FEBS Letters 580 (2006) 3900–3904

Evidence for a secretory pathway Ca^{2+}-ATPase in sea urchin spermatozoa

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Received 9 May 2006; revised 3 June 2006; accepted 8 June 2006

Available online 19 June 2006

Edited by Maurice Montal

Abstract Plasma membrane, sarco-endoplasmic reticulum and secretory pathway Ca^{2+}-ATPases (designated PMCA, SERCA and SPCA) regulate intracellular Ca^{2+} in animal cells. The presence of PMCA, and the absence of SERCA, in sea urchin sperm is known. By using inhibitors of Ca^{2+}-ATPases, we now show the presence of SPCA and Ca^{2+} store in sea urchin sperm, which refills by SPCA-type pumps. Immunofluorescence shows SPCA localizes to the mitochondrion. Ca^{2+} measurements reveal that ~75% of Ca^{2+} extrusion is by Ca^{2+} ATPases and 25% by Na^{+} dependent Ca^{2+} exchanger/s. Bisphenol, a Ca^{2+} ATPase inhibitor, completely blocks the acrosome reaction, indicating the importance of Ca^{2+}-ATPases in fertilization.

Keywords: Bisphenol; Intracellular calcium; Invertebrate fertilization; PMCA; SPCA

1. Introduction

In animal cells, three classes of P-type Ca^{2+} transporting ATPases are known to mediate intracellular Ca^{2+} homeostasis: plasma membrane calcium ATPase (PMCA), sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and secretory pathway Ca^{2+} ATPase (SPCA) [1]. PMCAs transport Ca^{2+} extracellularly, whereas the other two, which are localized to intracellular membrane vesicles, sequester Ca^{2+} into vesicles. Despite the great importance of Ca^{2+} ATPases in all other cell types, there are few studies on these pumps in sperm. Mammalian spermatozoa express PMCA, which is localized in the principle piece of flagellum [2]. Sea urchin sperm express PMCA, but it localizes to the sperm head [3]. Recently, a human sperm SPCA type Ca^{2+} ATPase was shown to be localized to the posterior sperm head and midpiece [4].

Evidence for the participation of Ca^{2+} ATPases in sperm physiology is mainly derived from studies using calcium ATPase inhibitors. For example, carboxyeosin (CE), which is a PMCA inhibitor [5], has been used to study the role of PMCA in both mouse and sea urchin sperm [2,3]. These studies show that 20 μM CE blocks sperm motility. SERCA is specifically inhibited by thapsigargin (TG) at less than 1 μM [6]. The concentration of less than 20 μM cyclopiazonic acid (CPA) also specifically inhibits SERCA [7]. There are currently no specific blockers for the TG-resistant SPCA. However, bisphenol (BP) inhibits both the TG-sensitive SERCA type and the TG-resistant SPCA type pumps of microsomal membranes [8,9]. Forty micromolar BP causes complete inhibition of TG-resistant Ca^{2+} uptake and it has no effect on Ca^{2+} exchangers [8]. Harper and coworkers showed the presence of a SPCA in human sperm using these blockers [4]. Here, we present evidence for the presence of SPCA in sea urchin sperm using the inhibitors and commercial antibody to SPCA. The study shows that Ca^{2+} pumps are required for sperm to be competent to undergo an acrosome reaction (AR). To our knowledge, except for CE [6–8] there are no studies on Ca^{2+} ATPase blockers in sperm functions.

2. Materials and methods

2.1. Materials

Sea urchin (*S. purpuratus*) gametes were spawned by injecting 0.5 M KCl into adults. Undiluted sperm (4×1010 spermatozoa per ml) were collected and kept on ice for no longer than 24 h. Egg jelly (EJ) was prepared and quantified as described [10]. Artificial seawater (ASW) was formulated as follows: 486 mM NaCl/10 mM CaCl2/10 mM KCl/27 mM MgCl2/29 mM MgSO4/2.5 mM NaHCO3/10 mM HEPES, adjusted to pH 7.9 with 1 N NaOH. Na^{+}-free seawater (NaFSW) was formulated as follows 486 mM choline chloride/10 mM CaCl2/7.5 mM KCl/27 mM MgCl2/29 mM MgSO4/2.5 mM KHCO3/10 mM Hepes, adjusted to pH 7.9 with 1 M *N*-methyl-D-glucamine for potassium dependent sodium calcium exchanger (NCKX) activity experiments. The Ca^{2+} ATPase inhibitors, 5-(-6)-carboxyeosin (CE; Sigma), thapsigargin (TG, Sigma), cyclopiazonic acid (CPA, Sigma) and bis(2-hydroxy-3-*tert*-butyl-5-methylphenyl)methane (bisphenol, BP, Pfaltz and Bauer, Waterbury, CT) were dissolved in dimethylsulfoxide (DMSO) to 5 mM.

