Secretory pathway Ca^{2+}-ATPase (SPCA1) Ca^{2+} pumps, not SERCAs, regulate complex $[Ca^{2+}]_i$ signals in human spermatozoa

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Summary

The sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors thapsigargin (0.1-1 μM) and cyclopiazonic acid (10 μM), failed to affect resting $[Ca^{2+}]_i$ in human spermatozoa. Slow progesterone-induced $[Ca^{2+}]_i$ oscillations in human spermatozoa, which involve cyclic emptying-refilling of an intracellular Ca^{2+} store were also insensitive to these inhibitors. Non-selective doses of thapsigargin (5-30 μM, 50-300 times the saturating dose for SERCA inhibition), caused elevation of resting $[Ca^{2+}]_i$ and partial, dose-dependent disruption of oscillations. A 10-40 μM concentration of bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)methane (bis-phenol), which inhibits both thapsigargin-sensitive and -insensitive microsomal Ca^{2+} ATPases, caused elevation of resting $[Ca^{2+}]_i$ and inhibition of $[Ca^{2+}]_i$ oscillations at doses consistent with inhibition of thapsigargin-resistant, microsomal ATPase and liberation of stored Ca^{2+}. Low doses of bis-phenol had marked effects on $[Ca^{2+}]_i$ oscillation kinetics. Application of the drug to cells previously stimulated with progesterone had effects very similar to those observed when it was applied to unstimulated cells, suggesting that the sustained Ca^{2+} influx induced by progesterone is not mediated via mobilisation of Ca^{2+} stores. Western blotting for human sperm proteins showed expression of secretory pathway Ca^{2+} ATPase (SPCA1). Immunolocalisation studies revealed expression of SPCA1 in all cells in an area behind the nucleus, extending into the midpiece. Staining for SERCA, carried out in parallel, detected no expression with either technique. We conclude that: (1) intracellular Ca^{2+} store(s) and store-dependent $[Ca^{2+}]_i$ oscillations in human spermatozoa rely primarily on a thapsigargin/cyclopiazonic acid-insensitive Ca^{2+} pump, which is not a SERCA as characterised in somatic cells; (2) effects of high-dose thapsigargin on spermatozoa primarily reflect non-specific actions on non-SERCAs and; (3) secretory pathway Ca^{2+} ATPases contribute at least part of this non-SERCA Ca^{2+} pump activity.

Key words: Sperm, Ca^{2+}-store, SERCA, SPCA, Thapsigargin, bis-phenol

Introduction

Despite the great importance of $[Ca^{2+}]_i$ signalling in spermatozoa (Florman et al., 1998; Suarez and Ho, 2003), regulation of $[Ca^{2+}]_i$ in human and other spermatozoa is poorly understood compared with equivalent processes in somatic cells. Human spermatozoa contain neither endoplasmic reticulum nor Golgi (both of which act as Ca^{2+} stores in somatic cells), the only major membranous organelles being the nucleus, the acrosome (a Golgi-derived, lysosome-like vesicle which lies in the apex of the sperm head) and the mitochondria in the midpiece. In response to agonist stimulation, sperm generate $[Ca^{2+}]_i$ signals dependent upon Ca^{2+} influx, a finding consistent with this 'simple' structure. However, there is considerable evidence that Ca^{2+} stores exist in mammalian spermatozoa and play important roles in $[Ca^{2+}]_i$ signalling that controls motility and acrosome reaction (O'Toole et al., 2000; Jungnickel et al., 2001; Ho and Suarez, 2001; Ho and Suarez, 2003; Harper et al., 2004), the two major functions of spermatozoa prior to fertilisation.

The primary candidate for a sperm internal Ca^{2+} store is the acrosome (Herrick et al., 2005). Receptors for inositol trisphosphate (IP3) have been localised to the acrosomal membrane (Walensky and Snyder, 1995; Kuroda et al., 1999) and IP3-mediated release of Ca^{2+} from the acrosome has been demonstrated in permeabilised human spermatozoa (De Blas et al., 2002). Recently, the existence of a second Ca^{2+} store in the redundant nuclear envelope has been proposed (Naaby-Hansen et al., 2001; Ho and Suarez, 2003). The zona pellucida-induced acrosome reaction in murine sperm, though primarily dependent upon Ca^{2+} influx, is believed to require emptying of an intracellular store and consequent activation of capacitative Ca^{2+} entry (O'Toole et al., 2000). However, evidence for the participation of a Ca^{2+} store in the functioning of intact sperm has been derived primarily from studies using blockers of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases

