CaMKIIα interacts with multi-PDZ domain protein MUPP1 in spermatozoa and prevents spontaneous acrosomal exocytosis

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Summary

The success of acrosomal exocytosis, a complex process with a variety of inter-related steps, relies on the coordinated interaction of participating signaling molecules. Since the acrosome reaction resembles Ca^{2+}-regulated exocytosis in neurons, we investigated whether cognate neuronal binding partners of the multi-PDZ domain protein MUPP1, which recruits molecules that control the initial tethering and/or docking between the acrosomal vesicle and the plasma membrane, are also expressed in spermatozoa, and whether they contribute to the regulation of acrosomal secretion. We observed that CaMKIIα colocalizes with MUPP1 in the acrosomal region of epididymal spermatozoa where the kinase selectively binds to a region encompassing PDZ domains 10-11 of MUPP1. Furthermore, we found that pre-treating mouse spermatozoa with a CaMKII inhibitor that directly blocks the catalytic region of the kinase, as well as a competitive displacement of CaMKIIα from PDZ domains 10-11, led to a significant increase in spontaneous acrosomal exocytosis. Since Ca^{2+}-calmodulin releases CaMKIIα from the PDZ scaffolding protein, MUPP1 represents a central signaling platform to dynamically regulate the assembly and disassembly of binding partners pertinent to acrosomal secretion, thereby precisely adjusting an increase in Ca^{2+} to synchronized fusion pore formation.

Key words: Acrosome reaction, CaMKII, MUPP1, Calcium-regulated exocytosis, Spermatozoa, PDZ domain, Lipid rafts, Scaffolding protein

Introduction

Ca^{2+}-regulated exocytosis, a series of molecular events including vesicle recruitment, tethering, docking, priming, and the final vesicle fusion event itself, is adapted to deliver molecules with high reliability and with the exact moment-to-moment requirements of a cell (Chapman, 2008; Jahn, 2004; Malsam et al., 2008; Rettig and Neher, 2002; Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009; Wojcik and Brose, 2007). The core fusion machinery consists of a super-family of conserved membrane-bridging proteins, called SNAREs (soluble *N*-ethylmaleimide-sensitive attachment protein receptor), which form a stable membrane-trafficking complex composed of a complementary set of vesicular synaptobrevin (v-SNARE), and plasma membrane-associated syntaxin and SNAP25 (t-SNAREs) (for reviews, see Jahn and Scheller, 2006; Lang and Jahn, 2008). At the subcellular level, the precision and adjustability of Ca^{2+}-regulated exocytosis is brought about by a dual protein-assembly strategy of components involved in the fusion reaction sequence. First, this includes clustering of SNAREs and assisting regulatory proteins (Malsam et al., 2008) in liquid-ordered membrane microdomains, commonly termed 'lipid rafts' (Lang, 2007) which have been implicated in the recruitment and regulation of signal transduction and membrane traffic processes (Ikonen and Simons, 1998). Second, components of the fusion machinery interact directly with each other (Mochida, 2000; Sudhof, 1995), and/or with specialized scaffolding proteins (Fejtova and Gundelfinger, 2006), thereby forming complex signaling frameworks below the plasma membrane (Schoch and Gundelfinger, 2006). Interestingly, a special mode of competitive protein-protein interaction between the Ca^{2+}-sensor protein synaptotagmin (Koh and Bellen, 2003) and the SNARE-associated complexin, also called synaphin (Brose, 2008; McMahon et al., 1995), has recently been shown to clamp the fully assembled fusion machinery at an intermediate pre-fusion stage (Sudhof and Rothman, 2009), thereby preventing spontaneous SNARE-promoted membrane fusion (Weber et al., 1998).

A special form of Ca^{2+}-regulated exocytosis is the sperm acrosome reaction, a mandatory event in the fertilization process, initiating two essential events: release of hydrolyzing enzymes, necessary for sperm to penetrate the egg glycoprotein matrix, the zona pellucida (ZP) (Wassarman and Litscher, 2008), and exposure of the inner acrosomal membrane, which thereby becomes accessible for the egg plasma membrane during the ultimate fusion event (Florman et al., 2008; Harper et al., 2008). Although acrosomal exocytosis in sperm cells differs from exocytotic events in other cellular systems, mainly because the acrosome is a huge and single secretory vesicle, certain remarkable parallels have been outlined between the acrosome reaction in sperm and vesicle fusion in neurons and neuroendocrine or exocrine cells (Mayorga et al., 2007; Tulsiani and Abou-Haila, 2004). First, zona-pellucida-induced secretion of the acrosomal contents is accomplished by an elevation in intracellular Ca^{2+} (Florman et al., 2008; Publicover et al., 2007), characterized by two sequential Ca^{2+} transients (Darszon et al., 2005). Furthermore, isoforms of the SNARE protein family, as well as assisting regulatory proteins that are also associated with lipid

raft-derived signaling platforms (Boerke et al., 2008; Travis et al., 2001; Tsai et al., 2007), are expressed in mammalian spermatozoa (Gerst, 1999; Kierszenbaum, 2000; Mayorga et al., 2007; Tomes, 2007). Beside these parallels of acrosomal secretion and regulated exocytosis in other secretory systems, there are some unique features of the acrosome reaction, whose underlying molecular mechanisms are still unclear. One of these characteristics is the formation of hundreds of fusion points between the plasma membrane and the outer acrosomal membrane, ensuring the efficient delivery of the entire acrosomal contents (Barros et al., 1967; Michaut et al., 2000; Zanetti and Mayorga, 2009). Although there are currently no experimental data to explain this special feature of synchronous membrane pore formation during acrosomal secretion, an intriguing solution for this conundrum could be a scenario in which distinct pre-assembled SNARE complexes (De Blas et al., 2005; Roggero et al., 2007; Tomes et al., 2005; Zarelli et al., 2009) are mechanically coupled in a functional protein network between the outer acrosomal membrane and the adjacent plasma membrane. However, if mechanically coupled SNARE complexes are the basis of the observed synchronized fusion pore formation, a single 'unintended' fusion event could be able to trigger a 'domino' effect. This would initiate a 'zipper-like' biochemical chain reaction, which would ultimately lead to an irreversible and total loss of the one and only secretory vesicle. Therefore, mechanisms might exist to prevent a spontaneous acrosome reaction in resting sperm.

We have recently observed that the multi-PDZ domain protein MUPP1, which contains 13 potential protein-binding motifs and is abundantly expressed in brain tissue, is also present in the acrosomal region of spermatozoa of different mammalian species (Heydecke et al., 2006). In these cells, MUPP1 is involved in recruiting molecules that control the initial tethering and/or docking of the acrosomal vesicle at specific sites of the plasma membrane (Ackermann et al., 2008). In vitro, MUPP1 interacts with distinct isoforms of the neuronal Ca^{2+}-calmodulin kinase II (CaMKII) (Krapivinsky et al., 2004), a holoenzyme of several protein subunits (α, β, γ and δ), encoded by four closely related genes (Hudmon and Schulman, 2002b). In analogy to the fact that the CaMKII is functionally active in recruiting synaptic vesicles to the active zone of the presynaptic nerve terminal (Greengard et al., 1993; Leal-Ortiz et al., 2008), the MUPP1-controlled recruitment of the acrosomal vesicle to the plasma membrane (Ackermann et al., 2008) might be mediated by MUPP1-associated CaMKII (Krapivinsky et al., 2004). However, it is also conceivable that a CaMKII-catalyzed phosphorylation reaction is responsible for 'freezing' the acrosomal vesicle in an intermediate pre-assembled fusion state (Wang, 2008), thereby preventing accidental spontaneous acrosomal secretion. Therefore, in the present study, we investigated whether the MUPP1 binding partners CaMKIIα and/or CaMKIIβ (Krapivinsky et al., 2004) are expressed in spermatozoa, and whether these kinases contribute to the regulation of acrosomal secretion. We show that a CaMKIIα-MUPP1 complex is present in the acrosomal region of spermatozoa, and that this complex serves a crucial role in clamping the acrosomal fusion machinery. The observed mechanism could be of pivotal importance to preserve the fertilization ability of spermatozoa.

Results

Identification of CaMKIIα in rodent spermatozoa

To investigate whether CaMKII isozymes found to interact with MUPP1 in vitro (Krapivinsky et al., 2004), are expressed in rodent spermatozoa, subtype-specific antibodies recognizing specific sequences within the C-terminal association region of either the α- or the β-isoform (Hudmon and Schulman, 2002b) were used. Western blot analyses were performed with *Rattus norvegicus* (rat) and *Mus musculus* (mouse) brain cortex, cortex-, testis- and epididymis-derived protein fractions and whole-sperm lysates of both rodent species. The anti-CaMKIIα antibody detected two predominant immunoreactive bands in rat (Fig. 1A, left panel) and mouse (Fig. 1A, middle panel) testicular tissue and in control extracts from brain cortex. Although the 52 kDa band probably represents the predominant neuronal CaMKII isoform (Wayman et al., 2008; Yamauchi, 2005), the 65 kDa protein might represent an α-isozyme created by alternative splicing (Hudmon and Schulman, 2002a). In lysates of isolated epididymal spermatozoa of rat and mice, the major neuronal CaMKII isoform was present in both rodent species; in addition, a degradation product with a molecular mass of about 40 kDa was detectable in mouse spermatozoa (Fig. 1A, right panel).

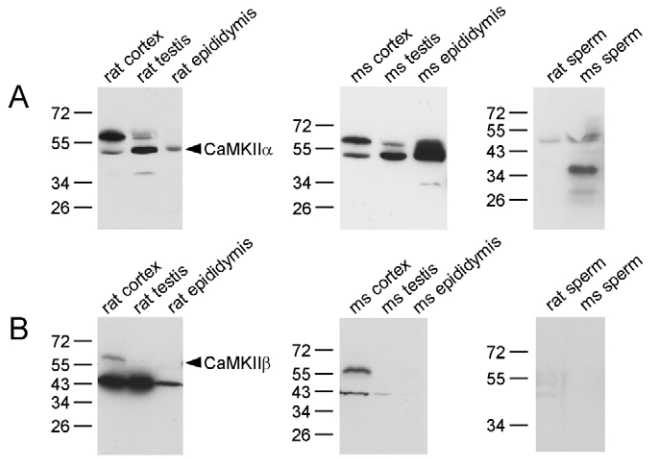

Fig. 1. Identification of CaMKII isoforms in rodent spermatozoa by immunoblot analyses. Equal amounts of rat (left panel) and mouse (ms, middle panel) tissue preparations from cortex, testis and epididymis as well as lysates of isolated epididymal spermatozoa (right panel) were separated by SDS-PAGE and probed with an anti-CaMKIIα (A) and an anti-CaMKIIβ antibody (B). Note that the major neuronal α-isoform with the predicted size of 52 kDa was present in rat as well as in mouse spermatozoa (A, right panel) whereas for the β-isozyme, there was no visible immunoreactivity in the separated sperm preparation (B, right panel). Representative results of at least three experiments with independent tissue preparations are shown. Positions of the molecular mass (in kDa) of standard proteins for each immunoblot are indicated on the left.