2.2. Fluorometric measurement of intracellular Ca^{2+}

Sea urchin sperm were loaded with indo-1 AM (Molecular Probes) and washed as described for fura-2 AM [11]. For Ca^{2+} measurements, 50 μl of indo-1 loaded sperm (4×10^{8} cells per ml) were diluted into 1.45 ml of ASW or CaFSW or NaFSW in a stirred cuvette in a Fluoro-Max-2 fluorometer with excitation at 340 nm and emission at 400 nm and 500 nm (16 °C). DMSO was used as a negative control and the volume of DMSO added to the cuvette never exceeded 0.6% (v/v). For Ca^{2+} free seawater (CaFSW) experiments, indo loaded sperm was washed with CaFSW and was resuspended in CaFSW. For NCKX activity experiments, indo loaded sperm washed with and resuspended in NaFSW was used. The ratio of intensities at 400 and 500 nm (F400/F500) reports relative intracellular Ca^{2+} concentrations. Here, the intracellular Ca^{2+} is expressed as the F400/F500 ratio.

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Abbreviations: AR, acrosome reaction; ASW, artificial seawater; BP, bisphenol; CaFSW, Ca^{2+} free artificial sea water; NaFSW, Na^{2+} free artificial sea water; CE, 5-(-6-)-carboxyeosin; CPA; cyclopiazonic acid; DMSO; dimethylsulfoxide; EJ, egg jelly; NCKX; K^{+}-dependent Na^{+}/Ca^{2+} exchanger; NCX, Na^{+}/Ca^{2+} exchanger; PMCA, plasma membrane calcium ATPase; SPCA, secretory pathway calcium ATPase; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; TG, thapsigargin

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.019

Control runs with indo-1 and without sperm showed that the inhibitors did not affect indo-1 fluorescence. Each experiment was repeated for at least three times.

2.3. Immunoblotting

Semen were dissolved in 10% SDS, boiled for 5 min and precipitated with 80% acetone. Protein was quantified by the BCA method, separated on SDS/PAGE (10%) and electrotransferred to nitrocellulose membranes. The blot was blocked with 5% non-fat dry milk in TBST (25 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) for 1 h, followed by incubation in H-200 anti-PMR1 antibody (Santa Cruz Biotechnology, Catalog #sc-5548, Santa Cruz, CA) for either 2 h at room temperature or overnight at 4 °C. Bands were detected with an HRP-conjugated goat anti-rabbit secondary antibody and developed with SuperSignal West Dura Extended Duration Substrate (Pierce Chemical Co., Rockford, IL) according to manufacturer's protocol.

2.4. Immunolocalization

Sperm were electrostatically bound to protamine-sulfate coated coverslips and fixed in seawater containing 3% paraformaldehyde/0.1% glutaraldehyde. Fixed cells were blocked in 150 mM NaCl/10 mM HEPES (pH 7.8) containing 5 mg/ml BSA and 5% normal goat serum (Sigma). Washes were in 150 mM NaCl/10 mM HEPES, pH 7.8 with 0.1% Tween-20 to permeabilize the cells. Coverslips were incubated 1 h in a range of dilutions of anti-PMR antibody. After five washes in Tween-saline, the coverslips were incubated 1 h in a 1:400 dilution of Alexa-Fluor 546 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

2.5. Acrosome reaction

Undiluted semen was diluted 1:200 in ASW. Sperm suspensions were mixed with various concentration of bisphenol for 5 min before exposure to crude EJ for 2 min (16 °C). AR was scored as described [12,13].

