3B). The finding of this single, common transcript by both PCR and Northern blots suggests that the polymorphic mol. wt forms of sperm CRT visualized in 2-D gels reflect post-translational modifications.

Overexpression of CRT has recently been shown to have an inhibitory effect on repetitive IP3-induced Ca2+ waves in Xenopus oocytes (Camacho and Lechleiter, 1995). The authors suggested that the inhibitory effect was due to interactions between CRT and either the IP3R or Ca2+-ATPases. Since IP3 serves as a second messenger in mammalian spermatozoa (Byrd and Wolf, 1989), we postulated that the presence of CRT indicated the presence of a calcium store and that interaction between CRT and IP3R might be involved in the regulation of cytosolic calcium oscillations from this storage site. As such an interaction implies co-localization, we examined the localization of CRT and IP3R in human spermatozoa.

Double immunolabelling experiments showed co-localization of CRT and IP3R over the acrosomal cap, in the equatorial segment and in vesicular structures in the cytoplasmic droplet of human spermatozoa (Figure 4: A, combined image; B, rhodamine secondary antibody to anti-IP3R; and C, FITC secondary antibody to anti-CRT). The most intense staining was observed in the equatorial segment and cytoplasmic droplet (Figure 4A–C). Equatorial segment co-localization was detected in 94% of the cells (100 cells from a mixture of five ejaculates were scored).
Calreticulin and IP₃ receptor in human spermatozoa

Figure 3. Reverse transcription-polymerase chain reaction (RT-PCR) and Northern analysis of calreticulin (CRT). (A) RT-PCR: optimized, degenerate primers based on amino acid microsequences generated a single 396 bp product after 40 RT-PCR cycles. Products of the RT-PCR reactions were separated on a 2.4% agarose/1XTAE gel and stained with ethidium bromide. Annealing temperatures of standard PCR reactions were varied from 38°C (lanes 2–4), 44°C (lanes 5–7), 50°C (lanes 8–10) to 56°C (lanes 11–13). An optimal annealing temperature of 50°C was determined. Lanes 2, 5, 8 and 11 contained human testicular poly-(A)/H11001 RNA; lanes 3, 6, 9 and 12 contained no RNA; while lanes 4, 7, 10 and 13 contained the products from input-cDNA-minus reactions. Lane 1 contains HindIII markers; lane 14 contains ØX-174 markers. Sizes (Kb) are designated at left and right. (B) and (C) Northern analysis: 50 ng of RT-PCR-generated calreticulin (B) and β-actin (C) DNA were radiolabelled (Feinberg and Vogelstein, 1984) and hybridized to a Human Multiple Tissue Northern (Clontech) in ExpressHyb (Clontech). Each lane contained 2 mg of poly-(A)⁺ mRNA from the indicated tissues.

The two antigens co-localized to structures in the cytoplasmic droplet of 48% of the cells; these structures were not stained in the remaining cells. Finally, co-localization was observed over the acrosomal cap in 84% of the human spermatozoa (Figure 4A). A subset of spermatozoa (11%) revealed exclusive staining of the equatorial segment (arrowhead Figure 4A), suggesting that these had undergone the AR. The same staining patterns were seen with both the rabbit anti-CRT (PA3-900) and the goat anti-CRT antibodies.

Post-embedding immunogold labelling with anti-CRT antibodies was performed to localize CRT (Figure 5A,B) at the ultrastructural level. CRT was localized to two types of membrane-bound vesicles located within the cytoplasmic droplet (CD). One type of vesicle, designated a dense CRT-containing vesicle (dCCV), contained electron-dense amorphous material and a high density of gold particles indicating an abundance of CRT (Figure 5B). The second type of vesicle, designated a light CRT-containing vesicle (lCCV), exhibited a less electron-dense interior and fewer gold particles (Figure 5B). Both dCCV and ICCV were situated in close apposition to the plasma membrane in the cytoplasmic droplet adjacent to the neck and midpiece regions. In both types of vesicles, CRT staining was most intense in regions containing electron-dense amorphous material (enlargements in Figure 5B). Some vesicles contained compartments of varying electron density as well as whorls of membrane (black arrowheads in Figure 5A,B). In such compartmentalized vesicles CRT appeared localized to the non-membranous, more electron-lucent compartments. In addition, small vesicles in the post-acrosomal region adjacent to the nuclear envelope were immunolabelled with anti-CRT antibodies (upward arrow in Figure 5A). Post-embedding labelling of spermatozoa with anti-CRT antibody did not show significant labelling of the acrosome or equatorial segment, presumably because of masking of epitopes or to a sensitivity of the epitopes in this region to aldehyde fixation, dehydration, or the embedding medium. These are commonly observed problems with post-embedding localization of some antigens. Staining that was observed might occur in those regions with apparently higher concentrations of CRT, i.e. the ICCV and dCCV, where not all of the antibody binding sites were compromised by the post-embedding procedure.

