Structure and Function of Caltrin (Calcium Transport Inhibitor) Proteins

Biochemistry Insights
Volume 10: 1–8
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DOI: 10.1177/1178626417745822

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ABSTRACT: Caltrin (calcium transport inhibitor) is a family of small and basic proteins of the mammalian seminal plasma which bind to sperm cells during ejaculation and inhibit the extracellular Ca^{2+} uptake, preventing the premature acrosomal exocytosis and hyperactivation when sperm cells ascend through the female reproductive tract. The binding of caltrin proteins to specific areas of the sperm surface suggests the existence of caltrin receptors, or precise protein-phospholipid arrangements in the sperm membrane, distributed in the regions where Ca^{2+} influx may take place. However, the molecular mechanisms of recognition and interaction between caltrin and spermatozoa have not been elucidated. Therefore, the aim of this article is to describe in depth the known structural features and functional properties of caltrin proteins, to find out how they may possibly interact with the sperm membranes to control the intracellular signaling that trigger physiological events required for fertilization.

KEYWORDS: caltrin, sperm cells, seminal plasma, fertilization, acrosome reaction, hyperactivation, extracellular calcium uptake

RECEIVED: September 6, 2017. **ACCEPTED:** November 10, 2017.

TYPE: Review

FUNDING: This work was supported by grants from Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP No. 11220120100617CO, Argentina to C.E.C.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

At ejaculation, mature epididymal spermatozoa are pumped through the vas deferens along the male genital tract where secretions of the seminal vesicles (~70% of seminal fluid volume), the bulbourethral gland, and the prostate (~20% of seminal fluid volume) dilute them while providing metabolic substrates, ions, and stabilizing factors.[1,2] Nevertheless, fresh mammalian sperm cells are not able to fertilize oocytes at the time of ejaculation; they become fertilization-competent in the female reproductive tract, where they leave behind the seminal plasma and undergo a series of biochemical and physiological changes in the plasma membrane, such as cholesterol efflux, alteration in the protein composition, and changes in the distribution of intramembranous particles and intracellular Ca^{2+}.[3] All these changes, collectively designated capacitation, increase the permeability of the sperm membrane to ions and, consequently, promote two Ca^{2+}-dependent processes required for fertilization: hyperactivation and the acrosome reaction.[3] The rapid uptake of extracellular Ca^{2+} that precedes these events in bovine epididymal sperm did not occur in spermatozoa separated from ejaculates.[4] On ejaculation, the spermatozoa are suspended in the seminal plasma and exposed to proteins and other molecules secreted by the reproductive accessory glands, mainly by the seminal vesicles and prostate, which may affect the physiological properties of the semen.[1,3] Thus, the seminal plasma contains proteins for specific processes, such as coagulation and liquefaction of semen, and others which are able to bind to the sperm surface and influence specific cellular processes associated with fertilization.[1] Among these molecules, caltrin (calcium transport inhibitor), a small basic protein secreted by the seminal vesicles, binds to epididymal spermatozoa and inhibits extracellular Ca^{2+} uptake.[5] Although the mechanisms of this inhibition have not been fully elucidated, bovine caltrin was detected on the anterior portion of the head and on the principal section of the tail of ejaculated sperm cells. The same distribution was observed incubating epididymal spermatozoa with purified caltrin.[6] Figure 1 shows a diagram of the mammalian spermatozoa indicating the regions where caltrin proteins bind. The specific distribution on the surface of ejaculated spermatozoa suggested the existence of specific caltrin receptors restricted to the regions where Ca^{2+} influx may take place. However, the presence of such specific caltrin receptors in the sperm plasma membrane has not been at present described. It is well known that Ca^{2+} influx into the head and tail of sperm cells is required for supporting the acrosome reaction and hyperactivation. As the concentration of Ca^{2+} in the fluids of the female reproductive tract is high enough to promote the onset of these two Ca^{2+}-dependent processes at any time during sperm transit, Ca^{2+} uptake of sperm cells must be under strict control to avoid premature acrosomal exocytosis and the earlier hyperactivated motility far from the oviduct. By inhibiting extracellular Ca^{2+} uptake, caltrin proteins prevent premature acrosomal exocytosis and hyperactivation when sperm cells ascend through the

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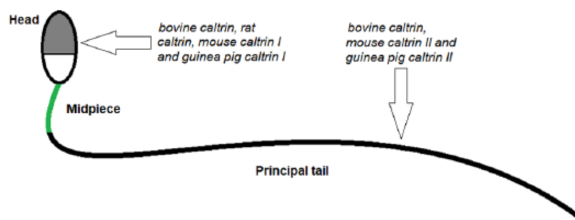

Figure 1. General diagram of the sperm cell depicting the head, midpiece, and principal tail. Arrows indicate the regions where caltrin proteins bind.

female reproductive tract.[7] Consequently, hydrolytic acrosomal enzymes are stored in the organelle until needed, and the sperm cells can keep moving forward to reach the oviduct, where they are trapped in the distal portion of the isthmus and reside under protective conditions until ovulation.[8]

The aim of this work is to describe in depth the known structures and functions of caltrin proteins at present studied (bovine, rat, mouse, and guinea pig) to understand how they may interact with the sperm membranes to influence the molecular mechanisms of intracellular signaling that occur in spermatozoa during the fertilization process.