The CaMKIIβ isoform showed a different expression pattern in rat and mouse tissues: In rat (Fig. 1B, left panel) and mouse (Fig. 1B, middle panel) brain cortex, a β-immunoreactive band with the predicted molecular mass of the prevalent neuronal β-isozyme of about 60 kDa (Yamauchi, 2005) was detectable. However, in testicular tissue of both rodent species, the antibody only recognized a smaller 45 kDa immunoreactive band (Fig. 1B, left and middle panel); in isolated rat and mouse sperm lysates, the β-isoform was not detectable (Fig. 1B; right panel).

Colocalization of CaMKIIα and MUPP1

To examine whether one of the two CaMKII isozymes found to associate with MUPP1 are detectable at the cellular level, double immunofluorescence staining was performed by incubating epididymal mouse and rat spermatozoa with anti-MUPP1 and anti-

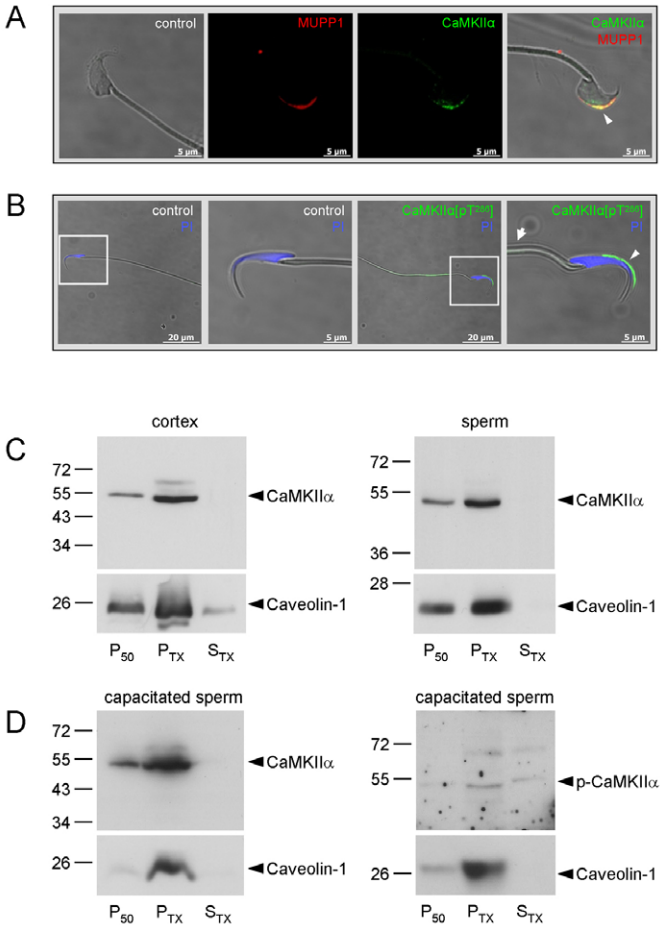

Fig. 2. Colocalization of CaMKIIα and MUPP1 in rodent spermatozoa. To determine the subcellular localization of MUPP1 and CaMKIIα in spermatozoa, freshly isolated epididymal mouse sperm were simultaneously probed with a rabbit anti-MUPP1 and a mouse anti-CaMKIIα antibody (A). Note the colocalization of MUPP1 (red) and CaMKIIα (green) in some regions of the crescent-shaped acrosomal region. Samples incubated with only the secondary antibodies were unstained (control). A polyclonal anti-CaMKIIα-Thr286-*P* antibody was used to visualize the subcellular localization of the identified CaMKIIα in rat spermatozoa (B, CaMKIIα-[pT^{286}]). Immunostaining was restricted to the midpiece region of the sperm tail (arrow) and to the convex side of the sperm head, which represents the acrosomal region (arrowhead). Negative controls represent samples incubated with the secondary antibody only (control). The boxes indicate regions that are magnified in panels to the right. Experiments were repeated with at least three independent sperm preparations, which showed very similar results. To examine whether CaMKIIα is associated with detergent-insoluble membrane microdomain clusters, cortical rat brain tissue (C, left panel) and freshly isolated (C, right panel) or capacitated epididymal rat spermatozoa (D, left and right panels) were extracted with ice-cold PBS, supplemented with 1.5% Triton X-100; subsequently equal amounts of total membrane starting material (P_{50}) and Triton X-100 insoluble membrane (P_{TX}) as well as detergent-soluble proteins (S_{TX}) were separated by SDS-PAGE and subjected to western blot analysis using an anti-CaMKIIα antibody generated against the C-terminal association region (CaMKIIα), or an anti-CaMKIIα antibody recognizing Thr286-*P* kinase (p-CaMKIIα). For in vitro phosphorylation of CaMKII, microsomal membrane fractions (P_{50}) of epididymal or capacitated spermatozoa, resuspended in PBS, were incubated for 15 minutes at 30°C in the presence of 2 mM $CaCl_2$, 1.2 μM calmodulin and 100 μM ATP, and subsequently samples were applied to Triton X-100 extraction. Note that the bulk of CaMKIIα immunoreactivity in cortex-derived fractions is only detectable in the Triton-X-100-insoluble pellet (P_{TX}), whereas the soluble fraction (S_{TX}) shows no significant staining (C, top left panel). The same strict raft-association of CaMKII was visible in freshly isolated (C, top right panel) and capacitated sperm cells (D, top left panel), and was also detectable for the Thr286-*P* form of CaMKIIα (D, top right panel), although a faint immunoreactivity was also visible in the soluble protein fraction (S_{TX}). To ensure isolation of detergent-resistant membrane microdomains, an antibody recognizing the raft marker protein caveolin-1 was applied to control immunoblots, corresponding to each individual preparation (lower panels in each figure). Representative results of at least three experiments with independent tissue preparations are shown. The positions of the molecular mass standards (in kDa) for each western blot are indicated on the left.

CaMKIIα or anti-CaMKIIβ antibodies (Fig. 2). Previous studies have shown that an antibody that recognizes both the CaMKIIβ and CaMKIIγ isoforms led to a distinct staining of the principal piece of the flagellum of mouse spermatozoa (Schlingmann et al., 2007); however, application of a subtype-specific anti-CaMKIIβ antibody did not reveal a specific β-immunolabeling, in neither mouse nor rat spermatozoa (data not shown), thus confirming the observations made by immunoblot analyses (Fig. 1B). In contrast to the lack of CaMKIIβ expression, experiments with the CaMKIIα-specific antibody verified the expression of the CaMKIIα isozyme in mouse spermatozoa detected by immunoblotting (Fig. 1A). Immunostaining was seen in sperm heads and was restricted to the hook-shaped acrosomal region (Fig. 2A). The same crescent-shaped acrosomal labeling was obtained with the anti-MUPP1 antibody (Fig. 2A). Overlay of the confocal microscopy pictures (orange-yellow) indicated that MUPP1 and CaMKIIα were colocalized within the acrosomal region of mouse spermatozoa (Fig. 2A).

To investigate whether CaMKIIα showed a comparable subcellular localization in rat sperm cells, isolated epididymal rat sperm were subjected to indirect immunostaining using the anti-CaMKIIα antibody that recognizes the association region of the kinase (Fig. 1A, left panel). However, no apparent labeling was observed (data not shown) by the latter antibody and by two other antibodies that were also raised against the C-terminal region of CaMKIIα (data not shown), suggesting that in rat spermatozoa, the association domain of CaMKIIα is not immunocytochemically accessible. To test this assumption, an antibody directed against residues surrounding the phosphorylated Thr286 in the regulatory domain of the kinase was chosen (Hudmon and Schulman, 2002a; Hunter and Schulman, 2005). Positive immunostaining would not only confirm expression of this kinase isoform in rat spermatozoa, but would also indicate whether CaMKIIα is already in an active conformation. Fig. 2B illustrates the fluorescence labeling pattern of rat sperm stained with the anti-CaMKIIα-Thr286-*P* antibody. A crescent-shaped acrosomal staining and a faint labeling of the flagellum restricted to the midpiece region of the sperm tail (Fig. 2B) was discerned.

In cultured neurons (Du et al., 2006) and in transfected human embryonic kidney 293 cells (Suzuki et al., 2008) CaMKIIα is associated with raft-derived detergent-resistant membrane fractions. Since MUPP1, which binds CaMKIIα in in vitro translated systems (Krapivinsky et al., 2004), is also concentrated in Triton-X-100-insoluble plasma membrane microdomains derived from brain and sperm preparations (Ackermann et al., 2008; Krapivinsky et al., 2004), we asked whether both proteins were colocalized to detergent-insoluble membrane clusters in sperm cells. Therefore, we extracted rat brain cortex tissue and freshly isolated epididymal rat spermatozoa with Triton X-100. Subsequently, equivalent amounts of total microsomal starting material (P_{50}) and detergent-insoluble (P_{Tx}) and soluble fractions (S_{Tx}) were separated by SDS-

PAGE and probed by immunoblotting using the anti-CaMKIIα antibody generated against the C-terminal association region. Fig. 2C shows that in cortex- (left panel) and in sperm-derived preparations (right panel), total CaMKIIα as well as the raft marker protein caveolin-1 (Anderson, 1998) co-migrated with the Triton-X-100-insoluble pellet, indicating colocalization with MUPP1 in detergent-resistant membrane fractions (Ackermann et al., 2008). A similar lipid raft targeting was also observed for the sperm-derived Thr286-*P* CaMKIIα (data not shown).

Recent studies have shown that, during capacitation, lipid-raft-associated key proteins of the sperm exocytotic machinery e.g. SNARE proteins, accumulate at the apical tip of the sperm head, a location that reflects their functional role in acrosomal secretion (Gadella et al., 2008; Nixon et al., 2009). Since raft association upon capacitation might thus correlate with a physiological function of a molecule in acrosomal exocytosis, raft targeting of CaMKIIα was assessed in capacitated spermatozoa. Fig. 2D (left panel) shows that capacitation of rat sperm did not reduce association of CaMKIIα with the detergent-insoluble pellet. Correspondingly, the major portion of Thr286-*P* CaMKIIα was raft-associated in fully capacitated spermatozoa (Fig. 2D, right panel), although the non-raft membrane sample of capacitated sperm also showed minor immunoreactivity.

