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Evidence for the involvement of proline-rich tyrosine kinase 2 in tyrosine phosphorylation downstream of protein kinase A activation during human sperm capacitation

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ABSTRACT: Sperm capacitation involves an increase in intracellular Ca^{2+} concentration as well as in protein kinase A (PKA)-dependent protein tyrosine (Tyr) phosphorylation. Interestingly, in humans, a decrease in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) during capacitation induces an increase in Tyr phosphorylation indicating the complexity of Ca^{2+} signaling during this process. In view of this, in the present study we further investigated the Ca^{2+}-mediated signaling pathways implicated in Tyr phosphorylation during human sperm capacitation. Results revealed that sperm incubation in a medium without added Ca^{2+} ($\ominus Ca^{2+}$) increased Tyr phosphorylation but did not modify PKA-mediated phosphorylation. Moreover, inhibition of either PKA or Src family kinase signaling cascades in $\ominus Ca^{2+}$ down-regulated both PKA substrate and Tyr phosphorylations, indicating that the $[Ca^{2+}]_e$ effects on Tyr phosphorylation depend on PKA targets. Inhibition of calmodulin or Ser/ Thr protein phosphatase 2B also increased Tyr phosphorylation without affecting PKA-mediated phosphorylation, supporting the potential role of these Ca^{2+} downstream effectors in the increase in Tyr phosphorylation observed in $\ominus Ca^{2+}$. Experiments aimed to identify the kinase responsible for these observations revealed the presence of proline-rich tyrosine kinase 2 (PYK2), a focal adhesion kinase (FAK) family member, in human sperm, and the use of PF431396, an FAK inhibitor, supported the involvement of PYK2 in Tyr phosphorylation downstream of PKA activation. Results also showed that PYK2 was activated in $\ominus Ca^{2+}$ as well as during capacitation and that PF431396 affected capacitated sperm motility, acrosome reaction and ability to penetrate both mouse cumulus matrix and zona-free hamster eggs. Together, our observations support PYK2 as an intermediary component of Ca^{2+} signaling between PKA-mediated and Tyr phosphorylations that is required for achieving functional human sperm capacitation.

Key words: calcium / capacitation / human / sperm / tyrosine phosphorylation

Introduction

Sperm are highly specialized cells with only one commitment, that is to reach and fertilize the egg. To accomplish this, mammalian ejaculated sperm require first to undergo a process called capacitation which occurs during their transit through the female reproductive tract towards the site of fertilization. The capacitation process encompasses structural and functional changes in sperm that confer the ability to undergo both the acrosome reaction (AR) and hyperactivated motility, key events

for penetration of the egg investments (i.e. *Cumulus oophorus* and zona pellucida) and gamete fusion (Chang, 1951; Austin, 1952). The bulk of evidence shows that Ca^{2+} signaling plays a major role in all these important events (Yanagimachi, 1994; Darszon et al., 2011). During capacitation, there is an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ruknudin and Silver, 1990; Baldi et al., 1991) which is attained and decoded by a complex set of molecules present in mature sperm (Jimenez-Gonzalez et al., 2006; Publicover et al., 2007). Whereas Ca^{2+} influx is achieved via voltage-, stored-operated and ligand-gated

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channels (i.e. VOCCs, SOCs and CatSper), its clearance relies on ATP-driven Ca^{2+} pumps (i.e. plasma membrane Ca^{2+} ATPases) and Na^+/Ca^{2+} exchangers. Intracellular Ca^{2+} is mobilized to or from internal stores present in the head (acrosome) as well as in the neck (redundant nuclear envelope), likely through inositol triphosphate and ryanodine receptors and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (Darszon et al., 2011). In addition, $[Ca^{2+}]_i$ is sensed by calmodulin (CaM), an ubiquitous small protein which regulates a variety of Ca^{2+}/CaM-dependent sperm proteins such as kinases (i.e. CaM kinases (CaMKs) and a Src family kinase (SFK) member (c-yes)), phosphodiesterases (PDE1) and phosphatases (i.e. protein phosphatase 2B (PP2B)) (Wasco and Orr, 1984; Tash et al., 1988; Carrera et al., 1996; Leclerc and Goupil, 2002; Marin-Briggiler et al., 2005). Interestingly, other proteins, such as the testis-specific soluble adenylyl cyclase (Adcy10, also known as sAC) (Chen et al., 2000), are Ca^{2+}-dependent but CaM-independent (Jaiswal and Conti, 2003; Litvin et al., 2003), suggesting that Ca^{2+} affects capacitation via multiple pathways.

The molecular mechanisms involved in the Ca^{2+}-mediated signaling underlying capacitation are known to some extent. Upon entry of both Ca^{2+} and HCO_3^- into sperm, the levels of cAMP increase by the opposing activities of sAC and PDE, and activate a protein kinase A (PKA)-dependent phosphorylation of proteins in serine/threonine (Ser/Thr) residues (Visconti et al., 2011). In this regard, recent studies in mouse and humans revealed that PKA-dependent phosphorylation is regulated not only by the activation of the cAMP/PKA pathway but also by the SFK inactivation of Ser/Thr phosphatases (Krapf et al., 2010; Battistone et al., 2013). Phosphorylation of PKA substrates finally leads to protein phosphorylation in tyrosine (Tyr) residues in all the mammalian species studied so far (Visconti et al., 1995, 1999; Leclerc et al., 1996; Galantino-Homer et al., 1997; Osheroff et al., 1999; Da Ros et al., 2004). However, how PKA activation results in the increase in Tyr phosphorylation is still not well understood. Interestingly, in human sperm, a decrease in extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) during in vitro capacitation induces an increase in Tyr phosphorylation (Carrera et al., 1996; Luconi et al., 1996; Leclerc and Goupil, 2002; Marin-Briggiler et al., 2003; Baker et al., 2004), indicating the complexity of Ca^{2+} signaling during this process.

In view of this, in the present study we further explored the signaling pathways implicated in both PKA-dependent and Tyr phosphorylations in human sperm with the aim of identifying the Ca^{2+} signaling molecules involved in Tyr phosphorylation during capacitation. Our results support a role for proline-rich tyrosine kinase 2 (PYK2), a member of the focal adhesion kinase (FAK) family, as a Ca^{2+} signaling component implicated in Tyr phosphorylation but not in PKA substrate phosphorylation and required for achieving functional human sperm capacitation.

Materials and Methods

Materials

The following chemicals (catalog number in brackets) were used: bovine serum albumin (BSA) (A7906), cyclosporin A (CsA) (C-3662), igepal CA-630 (I-3021), ethylene glycol-bis(2-aminoethylether)-N,N,N,N' tetraacetic acid (EGTA) (E-3889), N6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP) (D-0627), 3-isobutyl-1-methylxanthine (IBMX) (I-5879), N-[2-[[[2-[(2,3-dihydro-2-oxo-1H-indol-5-yl)amino]-5-(trifluoromethyl)-4-pyrimidinyl]amino]methyl]phenyl]-N-methyl-methanesulfonamide hydrate (PF431396) (PZ0185), Pisum sativum agglutinin coupled

to fluorescein isothiocyanate (PSA-FITC) (L0770), anti-β-tubulin antibodies (clone D66) and hCG (C610) from Sigma (St. Louis, MO, USA). N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) (681629), N-(6-aminohexyl)-1-naphthalenesulfonamide (W5) (681625) and N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89) (10010556) from Calbiochem (Billerica, MA, USA). 4-(2,4-dichloro-5-methoxyphenylamino)-6-methoxy-7-(3-(4-methylpiperazin-1yl)propoxy) quinoline-3-carbonitrile (SKI606) (S1014) from Selleck Chemical (Houston, TX, USA). 9,10-deepithio-9,10-didehydroacanthifolicin (okadaic acid, OA) (495604) from Cayman Chemical Company (Cayman, MI, USA). Equine chorionic gonadotrophin (eCG) (NV4811) from Syntex (Buenos Aires, Argentina). Anti-phosphotyrosine antibody (clone4G10) from Millipore (Billerica, MA, USA), anti-phospho-PKA substrates (clone100G7E), anti-PYK2 (3292s), anti-FAK (3285), anti-phospho-PYK2 (3291s) antibodies from Cell Signaling (Danvers, MA, USA) and anti-FAK (C-20, sc-558) antibody from Santa Cruz Biotechnology (Dallas, TX, USA).

Culture media

The modified Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971) used in this study contained (in mM): $NaHCO_3$ 25, NaCl 94.5, KCl 4.8, $CaCl_2$ 1.7, KH_2PO_4 1.17, $MgCl_2$ 1.22, Na-Pyruvate 0.3, Na-lactate 25.7, glucose 5.5 and HEPES 10 supplemented with BSA (2.6% p/v). This medium has been proved to successfully support human sperm capacitation (Battistone et al., 2013). Because medium without Ca^{2+} still contains micromolar concentrations of the ion, it is considered a nominal zero Ca^{2+} medium, indicated here by $\ominus$ Ca^{2+}. All media were adjusted to pH 7.4.