Structural properties of caltrin proteins

Bovine caltrin, the first caltrin protein isolated, has 47 amino acid residues and is cationic (pI = 8.3) and small (Mr = 5411).[5,9] Using antibovine caltrin antiserum, two caltrin proteins were detected and purified from guinea pig seminal vesicle secretions: they were designated guinea pig caltrin I (45 amino acid residues, Mr = 5084, pI = 9.5) and guinea pig caltrin II (55 amino acid residues, Mr = 6255, pI = 10.2).[10] Rat caltrin composed of 56 amino acid residues (Mr = 6217) is a basic protein (pI = 9.3)[11] and its biosynthesis is androgen dependent.[12] Two caltrin proteins from mouse seminal vesicle secretions were purified and identified as mouse caltrin I or P12 (57 residues, Mr = 6126, pI = 9.5)[13,14] and mouse caltrin II or SVS VII (75 amino acid residues, Mr = 8476, pI = 10.6).[11,15] Using ExPASy—ProtParam tools,[16] theoretical physicochemical parameters such as extinction coefficient, instability index, and hydropathy were calculated for caltrin proteins (Table 1). Table 2 shows the amino acid sequences of the caltrin proteins, and the secondary structures predicted using PSIPRED v 3.3 server are shown in Figure 2 (PSIPRED predicts secondary structure based on the protein sequence).[18] The 3-dimensional (3D) models shown for bovine caltrin and mouse caltrin I were previously published,[13,19] whereas those for guinea pig caltrin I and mouse caltrin II are preliminary results of homology modeling from our laboratory.

Bovine caltrin binds to the principal tail and the acrosome region of the sperm cells[6] affecting sperm extracellular Ca^{2+} transport (as will be discussed in the next section). This protein also expresses an antibacterial activity[20] that can increase the fluidity of the membranes, promoting the release of soluble intracellular enzymes, as reported by San Agustin and Lardy.[21] Based on their sequences, we developed 3D models for bovine caltrin and for mouse caltrin I proteins[19] (Figure 3). The predicted secondary structure of bovine caltrin, based on the template,[22] showed two α-helical segments: one stretched from S16 to A19 with 6.0-Å length (9.0 Å including residues L15 and K20 used for default axial length calculation, as shown in the work by Grasso et al.[19]), and the other from A26 to G43 residues (28.8-Å length). A β-turn from L21 to N23 residues was also reported.[19] Only the larger helical segment predicted for bovine caltrin showed hydrophobic stabilization arcs (residues L5, L6, F9, L10, and W13).[19] In addition, the negative Wimley-White whole-residue hydrophobicity value calculated suggests a clear amphiphilic structure for this protein.

The secondary structure of bovine caltrin was also examined by circular dichroism (CD) in the presence and absence of detergent[23] because its structure is dependent on the polarity of the environment, as was previously observed by tryptophan fluorescence spectral shifts.[21] The spectrum without detergent is characteristic of a random-coil structure. Following the addition of detergent, negative bands with maxima at 208 and 222 nm appeared indicating the presence of α-helix conformations.[23] In agreement with this, a fragment of 13 residues of bovine caltrin showing two negative bands with maximum at approximately 205 and 220 nm and a crossover at 200 nm was reported by Sitaram and Nagaraj.[20] Both CD spectra described are characteristic of a predominantly α-helical conformation.[24] Results suggesting this structural conformation were previously obtained by San Agustin and Lardy analyzing the fluorescence spectrum of bovine caltrin in the presence of phospholipids. The authors observed a blue shift and increase in fluorescence of the tryptophanyl residue when the protein interacts with phosphatidylserine vesicles indicating an increase in its α-helical content.[21] Thus, the secondary structure shown in our molecular model for bovine caltrin[19] accounts for the CD spectra and fluorescence profiles obtained previously.