Epididymal spermatozoa already exhibit Thr286-*P* CaMKIIα

So far, our results illustrate that the α-isoform of CaMKII exhibits the same subcellular expression pattern in mouse and rat spermatozoa and additionally indicate that a Thr286-*P* and thus autonomously active conformation of CaMKIIα (Hudmon and Schulman, 2002b) already exists in non-capacitated epididymal sperm. The complex transformation process of sperm capacitation is characterized by numerous phosphorylation reactions, and an increase in cytosolic Ca^{2+} (Breitbart and Naor, 1999; Tulsiani et al., 2007; Witte and Schafer-Somi, 2007). Since both of these reactions are necessary for CaMKII activation (Hunter and Schulman, 2005; Yamauchi, 2005), the question arises as to whether the proportion of phosphorylated CaMKII increases during the process of capacitation. To address this question systematically, Thr286-*P* CaMKIIα was determined in rat epididymal spermatozoa isolated in HS buffer devoid of capacitation-inducing $NaHCO_3$-BSA (Fig. 3A, left panel) as well as in HS buffer supplemented with $NaHCO_3$ and BSA (Fig. 3A, right panel). In addition Ca^{2+}-calmodulin (Ca^{2+}/CaM)-inducible phosphorylation of CaMKIIα (Hudmon and Schulman, 2002b; Yang and Schulman, 1999) was assessed for non-capacitated and in vitro capacitated sperm. To test whether the total amount of CaMKIIα changed upon capacitation, the anti-CaMKIIα antibody, which was successfully applied for western blot analysis (Fig. 1A) was used (Fig. 3C). Representative immunoblots shown in Fig. 3A confirm that Thr286-*P* CaMKIIα is already present in freshly isolated uncapacitated rat spermatozoa (Fig. 3A, left panel). Furthermore, we observed that neither capacitation nor application of Ca^{2+}-calmodulin caused a significant increase in the proportion of Thr286-*P* kinase in rat germ cells (Fig. 3A, right panel). To further support the notion that CaMKIIα is already in an active conformational state before capacitation, mouse spermatozoa were also examined for CaMKIIα phosphorylation. Freshly isolated epididymal mouse sperm also contained a phosphorylated and catalytically active state of CaMKIIα whose amount did not increase upon Ca^{2+}-calmodulin incubation (data not shown), indicating that in epididymal uncapacitated spermatozoa of both rodent species, CaMKIIα is already in an autonomous activation state.

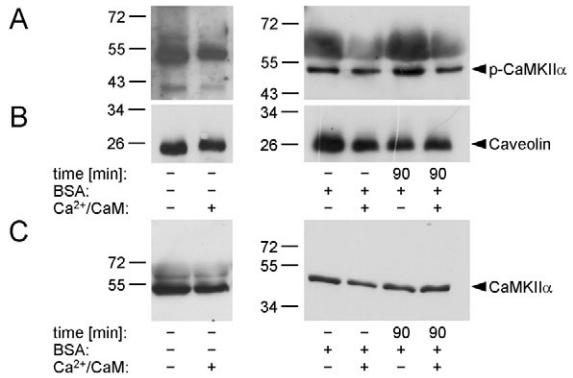

Fig. 3. CaMKIIα phosphorylation in rat spermatozoa is not increased upon sperm capacitation. Freshly isolated lysates of epididymal rat spermatozoa or capacitated sperm cells were incubated for 15 minutes at 30°C in HS buffer supplemented with Ca^{2+}-calmodulin (Ca^{2+}/CaM), and probed for CaMKIIα phosphorylation (A, right panel, p-CaMKIIα). In addition, phosphorylated CaMKIIα was detected in epididymal spermatozoa harvested in HS solution (A, left panel). Note that neither 90 minutes in vitro capacitation (90 min) nor the addition of Ca^{2+}-calmodulin (Ca^{2+}/CaM) significantly increased the level of Thr286-*P* CaMKIIα in sperm preparations. Equivalent protein loading was verified by staining the lower part of the nitrocellulose membrane with an antibody against caveolin-1 (B). To verify that the total amount of CaMKIIα did not change upon capacitation, the bulk of CaMKIIα was determined using the specified anti-CaMKIIα antibody that recognizes the association domain of the kinase (C, CaMKIIα). Arrowheads indicate the molecular mass of the protein detected by the primary antibody; diffuse staining at about 65 kDa is due to the addition of BSA during incubation. Representative results of at least three experiments with independent tissue preparations are shown. Positions of the molecular mass (in kDa) of standard proteins of each immunoblot are indicated on the left.

CaMKII inhibition increases spontaneous acrosomal secretion rates

Since the acrosomal location (Fig. 2A,B) and targeting of CaMKIIα to detergent-resistant membrane microdomains upon capacitation (Fig. 2D) suggest that CaMKIIα is involved in acrosomal exocytosis (Gadella et al., 2008; Nixon et al., 2009), initial experiments were created examining the effect of the anti-CaMKIIα antibody recognizing the C-terminal association domain on acrosomal secretion. Furthermore, the effect of commonly used membrane-permeable CaMKII inhibitors (KN62, KN93), which prevent Ca^{2+}-calmodulin binding and thereby activation (Hudmon and Schulman, 2002a; Hunter and Schulman, 2005), was assessed. However, neither the anti-CaMKIIα antibody, nor the KN drugs KN62 or KN93 (data not shown) significantly affected the rate of acrosomal secretion. In addition, the CaMKII inhibitor autocamtide-2 inhibitory peptide II (AIPII) was used, because capacitated mouse sperm already contain a Thr286-*P* CaMKII holoenzyme (data not shown), so that KN drugs are unable to competitively interfere with the calmodulin-binding site, and the antibody-recognition region might be masked upon oligomerization of the association region during kinase activation (Rosenberg et al., 2006). AIPII mimics the autoinhibitory region within the regulatory region of CaMKII and thus competitively binds and inhibits the catalytic domain of CaMKII (Hudmon and Schulman, 2002a; Ishida et al., 1998; Malinow et al., 1989). Fig. 4A summarizes the quantified effect of different concentrations of AIPII (Gardner et al., 2007; Hojjati et al., 2007) on spontaneous acrosomal exocytosis and the rate of acrosomal secretion induced by the Ca^{2+} ionophore A23187. Maintenance of CaMKII in a catalytically inactive state did not affect Ca^{2+}-induced

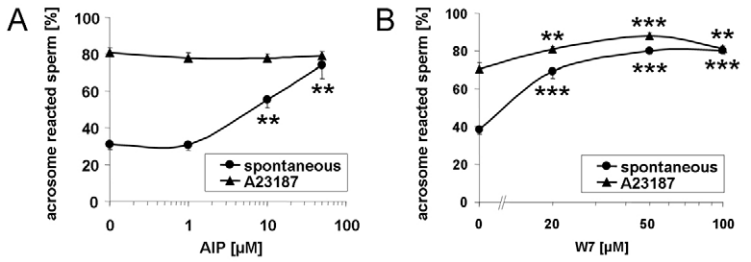

Fig. 4. CaMKII inhibition is enhancing spontaneous rate of acrosomal exocytosis in mouse spermatozoa. To examine the functional role of CaMKII during acrosomal secretion, in vitro capacitated and non-permeabilized mouse sperm were pre-treated with different concentrations of the membrane-permeable CaMKII peptide inhibitor AIPII (A) or the calmodulin antagonist W7 (B). Note that W7 increased both the spontaneous rate of acrosomal secretion (spontaneous) and acrosomal secretion elicited by the Ca^{2+} ionophore A23187 in a concentration-dependent manner (B), whereas for AIPII, potentiation was only detectable for the agonist-independent secretion rates (A). Data represent the mean values ± s.e.m. of 7 (A) or 14 (B) independent experiments of different mouse sperm preparations. $^{**}P<0.01$; $^{***}P<0.001$, for samples incubated under control conditions or treated with A23187.

acrosomal secretion. However, the spontaneous rate of acrosomal exocytosis was enhanced in a dose-dependent manner. A similar observation was made after pre-incubating capacitated mouse spermatozoa with different concentrations of the calmodulin antagonist W7 (Fan et al., 2003; Hidaka et al., 1981; Si and Olds-Clarke, 2000): Fig. 4B shows that W7 dose-dependently triggered spontaneous acrosomal secretion. However, in contrast to AIPII (Fig. 4A) the calmodulin antagonist also significantly enhanced acrosomal exocytosis induced by the Ca^{2+} ionophore, suggesting that calmodulin modulates more than one reaction event during the sequential process of acrosomal exocytosis (Mayorga et al., 2007).

Testicular CaMKIIα binds to PDZ domains 10-11 of MUPP1

In synapses of hippocampal neurons, CaMKII and the GTPase-activating protein SynGAP are brought in close proximity by direct physical interaction with the PDZ domains of MUPP1 (Krapivinsky et al., 2004). The observation of a colocalization of CaMKIIα and MUPP1 in the acrosomal region of rodent spermatozoa (Fig. 2A), together with the finding of co-migration of the two proteins in sperm-derived detergent-resistant membrane microdomains (Fig. 2C,D), raises the question of whether CaMKII and MUPP1 form stable protein complexes in testicular tissue. To address this, GST pull-down experiments were performed with lysates of testicular tissue or isolated spermatozoa and various GST-fused MUPP1 fragments that collectively represent the entire MUPP1-scaffolding protein (Lee et al., 2000) (Fig. 5A). Fig. 5B shows that in testicular tissue, the anti-CaMKIIα antibody, which was generated against subtype-specific sequences within the association domain of CaMKIIα, detected two α-immunoreactive bands, as described above (Fig. 1A). Examining the CaMKIIα-binding ability of MUPP1, we found that MUPP1 interacts with testicular CaMKIIα. Binding was only detectable with a GST-fusion construct comprising PDZ domains 10-11 of MUPP1 (GST M10-11) (Fig. 5B,D), whereas other MUPP1 PDZ-binding domains did not show any detectable interaction. Since CaMKII binds MUPP1 only in its Ca^{2+}-calmodulin free state in vitro (Krapivinsky et al., 2004), half of the testicular preparation used for the GST pull-down in Fig. 5B was pre-treated with Ca^{2+}-calmodulin, and subsequently, MUPP1-precipitated proteins were assayed for CaMKIIα binding. Fig. 5C documents that in the presence of Ca^{2+}/calmodulin, GST M10-11 failed to pull down CaMKIIα, suggesting that Ca^{2+}-occupied calmodulin dissociates CaMKII from MUPP1. To test whether CaMKIIα binding to MUPP1 depends on the activity of the kinase, half of the testicular tissue in Fig. 5D was pre-incubated with the CaMKII inhibitor AIPII. The results presented in Fig. 5E show that AIPII did not affect binding of CaMKIIα to GST M10-11, suggesting that recruitment of the kinase by MUPP1 does not depend on the catalytic activity of the kinase.

