Junctate, an inositol 1,4,5-triphosphate receptor associated protein, is present in rodent sperm and binds TRPC2 and TRPC5 but not TRPC1 channels.

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Abstract

During acrosome reaction, the first step of the fertilization, calcium influx trough Canonical Transient Receptor Potential type 2 channels (TRPC2) is responsible of the calcium plateau, allowing acrosomal exocytosis. Activation of TRPC channels is a debated question in general and more particularly in sperm, where little is known concerning the molecular events leading to TRPC2 activation. From the discovery of IP3R binding domains on TRPC2, it has been suggested that TRPC channel activation may be due to a conformational coupling between IP3R and TRPC channels. Moreover, recent data demonstrate that junctate, an IP3R associated protein, participates also in the gating of some TRPC. In this study, we focused on the presence of junctate in sperm and its potential role in TRPC2 activation. We demonstrate that junctate is expressed in sperm and co-localizes with the IP3R in the acrosomal crescent of the anterior sperm head of rodent. We also show that the N-terminus of junctate interacts with the C-terminus of TRPC2, both in vitro and in a heterologous expression system. We show that junctate binds to TRPC2 independently of the calcium concentration and that junctate binding site does not overlap with the common IP3R/Calmoduline binding sites.

TRPC2 gating is downstream phospholipase C activation, which is a key and necessary step during acrosome reaction. TRPC2 may then be activated directly by DiAcylGlycerol (DAG), as in neurons of the vomeronasal organ. In the present study, we investigated whether DAG could promote the acrosome reaction. We found that 100 μM OAG, a permeant DAG analogue, was unable to trigger the acrosome reaction.

All together, these results provide a new hypothesis concerning sperm TRPC2 gating: TRPC2 activation may be due to modifications of the bindings of both junctate and IP3R on TRPC2 induced by acrosome depletion.

Introduction

The acrosome reaction (AR), the first step of fertilization, is an exocytotic event allowing sperm to cross the zona pellucida and to become competent for fusion with the oocyte. The sperm AR, as all exocytotic events, is regulated by a multifaceted intracellular calcium rise. This calcium increase is due to the consecutive openings of three different types of calcium channels: (i) a voltage activated channel, (ii) the inositol 1,4,5-triphosphate receptor (IP3R) and finally (iii) a store-operated channel. The first channel type to be activated is on the plasma membrane, belongs to the low-voltage activated calcium channels family (Arnoult et al., 1996), and our recent data strongly suggest that this channel is a $Ca_v3.2$ channel (Stamboulian et al., 2004). This channel is activated few hundred of milliseconds after the binding of the zona pellucida glycoprotein ZP3 on its yet uncharacterized receptor and is responsible of a short calcium transient (Arnoult et al., 1999). The role of the transient calcium entry via $Ca_v3.2$ channel is still a matter of debate. It may modulate other calcium channels involved in the downstream intracellular calcium rise (Stamboulian et al., 2002). The second channel type to be activated is the intracellular IP3R, present in the outer membrane of the sperm acrosome (Walensky and Snyder, 1995), contrary to other cell types where it is present in the endoplasmic reticulum. Then, the acrosome, known to be a vesicle of secretion, plays also a of role calcium store (De Blas et al., 2002; Herrick et al., 2004). ZP3 activates, via a G protein, a phopholipase C (PLC), an enzyme that produces two secondary

messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). PLC activation is a necessary step since inhibitors of PLC block the AR (O'Toole et al., 2000). The production of IP3 by PLC activates IP3R and leads to the emptying of the specific sperm calcium store, the acrosome. Finally, the third channel, a store-operated calcium channel, is activated.

Store-operated calcium channels are activated by the depletion of intracellular calcium stores. In vertebrates, the seven members of TRPC proteins (TRPC1–7) are proposed to be classical representatives of these channels (Montell, 2001). In sperm, different types of TRPC channels are present such as TRPC1, 2, 3, 5 (Jungnickel et al., 2001;Sutton et al., 2004). Among them, it is accepted that TRPC2 plays the major role during the acrosome reaction because specific inhibition of TRPC2 blocks the slow calcium rise and the sperm exocytosis (Jungnickel et al., 2001). However, TRPC2 deficient mice do not present fertility troubles (Stowers et al., 2002), suggesting that other members of the TRPC family present in sperm may be up regulated to compensate the lack of TRPC2.

The mechanism of activation of TRPC channels is still a matter of debate. To summarize, three different ways of activation have been described: 1/TRPC are activated via secondary messenger like Calcium Influx Factor (CIF) (Randriamampita and Tsien, 1993) 2/TRPC activation depends on some extent on exocytosis (Yao et al., 1999) and 3/ the IP3R, localised vis a vis the plasma membrane in microdomains of the ER, interacts with TRPC allowing its direct activation after calcium store emptying (Kiselyov et al., 1998), in a mechanism similar to what already described for the coupling between ryanodine receptor and the dihydropyridine receptor in skeletal muscle cells. In agreement with the third hypothesis, we have recently shown that TRPC3 activation is modulated by junctate, an endoplasmic membrane protein which is a partner of the IP3R (Treves et al., 2004). Because the lumen part of the junctate has calcium binding domains, it has been hypothesized that junctate could be the sensor of store depletion and that structural modification of junctate may be the first molecular event leading to TRPC3 activation. Finally, it is important to point out that some TRPC channels are not activated by calcium store depletion but rather by DAG and to complicate the debate, that a same type of TRPC channel can be activated in different ways depending on its tissues distribution or density expression (Vazquez et al., 2003).

The TRPC2 calcium channel is a functional channel in rodent (Vannier et al., 1999), specifically expressed in vomeronasal organ where it plays a key role in pheromone detection (Stowers et al., 2002), in testis where it plays a key role in AR (Jungnickel et al., 2001) and in erythroblast where it controls erythropoietin-induced differentiation and proliferation (Chu et al., 2002). Biochemical results demonstrating the presence of IP3R binding sites on TRPC2 strongly suggest that TRPC2 could be activated by a direct interaction with IP3R (Tang et al., 2001). However, the mode of activation of TRPC2 appears quite variable depending of the cell type considered. In neurons of the vomeronasal organ, TRPC2 is clearly activated directly by DAG, independently of the IP3R activation (Lucas et al., 2003). In other hand, in erythroblast the TRPC2 activation requires a functional interaction with IP3R since mutations of the known binding sites on TRPC2 for IP3R lead to an inhibition of TRPC2 activation by erythropoietin (Tong et al., 2004). In contrast to these cell types, the mechanism of activation of TRPC2 in sperm is less understood. It has been showed that IP3R and TRPC2 are localized in the same sperm region, the acrosomal crescent (Jungnickel et al., 2001). Moreover, calcium release via IP3R is necessary for Rab3-activated acrosome reaction (De Blas et al., 2002) and finally the calcium entry induced by thapsigargin, a classical inhibitor of SERCA type calcium ATPase, is inactivated by specific antibodies against TRPC2 (Jungnickel et al., 2001), suggesting that the acrosomal calcium emptying is sufficient to activate TRPC2. These results, taken together, suggest that the store depletion via IP3R activation is an important step in TRPC2 activation.