Mouse caltrin I or P12 is expressed in the male accessory glands (seminal vesicles and dorsal prostate)[25,26] and binds to the sperm acrosomal region[27] in a single-type mouse caltrin I–binding site (1.49×10^6 sites/cell) with a K_d value of 70 nM.[13] Mouse caltrin I, guinea pig caltrin I, and rat caltrin are also serine protease inhibitors Kazal type (SPINK), characterized by a well-preserved amino acid sequence containing three disulfide bridges.[14] Using both homology modeling[28] and threading[29] methods, we predicted a mouse caltrin I model which shows the three disulfide bridges (C10, C17, and C25 cross-linked to C39, C36, and C57, respectively)[19] in agreement with the model reported by Luo et al[27] and Lin et al.[30] We also predicted a model for rat caltrin that shows three disulfide bridges (C19, C16, and C24 cross-linked to C38, C35, and C56, respectively) (Grasso et al, unpublished results). Based on the model prediction (using ESPript 3.0)[22] for mouse caltrin I, around 42% of the total amino acid residues formed secondary structures, which included one α-helix of 10 residues and 17.3-Å length (ECVLCFENRK residues from

Table 1. Physicochemical properties of caltrin proteins.

	Mr	pI	ε[a]	INST. INDEX	GRAVY[b]
Bovine	5411	8.3	6990	24.86	−0.994
Rat	6217	9.3	3355	36.12	−0.432
Mouse I	6126	9.5	3355	51.99	−0.161
Mouse II	8476	10.6	3605	51.02	−0.478
Guinea pig I	5084	9.5	4595	45.68	−0.163
Guinea pig II	6255	10.2	6460	73.57	−1.036

Abbreviation: GRAVY, grand average of hydropathy.
[a]Extinction coefficients (ε) are in units of $M^{-1}cm^{-1}$, at 280 nm measured in water. A protein with an instability index smaller than 40 is predicted as stable, whereas a value above 40 predicts that the protein may be unstable in aqueous solutions.[16]
[b]The negative values ($kcal\,mol^{-1}$) of GRAVY indicate that caltrin proteins are mainly hydrophilic.[17]

Table 2. Sequences of caltrin proteins.

Rat caltrin	KVIGKKANCPNTLVGCPRDYDPVCGTDGKTYANECILCFENRKFGTSIRIQRRGLC
Mouse caltrin I	AKVTGKEASCHDAVAGCPRIYDPVCGTDGITYANECVLCFENRKRIEPVLIRKGGPC
Mouse caltrin II	LICNSCEKSRDSRCTMPQSRCVAKPGESCSTVSHFVGTKHVYSKQMCSPQCK-EKQLNTGKKLIYIMCCEKNLCNSF
Guinea pig caltrin I	AFAPSKVDSDRPNCSRYVQHLYMCTKELDPVCGTDGHTYGNRSIF
Guinea pig caltrin II	RRLHGQAINRPGSCPRVMIYCPARHPPNKCTSDYDCPKPQKCCPGYCGKQCYQPE
Bovine caltrin	SDEKASPDKHHRFSLSRYAKLANRLANPKLLETFLSKWIGDRGNRSVK

Amino acid sequences of caltrin proteins obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/) in FASTA format and used for further analyses. Search criteria were "caltrin; seminalplasmin; SVS VII; SPINK3; P12."

positions 35-44), three β-sheets that involve 14 residues (PVCGT, ITY, and LIRKGG residues from positions 23 to 27, 30 to 32, and 50 to 55, respectively), a β-turn at D28G29 and random structures, and including loops within 33 residues which represent 58% of the total structure.[19] In addition, there appeared to be a type I reverse turn centered on G26 and T27, an irregular reverse turn at the C-terminus of the α-helix, where R43K44R45 allowed a sharp inversion of the chain path, and a reverse turn at the C-terminal region, all stabilized by a disulfide bond between C25 and C57.[27] It is obvious that the secondary structures of mouse caltrin I shown in Figure 2 (PSIPRED) are different when comparing with the 3D model. Thus, despite that secondary structure prediction based in the protein sequences is a well-established method, the development of 3D models using the homology modeling,[28] threading,[29] or molecular dynamics simulations should provide a more accurate secondary structural analysis.[19]

The predicted 3D model for mouse caltrin I[13,19] has experimental support. The CD spectrum of this protein in the wavelengths of 200 to 250 nm showed two negative bands with magnitudes of -8.1×10^3 and -1.31×10^4 $deg\,cm^2\,dmol^{-1}$ around 220 nm (band I) and 205 nm (band II), respectively, which suggests a considerable amount of ordered structures including a helix and a mixture of β-forms and β-turns.[13] Similar CD spectra were obtained for native rat caltrin protein, dissolved in ultrapure water and in phosphate-buffered saline (Grasso et al, unpublished results).