(SERCAs). Thapsigargin elevates internal Ca^{2+} in human sperm (Blackmore, 1993; Bonaccorsi et al., 1995; Williams and Ford, 2003) and stimulates acrosomal exocytosis in both human spermatozoa (Meizel and Turner, 1993; Rossato et al., 2001) and sperm of other mammals (Llanos, 1998; Dragileva et al., 1999). Specific inhibition of SERCAs by thapsigargin is beyond dispute (Thastrup et al., 1990), and has been well documented in many somatic cell types (Treiman et al., 1998), but data from studies on the action of thapsigargin on sperm are inconsistent. Although effects of this drug on $[Ca^{2+}]_i$ or acrosome reaction at doses appropriate for inhibition of SERCAs have been reported (Rossato et al., 2001; Meizel and Turner, 1993), dose-dependence studies typically show that concentrations of 1-30 μM thapsigargin are needed to induce significant Ca^{2+} mobilisation and/or acrosome reaction (Blackmore, 1993; Walensky and Snyder, 1995; Llanos, 1998; Dragileva et al., 1999; O'Toole et al., 2000; Williams and Ford, 2003). These concentrations are 10-300 times greater than the concentrations required to effect complete block of SERCAs in somatic cells (Treiman et al., 1998). Furthermore, although small responses to thapsigargin have been reported in spermatozoa incubated under 'Ca^{2+}-free' conditions (O'Toole et al., 2000; Rossato et al., 2001; Williams and Ford, 2003), responses to this drug are often $[Ca^{2+}]_o$-dependent (Blackmore, 1993; Dragileva et al., 1999) and lack the initial $[Ca^{2+}]_i$ transient characteristic of store mobilisation in somatic cells (Blackmore, 1993; Bonaccorsi et al., 1995; Williams and Ford, 2003). CPA (cyclopiazonic acid), the other 'diagnostic' inhibitor of SERCAs shows similar variability of effect. A dose-dependent elevation of $[Ca^{2+}]_i$ in CPA-treated human spermatozoa was reported to be maximal at 100 μM (Rossato et al., 2001), but other authors (Williams and Ford, 2003) found no effect of the drug on $[Ca^{2+}]_i$ at doses up to 60 μM (well in excess of a typical saturating dose for Ca^{2+} mobilisation in intact cells) (e.g. Demaurex et al., 1992) observing only a transient decrease in $[Ca^{2+}]_i$. In a recent study thapsigargin and CPA were used to assess contributions of various components to Ca^{2+} clearance in mouse sperm and no significant contribution from SERCAs was detectable (Wennemuth et al., 2003). The nature (and possibly existence) of Ca^{2+} stores in human (and other mammalian) spermatozoa is still, therefore, far from being resolved.

Stimulating human sperm with an increasing ramp of progesterone concentration induces large, slow $[Ca^{2+}]_i$ oscillations (and consequent modulation of flagellar activity) in more than one-third of cells (Harper et al., 2004). Similar oscillations occur in response to a 'stepped' addition of micromolar progesterone, but in far fewer cells (Harper et al., 2004; Kirkman-Brown et al., 2004). These oscillations are generated from an intracellular Ca^{2+} store that is insensitive to thapsigargin and CPA at selective doses (Harper et al., 2004). We have therefore investigated the activity and expression of SERCAs and other store Ca^{2+} ATPases and their participation in complex $[Ca^{2+}]_i$ signalling in human spermatozoa.

Materials and Methods

Preparation and capacitation of spermatozoa

Cells from ten different donors were used, all of whom were recruited at Birmingham Women's Hospital (HFEA Centre 0119), in accordance with the Human Fertilisation and Embryology Authority 6th Code of Practice. Donors gave informed consent. Highly motile spermatozoa were harvested into sEBSS (0.3% BSA) as described previously (Harper et al., 2003) and were left to capacitate for 6 hours at 37°C, 5% CO_2. All subsequent experimentation was carried out with cells suspended in sEBSS or 'Ca^{2+}-free' sEBSS (no added Ca^{2+}, $[Ca^{2+}] < 5$ μM).

Fluorimetric measurements of $[Ca^{2+}]_i$

Fluorimetric studies were carried out as described previously (Harper et al., 2003). Briefly, 2 ml aliquots (6×10^6 cells/ml) were labelled with Fura-2 AM, resuspended in sEBSS and $[Ca^{2+}]_i$ was monitored in a Perkin-Elmer LS50B fluorescence spectrofluorimeter (cuvette maintained at 37°C). Excitation alternated between 340 nm and 380 nm and emission was measured at 510 nm (sampling rate 12.5 Hz). Calibration of $[Ca^{2+}]_i$ was performed at the end of each experiment according to the equation of Grynkiewicz et al. (Grynkiewicz et al., 1985) assuming a K_d of 224 nM, maximum and minimum values being obtained by sequential addition of digitonin (50 μM) and EGTA (20 mM).

Manganese quench

To investigate uptake of Mn^{2+} into intracellular stores we used fluorimetry (essentially as described above) modified to observe quenching of Fura-2 fluorescence. The excitation wavelength was held at 360 nm (isobestic point). 1 mM $MnCl_2$ was added once a stable resting baseline fluorescence had been achieved. 3 μM progesterone was then added to induce Mn^{2+} influx (and consequent quenching of Fura-2 fluorescence) followed after 5 minutes by 1 mM $LaCl_3$ to terminate Mn^{2+} influx. After incubation in capacitating media cells were resuspended in a HEPES-buffered saline (without HCO_3^-) immediately before beginning the experiment to avoid precipitation of lanthanum.

Imaging

200 μl aliquots (6×10^6 cells/ml) were loaded with Oregon Green BAPTA 1-AM and imaged in a continuously-perfused chamber as described previously (Harper et al., 2003). Progesterone and drugs were applied by addition to the perfusion header. All experiments were carried out at 25°C. Data acquisition and storage were controlled by a PC running AQM Orca 2001 (Kinetic Imaging, Nottingham, UK).

Single cell data processing

Data were processed offline using AQM Orca 2001 as described previously (Harper et al., 2003). Raw intensity values from the caudal part of the head of each sperm were imported into Microsoft Excel and normalised to pre-stimulus values. Traces were then examined separately for each cell. Cells displaying oscillations in $[Ca^{2+}]_i$ were identified directly from time-fluorescence intensity plots. Only cells in which cyclical changes in fluorescence, comprising repeated events of consistent characteristics, were categorised as oscillators. At each time point the normalised fluorescence intensity values (R) for each cell were compiled to generate an overall average normalised head fluorescence (R_{tot}). The total series of R_{tot} could then be plotted to give the mean normalised response of head fluorescent intensity for that experiment.