To examine whether CaMKIIα in spermatozoa was also associated with MUPP1, GST pull-down experiments were performed with isolated epididymal sperm cells, and subsequently, precipitates were probed for CaMKIIα binding. Fig. 5F shows that CaMKIIα also interacts with MUPP1 in isolated germ cells and that this interaction is also focused to GST M10-11.

To examine the functional significance of the observed MUPP1-CaMKIIα interaction for acrosomal secretion, the MUPP1-CaMKII complex was disrupted by pre-treating permeabilized spermatozoa with the purified MUPP1-M10-11 GST fusion protein, thereby competitively displacing CaMKIIα from GST M10-11 of MUPP1, and subsequently the spontaneous rate of acrosomal secretion was quantified. Since GST alone slightly but not significantly increases acrosomal secretion (Lopez et al., 2007) (data not shown), the specificity of CaMKIIα-MUPP1 GST M10-11 interaction was assessed in parallel approaches in which permeabilized sperm cells were pre-incubated with equal amounts of the MUPP1-M12-13 GST fusion protein. Fig. 6 shows that prevention of MUPP1-CaMKII interaction led to a comparable effect to that observed upon inhibition of CaMKII by AIPII (Fig. 4A) and blockage of calmodulin by W7 (Fig. 4B). Competitive displacement of CaMKIIα from endogenous MUPP1 led to a significant enhancement of spontaneous acrosome reaction ($P<0.01$), which was not detectable for the MUPP1-M12-13 GST fusion protein.

Discussion

The acrosome reaction, a multistage reaction process that resembles Ca^{2+}-regulated exocytosis in somatic cells, is specialized to maintain the reliable fusion of the large acrosomal vesicle by a zipper-like fusion pore formation spread out from the initial contact site with the egg glycoprotein matrix. However, the molecular mechanisms governing precisely timed, Ca^{2+}-triggered synchronous membrane fusion, has only been partially defined. The results of the present study provide evidence that the CaMKIIα isozyme forms a protein complex with the multi-PDZ domain protein MUPP1 in the acrosomal region of rodent spermatozoa and that this complex is involved in preventing 'accidental' agonist-independent acrosomal secretion.

The subcellular localization of CaMKIIα in the flagellum (Fig. 2B) and the head of spermatozoa (Fig. 2A,B) is consistent with a functional role of Ca^{2+}-calmodulin kinase II in sperm motility and/or acrosomal secretion. The results of the present work demonstrate that autonomously active CaMKIIα negatively regulates acrosomal

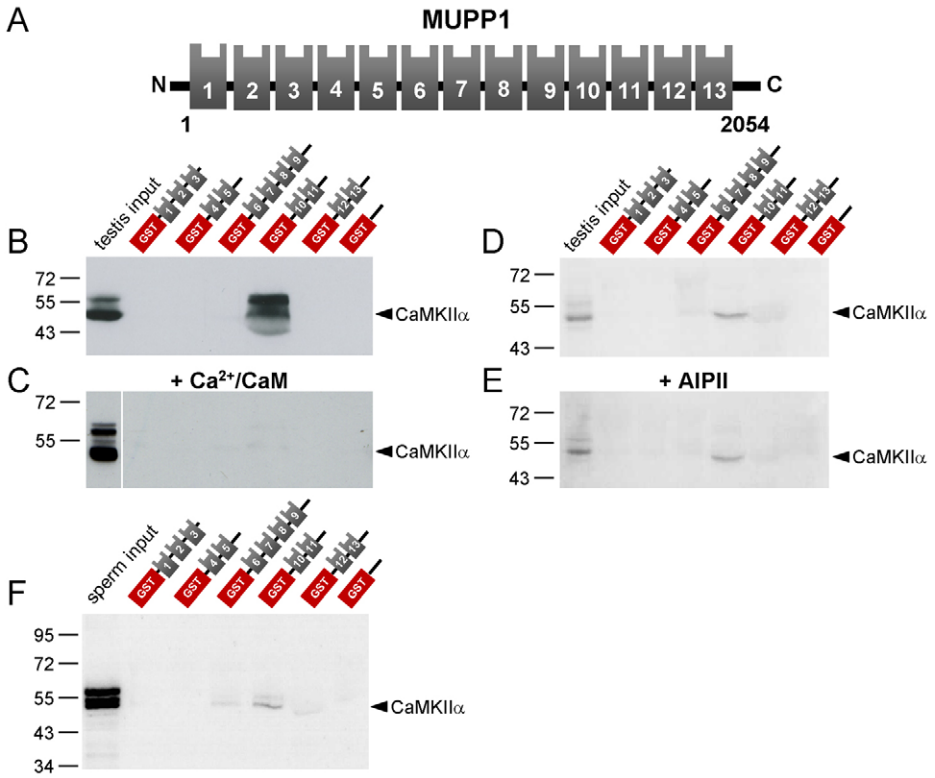

Fig. 5. CaMKIIα interacts with PDZ10-11 of MUPP1 in rat testicular tissue and isolated spermatozoa. To assess whether CaMKIIα interacts with MUPP1, and whether its binding depends on Ca^{2+}-calmodulin or the activation state of the CaMKII, GST pull-down experiments were performed with purified and immobilized MUPP1-GST fusion proteins (GST-M1-3, GST-M4-5, GST-M6-9, GST-M10-11 and GST-M12-13) (schematic diagram, A), GST alone (GST) and rat testis tissue (B-E) or isolated rat epididymal spermatozoa (F). Equivalent loading of the glutathione-Sepharose 4B beads with the purified fusion protein fragments was verified by staining the nitrocellulose sheets with Ponceau S before antibody binding; in addition, blotted membranes that were already developed with the anti-CaMKIIα antibody were stripped and re-probed with an anti-GST antibody. Note that among the 13 PDZ domains, MUPP1-CaMKIIα interaction was only detectable for the PDZ10-11 in testicular tissue (B,D) as well as in spermatozoa (F). However, if tissue preparations were pre-treated with Ca^{2+}-calmodulin (Ca^{2+}/CaM), MUPP1 failed to interact with the kinase (C). By contrast, GST pull-down experiments using lysates prepared in the presence of the CaMKII inhibitor AIPII (E) revealed no obvious discrepancy regarding CaMKIIα-MUPP1 interaction compared with the corresponding control (D). GST pull-down experiments were repeated with at least three independent rat sperm preparations, which show very similar results. The molecular mass (in kDa) of standard proteins is indicated on the left of each panel.

exocytosis in spermatozoa (Fig. 4). The finding that CaMKII prevents ZP-independent acrosomal exocytosis, and the fact that the kinase is already active in uncapacitated epididymal spermatozoa (Fig. 2B, Fig. 3) might explain the described relatively low rate of spontaneous loss of the acrosomal vesicle (Wassarman and Litscher, 2008). In presynaptic terminals, CaMKII is known to regulate synaptic transmission (Benfenati et al., 1993; Chi et al., 2003; Leal-Ortiz et al., 2008; Schiebler et al., 1986) by phosphorylating serine or threonine residues of target proteins, thereby modulating the function of these proteins (Wayman et al., 2008). An inhibitory effect of CaMKII on vesicle fusion is postulated to be mainly realized by the activation of large-conductance Ca^{2+}-activated K^+ channels [big potassium (BK) channels] (Wang, 2008), which represent negative key regulators of synaptic transmission (Liu et al., 2007). Although different types of K^+ channels have been found to be expressed in mammalian spermatozoa (Darszon et al., 2006; Navarro et al., 2008), including BK channels encoded by the Slo gene family (Martinez-Lopez et al., 2009; Navarro et al., 2007; Schreiber et al., 1998), further experiments are needed to investigate whether K^+ channels in germ cells are actually phosphorylated by CaMKII, and whether such covalent modification has any in vivo relevance for acrosomal exocytosis.

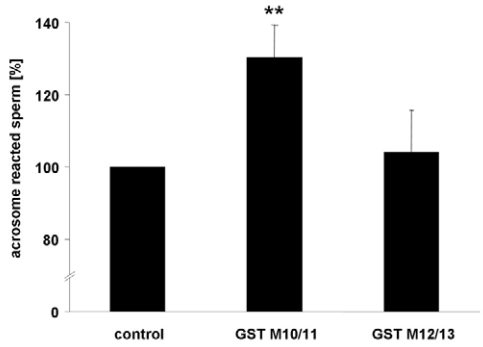

Fig. 6. Inhibition of CaMKIIα-MUPP1 interaction enhances spontaneous rate of acrosomal secretion in mouse sperm cells. Isolated and capacitated mouse sperm were permeabilized with the bacterial toxin Streptolysin O, treated for 20 minutes at 37°C with 0.5 μM of different purified MUPP1 PDZ regions fused to GST (GST M10/11, GST M12/13) and subsequently, acrosome-reacted sperm were quantified. Note that the competitive displacement of CaMKIIα from MUPP1 by a pre-incubation with the MUPP1-GST M10-11 significantly enhanced spontaneous loss of the acrosomal vesicle, whereas MUPP1-GST M12-13 had no effect. Data represent the mean values ± s.e.m. of 5 (GST M12-13) or 12 (GST M10-11) independent experiments with different mouse sperm preparations *P<0.01, compared with control. Actual percentages of acrosome reacted sperm for spontaneous acrosome reaction was 27.15±3.60.