The sequence of mouse caltrin II is quite different from that of mouse caltrin I (Table 2) and apparently belongs to a family of proteins that evolved very rapidly in rodents due to the effects of positive selection, which is thought to be a form of adaptive evolution resulting in an increase in the fitness of the organism.[31] Rat and mouse caltrin proteins are not reactive to Ellman's reagent,[32] indicating that the sulfhydryl groups of the cysteines are disulfides in their native forms. However, these proteins are able to form dimers in aqueous solution.[11]

The CD spectrum of mouse caltrin II showed at least seven bands in the wavelength region between 200 and 300 nm. Bands I to VI in the near-UV region arose from nonpeptide chromophores, and band VII in the UV region was mainly due to peptide chromophores. Band VII was negative with a minimum at 217 nm. In addition, a positive band appeared as the CD profile extended below 200 nm.[15] Such a spectrum is characteristic of a predominantly β-sheet conformation.[33,34] Nevertheless, the predicted secondary structure for mouse caltrin II shown in Figure 1 displays an α-helix at residues Q45M46C47S48. It may be that some ordered structure other than the α-helix, probably a mixture of β-form, β-turn, and

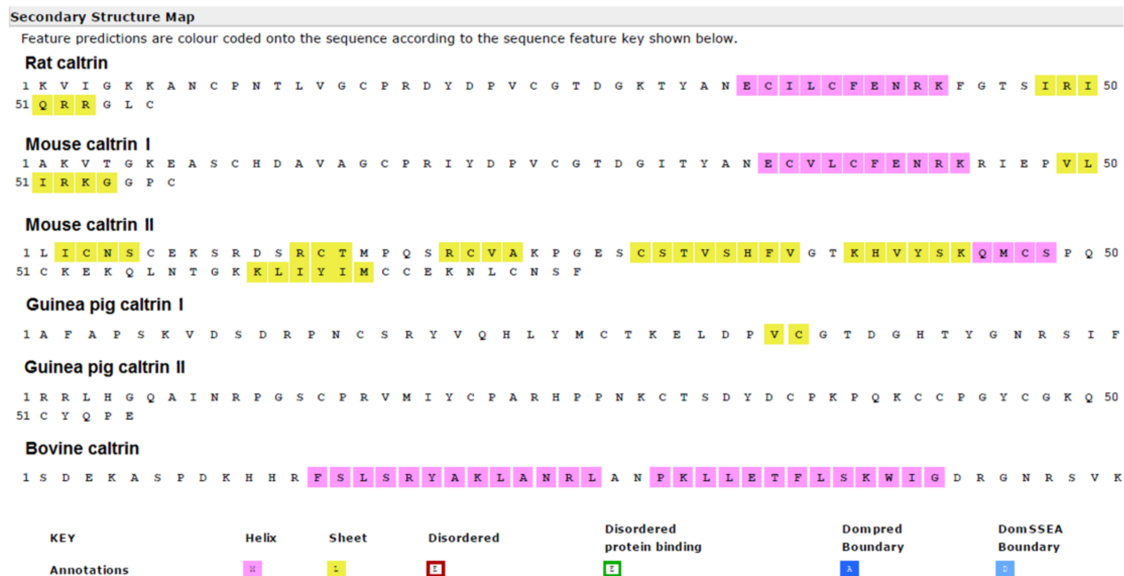

Figure 2. Secondary structures of caltrin proteins predicted using PSIPRED v 3.3 based on the amino acid sequences.[18] Note that for mouse caltrin I and bovine caltrin, several differences were observed when comparing the PSIPRED prediction vs the prediction based on the 3-dimensional model (see the work by Grasso et al[19] and discussion in the text).

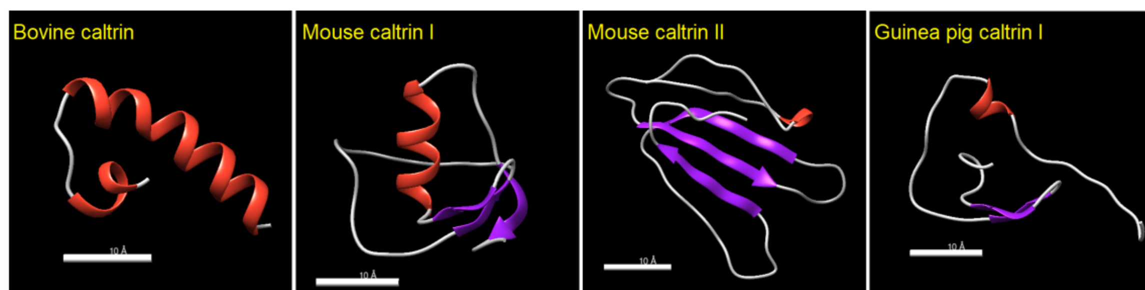

Figure 3. The 3-dimensional (3D) models predicted for bovine caltrin, mouse caltrins I and II, and guinea pig caltrin I. The 3D models for mouse caltrin II and guinea pig caltrin I are actually unpublished and under a systematic structural analysis. Scale bars = 10 Å.

unordered forms, is also formed in the molecular population.[15] We are currently working in the development of a 3D model for mouse caltrin II, and some preliminary results are shown in Figure 3.