Ethical approval

Approval for the study protocol was obtained from the Bioethics Committee of the Institute of Biology and Experimental Medicine from the National Research Council (CONICET, Buenos Aires, Argentina), and the Institutional Review Board of the University of Massachusetts (Amherst, MA, USA). All human donors were provided with written information about the study prior to giving informed consent. Experiments involving animals were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health.

Sperm preparation and capacitation

All procedures involving human semen samples were conducted according to the World Health Organization recommendations (WHO, 2010). Ejaculated human semen were obtained by masturbation from 15 healthy donors (aged 20–35 years) with no known fertility problems, collected into a sterile plastic container and transported immediately to the laboratory. After liquefaction, motile sperm were recovered by standard swim-up technique in medium without HCO_3^-, diluted to a final concentration of 1×10^7 cells/ml and incubated for different periods (1–18 h) at 37°C as previously described (Battistone et al., 2013). Alternatively, incubation was carried out in media containing the following compounds: a CaM antagonist, W7 (0–100 μM); a PP2B inhibitor, CsA (0–20 μM); a cAMP analog, dbcAMP (5 mM) and an inhibitor of PDE, IBMX (0.2 mM); a PKA inhibitor, H89 (30 μM); a SFK inhibitor, SKI606 (30 μM); a Ser/Thr phosphatase inhibitor, OA (100 nM) or an FAK family inhibitor, PF431396 (0–30 μM). At the end of the incubation, aliquots were recovered and sperm were analyzed for the following end-points: (i) protein phosphorylation (both PKA substrate and Tyr phosphorylations), (ii) functional parameters (motility, hyperactivation and AR) and (iii) fertilization competence (ability to penetrate mouse cumulus-oocyte complex (COCs) and zona-free hamster oocytes) as described below.

Sperm viability was assessed by dye exclusion using 0.5% (v/v) eosin Y (Sigma) and the percentage of viable sperm was calculated as the number

of sperm that did not incorporate the dye over the total number of sperm counted under the light microscope ($\times 400$).

Protein extracts

Sperm ($1-2 \times 10^6$) were washed and proteins were solubilized in Laemmli sample buffer (Laemmli, 1970) and heated at $100°C$ for 5 min. For immunoprecipitation experiments, sperm (1×10^7) were lysed in a buffer containing (in mM) Tris−HCl 50 pH 7.5, EDTA 1, EGTA 1, NaCl 150, MgCl$_2$ 1 and glycerol 10% (v/v), Triton X-100 1% (v/v), then centrifuged at 13 400g for 10 min, and the soluble and insoluble fractions were incubated with Laemmli sample buffer and boiled at $100°C$ for 5 min. As PYK2 was insoluble after this treatment, sperm were lysed in the same buffer but containing Igepal 1% (v/v) and sodium dodecyl sulfate (SDS) 0.1% (w/v) instead of Triton X-100, incubated at $100°C$ for 5 min and then subjected to the same procedures, as described above.

Western blotting analysis

The phosphorylation status of proteins was assessed by SDS−PAGE (7.5% polyacrylamide) and western blotting as previously described (Battistone et al., 2013) using anti-phospho-PKA substrate (1:1000) or anti-phosphoTyr (1:3000) antibodies. The specificity of these antibodies has been shown in previous work. For the detection of FAK family proteins, the following antibodies were used: anti-FAK (1:1000), anti-PYK2 (1:200) or anti-phospho-PYK2 (1:1000) antibodies. The membranes blotted with anti-PYK2 were then stripped and reprobed with anti-phospho-PYK2 (Tyr 402) or vice versa. Protein loading was analyzed by β-tubulin immunoblot (anti-β-tubulin antibody: α-tub; 1:5000). In all the cases, the reactive bands were detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA) and images captured with G:BOX GENI (Syngene, Synoptics, Ltd, Cambridge, England) according to the manufacturer's instructions. A representative blot from each set of experiments is shown.

Immunoprecipitation

Solubilized proteins were incubated with anti-PYK2 antibodies (2 μg) with constant agitation for 18 h at $4°C$, and subsequently incubated with 20 μl of protein A-Agarose (Santa Cruz Biotechnology). The proteins bound to the agarose−antibody complexes were recovered by centrifugation at 13 400g for 10 min, washed three times and eluted with Laemmli sample buffer, boiled at $100°C$ for 5 min and subjected to SDS−PAGE and western blotting as described above.

Computer-assisted sperm analysis

Aliquots of sperm incubated under different conditions for 18 h were placed in a pre-warmed 20 μm chamber (Leja Slide, Spectrum Technologies, Healdsburg, CA, USA) and examined using the CEROS computer-assisted sperm analysis (CASA) system (Hamilton Thorne, Inc., Beverly, MA, USA) (Mortimer et al., 1998) at $37°C$. For each sample, a minimum of 200 cells distributed in at least 20 different microscopy fields were scored (30 frames acquired at 60 Hz for each measurement). The following parameters were measured: average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), linearity (LIN, %), amplitude of lateral head displacement (ALH, μm) and straightness (STR, %) as previously described (Battistone at al., 2013). Sperm were considered progressively motile when presenting VAP > 25 and STR > 80%, and hyperactivated when exhibiting VCL ≥ 100, LIN < 60% and ALH ≥ 5 (Mortimer and Mortimer, 1990). A minimum of four independent experiments per treatment was carried out, using sperm from different donors each time.

AR assays

Sperm were incubated under different conditions for 18 h and then evaluated for both spontaneous and induced AR. For AR induction, sperm were exposed to 25 μM progesterone during the last 30 min of the incubation. Acrosomal integrity was evaluated as previously described (Battistone et al., 2013) using the acrosomal content marker PSA coupled to FITC. PSA labels acrosome-intact sperm and it is absent in acrosome-reacted sperm, as described elsewhere (Cross, 1996). Two hundred spermatozoa were evaluated in each treatment slide under a Nikon Optiphot microscope equipped with epifluorescence optics ($\times 1250$), and the percentage of spermatozoa without an intact acrosome was scored.

Cumulus penetration analysis

Adult ($30-60$ days old) female B6129F2 mice were maintained with food and water *ad libitum* in a temperature controlled room with light:dark (12:12) cycle. Females were superovulated by an i.p. injection of 5 IU of eCG, followed by i.p. administration of 5 IU of hCG 48 h later. The COCs recovered from the oviducts $14-16$ h after hCG administration were washed in BWW and distributed in groups of $10-15$ per treatment. An aliquot of human sperm incubated for 18 h in BWW either alone or containing PF431396 (10 or 30 μM) or in $\ominus$ Ca^{2+} was incubated with Hoechst 33342 (3 μg/ml final concentration, Sigma) for 10 min. After washing, sperm (1×10^4) were added to each group of COCs and co-incubation proceeded for 15 min (at $37°C$ and 5% CO$_2$) in the same medium used for capacitation in each treatment group. In those cases in which sperm were capacitated in $\ominus$ Ca^{2+}, co-incubation was carried out in complete BWW. Sperm exposed to PF431396 (30 μM) only during gamete co-incubation was used as a control. At the end of co-incubation, cells were washed and fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min at room temperature. After washing, cells were mounted on slides and examined under a Nikon Optiphot microscope equipped with epifluorescence optics ($\times 250$) to score the number of fluorescent human sperm heads within the *C. oophorus*.

Zona-free hamster oocyte penetration assay

Analysis of zona-free hamster oocyte penetration was performed as previously described (Cohen et al., 2001). Briefly, zona-free oocytes were prepared from COCs recovered from superovulated immature hamsters (*Mesocricetus aureatus*) maintained with food and water *ad libitum* in a temperature controlled room with light:dark (14:10) cycle. An aliquot of motile human sperm (2×10^5 cells/20 μl) incubated for 18 h in BWW either alone or containing PF431396 (30 μM) or in $\ominus$ Ca^{2+} was added to $10-15$ hamster oocytes in BWW (80 μl). Sperm exposed to 6 μM PF431396 (concentration calculated based on the residual amount of inhibitor present in the insemination drop) only during gamete co-incubation was used as a control. After 2.5 h of co-incubation, cells were fixed with 2.5% glutaraldehyde, stained with 1% aceto-carmine solution, observed under the microscope ($\times 400$) and the number of oocytes presenting either decondensing sperm heads or pronuclei and sperm tails in the ooplasm was recorded.

