

Original Research Article

Biphasic Role of Calcium in Mouse Sperm Capacitation Signaling Pathways[†]

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ABSTRACT

Mammalian sperm acquire fertilizing ability in the female tract in a process known as capacitation. At the molecular level, capacitation is associated with up-regulation of a cAMP-dependent pathway, changes in intracellular pH, intracellular Ca^{2+} and an increase in tyrosine phosphorylation. How these signaling systems interact during capacitation is not well understood. Results presented in this study indicate that Ca^{2+} ions have a biphasic role in the regulation of cAMP-dependent signaling. Media without added Ca^{2+} salts (nominal zero Ca^{2+}) still contain micromolar concentrations of this ion. Sperm incubated in this medium did not undergo PKA activation or the increase in tyrosine phosphorylation suggesting that these phosphorylation pathways require Ca^{2+} . However, chelation of the extracellular Ca^{2+} traces by EGTA induced both cAMP-dependent phosphorylation and the increase in tyrosine phosphorylation. The EGTA effect in nominal zero Ca^{2+} media was mimicked by two calmodulin antagonists, W7 and calmidazolium, and by the calcineurin inhibitor cyclosporine A. These results suggest that Ca^{2+} ions regulate sperm cAMP and tyrosine phosphorylation pathways in a biphasic manner and that some of its effects are mediated by calmodulin. Interestingly, contrary to wild type mouse sperm, sperm from *CatSper1* KO mice underwent PKA activation and an increase in tyrosine phosphorylation upon incubation in nominal zero Ca^{2+} media. Therefore, sperm lacking *Catsper* Ca^{2+} channels behave as wild-type sperm incubated in the presence of EGTA. This latter result suggests that *Catsper* transports the Ca^{2+} involved in the regulation of cAMP-dependent and tyrosine phosphorylation pathways required for sperm capacitation. This article is protected by copyright. All rights reserved

INTRODUCTION

Mammalian sperm acquire the ability to fertilize the egg in the female tract in a process known as capacitation (Austin, 1959; Chang, 1951). Functionally, capacitation is associated with changes in the sperm motility pattern (i.e. hyperactivation) and with their ability to undergo the acrosome reaction (Visconti et al., 2011; Yanagimachi, 1994). At the molecular level, sperm capacitation correlates with: 1) activation of a cAMP/PKA pathway (Harrison, 2004; Krapf et al., 2010); 2) increase in intracellular pH (pH_i) (Zeng et al., 1996); 3) increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Ruknudin and Silver, 1990); 4) hyperpolarization of the sperm plasma membrane potential (E_m) (Escoffier et al., 2012; Zeng et al., 1995); 5) loss of cholesterol (Cross, 1996; Davis et al., 1980) and other lipid modifications (Gadella and Harrison, 2000); and 6) increase in protein tyrosine phosphorylation (Krapf et al., 2010; Visconti et al., 1995a). How these signaling pathways interact to induce hyperactivation and to prepare the sperm for the acrosome reaction is not well understood.

In all cell types, Ca^{2+} plays essential roles as second messenger controlling a battery of cellular processes. In sperm, pharmacological and genetic loss-of-function experiments have shown a central role of this ion in the regulation of sperm motility and the acrosome reaction. However, little is known about the mechanisms involved in controlling $[Ca^{2+}]_i$ as well as the identity of Ca^{2+} targets inside the sperm. Two Ca^{2+} transport systems have been conclusively identified in sperm: 1) the Ca^{2+} channel complex Catsper, composed by at least 7 subunits. Knock out (KO) of each of them results in degradation of all the other subunits (Qi et al., 2007). Studies using these mice revealed that Catsper is essential for hyperactivation and fertilization; 2) Ca^{2+} extrusion systems, composed by the Na^+/Ca^{2+} Exchanger and the Plasma Membrane Ca^{2+} -ATPase (PMCA) protein families. Sperm from *PMCA4b* KO mice are deficient in both progressive and hyperactivated motility resulting in sterility (Okunade et al., 2004). Regarding Ca^{2+} targets, Ca^{2+} -dependent enzymes may be activated directly by Ca^{2+} or indirectly by Ca^{2+} /Calmodulin (CaM) interaction. Calmodulin (Wasco et al., 1989) and several Ca^{2+} /Calmodulin targets,

including phosphodiesterase I (Baxendale and Fraser, 2005), calcineurin (Tash et al., 1988) and Calmodulin Kinase II and IV (Ignatz and Suarez, 2005; Marin-Briggiler et al., 2005; Wu and Means, 2000), have been shown to be present in mammalian sperm. However, their roles in the regulation of sperm function have not been established.

The present work focuses on studying the role of Ca^{2+} ions in the regulation of the capacitation-associated increase in tyrosine phosphorylation. We have previously shown (Marin-Briggiler et al., 2005) that incubation of mouse sperm in the absence of added extracellular Ca^{2+} (nominal zero Ca^{2+} concentration, $\ominus \text{Ca}^{2+}$ media) blocked the capacitation-associated increase in tyrosine phosphorylation. However, $\ominus \text{Ca}^{2+}$ media still contain contaminant Ca^{2+} at micromolar concentrations (Marin-Briggiler et al., 2005). Thus, we added EGTA to test the effect of further lowering the extracellular Ca^{2+} . Surprisingly, chelation of the contaminant extracellular Ca^{2+} induced a robust increase in tyrosine phosphorylation. We hypothesized that EGTA released a negative control of those pathways leading to the increase in tyrosine phosphorylation.

Consistent with this hypothesis, when instead of EGTA sperm were incubated in $\ominus \text{Ca}^{2+}$ media in the presence of calmodulin antagonists (W7 and calmidazolium) or in the presence of inhibitors of some calmodulin-dependent enzymes, the increase in tyrosine phosphorylation was also observed. As part of this work we also tested whether the Ca^{2+} needed for the regulation of tyrosine phosphorylation pathways was entering the sperm through *Catsper* ion channels. Our data show that sperm from *Catsper* null mice undergo tyrosine phosphorylation even in $\ominus \text{Ca}^{2+}$ media, suggesting that sperm from these mice behave as wild-type sperm in the presence of EGTA. Altogether these data suggest a biphasic role of Ca^{2+} in the regulation of phosphorylation pathways.

MATERIALS AND METHODS

Materials: Chemicals were purchased from the following sources (codes between parenthesis indicate the catalog number of the respective compound): Bovine serum albumin (BSA, fatty acid-free) (A0281), H-89 (B1427), Cyclosporin A (C-3662), Ethylene glycol-bis (2-aminoethylether)-N,N,N,N'tetraacetic acid (EGTA) (E-3889), KN93 (K-1385), KN-62 (K-102), N6,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (D-0627), 3-Isobutyl-1-methylxanthine (I-5879), Tween-20 (P7949), ionomycin (I-0634), fish skin gelatin (G7765), and human chorionic gonadotropin (CG5), were purchased from Sigma (St. Louis, MO). N-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide (W7) (681629), and Calmidazolium Chloride (208665) were acquired from Calbiochem (Billerica, MA). Anti-phosphotyrosine (anti-PY) monoclonal antibody (clone 4G10) was obtained from Millipore (Billerica, MA). Rabbit monoclonal anti-phosphoPKA substrates (anti-pPKAS) (clone 100G7E), anti-pan-CalcieneurinA polyclonal (2614), anti-cyclophilin A polyclonal (2175) were purchased from Cell Signaling (Danvers, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgGs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and GE Life Sciences, respectively. Acrylamide (30%) and β -Mercaptoethanol were obtained from Biorad (Hercules, CA).

Mouse sperm preparation: Animals were euthanized in accordance with the Animal Care and Use Committee (IACUC) guidelines of UMass-Amherst. Cauda epididymal mouse sperm were collected from CD1 retired male breeders (Charles River Laboratories, Wilmington, MA) from young adult (7–8 week-old mice), and *CatSper* KO (Ren et al., 2001) mice. Each cauda epididymis was placed in 500 μ L of a modified Krebs-Ringer medium (Whitten's HEPES-buffered medium) (Moore et al., 1994) in the presence or absence of Ca^{2+} as described for each experiment. Briefly, this medium contained the following compounds (concentrations are given in parenthesis): NaCl (10 mM), KCl (4.4 mM), KH_2PO_4 (1.2 mM), MgSO_4 (1.2 mM), Glucose (5.4

mM), Pyruvic Acid (0.8 mM), Lactic Acid (2.4 mM), Hepes Acid (20 mM). This medium does not support mouse sperm capacitation. After 10 min incubation at 37 °C (swim-out), epididymis were removed, and the suspension adjusted with non-capacitating medium to a final concentration of $1-2 \times 10^7$ cells/ml before dilution of four times in the experimentally appropriate medium. The sperm were then incubated at 37 °C for at least 1 h in media containing increasing Ca^{2+} concentrations in the absence or in the presence of 15 mM $NaHCO_3$ and 5 mg/ml BSA. To test the effect of inhibitors, sperm were incubated under the conditions described above in the presence of increasing concentrations of inhibitors. These compounds were assayed for 1 h and were added from the beginning of the incubation period. For *in vitro* fertilization (IVF) assays, sperm were first incubated in Whitten's medium without HEPES containing 25 mM $NaHCO_3$ and 5 mg/ml BSA in the absence or in the presence of Ca^{2+} as indicated. The medium was previously equilibrated in a humidified atmosphere of 5% CO_2 at 37° C (Wertheimer et al., 2008).