Pre-embedding immunogold labelling of acrosome-reacted spermatozoa demonstrated localization of CRT to the equatorial segment (Figure 5D). Gold particles appeared to be associated with electron-dense material (acrosomal matrix) in this region (Figure 5D). No staining in the equatorial segment was observed in acrosome-intact spermatozoa (Figure 5E), which had been labelled by the pre-embedding method.

Discussion

The present study is the first to employ the ⁴⁵Ca overlay technique on 2-D gel-separated extracts of human sperm proteins to identify and characterize the CBPs. CBPs with mol. wt between 12.5 and 115 kDa and pI between 3.8 and 5.3 were distinguished by ⁴⁵Ca overlay followed by autoradiography. No sperm CBPs were detected in the neutral pH range (5.5 < pI > 8.5; data not shown). The ⁴⁵Ca overlay procedure (Maruyama et al., 1984) has previously been shown to identify both high affinity and low affinity CBPs (Macer and Koch, 1988). In the present study the specificity of the
Figure 4. Co-localization of calreticulin (CRT) and inositol trisphosphate receptor (IP₃R) demonstrated by dual fluorescence staining. (A) Double labelling of human spermatozoa revealed co-localization (yellow staining) of CRT and IP₃R. Co-localization was observed over the acrosomal cap, in the equatorial segment (arrowheads) and in vesicular structures in the neck region (arrows). (B) The same field as in (A) showing IP₃R detected with the rhodamine-conjugated secondary antibody (red staining). (C) The same field as in (A) with CRT detected by fluorescein isothiocyanate-conjugated secondary antibody (green staining). (D) Differential interference contrast (DIC) image corresponding to (A–C). (E) Control: secondary antibodies alone. (F) DIC image corresponding to (E). Both primary antibodies were also incubated individually with each of the secondary antibodies. No cross-reactivity occurred with the other secondary antibody and the same staining patterns noted in (A) were observed with the appropriate secondary antibody (data not shown).

⁴⁵Ca overlay procedure was validated by the identification of CRT, calmodulin and BiP as CBPs of the human spermatozoa. The calcium binding properties of CRT and calmodulin are well characterized, while BiP previously has been shown to bind low amounts of ⁴⁵Ca in an overlay assay (Macer and Koch, 1988). The ability of immobilized CRT to bind ⁴⁵Ca is
Calreticulin and IP3 receptor in human spermatozoa

Figure 5. Localization of human sperm calreticulin (CRT) at the ultrastructural level. Electron micrographs of human spermatozoa labelled with gold-conjugated anti-CRT antibodies (A, B, D and E) or secondary antibody alone controls (C, F). Post-embedding immunogold labelling was performed on sections A, B, and C (top row). (A) Longitudinal section through the posterior portion of the nucleus (n), basal plate, connecting piece (c), axoneme (AX), mitochondrial sheath (mit) and cytoplasmic droplet, within which arrows delineate CRT-containing vesicles (CCV). Gold particles were observed in small membrane-bound vesicles in the anterior region of the cytoplasmic droplet adjacent to the neck (upward arrow). CRT-containing vesicles in the cytoplasmic droplet adjoining the midpiece were larger and often compartmentalized into regions of varying density (oblique arrow). Not all vesicle compartments were labelled for CRT. The black arrowheads in (A) and (B) mark vesicular domains, which did not stain for calreticulin. (B) Cross-section through the cytoplasmic droplet (CD) of the proximal midpiece region. Two types of membrane-bounded CCV were observed in the CD: light CCV (lCCV) and dense CCV (dCCV). Both types contained immunogold particles, although dCCV exhibited a higher concentration of particles (compare magnified boxes). In both types of CCV the gold particles were concentrated in areas with the highest electron density. (C) Sagittal section of the CD labelled with secondary antibody only shows no labelling of CCV. Pre-embedding labelling of acrosome-reacted (D) and acrosome-intact (E) spermatozoa was also performed (bottom row). (D) Gold particles were observed on acrosomal matrix-like material in the equatorial segment (es, arrows). (E) Sagittal section of an acrosome-intact spermatozoon demonstrating no gold labelling; a, acrosome; n, nucleus. (F) No gold particles in a section of an acrosome-reacted spermatozoa labelled with secondary antibody alone. Original magnifications: (A) 33 000; (B) 30 800; (C) 34 000; (D) 46 500; (E) 41 100; (F) 53 000.