3. Results

3.1. Measurement of Intracellular Ca^{2+}

Four inhibitors were used to explore the possible role of Ca^{2+} ATPases in sperm. Relative changes in intracellular

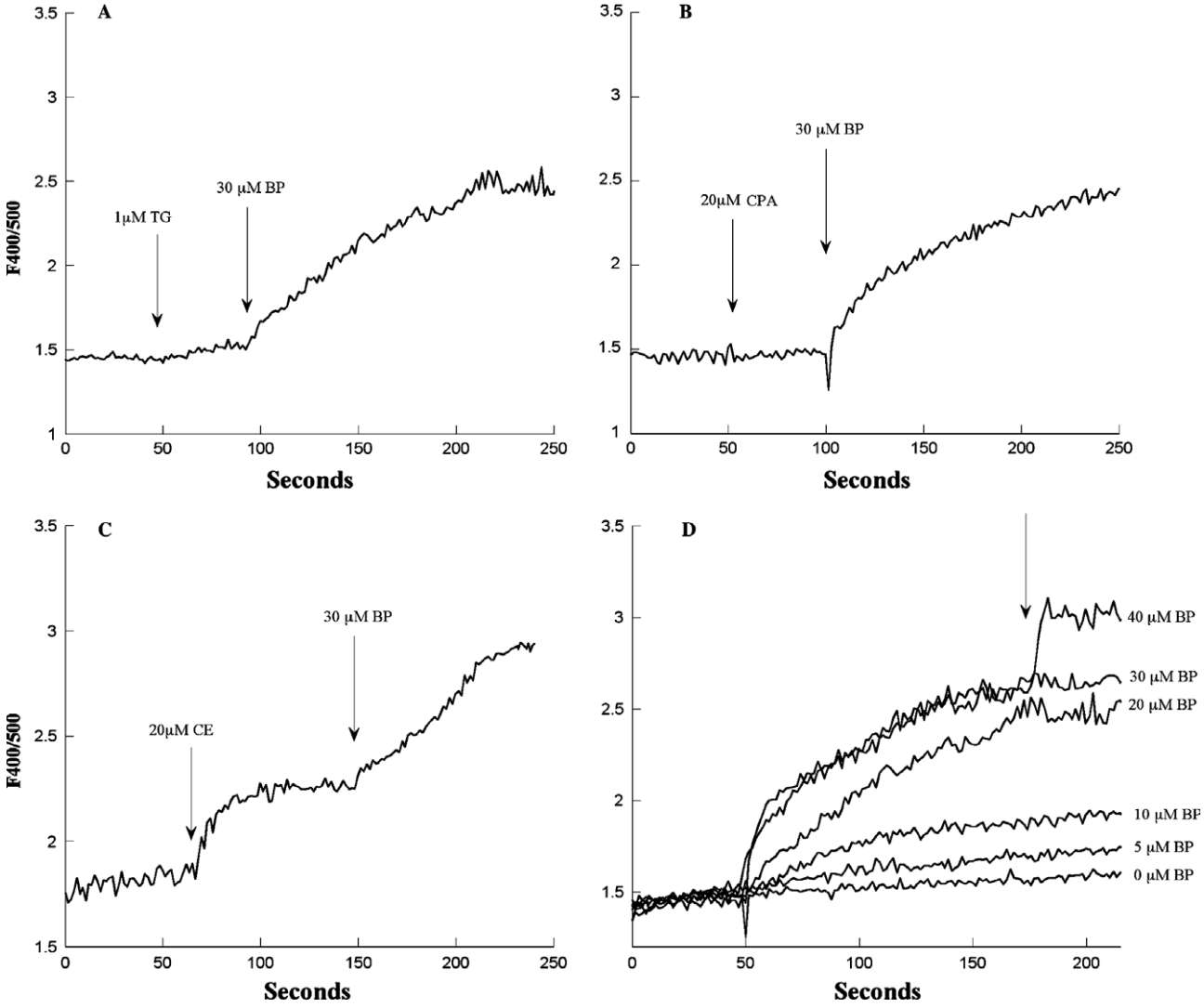

Fig. 1. Effects of Ca^{2+} ATPase inhibitors on intracellular Ca^{2+} in sea urchin sperm. Each inhibitor was added to indo-1 loaded sperm in ASW at the arrow. (A) One micromolar TG has no effect on intracellular Ca^{2+} levels, but 30 μM BP causes a sustained increase in Ca^{2+}. (B) Twenty micromolar CPA has no effect on intracellular Ca^{2+} levels, but BP causes Ca^{2+} elevation. The results in panels A and B show that SERCA is not in these cells. (C) BP elevates sperm intracellular Ca^{2+} levels at concentrations where PMCA is completely inhibited by CE. (D) BP inhibits Ca^{2+} ATPase activity in dose dependent manner. Maximum increase in intracellular Ca^{2+} was recorded at 30 μM BP. Ionomycin was added to the 40 μM sample BP, (arrow) to 10 μM to be certain that indo-1 was not exhausted.

