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The development of signal transduction pathways during epididymal maturation is calcium dependent

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Keywords

signal, dependent, development, transduction, maturation, during, pathways, calcium, epididymal, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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**The development of signal transduction pathways during epididymal maturation
is calcium dependent**

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Short Title: Tyrosine Phosphorylation and Epididymal Maturation

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Abstract

Capacitation has been correlated with the activation of a cAMP/PKA-dependent signaling pathway leading to protein tyrosine phosphorylation. The ability to exhibit this response to cAMP matures during epididymal maturation in concert with the ability of the spermatozoa to capacitate. In this study, we have addressed the mechanisms by which spermatozoa gain the potential to activate this signaling pathway during epididymal maturation. In a modified Tyrode's medium containing 1.7 mM calcium, caput spermatozoa had significantly higher $[Ca^{2+}]_i$ than caudal cells and could not tyrosine phosphorylate in response to cAMP. However, in calcium-

10 depleted medium both caput and caudal cells could exhibit a cAMP-dependent phosphorylation response. The inhibitory effect of calcium on tyrosine phosphorylation was also observed in caudal spermatozoa using thapsigargin, a Ca^{2+} -ATPase inhibitor, that increased $[Ca^{2+}]_i$ and precipitated a corresponding decrease in phosphotyrosine expression. We also demonstrate that despite the activation of tyrosine phosphorylation in caput spermatozoa, these cells remain non-functional in terms of motility, sperm-egg recognition and acrosomal exocytosis. These results demonstrate that the signaling pathway leading to tyrosine phosphorylation in mouse spermatozoa is negatively regulated by $[Ca^{2+}]_i$, and that maturation mechanisms that control $[Ca^{2+}]_i$ within the spermatozoon are critically important during epididymal

20 transit.

Key Words: Calcium, Epididymis, Signal transduction, Sperm capacitation, Sperm maturation, Tyrosine phosphorylation, Capacitation

Introduction

When spermatozoa leave the testis they are not capable of progressive movement or the cascade of cellular interactions that result in fertilization of the oocyte. They acquire the potential to express these properties as they pass through the epididymis (Yanagimachi, 1994), but it is only after a further period of post-
30 epididymal maturation known as ‘capacitation’, that this potential is fully realized (Austin, 1951; Chang, 1951). Capacitation involves a time dependent series of physiological and biochemical changes that enables spermatozoa to recognize and bind to the zona pellucida, undergo the acrosome reaction and initiate fusion with the oocyte (Ward and Storey, 1984; Florman and Babcock, 1991; Kopf and Gerton, 1991). A correlate of this process is the activation of a cAMP/protein kinase A (PKA)-dependent signaling pathway that leads to the induction of protein tyrosine phosphorylation, which has been identified in all species studied to date (Aitken *et al.*,
1995; Visconti *et al.*, 1995a; Visconti *et al.*, 1995b; Galantino-Homer *et al.*, 1997; Mahony and Gwathmey, 1999; Tardif *et al.*, 1999; Visconti *et al.*, 1999; Lewis and
40 Aitken, 2000). This protein tyrosine phosphorylation is thought to play a significant role in the control of capacitation-dependent events, such as hyperactivated motility (Mahony and Gwathmey, 1999; Si and Okuno, 1999; Yeung *et al.*, 1999) and sperm-egg recognition (Flesch *et al.*, 2001; Urner *et al.*, 2001).

Calcium is a key regulator of sperm activity, including motility activation (Morton *et al.*, 1974; Armstrong *et al.*, 1994; Wade *et al.*, 2003), capacitation (Fraser, 1987; DasGupta *et al.*, 1993; Visconti *et al.*, 1995a) and the acrosome reaction (Yanagimachi and Usui, 1974; Thomas and Meizel, 1989; Florman, 1994). Importantly, calcium has also been shown to impact on the signaling pathway leading to tyrosine phosphorylation (Visconti *et al.*, 1995a; Carrera *et al.*, 1996; Luconi *et al.*,

50 1996). Intracellular free calcium, $[Ca^{2+}]_i$, is higher, and the rate of calcium uptake is 2-4 times greater, in spermatozoa from the caput than cauda epididymis (Vijayaraghavan *et al.*, 1989; White and Aitken, 1989). However, it is not known whether such differences in $[Ca^{2+}]_i$ impacts on the signaling pathway leading to tyrosine phosphorylation in the immature gamete. Moreover, whilst this tyrosine phosphorylation event has been documented in many species, the mechanism by which spermatozoa acquire the potential to exhibit this signaling pathway during epididymal maturation remains to be elucidated. Studies on the rat (Lewis and Aitken, 2001) have demonstrated that this cAMP-signal transduction pathway is not functional in spermatozoa from the caput epididymis, despite the availability of
60 cAMP, and the presence of both PKA and its associated regulatory subunits. In this study we have investigated the mechanisms controlling the ability of spermatozoa to tyrosine phosphorylate during epididymal maturation and have identified $[Ca^{2+}]_i$ as being a key regulator of this process.

Materials and Methods

Reagents

The following reagents were used: bovine serum albumin (BSA, Research Organics, Cleveland, OH), HEPES, penicillin, and streptomycin (Gibco, Paisley, UK), thapsigargin and mowiol (Calbiochem, San Diego, CA), sulfo-NHS-LC-biotin and bicinchoninic acid (BCA) kit (Pierce, Rockford, IL), streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin, Dynal, Oslo, Norway), pregnant mares serum gonadotrophin (PMSG; Folligon) and human chorionic gonadotrophin (hCG; Chorulon) (Intervet, Castle Hill, NSW, Australia), clone 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), goat anti-mouse antibody (Santa Cruz, Santa Cruz, CA), goat serum (Hunter Antisera, Jesmond, NSW, Australia), Fura-2AM (tetraacetoxymethyl ester) and Fluo-3AM (Molecular Probes, Eugene, OR), ammonium persulphate, 2-mercaptoethanol and N, N, N1-tetra-methylethylenediamine (Temed) (Biorad, Herts, UK), nitrocellulose hybond super-C membrane and enhanced chemiluminescence (ECL) kit (Amersham International, Buckinghamshire, UK) and other reagents (Sigma, St. Louis, MO), unless otherwise stated.