Since junctate is an important actor of calcium signaling involving TRPC channels in heterologous expression systems (Treves et al., 2004), we decided to investigate the presence of junctate in sperm. In order to evaluate its potential involvement in sperm calcium signaling during acrosome reaction, we explored the possibility for junctate to be a molecular partner of TRPC2 channel.

In this paper, we show that junctate, an important IP3R-associated protein, recently described to control calcium influx induced by IP3R activation, is present in mouse and rat sperm and is localized in the acrosomal crescent of the anterior sperm head. Junctate is not only an IP3R associated protein but binds also directly on the carboxyl-terminal domain of TRPC2 (TRPC2 Cter). The binding site of junctate on TRPC2 is different than 1/the CIRB (**C**almodulin **IP3R B**inding site) IP3R binding site described earlier (Tang et al., 2001) and 2/the calmodulin binding sites. The presence of junctate in the acrosomal region, the biochemical evidences that junctate and TRPC2 are able to interact tightly and the fact that DAG does not promote acrosome reaction provide new hypothesis concerning TRPC2 activation in sperm: TRPC2 activation may be due to modifications of the bindings of both junctate and IP3R on TRPC2 induced by store depletion.

Materiels and methods

Cell Culture and transfection

HEK-293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin and transiently transfected with pEGFP-junctate, using JetPEI from Qbiogene according to the instruction

of the manufacturer. Two days after transfection, transfected and control cells were collected and re-suspended in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP 40, 0.5% DOC) complemented with a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche).

Acrosomal membrane preparation

Sperm were obtained from OF1 mice (16 weeks old, Charles River) by manual trituration of caudae epididymis.

To obtain an acrosomal-membrane enriched fraction, sperm were capacitated in M2 (Sigma) supplemented with BSA (20 mg/ml, pH 7.4) for 1 h at 37°C. Sperm were pelleted (500 g, 10 min) and treated with 10 μM A23187 during 30 min at 37°C in the presence of a cocktail of protease inhibitors. Sperm were then centrifuged 5 min at 1000 g. The supernatant was collected and subsequently ultra-centrifuged at 100 000 g for 1h at 4°C. The pellet was re-suspended in RIPA buffer containing: 50 mM Tris-Ph 7.5, 150 mM NaCl, 0.1% SDS, 1% NP 40, 0.5% DOC, complemented with a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche).

DNA construct

Expression of the recombinant proteins

An His-Tag (6 His) fusion protein, starting three amino acids before the TRP box, containing the full COOH-terminal domain of TRPC2 was obtained as follow: a 747 bp cDNA corresponding to amino acid residues 925–1172 (NM_011644) was obtained by PCR using the following primers: forward primer 5′-CATGCCATGGTCAAGCTTCAGAAGATCGAGGATGATGCTG-3′ and reverse primer 5′-GCTTCTAGAGTTAGGACTCGCCCTTGGTCTCCAG-3. This construct was then inserted in frame into the multiple cloning sites of the His-tag pMR78 vector (Arnaud et al., 1997) and verified by sequencing. The His tag is located at the N-terminus of the fusion protein. The recombinant plasmid was then transformed into E. Coli BL 21 (Invitrogen) in order to express the His-TRPC2-Cter fusion protein. The fusion protein was purified with a Ni-NTA agarose column (Qiagen) according to the instructions of the manufacturer.

GFP-junctate was constructed as previously described (Treves et al., 2004)

RT-PCR amplification

Messenger RNA from adult mice testes and brains were prepared from tissue with the Dynabeads isolation kit (Dynal). Each RT-PCR reaction was performed in a total volume of 25 μl in the presence of 30 ng of mRNA using the Superscript TM One step RT-PCR system (Invitrogen).

Sense and reverse primers were respectively 5′-TTTGTGCATGGATTGAAGAA-3′ (nucleotides 40–61 of junctate AF302653) and 5′-TCGACCAAGTCAAACCACAC-3′ (nucleotides 130–149 of junctate). Reverse transcription was achieved within 30 min of incubation at 45°C. Amplification was obtained after 40 cycles of temperature: 30 sec at 94°C, 30 sec at 48°C, and 30 sec at 72°C. Elongation was done at 72°C during 10 min.

A 185 bp product was obtained in both tissues and purified using Nucleospin (Macherey Nagel). Theses cDNA product were amplified in the same conditions as above and subsequently sequenced.

As control for the mRNA preparation and quantification, a 192 bp cDNA product from Hypoxanthine Phospho Ribosyl Transferase (HPRT) cDNA was amplified using the forward primers 5′-TGTAATGACCAGTCAACAGGG-3′ and the reverse primer 5′-TGGCTTATATCCAACACTTCG-3 (data not shown)

Western blot analysis

Proteins were separated on 12% polyacrylamide denaturing gels and electro-transferred for 90 min at 350 mA to Immobilon P transfer membrane (Millipore). The membranes were then blocked 60 min with 4% non-fat dry milk (Biorad) in PBS Tween 0.1%. The primary antibody was added and incubated overnight at 4°C. After washing in PBS Tween 0.1%, the secondary antibody (anti-rabbit Jackson lab.) was added at 1:10,000 during 3 h at room temperature. The membrane was washed and incubated 1 min in HRP substrat (Western Lightning, Perkin Elmer Life Science). The reactive proteins were detected using Chemiluminescence assay followed by exposure to Biomax film (Kodak).

The presence of junctate in sperm membrane fraction and in acrosomal enriched fraction was tested using antibody raised against its carboxyl-terminus (Treves et al., 2000) at a final concentration of 1 μg/ml.

Antibodies against Green Fluorescent Protein (GFP) (Santa Cruz Biotechnology) was used to purify junctate during immunoprecipitation and for Western blotting diluted at 1:5,000.

Monoclonal anti-polyhistidin (Sigma) was used at a dilution of 1:10,000.

Immunoprecipitation and pull-down experiments

Immunoprecipitation of TRPC2 CTer with GFP-junctate

GFP antibody (0.8 μg/ml) was incubated with protein A (Dynabeads Protein A, Dynal) 30 min at room temperature. Beads were rinsed with 50 mM Tris, 100 mM NaCl, 5 mM CaCl$_2$, 5 mM EGTA (free calcium concentration **=**10 μM) pH 7.5, three times and incubated overnight with total protein extracts from HEK-293 cells, transiently transfected with pEGFP-junctate (Treves et al., 2004). After three washes, beads were incubated with the fusion protein TRPC2-Cter containing the His-tag during 2 h at 4°C. Beads are then washed five times and proteins bounds were eluted by boiling 5 min at 95°C in Laemmli sample buffer. Control was performed with total protein extract from untransfected HEK-293 cell.

Immunoprecipitation of TRPC2 Cter with biotinylated junctate peptide on streptavidin beads

Biotinylated peptide corresponding to the amino terminus of junctate (MAEDKETKHGGHKNGRKGGLSGTSK-biotin) or biotin were incubated 30 min at room temperature with streptavidin beads (Dynabeads M280 Streptavidin, Dynal).