Rat caltrin and guinea pig caltrin I bind to the sperm acrosome[14,35] and share a segment of 13 amino acids of almost identical sequence in the two molecules (residues 21-33 in the rat caltrin and 29-41 in guinea pig caltrin I). There are only two different amino acids in this segment: K in position 29 in the rat protein is replaced by H in position 37 in the guinea pig sequence, whereas A in position 32 in the rat protein is replaced by G in position 40 in the guinea pig sequence[14] (Figure 4). Guinea pig caltrin II binds to the principal tail but not to the acrosome of the sperm cells.[35] It has eight conserved cysteine residues in the position characteristics of the whey acidic protein (WAP) motif found in some serine protease inhibitors[37–39] and antibacterial proteins.[40–43] As shown in Figure 2, guinea pig caltrin II does not display any predicted secondary structures, whereas guinea pig caltrin I shows only a β-sheet at residue V31C32. Using RONN v 3.2 server,[44] we observed that a great part of the guinea pig caltrin II secondary structure is predicted disordered (Figure 5) and, consequently, a 3D model for the protein is very difficult to be achieved. Nevertheless, the absence of specific 2D/3D predicted structures is not an impediment to the functionality of this protein. In fact, many proteins contain local regions of such disorder, and some appear to be totally unfolded in their native states.[45] There are examples of disorder-to-order transitions on binding, in which natively unfolded proteins form complexes with their cognate partners,[46] but whether this occurs in the guinea pig caltrin II binding to sperm cells remains to be elucidated. On the contrary, RONN v 3.2 server predicted that guinea pig caltrin I structure is ordered, and, unlike guinea pig caltrin II, a 3D model was obtained for this protein (Figure 3).

Both guinea pig caltrin proteins contain carbohydrate residues, as detected with concanavalin A.[10] According to the sequence of both molecules, there are two possible sites of glycosylation. In the case of guinea pig caltrin I, these sites would be located in residues NCS and NRS at positions 13 to 15 and 41 to 43, respectively, and, in the case of guinea pig caltrin II, in the guanidine group of arginine at the NH_2 terminus and also in NKCT residues at positions 28 to 31.[10]

Score	Expect	Method	Identities	Positives	Gaps
35.4 bits(80)	3e-10	Compositional matrix adjust.	18/40(45%)	21/40(52%)	3/40(7%)

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Query (g. pig caltrin I)  KVDSDRPNCSRYVQHLYMCTKELDPVCGTDGHTYGNRSIF
                          KV    + NC       L  C ++ DPVCGTDG TY N   I
Sbjct (rat caltrin)       KVIGKKANCP---NTLVGCPRDYDPVCGTDGKTYANECIL
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Figure 4. Protein to protein BLAST[36] between rat caltrin and guinea pig caltrin I sequences. The greatest degree of overall homology between rat caltrin and guinea pig caltrin I sequences is found in the fragment of 13 residues DPVCGTDGK/HTYA/GN (residues 21-33 in rat caltrin and 29-41 in guinea pig caltrin I), whose sequence resembles that of a highly conserved segment in pancreatic secretory trypsin inhibitors (PSTIs) and trypsin/acrosin inhibitors from seminal secretions.[14]