Calculations and statistical analysis

All the experiments were repeated at least three times and values are presented as mean ± SEM. Calculations were performed using the Prism 3.0 software (GraphPad Software, La Jolla, CA, USA). Statistical significance of the data was analyzed using the χ^2 test for the percentages of acrosome-reacted sperm and penetrated oocytes, one-way analysis of variance for the CASA determinations and Student's t-test for number of sperm within the cumulus. In all cases, P-values <0.05 were considered statistically significant.

Results

Effect of $[Ca^{2+}]_e$ on protein phosphorylation

To study the effects exerted by Ca^{2+} during human sperm capacitation, different signaling events associated with this process were evaluated as a function of $[Ca^{2+}]_e$. After 6-h incubation, PKA substrate phosphorylation, which had not yet been analyzed in relation to $[Ca^{2+}]_e$, as well as Tyr phosphorylation were examined by western blotting using the corresponding specific antibodies. As previously reported (Osheroff et al., 1999; Battistone et al., 2013), incubation of human sperm in complete or HCO_3^--free BWW promoted or abrogated both PKA-dependent and Tyr phosphorylations, respectively (Fig. 1A). However, whereas similar patterns of PKA substrate phosphorylation were observed at all $[Ca^{2+}]_e$ tested, Tyr phosphorylation levels increased as a function of decreasing $[Ca^{2+}]_e$ (Fig. 1A). Similar results were obtained for sperm capacitated in BWW without added Ca^{2+} (nominal zero Ca^{2+} medium here indicated by $\ominus$ Ca^{2+}) and containing EGTA to chelate residual Ca^{2+} (Fig. 1B). When sperm were incubated in $\ominus$ Ca^{2+} containing different HCO_3^- concentrations to modulate the induction of capacitation, once again PKA-dependent phosphorylation did not show substantial modifications under any condition assayed, whereas Tyr phosphorylation positively correlated with HCO_3^- concentrations showing bands more intensively stained than those corresponding to sperm incubated in regular BWW (Fig. 1C). Finally, when different incubation periods (1 min, 1 h, 6 h or 18 h) were evaluated, no significant modifications in PKA substrate phosphorylation were observed in either BWW or $\ominus$ Ca^{2+}, whereas Tyr-phosphorylated proteins were detected earlier in $\ominus$ Ca^{2+} than in control medium (Fig. 1D). As no differences in phosphorylation levels were observed between 6 and 18 h of incubation, subsequent signaling experiments were performed using a 6-h incubation period. Together, these results indicate that $[Ca^{2+}]_e$ modulates the level of Tyr phosphorylation without directly affecting PKA-dependent phosphorylation.

The increase in Tyr phosphorylation observed in the absence of Ca^{2+} might occur either independently of PKA activation or mediated by a Ca^{2+}-dependent mechanism downstream of PKA. To evaluate these hypotheses, PKA activation was inhibited by two different approaches. First, PKA was directly inhibited by exposing sperm to H89 in either $\ominus$ Ca^{2+} or BWW. Results showed that H89 (30 μM) abolished both PKA substrate and Tyr phosphorylations in $\ominus$ Ca^{2+} as well as in complete media (Fig. 2A). As an alternative approach, sperm were incubated in $\ominus$ Ca^{2+} or BWW containing SKI606, an SFK inhibitor which affects PKA substrate phosphorylation (Krapf et al., 2010; Battistone et al., 2013). Similar to H89, SKI606 (30 μM) inhibited PKA substrate phosphorylation as well as the increase in Tyr phosphorylation in both media (Fig. 2B). Together, these results indicate that the increase in Tyr phosphorylation observed in $\ominus$ Ca^{2+} is downstream of PKA activation.

We next evaluated the potential involvement of Ca^{2+} downstream effectors such as CaM and one of its targets, PP2B, on both PKA-dependent and Tyr phosphorylations. For this purpose, sperm incubations were carried out in BWW containing the CaM antagonist W7 (using W5 as a control) (Fig. 3A) or the PP2B inhibitor CsA (Fig. 3B). Under these conditions, although no changes in PKA-dependent phosphorylation were observed, Tyr phosphorylation positively correlated with increasing concentrations of each inhibitor, supporting a role for both CaM and PP2B in a Ca^{2+}-dependent signaling cascade leading to Tyr phosphorylation.

Involvement of PYK2 in protein phosphorylation

Activation of FAK proteins during stallion sperm incubation in $\ominus$ Ca^{2+} (Gonzalez-Fernandez et al., 2013) led us to investigate whether these proteins could be responsible for the Tyr phosphorylation increase observed in human sperm. Western blotting analysis using different specific antibodies against each of the two family members, i.e. FAK and PYK2 revealed the presence of PYK2 but not of FAK in human ejaculated sperm (Fig. 4A). Based on this, we next evaluated the effect of PF431396 (0–30 μM), a FAK/PYK2 inhibitor (Buckbinder et al., 2007; Han et al., 2009), on Tyr phosphorylation in $\ominus$ Ca^{2+} with the premise that PYK2 inhibition could prevent the increase in Tyr phosphorylation observed under this condition. Results showed that PF431396 concentrations >3 μM produced a slight increase in PKA-dependent phosphorylation, while blocking almost completely the increase in Tyr phosphorylation (Fig. 4B). To confirm that PF431396 was indeed inhibiting sperm PYK2, we evaluated the PYK2 activation state by determining the trans/autophosphorylation levels of this kinase (Avraham et al., 2000). Western blotting results using commercial antibodies against total PYK2 and phospho-PYK2 (Tyr 402) showed that when PF431396 was present in the medium, PYK2 levels remained constant while anti-phospho-PYK2 signal decreased (Fig. 4C). Interestingly, the intensity of the phospho-PYK2 band increased in $\ominus$ Ca^{2+} (Fig. 4C) as well as in BWW containing the CaM antagonist W7 (50 μM) (Fig. 4D). Altogether, these results support PYK2 as the candidate Tyr kinase responsible for the phosphorylation cascade observed in $\ominus$ Ca^{2+} in human sperm.

Based on these observations, we then explored the behavior of PYK2 during sperm capacitation in complete media. Western blotting results showed that phospho-PYK2 was not detected in fresh ejaculated sperm but increased in a time-dependent manner during capacitation, whereas total PYK2 levels remained constant (Fig. 5A). To confirm that the band detected was indeed PYK2, sperm extracts were immunoprecipitated with anti-PYK2 antibody and then analyzed by western blotting using the anti-phospho-PYK2 antibody. When Triton X-100 was used for sperm extract preparation, the bands corresponding to both total PYK2 and phospho-PYK2 were detected in the insoluble but not in the soluble fraction (Fig. 5B). Due to the insolubility of PYK2 in Triton X-100, sperm proteins were extracted using more stringent conditions (boiling of samples in 0.1% SDS and 1% Igepal), which allowed the solubilization of PYK2 as judged by its detection only in the soluble fraction (Fig. 5C). When this solubilized fraction was subjected to immunoprecipitation with anti-PYK2 and the immunoprecipitate analyzed by western blotting, a band corresponding to phospho-PYK2 was detected in capacitated but not in non-capacitated cells (Fig. 5D), confirming the previous direct western blot results indicating that PYK2 is indeed activated during sperm capacitation. This was further supported by the finding that PF431396 inhibited PYK2 phosphorylation without affecting total PYK2 (Fig. 5E).