Detection of Ca^{2+}-ATPases

Spermatozoa were centrifuged (5 minutes, 500 ***g***) to achieve a concentration of 2×10^6 cells in 12 μl. Aliquots of 12 μl were treated with solubilisation buffer (2% SDS, 10% glycerol, 1.4% dithiothreitol, 62.5 mM Tris-HCl, pH 6.8). Samples were heated at

100°C for 5 minutes, sonicated for 10 seconds and dispersed by flicking. Proteins were separated by electrophoresis on 7.5% SDS-polyacrylamide gels (Laemmli, 1970) and electrotransferred onto nitrocellulose (Gershoni et al., 1985). Non-specific binding sites were blocked with 5% dried skimmed milk in 0.05% Tween-20 supplemented Tris-buffered saline (TTBS; 25 mM Tris HCl, pH 8.0, 150 mM NaCl). The nitrocellulose membrane was cut and separate parts incubated for 1.5 hours at room temperature with the monoclonal Y1F4 antibody (a gift from J. M. East, Southampton University, which recognises the epitope KMFVK present in all SERCA isoforms) (Tunwell et al., 1991) or the polyclonal SPCA antibody (raised against amino acid sequence 503-516 of the human SPCA1) (Wootton et al., 2004). The membranes were then washed extensively in TTBS and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for Y1F4 or goat anti-rabbit IgG for SPCA1, for 1 hour at room temperature, following by extensive washing in TTBS. Positive immunoreactive bands were detected by chemiluminescence using SuperSignal® West Pico according to the manufacturer's instructions (Pierce Biotechnology). Tubulin was run as a positive control for successful transfer of sperm proteins.

Assay of Ca^{2+}-dependent ATPase activity

Ca^{2+}-dependent ATPase activity of pig cerebellar microsomes was measured by monitoring the hydrolysis of ATP using the method described (Hughes et al., 2000). To study thapsigargin-insensitive uptake, microsomes were preincubated for 5 minutes with 1 μM thapsigargin.

In situ immunolocalisation

Spermatozoa were washed in PBS (128 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 1 mM sodium azide pH 7.5) and dispersed on microscope slides previously treated with poly-L-lysine and air-dried for 5-10 minutes. Spermatozoa were then fixed in 4% formaldehyde for 6 minutes at room temperature followed by permeabilisation with PBS containing 1% (v/v) Triton X-100 for 6 minutes, and washed three times (5 minutes each) with PBS containing 0.1% Triton X-100 (Triton-PBS). To minimise non-specific binding, sperm were preincubated with 5% (v/v) goat serum in Triton-PBS for 1 hour before an overnight incubation with the primary anti-polyclonal antibody at 4°C. After extensive washing with Triton-PBS, sperm were further incubated for 1 hour with a biotin-SP-conjugated affiniPure goat anti-rabbit antibody (Jackson Immuno Research Laboratories) and then for 1 hour with streptavidin-conjugated Alexa 488 (5 μg/ml in TPBS; Molecular Probes). Following extensive washing with Triton-PBS, slides were finally mounted in Prolong Antifade (Dako). A control for the secondary antibody was also performed in which sperm preparations were incubated with the biotinylated goat anti-rabbit antibody followed by Alexa 488 conjugated to streptavidin as described above.

Materials

Oregon Green BAPTA 1-AM and Fura-2 AM were from Molecular Probes (Cambridge Bioscience, UK). Fatty acid-free BSA was from JRH Biosciences Lenexa, KS (type 85041). Cyclopiazonic acid (CPA), DMSO, EGTA, progesterone, PBS, pluronic F-127, A23187, poly-D-lysine were from Sigma-Aldrich (Poole, UK). bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)methane (bis-phenol) was purchased from Pfaltz and Bauer, Waterbury, CT. To ensure that lack of effect was not batch-specific, thapsigargin from three sources (Sigma, Poole, UK; Alomone Laboratories, Jerusalem, Israel and Calbiochem, Nottingham, UK) was used. All chemicals were cell culture-tested grade where available.

Statistical analysis

Values in text are stated as mean±s.e.m. unless stated otherwise. Mean percentage values were compared using student's *t*-test on arcsine-transformed data.

Results

Effect of Ca^{2+}-store ATPase inhibitors on resting $[Ca^{2+}]_i$

Fluorimetric analysis

Initially we examined the effects of SERCA inhibitors on Fura-2-loaded cell populations to determine whether $[Ca^{2+}]_i$ in spermatozoa prepared in our laboratory showed the insensitivity to SERCA inhibition reported by others (see Introduction). At 100 nM, thapsigargin had no effect on the resting $[Ca^{2+}]_i$ concentration but a dose of 1 μM (ten times the typical saturating dose for SERCA inhibition) (Treiman et al., 1998) caused a sustained increase in $[Ca^{2+}]_i$ (50-100 nM) in three out of eight experiments. In two of these experiments a small $[Ca^{2+}]_i$ transient of 1-2 minutes duration preceded the sustained rise (Fig. 1A). On other occasions we observed no effect or a small decrease in the calibrated $[Ca^{2+}]_i$ upon application of 1 μM thapsigargin (Fig. 1A inset). At 10 μM, thapsigargin regularly caused a sustained increase in $[Ca^{2+}]_i$ (typically 200-300 nM) as reported previously (Blackmore, 1993; Williams and Ford, 2003) (Fig. 1A). At 10 μM, cyclopiazonic acid (CPA) appeared to cause a slight reduction in resting $[Ca^{2+}]_i$, which recovered within 1 minute, an observation consistent with another report (Williams and Ford, 2003) (data not shown). There are currently no specific inhibitors of the thapsigargin-resistant secretory pathway Ca^{2+} ATPases (SPCAs) (Wuytack et al., 2003). However, in tissues that are abundant in SPCA, bis-phenol (10-40 μM) inhibits both the thapsigargin-sensitive and thapsigargin-resistant components of microsomal Ca^{2+}-ATPase activity (Brown et al., 1994; Wootton et al., 2004), showing that this compound inhibits both SERCA and SPCA Ca^{2+}-pumps. At 10 μM, bis-phenol caused a sustained increase in $[Ca^{2+}]_i$ of 30-60 nM, which was usually preceded by a transient increase in $[Ca^{2+}]_i$ of 1-2 minutes duration (Fig. 1B), resembling the transient occasionally observed upon application of 1 μM thapsigargin (Fig. 1A). Increasing the dose of bis-phenol to 40 μM (sufficient to cause complete inhibition of thapsigargin-resistant Ca^{2+} uptake) (Brown et al., 1994) caused a further sustained rise in $[Ca^{2+}]_i$, giving total increment of 100-200 nM (Fig. 1B,C). In preparations where 1 μM thapsigargin did not cause elevation of $[Ca^{2+}]_i$, subsequent application of bis-phenol had a clear effect (Fig. 1C), indicating that bis-phenol acts on a thapsigargin-insensitive target.