Ca^{2+} concentrations were measured in indo-1 loaded sperm before and after exposure to these inhibitors. Fluorescence measurements began 1 min before inhibitor addition. Fig. 1A and B show that SERCA specific inhibitors (1 μM TG and 20 μM CPA) have no effect on intracellular Ca^{2+} concentrations, but there is a sustained increase in intracellular Ca^{2+} after the addition of 30 μM bisphenol (BP). The addition of 20 μM CE, followed by 30 μM BP, caused a further increase in intracellular Ca^{2+} (Fig. 1C), suggesting the presence of another Ca^{2+} ATPase. As SERCA is absent in these cells, a SPCA would be this Ca^{2+} ATPase. BP treatment resulted in a concentration-dependent increase in intracellular Ca^{2+} in these sperm (Fig. 1D). Forty micromolar BP yields the same Ca^{2+} increase curve as 30 μM, showing that the Ca^{2+} ATPases in sperm are maximally inhibited at this BP concentration (Fig. 1D). To be certain that the indo-1 reporter was not exhausted, the cation ionophore, ionomycin, was added to the 40 μM BP sample at the plateau. Ionomycin gave a further increase in Ca^{2+}, showing that the result of Fig. 1D is not due to lack of indo-1.

Intracellular Ca^{2+} measurements were done under low Ca^{2+} conditions ("Ca^{2+} free conditions") Fig. 2. In low extracellular Ca^{2+} (<33 μMCa^{2+}), 30 μM BP still increased intracellular Ca^{2+}. However, the final height of Ca^{2+} increase in Ca^{2+} low conditions is approximately one-third of that observed in sea water of 10 mM Ca^{2+}.

The percentage contribution of each calcium extrusion mechanism was estimated using Na^+ free condition, which blocks NCKX and NCX activities. Thirty micromolar BP blocks all Ca^{2+} ATPases without affecting Ca^{2+} exchanges (Fig. 3A). The difference of intracellular Ca^{2+} measurements between sperm suspended in NaFSW and sperm in seawater indicates the contribution of Ca^{2+} exchanges (NCKX/NCX). This result was confirmed by a deferent experiment where NCKX/NCX activities are resumed by addition of NaCl to the cells in NaFSW (Fig. 3B). These results indicate that potentially ~25% of Ca^{2+} could be expelled from NCKX/NCX, while Ca^{2+} ATPases could be responsible for ~75% of the Ca^{2+} removal. This estimation of Ca^{2+} extrusion percentage by ATP driven Ca^{2+} pumps could be only from the plasma membrane, or a combination of plasma membrane and mitochondrial transporters.

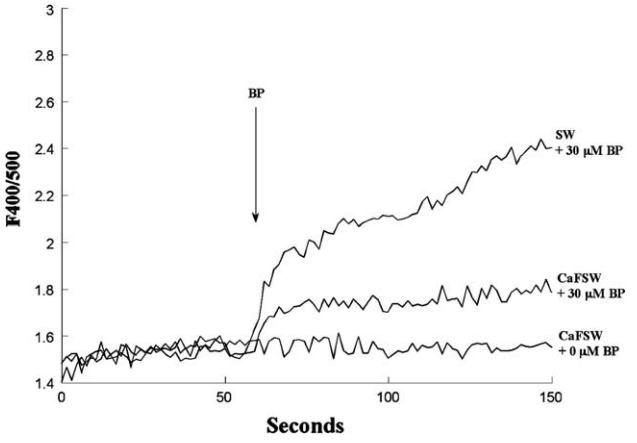

Fig. 2. The presence of Ca^{2+} stores in sperm. Thirty micromolar BP was added to indo-1 loaded sperm in ASW and CaFSW at the arrow. In CaFSW, BP elevated intracellular Ca^{2+} level although there are one third of elevation in ASW. These results indicate that sea urchin sperm would contain internal Ca^{2+} stores.