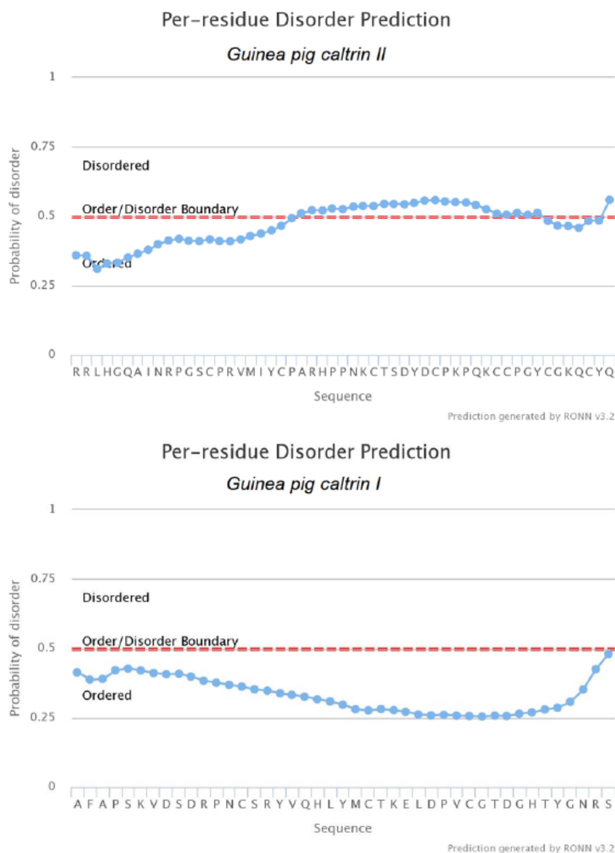

Figure 5. In silico prediction of disordered regions of guinea pig caltrin I and II proteins using RONN v3.2.

Functional properties of caltrin proteins

Caltrin proteins are cationic and small, as mentioned above. The unique molecular form of bovine caltrin binds to the sperm plasma membrane over the acrosome and the principal tail but not to the distal postacrosomal portion of the sperm head or to the midpiece.[6] This protein has a bimodal behavior in relation to Ca^{2+} movement across the sperm plasma membrane: bovine caltrin is a Ca^{2+} transport inhibitor but it can be converted to a Ca^{2+} transport enhancer.[21] Anionic cofactors of the seminal plasma stabilized the inhibitory form of bovine caltrin. These molecules were isolated and identified by high-performance liquid chromatography as citric and L-lactic acids and as phosphatidylserine, phosphatidylglycerol, and cardiolipin by thin-layer chromatography in silica gel.[21] Of these anionic cofactors identified in the bovine seminal plasma, only phosphatidylserine converted the enhancer form of caltrin into the inhibitory molecule at pH 7.4. This was associated with conformational changes in the protein, due to the binding of the phospholipid, forming a protein-phospholipid complex.[21] As this interaction seems to be noncovalent, it is possible that the net positive electric field generated in the caltrin molecule[19] accounts for the binding of phosphatidylserine to the protein. Loss of phosphatidylserine from the caltrin-phospholipids inhibitor complex stabilizes the enhancer Ca^{2+} uptake form of bovine caltrin.[21]

Another interesting biological feature of this protein is its bactericide activity by permeabilizing the bacterial membranes, as described by Sitaram and Nagaraj.[20] They constructed a synthetic peptide of 27 amino acids with antimicrobial activity comparable with that of the whole molecule of bovine caltrin. This peptide contained two segments of 13 amino acids located between residues 14 to 26 (SLSRYAKLANRLA) and 28 to 40 (PKLLETFLSKWIG) in the bovine caltrin sequence, which were indicated as responsible for caltrin-membrane interaction.[20] Sitaram and Nagaraj[20] and ourselves,[19] separately, used the Kyte and Doolittle scale[17] to evaluate the hydropathy profile of bovine caltrin. Based on the KD scores, which indicate that amino acids with KD values above 0 kcal mol^{-1} are located in a hydrophobic region of the polypeptide chain, it is clear that the 27 residues segment of bovine caltrin has the ability to spontaneously partition into the lipid bilayer of membranes, inducing reorganization of the fatty acid acyl chain arrangement, thereby altering the physical properties of the membrane. Likewise, San Agustin and Lardy[47] reported lysogenic activity of bovine caltrin in the Ca^{2+} transport enhancer form. They showed that bovine caltrin at 37 μM (200 μg/mL) was able to release about 30% of the total hyaluronidases of the acrosome and 50% of the cytosolic lactate dehydrogenase from epididymal spermatozoa. This was prevented by phosphatidylserine, probably by caltrin-phospholipid complex formation. In contrast, phosphatidylcholine was ineffective.[47]

In the guinea pig, the two different caltrin molecules purified from the seminal vesicle secretion were equally effective as inhibitors of sperm Ca^{2+} uptake, although they participate in different cell processes.[10] The smaller molecule, designated caltrin I, binds to the head and prevents the release of acrosomal enzymes of epididymal spermatozoa incubated in the presence of Ca^{2+}, as a consequence of the inhibitory action on extracellular Ca^{2+} uptake; caltrin II, the larger one, binds to the main portion of the sperm tail and delays the onset of hyperactivated motility by inhibition of Ca^{2+} influx.[35] Total inhibition of sperm extracellular Ca^{2+} uptake, measured independently, was

40% for guinea pig caltrin I and 48% for guinea pig caltrin II.[10] These two guinea pig caltrin molecules contain carbohydrate residues, which were detected with concanavalin A, and were completely removed by chemical deglycosylation with trifluoromethanesulfonic acid. Both deglycosylated proteins enhanced the rate of calcium uptake by guinea pig epididymal sperm. Thus, they become enhancer molecules, like the bovine caltrin, but using different molecular mechanisms.