Single cell imaging

To determine whether the population responses were representative of the responses occurring in individual cells, we used imaging of Oregon Green BAPTA 1-loaded sperm. Superfusion with 1 μM thapsigargin had no effect on resting $[Ca^{2+}]_i$ in >95% of cells. An increase in fluorescence was observed in only 4.7% of cells (338 cells, two experiments) during the period following application of the drug (Fig. 2A). These events were small (mean amplitude 10±3% increase in fluorescence) and were poorly synchronised such that the mean

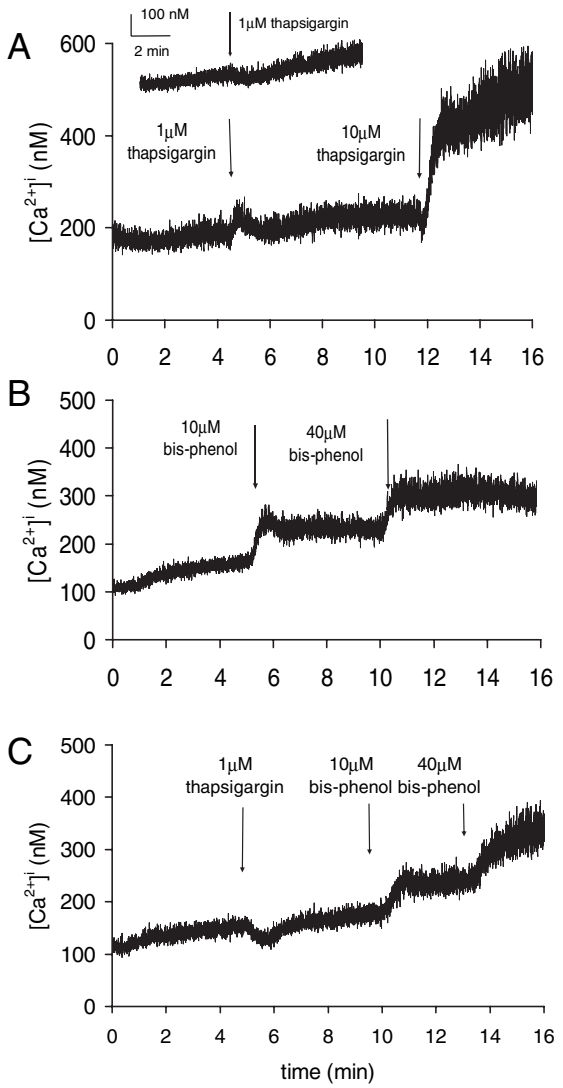

Fig. 1. Effects of store Ca^{2+} ATPase inhibitors on resting $[Ca^{2+}]_i$ in human sperm populations loaded with Fura-2. Horizontal axes in all traces show time in minutes. (A) Main trace shows effect of sequential additions of 1 μM and 10 μM (total final concentration) thapsigargin (arrows). 10 μM thapsigargin regularly caused a sustained increase in $[Ca^{2+}]_i$ of 100-300 nM. In most experiments 1 μM thapsigargin had no discernible effect or apparently caused a slight fall in $[Ca^{2+}]_i$ (inset). Occasionally we saw a small sustained increase of 20-30 nM and/or (in two experiments) a small (10-20 nM) transient increase in $[Ca^{2+}]_i$ as shown here. (B) treatment of cell populations with 10 μM and 40 μM (total final concentration) bis-phenol (arrows) caused a dose-dependent, sustained increase in $[Ca^{2+}]_i$; the first application usually inducing an initial transient elevation of $[Ca^{2+}]_i$ lasting 1-2 minutes. (C) A normal response to 10 μM and 40 μM bis-phenol was seen in cell suspensions that generated no elevation of $[Ca^{2+}]_i$ (or a transient decrease) in response to 1 μM thapsigargin. Arrows indicate times of drug additions.