3.2. Immmunoblots and immmunofluorescence

Since the inhibitor studies show that the possibility of an SPCA in sea urchin sperm, immmunoblots and immmunofluorescence were performed using the commercial antibody to human SPCA1 (GenBank Accession # AAP30008)(H-200 antiPMR1 rabbit polyclonal antibody), which is 68% identical to SPCA (Fig. 4A) that we have cloned from sea urchin testis cDNA (GenBank Accession # DQ150587). Immmunoblots of a whole sperm lysate detected a single band at about 110 kDa (Fig. 4B), which is slightly above the calculated molecular weight (100.60 kDa) of sea urchin SPCA. Immmunofluorescence of whole sperm showed a strong reaction to only the single, gaint mitochondrion (Fig. 4C).

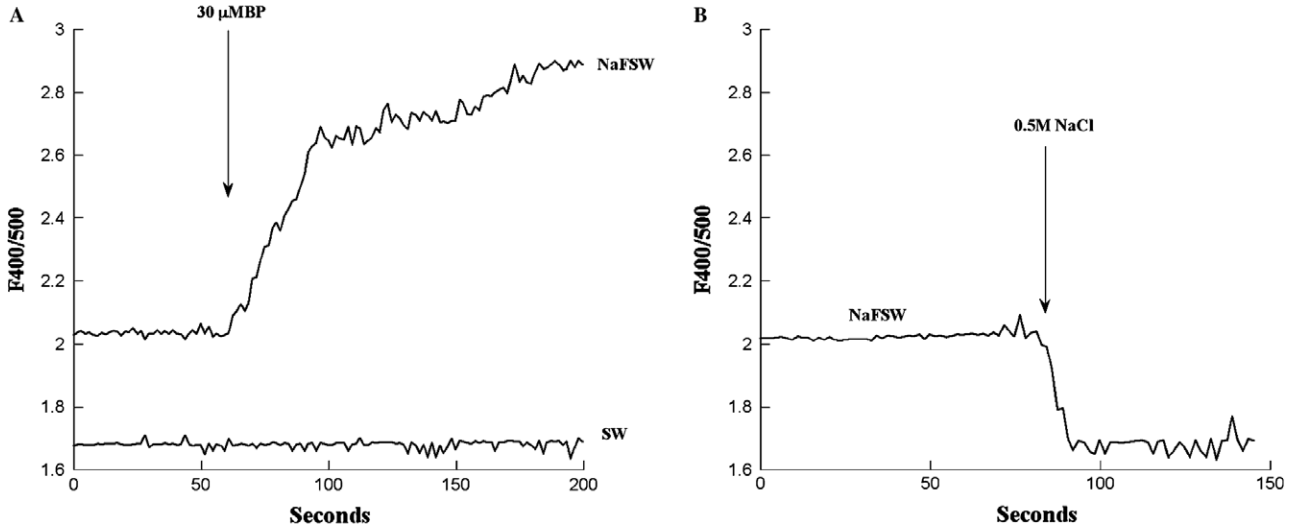

Fig. 3. Estimation of percent activity of NCKX (and maybe NCX) and Ca^{2+} ATPases in sperm. (A) Thirty micromolar BP was added to indo-1 loaded sperm in ASW and NaFSW where NCKX/NCX would not be active. The difference between two conditions corresponds to the contribution of NCKX/NCX in sperm Ca^{2+} extrusion. The degree of elevation after addition of BP indicates the contribution of Ca^{2+} ATPases. (B) NaCl was added to indo-1 loaded sperm in NaFSW to a final concentration of 0.5 M, which resumes NCKX activity. The decrease in intracellular Ca^{2+} indicates the NCKX activity. Note that levels of intracellular Ca^{2+} in ASW and NaFSW plus 0.5 M NaCl are the same.