To study the involvement of PYK2 in capacitation-induced phosphorylation, sperm were incubated in BWW with or without PF431396 and both PKA-dependent and Tyr phosphorylations were evaluated. Results showed that although PKA substrate phosphorylation slightly increased as a function of the inhibitor concentration, Tyr phosphorylation
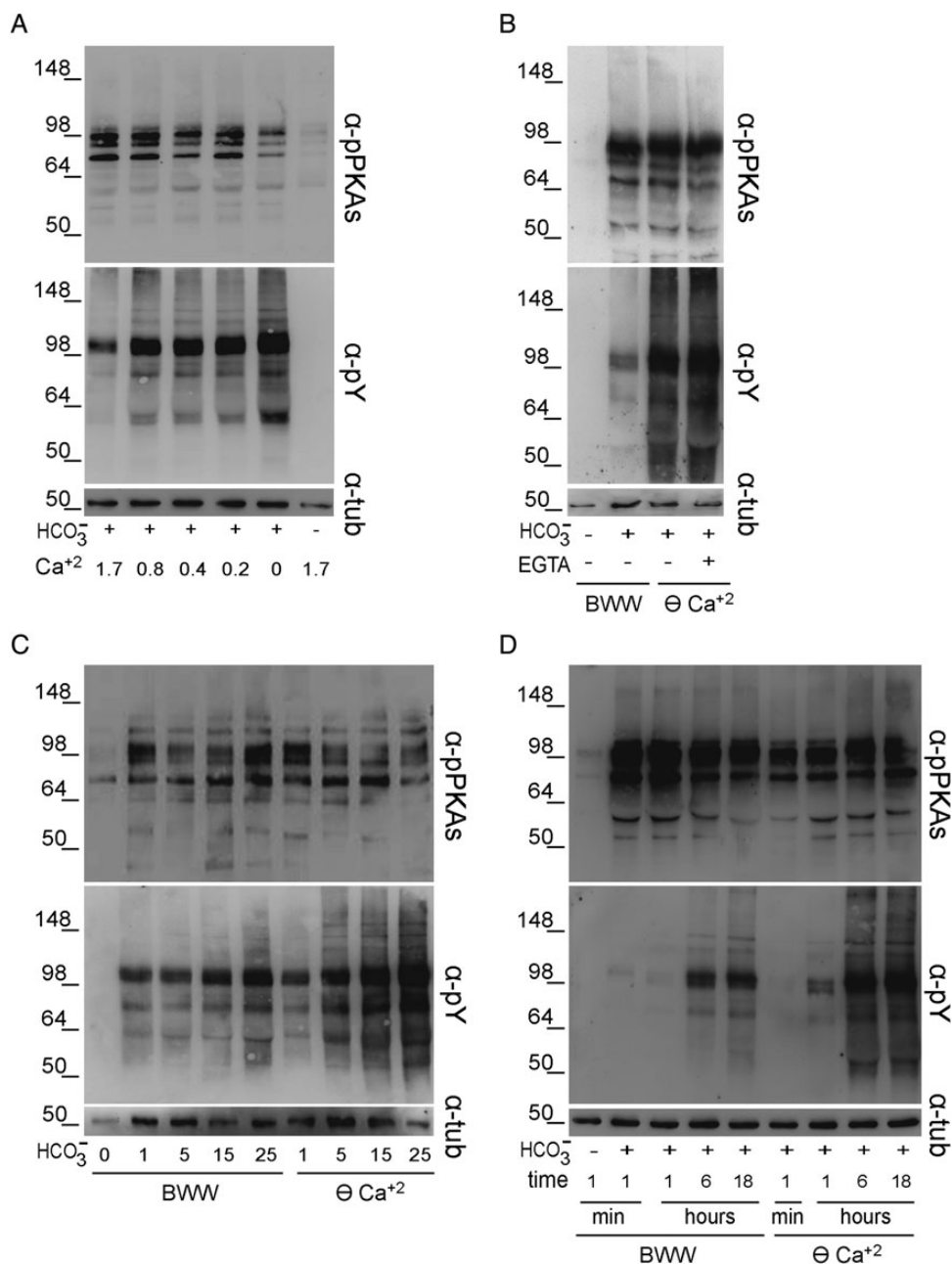
Figure 1 Effect of $[Ca^{2+}]_e$ on both PKA substrate and Tyr phosphorylations. (**A**) Human sperm were incubated for 6 h in BWW either lacking HCO_3^- or containing different concentrations of Ca^{2+} ($n = 4$). (**B**) Sperm were incubated for 6 h in BWW (1.7 mM Ca^{2+}) or in BWW without added Ca^{2+} (nominal zero Ca^{2+} medium indicated by $\ominus Ca^{2+}$) with or without EGTA (1 mM) ($n = 4$). (**C**) Sperm were incubated for 6 h in BWW or in $\ominus Ca^{2+}$ containing different concentration of HCO_3^- ($n = 3$). (**D**) Sperm were incubated in BWW or in $\ominus Ca^{2+}$ for different time periods ($n = 5$). In all cases, protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively. β-Tubulin was used as the protein loading control.

decreased and was completely abolished at concentrations >5 μM (Fig. 5F), indicating a role for PYK2 downstream of PKA activation. Indeed, the inhibition of PKA activity by H89 reduced PYK2 phosphorylation, whereas total PYK2 levels remained constant (Fig. 5G).

To gain insights into the mechanisms leading to PYK2 activation during capacitation, sperm were incubated under different conditions known to promote Tyr phosphorylation and the effect of PYK2 inhibition on both

PKA-dependent and Tyr phosphorylations was evaluated. Results showed that exposure of sperm to dbcAMP and IBMX (5 mM and 200 μM, respectively), which stimulates the cAMP/PKA signaling cascade, produced an enhancement of both PKA-dependent and Tyr phosphorylations (Fig. 6A). However, whereas PF431396 did not modify the stimulated PKA substrate phosphorylation, it substantially reduced the increase in Tyr phosphorylation (Fig. 6A).
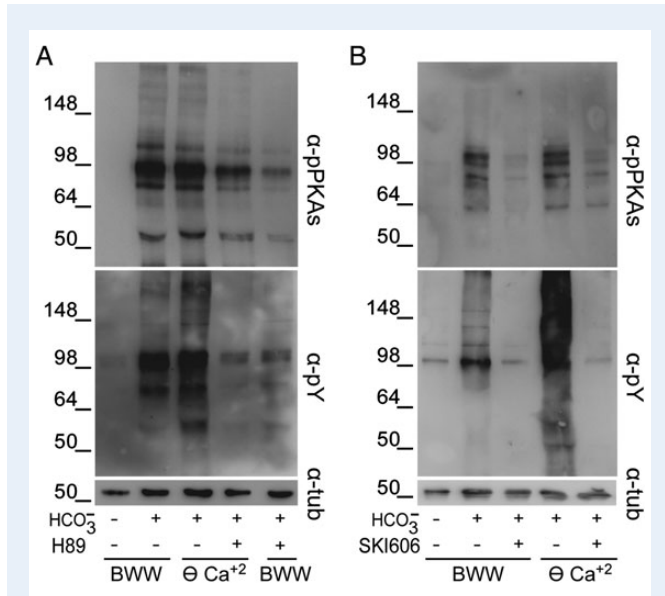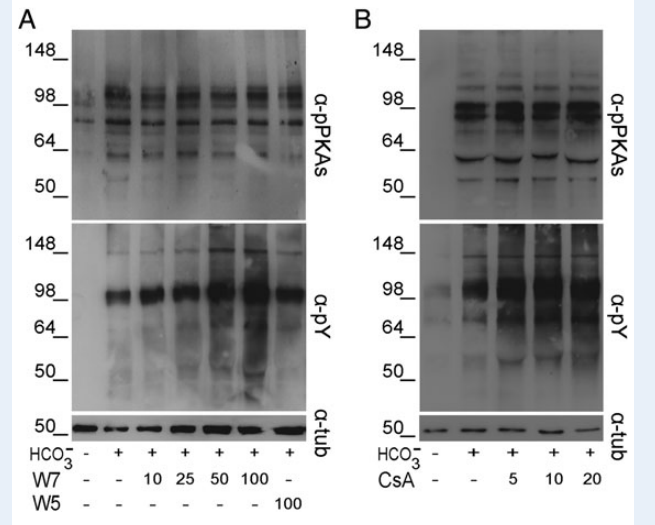
Figure 2 Effect of the inhibition of cAMP/PKA or SFK/phosphatase signaling pathways on the $[Ca^{2+}]_e$-dependent increase in protein phosphorylation. Human sperm were incubated for 6 h in BWW or in $\ominus$ Ca^{2+} containing either (**A**) the PKA inhibitor H89 (30 μM) or (**B**) the SFK inhibitor SKI606 (30 μM). In all cases, protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively ($n = 3$). β-Tubulin was used as the protein loading control.


Figure 3 Effect of CaM or PP2B inhibitors on protein phosphorylation. Human sperm were incubated for 6 h in BWW containing either (**A**) different concentrations of the CaM antagonist W7 or its inactive analog W5 or (**B**) different concentrations of the PP2B inhibitor CsA. In all cases, protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively ($n = 5$). β-Tubulin was used as the protein loading control.

To analyze whether the PF431396 effects could be overcome by exposure of sperm to OA, a Ser/Thr phosphatase inhibitor that increases Tyr phosphorylation (Krapf et al., 2010; Battistone et al., 2013), the effect of the simultaneous addition of both inhibitors on PKA-dependent and Tyr phosphorylations was evaluated. Contrary to our observations using the SFK inhibitor, PF431396 did not block PKA substrate phosphorylation and exposure to relatively high concentrations of OA (100 nM) could not overcome the inhibitory effect on Tyr phosphorylation (Fig. 6B). Altogether, these results indicate that the inhibition of PYK2 blocks the increase in Tyr phosphorylation downstream of PKA substrate phosphorylation.