SDS-PAGE and immunoblotting: Sperm were collected by centrifugation, washed in 1 ml of phosphate buffer solution (PBS), resuspended in Laemmli sample buffer (Laemmli, 1970), boiled for 5 min and centrifuged once more. Supernatants were then supplemented with 5% β -mercaptoethanol and boiled again for 3 min. Protein extracts equivalent to $1-2 \times 10^6$ sperm were loaded per lane and subjected to SDS-PAGE an electro-transfer to PVDF membranes (Bio-Rad) at 250 mA for 60 min on ice. Immunoblotting with either anti-pPKAS antibodies (clone 100G7E) or with anti-PY (clone 4G10) was carried out as previously described (Krapf et al., 2010). For immunodetection of mouse sperm calcineurin A and cyclophilin, PVDF membranes were blocked with 5% fat-free milk in TBS containing 0.1% Tween 20 (T-TBS) and antibodies used at 1:1,000 final concentration. Secondary antibodies were diluted in T-TBS (1:10,000). An enhanced chemiluminescence ECL plus kit (GE Healthcare) was used for detection. Hexokinase, a protein known to be constitutively phosphorylated on tyrosine residues in mouse sperm, served as a loading control (Porambo et al., 2012; Visconti et al., 1995a). When

necessary, PVDF membranes were stripped at 65° C for 15 min in 2% SDS, 0.74% β -mercaptoethanol, 62.5 mM Tris, pH 6.5, and washed six times for 5 min each in T-TBS. For all experiments, molecular masses are expressed in kDa. Image analysis was conducted using the freeware ImageJ v1.48, downloaded from the NIH website (<http://imagej.nih.gov/ij/download.html>). The bar at the left of the graph labeled the region from the Western blot used for quantification. In all cases, results were normalized arbitrarily considering the Θ Ca²⁺ lane as the unit value. After normalization using at least three independent blots, results were reported as mean \pm standard error of the mean (SEM).

Sperm motility analysis: Sperm suspensions (25 μ l) were loaded into pre-warmed chamber slides (depth, 100 μ m) (Leja slides, Spectrum Technologies) and placed on a microscope stage at 37 °C. Sperm movements were examined using the CEROS computer-assisted semen analysis (CASA) system (Hamilton Thorne Research, Beverly, MA). The default settings included the following: frames acquired: 90; frame rate: 60 Hz; minimum cell size: 4 pixels; static head size: 0.13-2.43; static head intensity: 0.10-1.52; static head elongation: 5-100. Sperm with hyperactivated motility, defined as motility with high amplitude thrashing patterns and short distance of travel, were sorted using the criteria established by Goodson et al. (Goodson et al., 2011). The data was analyzed using the CASAnova software (Goodson et al., 2011). At least 20 microscopy fields corresponding to a minimum of 200 sperm were analyzed in each experiment.

Mouse eggs collection and IVF assays: Metaphase II-arrested eggs were collected from 6–8 week-old super ovulated C57BL/6 female mice (Charles River Laboratories) at 12 h after human chorionic gonadotropin intraperitoneal injection as previously described (Wertheimer et al., 2008). The cumulus-oocyte complexes (COCs) were placed into a well with 500 μ l of media (Whitten's media with or without calcium depending on the experiment) previously equilibrated in an incubator with 5% CO₂ at 37 °C. Fertilization wells containing 25-40 eggs were inseminated with sperm (final concentration of 2.5×10^6 cells/ml) that had been incubated for 1 h

and 20 min in a Whitten's medium supporting capacitation with or without calcium depending on the experiment. After 4 h of insemination, eggs were washed and put in fresh Whitten's media.

The eggs were evaluated 24 h post-insemination. To assess fertilization, the three following criteria were considered: 1) the formation of the male and female pronuclei, 2) the emission of the second polar body, and 3) two- cell stage events.

Ca²⁺ image recordings: Sperm were incubated in Whitten's medium containing 2 μ M Fluo-4 AM (Invitrogen, Life Technologies, NY) and 0.05 % pluronic acid for 45 min at 37° C and then washed by centrifugation. Sperm heads were then allowed to stick to coverslips previously coated with mouse laminin (2 mg/ml, Invitrogen, Life Technologies, NY). The coverslip with bound spermatozoa was placed at the bottom of the recording chamber (De La Vega-Beltran et al., 2012) containing Whitten's medium. Recordings were made for ~2 min in the presence of Θ Ca²⁺ media, thereafter this media was washed out and replaced by Θ Ca²⁺ media containing EGTA 1 mM during ~3 min. At the end of this time, Θ Ca²⁺ media was replaced during other ~2 min followed by a 2 mM Ca²⁺ addition for ~2 min more. At the end of the recording, 20 μ M of Ionomycin was added to increase intracellular Ca²⁺ as a functional control and for quantification purposes. Recordings were performed at 37° C using a temperature controller model 202A (Harvard Apparatus, Holliston, MA). Sperm were viewed using an inverted microscope (Nikon Eclipse Ti-U, Melville, NY) equipped with an oil immersion fluorescence Nikon plan Apo TIRF objective (60 x/1.45) DIC H/N2. An EL6000 Leica mercury short-arc reflector lamp with an integrated shutter was connected via a liquid light guide to the microscope. For excitation and emission collection of Fluo-4 the filter set GFP 96343, Dichroic: 495, Exc: 470/ 40 and Barrier 525/50 filters were used (Nikon, Melville, NY). Fluorescent images were acquired with an Andor Ixon3 EMCCD camera model DU-897D-C00#B (Andor Technology, UK) under protocols written in Andor iQ 1.10.2 software 4.0 version. Images were acquired at 0.4-0.5 Hz with exposure/illumination time of 30 ms. Movies were processed and analyzed with macros written

in Image J (Version 1.38, National Institutes of Health). The fluorescence changes measured in the different Ca^{2+} concentration conditions were normalized with respect to those induced by ionomycin.

Statistical analysis: Data are expressed as the means \pm S.E.M. The difference between mean values of multiple groups was analyzed by one-way analysis of variance followed by T-Tukey test. Statistical significances are indicated as described in the figure legends.

RESULTS

Previous knowledge indicated that sperm incubated in Θ Ca^{2+} media did not display tyrosine phosphorylation associated to capacitation, even in the presence of BSA and HCO_3^- . To further test the role of Ca^{2+} in the signaling pathways leading to tyrosine phosphorylation, mouse sperm were incubated for 1 h in Θ Ca^{2+} media or in the presence of increasing concentrations of Ca^{2+} or EGTA. Confirming previous results (Visconti et al., 1995a), the increase in tyrosine phosphorylation was dependent on Ca^{2+} and did not occur in Θ Ca^{2+} media (Fig. 1 A, lanes 2-8 and Fig. 1 B). To test lower Ca^{2+} concentrations, because Θ Ca^{2+} media still contain ~ 10 - 100 μM of contaminant Ca^{2+} (Marin-Briggiler et al., 2005), the incubation medium was supplemented with 1 mM EGTA to further decrease $[\text{Ca}^{2+}]_e$. In these conditions, protein-tyrosine phosphorylation was up regulated to levels comparable to those obtained in the presence of standard $[\text{Ca}^{2+}]_e$ (2.4 mM) (Fig. 1A, lane 1). Moreover, EGTA had a concentration-dependent effect on phosphorylation events (Fig. 1 C and D). To compare experimental replicates, the Western blots were quantified using Image J (Fig. 1 C and D). Similar to the onset of tyrosine phosphorylation in standard Ca^{2+} -containing media (Visconti et al., 1995a), the EGTA-induced tyrosine phosphorylation in mouse sperm was slow and required ~ 45 min. (Fig. 2 A and B). Also similar to sperm incubation in the presence of Ca^{2+} , the stimulatory effect of EGTA on tyrosine phosphorylation required HCO_3^- (Fig. 2 C and D) and BSA (Fig. 2 E and F) in the incubation media. Addition of EGTA reduced $[\text{Ca}^{2+}]_e$; however, how EGTA affects

intracellular Ca^{2+} is not known. It is predicted that EGTA addition decreases $[\text{Ca}^{2+}]_i$. To test this hypothesis, sperm were loaded with Fluo-4 AM, and $[\text{Ca}^{2+}]_i$ measurements were conducted in single sperm before and after addition of EGTA (Fig. 3 A and B). As shown, addition of EGTA decreased intracellular Ca^{2+} significantly. Conversely, when EGTA was removed and replaced first with ΘCa^{2+} media, Ca^{2+} remained by a 2 mM Ca^{2+} solution, sperm responded with an increase in $[\text{Ca}^{2+}]_i$ (Fig. 3 A, B and C).

Considering that cAMP is necessary for the increase in tyrosine phosphorylation (Visconti et al., 1995b), the Ca^{2+} stimulatory effect can be explained considering that this ion stimulates Adcy10 and promotes cAMP synthesis (Jaiswal and Conti, 2003; Kleinboelting et al., 2014; Litvin et al., 2003; Steegborn et al., 2005). On the other hand, it is not clear why lowering $[\text{Ca}^{2+}]_i$ with EGTA activates tyrosine phosphorylation. One possibility is that other Ca^{2+} -dependent enzymes negatively regulate tyrosine phosphorylation. In this case, addition of EGTA would block the activation of those enzymes and result in an increase in protein tyrosine phosphorylation as observed in Fig. 1 and 2. Ca^{2+} -dependent enzymes involved in these negative feedback mechanisms could be activated directly by Ca^{2+} or indirectly by Ca^{2+} /Calmodulin (CaM) interaction. To evaluate the possible role of CaM on tyrosine phosphorylation, instead of adding EGTA, sperm incubated in ΘCa^{2+} medium were treated with increasing concentrations of either W7 (Fig. 4 A) or calmidazolium (Fig. 4 B), two well-characterized CaM inhibitors. As observed in this figure, both compounds mimicked the presence of EGTA, stimulating an increase in tyrosine phosphorylation (Fig. 4 A and B).