plot (R_{tot}) for these experiments showed no response (Fig. 2A, inset). As a small proportion of cells show spontaneous $[Ca^{2+}]_i$ transients or ripples (Kirkman-Brown et al., 2004), it is likely that 4.7% is an exaggeration of the 1 μM thapsigargin-responsive population. In contrast, 100 nM-1 μM thapsigargin (diluted from the same stocks) consistently caused store emptying in primary rat osteoblasts in our laboratory (Foreman et al., 2005). Superfusion of spermatozoa with 10 μM thapsigargin produced an increase in baseline $[Ca^{2+}]_i$ in 35±8% of cells (253 cells, three experiments) that was synchronised such that the effect was also visible in the mean plot (Fig. 2B, inset). In most of these cells this was a slowly developing, sustained elevation of $[Ca^{2+}]_i$ (mean amplitude 13±2% increase in fluorescence 8 minutes after application of thapsigargin; Fig. 2B) but in a small proportion (<20% of the responsive cells) there was a transient increase in $[Ca^{2+}]_i$, lasting 1-2 minutes which preceded the sustained phase (Fig. 2B, ■). CPA (10 μM) did not affect resting $[Ca^{2+}]_i$ in any cells (data not shown). Super-fusion with 10 μM bis-phenol caused an increase in $[Ca^{2+}]_i$ in 70% of cells (103/148). Of these, most showed a rise of 10-30% that was sustained to the end of the recording period. This often developed slowly (over 5-10 minutes) but in almost 50% of responsive cells there was an initial transient response, followed by a plateau (e.g. Fig. 2C, ■,▲). A similar effect was observed with 40 μM bis-phenol, which induced a clear rise in $[Ca^{2+}]_i$ in 85±9% of cells (Fig. 2C; 345 cells, four experiments). Although there was little difference in effect at the two doses, sequential treatment of cells with 10 μM and 40 μM bis-phenol showed that the higher dose was able to induce a further increment in $[Ca^{2+}]_i$ in most cells (not shown). When 40 μM bis-phenol was applied in 'Ca^{2+}-free' sEBSS the effect was similar to that seen in normal EBSS (Fig. 2D), 74% of cells (80/108) responding with a synchronised increase in $[Ca^{2+}]_i$, though the amplitude of the normalised response was larger (compare mean plots shown as insets in Fig. 2C,D), possibly due to the reduced resting $[Ca^{2+}]_i$ under these conditions. In most responsive cells (60%), the response comprised an initial transient (lasting 2-3 minutes) followed by a small, sustained elevation (Fig. 2D). When standard EBSS was then introduced into the chamber $[Ca^{2+}]_i$ rose rapidly in all cells (Fig. 2D, arrow).

Effect of Ca^{2+}-store ATPase inhibitors on the sustained phase of the progesterone-induced $[Ca^{2+}]_i$ response

It has been proposed that the sustained phase of the biphasic $[Ca^{2+}]_i$ response to progesterone in human spermatozoa may be generated by store mobilisation and activation of store-operated channels (Barratt and Publicover, 2001; Kirkman-Brown et al., 2002). As sustained elevation of $[Ca^{2+}]_i$ in human sperm by thapsigargin is believed to occur by this process, we investigated whether prior activation of the progesterone-induced sustained response might sensitise cells to stimulation with lower doses of thapsigargin or occlude the response to more effective, higher doses.

Cells were stimulated with 3 μM progesterone, followed after 10 minutes (during the sustained phase of the $[Ca^{2+}]_i$ response) by thapsigargin in the continuing presence of progesterone. Upon addition of 1 μM thapsigargin, approximately 2% of cells (3/163 in two experiments) showed an increase in $[Ca^{2+}]_i$, the response being a sustained elevation with no clear preceding transient (Fig. 3A, ■). No effect of thapsigargin was visible in mean (R_{tot}) plots. In four experiments (307 cells), 10 μM thapsigargin caused an increase in $[Ca^{2+}]_i$ in 31±4% of cells (Fig. 3B). Both the proportion of responsive cells and the size of the increase in fluorescence were similar to those seen without progesterone

pre-treatment, but we did not observe transient responses preceding the sustained rise in fluorescence. CPA (10 μM) did not affect $[Ca^{2+}]_i$ during the progesterone-induced sustained signal (data not shown). When 40 μM bis-phenol was applied 10 minutes after progesterone stimulation it caused an increase in $[Ca^{2+}]_i$ in 74±10% of cells (Fig. 3C; 284 cells, three experiments; no significant difference compared to non-pretreated cells, $P>0.05$), with preceding transients occurring in <10% of cells (Fig. 3C, ■). At 40 μM, bis-phenol was equally effective when applied to cells that had been stimulated first with progesterone and then exposed to 1 μM thapsigargin (Fig. 3D). In these experiments, bis-phenol caused a large and immediate increase in $[Ca^{2+}]_i$ in 61±6% of cells (no significant difference compared to cells without thapsigargin treatment, $P>0.05$; 257 cells, three experiments), nearly all of which had failed to respond to thapsigargin (Fig. 3D). Clear transient responses were not seen in these cells.

Effect of Ca^{2+} store ATPase inhibitors on progesterone-induced $[Ca^{2+}]_i$ oscillations

We have reported previously that, upon exposure to 3 μM progesterone, 10-14% of human spermatozoa generate large, slow oscillations in $[Ca^{2+}]_i$ (period typically 1-4 minutes) (Kirkman-Brown et al., 2004; Harper et al., 2004). These oscillations are resistant to reduction of $[Ca^{2+}]_o$, showing that they are generated primarily upon cyclic emptying-refilling of an intracellular store (Harper et al., 2004). At 1 μM, thapsigargin had no discernible effect on progesterone-induced $[Ca^{2+}]_i$ oscillations (four experiments, 410 cells; not shown). At a dose of 5 μM, thapsigargin caused a reduction in oscillation amplitude in 2.5±0.8% of oscillating cells, due to elevation of the baseline $[Ca^{2+}]_i$ between cycles (three experiments; 453 cells; Fig. 4A, ■) but oscillations did not completely arrest. At 10 μM thapsigargin (100 times the saturating concentration for SERCA inhibition), a decrease in