hardly surprising as its high capacity Ca\(^{2+}\) binding domain consists of a stretch of polyacidic clusters (Krause and Michalak, 1997), presumably unaffected by minor structural changes. However, the significant \(^{45}\)Ca binding capacity of immobilized calmodulin suggests that its helix–loop–helix structures are resistant to the denaturing effect of sodium dodecyl sulphate during second dimension electrophoresis or that they are renatured at least in part during the subsequent mild electrotransfer, washing and blocking procedures.

An interesting horizontal staining pattern of the major 60.5 kDa sperm CRT form was observed in immunoblotting analysis with anti-CRT and anti-KDEL antibodies and by silver staining. Only the 60.5 kDa form bound sufficient amounts of \(^{45}\)Ca to be detected by autoradiography, even after prolonged exposure. Post-translational modification of a protein is the most frequent cause of charge shifts in 2-D electrophoresis, and CRT contains a potential N-glycosylation site (amino acid 344), a potential tyrosine phosphorylation site (amino acid 285), as well as several potential serine and threonine phosphorylation sites. However, sperm CRT did not bind a panel of lectins, with specificities towards a broad range of carbohydrate moieties, in affinity blotting analysis indicating that glycosylation is unlikely to be the reason for the observed charge heterogeneity. Both methylation and phosphorylation could account for some of the observed isoforms, and may participate in regulating the functional activity of CRT, although phosphorylated isoforms are expected to migrate at more acidic pI values than the unmodified form whereas the minor isoforms...
of the 60.5 kDa sperm CRT were predominantly basic. The observed isoforms might simply reflect sperm CRT that has bound various amounts of calcium during first dimensional separation, thus changing the net charge of the polyacidic clusters and resulting in a more basic migration in isoelectric focusing.

Two-dimensional immunoblotting analysis revealed four mol. wt forms of human sperm CRT with almost identical net charges. The results obtained by RT–PCR and Northern blot analysis suggest that only one human testicular CRT transcript is expressed, indicating that the observed mass differences are likely due to post-translational modifications. These results are consistent with observations made by others, namely that the somatic CRT gene exists as a single copy and there is no evidence for RNA splicing (Krause and Michalak, 1997).

CRT is synthesized with an NH2-terminal signal sequence and a KDEL ER retention signal at its C-terminus (Krause and Michalak, 1997). Edman degradation of the 60.5 kDa form demonstrated the expected N-terminus sequence immediately after the co-translationally processed leader sequence. The KDEL signal was found in all four sperm CRT forms, suggesting that the lower mol. wt forms (58.8 and 57.4 kDa) are generated by proteolytic modifications of the N-terminus. The high mol. wt CRT form migrating at ~60.8 kDa may reflect a small reservoir of unprocessed CRT or an alternative modification of the major 60.5 kDa form. The four CRT mass variants were observed in numerous replicates of the 2-D immunostaining experiment which employed a broad range of protease inhibitors during cell lysis, suggesting that they represent naturally occurring CRT forms rather than degradation products generated during protein solubilization. A further dissection of the molecular structures of these forms may prove interesting as they may relate to the various subcellular localizations of human sperm CRT.