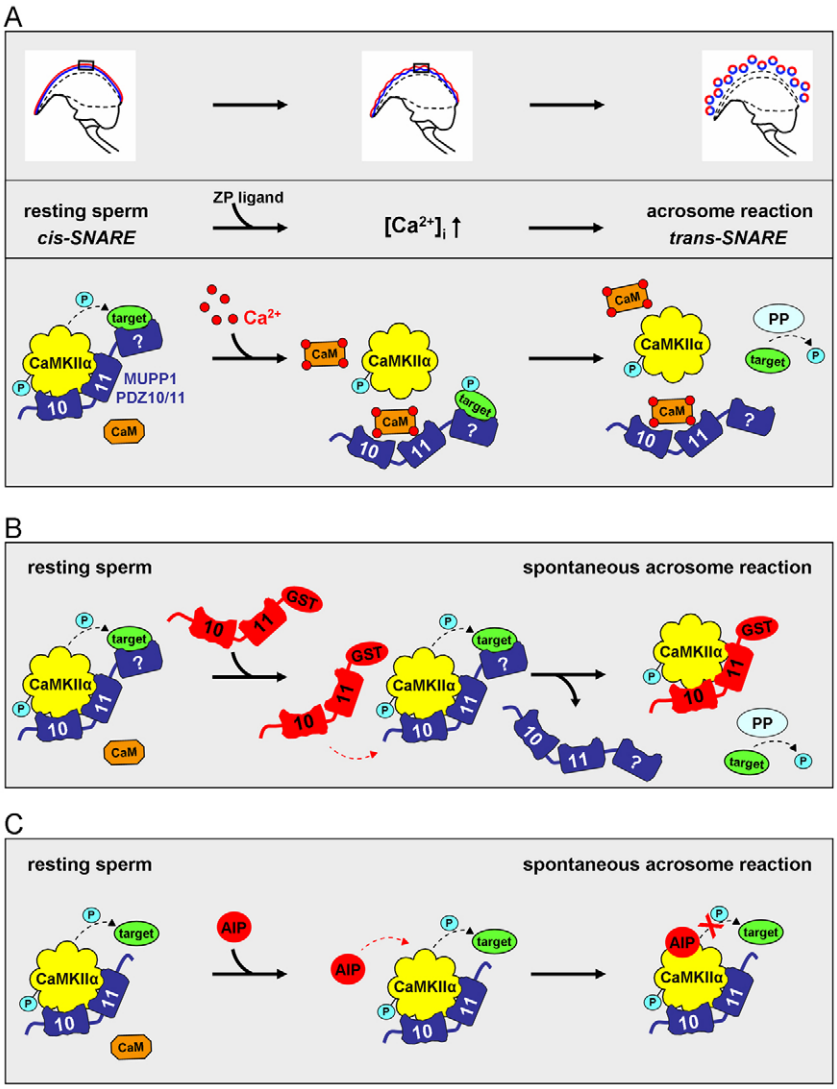

Fig. 7. Working model illustrating a possible functional role of the MUPP1-CaMKIIα complex in preventing spontaneous acrosome reaction. The model juxtaposes the morphological changes in the sperm acrosome region (A, upper panel), the sequence of events during acrosome reaction (A, middle panel) and the changes in the interaction of MUPP1 and CaMKIIα during the sequential fusion process (A, lower panel). In addition, a possible mode of action of a recombinant GST M10-11 fusion protein (B), and the CaMKII inhibitor autocamtide-2 inhibitory peptide II (AIP) (C) on acrosomal secretion are presented. Upon sperm-egg interaction, zona pellucida ligand(s) (ZP-ligand) are bound to receptors on the head of capacitated spermatozoa, thereby inducing a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ ↑), which triggers acrosomal secretion (A, upper and middle panels). In resting sperm in which SNARE-complexes are in a pre-assembled *cis*-configuration (A, middle panel), CaMKIIα is bound to GST M10-11, which localizes CaMKIIα in close proximity to its phosphorylation substrate(s) (target) (A, lower left panel). Whether these targets are also associated with PDZ domains of MUPP1 is not clear (indicated by '?'). This recruitment of the kinase by MUPP1 might enable well-directed phosphorylation and thus prevent an unintended spontaneous acrosome reaction. If MUPP1-CaMKIIα complex formation is inhibited by competitively displacing endogenous MUPP1 by a GST M10-11 fusion protein (red), recruitment of the kinase substrate(s) is prevented (B). The same suppression of phosphorylation of CaMKIIα (s) might be achieved by directly blocking the catalytic activity of the kinase by the peptide-derived CaMKII inhibitor AIPII (AIP) (C). The necessary dissociation of the CaMKIIα-MUPP1 complex and thus 'unfreezing' of the fusion clamp is elicited by Ca^{2+}-occupied calmodulin, which is formed upon the ZP-induced rise in cytosolic Ca^{2+} (A). Whether Ca^{2+}-calmodulin binds to MUPP1 and/or the CaMKIIα is not yet known. The release of CaMKIIα might lead to the delivery of recruited kinase target substrate(s), which now becomes accessible for phosphatases (PP). The catalyzed dephosphorlyation of CaMKII substrates mighty subsequently pave the way for the final steps of fusion pore building and formation of hybrid vesicles at multiple sites of the membrane (A, bottom panel).

An indispensable prerequisite for ultimate membrane fusion is the release of the CaMKIIα-controlled clamp by an increase in free Ca^{2+}. In this context, it is worth mentioning that Ca^{2+}-calmodulin has been found to dissociate CaMKIIα from MUPP1 (Krapivinsky et al., 2004) (Fig. 5C), whereas inhibition of the catalytic activity of the kinase (Fig. 5E) does not induce kinase release (Fig. 7). In sperm, the Ca^{2+} controlled 'unfreezing' of the MUPP1-CaMKII fusion clamp might either be triggered by the ZP3-induced initial fast and transient increase in Ca^{2+} that is mediated by voltage-gated Ca^{2+} channels (Arnoult et al., 1999), or by the second sustained efflux of Ca^{2+} from the acrosomal Ca^{2+} store, which is necessary to elicit ultimate fusion pore formation (De Blas et al., 2005; Herrick et al., 2005). Therefore, we suggest that CaMKIIα modulates proteins participating in distinct stages of the sequential Ca^{2+}-controlled secretion pathway. On the one hand, it is conceivable that CaMKIIα inactivates proteins of intracellular transduction cascade/s stimulated upon ZP binding (Florman et al., 2008), thereby stabilizing the inactive *cis*-SNARE-complex in capacitated resting sperm (Zarelli et al., 2009). On the other hand, it is also feasible that target substrates of CaMKIIα are operative in triggering the final fusion step(s) of acrosomal exocytosis, such as the inositol-1,4,5-trisphosphate receptor (Aromolaran et al., 2007; Bare et al., 2005), or the t-SNARE syntaxin (Ohyama et al., 2002). However, the complexin- or synaptotagmin-mediated fusion clamp of the primed SNARE fusion machinery (Sudhof and Rothman, 2009) might also be a promising CaMKII-controlled 'interlocking' target. Complexin is expressed in mammalian spermatozoa (Redecker et al., 2003; Roggero et al., 2007; Zhao et al., 2007); furthermore, synaptotagmin is known to present a target substrate for CaMKII phosphorylation (Abbott and Ducibella, 2001; Hilfiker et al., 1999). Thus, it will be important for future studies to investigate whether CaMKIIα phosphorylates and thus inactivates fusion components involved in Rab3A-elicited re-assembly of SNAREs leading to the primed membrane-bridging *trans*-SNARE complex (De Blas et al., 2005) (Zarelli et al., 2009), and/or whether target substrate(s) of CaMKII are involved in blocking ultimate fusion pore formation by either inhibiting the release of Ca^{2+} from the acrosome and/or by blocking the subsequent synaptotagmin-mediated release of the complexin interlock (Fig. 7).

An additional observation of the present work is that CaMKII is colocalized with MUPP1 in Triton-X-100-insoluble membrane fractions (Fig. 2C,D), and that direct interaction with a region consisting of the PDZ10-11 is necessary for the ability of CaMKII to prevent agonist-independent loss of the acrosomal vesicle (Figs

5, 6). Thus, MUPP1 might persistently organize activated CaMKII and possibly also its target substrate(s) in close proximity to a defined space between the outer acrosomal membrane and the plasma membrane (Fig. 7); a recruitment that might be further facilitated by lipid raft association (Fig. 2). The observation that lipid raft association and correct subcellular targeting are required for an effective and specific substrate phosphorylation of CaMKII has already been made (Strack et al., 1997; Tsui et al., 2005). Furthermore, binding to scaffolding proteins was found to prevent dephosphorylation and thus inactivation of CaMKII (Mullasseril et al., 2007). Although we do not know yet whether MUPP1 avoids kinase dephosphorlyation and whether CaMKIIα substrates also bind to MUPP1 (Fig. 7), the observation of a Ca^{2+}-controlled assembly and disassembly of a MUPP1-CaMKIIα complex, in conjunction with the 13 binding motifs of MUPP1, offers an intriguing model of how MUPP1 and possible other interaction partner(s) (Fig. 7) might translate an increase in Ca^{2+} into final fusion pore building. This regulatory mode of action is of utmost importance for spermatozoa that only have a single opportunity to release their acrosomal contents. In this context, MUPP1 might be involved in passing a Ca^{2+}-occupied calmodulin along the acrosomal reaction cascade. It has been suggested that calmodulin controls more than just a single step of the sequential membrane fusion process of the acrosome reaction, ranging from stimulation of T-type Ca^{2+} channels (Bendahmane et al., 2001; Lopez-Gonzalez et al., 2001) to inhibition of the final fusion event (Yunes et al., 2002). This hypothesis of multiple sites of action of calmodulin is extended by the present work demonstrating that the calmodulin antagonist W7 also increases spontaneous acrosomal secretion (Fig. 4B). We currently do not know whether Ca^{2+}-occupied calmodulin mediates the observed 'unlocking' of the fusion clamp by associating with either CaMKIIα or MUPP1 (Fig. 7). Nevertheless one might speculate that Ca^{2+}-calmodulin affects several consecutive steps of the acrosome reaction, with MUPP1 guiding it from the initial recruitment and assembly of SNARE proteins, to the regulation of Ca^{2+} channels, to the fusion block and its subsequent release, up to final fusion pore formation. However, whether MUPP1 also interacts with itself by direct PDZ-PDZ binding (Harris and Lim, 2001), thereby forming a mechanically coupled scaffolding network below the plasma membrane, and whether such a mechanism is responsible for synchronized fusion pore building at hundreds of distinct loci in the sperm membrane (Barros et al., 1967) has to be explored in future studies.

Materials and Methods

Antibodies and reagents

Male adult mice and rats were raised at the Philipps-University, Marburg, or purchased from Charles River (Sulzfeld, Germany). To identify the α- and β-isoforms of CaMKII, as well as phosphorylated CaMKIIα, the following antibodies were used: for the CaMKIIα isoform, an anti-mouse IgG generated against the C-terminal region of rat CaMKIIα (residues 448-460) (BD Bioscience, Heidelberg, Germany) as well as an equivalent polyclonal (Santa Cruz, Biotechnology, Heidelberg) and a monoclonal anti-CaMKIIα IgG (Santa Cruz Biotechnology) were used. To identify the CaMKIIβ isozyme, a corresponding antibody also raised against the subunit-specific C-terminal association domain was used (Santa Cruz Biotechnology). For the detection of phosphorylated CaMKIIα, a mouse monoclonal antibody generated against a peptide surrounding the Thr286-*P* of rat origin (Santa Cruz Biotechnology) and a rabbit polyclonal antibody matching Thr286-*P* of mouse, rat and human CaMKIIα (Abcam, Cambridge, UK) (Pezet et al., 2008) were used.