Relevance of PYK2 for sperm function

We next evaluated the functional relevance of PYK2 by testing the effect of its modulation on two well-known capacitation-associated events such as hyperactivation and AR. Results showed that similarly to what was observed in $\ominus$ Ca^{2+}, exposure of sperm to PF431396 significantly reduced most (i.e. all except LIN and STR) (10 μM) or all (30 μM) of the motility parameters examined as well as the percentage of hyperactivation without modifying total motility (Table I). Regarding the occurrence of the AR, although both the spontaneous and the progesterone-induced AR rates were reduced in $\ominus$ Ca^{2+}, addition of PF431396 (10 or 30 μM) during capacitation significantly inhibited the progesterone-induced AR without modifying the spontaneous AR rates (Fig. 7A). Evaluation of sperm viability at the end of the incubation with PF431396 30 μM confirmed the lack of inhibitor toxicity on sperm (inhibitor 92.0% ± 2.9 versus control 93.4% ± 5.4, $n = 4$, NS).

To evaluate the relevance of PYK2 for sperm fertilizing ability, two different functional assays were used. First, we set up a heterologous penetration assay that examines the ability of human sperm to penetrate the mouse cumulus oophorus matrix. Results showed that sperm incubated in non-capacitating media (i.e. $\ominus$ Ca^{2+} or HCO_3^--free BWW) showed significantly lower penetration ability than those incubated under capacitating conditions, validating the use of this assay for the evaluation of the sperm functional capacitation state (Fig. 7B). When this assay was carried out using sperm exposed to PF431396 (10 or 30 μM) during the whole capacitation period, a clear reduction in the ability of sperm to penetrate the mouse cumulus matrix was observed compared with controls (Fig. 7B), whereas no differences were observed when the inhibitor (30 μM) was added only during the 15 min-gamete co-incubation period (Fig. 7B). As a second approach to analyze the functional state of sperm, we evaluated the ability of human sperm to penetrate zona-free hamster oocytes (WHO, 2010). As expected, sperm incubated in $\ominus$ Ca^{2+} exhibited a significant decrease in their ability to penetrate zona-free hamster eggs compared with controls. An inhibitory effect was also observed when sperm were capacitated in the presence of PF431396 (30 μM) but not when the inhibitor was present only during the gamete co-incubation period (Fig. 7C). Altogether, these results are consistent with a role of PYK2 in the acquisition of human sperm fertilizing ability during capacitation.

Discussion

Ca^{2+}-mediated signaling has been extensively studied in relation to sperm physiology but only recently have these signaling components been associated with fertility problems (Khattri et al., 2012; Smith et al.,
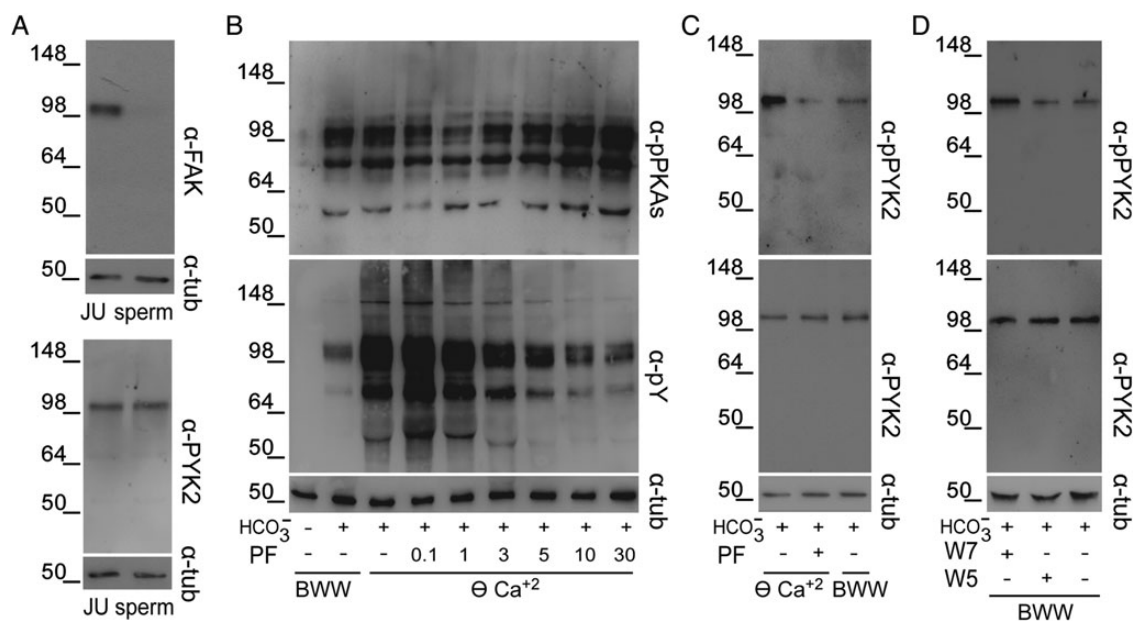
Figure 4 Presence of PYK2 in human sperm and its activation in $\ominus$ Ca^{2+}. (**A**) Jurkat cells (JU), as a positive control for FAK and PYK2 expression, or sperm protein extracts were analyzed by western blotting using α-FAK or α-PYK2 antibodies ($n = 3$). (**B**) Sperm were incubated for 6 h in $\ominus$ Ca^{2+} containing different concentrations of the FAK/PYK2 inhibitor PF431396, and protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively ($n = 4$). (**C**) Sperm were incubated for 6 h in BWW or in $\ominus$ Ca^{2+} with or without PF431396 (10 μM). Protein extracts were analyzed by western blotting using α-pPYK2 antibody and the membrane was then stripped and reprobed with α-PYK2 antibody ($n = 4$). (**D**) Sperm were incubated for 6 h in BWW with or without W7 (50 μM) or its analog W5 (50 μM) and protein extracts were analyzed by western blotting using α-PYK2 and then α-pPYK2 antibodies ($n = 4$). β-Tubulin was used as the protein loading control.

2013; Mohamed et al., 2013; Alasmari et al., 2013a; Tamburrino et al., 2014). In the present study, we explored the molecular mechanisms underlying the decodification of Ca^{2+} signals involved in human sperm capacitation which correlate with an increase in $[Ca^{2+}]_i$ (Baldi et al., 1991; Wall et al., 1994; Perry et al., 1997) and Tyr phosphorylation (Leclerc et al., 1996; Osheroff et al., 1999). As an attempt to elucidate these mechanisms, we first evaluated the impact of different $[Ca^{2+}]_e$ not only on Tyr phosphorylation but also on PKA-dependent phosphorylation which had not been previously analyzed. Our results show that PKA substrate phosphorylation did not exhibit major modifications in any of the assayed conditions which included media with different $[Ca^{2+}]_e$, HCO_3^- concentrations or incubation periods. Previous reports have shown that incubation of human sperm in media without added Ca^{2+} produced a significant decrease in both sAC activity and cAMP levels (Jaiswal and Conti, 2003; Torres-Flores et al., 2011) with no changes in PDE1 activity (Lefievre et al., 2002). Although these results seem to be in conflict with the lack of changes in PKA substrate phosphorylation observed in our study, it is possible that low levels of cAMP are sufficient to activate PKA and maintain a sustained PKA-dependent phosphorylation as previously proposed (Battistone et al., 2013). Alternatively, the lack of changes in PKA-dependent phosphorylation levels could be due to compensatory effects of Ca^{2+} on enzymes that play a role in cAMP-dependent pathways and which finally lead to a steady state of PKA-mediated phosphorylation. In agreement with previous works (Luconi et al., 1996; Carrera et al., 1996; Leclerc and Goupil, 2002; Marin-Briggiler et al., 2003; Baker et al., 2004), Tyr phosphorylation increased as a function of decreasing $[Ca^{2+}]_e$. In addition,

Tyr-phosphorylated proteins were detected either sooner or at lower HCO_3^- concentrations when sperm were incubated in $\ominus$ Ca^{2+}. A possible explanation for these observations is that the lack of added Ca^{2+} in the medium leads to an increase in Tyr kinase activity mediated by the higher levels of ATP observed in the absence of Ca^{2+} (Baker et al., 2004). This increase in ATP availability might result from a decrease in the activity of the ATP-consuming Ca^{2+}-extruding mechanism that operates in the sperm to maintain resting $[Ca^{2+}]_i$ as well as from the reduced sperm vigorous motility observed in medium lacking Ca^{2+}. Consistent with these hypotheses, exposure of sperm to thapsigargin, an endoplasmic Ca^{2+}-ATPase inhibitor that produces an increase in $[Ca^{2+}]_i$ (Dorval et al., 2003), generated a decrease in both ATP content (Baker et al., 2004) and Tyr phosphorylation (Luconi et al., 1996; Baker et al., 2004). Nevertheless, considering that the lack of Ca^{2+} in the medium is known to also produce a sodium-dependent plasma membrane depolarization as well as an increase in intracellular sodium concentration (Gonzalez-Martinez, 2003; Torres-Flores et al., 2008), we cannot exclude the possibility that other molecular mechanisms enhanced by low $[Ca^{2+}]_e$ could negatively affect Tyr phosphorylation.