In relation to the cysteine-rich WAP motif of guinea pig caltrin II, Futanari et al[48] reported a recombinant protein with inhibitory activity against porcine pancreatic elastase but with affinity much lower than those of other WAP-motif proteins, such as SLPI and trappin-2 (elafin), which have been established as specific elastase inhibitors.[49] These activities have not been detected in the native guinea pig caltrin II protein.

The biological properties of rat caltrin have also been thoroughly studied.[8,11,14] This protein binds to the sperm head of epididymal spermatozoa over both convex and concave surfaces, which are designated dorsal and ventral acrosome in accordance with Pikó's nomenclature.[50] Rat caltrin protein bound to spermatozoa inhibited Ca^{2+} uptake during in vitro capacitation experiments and, consequently, produced several effects on the cells: (a) it reduced the rate of spontaneous acrosomal exocytosis; (b) it did not alter the pattern of protein tyrosine phosphorylation, a key event that occurs during sperm capacitation[51]; and (c) it enhanced sperm binding to the oocyte zona pellucida (ZP).[8] Experimental results showed that epididymal sperm, capacitated with caltrin protein bound to their heads, binds more efficiently to the oocyte ZP than untreated cells. Significantly higher percentages of oocytes with bound sperm and higher average numbers of bound sperm per egg were detected. In addition to this important finding on sperm-egg interaction, spermatozoa maintaining the acrosomal integrity required to interact with the oviductal epithelium can reside there under protective conditions until the sperm-egg encounter occurs. Exposure of rat epididymal sperm to rat caltrin protein did not cause a detrimental effect on sperm motility.[8]

Rat caltrin and caltrin I proteins of the guinea pig and mouse are potent SPINK,[13,14] whereas bovine caltrin and caltrin II proteins from guinea pig and mice are not.[14] The synthesis and secretion of these three caltrins with protease inhibitory activity was studied by immunocytochemistry in seminal vesicle tissues.[12,13,52] Caltrin proteins were immunolocalized in cells of the secretory epithelium and stored in secretory granules which transport proteins to the lumen. The effect of androgen status on the synthesis and secretion of caltrin proteins was also examined in these studies. Data obtained by Western blotting and evaluation of trypsin inhibitory activity, before and after castration, and after 14 days of testosterone administration, proved to be androgen-dependent processes.[12] Similar results were reported by

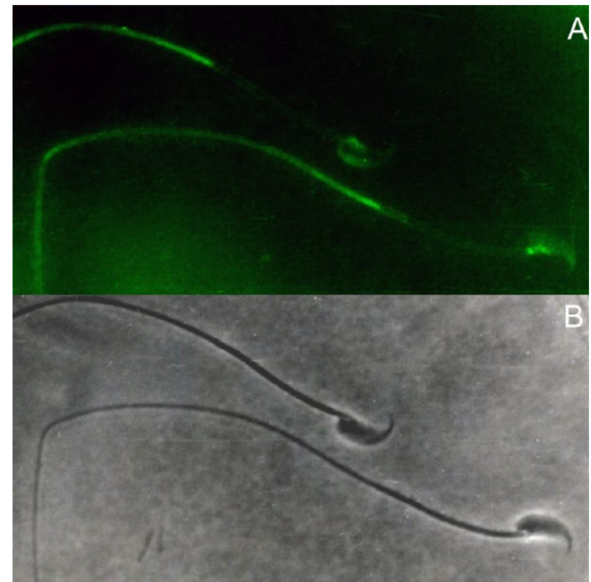

Figure 6. Immunolocalization of sperm-bound mouse caltrin II protein. Epididymal spermatozoa were treated with 0.5 mg of mouse caltrin II/10^8 cells for 1 hour and then washed twice with phosphate-buffered saline. Sperm cells were spread and dried on glass slides, treated in succession with rabbit monospecific mouse caltrin II antiserum, and goat antirabbit IgG labeled with fluorescein isothiocyanate. Slides were washed to remove protein excess and viewed with a Zeiss fluorescence microscope. Original magnification X1000. (A) Fluorescence photomicrograph showing immunoreaction mainly in the principal portion of the tail. Immunofluorescence is observed neither in the midpiece nor in the acrosome. (B) Phase contrast photomicrograph.