The sources of other applied antibodies and chemicals are as follows: anti-caveolin1 IgG and donkey anti-goat horseradish peroxidase (HRP)-labeled IgG were obtained from Santa Cruz Biotechnology. HRP-conjugated goat anti-rabbit IgG was obtained from Bio-Rad (Munich, Germany); HRP-conjugated goat anti-mouse IgG was purchased from Amersham-Pharmacia (Freiburg, Germany). A biotinylated anti-mouse IgG as well as fluorescein avidin were purchased from Vector Laboratories (Burlingame, CA); Alexa-Fluor-546-conjugated goat anti-rabbit antibody was from Invitrogen (Karlsruhe, Germany). Anti-MUPP1 antibody was generated against a protein region between PDZ domains 5 and 6 of MUPP1 (Ackermann et al., 2008). The KN-CaMKII inhibitors 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (KN93), as well as its inactive analogue 2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine phosphate (KN92) (Marley and Thomson, 1996), and 1-[*N*,*O*-bis-(5-Isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) (Tokumitsu et al., 1990), which competitively interact with the calmodulin-binding site of CaMKII (Sumi et al., 1991) were from Calbiochem-Novabiochem (Bad Soden, Germany); likewise, the non-cell-permeable non-phosphorylatable CaMKII-peptide-substrate inhibitor autocamtide-2 inhibitory peptide II (AIPII) was derived from the autoinhibitory region of CaMKII (Ishida et al., 1995; Ishida et al., 1998) and its cell-permeable analogue fused to the antennapedia transport peptide sequence (Passafaro et al., 1999), as well as the CaM antagonist W7 [8 (*N*-86-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (Hidaka et al., 1981), the phosphatase inhibitor okadaic acid (Cohen et al., 1990) and a protease inhibitor cocktail III. Recombinant calmodulin and ATP were from BioLabs (P6060S) (Hohenwestedt, Germany); the chemiluminescent substrates West Femto and West Pico ECL were from Thermo Scientific (Schwerte, Germany). The glutathione *S*-transferase (GST) fusion protein MUPP1-expression plasmids (pGEX-2T or pGEX-4T-1) presenting distinct MUPP1-PDZ domains [M1-3 (residues 118-504), M4-5 (residues 489-785), M6-9 (residues 780-1611); M10-11 (residues 1606-1830), M12-13 (residues 1825 to 2054)] were a gift from Ronald T. Javier (Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX), and have been described elsewhere (Barritt et al., 2000; Lee et al., 2000). The pGEX-2T plasmid expressing GST alone was provided by Michael Bölker (Department of Genetics, Faculty of Biology, Philipps-University, Marburg, Germany). All other reagents, unless specified otherwise, were either purchased from Sigma (Deisenhofen, Germany) or Carl Roth (Karlsruhe, Germany).

Sperm preparation, capacitation and immunocytochemistry

Adult mouse and rat spermatozoa were isolated as described previously (Heydecke et al., 2006). To obtain spermatozoa of different capacitation status, motile sperm of cauda epididymis were collected either in HS working solution (30 mM HEPES, 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM lactic acid and 1 mM pyruvic acid, adjusted to pH 7.4 with NaOH) or in capacitating buffer (HS, 0.5% BSA, 15 mM $NaHCO_3$) and incubated for 90 minutes at 37°C in 5% CO_2 (Ackermann et al., 2008). Indirect immunocytochemical approaches were performed as described (Fehr et al., 2007); control samples were incubated with 10% FCS in PBS. Double-fluorescence immunocytochemical staining of MUPP1 and CaMKIIα was performed by incubating samples of mouse spermatozoa for 48 hours with the mouse anti-CaMKIIα antibody recognizing the C-terminal association region and the rabbit anti-MUPP1 antibody (Ackermann et al., 2008). Subsequently excess of antibody was removed by three washes with PBS, and samples were incubated for 1 hour at room temperature with the biotinylated anti-mouse IgG. After three additional washes, a mixture of the Fluorescein-crosslinked Avidin and the Alexa-Fluor-546-conjugated anti-rabbit IgG was applied (1 hour at room temperature). After three additional washes in PBS, samples were coated with fluorescent mounting medium (DAKO, Cytomation, Hamburg, Germany) and examined under a Zeiss LSM 510 META laser scanning confocal microscope. The results of the double-staining experiments showed staining patterns and staining intensities similar to those obtained in performed single staining experiments.

Recombinant protein expression, purification and GST pull-down assay

GST fusion proteins (GST, GST-M1-3, GST-M4-5, GST-M6-9, GST-M10-11, GST-M12-13) (Lee et al., 2000) were obtained from isopropyl 1-thio-β-D-galactopyranoside (IPTG)-induced bacterial cultures and purified through affinity chromatography using glutathione-Sepharose 4B beads (Amersham-Pharmacia, Freiburg, Germany) according to the manufacturer's instructions. Purity of the eluates was verified by staining aliquot samples by Coomassie Brilliant Blue dye stained SDS-PAGE; protein concentration of pooled fractions was determined by Bradford (Bradford, 1976).

For GST pull-down assays, MUPP1-GST fusion-protein-coated glutathione-Sepharose 4B beads were used as matrix for binding of CaMKIIα prepared from rat testis or isolated spermatozoa, respectively. To this aim, each of the five purified MUPP1-GST fusion proteins (75 μg) covering the whole MUPP1 protein or GST alone were incubated overnight at 4°C with PBS-equilibrated glutathione-Sepharose beads (60 μl). Thereafter, the coated GST-glutathione beads were washed with PBS, and subsequently, the unspecific binding sites were blocked by incubating the beads for 1 hour at 4°C with 10% BSA in PBS. At the same time, tissue preparations were either sonicated in homogenization buffer containing 10 mM Tris-HCl, 3 mM $MgCl_2$, 2 mM EDTA, pH 7.4, protease inhibitor cocktail (testis) or in 50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, 0.5% NP40, pH 8.0, protease inhibitor cocktail, 0.1 mM phenylmethanesulfonyl fluoride solution (sperm), solubilized by stirring (30 minutes at 4°C) and centrifuged for 10 minutes at 1000 ***g*** to separate cell nuclei and intact organelles. The supernatants (S_1) were subsequently cleaned from cell debris

(10,000 ***g***, 10 minutes, S_2) and incubated overnight at 4°C under constant agitation on a rotary shaker together with fusion protein-loaded Sepharose beads. After extensive washing of the beads with PBS, samples were boiled in loading buffer (Laemmli, 1970) and subjected to SDS-PAGE and immunoblotting. To analyze the effect of Ca^{2+} and calmodulin on MUPP1-CaMKIIα interaction, one half of a freshly prepared S_2 fraction of testicular tissue was incubated for 10 minutes at 30°C with 2 mM $CaCl_2$, 1.2 μM calmodulin and 100 μM ATP (Yang and Schulman, 1999). To examine whether the CaMKIIα-MUPP1 interaction was activation dependent, one half of the testis tissue preparation was homogenized and solubilized in buffer supplemented with 50 μM non-cell permeable CaMKIIα inhibitor AIPII.

Acrosome reaction in intact and permeabilized spermatozoa

To induce acrosome reaction in unpermeabilized capacitated mouse spermatozoa, the Ca^{2+} ionophore A23187 was used to raise the intracellular Ca^{2+} concentration, as described recently (Heydecke et al., 2006). To elicit acrosome reaction in streptolysin-O (SLO)-permeabilized cells, the extracellular Ca^{2+} concentration was increased as described (Ackermann et al., 2008). Acrosome-reacted sperm were determined using Coomassie Brilliant Blue staining (Zeginiadou et al., 2000); for each experiment, at least 100 spermatozoa of each slide with blinded labels were scored using a Zeiss microscope (Axiovert 200M) or an Olympus microscope (CKX 31) equipped with bright-field light optics. Decoded samples were subsequently identified, and the percentage of spermatozoa with intact acrosome was calculated. To determine the functional role of CaMKII in the acrosome reaction, the effect of anti-CaMKIIα antibodies, a GST fusion protein containing the PDZ domains 10 and 11 of MUPP1 (GST M10-11, residues 1606-1830), one containing PDZ domains 12 and 13 (GST M12-13, residues 1825-2054) or GST alone, as well as a battery of cell-permeable protein kinase antagonists (KN93/KN92, KN62, AIPII, W7) were used. Stock solutions (10 mM) of KN92, KN93 and KN62 were prepared in DMSO and kept in the dark at –20°C until use; for each experiment, a control sample was included which was supplemented with DMSO concentrations equivalent to the maximum concentration of the applied inhibitor; final concentrations of DMSO never exceeded 0.01%. Non-membrane-permeable compounds or the anti-CaMKII antibodies were directly added after sperm permeabilization. After incubation for 20 minutes at 37°C, the acrosome reaction was induced and thereafter stopped by washing the germ cells. Spermatozoa were then fixed and their acrosomal status evaluated as described above. The spontaneous loss of acrosome was assessed by scoring the acrosomal status of sperm samples exposed only to the DMSO or SLO solution. In experiments in which sperm were pre-treated with SLO, experiments were included only when the rate of acrosome reaction induced by the addition of extracellular Ca^{2+} was higher than 5%, thus reflecting a successful permeabilization.

Statistical analysis

Values are presented as means ± s.e.m. of independent experiments with isolated sperm from different animals. Statistical analyses were performed using a paired Student's *t*-test. Levels of statistical significance were indicated by asterisks: $*P<0.05$; $**P<0.01$; $***P<0.001$.

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References

Abbott, A. L. and Ducibella, T. (2001). Calcium and the control of mammalian cortical granule exocytosis. *Front Biosci.* **6**, D792-D806.

Ackermann, F., Zitranski, N., Heydecke, D., Wilhelm, B., Gudermann, T. and Boekhoff, I. (2008). The Multi-PDZ domain protein MUPP1 as a lipid raft-associated scaffolding protein controlling the acrosome reaction in mammalian spermatozoa. *J. Cell Physiol.* **214**, 757-768.

Anderson, R. G. (1998). The caveolae membrane system. *Annu. Rev. Biochem.* **67**, 199-225.

Arnoult, C., Kazam, I. G., Visconti, P. E., Kopf, G. S., Villaz, M. and Florman, H. M. (1999). Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *Proc. Natl. Acad. Sci. USA* **96**, 6757-6762.