Sperm preparation

Spermatozoa were collected from Swiss mice (8-14 week old, bred in the University's Animal Facility) by backflushing the distal cauda epididymidis with water-saturated paraffin oil. The samples were stored under oil at 37°C until use. The spermatozoa were then activated by adding 200 µL of BWW/PVA-Ca²⁺, a modification of BWW (Biggers *et al.*, 1971: 915 mM NaCl, 44 mM sodium lactate, 25 mM NaHCO₃, 20

mM HEPES, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2
90 mM MgSO₄·7H₂O, 0.27 mM sodium pyruvate, 0.3% w/v BSA, 5 U/mL penicillin, 5
µg/mL streptomycin, pH 7.4) that lacked Ca²⁺ and contained 1mg/ml polyvinyl-
alcohol instead of BSA. This medium was used as it minimizes the loss of cholesterol
from the sperm plasma membrane (Davis *et al.*, 1979; Davis, 1981) and slows
tyrosine phosphorylation (Visconti *et al.*, 1995a). Sodium chloride was used to adjust
the osmolarity. Spermatozoa from the caput epididymidis were collected by
dissecting the region free of fat, blotting it free of blood and finally immersing the
tissue in 500 µL of BWW/PVA-Ca²⁺ under water-saturated paraffin oil. Spermatozoa
were released by piercing the duct 5-8 times with a 30-gauge needle and incubating
for 10 min at 37°C. The concentration of sperm was determined using a Neubauer
100 haemocytometer. Unless otherwise stated, cells were then diluted in either
BWW/BSA or BWW/BSA-Ca²⁺ (i.e. the medium contained BSA instead of PVA) at a
final concentration of 1 x 10⁶ /mL and incubated at 37°C under an atmosphere of 5%
CO₂ and 95% air. Measurements of the amount of the Ca²⁺ present in medium in
which calcium chloride was omitted were made by atomic absorption spectroscopy
and were found to be 2 ± 0.3 µM. Thus, the medium is designated 'calcium-depleted'
in the text.

The ability of spermatozoa to undergo the acrosome reaction was assessed
after 60 min incubation. A sub-sample from each treatment was treated with 5 µM
A23187 and incubated for 30 min. Untreated 'control' samples were treated with
110 DMSO, the vehicle for A23187. After incubation, sperm were fixed with ice-cold
methanol and stored at -20°C. Samples were coded and arranged in random order to
avoid bias, plated onto poly-L-lysine coated slides and allowed to dry. The presence

of the acrosome was assessed by adding phosphate-buffered saline (PBS, pH 7.4) containing fluorescein-labeled *Arachis hypogaea* lectin (1 mg/mL), incubated for 15 min at 4°C, rinsed in PBS and viewed under a Zeiss Axioplan 2 microscope equipped with FITC filters. Spermatozoa were examined for the occurrence of the acrosome reaction, and a minimum of 200 cells were scored per treatment.

Protein Extraction, SDS-PAGE and Immunoblotting

120 Following incubation, the spermatozoa were centrifuged (500g, 3 min) and washed in 1 mL PBS. Sperm proteins were extracted with 2%-SDS buffer (0.375 M Tris-HCL and 1% (w/v) sucrose, pH 6.8), containing 1 mM sodium orthovanadate, heated to 100°C (5 min), centrifuged (20,000g, 10 min), and the supernatant boiled in SDS-buffer containing 2% (v/v) 2-mercaptoethanol. Protein concentration was determined on microtitre plates using a BCA kit and for each sample the same amount of protein was loaded onto the gels. Sperm proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels (Laemmli, 1970) and transferred onto nitrocellulose hybond super-C membrane (Towbin *et al.*, 1979), blocked with 3% (w/v) BSA in Tris-buffered saline (TBS, pH 7.6), and incubated for 2 h at room temperature with
130 monoclonal anti-phosphotyrosine antibody (1:4,000) in TBS containing 1% (w/v) BSA and 0.1% (v/v) Tween-20. The blots were washed 4 times for 5 min with TBS containing 0.01% (v/v) Tween-20, and incubated for 1 h at room temperature in TBS containing goat anti-mouse immunoglobulin G conjugated to HRP (1:3000), 1% (w/v) BSA and 0.1% (v/v) Tween-20. The membrane was washed and the phosphorylated proteins detected using an enhanced chemiluminescence (ECL) kit according to the manufacturers instructions. To ensure antibody specificity, separate membranes were

also incubated with anti-phosphotyrosine antibody that was absorbed with 20 mM *O*-phospho-L-tyrosine (1 h at room temperature and constant rotation) prior to immunoblotting. Western blots shown in the Results are representative of the 3 or
140 more replicates carried out for each experiment.

Indirect Immunofluorescence of Tyrosine Phosphorylated Proteins

Spermatozoa were fixed in 1% (w/v) paraformaldehyde (10 min), centrifuged (500g, 3 min), resuspended in PBS/glycine containing 0.01% (w/v) sodium azide and stored at 4°C. Samples were coded and randomized to avoid bias, plated onto poly-L-Lysine coated slides and permeabilized by exposure to 0.2% (v/v) Triton X-100 in PBS (room temperature, 10 min). The slides were washed 3 times with PBS and non-specific sites blocked with 10% (v/v) goat serum and 1% (w/v) BSA in PBS (1 h at 37°C in a humid chamber), washed 3 times with PBS, swamped with anti-
150 phosphotyrosine antibody (1:100) in PBS, incubated for 1 h (37°C in a humid chamber), washed 3 times with PBS-Tween, incubated in the presence of goat anti-mouse antibody (1:200) for 1h (37°C in a humid chamber), then washed again and allowed to air dry. The slides were mounted in mowiol containing 2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO), and the percentage of cells that displayed tail fluorescence scored (minimum of 200 cells per treatment). Phosphorylated proteins were evident over just the principal piece of the sperm's flagellum, or over both the principal piece and midpiece. 'Negative controls' either did not receive the primary antibody or the anti-phosphotyrosine antibody was pre-incubated in 20 mM *O*-phospho-L-tyrosine (1 h, room temperature).

Determination and Localization of Intracellular Calcium

[Ca²⁺]_i was assessed using the fluorescent calcium indicator Fura-2AM, while intracellular calcium was imaged using Fluo-3AM. Spermatozoa were loaded with either 2 μM Fura-2AM or 2 μM Fluo-3AM (20 min, 37°C) in either BWW/PVA or BWW/PVA-Ca²⁺, then centrifuged (300g, 10 min, 37°C) through 60% Percoll and resuspended in BWW/BSA, or BWW/BSA-Ca²⁺. [Ca²⁺]_i was determined by loading 1 mL of spermatozoa into a prewarmed cuvette, and recording the fluorescence signal using a Shimadzu RF-5301PC spectrofluorophotometer (Tokyo, Japan) following excitation at 340 nm and 380 nm, and emission at 510 nm, and calculating the fluorescence ratio (F). [Ca²⁺]_i was calculated using the equation (Grynkiewicz *et al.*, 1985) $[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$, where $K_d = 224$ nM. F_{max} and F_{min} were recorded at the end of each incubation. F_{max} was determined after the addition of 1% (v/v) Triton X-100 (prepared in complete BWW) and F_{min} was determined after addition of 5mM EGTA (pH 10) to the cuvette. In some experiments, CaCl₂ (2 mM) was added to the medium following addition of Triton in order to ensure that F_{min} was measured under saturating Ca²⁺ conditions.