The aforementioned experiments suggest that CaM pathways play a role in the regulation of PKA activity. CaM has several possible targets in sperm, many of them unknown. However, three of the CaM targets that have been described in mature sperm are phosphodiesterase 1 (PDE 1) (Baxendale and Fraser, 2005), Calmodulin Kinase II and IV (CaMK II and IV) (Ignatz and Suarez, 2005; Marin-Briggiler et al., 2005; Wu and Means, 2000) and Ca^{2+} /CaM-dependent

phosphatase PPP3C (aka calcineurin) (Tash et al., 1988). To test whether these enzymes mediated the CaM effect on sperm phosphorylation pathways, sperm were incubated in Θ Ca^{2+} media with different concentration of inhibitors for each of these enzymes. Considering that PDE is the enzyme responsible for cAMP hydrolysis, it was not surprising that IBMX, a broad spectrum PDE inhibitor, stimulated tyrosine phosphorylation (Fig. 4 C). On the other hand, CaMK II and IV inhibitors KN62 and KN93 did not increase tyrosine phosphorylation (Fig. 4 D and E). To test the role of calcineurin, the specific calcineurin inhibitor cyclosporin A (CSA) was used. In Θ Ca^{2+} , increasing CSA concentrations stimulated tyrosine phosphorylation (Fig. 4 F). All blots in Fig. 4 were quantified using Image J (see Fig. S1) as described above in the Methods section.

It is known that calcineurin inhibition by CSA is mediated by binding to cyclophilin (Liu et al., 1991). Consistent with the effect of CSA on sperm phosphorylation, anti-cyclophilin antibodies recognized a single band in mature mouse sperm (Fig. 5 A). In addition, Western blot analysis using anti-calcineurin A antibodies also confirmed the presence of this enzyme in sperm (Fig. 5 B). Compared to sperm incubated under control conditions in complete capacitation medium (containing 2.4 mM Ca^{2+}), neither the viability nor the percentage of motile cells was affected when sperm were incubated in Θ Ca^{2+} or in the presence of EGTA, IBMX, W7, calmidazolium-CZ or CSA (data not shown). It is well-established that, in addition to hyperactivation, Ca^{2+} is needed for a physiological acrosome reaction, for hyperactivation and for fertilization (Yanagimachi, 1994). Therefore, although in all these conditions tyrosine phosphorylation was stimulated in Θ Ca^{2+} medium, it was not surprising that none of these compounds were able to enhance sperm hyperactivation over control samples incubated in Θ Ca^{2+} medium (Fig. 5 C). Also it was not surprising that in the continuous presence of EGTA, *in vitro* fertilization failed (Fig. 5 D). However, when EGTA was washed out by centrifugation after sperm had been incubated for 1 h and then suspended in Whitten's media containing Ca^{2+} ,

fertilization rate was recovered (Fig. 5 D). These data indicate that once EGTA is removed, sperm can recover their functionality.

Because of the established role of HCO_3^- in cAMP synthesis by Adcy10 regulation, the HCO_3^- -dependence of the EGTA-induced tyrosine phosphorylation suggested that PKA is involved in the process. To further test this possibility, we analyzed how Ca^{2+} and calmodulin affected PKA activation. The PKA pathway was monitored by Western blotting using anti-phospho PKA substrate antibodies as described (Krapf et al., 2010) (Fig. 5 E). Although in $\ominus \text{Ca}^{2+}$ medium the level of phosphorylation was low, addition of EGTA, W7, calmidazolium or CSA increased phosphorylation of PKA substrates and tyrosine residues to levels similar to those obtained in the presence of Ca^{2+} . Also consistent with a role of PKA mediating the EGTA effect, addition of H-89, a PKA inhibitor, blocked both types of phosphorylation (Fig. 5 F).

Altogether, these data suggest that Ca^{2+} plays a biphasic role in the regulation of sperm phosphorylation pathways. These results, however, are silent regarding the molecule(s) involved in Ca^{2+} transport early in the signaling cascade. One possible candidate for this early influx is the sperm-specific Ca^{2+} channel complex CatSper (Ren et al., 2001). However, sperm from *CatSper* KO mice are able to undergo the increase in tyrosine phosphorylation (Carlson et al., 2005; Wennemuth et al., 2003) suggesting that Catsper is not involved in the regulation of tyrosine phosphorylation. On the other hand, in the present work we show that EGTA can induce PKA activation and tyrosine phosphorylation in $\ominus \text{Ca}^{2+}$ medium. Moreover, it has been recently shown (Chung et al., 2014) that tyrosine phosphorylation is enhanced in *Catsper* KO sperm. Taken together, these data suggested to us an alternative hypothesis to explain the increase in tyrosine phosphorylation observed in *Catsper* null sperm, as follows: in the absence of Catsper, Ca^{2+} cannot enter the sperm; therefore, KO sperm is expected to behave similarly to wild type mouse sperm incubated in $\ominus \text{Ca}^{2+}$ medium with EGTA. To test this possibility, *CatSper* null sperm were incubated in $\ominus \text{Ca}^{2+}$ medium with increasing Ca^{2+} concentrations or EGTA and

assayed for phosphorylation of PKA substrates and for tyrosine phosphorylation. Contrary to the response seen in wild type control sperm incubated in \ominus Ca^{2+} medium (Fig. 6 A, left panel), CatSper KO sperm in the same conditions underwent tyrosine phosphorylation (Fig. 6 A, right panel). To compare different repeats, the Western blots were quantified using Image J and normalized against the respective EGTA lane (Fig. 6 B). In addition, similar to their behavior in standard Ca^{2+} containing media (Chung et al., 2014), we found that in \ominus Ca^{2+} medium, the increase in tyrosine phosphorylation, absent in wild type sperm (Fig. 6 C, left panel) started around 15 min after addition of HCO_3^- (Fig. 6 C, right panel). Finally, it is worth noticing that either in the absence of HCO_3^- , in the presence of the PKA inhibitor H-89 or in the presence of the Adcy10 inhibitor KH7, the increase in tyrosine phosphorylation did not occur (Fig. 6 D) indicating that in *Catsper* null sperm, the increase in tyrosine phosphorylation is also mediated by a PKA-dependent pathway.

DISCUSSION

Different knock-out (KO) mice models have revealed that cAMP (i.e. KOs for the atypical soluble adenylyl cyclase (*Adcy10*) and for the sperm specific splicing variant of the PKA catalytic subunit $\text{Ca}2$) (Hess et al., 2005; Morgan et al., 2008; Nolan et al., 2004; Xie et al., 2006), pH_i (Na^+/H^+ exchanger KO) (Wang et al., 2003), sperm membrane hyperpolarization (the sperm specific K^+ channel SLO3 KO) (Santi et al., 2010), and Ca^{2+} dependent signaling pathways (CatSper KOs) (Qi et al., 2007; Quill et al., 2001; Ren et al., 2001) are essential for capacitation. All these mice are infertile *in vivo* and *in vitro* without presenting observable defects either in spermatogenesis or in epididymal maturation. These loss-of-function genetic experiments have confirmed previous pharmacological approaches and conclusively demonstrated that all these pathways are required for fertilization. However, the hierarchical interaction between cAMP, pH_i , $[\text{Ca}^{2+}]_i$ and changes in the membrane potential are not well understood.

A very early event in sperm capacitation is the activation of a cAMP pathway (Visconti, 2009). The activation of cAMP synthesis occurs immediately after sperm are released from the epididymis and get into contact with high HCO_3^- and Ca^{2+} present in the seminal fluid (Carlson et al., 2005; Wennemuth et al., 2003). Plasma membrane transport of these ions regulates sperm cAMP metabolism through stimulation of Adcy10 (Hess et al., 2005; Xie et al., 2006). Adcy10 activation elevates intracellular cAMP and stimulates PKA, which then phosphorylates target proteins and initiate several signaling pathways including the increase in protein tyrosine phosphorylation (Visconti et al., 1995a). In sperm exposed to HCO_3^- , cAMP rises to a maximum within 60 sec, followed closely by PKA activation (Morgan et al., 2008). We have shown that in mouse sperm the increase in tyrosine phosphorylation is dependent on $[\text{Ca}^{2+}]_e$ (Visconti et al., 1995a). Those published experiments were conducted using media prepared without the addition of Ca^{2+} salts ($\ominus \text{Ca}^{2+}$). However, it is known that $\ominus \text{Ca}^{2+}$ media still contains contaminating Ca^{2+} (Marin-Briggiler et al., 2005). In the present work we evaluated the effect of further decreasing Ca^{2+} in the sperm incubation medium by using EGTA. This chelating agent induced an increase in PKA activity and in tyrosine phosphorylation when HCO_3^- and BSA were present in the media. The EGTA-dependent increase followed a similar kinetics to the one depicted in standard Ca^{2+} containing media. Considering the role of HCO_3^- in Adcy10 activation, these data suggest that the EGTA effect was mediated by PKA. Consistently, the PKA inhibitor H-89 blocked the EGTA induced phosphorylation of PKA substrates.