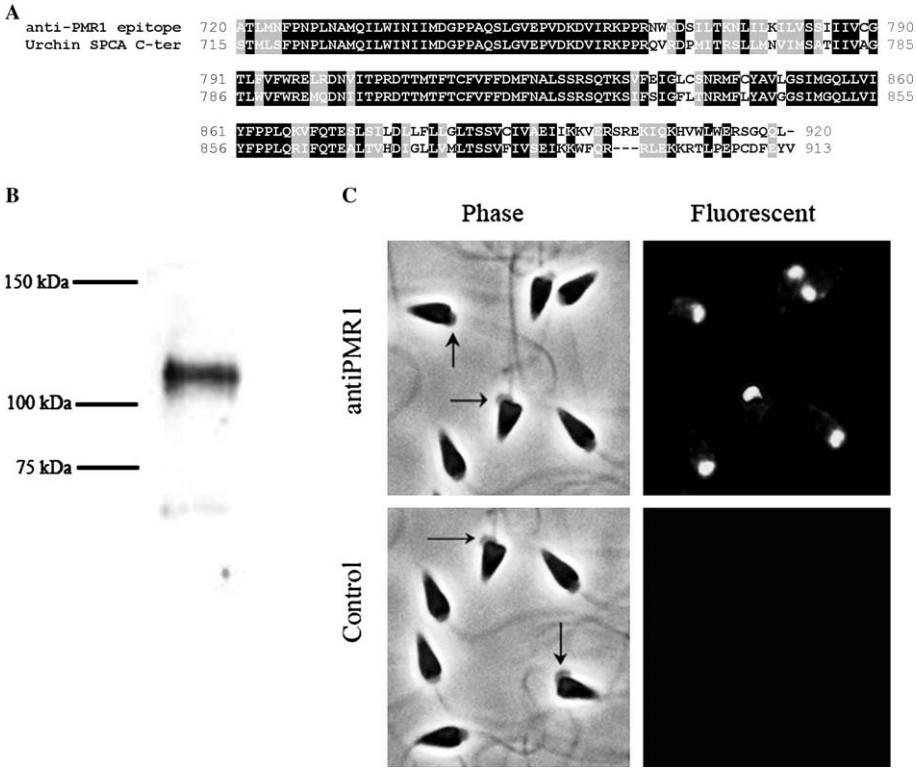

Fig. 4. Immunoblot and immunolocalization with anti-PMR1 antibody. (A) The sequence alignment of PMR1 antibody epitope region (human SPCA1) and the relavent region of sea urchin SPCA. The alignment shows that there is 68% sequence identity between the two. (B) A single band at ~110 kDa appears in the sperm lysate. (C). Immunolocalization of Sp-SPCA with anti-PMR1 antibody. Upper two panels are cells reacted with anti-PMR1. Lower two panels are cells reacted with normal rabbit serum. Left panels are phase contrast images and right panels the immunofluorescent images. Arrows point to mitochondria that are displaced to the side of the sperm head. The width of each sperm head is ~1 μm.

3.3. Bisphenol blocks acrosome reaction

To observe the effects of BP on the AR, sperm were incubated with various concentrations of BP (Fig. 5) for 5 min before the addition of EJ (final concentration 700 ng fucose/ml). Two minutes after EJ addition, sperm were fixed and stained with Alexa Fluor 488 phalloidin for AR scoring. At 30 μM BP, AR decreased from 96% to 8%. In 5 μM BP, AR decreased from 96% to 24%. These results suggest that blocking Ca^{2+} extrusion pumps alters sperm ion balance in ways that prevent the signaling pathways leading to the AR.

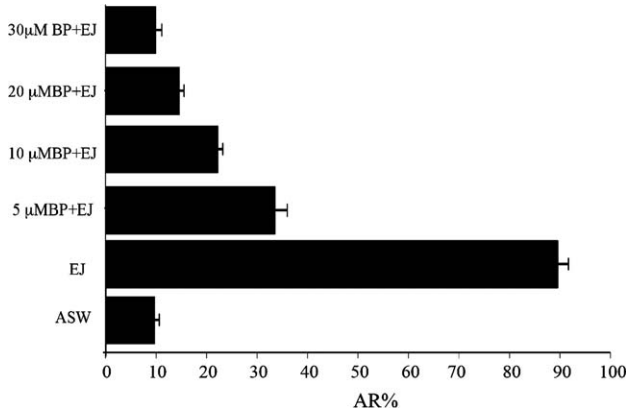

Fig. 5. Bisphenol (BP) inhibits the EJ-induced AR. Alexa Fluor 488 phalloidin was used to visualize the AR (acrosomal processes with their filamentous actin appear as fluorescent rods, [13]). Sperm were incubated with various concentrations of BP for 5 min before the addition of egg jelly (final concentration 700 ng fucose/ml). Two minutes after egg jelly addition, sperm were fixed and stained with Alexa Fluor 488 phalloidin for AR scoring. In the 5–30 μM range BP is a potent inhibitor of the AR. Error bars indicate SEs from at least three experiments.