Unbound intracellular calcium in mouse epididymal spermatozoa was imaged using Fluo-3AM and detected with a Zeiss Axiovert confocal laser-scanning microscope LSM 510 using an oil immersion lens (100/1.3). Fluorescence was examined in a wet mount on prewarmed poly-L-lysine coated glass slides excited with an argon-krypton laser and viewed at wavelengths >515 nm.

Effect of Thapsigargin on $[Ca^{2+}]_i$ and the Acrosome Reaction

The effect of thapsigargin on $[Ca^{2+}]_i$ was assessed by adding 1-10 μ M directly to the cuvette containing 1 mL of spermatozoa and determining the increase in F after 3 min. F_{max} and F_{min} were then determined as described above. The ability of thapsigargin to induce the acrosome reaction and loss of dye into the supernatant was also determined. The induction of the acrosome reaction was assessed on a sub-sample of spermatozoa from each treatment as described above. The loss of calcium
190 into the supernatant was assessed by centrifuging the sample (300g, 3min) and measuring F and F_{min} in the supernatant. The average increase in $F - F_{min}$ relative to 'control samples' (i.e. only exposed to the vehicle for thapsigargin, DMSO) in the treatment group, is reported as the measure of response.

Solubilized Zona-Pellucida Binding Assay

Female Swiss mice (6 week old) were superovulated with 10 IU PMSG followed 48 h later with 10 IU hCG. Oocyte-cumulus mass complexes were collected 12-15 hours after hCG injection by releasing them into 0.1% (w/v) hyaluronidase and incubating for 15 min at 37°C. Oocytes were dissociated from cumulus cells by
200 gently pipetting up and down, and washed in Ca^{2+} - and Mg^{2+} -free Hanks buffered salt solution (7.2 mM KCl, 0.3 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 4.2 mM $NaHCO_3$, 139 mM NaCl, 5.6 mM D-glucose, 20 mM HEPES) containing 0.1% (w/v) PVA (HBSS/PVA, pH 7.4). The oocytes (~200/experiment) were incubated (30 min at 37°C) in HBSS/PVA containing 1 mg/mL sulfo-NHS-LC-biotin, washed 3 times to remove unconjugated biotin, and the zona pellucidae solubilized by incubating (15 min at 37°C) in HBSS/PVA (pH 2.0, adjusted with 1 M HCl). Solubilized zona

proteins were removed from the insoluble oocyte material by aspiration with a fine bore pipette and then adjusted to pH 7.4 with 1 M NaOH.

210 In order to assess sperm-zona binding, epididymal spermatozoa isolated as described above were incubated in either BWW/BSA or BWW/BSA-Ca²⁺ supplemented with 1.8 mM Sr²⁺. Strontium was included in the calcium deficient medium as it facilitates sperm-zona binding without altering tyrosine phosphorylation (see Results). Spermatozoa were incubated (30 min at 37°C) in a preparation equivalent to 10 biotinylated ZP/μL and transferred to a droplet of streptavidin-coated magnetic beads, incubated (30 min at 37°C) and stained with 2 μg/mL propidium iodide for assessment of cell viability. The percentage of viable spermatozoa bound to streptavidin beads was assessed by phase contrast and fluorescence microscopy with a Zeiss Axioplan 2 microscope.

220 *Statistics*

All experiments were replicated with samples from at least three different mice and the statistical significance of differences between group means was determined by analysis of variance. Before testing, percentage data was arcsine transformed, and determinations of [Ca²⁺]_i following addition of thapsigargin were transformed to logarithms. Fisher's Protected Least Significant Difference (PLSD) was used to test the statistical significance of differences between two means. Where appropriate, data are shown as means ± SEM. The number of experiments is indicated in the figure legends.

230 **Results***Tyrosine Phosphorylation of Sperm is Negatively Regulated by Calcium*

Figure 1A shows the effect on tyrosine phosphorylation of incubating spermatozoa from the caput and cauda epididymis for 90 min in BWB/BSA. Only 1 protein band (116 kDa) was tyrosine phosphorylated in all groups. It corresponds to a hexokinase that has been identified previously as a constitutively tyrosine phosphorylated protein in mouse spermatozoa, and is probably unique to these cells (Kalab *et al.*, 1994). It was therefore used in this study in order to confirm equal protein loading of samples. Upon addition of dibutyryl cAMP (dbcAMP) and pentoxifylline (Ptx) there was a slight increase in the tyrosine phosphorylation of a
240 broad range of proteins (M_r 55-220 kDa) in caput spermatozoa. In caudal spermatozoa, higher levels of tyrosine phosphorylation were observed. Clear qualitative differences were evident between caput and caudal spermatozoa in the profile of proteins tyrosine phosphorylated, both under control conditions and following cAMP stimulation. These differences were confirmed by immunocytochemical analysis of phosphotyrosine expression in mouse epididymal spermatozoa. Thus, immunolocalization studies demonstrated fluorescence over the acrosomal region in 80% of caput spermatozoa, but only 3% of caudal cells. By contrast, there was no fluorescence over the tail of caput spermatozoa, but flagellar staining was recorded in 45% of caudal cells. Inclusion of dbcAMP and Ptx in the
250 incubation medium resulted in a significant ($P < 0.001$) increase in the percentage of caput cells displaying fluorescence over the tail, to approximately 18% of spermatozoa, but had no effect on the incidence of phosphotyrosine expression in caudal sperm flagella. No phosphotyrosine expression was detected by either western

blot or immunocytochemistry following pre-incubation of the anti-phosphotyrosine antibody with 20 mM *O*-phospho-L-tyrosine.

Figure 2 shows the effects of omitting calcium from the incubation medium on tyrosine phosphorylation expression of mouse epididymal spermatozoa. In accordance with the results shown in Figure 1, including dbcAMP and Ptx in calcium-supplemented medium caused an increase in tyrosine phosphorylation of caput spermatozoa ($P < 0.001$), but this increase was much greater when the spermatozoa were incubated in calcium-deficient medium (Fig. 2A). This increased signal was associated with a significant ($P < 0.001$) increase in the percentage of cells that displayed complete tyrosine phosphorylation over the sperm tail, such that the proportion was similar to that observed in caudal spermatozoa (Fig.2B). In contrast to caput cells, incubation of caudal spermatozoa in calcium-depleted medium alone caused a marked increase in tyrosine phosphorylation as assessed by Western blot, but this level could not be increased further by adding dbcAMP and Ptx (Fig. 2A). The increase in tyrosine phosphorylation in caudal cells exposed to calcium-depleted medium was not due to an increase in the percentage of cells displaying fluorescence over their tail (Fig. 2B), but rather an increase in the intensity of the phosphotyrosine signal observed in positive cells. In contrast, whilst addition of dbcAMP and Ptx to calcium-depleted medium was not found to increase tyrosine phosphorylation as assessed by Western blot (Fig. 2A), it did result in a significant ($P < 0.01$) increase in the percentage of cells that showed phosphotyrosine expression over the tail (Fig. 2B).