Altogether, these results suggest that Ca^{2+} has both positive and negative roles in the regulation of tyrosine phosphorylation and capacitation associated pathways. It is known that Ca^{2+} modulates many different enzymes either directly or indirectly through interaction with calmodulin. Regarding the cAMP pathway, Ca^{2+} positively regulates Adcy10 (Jaiswal and Conti, 2003; Litvin et al., 2003) what would explain the higher phosphorylation of PKA substrates and tyrosine residues as a consequence of increasing Ca^{2+} concentration. On the other hand,

negative regulation of cAMP-dependent pathways can be explained by the activation of Ca^{2+} /CaM-dependent enzymes such as the phosphodiesterase PDE1 or the ser/thr phosphatase calcineurin. Consistent with this hypothesis, two CaM antagonists (W7 and calmidazolium) increased tyrosine phosphorylation in sperm incubated in $\ominus \text{Ca}^{2+}$ medium to similar levels to the ones obtained by Ca^{2+} chelation with EGTA. Furthermore, the PDE inhibitor IBMX, and cyclosporine A (CSA), which associates to cyclophilin and specifically blocks calcineurin activity, were also able to induce an increase in phosphorylation pathways in $\ominus \text{Ca}^{2+}$ conditions. On the contrary, addition of KN93, which blocks calmodulin-dependent kinases present in sperm (CaMKII and CaMKIV) (Ignotz and Suarez, 2005; Marin-Briggiler et al., 2005), had no effect.

Interestingly, it has been reported that contrary to mouse sperm, human (Battistone et al., 2014; Carrera et al., 1996), and horse (Gonzalez-Fernandez et al., 2013) sperm incubated in $\ominus \text{Ca}^{2+}$ media undergo tyrosine phosphorylation. Although these results suggest species-specific differences, in view of the effect of EGTA in mouse sperm, the differences observed are likely to be due to differences in the effective Ca^{2+} concentration needed for negative and positive feedback regulation of tyrosine phosphorylation more than to differences in the identity of Ca^{2+} -dependent molecules mediating this signaling pathway. This idea is reinforced by experiments showing that addition of W7 significantly up-regulated protein tyrosine phosphorylation in stallion sperm (Gonzalez-Fernandez et al., 2013).

The combination of positive and negative feedback loops involving Ca^{2+} highlights the relevance of this ion in sperm physiology. However, the molecular mechanisms that control $[\text{Ca}^{2+}]_i$ in sperm are not well established. The best-characterized Ca^{2+} channel in these cells is CatSper, a complex of at least 7 subunits (CatSper 1, 2, 3, 4, β , γ , and δ) localized to the plasma membrane surrounding the sperm principal piece. Null mice for any of these subunits result in lack of CatSper functionality giving rise to a sterile phenotype (Qi et al., 2007; Ren et

al., 2001; Ren and Xia, 2010). Sperm from these mice are unable to undergo hyperactivation and are also infertile *in vitro*. Interestingly, the capacitation-associated increase in tyrosine phosphorylation is not affected in Catsper KO sperm (Carlson et al., 2003). A first approximation to these results suggests that Catsper is not responsible for the increase in tyrosine phosphorylation. However, this straightforward interpretation is challenged by our new data indicating that the Ca^{2+} effect on the activation of the tyrosine phosphorylation pathway is biphasic. Results using Catsper KO sperm could also be explained considering that even in the presence of high $[\text{Ca}^{2+}]_e$, Catsper null sperm behave similarly to wild type sperm when incubated in $\ominus \text{Ca}^{2+}$ media with EGTA. If this hypothesis is correct, sperm from Catsper null mice should undergo tyrosine phosphorylation in $\ominus \text{Ca}^{2+}$ media. On the other hand, if CatSper is not involved in the initial Ca^{2+} response, the Ca^{2+} biphasic response would still be observed and CatSper KO sperm would not undergo phosphorylation pathways in $\ominus \text{Ca}^{2+}$ media. In the present manuscript, we show that contrary to wild type sperm, CatSper KO sperm undergo PKA activation and tyrosine phosphorylation in $\ominus \text{Ca}^{2+}$. These results indicate that Catsper KO sperm response is similar to the one obtained when wild type sperm are incubated with EGTA and strongly suggest that CatSper mediates the Ca^{2+} transport involved in the regulation of tyrosine phosphorylation. Using super resolution microscopy, recent work has revealed that CatSper acts as a signaling dock establishing four linear rows throughout the flagellum (Chung et al., 2014). The authors show that in sperm from CatSper KO mice, the increase in tyrosine phosphorylation is significantly up regulated. These results are consistent with our findings that contrary to wild type sperm, in CatSper KO sperm, cAMP-dependent pathways are activated in the absence of added external Ca^{2+} . Interestingly, these authors also showed that calcineurin and Adcy10 are both found in the linear signaling array.

The crosstalk between Ca^{2+} , CaM and cAMP pathways is summarized in a working model (Fig. 7). This Figure depicts only the pathways explored as part of this manuscript and it is not

an exhaustive analysis of possible Ca^{2+} or $\text{Ca}^{2+}/\text{CaM}$ targets. Briefly, *Adcy10* activity is positively regulated by Ca^{2+} and HCO_3^- (Litvin et al., 2003) (Jaiswal and Conti, 2003) resulting in cAMP synthesis and activation of PKA, both of them present in the sperm flagellum (Hess et al., 2005; Wertheimer et al., 2013). Ca^{2+} also activates CaM, which mediates the stimulation of PDE and calcineurin. While PDE catalyzes cAMP degradation to 5'AMP, and therefore has only a negative role in cAMP-dependent pathways, calcineurin is involved in de-phosphorylation of ser/thr residues including those present in PKA substrates. As part of the present work, our results indicate that in sperm from *CatSper 1* KO mice, both PKA activity and the pathway leading to tyrosine phosphorylation is up-regulated in $\ominus \text{Ca}^{2+}$. These data suggest that in addition to having an essential role in later capacitation processes such as the regulation of sperm hyperactivated motility, *CatSper* is also involved in the initial crosstalk between Ca^{2+} and cAMP-dependent signaling.

Finally, as mentioned above, abrogation of enzymes involved in the cAMP pathway clearly demonstrate that cAMP synthesis and PKA are absolutely required for the sperm to undergo capacitation and to fertilize. However, it is worth clarifying that although necessary, the increase in cAMP levels and the consequent increase in tyrosine phosphorylation are not sufficient to induce sperm fertilizing capacity. Similar results can be observed when PKA is activated with cAMP permeable agonists in the absence of Ca^{2+} . In these conditions, PKA and tyrosine phosphorylation are up-regulated but the sperm does not achieve fertilizing capacity (data not shown). In addition, we should emphasize that motility hyperactivation, the acrosome reaction and the ability to achieve fertilization do not occur in Ca^{2+} -free media. Consistent with a role of Ca^{2+} for these events, we have recently shown that a short exposure to the Ca^{2+} ionophore A23187 is sufficient to induce hyperactivation, acrosome reaction and fertilizing ability in mouse sperm even when the cAMP pathway is completely inhibited (Tateno et al., 2013). Altogether, these results suggest that, in addition to phosphorylation changes, the acquisition of

fertilizing capacity requires processes downstream PKA activation, thus demanding a more robust increase in intracellular Ca^{2+} through Catsper or other Ca^{2+} channels and/or intracellular Ca^{2+} stores.

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FIGURE LEGENDS

Figure 1. Ca²⁺ involvement in capacitation-associated, tyrosine phosphorylation response is biphasic.

Cauda epididymal sperm were recovered by swim-out in media without Ca²⁺ (nominal zero Ca²⁺, indicated by Θ Ca²⁺), HCO₃⁻ or BSA as described in Methods. Aliquots of this sperm suspension containing $\sim 10^6$ sperm were then diluted in media supplemented with HCO₃⁻ and BSA (15 mM and 5 mg/ml, respectively) and in the presence of either EGTA (1 mM), increasing Ca²⁺ concentrations (from Θ to 2.4 mM) (**A and B**); or with increasing EGTA concentrations (from 0.01-1 mM) (**C and D**). After 1 h incubation, sperm were processed for the analysis of protein phosphorylation with anti-PY antibodies. Images shown on the left are representative of experiments repeated at least three times. For each experiment, Western blots were scanned and analyzed using Image J. For comparison between blots, pixels for each lane contained in the region marked by # (proteins in a 50-150 KDa range) were quantified and normalized using the Θ Ca²⁺ lane as reference (**B and D** correspond to Western blots in panels A and C, respectively). Bars represent the average \pm SEM of the normalized values (*p<0.05; **p<0.01; *** p<0.001).

Figure 2. Analysis of the EGTA-dependent increases in tyrosine phosphorylation.