Beads were washed three times with a buffer containing in mM: Tris 50, NaCl 100, CaCl$_2$ 5, EGTA 5 pH 7.5 and 100 μM or 10 nM free Ca^{2+}. Beads were blocked during 1 h with BSA (0.2 mg/ml) and then incubated with purified His-TRPC2-Cter overnight at 4°C in the presence of a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche). Beads were washed two times and proteins bound were eluted by boiling 5 min in Laemmli sample buffer.

Immunoprecipitation of TRPC2 Cter with a calmodulin sepharose beads

Calmodulin Sepharose 4B (Amersham Biosciences) was incubated 45 min with TRPC2-Cter diluted in a solution containing in mM: Tris 50, NaCl 100, CaCl2 5, EGTA 5 and BSA 2 mg/ml pH 7.5. Bound proteins were eluted by boiling 5 min in Laemmli sample buffer.

For competition studies F2q peptide (amino acids 669–698 of IP3R) was synthesized by Neosystem-Strasbourg F.

Immunohistochemistry and indirect immunofluorescence

Sperm were harvested from the caudae epididymis, washed in PBS and fixed in 4% PFA for 30 min on ice. Fixed sperm were allowed to air-dry on poly L Lysine coated slides. The slides were washed in PBS (3×5 min), 50 mM NH4C1 (2×15 min), PBS (3×5 min), 0.1% triton X-100 (15 min) and PBS (3×5 min). Slides were blocked with 1% BSA and 2% normal goat serum during 60 min at room temperature. Slides were incubated overnight at 4°C, in the presence of an antibody against junctate (Treves et al., 2000) and/or an antibody against IP3R (gift from Dr Mikoshiba Tokyo-Japan), diluted in the blocking solution at 1/100 and 1/2000 respectively. Slides were then incubated 60 min with a secondary antibody (alexa fluor 546 or alexa fluor 488 - Molecular probes), diluted at 1/800 and washed in PBS (3×5 min). Slides were analyzed on a confocal laser scanning microscope (Leica TCS-SP2, Mannheim)

BIAcore Analysis

Real time surface plasmon resonance (SPR) experiments were performed on a BIAcore biosensor system 1000 at 25 °C with a constant flow rate of running buffer 100 μM free Calcium (Tris 50 mM 100 mM NaCl 5 mM CaCl2 5mM EGTA pH 7.5) or with a buffer containing 10 nM free Calcium (Tris 50 mM 100 mM NaCl 5 mM EGTA pH 7.5). Biotinylated junctate peptide or biotin were immobilized on the sensor chip surface coated with streptavidin (sensor chip SA). Various concentrations of purified TRPC2 Cter were injected onto the coupled surfaces. Regeneration of the sensor chip for subsequent injections was accomplished by injecting SDS 0.01%, 0.02% and 0.03%.

Acrosome reaction assays

Sperm were harvested from OF1 mice, and allowed to swim in M2 medium for 10 min. Sperm were then capacitated for 45 min at 37° C in M16 medium containing 20 mg/ml BSA. The different modulators of acrosome reaction (A23187, OAG) were added in the capacitation medium and sperm were incubated for further 30 min. Sperm were then fixed in 4% PFA and stained with coomassie blue.

M2 and M16 medium, 1-oleoyl-2-acetyl-sn-glycerol (OAG), A23187 were from Sigma.

Results

Junctate is present in rodent sperm and localized in the acrosomal crescent of the anterior sperm head

Because IP3 production was shown to be necessary for acrosome reaction and junctate was shown to be an important regulating protein of IP3R-dependent calcium influx, we decided to seek this protein in mature sperm cells. Three different approaches have been used to demonstrate the presence of junctate in sperm. First, we checked the presence of junctate in mouse testis by RT-PCR, using a sense primer designed in the non-codant ammo-terminal part of the protein and an antisense primer spanning across transmembrane and luminal

part of the protein. The PCR product amplified from mRNA has a size of 180 bp, as expected (Figure 1A). Identity of the product was confirm by sequence analysis of the band (data not shown).

Our second approach was to demonstrate the presence of the protein junctate by Western blot using a polyclonal antibody (Treves et al., 2000). Junctate, as IP3R, is generally present in the endoplasmic membranes. However, in mature sperm cells, where the endoplasmic reticulum is absent, endoplasmic membrane proteins like IP3R are localized in the acrosomal membrane. Thus, membrane preparation enriched in acrosomal membrane should present a higher concentration of junctate than in a sperm crude membrane extract. To obtain a membrane preparation enriched with outer acrosomal membranes, capacitated sperm were treated with the calcium ionophore A23187 to promote AR. Secretion vesicles, corresponding to plasma membrane and outer acrosomal membranes merged together, were purified by centrifugation (see Methods section) and the presence of junctate studied by Western blot analysis (figure 1B). For mouse sperm, 3 bands were stained with apparent molecular weight (MW) of 41, 43 and 45 kDa respectively. We also checked the presence of junctate in similar membrane preparation obtained from sperm rat. In rat, the antibody immunodecorated only one band at 43 kDa (figure 1B). The 3 bands observed in mouse preparation likely correspond to different isoforms of junctate. Three junctate isoforms have indeed already been described in mouse cardiac cells with apparent molecular weight between 40–53 kDa.

The acrosomal localization of junctate was confirmed by immunostaining experiments of rat and mouse sperm cells. In both species, the polyclonal antibody against junctate stained the acrosomal crescent of the anterior sperm head (Figure 2A–2B). Such result is particularly interesting since the IP3R was shown to be localized in the same subcellular area (Walensky and Snyder, 1995). We, then, performed co-localization experiments using antibodies directed against IP3R and junctate. Figure 2C–F show that both proteins co-localize in the same subcellular area, that is the acrosomal crescent.

All together, these results demonstrate that junctate is present in sperm and sub-localized in the acrosomal crescent of the anterior sperm head, in the vicinity of the IP3R.

Junctate binds to the carboxyl-terminus of TRPC2

The presence of junctate in the sperm acrosomal region, and the facts that junctate binds to TRPC3 and regulates its activation by carbachol (Treves et al., 2004), raise the question of a direct interaction of junctate with TRPC2. Junctate tagged by GFP was expressed in HEK-293 cells. As expected, in a Western blot, an antibody against GFP immunodecorated a band around 70 kDa corresponding to GFP-junctate (Figure 3A). Then, expressed GFP-Junctate was immobilized on sepharose beads coated with an antibody directed against GFP and incubated with a fusion protein corresponding to the full carboxyl-terminus of TRPC2 (TRPC2-Cter). In this experiment and in the following, the TRPC2-Cter fusion protein was tagged with histidin residues at its N-terminus (His-TRPC2-Cter), for purification and was evidenced in Western blots with an antibody against its histidin tag. Figure 3B shows that the fusion protein corresponding to the carboxyl-terminus of TRPC2 was bound to immobilized GFP-junctate (Figure 3B lane 3), whereas the non-bound fraction (lane 2) was highly depleted in His-TRPC2-Cter. The bound fraction on sepharose beads incubated with cell extracts of control HEK-293 did not contain His-TRPC2-Cter (Figure 3B lane 5), all the fusion protein being present in the non-bound fraction (lane 4). Our result clearly demonstrate that TRPC2, and more precisely its C-terminal cytosolic domain, physically interacts with junctate.