Epididymal Spermatozoa and $[Ca^{2+}]_i$

Figure 3A shows representative ratiometric traces of the fluorescence ratio (F) for caput and caudal spermatozoa incubated in calcium-supplemented and calcium-depleted medium. The figure shows that upon addition of Triton there was an increase in the fluorescent ratio and in general this increase was larger for the cells incubated in calcium-deficient medium. The fluorescent ratio did not increase further upon addition of $CaCl_2$ to the incubation medium, indicating that the Fura-2 was saturated upon addition of Triton. The fluorescent signal was quenched by addition of EGTA. In medium that did not contain spermatozoa the fluorescence ratio did not change upon addition of either Triton or EGTA (data not shown). These ratios were used to calculate the $[Ca^{2+}]_i$ of epididymal spermatozoa. Figure 3B shows that including calcium in the medium increased $[Ca^{2+}]_i$ 4-fold in caput sperm ($P < 0.001$), but only 2-fold in caudal sperm ($P < 0.001$). Furthermore, in calcium-supplemented medium, $[Ca^{2+}]_i$ was 3- to 4-times greater in caput than caudal cells ($P < 0.001$).

Fluorescence analyses using Fluo-3AM as the probe revealed that high levels of intracellular calcium are localized in the acrosome, midpiece and cytoplasmic droplet of caput epididymal spermatozoa (Fig. 4A-C). Less intense fluorescence was found over the post acrosomal region of the sperm head, and even less over the principal piece. Caudal spermatozoa also displayed intense fluorescence over the acrosome and midpiece (Fig. 4D-F). Similarly, caudal spermatozoa had less intense fluorescence over the principal piece. In contrast to caput cells, caudal spermatozoa displayed little or no fluorescence over the postacrosomal region of the head and no cytoplasmic droplets were present.

300 *Effect of Thapsigargin on $[Ca^{2+}]_i$, the Acrosome Reaction and Tyrosine Phosphorylation*

In order to further examine the effect of $[Ca^{2+}]_i$ on phosphotyrosine expression in mouse epididymal spermatozoa, thapsigargin was added to medium which contained dbcAMP and Ptx. Figure 5 shows representative traces of the fluorescence ratio (F) for caput and cauda spermatozoa incubated in calcium-supplemented and calcium-depleted medium, before and after addition of thapsigargin (5 μ M), 1% Triton X-100 and 5 mM EGTA. Addition of 5 μ M thapsigargin failed to induce an increase in the fluorescence ratio of caput spermatozoa, however the ratio increased upon addition of Triton and was quenched by EGTA. Caudal spermatozoa incubated
310 in calcium-deficient medium showed a slight increase in the fluorescence ratio after addition of 5 μ M thapsigargin, and this increased further upon addition of Triton and was quenched by EGTA. Caudal spermatozoa incubated in calcium-supplemented medium showed a larger increase in the fluorescence ratio upon addition of 5 μ M thapsigargin

Figure 6 shows that inclusion of 1-10 μ M thapsigargin in the incubation medium did not change $[Ca^{2+}]_i$ or the level of tyrosine phosphorylation in caput spermatozoa. In keeping with previous results, when caput spermatozoa were incubated in calcium-supplemented medium they had high $[Ca^{2+}]_i$ (Fig. 6A) and low levels of phosphotyrosine expression (Fig. 6B & C) at all doses of thapsigargin
320 examined. In contrast, caput cells incubated in calcium-depleted medium exhibited low $[Ca^{2+}]_i$, and tyrosine phosphorylation levels that were correspondingly higher (Fig 6B & C).

Addition of 1-10 μM thapsigargin to caudal spermatozoa incubated in calcium-supplemented medium resulted in a dose-dependent increase in $[\text{Ca}^{2+}]_i$ ($P < 0.05$) (Fig. 6A). Further, 5 μM thapsigargin also resulted in a significant decrease in tyrosine phosphorylation as assessed by Western blot (Fig. 6B) and immunocytochemistry ($P < 0.05$) (Fig. 6C) reinforcing the concept that calcium negatively regulates tyrosine phosphorylation. In calcium-depleted medium, only 10 μM thapsigargin increased $[\text{Ca}^{2+}]_i$ in caudal spermatozoa ($P < 0.01$) (Fig. 6A), and
330 produced a corresponding decrease in phosphotyrosine expression ($P < 0.01$) (Fig. 6B & C).

In contrast to the above results revealing a reciprocal relationship between $[\text{Ca}^{2+}]_i$ and phosphotyrosine expression, addition of high concentrations of thapsigargin (10 μM) to caudal spermatozoa in the presence of calcium, caused a significant increase in $[\text{Ca}^{2+}]_i$ ($P < 0.001$) (Fig. 6A), but did not effect the level of tyrosine phosphorylation in these cells (Fig. 6B & C). In order to resolve this paradox, studies were conducted in order to determine whether this increase in Fura-2 fluorescence represented a real increase in the $[\text{Ca}^{2+}]_i$ or was due to the release of dye into the extracellular medium, as a consequence of the ability of 10 μM thapsigargin
340 to induce the acrosome reaction. In keeping with this hypothesis, addition of 10 μM thapsigargin to these cells resulted in a significant increase in the occurrence of the spontaneous acrosome reaction ($P < 0.01$) (Fig. 7A), and the presence of high levels of Fura-2 associated fluorescence in the supernatant ($P < 0.01$) (Fig. 7B). Together these results suggest that the increase in Fura-2 fluorescence observed upon addition of 10 μM thapsigargin (Fig 6A) is due to the leakage of dye into the extracellular space as a consequence of the ability of this treatment to induce the acrosome reaction (Fig. 7). This accounts for the failure of this concentration of thapsigargin to effect

the levels of tyrosine phosphorylation in these cells (Fig. 6B & C) despite the increased Fura-2 signal.

350 In contrast to caudal spermatozoa incubated in calcium-supplemented medium, addition of 1-10 μ M thapsigargin to caput cells, or cauda spermatozoa incubated in calcium-depleted medium had no effect on the occurrence of the spontaneous acrosome reaction (Fig. 7A). Also, it did not result in significant levels of calcium associated Fura-2 fluorescence in the supernatant (Fig. 7B) in these cells.

Functionality of Tyrosine Phosphorylated Caput Sperm

In order to examine the functionality of the tyrosine phosphorylated caput spermatozoa, 1.8 mM strontium was added to the calcium-deficient medium as it facilitates binding to zona glycoproteins. Figure 8A shows that upon addition of
360 strontium, both caput and caudal spermatozoa maintained the high levels of tyrosine phosphorylation associated with calcium-deficient medium supplemented with dbcAMP and Ptx (see Fig. 2). As caput spermatozoa do not display progressive forward motility, a solubilized zona pellucida assay was performed in order to examine their interaction with zona glycoproteins (Fig. 8B). In addition, the ability of these cells to undergo the acrosome reaction when exposed to the divalent cation A23187 was examined (Fig. 8C). The results show that even under conditions that induce tyrosine phosphorylation, caput spermatozoa were immotile (data not shown), unable to bind to solubilized zona glycoproteins, and incapable of undergoing an A23187-induced acrosome reaction. On the other hand, caudal cells incubated with
370 dbcAMP and Ptx in the medium in which calcium had or had not been added were vigorously motile (data not shown), readily bound to solubilized zona-pellucida

proteins and underwent the acrosome reaction when exposed to A23187 ($P < 0.05$) (Fig. 8B & C).