4. Discussion

In previous studies, we showed that there are at least two extrusion mechanisms in sea urchin spermatozoa that maintain low internal Ca^{2+}: PMCA [3] and the potassium dependent sodium calcium exchanger (NCKX) [11]. An NCX activity may also be present, but to date it has not been demonstrated in these cells. We now show the presence of another Ca^{2+} ATPase other than PMCA (Fig. 1C). Previously we showed that SERCA is not in these sperm [14]. Thus, a member of the SPCA family would be the other Ca^{2+} ATPase activity demonstrated herein. Further, immunoblot and immunofluorescence support this observation. Human spermatozoa also express an SPCA, but not a SERCA pump [4]. Hence, human and sea urchin sperm share similar energy driven Ca^{2+} homeostasis mechanisms that have been maintained during the course of deuterostome evolution.

Immunolocalization shows that sea urchin SPCA is present on the giant mitochondrion that forms the midpiece of sea urchin sperm. In somatic cells SPCA is localized mainly to golgi-membranes. Immunofluorescence comparison with phase contrast images (Fig. 4 arrows) shows that in some of the cells the giant mitochondrion is displaced to the side of the cell, out

of its normal linear alignment with the flagellum. This mitochondrial displacement is known to occur when sperm are injured, or when they undergo the acrosome reaction [15]. The coincident superimposition of the fluorescence with the displaced mitochondrion is evidence that the SPCA is localized in the mitochondrial membrane and not in the overlying plasma membrane. The mitochondrion of the sea urchin sperm was shown to sequester high concentrations of Ca^{2+} following the acrosome reaction [16]. In human spermatozoa, the SPCA localizes to an area behind the nucleus and also in the midpiece, which is where the mitochondria reside [4]. In sea urchin sperm, the only major membranous organelles are the nucleus, the acrosome and the mitochondrion. Here, we show that BP causes intracellular Ca^{2+} elevation even under extracellular Ca^{2+} low conditions of <33 μM. These data are evidence for the presence of a Ca^{2+} store in sea urchin sperm that can be released by BP inhibition. Binding of the anti-SPCA IgG to the mitochondrion indicates this Ca^{2+} store could be this organelle. This does not exclude the nucleus and acrosome vesicle as other potential Ca^{2+} stores.

Thapsigargin at 5–20 μM and 50 μM CPA elevate Ca^{2+} in sea urchin sperm [17,18]. However, these concentrations are non-SERCA-specific [6,7] and mobilize stored Ca^{2+} in sperm by acting on other targets. Williams and Ford showed no effect of CPA on intracellular Ca^{2+} in human sperm at doses up to 60 μM observing only a transient decrease [19]. Although these inhibitors increase Ca^{2+} levels in sperm, they do not induce the AR [17,18]. One observation in our study is that BP increases intracellular Ca^{2+}, but inhibits the AR instead of inducing it. Simultaneous addition of NH_4Cl to TG treated sperm increases AR 2.5-fold [18], suggesting that intracellular pH is not optimal despite having sufficient Ca^{2+} to induce AR. In our study 30 μM BP had no effect on intracellular pH in sea urchin sperm (data not shown). It was shown that the fragmented fucose sulfate polymer increases intracellular Ca^{2+} to the same levels as the intact polymer, but does so at a slower rate which does not induce the AR. Clearly, the pathway and rate of Ca^{2+} increase in sperm are critical for induction of the complete AR [20].

Ca^{2+} measurements indicate that ~25% of Ca^{2+} extrusion is by NCKX, while Ca^{2+} ATPases are responsible for ~75%, by PMCA and SPCA. In summary, the known Ca^{2+} extruding systems of sea urchin sperm are each concentrated in the three major anatomically distinct regions of the cell: PMCA is concentrated in the sperm head plasma membrane [3], NCKX in the flagellar plasma membrane [11], and SPCA in the mitochondrion.

Acknowledgement: This work was supported by NIH HD12986 to VDV.

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