The first evidence for the existence of mobilizable stores of intracellular Ca2+ in mammalian spermatozoa arose from studies employing thapsigargin, a specific inhibitor of the ER Ca2+/ATPase Ca2+ pump. This pump moves cytosolic calcium back to the endoplasmic lumen and its inhibition leads to an increase in the free cytosolic Ca2+ concentration in other cell types. It has been demonstrated that the AR in capacitated human spermatozoa could be initiated by treatment with thapsigargin, suggesting the existence of thapsigargin-sensitive intracellular Ca2+ stores in either the cytoplasmic droplets, the nucleus or the acrosome (Mezel and Turner, 1993). This finding was confirmed with bovine spermatozoa and showed that the outer acrosomal membrane indeed contains a thapsigargin-sensitive Ca2+ pump (Spungin and Breitbart, 1996). Our demonstration of the major calcium storage protein CRT in the acrosome, and its concentration in the equatorial segment, strongly supports the existence of a mobilizable store of calcium in the human sperm acrosome. The localization of the IP3 receptor to the acrosome (Mezel and Turner, 1993) suggests that Ca2+ may be mobilized from this store at least partly via the IP3 pathway and subsequently pumped back via the above-mentioned thapsigargin-sensitive ATPase.

The existence of two or more regulatory stores for intracellular calcium in spermatozoa, one for the tail and one for the head, was suggested by fluorimetric studies using calcium indicators such as Indo-1 and Fura-2. Cytoplasmic Ca2+ oscillations in spermatozoa are significantly more rapid than oscillations detected in other cell types (Suarez et al., 1993). Intracellular Ca2+ is elevated in hyperactivated spermatozoa and a correlation between Ca2+ oscillation frequency and flagellar-bending frequency has been demonstrated in the proximal flagellar midpiece (Camacho and Lechleiter, 1995). Intracellular Ca2+ reaches two different elevated levels in intact moving hamster spermatozoa, first with the achievement of hyperactivation and second with completion of the AR (Camacho and Lechleiter, 1995). Moreover, the increase of intracellular Ca2+ is greater in the midpiece than in the head of hyperactivated spermatozoa, while the reverse is true for acrosome-reacted spermatozoa (Camacho and Lechleiter, 1995). Our demonstration shows three localization sites for CRT: (i) the acrosome with concentration in the equatorial segment; (ii) small CCV in the postacrosomal, perinuclear area; and (iii) light and dark CCV in the neck and midpiece. This localization may provide an explanation for the complex spatio-temporal patterns of calcium oscillations observed during the different stages of the capacitation process.

As the IP3R was shown to co-localize with CRT in the acrosome and in the neck CCV by immunofluorescence, two putative sites for capacitative Ca2+ entry appear, namely the plasma membrane overlaying the acrosome and the plasma membrane overlaying the CCV in the neck. Interactions between store-operated calcium channels in the plasma membrane and the acrosomal IP3R would depend on the dispersion of the F-actin barrier, which has been shown to intervene between the outer acrosomal and the overlying plasma membranes (Spungin and Breitbart, 1996). The activity of the actin severing proteins involved in such a process appear to be regulated by calcium (Spungin and Breitbart, 1996), suggesting that mobilization of calcium from the putative stores in the acrosome, the postacrosomal area, and neck, as well as activation of store-operated calcium channels by the CCV in the neck region, may contribute to the increase in mean [Ca2+]i over the acrosomal cap, necessary for the final stages of the AR to occur. In this respect it is intriguing to speculate that spermatozoa with CCV may have different fertilizing potential versus spermatozoa lacking CCV and to note that immunofluorescence for CRT was observed in the cytoplasmic droplet in approximately half of the cells.

The identification and co-localization of CRT and IP3R may foster a more comprehensive model for [Ca2+]i regulation in spermatozoa and lead to better understanding of the complex spatio-temporal patterns of calcium oscillations observed during hyperactivation and the AR. Such a model must include several sites of mobilizable Ca2+ stores within the human spermatozoon, and regional activation of phosphoinosidade kinases and phospholipases in combination with sequential activation of Ca2+ channels in the various sperm membranes. 

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Calcreticulin and IP3 receptor in human spermatozoa