Discussion

This report demonstrates for the first time that the signal transduction pathway leading to tyrosine phosphorylation in mouse epididymal spermatozoa is negatively regulated by calcium, and that maturation of the mechanisms that control $[Ca^{2+}]_i$ must be an essential part of extratesticular sperm maturation. The high $[Ca^{2+}]_i$ associated with the inhibition of tyrosine phosphorylation of caput spermatozoa is due, in part, to the presence of the cytoplasmic droplet and relatively high levels of calcium in the post-acrosomal region of the head. This may be due to immaturity of the calcium regulatory pathways in caput spermatozoa (Hall *et al.*, 1991; Okamura *et al.*, 1992) and the permeability of the plasma membrane of the immature gamete to this cation (Vijayaraghavan *et al.*, 1989). It is likely that such mechanisms may be biologically significant *in vivo*, since studies of the rat show that the concentration of free ionic calcium in caput epididymal fluid is approximately 3-fold higher than that from the distal cauda (810 ± 90 compared to 250 ± 60 μ M respectively) (Jenkins *et al.*, 1980). Decreasing the $[Ca^{2+}]_i$ of caput spermatozoa to that of caudal spermatozoa allowed the cAMP-mediated signal transduction pathway to be switched on and tyrosine phosphorylation of the tail to proceed, as it does in capacitating caudal spermatozoa. Together, our results suggest that an essential part of the sperm maturational process involves the attainment of a thapsigargin sensitive calcium store, and loss of the cytoplasmic droplet during epididymal maturation.

This report confirms work on the rat (Lewis and Aitken, 2001) showing that caput spermatozoa contain all the components of a functional signaling pathway leading to tyrosine phosphorylation. Consequently, it is concluded that extratesticular sperm maturation does not involve development of this pathway. Further, it may be concluded that activation of tyrosine phosphorylation is not the *sine qua non* of sperm

400 capacitation since our findings show that even when the process was activated in caput spermatozoa they remained non-functional in terms of motility, sperm-egg recognition and acrosomal exocytosis. These findings highlight the need for functional endpoints of capacitation to be assessed when examining the effects of various treatments on tyrosine phosphorylation.

Our observations that calcium is a negative regulator of the signaling pathway leading to tyrosine phosphorylation in mouse spermatozoa is not in agreement with a previous study by Visconti *et al.*, (1995a). However, they are consistent with studies on human (Carrera *et al.*, 1996; Luconi *et al.*, 1996), pig (Kalab *et al.*, 1998), and bull spermatozoa (Vijayaraghavan *et al.*, 1997). All these reports demonstrated that

410 omission of calcium from incubation medium results in an increase in protein tyrosine phosphorylation associated with capacitation in these species. The differences in the results of this present study and that of Visconti and colleagues (Visconti *et al.*, 1995a) is also not attributable to the method of collection of spermatozoa, since we have found tyrosine phosphorylation to be higher in caudal spermatozoa regardless of the method of collection. Differences with the previous work may be attributable to the alternative method of processing the spermatozoa before incubation: Visconti and colleagues washed and centrifuged the spermatozoa prior to incubation (thereby removing factors loosely associated with the sperm membrane), whereas we have simply diluted the spermatozoa into medium. Thus, factors arising from the cauda

420 epididymal fluid may play significant roles in regulating the response of caudal spermatozoa to extracellular calcium.

Recent work from our laboratory suggests that the mode of calcium regulation is downstream of cAMP metabolism. It is envisaged that calcium may either have a direct action on the kinase/phosphatase system leading to tyrosine phosphorylation or

act indirectly via regulation of calcium-dependent channels in the sperm plasma membrane. Our current studies are aimed at elucidating the molecular targets regulated by changes in $[Ca^{2+}]_i$.

The ability of thapsigargin to increase $[Ca^{2+}]_i$, and decrease protein tyrosine phosphorylation in caudal spermatozoa confirms the ability of this cation to negatively regulate this signal transduction pathway. Thapsigargin-sensitive calcium stores have been primarily localized to the acrosome (Spungin and Breitbart, 1996; Dragileva *et al.*, 1999), and thapsigargin is able to induce the spontaneous acrosome reaction (Meizel and Turner, 1993; Walensky and Snyder, 1995; Dragileva *et al.*, 1999; Jungnickel *et al.*, 2001). This may be as a result of triggering capacitative calcium entry into the cell due to its requirement on extracellular calcium (Putney, 1986; Llanos, 1998; Dragileva *et al.*, 1999). In this study, treatment of caudal spermatozoa in calcium-supplemented medium with 10 μ M thapsigargin induced the spontaneous acrosome reaction and, as a result, the release of Fura-2 into the surrounding medium. This appeared to account for the 4-fold increase in $[Ca^{2+}]_i$ in the absence of any concomitant change in the levels of phosphotyrosine expression (Fig. 5 & 6).

The $[Ca^{2+}]_i$ of mouse caudal spermatozoa reported here is higher than that reported previously (Ref\$\$), but approximate that reported by others (535 ± 49 nM: Rockwell and Storey, 1999). In any case, the trends in the data support studies that have shown that the $[Ca^{2+}]_i$ of caput spermatozoa is 2 to 4-fold that of caudal cells (White and Aitken, 1989; Vijayaraghavan and Hoskins, 1990). Calculations of $[Ca^{2+}]_i$ in this study are based on a dissociation constant (K_d) of 224 nM for Fura-2 (Grynkiewicz *et al.*, 1985). However, due to the effect of ionic strength, pH and

protein concentration on K_d , absolute values of $[Ca^{2+}]_i$ from this study should be
450 interpreted with caution.

In addition, these results show that the increase in tyrosine phosphorylation observed in caudal spermatozoa in calcium-depleted medium by Western blot analysis (Fig. 2A) does not correlate with an increase in the percentage of functional spermatozoa within a population (Fig. 8). Rather, the parallel immunocytochemical analysis revealed that this change was due to more intense phosphorylation in cells in which this signaling pathway is already switched on.

In conclusion, the results of this study show that the signaling pathway leading to tyrosine phosphorylation in mouse epididymal spermatozoa is negatively regulated by calcium. High $[Ca^{2+}]_i$ in caput spermatozoa, due to the presence of the cytoplasmic
460 droplet and limited capacity of the cell to remove calcium by sequestering it into thapsigargin-sensitive stores or pumping it across the membrane, therefore acts to inhibit this signal transduction pathway in the immature gamete. As well, we show that despite induction of tyrosine phosphorylation in caput spermatozoa, these cells are still unable to engage in other correlates of capacitation. Thus, other processes during epididymal maturation are important in conferring upon the spermatozoa the potential to fertilize. It is envisaged that studies aimed at elucidating these mechanisms, as well as the manner by which calcium is able to regulate the signaling pathway leading to tyrosine phosphorylation, will contribute significantly to our understanding of the sperm maturation process.