Interestingly, although $[Ca^{2+}]_e$ does not directly affect PKA substrate phosphorylation levels, its effect on Tyr phosphorylation depends on PKA targets as judged by the fact that pharmacological inhibition of either PKA or SFK signaling pathways (which control PKA substrate phosphorylation status) abrogated both PKA-mediated and Tyr phosphorylations even in $\ominus$ Ca^{2+}. These results suggest that Ca^{2+} target(s) are directly or indirectly linked to phosphorylation of PKA substrates. In order to gain further insights into the signaling pathway through
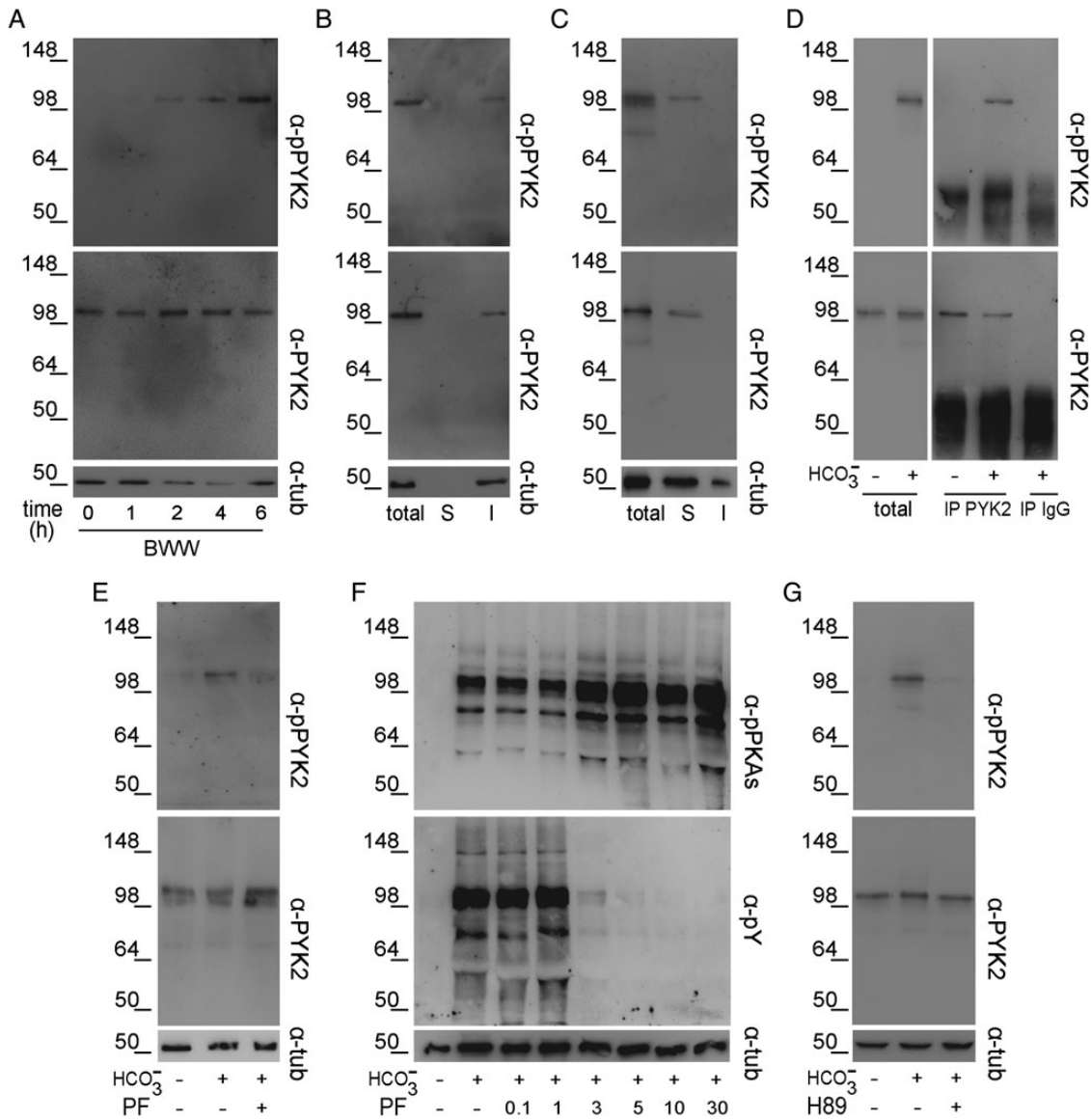
Figure 5 Activation of PYK2 during human sperm capacitation. (**A**) Sperm were incubated in BWW for different time periods. Protein extracts were analyzed by western blotting using α-pPYK2 antibody and the membrane was then stripped and reprobed with α-PYK2 antibody (n = 3). (**B**) Sperm were incubated in BWW for 6 h and proteins were extracted with 1% Triton X-100. The soluble (S) and insoluble (I) fractions were analyzed by western blotting using α-pPYK2 and then α-PYK2 antibodies (n = 3). (**C**) Sperm were incubated in BWW for 6 h and proteins were extracted with 0.1% SDS/1% Igepal. The soluble (S) and insoluble (I) fractions were analyzed by western blotting using α-pPYK2 and then α-PYK2 antibodies (n = 4). (**D**) Sperm were incubated for 6 h in complete or HCO_3^--free BWW, proteins were extracted with 0.1% SDS/1% Igepal and then subjected to immunoprecipitation with α-PYK2 antibody or rabbit IgG as a control. The immunoprecipitates (IP) were first analyzed using α-pPYK2 and then reblotted using α-PYK2 antibodies. (**E**) Sperm were incubated for 6 h in BWW with or without PF431396 (5 μM) and protein extracts were analyzed by Western blotting using α-PYK2 and then α-pPYK2 antibodies (n = 4). (**F**) Sperm were incubated for 6 h in BWW in the presence of PF431396, and protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively (n = 4). (**G**) Sperm were incubated for 6 h in BWW with or without H89 (30 μM) and protein extracts were analyzed by western blotting using α-PYK2 and then α-pPYK2 antibodies (n = 3). β-Tubulin was used as the protein loading control.

which Ca^{2+} affects Tyr phosphorylation, we studied if CaM, a universal $[Ca^{2+}]_i$ sensor, was required for Tyr phosphorylation in human sperm. Our results showed that inhibition of CaM by W7 maintained PKA substrate phosphorylation at a constant level but, in agreement with previous reports (Carrera et al., 1996: Leclerc et al., 1998), it produced a dose-dependent increase in Tyr phosphorylation. Based on the finding

that direct inhibition of CaMK, one of the CaM targets, did not affect Tyr phosphorylation in either human (Marin-Briggiler et al., 2005) or stallion (Gonzalez-Fernandez et al., 2013) sperm, we tested the effect of pharmacologically inhibiting another CaM target, namely PP2B, detecting no modifications of PKA target phosphorylation and an increase in Tyr phosphorylation. The effect of PP2B inhibition on Tyr phosphorylation
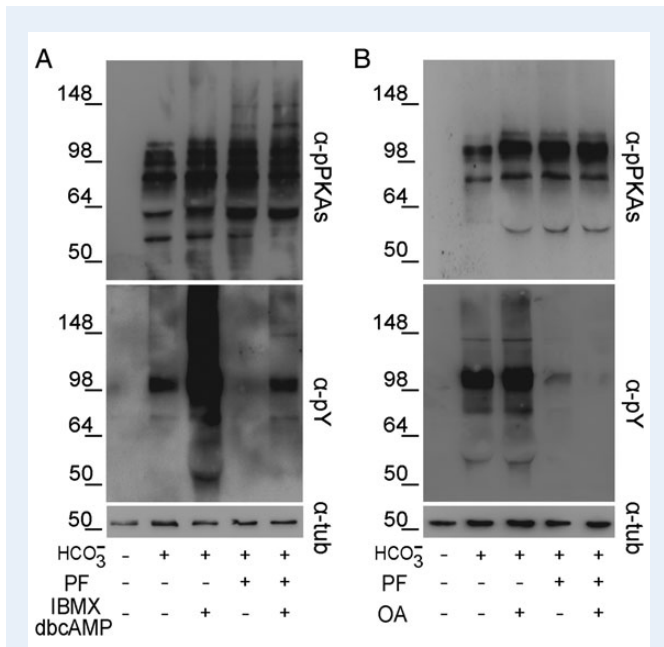
is in agreement with previous observations in human sperm (Carrera et al., 1996) but not with those recently reported in stallion (Gonzalez-Fernandez et al., 2013) probably due to inherent differences in species specificity. The fact that inhibition of both CaM and PP2B mimics the effects observed on both PKA-dependent and Tyr phosphorylations in $\ominus$ Ca²⁺ suggests that CaM and PP2B may be involved in the Ca²⁺ signaling pathway regulating Tyr phosphorylation. Moreover, the finding that similar results were obtained when inhibition of CaM and PP2B was carried out in $\ominus$ Ca²⁺ (data not shown) suggests that these Ca²⁺ downstream effectors could also mediate the increase in Tyr phosphorylation observed under this condition. In this regard, it is interesting to mention

Figure 6 Crosstalk between the PYK2 and cAMP/PKA or SFK/phosphatase signaling pathways. Human sperm were incubated for 6 h in BWW with or without PF431396 (10 μM) in the absence or presence of (**A**) the cAMP analog dbcAMP and the phosphodiesterase inhibitor IBMX (5/0.2 mM), or (**B**) the Ser/Thr phosphatase inhibitor OA (100 nM). In all cases, protein extracts were analyzed for both PKA substrate and Tyr phosphorylations by western blotting using α-pPKAs or α-pY antibodies, respectively (n = 4). β-Tubulin was used as the protein loading control.

previous results postulating that CaM levels decrease during human sperm capacitation (Leclerc et al., 1998) which, according to our observations, could lead to an increase in Tyr phosphorylation.