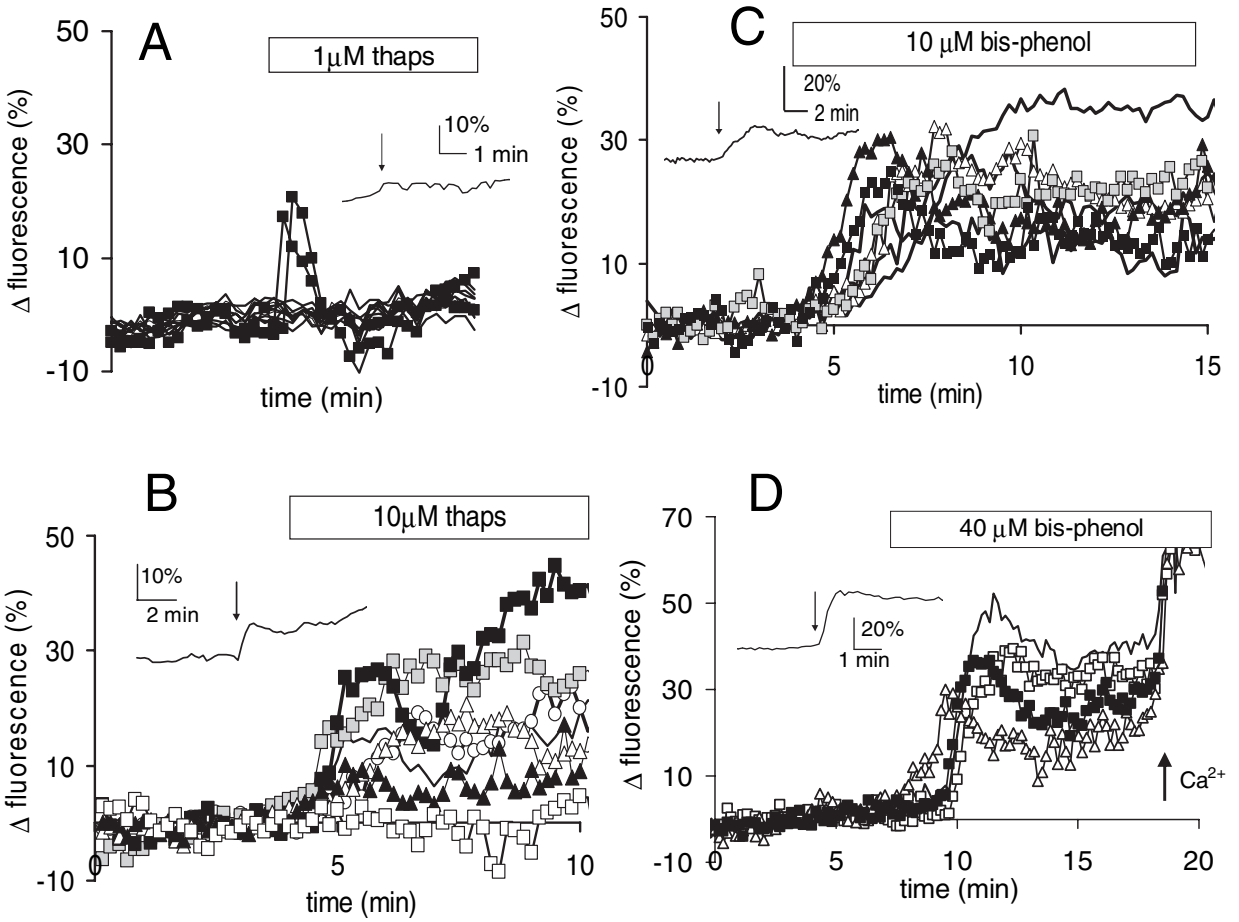

Fig. 2. Effects of store Ca^{2+}-ATPase inhibitors on resting $[Ca^{2+}]_i$ in individual human sperm. (A) Responses of eight cells to 1 μM thapsigargin (thaps, bar above traces). Most cells failed to respond at this dose (lines without symbols) but a small proportion (approx 5%) generated a brief increase in fluorescence lasting approximately 1 minute (■). Inset shows mean response of all cells in this experiment (thapsigargin added at arrow). (B) Responses of seven cells to 10 μM thapsigargin At this concentration thapsigargin (bar above traces) caused a sustained elevation of $[Ca^{2+}]_i$ in 35% of cells, a small proportion generating an initial transient (■). An example of a cell that failed to respond to 10 μM thapsigargin is shown (□). Inset, mean response of all cells in this experiment (thapsigargin added at arrow). (C) Responses of six cells to 10 μM bis-phenol (bar above traces). Bis-phenol caused a clear rise in $[Ca^{2+}]_i$ in 70% of cells. Most cells generated a sustained response that was preceded by a transient elevation (■,▲). Inset, mean response of all cells in this experiment (bis-phenol added at arrow). (D) Responses of four cells to 40 μM bis-phenol (bar above traces) in 'Ca^{2+}-free' sEBSS (no added Ca^{2+}). In this saline bis-phenol caused a clear rise in $[Ca^{2+}]_i$ in 74% of cells. Responses resembled those in standard EBSS. Upon reintroduction of standard EBSS containing Ca^{2+} (upward arrow) all cells showed a marked rise in $[Ca^{2+}]_i$. Inset, mean response of all cells in this experiment (bis-phenol added at arrow).