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Figure Legends

FIG. 1. The effect on protein tyrosine phosphorylation of incubating caput or caudal epididymal spermatozoa for 90 min in the presence or absence of dbcAMP (5 mM) + Ptx (3 mM). Tyrosine phosphorylation was assessed by Western blot analysis (A), and immunocytochemistry, where the response was the percentage of spermatozoa that displayed fluorescence over the tail (B) (n = 5). *** P < 0.001 compared to caput cells incubated in the absence of dbcAMP + Ptx.

FIG. 2. The effect on protein tyrosine phosphorylation of incubating caput and caudal epididymal spermatozoa for 90 min in medium with or without (calcium-depleted) added calcium, and including dbcAMP (5 mM) and Ptx (3 mM). Tyrosine phosphorylation was assessed by Western blot (A) and immunocytochemistry, where

the response was the percentage of spermatozoa that displayed fluorescence over the tail (B) (n = 5). ** P < 0.01 compared to the caudal spermatozoa incubated in calcium-deficient medium and *** P < 0.001 compared to the caput cells incubated in the absence of dbcAMP + Ptx, and compared to caput spermatozoa incubated in calcium-supplemented medium containing dbcAMP + Ptx respectively.

FIG. 3. The effect on $[Ca^{2+}]_i$ of caput and caudal epididymal spermatozoa by excluding extracellular calcium from the medium. (A) The fluorescence ratio (F) of mouse epididymal spermatozoa loaded with Fura-2AM and then incubated in calcium-supplemented or calcium-deficient medium. The figure shows a representative trace of the fluorescence ratio (340/380 nm) of caput and caudal spermatozoa before and after addition of 1% Triton, 2 mM $CaCl_2$ and 5 mM EGTA. (B) The $[Ca^{2+}]_i$ was determined as described in the Materials and Methods (n = 16, 16, 25 and 19 respectively). *** P < 0.001 for comparison of group means indicated in the figure.

FIG. 4. The localization of intracellular free calcium in mouse epididymal spermatozoa. Caput (A-C) and cauda (D-F) sperm were loaded with 2 μ M Fluo-3AM and imaged using a Zeiss confocal laser scanning microscope LSM 510 in a wet mount on prewarmed poly-L-lysine coated glass slides, following excitation with an argon-krypton laser.

FIG. 5. The effect of thapsigargin on the fluorescence ratio (F) of mouse epididymal spermatozoa loaded with Fura-2AM and incubated in calcium-supplemented or calcium-deficient medium. The figure shows a representative trace of the

fluorescence ratio (340/380 nm) of caput and caudal spermatozoa before and after addition of thapsigargin (5 μ M), 1% Triton and 5 mM EGTA.

FIG. 6. The effect on $[Ca^{2+}]_i$ and tyrosine phosphorylation of thapsigargin on mouse epididymal spermatozoa incubated in medium containing dbcAMP (5 mM) + Ptx (3 mM), with or without added calcium. $[Ca^{2+}]_i$ was determined 5 min after addition of thapsigargin (1-10 μ M) to the cuvette (A) (n = 10 (caput) and 12 (cauda) respectively). Tyrosine phosphorylation was assessed after 90 min incubation in the presence of thapsigargin by Western blot (B) and immunocytochemistry, where the response was the percentage of spermatozoa that displayed fluorescence over the tail (C) (n = 4). * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to the 0 μ M thapsigargin treatment for that group.

FIG. 7. The effect on the spontaneous acrosome reaction and the presence of Fura-2 fluorescence in the supernatant, of mouse epididymal spermatozoa after treatment with thapsigargin. Caput and cauda sperm were prepared in medium containing dbcAMP (5 mM) and Ptx (3 mM) with or without added calcium, and then exposed to thapsigargin (1-10 μ M) for 5 min. The occurrence of the acrosome reaction following thapsigargin treatment was assessed using fluorescein-labeled *Arachis hypogaea* lectin (A) (n = 4). Fura-2 fluorescence in the supernatant following thapsigargin treatment was determined using spectrofluorimetry. F_{min} was recorded and the relative increase in $F - F_{min}$ over the 0 μ M thapsigargin 'control' for that treatment is shown (B) (n = 5). ** P < 0.01 compared to the 0 μ M thapsigargin 'control' for that group.

FIG. 8. Assessment of the functionality of tyrosine phosphorylated mouse epididymal spermatozoa. Caput and cauda epididymal sperm were collected and incubated alone or in the presence of Sr^{2+} (1.8 mM), dbcAMP (5 mM) and Ptx (3 mM), in calcium-supplemented or calcium-deficient medium. (A) The effect of adding Sr^{2+} to calcium-deficient medium on tyrosine phosphorylation of mouse epididymal spermatozoa. (B) The percentage of spermatozoa bound to solubilized biotinylated zona pellucida proteins (prepared as described in the Materials and Methods) was assessed following incubation with streptavidin-coated magnetic beads (n = 3). (C) The occurrence of the acrosome reaction following treatment with 5 μM A23187 or DMSO, the vehicle for A23187, was assessed using fluorescein-labeled *Arachis hypogaea* lectin (n = 4). * P < 0.05 compared to the DMSO 'control'.