Based on a recent report in stallion showing the activation of FAK family members (i.e. FAK and PYK2) in sperm incubated in a medium without added Ca²⁺ (Gonzalez-Fernandez et al., 2013), we investigated whether FAK proteins could mediate the phosphorylation cascade observed in our studies. In this regard, whereas the work in stallion involved only the use of antibodies against the phospho-FAK proteins, our studies were carried out using also the antibodies against the non-phosphorylated proteins. Results revealed, for the first time, the presence of PYK2 but not its homolog FAK in human sperm. Moreover, we observed that the addition of PF431396, a specific ATP-competitive inhibitor of FAK family proteins (Buckbinder et al., 2007; Han et al., 2009), prevented the increase in Tyr phosphorylation observed in $\ominus$ Ca²⁺, producing only a slight increase in PKA substrate phosphorylation. Accordingly, PF431396 inhibited the trans/autophosphorylation at Tyr402 of PYK2, which was enhanced in $\ominus$ Ca²⁺, arguing in favor of an increase in Tyr kinase activity in this medium as previously proposed (Luconi et al., 1996; Baker et al., 2004). As the SFK member c-*yes* is present in human sperm and is inhibited by Ca²⁺, it was postulated as the kinase involved in the Ca²⁺-mediated increase in Tyr phosphorylation (Leclerc and Goupil, 2002). However, the fact that PF431396 completely abolished the Tyr phosphorylation increase in $\ominus$ Ca²⁺ supports the role of a PF431396-sensitive Tyr kinase, such as PYK2, as the molecule responsible for inducing Tyr phosphorylation under this condition. In agreement with previous observations in both stallion sperm (Gonzalez-Fernandez et al., 2013) and cytotoxic T lymphocytes (Lysechko et al., 2010), our results showed that CaM inhibition by W7 enhanced phospho-PYK2. The finding that W7 produced an increase in both PYK2 activation and Tyr phosphorylation suggests a CaM-mediated increase in human sperm PYK2 phosphorylation in $\ominus$ Ca²⁺. In this regard, although a CaM-binding sequence has been identified in PYK2 (Kohno et al., 2008), it is likely that CaM indirectly regulates sperm PYK2 activation. In addition to this, we cannot exclude the possibility that PYK2 is activated in $\ominus$ Ca²⁺ by other mechanisms (i.e. an increase in ATP) as this kinase is known to respond to stress and extracellular ATP in other cell types (Block et al., 2011; Martel-Gallegos et al., 2013).

In light of our observations, we next performed a series of studies to investigate the potential involvement of PYK2 in sperm capacitation. Our results revealed that PYK2 is activated during capacitation as judged by the time-dependent increase in phospho-PYK2. As PYK2 has been
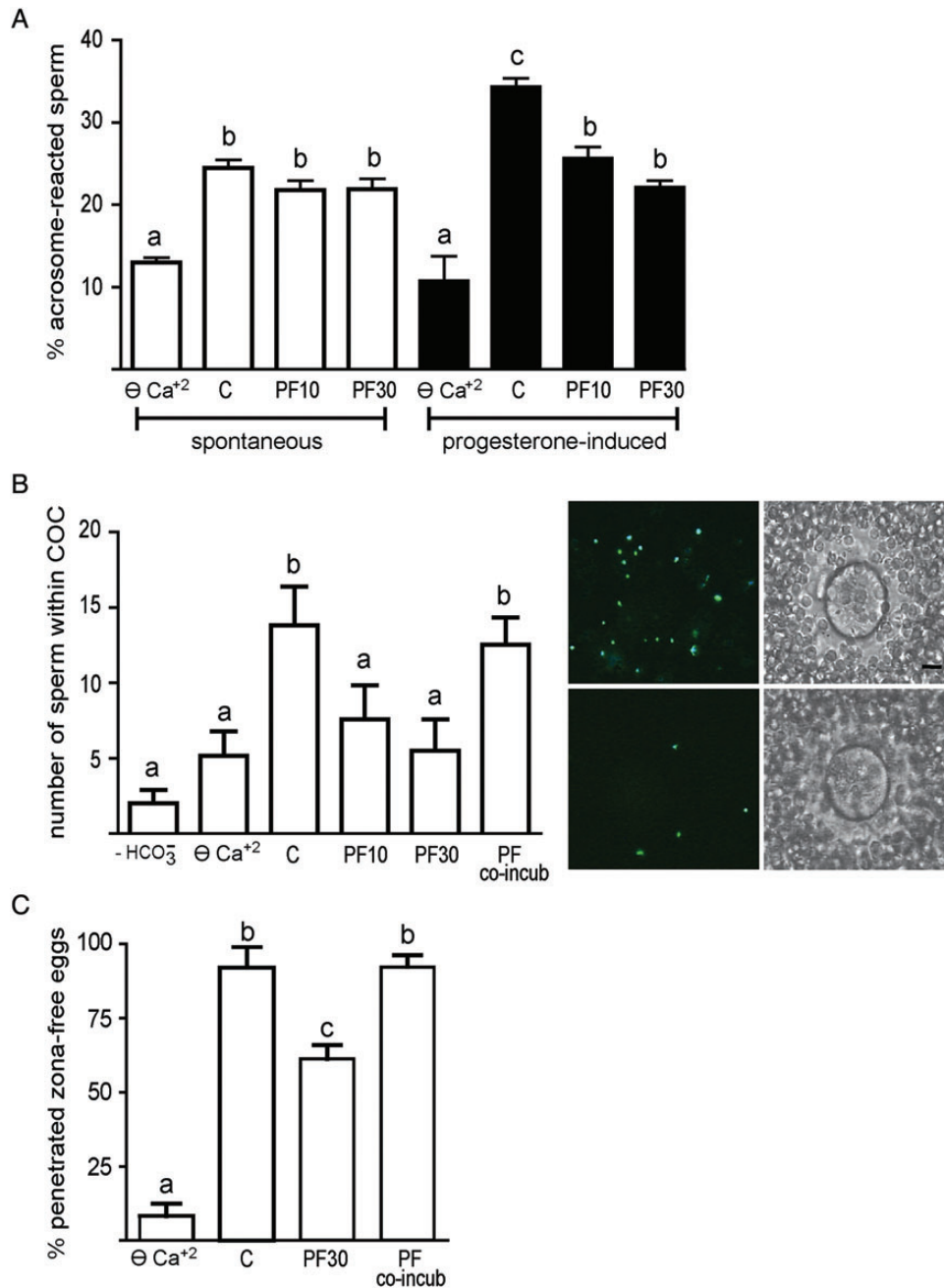
Table I Relevance of PYK2 for human sperm motility during capacitation.

|	VAP	VCL	VSL	ALH	LIN	STR	% Motility	% Progressive motility	% Hyperactivation
C	53.8 ± 2.4	85.2 ± 2.3	47.2 ± 2.5	4.1 ± 0.1	52.6 ± 1.8	82.8 ± 1.2	97.2 ± 0.2	68.3 ± 2.4	12.2 ± 2.4
$\ominus$ Ca²⁺	24.5 ± 1.3*	53.2 ± 6.1*	15 ± 1*	2.9 ± 0.1*	29.2 ± 1.6*	58.9 ± 0.6*	94.8 ± 0.3	15.5 ± 1.9*	1.6 ± 0.3*
PF 10 μM	34.5 ± 3.4*	59.2 ± 2.6*	28.1 ± 2.8*	3.1 ± 0.1*	43.3 ± 3.9	73.9 ± 3.5	97.2 ± 0.2	39.9 ± 7.4*	2.5 ± 0.6*
PF 30 μM	29.0 ± 2.5*	54.9 ± 2.9*	21.3 ± 2.5*	3.2 ± 0.1*	35.6 ± 1.8*	66.8 ± 1.7*	94.6 ± 1.1	25 ± 4.5*	2.4 ± 0.4*

Computer-assisted sperm analysis was performed in sperm incubated for 18 h in BWW (C), or in BWW without added Ca²⁺ ($\ominus$ Ca²⁺) or in BWW containing the FAK/PYK2 inhibitor PF431396 (PF 10 μM or 30 μM). VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; ALH, amplitude of lateral head displacement; LIN, linearity; STR: straightness (n = 4). Statistical significance of the data was analyzed using one-way analysis of variance. *versus C, P < 0.05.