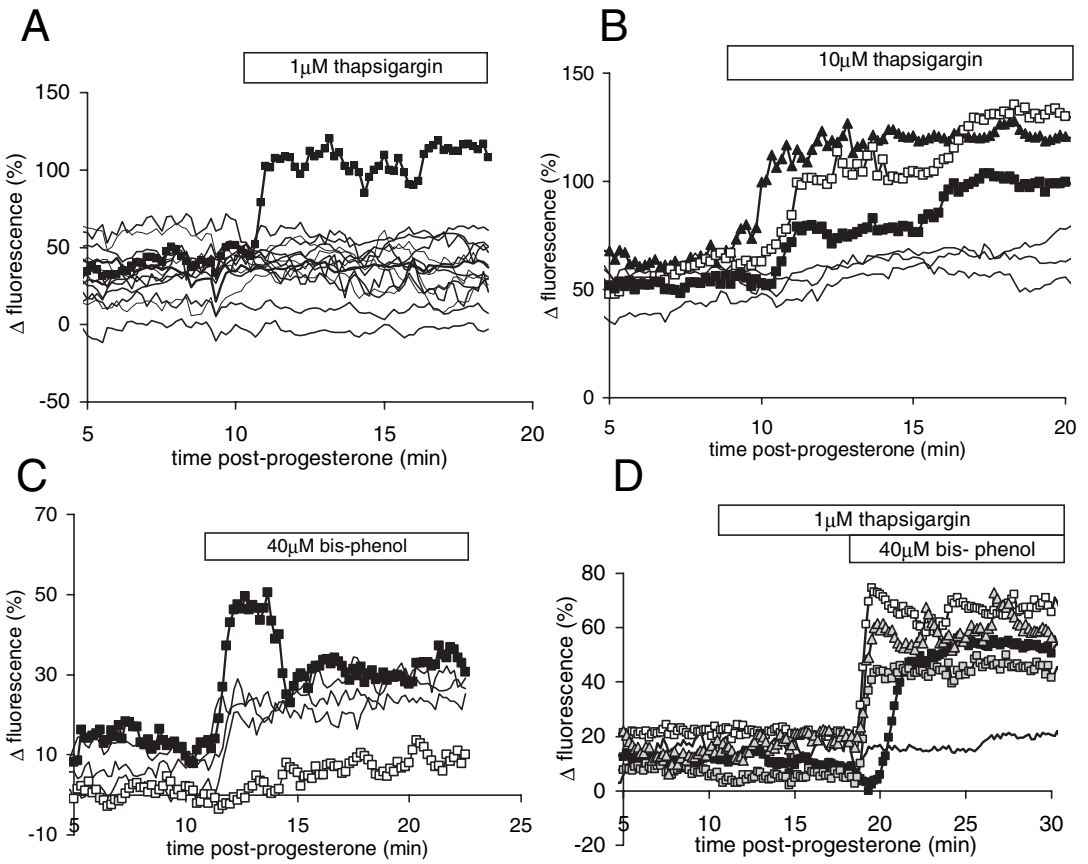

Fig. 3. Effects of store Ca^{2+}-ATPase inhibitors on $[Ca^{2+}]_i$ in individual cells after establishment of the sustained phase of the progesterone-induced response. (A) Application of 1 μM thapsigargin (bar above traces) had no effect on $[Ca^{2+}]_i$ in most cells (lines without symbols) but in approximately 2% of cells there was a sustained increase in $[Ca^{2+}]_i$ (■). Responses of 15 cells are shown. (B) 10 μM thapsigargin (bar above traces) was similarly ineffective in most cells (lines without symbols) but induced a sustained elevation of $[Ca^{2+}]_i$ in approximately one third of cells (▲,□,■). Responses of six cells are shown. (C) 40 μM bis-phenol added 10 minutes after progesterone (arrow) caused a sustained elevation of $[Ca^{2+}]_i$ in 74% of cells (lines without symbols), occasionally preceded by a $[Ca^{2+}]_i$ transient (■), a few cells showing no clear response (□). Responses of six cells are shown. (D) After application of 1 μM thapsigargin (bar above traces), subsequent application of 40 μM bis-phenol (bar above traces) caused a sustained rise in $[Ca^{2+}]_i$ in 40% of thapsigargin-insensitive cells. Responses of five cells are shown. Trace without symbols is a non-responsive cell.

amplitude of oscillations was observed in approximately 40% of cells owing to elevation of baseline $[Ca^{2+}]_i$. A total of 21±5% of oscillating cells arrested with $[Ca^{2+}]_i$ 'jammed' at or close to the oscillation peak (Fig. 4B; six experiments, 616 cells). In many cells, subsequent application of 10 μM A23187 caused a significant further increase in $[Ca^{2+}]_i$, showing that this stabilisation of $[Ca^{2+}]_i$ was not due to saturation of the fluorescent probe (Fig. 4C). A23187 halted oscillations in all cells. To investigate whether the cells in which oscillations were sensitive to 10 μM thapsigargin were a 'thapsigargin-sensitive' subpopulation, we applied 5 μM, 10 μM and 30 μM thapsigargin sequentially. The majority of cells in which oscillations persisted in 10 μM thapsigargin arrested at 30 μM (18% block at 10 μM; 70% block at 30 μM; two experiments, 210 cells; Fig. 4D). Treatment with 10 μM CPA had no effect on progesterone-induced oscillations (not shown).