Aromolaran, A. S., Zima, A. V. and Blatter, L. A. (2007). Role of glycolytically generated ATP for CaMKII-mediated regulation of intracellular Ca^{2+} signaling in bovine vascular endothelial cells. *Am. J. Physiol. Cell Physiol.* **293**, C106-C118.

Bare, D. J., Kettlun, C. S., Liang, M., Bers, D. M. and Mignery, G. A. (2005). Cardiac type 2 inositol 1,4,5-trisphosphate receptor: interaction and modulation by calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **280**, 15912-15920.

Barritt, D. S., Pearn, M. T., Zisch, A. H., Lee, S. S., Javier, R. T., Pasquale, E. B. and Stallcup, W. B. (2000). The multi-PDZ domain protein MUPP1 is a cytoplasmic ligand for the membrane-spanning proteoglycan NG2. *J. Cell Biochem.* **79**, 213-224.

Barros, C., Bedford, J. M., Franklin, L. E. and Austin, C. R. (1967). Membrane vesiculation as a feature of the mammalian acrosome reaction. *J. Cell Biol.* **34**, C1-C5.

Bendahmane, M., Lynch, C., 2nd and Tulsiani, D. R. (2001). Calmodulin signals capacitation and triggers the agonist-induced acrosome reaction in mouse spermatozoa. *Arch. Biochem. Biophys.* **390**, 1-8.

Benfenati, F., Valtorta, F., Rossi, M. C., Onofri, F., Sihra, T. and Greengard, P. (1993). Interactions of synapsin I with phospholipids: possible role in synaptic vesicle clustering and in the maintenance of bilayer structures. *J. Cell Biol.* **123**, 1845-1855.

Boerke, A., Tsai, P. S., Garcia-Gil, N., Brewis, I. A. and Gadella, B. M. (2008). Capacitation-dependent reorganization of microdomains in the apical sperm head plasma membrane: functional relationship with zona binding and the zona-induced acrosome reaction. *Theriogenology* **70**, 1188-1196.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Breitbart, H. and Naor, Z. (1999). Protein kinases in mammalian sperm capacitation and the acrosome reaction. *Rev. Reprod.* **4**, 151-159.

Brose, N. (2008). For better or for worse: complexins regulate SNARE function and vesicle fusion. *Traffic* **9**, 1403-1413.

Chapman, E. R. (2008). How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem.* **77**, 615-641.

Chi, P., Greengard, P. and Ryan, T. A. (2003). Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. *Neuron* **38**, 69-78.

Cohen, P., Holmes, C. F. and Tsukitani, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**, 98-102.

Darszon, A., Acevedo, J. J., Galindo, B. E., Hernandez-Gonzalez, E. O., Nishigaki, T., Trevino, C. L., Wood, C. and Beltran, C. (2006). Sperm channel diversity and functional multiplicity. *Reproduction* **131**, 977-988.

Darszon, A., Nishigaki, T., Wood, C., Trevino, C. L., Felix, R. and Beltran, C. (2005). Calcium channels and Ca^{2+} fluctuations in sperm physiology. *Int. Rev. Cytol.* **243**, 79-172.

De Blas, G. A., Roggero, C. M., Tomes, C. N. and Mayorga, L. S. (2005). Dynamics of SNARE assembly and disassembly during sperm acrosomal exocytosis. *PLoS Biol.* **3**, e323.

Du, F., Saitoh, F., Tian, Q. B., Miyazawa, S., Endo, S. and Suzuki, T. (2006). Mechanisms for association of Ca^{2+}/calmodulin-dependent protein kinase II with lipid rafts. *Biochem. Biophys. Res. Commun.* **347**, 814-820.

Fan, H. Y., Huo, L. J., Meng, X. Q., Zhong, Z. S., Hou, Y., Chen, D. Y. and Sun, Q. Y. (2003). Involvement of calcium/calmodulin-dependent protein kinase II (CaMKII) in meiotic maturation and activation of pig oocytes. *Biol. Reprod.* **69**, 1552-1564.

Fehr, J., Meyer, D., Widmayer, P., Borth, H. C., Ackermann, F., Wilhelm, B., Gudermann, T. and Boekhoff, I. (2007). Expression of the G-protein alpha-subunit gustducin in mammalian spermatozoa. *J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol.* **193**, 21-34.

Fejtova, A. and Gundelfinger, E. D. (2006). Molecular organization and assembly of the presynaptic active zone of neurotransmitter release. *Results Probl. Cell Differ.* **43**, 49-68.

Florman, H. M., Jungnickel, M. K. and Sutton, K. A. (2008). Regulating the acrosome reaction. *Int. J. Dev. Biol.* **52**, 503-510.

Gadella, B. M., Tsai, P. S., Boerke, A. and Brewis, I. A. (2008). Sperm head membrane reorganisation during capacitation. *Int. J. Dev. Biol.* **52**, 473-480.

Gardner, A. J., Knott, J. G., Jones, K. T. and Evans, J. P. (2007). CaMKII can participate in but is not sufficient for the establishment of the membrane block to polyspermy in mouse eggs. *J. Cell Physiol.* **212**, 275-280.

Gerst, J. E. (1999). SNAREs and SNARE regulators in membrane fusion and exocytosis. *Cell Mol. Life Sci.* **55**, 707-734.

Greengard, P., Valtorta, F., Czernik, A. J. and Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780-785.

Harper, C. V., Cummerson, J. A., White, M. R., Publicover, S. J. and Johnson, P. M. (2008). Dynamic resolution of acrosomal exocytosis in human sperm. *J. Cell Sci.* **121**, 2130-2135.

Harris, B. Z. and Lim, W. A. (2001). Mechanism and role of PDZ domains in signaling complex assembly. *J. Cell Sci.* **114**, 3219-3231.

Herrick, S. B., Schweissinger, D. L., Kim, S. W., Bayan, K. R., Mann, S. and Cardullo, R. A. (2005). The acrosomal vesicle of mouse sperm is a calcium store. *J. Cell Physiol.* **202**, 663-671.

Heydecke, D., Meyer, D., Ackermann, F., Wilhelm, B., Gudermann, T. and Boekhoff, I. (2006). The multi PDZ domain protein MUPP1 as a putative scaffolding protein for organizing signaling complexes in the acrosome of mammalian spermatozoa. *J. Androl.* **27**, 390-404.

Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fujii, Y. and Nagata, T. (1981). N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. USA* **78**, 4354-4357.

Hilfiker, S., Pieribone, V. A., Nordstedt, C., Greengard, P. and Czernik, A. J. (1999). Regulation of synaptotagmin I phosphorylation by multiple protein kinases. *J. Neurochem.* **73**, 921-932.

Hojjati, M. R., van Woerden, G. M., Tyler, W. J., Giese, K. P., Silva, A. J., Pozzo-Miller, L. and Elgersma, Y. (2007). Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. *Nat. Neurosci.* **10**, 1125-1127.

Hudmon, A. and Schulman, H. (2002a). Neuronal Ca^{2+}/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu. Rev. Biochem.* **71**, 473-510.

Hudmon, A. and Schulman, H. (2002b). Structure-function of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II. *Biochem. J.* **364**, 593-611.

Hunter, T. and Schulman, H. (2005). CaMKII structure-an elegant design. *Cell* **123**, 765-767.

Ikonen, E. and Simons, K. (1998). Protein and lipid sorting from the trans-Golgi network to the plasma membrane in polarized cells. *Semin. Cell Dev. Biol.* **9**, 503-509.

Ishida, A., Kameshita, I., Okuno, S., Kitani, T. and Fujisawa, H. (1995). A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem. Biophys. Res. Commun.* **212**, 806-812.

Ishida, A., Shigeri, Y., Tatsu, Y., Uegaki, K., Kameshita, I., Okuno, S., Kitani, T., Yumoto, N. and Fujisawa, H. (1998). Critical amino acid residues of AIP, a highly specific inhibitory peptide of calmodulin-dependent protein kinase II. *FEBS Lett.* **427**, 115-118.

Jahn, R. (2004). Principles of exocytosis and membrane fusion. *Ann. N. Y. Acad. Sci.* **1014**, 170-178.

Jahn, R. and Scheller, R. H. (2006). SNAREs-engines for membrane fusion. *Nat. Rev. Mol. Cell. Biol.* **7**, 631-643.

Kierszenbaum, A. L. (2000). Fusion of membranes during the acrosome reaction: a tale of two SNAREs. *Mol. Reprod. Dev.* **57**, 309-310.

Koh, T. W. and Bellen, H. J. (2003). Synaptotagmin I, a Ca^{2+} sensor for neurotransmitter release. *Trends Neurosci.* **26**, 413-422.

Krapivinsky, G., Medina, I., Krapivinsky, L., Gapon, S. and Clapham, D. E. (2004). SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. *Neuron* **43**, 563-574.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lang, T. (2007). SNARE proteins and 'membrane rafts'. *J. Physiol.* **585**, 693-698.

Lang, T. and Jahn, R. (2008). Core proteins of the secretory machinery. *Handb. Exp. Pharmacol.* pp. 107-127.

Leal-Ortiz, S., Waites, C. L., Terry-Lorenzo, R., Zamorano, P., Gundelfinger, E. D. and Garner, C. C. (2008). Piccolo modulation of Synapsin1a dynamics regulates synaptic vesicle exocytosis. *J. Cell Biol.* **181**, 831-846.

Lee, S. S., Glaunsinger, B., Mantovani, F., Banks, L. and Javier, R. T. (2000). Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. *J. Virol.* **74**, 9680-9693.

Liu, Q., Chen, B., Ge, Q. and Wang, Z. W. (2007). Presynaptic Ca^{2+}/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at Caenorhabditis elegans neuromuscular junction. *J. Neurosci.* **27**, 10404-10413.

Lopez, C. I., Belmonte, S. A., De Blas, G. A. and Mayorga, L. S. (2007). Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. *FASEB J.* **14**, 4121-4130.

Lopez-Gonzalez, I., De La Vega-Beltran, J. L., Santi, C. M., Florman, H. M., Felix, R. and Darszon, A. (2001). Calmodulin antagonists inhibit T-type Ca(2+) currents in mouse spermatogenic cells and the zona pellucida-induced sperm acrosome reaction. *Dev. Biol.* **236**, 210-219.

Malinow, R., Schulman, H. and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245**, 862-866.

Malsam, J., Kreye, S. and Sollner, T. H. (2008). Membrane fusion: SNAREs and regulation. *Cell Mol. Life Sci.* **18**, 2814-2832.