Figure 7 Relevance of PYK2 for human sperm function. (**A**) Sperm were capacitated for 18 h in $\ominus$ Ca^{2+} or in BWW either alone (C) or containing PF431396 (10 or 30 μM) and both the spontaneous and progesterone (25 μM)-induced AR were evaluated by staining sperm with *P. sativum* agglutinin coupled to fluorescein isothiocyanate ($n = 5$). Statistical significance of the data was analyzed using the χ^2 test. (**B**) Sperm were incubated for 18 h in $\ominus$ Ca^{2+} or in BWW either with (C) or without HCO$_3^-$ or containing PF431396 (10 or 30 μM), then co-incubated with mouse COCs for 15 min in BWW with or without PF431396, and the number of sperm within the COCs determined under microscope. Sperm exposed to PF431396 (30 μM) only during gamete co-incubation was used as a control (PF co-incub) ($n = 4$). Statistical significance of the data was analyzed using the Student's *t*-test. The images on the right show representative fluorescent (left) and phase-contrast (right) micrographs of COCs inseminated with either control (upper panel) or PF-treated (bottom panel) sperm (Hoechst-stained). The scale bar is 25 μm. (**C**) Sperm were incubated for 18 h in $\ominus$ Ca^{2+} or in BWW either alone (C) or containing PF431396 (30 μM). Sperm were then co-incubated with zona-free hamster eggs for 2.5 h in BWW, and the percentage of penetrated eggs determined. Sperm exposed to PF431396 only during gamete co-incubation was used as a control (PF co-incub) ($n = 4$). Statistical significance of the data was analyzed using the χ^2 test. In all cases, bars with different letters are significantly different, $P < 0.05$.

described as a Ca^{2+}-dependent kinase in other systems (Lev et al., 1995), its activation during capacitation might be a consequence of the increase in $[Ca^{2+}]_i$ that occurs during this process (Baldi *et al.*, 1991; Wall *et al.*, 1994; Perry *et al.*, 1997). PYK2 activation during capacitation was also indicated by the presence of the phosphorylated protein only in the immunoprecipitates obtained from capacitated cells. In this regard, it is interesting to note that phospho-PYK2 was detected in the insoluble fraction of Triton X-100-treated sperm consistent with the localization of the majority of the Tyr-phosphorylated proteins associated with the cytoskeleton (Visconti *et al.*, 1997; Chung *et al.*, 2014). The finding that PF431396 inhibited PYK2 phosphorylation and abrogated Tyr phosphorylation but not PKA substrate phosphorylation supports PYK2 as an intermediate component between both PKA-mediated and Tyr phosphorylations during human sperm capacitation (Fig. 8). This conclusion is further supported by the finding that H89 blocked PYK2 activation and by our observations that when the cAMP/PKA or SFK/phosphatase signaling pathways were stimulated by dbcAMP/IBMX or OA, respectively, the PYK2 inhibitor did not affect the induced PKA substrate phosphorylation but markedly inhibited Tyr phosphorylation. These results, together with the fact that both PYK2 and Tyr phosphorylations are downstream of PKA activation in all the conditions tested, indicate that PYK2 activity directly or indirectly depends on PKA-mediated phosphorylation. Moreover, the finding that blockage of both PYK2 and Tyr phosphorylations by PF431396 resulted in an enhancement of PKA target phosphorylation suggests the existence of a negative feedback mechanism that inhibits PKA-mediated phosphorylation during capacitation. In this regard, a recent article showing that PKA is Tyr-phosphorylated during capacitation (Chung *et al.*, 2014) opens the possibility that this phosphorylation could play a role in the negative feedback suggested by our results.

The analysis of the functional relevance of our observations showed that PF431396 affected the development of hyperactivation during capacitation as well of the sperm ability to acrosome reaction in response to progesterone, similarly to what we observed by inhibiting the SFK signaling pathway (Battistone *et al.*, 2013). In addition, exposure of human sperm to PF431396 during capacitation affected their ability to both penetrate mouse cumulus matrix and fertilize zona-free hamster eggs. Considering that sperm need to exhibit a vigorous motility to penetrate the viscous cumulus matrix and to undergo a functional AR to fuse with the plasma membrane, our results support the idea that PYK2 plays an important role in the acquisition of human sperm fertilizing ability during capacitation. In addition, our findings showing that the different functional parameters evaluated were also affected by incubation of sperm in $\ominus$ Ca^{2+} are consistent with previous evidence supporting the biological relevance of Ca^{2+} signaling for human fertilization (Ahmad *et al.*, 1995; Marin-Briggiler *et al.*, 2003; Shahar *et al.*, 2011). In this regard, the fact that low $[Ca^{2+}]_e$ also produces an increase in Tyr phosphorylation suggests that low resting $[Ca^{2+}]_i$ could be sufficient for triggering Tyr phosphorylation but not for the acquisition of human sperm fertilizing ability. The latter might require a higher Ca^{2+} influx likely through CatSper channel which both produces a rapid transient $[Ca^{2+}]_i$ increase followed by a slow sustained $[Ca^{2+}]_i$ elevation after stimulation (Strunker *et al.*, 2011), and may activate Ca^{2+} stores or store-operated channels (Lefievre *et al.*, 2012). In this regard, a recent report proposed that human sperm penetration through a viscous medium (comparable to our cumulus matrix penetration assay) is enhanced by CatSper stimulation, whereas hyperactivated
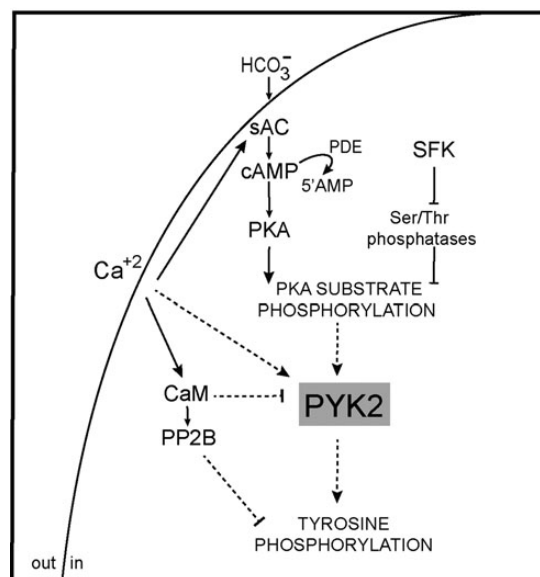
Figure 8 Working model for the signaling pathways proposed to be involved in Tyr phosphorylation during human sperm capacitation. In addition to the previously reported signaling pathways involved in Tyr phosphorylation (i.e. cAMP/PKA and SFK/phosphatase), we propose (dashed lines) PYK2 as a Ca^{2+} signaling component involved in Tyr phosphorylation downstream of PKA activation during human sperm capacitation.

motility rather depends on the mobilization of intracellular stored Ca^{2+} (Alasmari *et al.*, 2013b). All these observations reveal the spatio-temporal complexity of the Ca^{2+}-mediating signaling that may lead to different sperm behaviors depending on the Ca^{2+} source (Alasmari *et al.*, 2013b). In view of this, and considering the inhibitory effect of PF431396 on different sperm functional parameters, it is possible that PYK2 could be acting as a platform that integrates signals coming from different Ca^{2+} sources and leading to different downstream functional events.

In conclusion, in the present study we provide evidence that PYK2 is a Ca^{2+} signaling component involved in Tyr phosphorylation downstream of PKA activation and is required for functional human sperm capacitation. These results contribute to a better understanding of the molecular mechanisms involved not only in physiological human sperm capacitation but also in pathological conditions such as those associated with impaired sperm Ca^{2+} signaling.

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Authors' roles

M.A.B., A.A., A.M.S. and V.G.D.R. carried out the experiments and analyzed the data. P.E.V., V.G.D.R. and P.S.C conceived the study, designed experiments, analyzed the data and wrote the article. M.A.B., A.A. and A.M.S. contributed in drafting the article.

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Conflict of interest

None declared.

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