A and B: Kinetics of EGTA-induced tyrosine phosphorylation. Sperm were obtained

in media devoid of Ca^{2+} (Θ), HCO_3^- or BSA. Aliquots containing $\sim 10^6$ sperm were then diluted in media containing HCO_3^- , BSA and EGTA (15 mM, 5 mg/ml and 0.3 mM, respectively). At different time periods, reactions were stopped by sperm centrifugation, washing with PBS and boiled in sample buffer. Protein extracts were then analyzed by Western blot with an anti-PY antibody. **C and D: EGTA-induced phosphorylation**

requires HCO_3^- . Cauda epididymal sperm were obtained as described in (A). Aliquots of this suspension containing $\sim 10^6$ sperm were diluted in ΘCa^{2+} media containing BSA (5 mg/ml), EGTA (0.3 mM) and increasing HCO_3^- concentrations (from 0 to 15 mM as indicated) (lanes 3 to 8). **E and F: EGTA-induced phosphorylation requires BSA.**

Sperm were obtained as in (A) and aliquots ($\sim 10^6$ sperm) diluted in ΘCa^{2+} media containing HCO_3^- (15 mM), EGTA (0.3 mM) and increasing BSA concentrations (from 0 to 5 mg/ml) (lanes 3 to 8). Both HCO_3^- and BSA concentration curves were analyzed after 1 h incubation by Western blots with anti-PY antibodies. In B and C panels, lane 1 contains extracts from sperm incubated for 1 h in ΘCa^{2+} media devoid HCO_3^- and BSA; lane 2 (Iuvone et al.) is a control containing extracts from sperm incubated for 1 h in standard Whitten's capacitation media containing Ca^{2+} (2.4 mM), HCO_3^- (15 mM) and BSA (5 mg/ml). All images are representative of experiments repeated at least three times. For each experiment, Western blots were scanned and analyzed using Image J. For comparison between blots, pixels for each lane contained in the region marked by # were quantified and normalized using the ΘCa^{2+} lane as reference (**B, D and F, respectively**). Bars represent the average \pm SEM of the normalized values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 3. EGTA decreases intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in ΘCa^{2+} media. A: Pseudocolored fluorescence images illustrate different $[\text{Ca}^{2+}]_i$ levels in a

Fluo4-loaded spermatozoa when exposed to different extracellular Ca^{2+} concentrations: Θ Ca^{2+} media, Θ Ca^{2+} media containing EGTA (1 mM), 2 mM Ca^{2+} and during Ionomycin application. **B**: Average fluorescence trace of several sperm corresponding to $[\text{Ca}^{2+}]_i$ changes exposed to different extracellular $[\text{Ca}^{2+}]_o$. Ionomycin was added at the end of each recording as control of maximum fluorescence. **C**: Percentage of sperm incubated in Θ Ca^{2+} media that manifest a $[\text{Ca}^{2+}]_i$ decrease after incubation in Θ Ca^{2+} media containing EGTA. The percentage of sperm that display this decrease is statistically significant. **D**: Percentage of fluorescence changes corresponding to $[\text{Ca}^{2+}]_i$ of sperm exposed to different extracellular $[\text{Ca}^{2+}]_o$. For quantification, fluorescence was normalized to the maximum fluorescence induced by ionomycin. The $[\text{Ca}^{2+}]_i$ decrease promoted by EGTA in Θ Ca^{2+} media is statistically significant. Bars represent the average \pm SEM of the normalized values (* $p < 0.05$) of measurements performed on the sperm head.

Figure 4. Effect of Calmodulin and Calmodulin targets inhibitors on the increase in tyrosine phosphorylation incubated in Θ Ca^{2+} media. Cauda epididymal sperm were recovered by swim-out in media without Ca^{2+} (Θ), HCO_3^- or BSA. Aliquots of this sperm suspension containing $\sim 10^6$ sperm were then incubated for 1 h in Θ Ca^{2+} media containing HCO_3^- and BSA (15 mM and 5 mg/ml, respectively) and in the absence or in the presence of either EGTA (0.3 mM) (negative and positive controls, respectively) or in the presence of increasing concentrations of W7 (1-100 μM) (**A**), calmidazolium (CZ) (0.5-2.5 μM) (**B**), IBMX (1-200 μM) (**C**), KN-62 (0.5 to 8 μM) (**D**), KN-93 (0.1 to 10 μM) (**E**), and CSA (0.5 to 3 μM) (**F**). Sperm extracts were analyzed by gel electrophoresis, transferred to PVDF membranes and Western blot using anti-PY antibodies were conducted. Data shown are representative of experiments repeated at least three times. For each experiment, Western blots were scanned and analyzed using Image J. For

comparison between blots, pixels for each lane contained in the region marked by # were quantified and normalized using the Θ Ca^{2+} lane as reference. Results of this quantification analysis are shown in **Supplemental Figure 1**).

Figure 5. A and B: Cyclophilin A and Calcineurin are present in mouse sperm.

Cauda epididymal sperm were obtained by swim-out as described. Protein extracts prepared from 10^6 sperm were analyzed by Western blot using anti-cyclophilin A (**A**), or anti-calcineurin A (**B**). As positive controls, protein extracts (10 μg) of either thymus or brain were included. All Western blots are representative of experiments repeated at least three times. **C: Effect of calmodulin and calmodulin-target inhibitors on sperm hyperactivation.** Mouse sperm were incubated in Θ Ca^{2+} media containing HCO_3^- (15 mM) and BSA (5 mg/ml) and in the absence or in the presence of EGTA (1 mM), IBMX (100 μM), W7 (100 μM), calmidazolium (CZ) (2.5 μM), or CSA (3 μM). Sperm incubated in medium containing Ca^{2+} (2.4 mM) (Iuvone et al.) (represented as Cap in the figure) was included as positive control. After 60 min of sperm incubation, sperm motility data was analyzed as described in Methods. The percentage of hyperactive sperm (%) was assessed using parameters described for mouse sperm by the CASAnova software algorithm. Data represents mean \pm S.E. of four independent experiments (** $p < 0.01$). **D: Sperm do not fertilize in the presence of EGTA but recover fertilizing capacity when the EGTA is washed out.** As controls, aliquots of 10^6 sperm were incubated for 1 h in Whitten's media containing 2.4 mM Ca^{2+} in the absence (NCap) or in the presence of 15 mM HCO_3^- and 5 mg/ml of BSA. After 1 h of incubation in the respective media, IVF was carried out in standard Whitten's media as described in Methods. To test the effect of EGTA, parallel sperm aliquots were incubated in Θ Ca^{2+} media containing 15 mM HCO_3^- and 5 mg/ml BSA in the presence of 1 mM EGTA (EGTA or EGTA washed). After

1 h, one of the aliquots was tested for IVF conducted in Θ Ca^{2+} media with 1 mM EGTA (EGTA). The other aliquot (EGTA washed), also incubated for 1 hour in the presence of 1 mM EGTA, was centrifuged and resuspended in standard Whitten's media containing 2.4 mM Ca^{2+} . IVF was then conducted in Ca^{2+} containing media. **E and F: EGTA and Calmodulin antagonists induce phosphorylation of PKA substrates.** Mouse sperm were incubated in Θ Ca^{2+} media containing HCO_3^- (15 mM) and BSA (5 mg/ml) and in the absence or in the presence of EGTA (1 mM), IBMX (100 μM), W7 (100 μM), calmidazolium (CZ) (2.5 μM), or CSA (3 μM). After 1 h incubation, sperm protein extracts were prepared and analyzed by Western blotting using anti-phospho PKA substrates antibodies (Fig. 5 E). Data shown are representative of experiments repeated at least three times. **F:** For each experiment, Western blots were scanned and analyzed using Image J. For comparison between blots, pixels for each lane contained in the region marked by # were quantified and normalized using the Θ Ca^{2+} lane as reference (Fig. 5 F). Bars represent the average \pm SEM of the normalized values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 6. Sperm from *CatSper* KO mice undergo an increase in tyrosine phosphorylation in Θ Ca^{2+} media. A and B: Calcium concentration-dependent response. Cauda epididymal sperm from wild type (left panel) or from *CatSper* 1 KO mice (right panel) were separately obtained by swim-out in media without Ca^{2+} (Θ), HCO_3^- or BSA. Aliquots of each sperm suspension containing $\sim 10^6$ sperm were then diluted in media containing HCO_3^- and BSA (15 mM and 5 mg/ml, respectively) and in the presence of either EGTA (0.3 mM) (as a positive control) or increasing Ca^{2+} concentrations (Θ -2.4 mM). After 1 h incubation, sperm were centrifuged, washed once with PBS by centrifugation and processed for the analysis of tyrosine phosphorylation by

Western blotting. Images shown are representative of experiments repeated at least three times. In Panel B Western blots for each experiment, were scanned and analyzed using Image J. For comparison between blots, pixels for each lane contained in the region marked by # were quantified and normalized using the respective EGTA lane as reference (represented by bars and arbitrarily given a value of 1). Values in the graph represent the average \pm SEM of the normalized values (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

C: Tyrosine phosphorylation kinetics in Θ Ca^{2+} . Sperm from wild type and *Catsper* 1 KO mice were obtained in media devoid of Ca^{2+} (Θ), HCO_3^- or BSA. Aliquots of this suspension were diluted in media containing HCO_3^- and BSA (15 mM and 5 mg/ml, respectively). After different time periods, sperm samples were centrifuged, washed with PBS and boiled in sample buffer. After boiling, the protein extracts were analyzed by Western blot with an anti-PY antibody. Images are representative of experiments conducted three times with similar results. **D: The increase in tyrosine**

phosphorylation observed in sperm from *Catsper* KO mice is downstream a cAMP-dependent pathway. Cauda epididymal sperm were recovered from *Catsper* KO mice by swim-out in media without Ca^{2+} (Θ), HCO_3^- or BSA. Aliquots of this sperm suspension containing $\sim 10^6$ sperm were then incubated for 1 h in Θ Ca^{2+} media containing HCO_3^- and BSA (15 mM and 5 mg/ml, respectively) and in the absence or in the presence of either H-89 (30 μM) or KH7 (75 μM). Protein extracts were analyzed by Western blot with an anti-PY antibody. Images are representative of experiments conducted three times with similar results.