Mirosevich et al,[26] who analyzed the levels of caltrin messenger RNA in prostate tissue of mice under identical treatment. In relation to the protease inhibitory activity of some caltrin proteins, it is known that the Kazal domain acts as a substrate analogue that stoichiometrically and competitively binds to the active site of the target proteinase forming a proteinase-proteinase inhibitor complex, much more stable than the Michaelis enzyme-substrate complex.[25,53] Almost complete inhibition of trypsin (95%) was registered with 0.22 mM of rat caltrin, whereas no inhibition was detected when the assay was performed with the reduced and alkylated protein. A similar inhibitory effect was determined with native guinea pig caltrin I, but more protein was required to obtain the highest inhibition.[14]

Zalazar et al found a recombinant mouse caltrin I protein expressed in *Escherichia coli*, which they called SPINK3, which inhibits trypsin activity as efficiently as the soybean trypsin inhibitor[54,55] and modulates sperm physiology through a downstream reduction in endogenous nitric oxide concentration.[54] Thus, it binds to epididymal sperm cells, reduces intracellular Ca^{2+} concentration, and modulates the acrosome reaction and protein tyrosine phosphorylation without altering sperm motility. The same results were detected with SPINK3 expressed as a GST-fusion protein (GST-SPINK3), but it lacked protease inhibitor activity.

Consequently, the authors assume that the serine protease activity of this mouse caltrin protein might not be necessary in a sperm intracellular signaling pathway that involves nitric oxide. This proposal agrees with the results of Ou et al[56] obtained with the recombinant mouse caltrin I protein carrying a point mutation at residue R19, which was indicated as essential for trypsin inhibition.

In relation to mouse caltrin II, Luo et al[15] showed that it is able to bind to epididymal spermatozoa with no specificity, covering the whole cells. Using polyclonal monospecific anti-mouse caltrin II antibodies developed in the rabbit, we have recently detected immunofluorescence only in the principal tail of mouse epididymal sperm (Figure 6, unpublished results). These new results suggest that mouse caltrin II might play a similar functional role as a regulator of sperm hyperactivated motility as it was demonstrated with guinea pig caltrin II.[35] Consequently, caltrin proteins (bovine and caltrin II of the mice and guinea pigs) could allow the sperm cells keep moving forward to reach the oviduct, where they are trapped in the distal portion of the isthmus to form the oviductal sperm reservoir and where they reside under protective conditions until ovulation.[8]

Conclusions

There are several factors that regulate mammalian reproduction.[3] Among these, secretory proteins of the seminal plasma seem to play an important role in the regulation of a number of sperm physiological processes associated with fertilization.[3,7] Proteomic studies on sperm cells and seminal fluids from different mammalian species have helped to identify molecules involved in the molecular mechanisms of generation and maintenance of fertile spermatozoa.[1] Thus, caltrin proteins, which are synthesized by the secretory epithelium of the seminal vesicles under strict androgenic control,[12,26,48] appear to be engaged in the fertilization process, regulating not only the extracellular Ca^{2+} uptake and, consequently, the sperm Ca^{2+}-dependent acrosomal exocytosis and hyperactivated motility but also the activity of sperm enzymes that might have a role in fertilization. The activity of caltrin modulating these physiological events which precede fertilization is very specific and takes place without disturbing the capacitation process.[8] Caltrin proteins inhibit around 50% of sperm Ca^{2+} uptake which seems to operate exclusively in the mechanisms of spontaneous acrosomal exocytosis and hyperactivation, as reported previously.[8] Thus, caltrin protects the acrosomal integrity and functionality required for sperm binding to the oviductal epithelium where they are retained under protective conditions until ovulation. As it was shown by Clark et al,[57] the protective action of caltrin on the sperm acrosome does not interfere with the physiologic acrosome reaction induced by the oocyte ZP which guarantees the fertilization success.

The functional activity of these proteins is performed through the specific binding to the sperm cells, probably by means of caltrin receptor molecules or precise protein phospholipids arrangements in the sperm membrane. The primary structure of all caltrin proteins at present detected has been described, but there are few systematic studies of the secondary and tertiary structures to understand the molecular mechanisms of recognition and interaction between caltrin and the sperm membranes.

In this article, we tried to connect the well-known biological properties of caltrin proteins with their molecular structures to explain how they interact with the spermatozoa. For this purpose, we also showed data of structural studies recently completed in our laboratory.

Acknowledgements

The authors are grateful to Joss Heywood (native speaker) for the careful revision of the manuscript.

Author Contributions

EJG wrote the first draft of the manuscript. CEC made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

Disclosures and Ethics

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