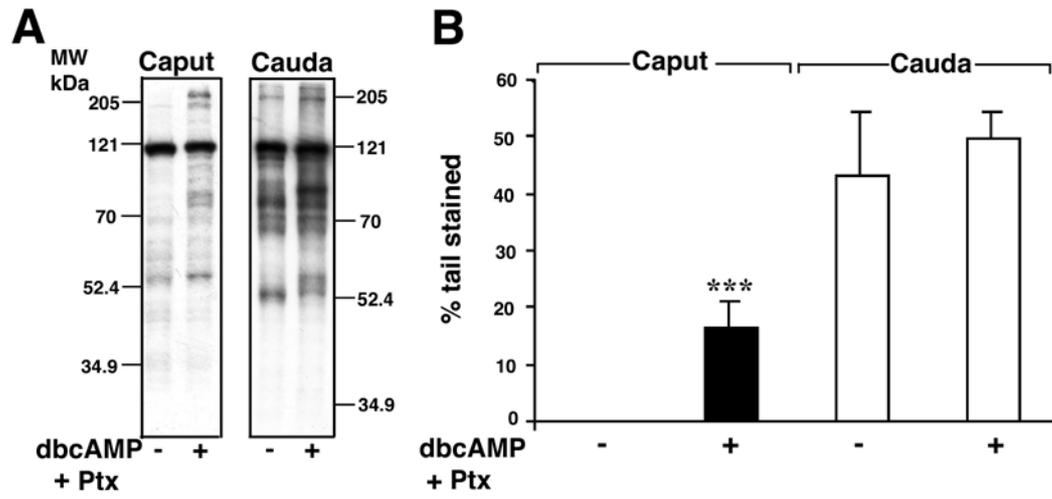


Figure 1

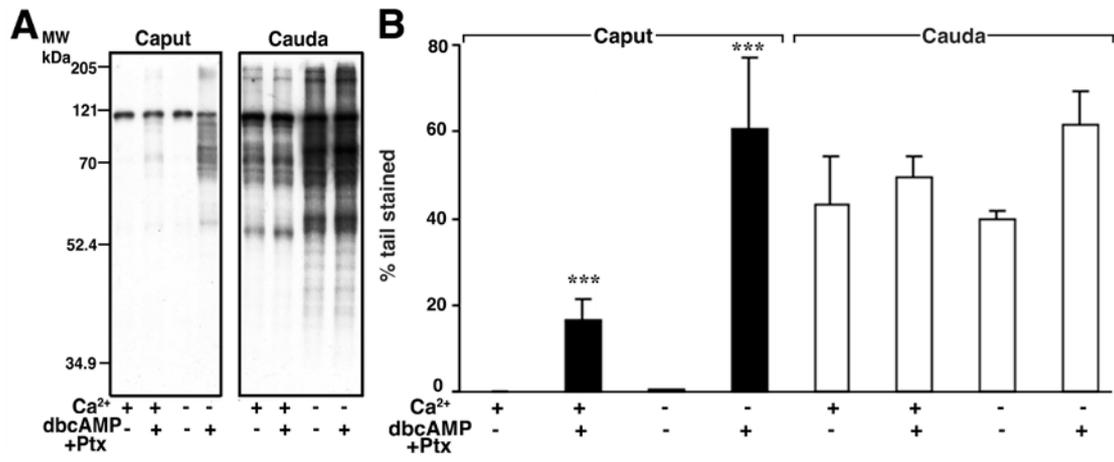


Figure 2

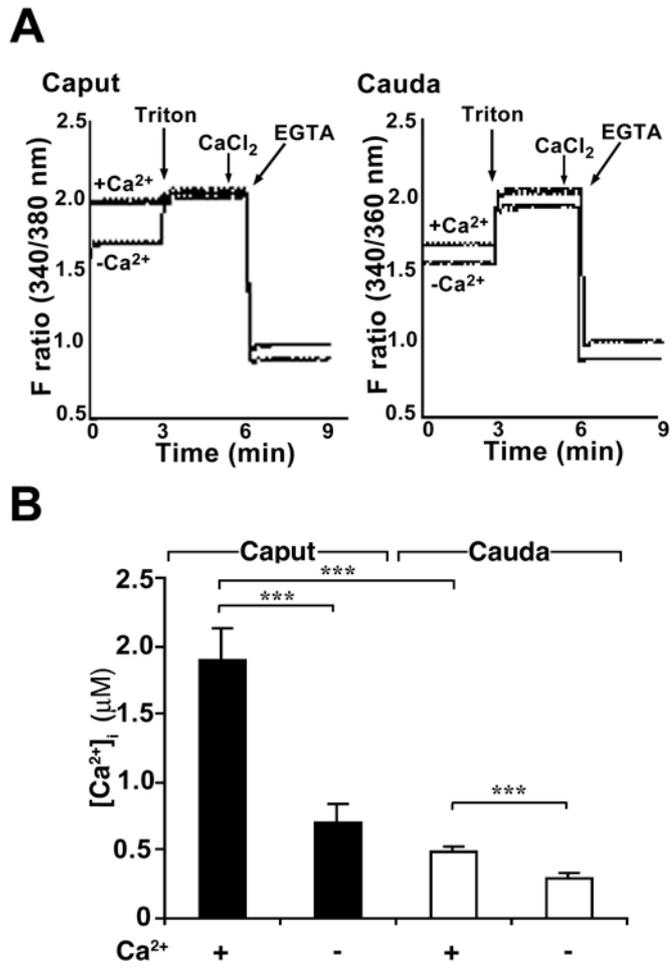


Figure 3

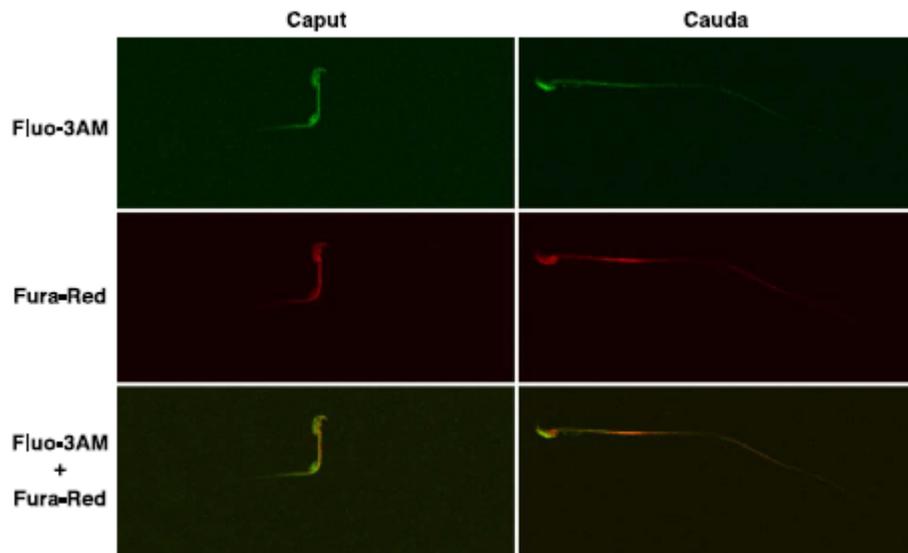


Figure 4