The amino terminus of junctate binds to carboxyl terminus of TRPC2 in a calcium independent manner

In order to confirm the binding of junctate on TRPC2 channels, and also to determine which part of junctate is involved in this binding, a peptide, encompassing the 30 amino acids of its amino-terminus (junctate-Nter), was synthesized, because only the junctate-Nter can potentially interacts with TRPC2, since its carboxyl-terminus is luminal. The junctate-N-ter peptide was tagged with biotin and was attached to streptavidin coated beads. Then His-TRPC2-Cter fusion proteins were incubated overnight with the coated beads. Figure 4A shows that beads specifically coated with junctate-Nter peptide retained TRPC2 fusion protein (figure 4A lane 1 and 3), whereas beads coated with biotin only did not retain the fusion protein (figure 4A lane 2 and 4). Because calcium is well known to modify binding of calcium-dependent proteins, we checked the binding of TRPC2 in two different calcium conditions: 10 nM and 100 μM free calcium. We shows that the binding of TRPC2 on junctate is calcium independent, since His-TRPC2-Cter binds on junctate-Nter in the presence of 100 μM (figure 4A lane 1) and in 10 nM (figure 4A lane 3) free calcium.

In order to confirm the binding of junctate-Nter on TRPC2, experiments using the surface plasmon resonance (SPR) technique (Biacore), have been carried out. Biotynilated-junctate-Nter was covalently bound on a streptavidin matrix and TRPC2 fusion protein was introduced in the running buffer. Figure 4B shows specific interaction of TRPC2 with biotynilated-junctate-Nter. Control experiments have been also carried out, using a matrix covered with only biotin (figure 4B: non specific binding). This experiment confirms clearly that carboxyl-terminus of TRPC2 binds to amino-terminus of junctate.

Altogether, our result show that the 30 N-terminal amino acids of junctate are sufficient to bind to the C-terminal domain of TRPC2, and that this interaction is calcium-independent.

TRPC2 has distinct binding sites for junctate and IP3R/calmodulin

It has previously been shown that TRPC2 carboxyl-terminus contains binding sites for the IP3R and calmodulin (CaM) (Tang et al., 2001). Tang and collaborators characterized two binding sites for IP3R on the carboxyl-terminus of TRPC2. The first one, localized between amino acids 901 and 936 of TRPC2, was named CIRB (**C**almodulin **I**P3**R B**inding site) because not only IP3R, but also CaM is able to bind to it. CaM and IP3R are in competition at the CIRB, and a small peptide from the IP3R, named F2v (amino acids 681–698 of the IP3R), blocks the interaction between CIRB and CaM with an IC_{50} of 21 μM. The second binding site for IP3R is localized between amino acids 944 and 1072 of TRPC2. Although CaM binds to the same region of the protein, no data are available concerning mutual exclusion of IP3R and CaM at this second binding site of TRPC2. We attempted to determine if junctate binding site and the IP3R and calmodulin binding sites overlap. To determine whether junctate and IP3R share the same interaction site, we used a peptide from the IP3R, named F2q (amino acids 669–698 of the IP3R), which is longer that F2v and known to interact also with both IP3R binding sites of TRPC2. In a first set of experiments, we confirmed whether F2q is able to interact with the full carboxyl-terminus of TRPC2 fusion protein. To check the binding of F2q on TRPC2, we used the property of F2q to inhibit the interaction of CaM on the CIRB sequence of TRPC2 (Tang et al., 2001).

His-TRPC2-Cter fusion protein was incubated with calmodulin sepharose beads and bound proteins were evidenced by Western blot. Figure 5A shows that His-TRPC2-Cter binds to CaM-sepharose in both 10 nM (lanes 1) and 100 μM (lanes 3) calcium concentrations tested. When 0.1 μM His-TRPC2-Cter fusion protein was preincubated with 20 μM F2q peptide, the interaction of His-TRPC2-Cter with CaM-sepharose was completely blocked (figure 5A lane 2 and 4). This experiment confirms previous data showing that F2q peptide and CaM bind to TRPC2 on a common site (CIRB domain).

We, then, examined the possibility that the junctate-Nter peptide modulates TRPC2 binding on CaM-sepharose beads (Figure 5B). The preincubation of His-TRPC2-Cter fusion protein with 25 μM junctate-Nter peptide did not prevent TRPC2 binding to CaM-sepharose (figure 5B - lane 2). This result strongly suggests that junctate binding site and CaM binding sites are different. We then examined the ability of junctate to bind on TRPC2 when both IP3R binding sites are supposedly occupied by F2q peptide (figure 5C): His-TRPC2-Cter fusion protein was incubated with 20 μM F2q and then challenged for the binding to streptavidin beads coated with biotin-junctate-Nter peptide. Figure 5C shows that F2q interaction with TRPC2 has no effect on His-TRPC2-Cter binding on junctate-Nter peptide, contrary to its action on the binding of TRPC2 onto CaM sepharose. These results demonstrate that the binding site of junctate on TRPC2 is different from the common binding sites for the IP3R and CaM.

DAG does not promote acrosome reaction

Currently, two modes for TRPC2 activation have been proposed: via the second messenger DAG in neurons of the vomeronasal organ, or via a direct interaction with the IP3R. The fact that TRPC2 has both IP3R and junctate as biochemical partners does not allow rejecting the hypothesis of a direct activation of TRPC2 by DAG in sperm, because activation of PLC is an important step during acrosome reaction. Indeed, U73122, an inhibitor of PLC, blocks ZP3-activated calcium influx. We evaluated the direct effect of DAG on sperm acrosome reaction. Figure 6 shows that 100 μM OAG does not increase the number of acrosome reacted sperm in comparison with a control medium containing no specific activators of the AR (figure 6 bar labeled OAG versus bar labeled control). We checked that a calcium ionophore increase the number of acrosome reacted sperm (figure 6-bar labeled A23187). The fact that the medium used to promote capacitation increases the level of spontaneous acrosome reacted sperm indicates that sperm are ready to be activated by physiological agonists of the AR (figure 6 bar labeled No Cap versus bar labeled control).

Discussion

In this paper, we demonstrate the following points. Firstly, in Rodent, junctate is expressed in sperm and localized in the same sub-cellular area as IP3R, i.e. the acrosomal crescent of the anterior sperm head. Secondly the amino-terminus of junctate interacts with the carboxyl-terminus of TRPC2. We also confirmed in this paper previous results regarding the TRPC2 interaction with IP3R and calmodulin. Finally, DAG is not sufficient to promote acrosome reaction by itself.