Figure 7. Proposed model for the crosstalk between Ca^{2+} and cAMP-dependent signaling pathways conducive to mouse sperm capacitation. See Discussion section for explanation of this Figure.

Figure S1. Image J quantification of Western blots depicted in Fig. 4. For comparison between blots depicted in Fig. 4, pixels for each lane contained in the region marked by # were quantified and normalized using the Θ Ca²⁺ lane as reference.

Fig. 1

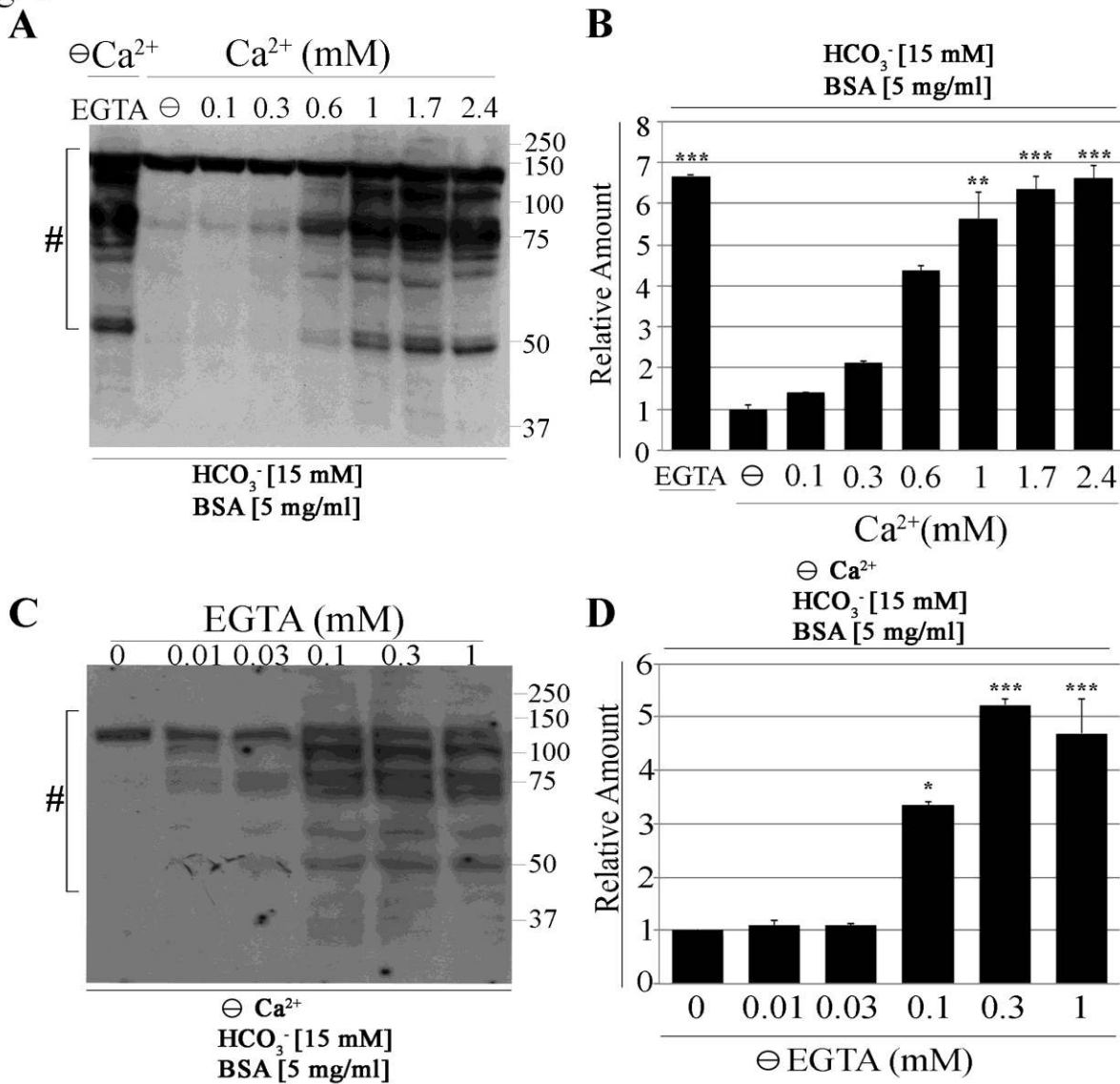


FIG 2

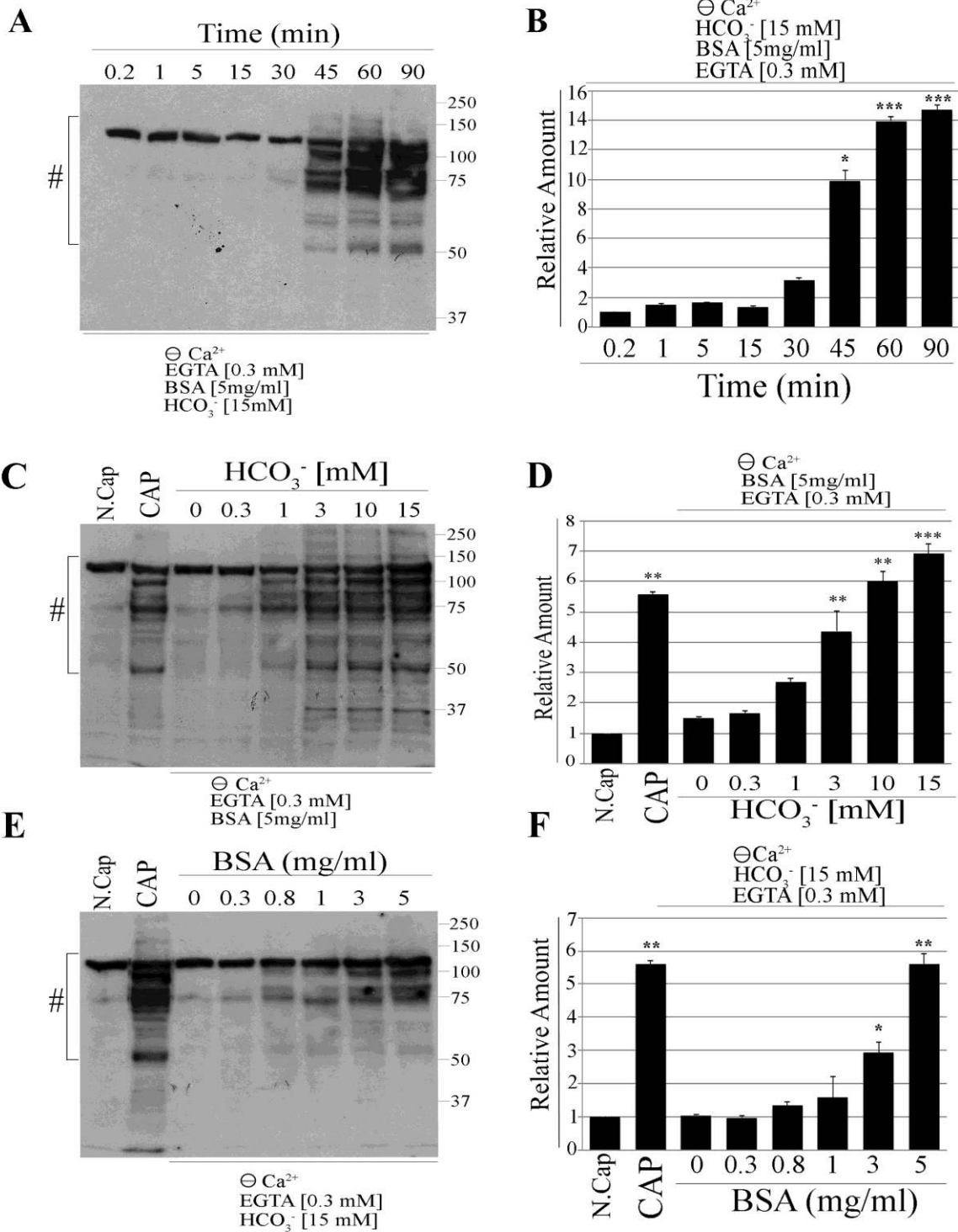
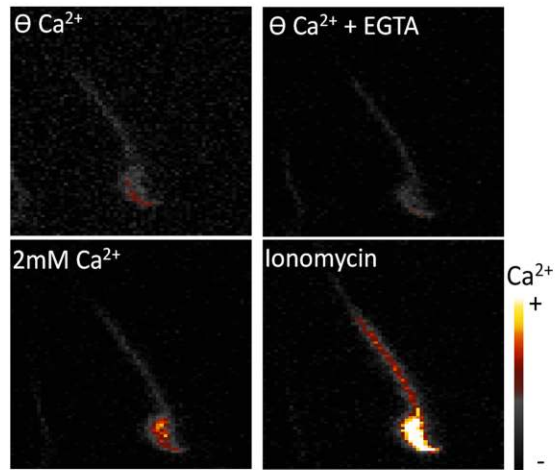
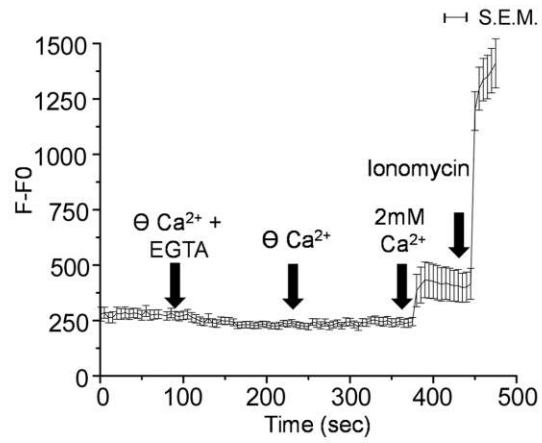