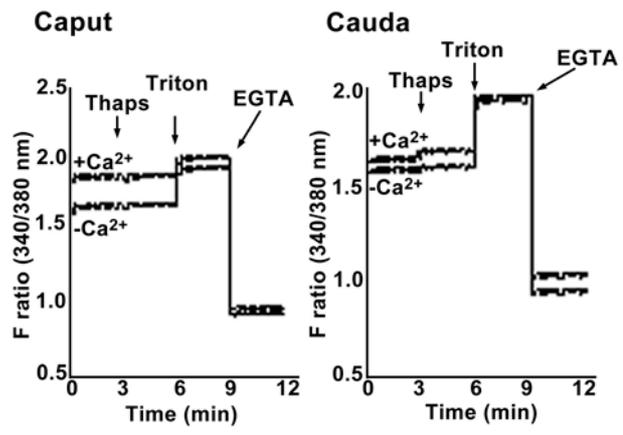


Figure 5

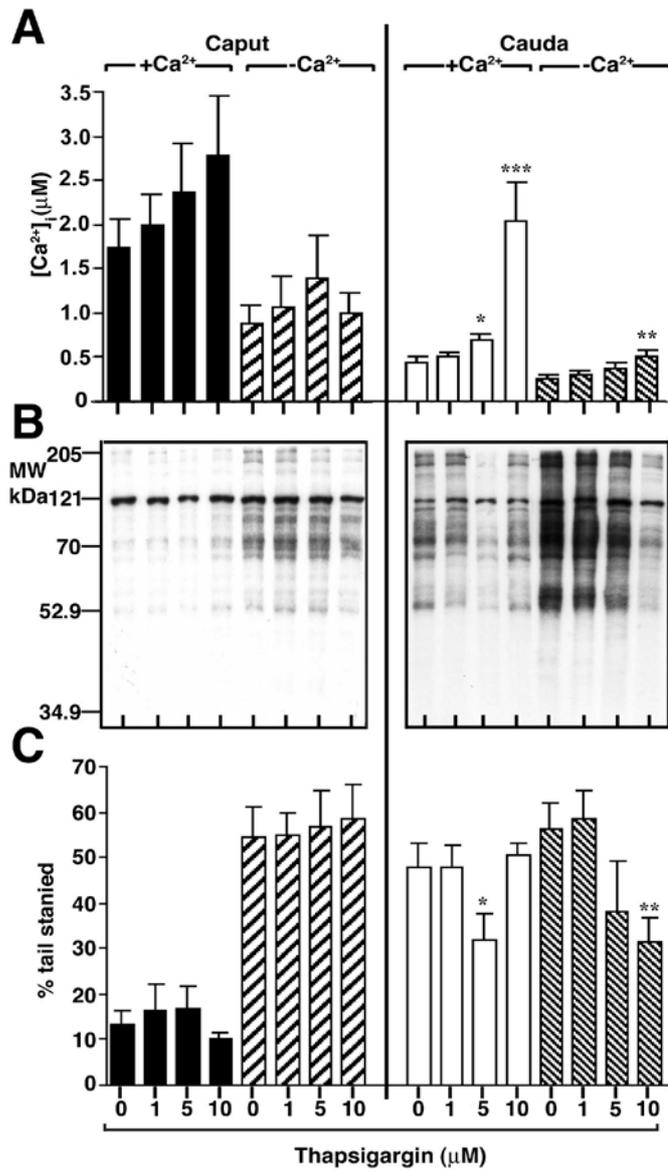


Figure 6

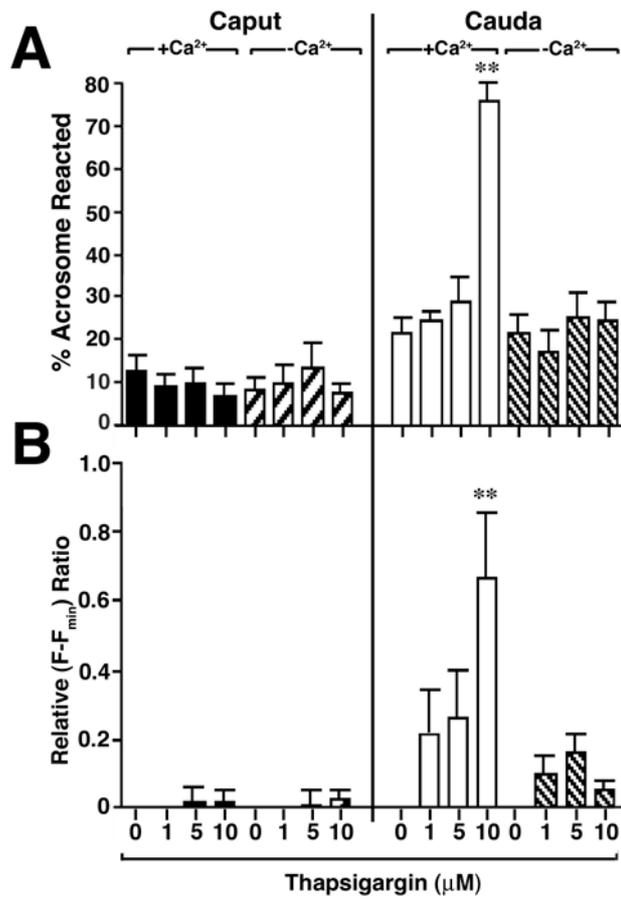


Figure 7

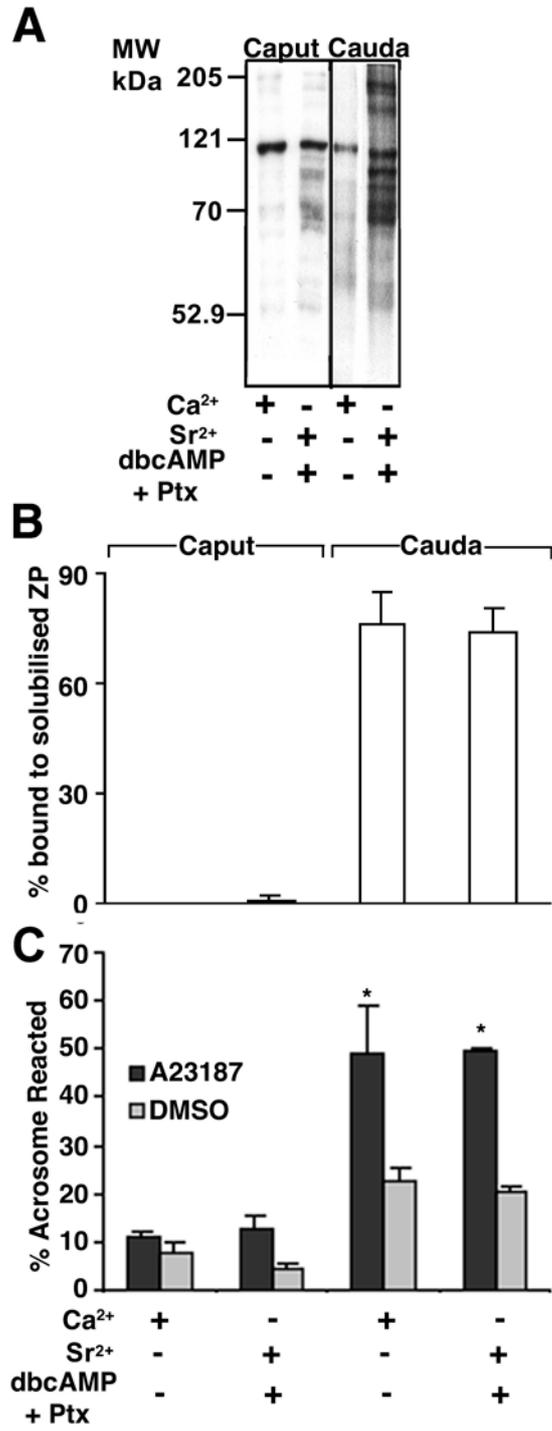


Figure 8