Junctate is present in sperm

The gene coding for junctate produces also two other proteins: junctine and aspartyl beta-hydroxylase. The three splice variants of the same gene, junctate, junctine and aspartyl beta-hydroxylase, have different patterns of expression and different cellular roles. Aspartyl beta-hydroxylase is expressed at different level in almost all tissues tested and also in testis (Dinchuk et al., 2000). This protein is involved in post-translational protein processing. Junctin is expressed in cardiac and skeletal muscles (Treves et al., 2000), in which it regulates the activation of the ryanodine receptor. Because of the choice of the primers, both junctate and junctin mRNA, but not aspartyl-beta-hydroxylase mRNA, were potentially amplified. However, junctin is not expressed in non-muscle tissue (Lim et al., 2000; Treves et al., 2000) and its expression as followed by real-time RT-PCR is null in testis (Dinchuk et al., 2000). On the other hand, junctate is expressed in all tissues tested, but skeletal muscles (Hong et al., 2001;Treves et al., 2000). The band amplified in sperm should thus correspond to junctate. In Western blot experiments, the antibody used has been designed against the non-catalytic part of the

aspartyl-beta-hydroxylase and then recognize specifically junctate and aspartyl-beta-hydroxylase, but not junctins (Treves et al., 2000). However, all expressed splice variants of aspartyl-beta-hydroxylase have a molecular weight higher than 60 kDa (Dinchuk et al., 2000) and bands stained below 50 kDa most likely correspond to junctate.

In mouse, Western blot analysis shows that the antibody against junctate immunodecorates three bands in the range of 40 to 45 kDa from acrosomal membrane extract. The fact that Western blots reveal different bands in the range of 40–50 kDa was expected, since it has already been described that there are different splice variants of junctate, especially in cardiac mouse cells (Hong et al., 2001). This result suggests that different isoforms of junctate are present in sperm, contrary to the rat where only one band is stained.

In rabbit kidney, a lower weight of 32 kDa for junctate has already been described (Treves et al., 2000). This difference may be due to murine specific glycosylation of junctate proteins. Indeed, the mouse cardiac isoforms show similar apparent molecular weights in the range of 40–53 kDa whereas their predicted MW from cDNA sequence are 23.7, 28.5 and 29.9 kDa (Hong et al., 2001) and humbug, a truncated transcript of aspartyl-beta-hydroxylase lacking the catalytic domain has an apparent MW of 60 kDa whereas its predicted MW from cDNA sequence is 35 kDa (Dinchuk et al., 2000). This glycosylation difference may be due to species differences since human junctate has 29 amino acids in excess over mouse junctate. This difference may also be due to testis specific splice variants, as known for many proteins that possess specific variant in testis.

Binding sites on carboxyl-terminus of TRPC2

So far, two proteins have been described to interact with carboxyl-terminus of TRPC2: the IP3R and calmodulin (Tang et al., 2001). Both proteins bind onto two domains: one is localized between amino acids 901–936 (CIRB, domain 1) and the other domain is localized between amino acids 944–1072 of TRPC2 (domain 2). In this study, we describe a third protein that interacts with the carboxyl-terminus of TRPC2, that is junctate. We demonstrate that the binding site of junctate is distinct from the CIRB (domain 1) and the domain 2. Moreover, our results give new insights concerning IP3R and CaM bindings on TRPC2.

Firstly, from studies on TRPC4 (Tang et al., 2001), it has been shown that there is no mutual exclusion of IP3R and calmodulin on domain 2, contrary to the domain 1 (CIRB domain). For TRPC2, no data are available concerning mutual exclusion of IP3R and CaM on domain 2. If we hypothesize that the binding of IP3R on domain 1 does not interfere with the binding of IP3R on domain 2, the fact, that the F2q peptide completely blocks the binding of the full carboxyl-terminus of TRPC2 on CaM-sepharose, indicates a mutual exclusion of IP3R and calmodulin on both domains 1 and 2. Therefore, TRPC2 would present two CIRB instead of one. Moreover, the fact, that the binding of His-TRPC2-Cter on CaM-sepharose in the presence of F2q is abolished, also suggest that these two domains are the only CaM-binding domains present in the carboxyl terminus of TRPC2. It may be important to notice that the absence of competition between the IP3R and CaM on the domain 2 of TRPC4 (localized between amino acids 781–864), may be due to the fact that the competition experiments have been performed using the F2v peptide, instead of the F2q peptide, which is 10 amino acids shorter.

Secondly, earlier studies, using a short fragment of TRPC4, encompassing domain 1 (CIRB domain, amino acids 901–936) showed that the binding of CaM on TRPC4 is calcium-dependent. The calcium-dependence of domain 2 was not tested (Tang et al., 2001). In this paper, we tested the full carboxyl-terminus of TRPC2, containing both CaM domains. In our conditions, the full carboxyl-terminus of TRPC2 binds to CaM in a calcium-independent manner.

In conclusion, we demonstrate in this study that TRPC2 behaves differently than TRPC4: firstly the binding of F2q on both domains 1 and 2 of TRPC2 blocks CaM binding and secondly, at least one CaM binding site of TRPC2 is calcium independent.

TRPC2 activation

We have shown that 1/junctate is localized in the same sub-cellular area than IP3R, the acrosomal crescent of the anterior sperm head and 2/ the amino-terminus of junctate interacts with the carboxyl-terminus of TRPC2. Moreover, we previously described that the amino-terminus of junctate binds also to the IP3R (Treves et al., 2004). All these results, taken together, suggest that calcium signaling during the AR involves a supra molecular complex of two calcium channels, one localized in the plasma membrane and the other one in the outer acrosomal membrane associated to at least two regulating proteins which are junctate and calmodulin. Because OAG, a permeant analogue of DAG, does not promote acrosome reaction, TRPC2 activation is likely due to molecular events downstream IP3 production by PLC. The fact that junctate, as IP3R, binds to TRPC2 suggest that junctate is involved in TRPC2 activation. This result provides new hypothesis concerning TRPC2 activation in sperm: TRPC2 activation may be due to modifications of the bindings of both junctate and IP3R on TRPC2 induced by store depletion.

Previous studies have already shown that the gating of TRPC channels is dependent on their level of expression in cell lines (Vazquez et al., 2003). This work points out the importance of studying TRPC channel activation and regulation in the physiological context of differentiated cells, like sperm. Indeed, in function of its tissue localization, TRPC2 is activated by different mechanisms: in sperm and erythroblast, TRPC2 activation appears dependent on store depletion, contrary to neurons of the vomeronasal organ, where TRPC2

activation is dependent on an increase of DAG concentration. In sperm and in erythroblasts, long splice variants were detected contrary to neurons of the vomeronasal organ, where only the short splice variant is expressed (Hoffmann et al., 2000). This difference in the mechanisms of activation is not due to the absence of the IP3R binding motif in the short splice variant since the carboxyl-terminus of both splice variants are identical. This difference is rather due to a specific targeting of TRPC2 in sperm in microdomains of plasma membrane specialized in interactions with the acrosome membrane. So far, no proteins have been described to bind to the first specific 300 amino acids of the long splice variants of TRPC2. Enkurin, a new partner of TRPC channels which bind to amino-terminus of TRPC (Sutton et al., 2004), is probably not be involved in specific targeting since this protein is expressed in both testis and vomeronasal organ.