FIG 3

A



B



C

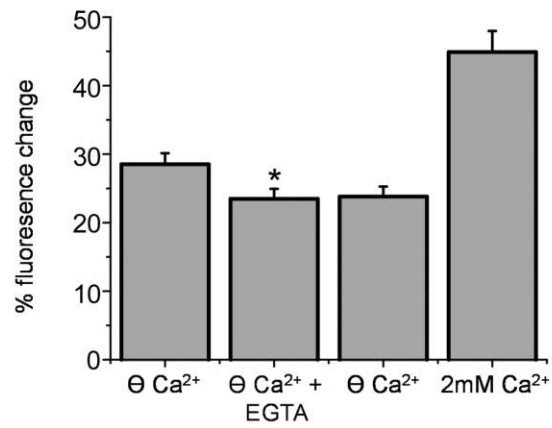


FIG 4

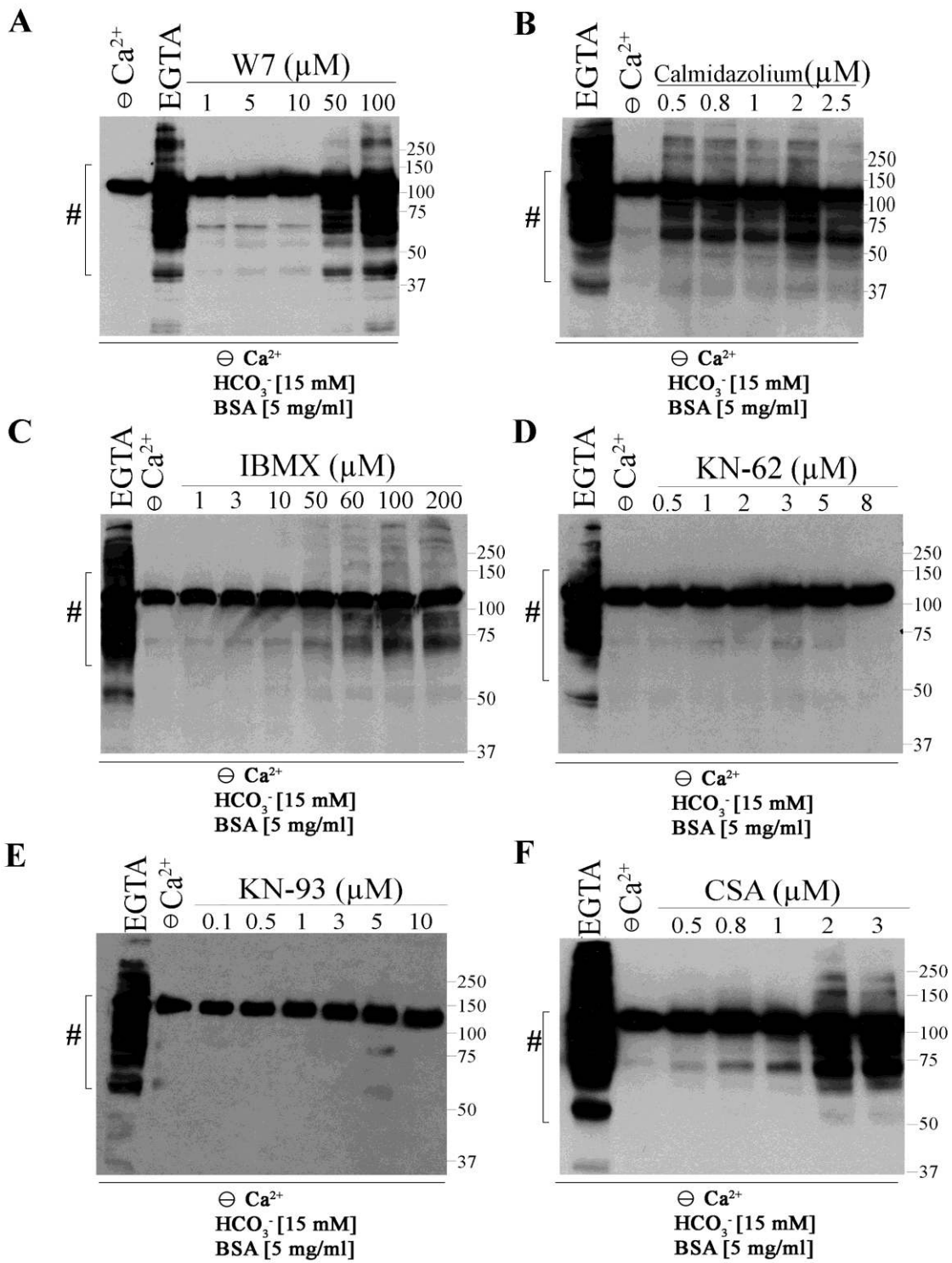


Fig. 5

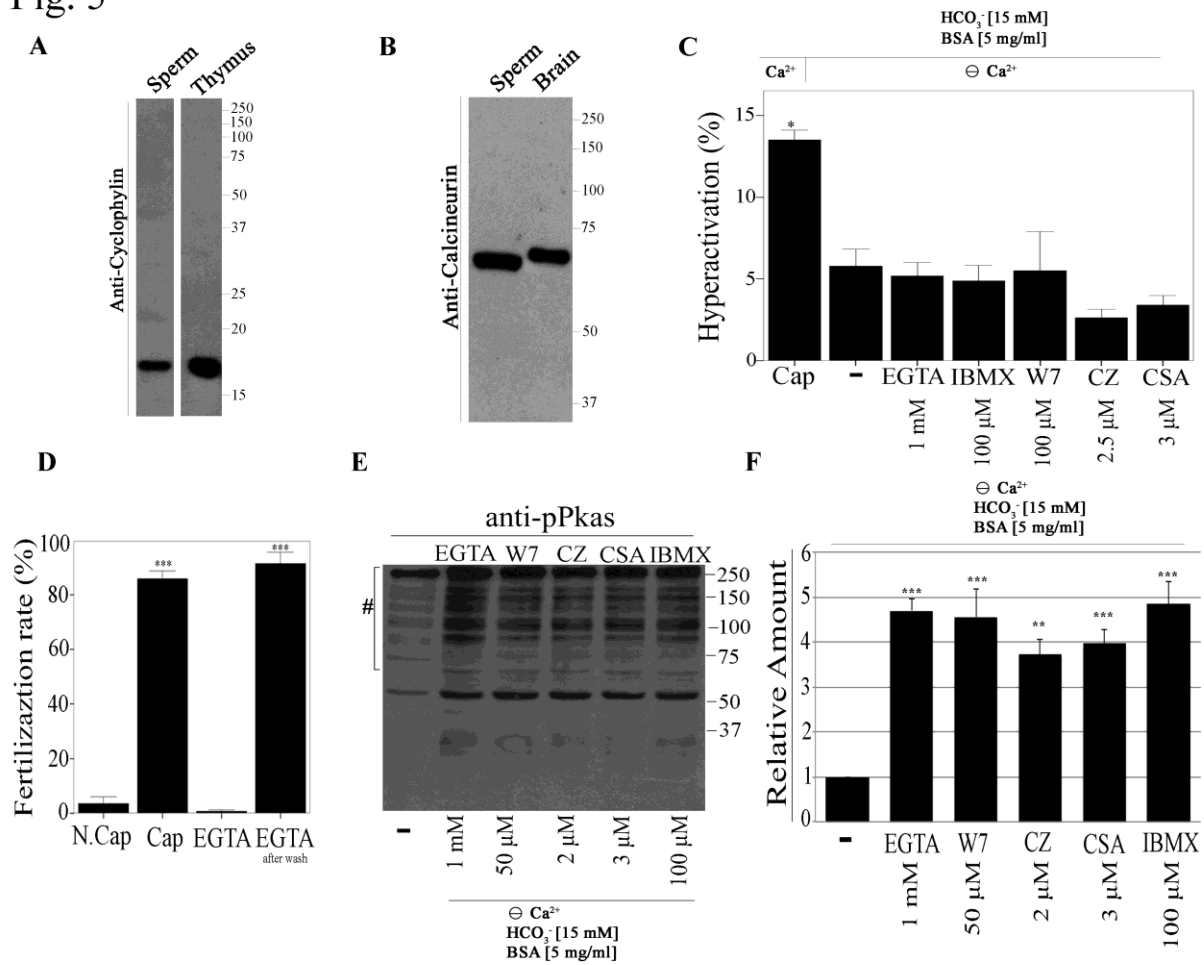
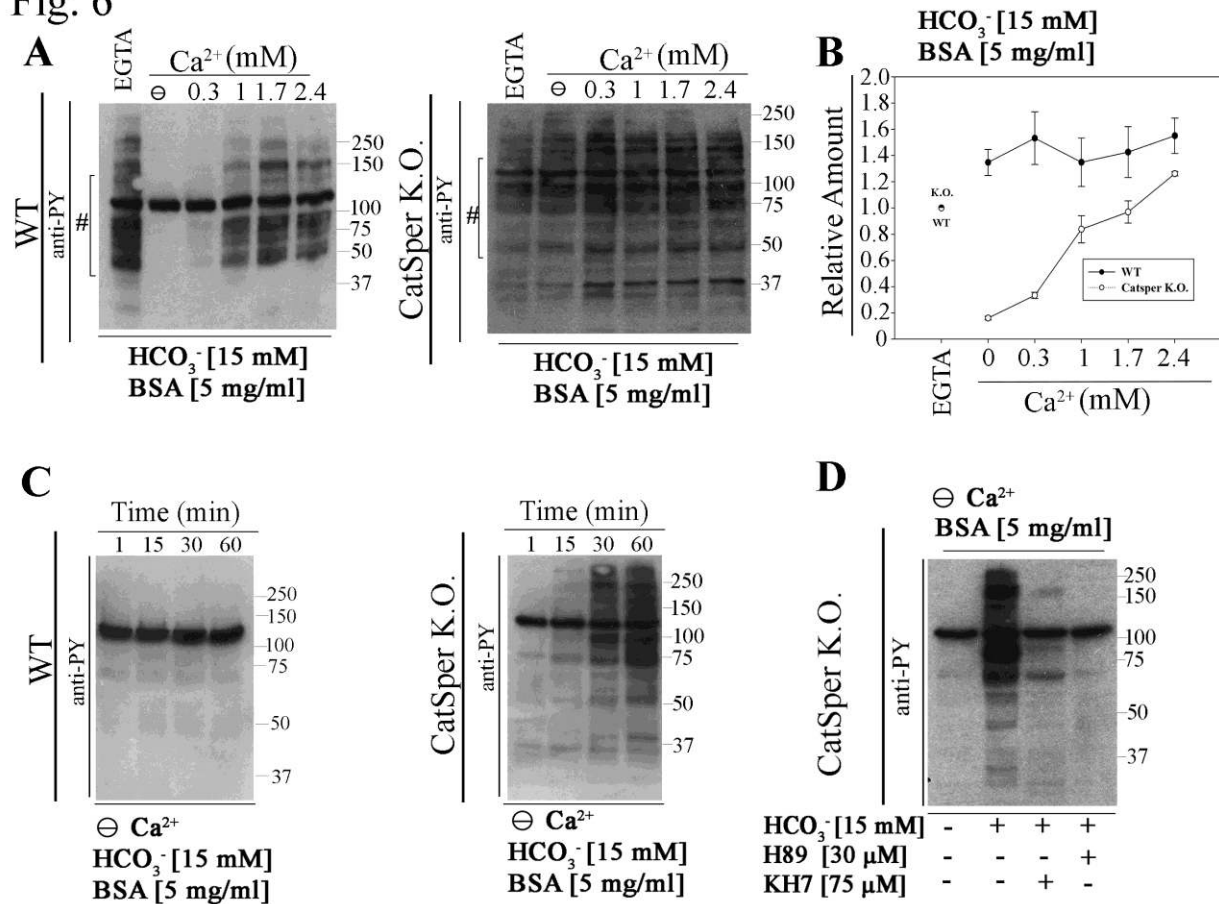