Marley, P. D. and Thomson, K. A. (1996). The Ca^{++}/calmodulin-dependent protein kinase II inhibitors KN62 and KN93, and their inactive analogues KN04 and KN92, inhibit nicotinic activation of tyrosine hydroxylase in bovine chromaffin cells. *Biochem. Biophys. Res. Commun.* **221**, 15-18.

Martinez-Lopez, P., Santi, C. M., Trevino, C. L., Ocampo-Gutierrez, A. Y., Acevedo, J. J., Alisio, A., Salkoff, L. B. and Darszon, A. (2009). Mouse sperm K+ currents stimulated by pH and cAMP possibly coded by Slo3 channels. *Biochem. Biophys. Res. Commun.* **381**, 204-209.

Mayorga, L. S., Tomes, C. N. and Belmonte, S. A. (2007). Acrosomal exocytosis, a special type of regulated secretion. *IUBMB Life* **59**, 286-292.

McMahon, H. T., Missler, M., Li, C. and Sudhof, T. C. (1995). Complexins: cytosolic proteins that regulate SNAP receptor function. *Cell* **83**, 111-119.

Michaut, M., Tomes, C. N., De Blas, G., Yunes, R. and Mayorga, L. S. (2000). Calcium-triggered acrosomal exocytosis in human spermatozoa requires the coordinated activation of Rab3A and N-ethylmaleimide-sensitive factor. *Proc. Natl. Acad. Sci. USA* **97**, 9996-10001.

Mochida, S. (2000). Protein-protein interactions in neurotransmitter release. *Neurosci. Res.* **36**, 175-182.

Mullasseril, P., Dosemeci, A., Lisman, J. E. and Griffith, L. C. (2007). A structural mechanism for maintaining the 'on-state' of the CaMKII memory switch in the post-synaptic density. *J. Neurochem.* **103**, 357-364.

Navarro, B., Kirichok, Y. and Clapham, D. E. (2007). KSper, a pH-sensitive K+ current that controls sperm membrane potential. *Proc. Natl. Acad. Sci. USA* **104**, 7688-7692.

Navarro, B., Kirichok, Y., Chung, J. J. and Clapham, D. E. (2008). Ion channels that control fertility in mammalian spermatozoa. *Int. J. Dev. Biol.* **52**, 607-613.

Nixon, B., Bielanowicz, A., McLaughlin, E. A., Tanphaichitr, N., Ensslin, M. A. and Aitken, R. J. (2009). Composition and significance of detergent resistant membranes in mouse spermatozoa. *J. Cell Physiol.* **218**, 122-134.

Ohyama, A., Hosaka, K., Komiya, Y., Akagawa, K., Yamauchi, E., Taniguchi, H., Sasagawa, N., Kumakura, K., Mochida, S., Yamauchi, T. et al. (2002). Regulation of exocytosis through Ca^{2+}/ATP-dependent binding of autophosphorylated Ca^{2+}/calmodulin-activated protein kinase II to syntaxin 1A. *J. Neurosci.* **22**, 3342-3351.

Passafaro, M., Sala, C., Niethammer, M. and Sheng, M. (1999). Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. *Nat. Neurosci.* **2**, 1063-1069.

Pezet, S., Marchand, F., D'Mello, R., Grist, J., Clark, A. K., Malcangio, M., Dickenson, A. H., Williams, R. J. and McMahon, S. B. (2008). Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. *J. Neurosci.* **28**, 4261-4270.

Publicover, S., Harper, C. V. and Barratt, C. (2007). [Ca(2+)](i) signalling in sperm-making the most of what you've got. *Nat. Cell Biol.* **9**, 235-242.

Redecker, P., Kreutz, M. R., Bockmann, J., Gundelfinger, E. D. and Boeckers, T. M. (2003). Brain synaptic junctional proteins at the acrosome of rat testicular germ cells. *J. Histochem. Cytochem.* **51**, 809-819.

Rettig, J. and Neher, E. (2002). Emerging roles of presynaptic proteins in Ca^{++}-triggered exocytosis. *Science* **298**, 781-785.

Rizo, J. and Rosenmund, C. (2008). Synaptic vesicle fusion. *Nat. Struct. Mol. Biol.* **15**, 665-674.

Roggero, C. M., De Blas, G. A., Dai, H., Tomes, C. N., Rizo, J. and Mayorga, L. S. (2007). Complexin/synaptotagmin interplay controls acrosomal exocytosis. *J. Biol. Chem.* **282**, 26335-26343.

Rosenberg, O. S., Deindl, S., Comolli, L. R., Hoelz, A., Downing, K. H., Nairn, A. C. and Kuriyan, J. (2006). Oligomerization states of the association domain and the holoenzyme of Ca^{2+}/CaM kinase II. *FEBS J.* **273**, 682-694.

Schiebler, W., Jahn, R., Doucet, J. P., Rothlein, J. and Greengard, P. (1986). Characterization of synapsin I binding to small synaptic vesicles. *J. Biol. Chem.* **261**, 8383-8390.

Schlingmann, K., Michaut, M. A., McElwee, J. L., Wolff, C. A., Travis, A. J. and Turner, R. M. (2007). Calmodulin and CaMKII in the sperm principal piece: evidence for a motility-related calcium/calmodulin pathway. *J. Androl.* **28**, 706-716.

Schoch, S. and Gundelfinger, E. D. (2006). Molecular organization of the presynaptic active zone. *Cell Tissue Res.* **326**, 379-391.

Schreiber, M., Wei, A., Yuan, A., Gaut, J., Saito, M. and Salkoff, L. (1998). Slo3 channels a novel pH-sensitive K^+ channel from mammalian spermatocytes. *J. Biol. Chem.* **273**, 3509-3516.

Si, Y. and Olds-Clarke, P. (2000). Evidence for the involvement of calmodulin in mouse sperm capacitation. *Biol. Reprod.* **62**, 1231-1239.

Strack, S., Choi, S., Lovinger, D. M. and Colbran, R. J. (1997). Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. *J. Biol. Chem.* **272**, 13467-13470.

Sudhof, T. C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653.

Sudhof, T. C. and Rothman, J. E. (2009). Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**, 474-477.

Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T. and Hidaka, H. (1991). The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochem. Biophys. Res. Commun.* **181**, 968-975.

Suzuki, T., Du, F., Tian, Q. B., Zhang, J. and Endo, S. (2008). Ca^{2+}/calmodulin-dependent protein kinase IIalpha clusters are associated with stable lipid rafts and their formation traps PSD-95. *J. Neurochem.* **104**, 596-610.

Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H. (1990). KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **265**, 4315-4320.

Tomes, C. N. (2007). Molecular mechanisms of membrane fusion during acrosomal exocytosis. *Soc. Reprod. Fertil.* **Suppl. 65**, 275-291.

Tomes, C. N., De Blas, G. A., Michaut, M. A., Farre, E. V., Cherhitin, O., Visconti, P. E. and Mayorga, L. S. (2005). alpha-SNAP and NSF are required in a priming step during the human sperm acrosome reaction. *Mol. Hum. Reprod.* **11**, 43-51.

Travis, A. J., Merdiushev, T., Vargas, L. A., Jones, B. H., Purdon, M. A., Nipper, R. W., Galatioto, J., Moss, S. B., Hunnicutt, G. R. and Kopf, G. S. (2001). Expression and localization of caveolin-1, and the presence of membrane rafts, in mouse and Guinea pig spermatozoa. *Dev. Biol.* **240**, 599-610.

Tsai, P. S., De Vries, K. J., De Boer-Brouwer, M., Garcia-Gil, N., Van Gestel, R. A., Colenbrander, B., Gadella, B. M. and Van Haeften, T. (2007). Syntaxin and VAMP association with lipid rafts depends on cholesterol depletion in capacitating sperm cells. *Mol. Membr. Biol.* **24**, 313-324.

Tsui, J., Inagaki, M. and Schulman, H. (2005). Calcium/calmodulin-dependent protein kinase II (CaMKII) localization acts in concert with substrate targeting to create spatial restriction for phosphorylation. *J. Biol. Chem.* **280**, 9210-9216.

Tulsiani, D. R. and Abou-Haila, A. (2004). Is sperm capacitation analogous to early phases of Ca^{2+}-triggered membrane fusion in somatic cells and viruses? *BioEssays* **26**, 281-290.

Tulsiani, D. R., Zeng, H. T. and Abou-Haila, A. (2007). Biology of sperm capacitation: evidence for multiple signalling pathways. *Soc. Reprod. Fertil.* **Suppl. 63**, 257-272.

Wang, Z. W. (2008). Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol. Neurobiol.* **38**, 153-166.

Wassarman, P. M. and Litscher, E. S. (2008). Mammalian fertilization:the eggs multifunctional zona pellucida. *Int. J. Dev. Biol.* **52**, 665-676.

Wayman, G. A., Lee, Y. S., Tokumitsu, H., Silva, A. and Soderling, T. R. (2008). Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron* **59**, 914-931.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H. and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-772.

Witte, T. S. and Schafer-Somi, S. (2007). Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Anim. Reprod. Sci.* **102**, 181-193.

Wojcik, S. M. and Brose, N. (2007). Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. *Neuron* **55**, 11-24.

Yamauchi, T. (2005). Neuronal Ca^{2+}/calmodulin-dependent protein kinase II-discovery, progress in a quarter of a century, and perspective: implication for learning and memory. *Biol. Pharm. Bull.* **28**, 1342-1354.

Yang, E. and Schulman, H. (1999). Structural examination of autoregulation of multifunctional calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **274**, 26199-26208.

Yunes, R., Tomes, C., Michaut, M., De Blas, G., Rodriguez, F., Regazzi, R. and Mayorga, L. S. (2002). Rab3A and calmodulin regulate acrosomal exocytosis by mechanisms that do not require a direct interaction. *FEBS Lett.* **525**, 126-130.

Zanetti, N. and Mayorga, L. S. (2009). Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. *Biol. Reprod.* **2**, 396-405.

Zarelli, V. E., Ruete, M. C., Roggero, C. M., Mayorga, L. S. and Tomes, C. N. (2009). PTP1B dephosphorylates N-ethylmaleimide-sensitive factor and elicits SNARE complex disassembly during human sperm exocytosis. *J. Biol. Chem.* **284**, 10491-10503.

Zeginiadou, T., Papadimas, J. and Mantalenakis, S. (2000). Acrosome reaction: methods for detection and clinical significance. *Andrologia* **32**, 335-343.

Zhao, L., Burkin, H. R., Shi, X., Li, L., Reim, K. and Miller, D. J. (2007). Complexin I is required for mammalian sperm acrosomal exocytosis. *Dev. Biol.* **309**, 236-244.