Finally, different TRPC channels are also present in acrosomal crescent of the anterior sperm head like TRPC1 and TRPC5 (Sutton et al., 2004). Therefore, it would be interesting to test the ability of junctate to bind to and regulate these different channels, as well.

Ackowledgements:

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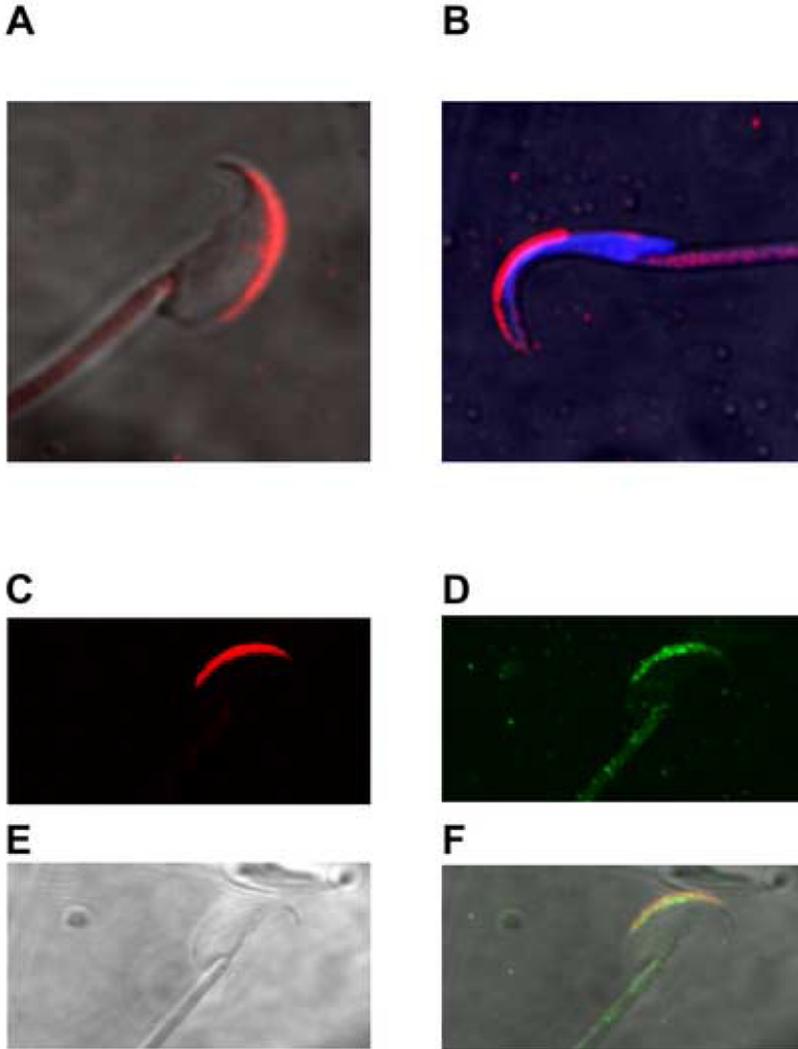**Figure 1**

Junctate is present in sperm

Panel A: cDNA products from brain (B) and testis (T) obtained after RT PCR on poly A mRNA using junctate specific primers. The testis 185 bp product sequence aligns with junctate sequence (data not shown). Panel B: Western blot showing the presence of junctate in a membrane fraction enriched with outer acrosomal membrane from both mouse and rat sperm, using anti-junctate specific antibody. On the left, visualization of proteins standards (lane truncated) used to estimate the molecular weight.
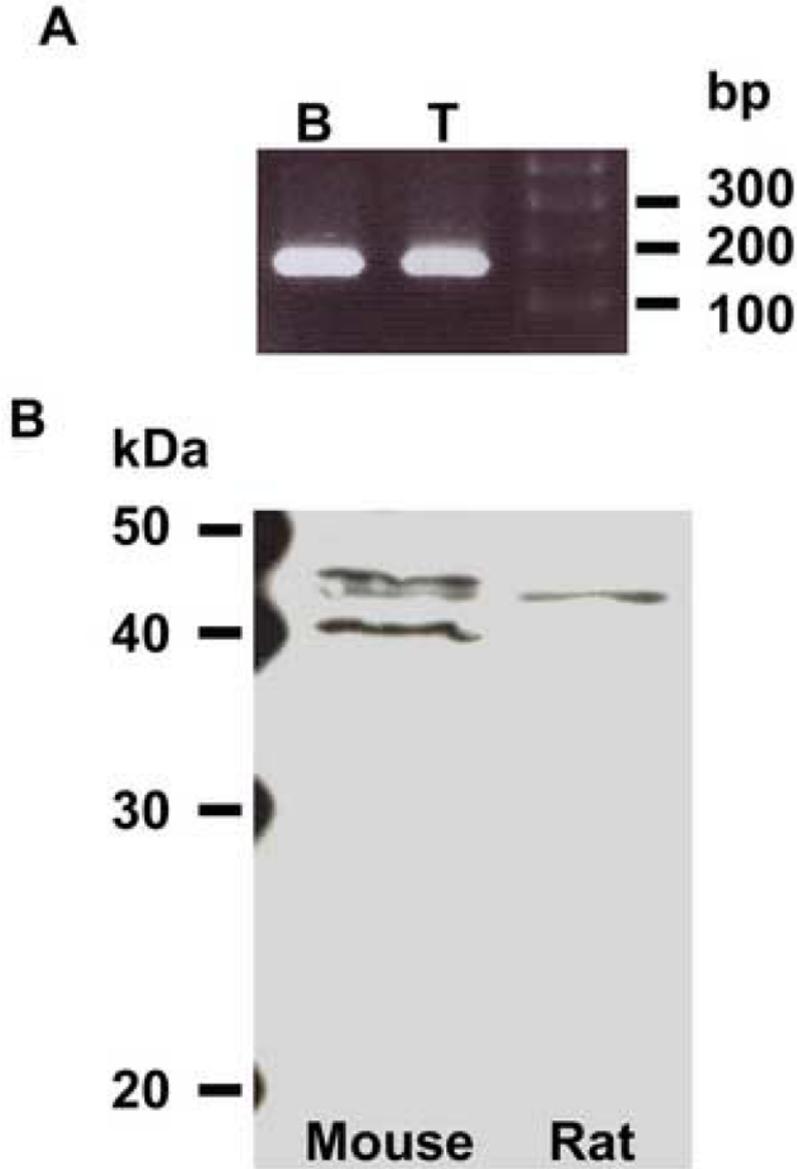
Figure 2