Fig. 6



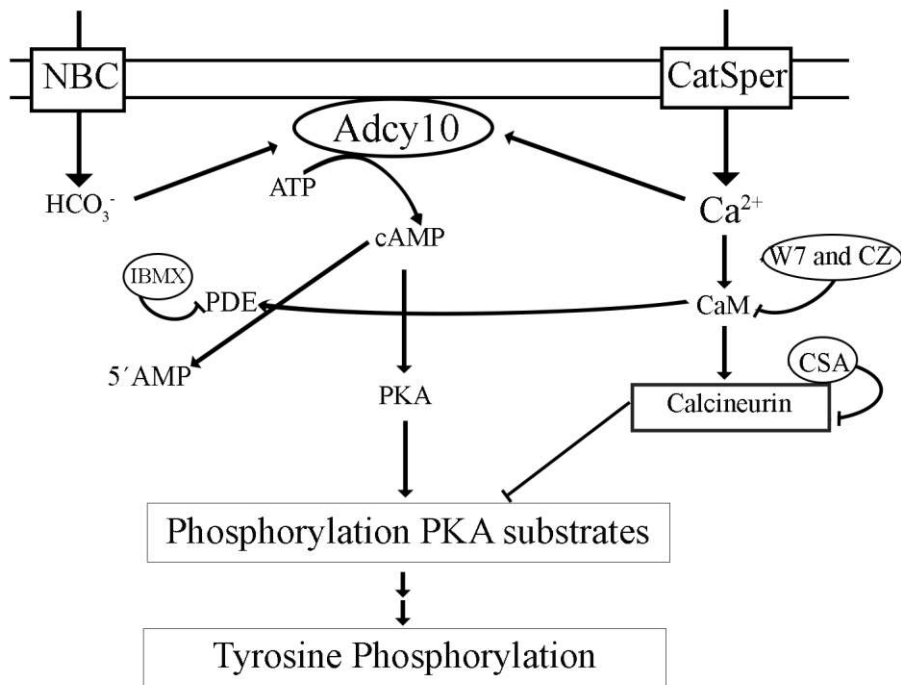
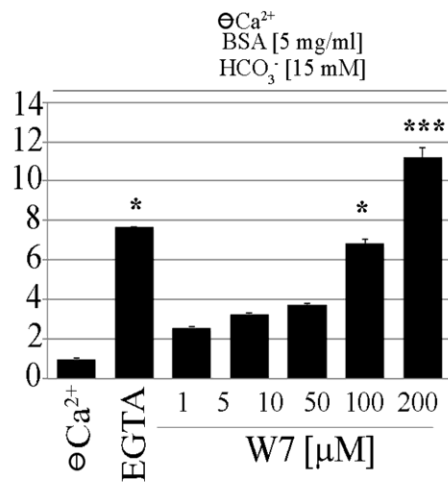


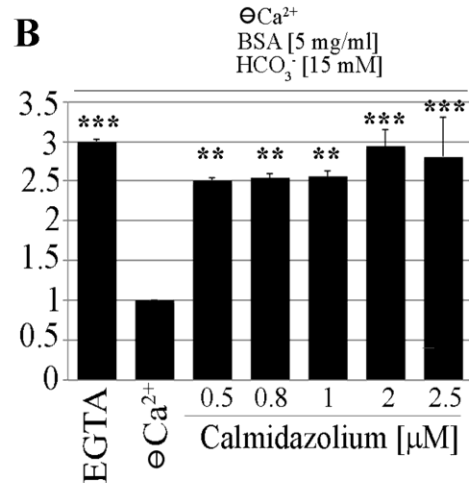
Fig. 7

Fig S1

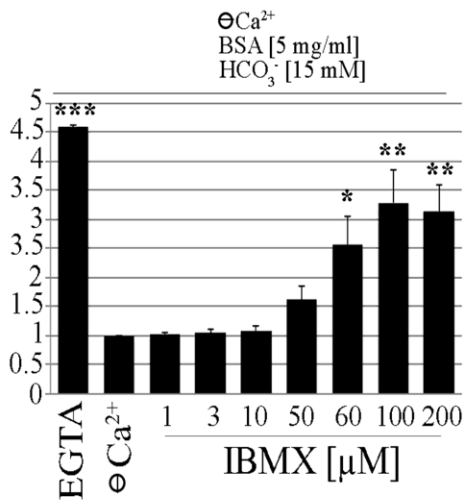
A



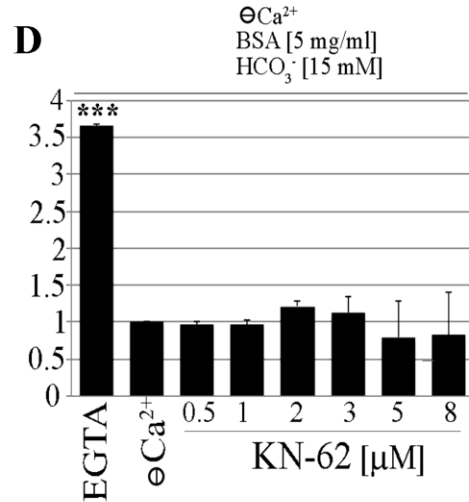
B



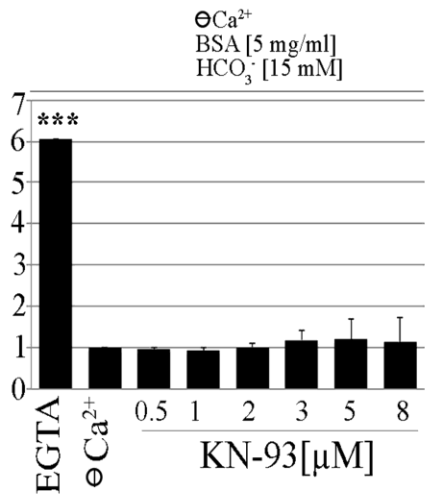
C



D



E



F