Junctate is present in the acrosomal crescent of the anterior head

Immunolocalization of junctate in sperm head of mouse (A) and rat (B) evidenced with a junctate specific antibody. Junctate is localized in the acrosomal crescent of the anterior head. Blue staining in rat sperm head correspond to the nucleus stained with TOPRO stain. Co-immunolocalization of junctate (C) and IP3R (D) in the same mouse sperm head. (E) transmitted light image (DIC) and (F) overlay of images presented in C, D and E, showing the co-localization of both proteins.
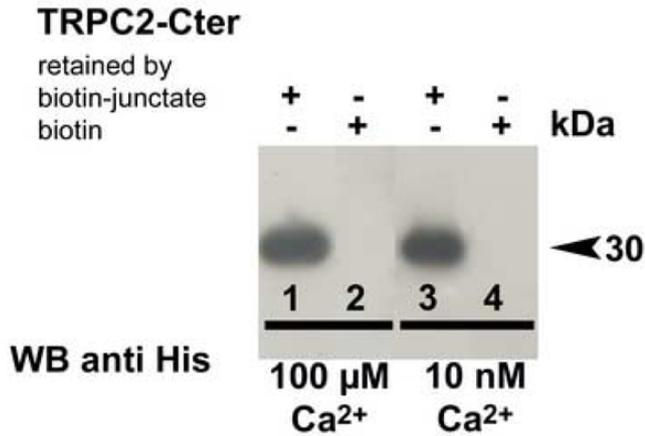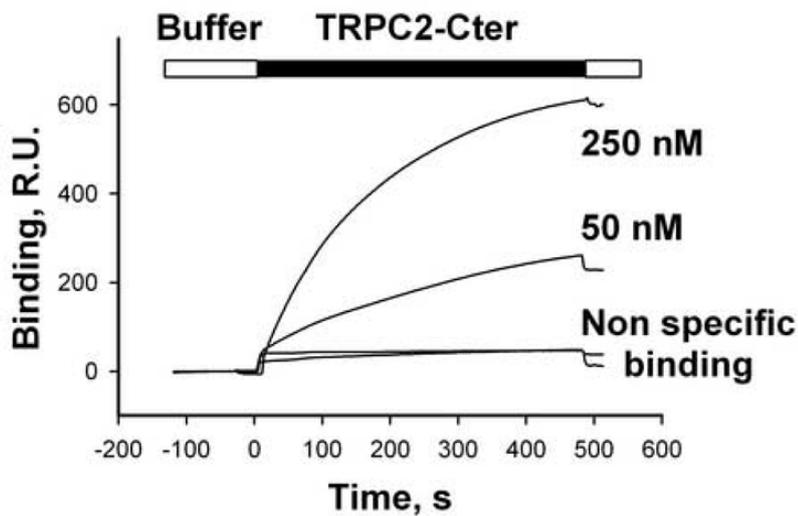
Figure 3

Junctate and TRPC2 CTER are partners

Panel A: Expression of GFP-junctate in HEK-293 cells. Western blot using anti-GFP antibody in HEK-293 cells. Lane 1: protein extract from non-transfected cells; lane 2: protein extract from HEK-293 cells transfected with GFP-junctate. Antibody against GFP immunodecores a protein of around 65 kDa, as expected, since GFP has a molecular weight of 27 kDa and junctate 40 kDa Panel B: Coprecipitation of TRPC2 CTER with GFP-junctate. Protein A beads were coated with an antibody against junctate. Then, two types of cell extracts were incubated with the beads: one from GFP-junctate transfected HEK cells (Labelled "GFP-junctate") and one from non-transfected HEK cells (labelled "control"). Finally, TRPC2 fusion protein (lane 1: Input) was incubated overnight with the beads. Only in the presence of junctate GFP, TRPC2 was immobilized on the beads (Lane 3 bound fraction (B)), the supernatant being depleted in TRPC2 fusion protein (lane 2, non bound fraction (NB)). Lane 4 and 5 correspond to the beads incubated with cell extract without GFP-junctate. TRPC2 is present in the non bound fraction (lane 4) and not in the bound fraction (lane 5).
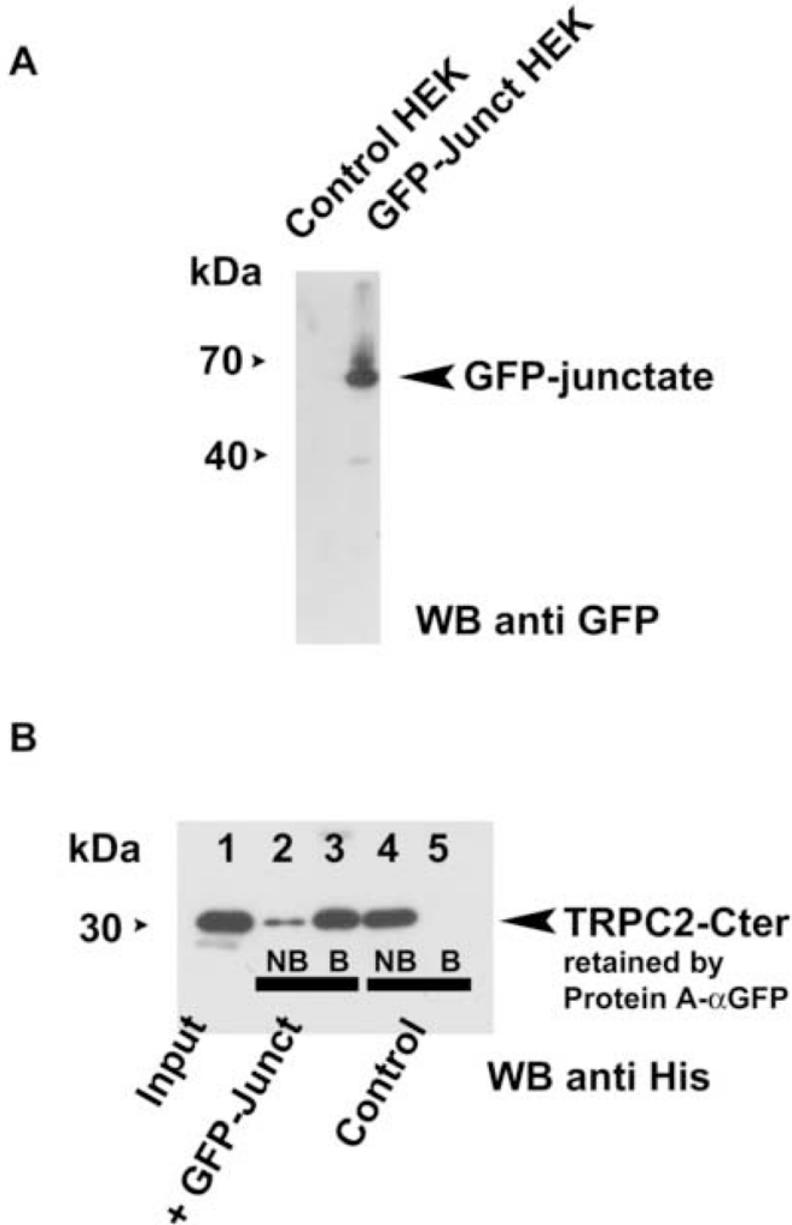
Figure 4

The junctate ammo-terminus interacts with the TRPC2 carboxyl-terminus and this binding is calcium independent

Panel A. Western blot of the bound fractions, showing the binding of TRPC2 on the beads, after pull down of His-TRPC2-Cter with biotinylated junctate-Nter peptide immobilized on streptavidin beads. Biotinylated junctate-Nter peptide was immobilized on streptavidin beads and then incubated with His-TRPC2-Cter. beads coated with biotin only correspond to a control experiment. His-TRPC2-Cter is immunodecorated with an anti-histidin antibody. This experiment was done in two different calcium concentrations: 100 μM (Lanes 1 and 2) and 10 nM (Lanes 3 and 4). Lanes 1 and 3 show the bound fraction in presence of biotinylated junctate-Nter peptide on beads. Lanes 2 and 4 show the bound fraction on beads coated with biotin only (control). Panel B. SPR measurements showing the interaction of TRPC2-Cter (at concentrations of 50 and 250 nM as indicated) with biotinylated junctate-Nter immobilized on the surface of the matrix (total). Controls traces (non specific binding) were obtained with biotin immobilized on the surface of the matrix in a different flow cell and correspond to the non specific TRPC2-Cter on the surface of the matrix (bulk +NS). The running buffer used contained 50 mM Tris, 150 mM- pH 7.4 NaCl and 10 nM free calcium concentration.

Figure 5

Binding site of junctate on TRPC2 is different of IP3 Receptor sites

Panel A: F2q peptide prevents TRPC2 binding on calmodulin-sepharose beads in a calcium-independent manner. Western blot of the bound fractions on the CaM-sepharose beads with an anti histidin antibody, showing the binding of TRPC2. When TRPC2-Cter was incubated with CaM-sepharose beads, TRPC2 was kept within the beads (Lane 1 with [Ca2+] = 100 μM and lane 3 with [Ca2+] = 10 nM). When TRPC2 Cter is preincubated with 20 μM F2q peptide 30 minutes before addition to CaM-sepharose beads, TRPC2 was no longer hold on the beads (Lane 2 with [Ca2+] = 100 μM and lane 4 with [Ca2+] = 10 nM). Panel B: 25 μM junctate peptide is unable to block TRPC2 binding on CaM-sepharose beads. Western blot of the bound fractions on CaM-sepharose beads with an anti histidin antibody, showing the binding of TRPC2. When His-TRPC2-Cter was incubated with CaM-sepharose beads, TRPC2 was kept within the beads (Lane 1 with [Ca2+] = 100 μM) Preincubation with 25 μM biotinylated-junctate-Nter peptide, 30 minutes before addition to CaM-sepharose beads, does not prevent TRPC2 binding on the beads (Lane 2). Panel C: F2q peptide is unable to modify His-TRPC2-Cter binding on biotinylated-junctate-Nter peptide. Biotinylated-junctate-Nter peptide was first bound on streptavidin beads. Western blot of the bound fractions on the streptavidin beads with an anti histidin antibody, showing the binding of TRPC2. This Western blot evidences the specific binding of His-TRPC2-Cter on biotynilated-junctate-Nter peptide immobilized on streptavidin beads (lane 1). Preincubation with 20 μM F2q peptide, 30 minutes before addition to streptavidin beads, does not prevent TRPC2 binding on the beads (Lane 2).
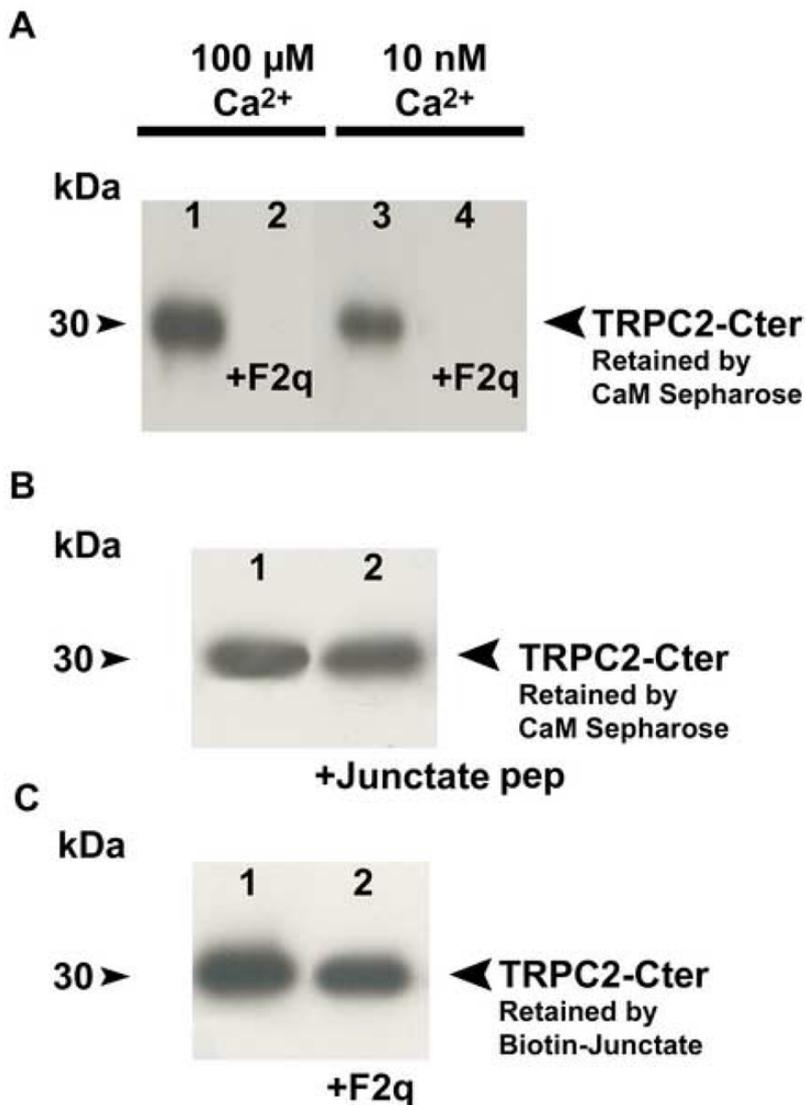
Figure 6

OAG does not promote acrosome reaction

Histograms showing the % of acrosome reacted sperms in different conditions: forty five min after the beginning of capacitation in a medium containing 20 mg/ml BSA, 100 μM OAG, a permeant analogue of DAG, was introduced in the medium of capacitation and sperm were incubated for further 30 min. Three different types of control experiments were performed: 1/sperm incubated during 75 min in a medium with a low concentration of BSA (0.1 mg/ml) that does not support capacitation (bar labeled No Cap), 2/sperm incubated 75 min in the medium supporting the sperm capacitation (with 20 mg/ml BSA) (bar labeled control) and 3/sperm incubated for further 30 min with 10 μM A23187, a calcium ionophore, after an initial 45 min incubation in the capacitation medium (bar labeled A23187). Sperm were then fixed and stained with coomassie G250 to assess the acrosomal status. The number of acrosome reacted sperm were counted. For each experiment, more than 150 sperm cells have been counted n=3 independent experiments. The difference between OAG and control bars is not statistically different (t-test).