When we applied bis-phenol to cells in which progesterone-induced $[Ca^{2+}]_i$ oscillations were established, we observed a clear dose-dependent sensitivity over the concentration range (10-40 μM) at which this compound inhibits intracellular store Ca^{2+} ATPases (Brown et al., 1994). At 10 μM, bis-phenol caused immediate arrest (or near complete attenuation) of oscillations (Fig. 5A) in 21% of oscillating cells, with a further 26% stopping within 3-5 minutes with $[Ca^{2+}]_i$ stabilised above resting levels, often close to the oscillation peak (Fig. 5B; total arrested cells=47±6%, five experiments, 694 cells). Washout of bis-phenol from the superfusing media (whilst maintaining the progesterone stimulus) resulted in a fall in resting $[Ca^{2+}]_i$ in most cells and resumption of oscillations in almost 50% of those cells in which treatment had caused arrest (Fig. 5A). In cells in which $[Ca^{2+}]_i$ oscillations persisted in the presence of 10 μM bis-phenol, the $[Ca^{2+}]_i$ peaks were often prolonged, (Fig. 5C,D). Fig. 5D shows mean transient responses from two cells recorded before (■) and during (□) superfusion with bis-phenol. Rising phases of the oscillations were little affected by bis-phenol, but decay was delayed, consistent with inhibition of a mechanism responsible for Ca^{2+} clearance during oscillations. Similar effects were sometimes seen with high doses of thapsigargin (e.g. see Fig. 4D, □). Application of 30 μM bis-phenol after 10 μM caused most cells in which $[Ca^{2+}]_i$ oscillations persisted to arrest at or near the $[Ca^{2+}]_i$ oscillation peak (Fig. 5E; two experiments, 242 cells). Subsequent application of 10 μM A23187 caused a further rise in fluorescence in most cells, confirming that the plateau was not merely a reflection of dye saturation (not shown). At a dose of 40 μM bis-phenol caused arrest of oscillations in 98±3% of oscillating cells (five experiments, 332 cells). To confirm that thapsigargin-insensitive oscillations could be blocked by bis-phenol, we treated progesterone-stimulated cells first with thapsigargin (1 μM) and then bis-phenol (40 μM). Oscillations were insensitive to thapsigargin but were completely blocked by bis-phenol (three experiments, 222 cells), with $[Ca^{2+}]_i$

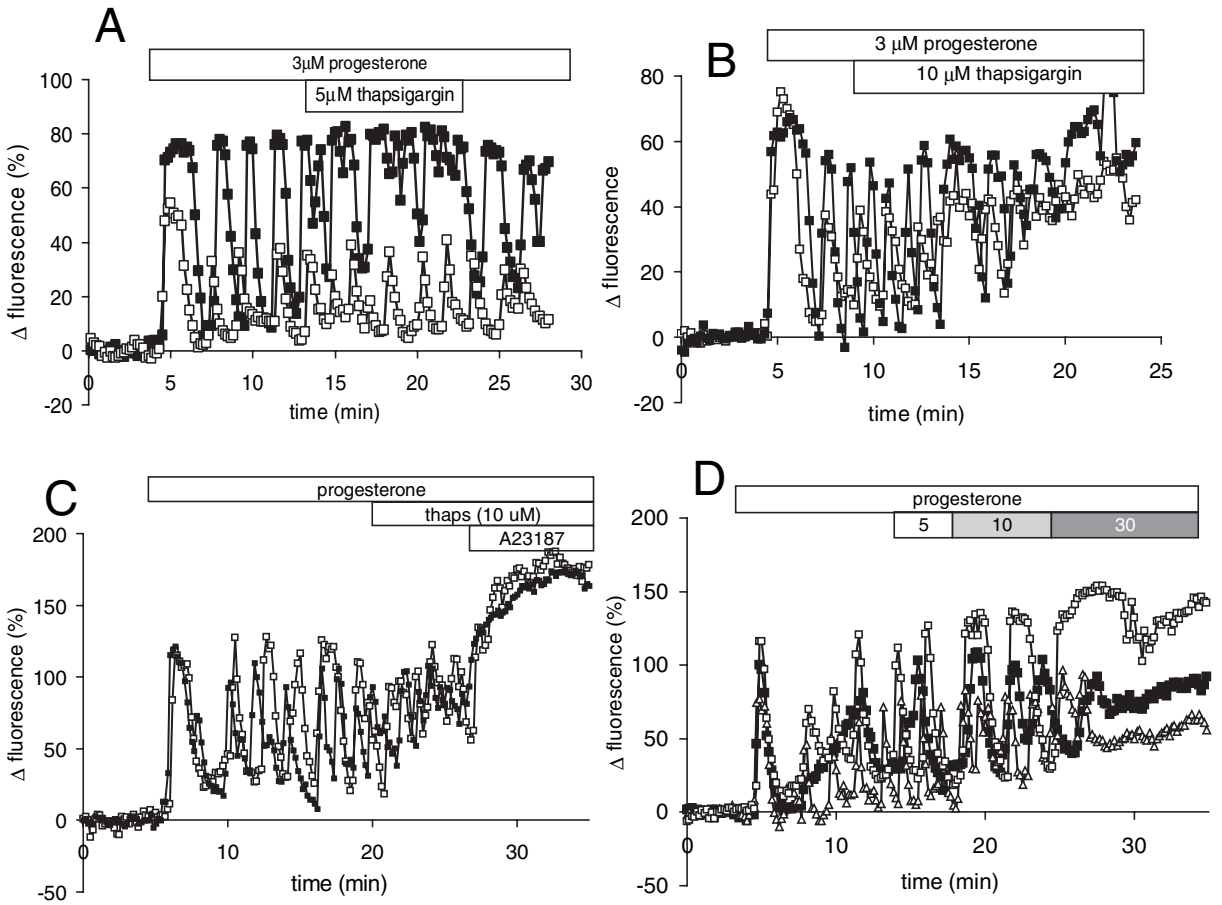

Fig. 4. Thapsigargin inhibits $[Ca^{2+}]_i$ oscillations at non-SERCA-specific doses. (A) Application of 5 μM thapsigargin to cells generating oscillations after progesterone stimulation had no effect in most instances (□). In just 2.5% of these cells there was a failure to clear Ca^{2+} from the cytoplasm between cycles, such that oscillation amplitude was reduced (■). Upon washout of thapsigargin the amplitude of oscillations recovered. (B) Two single cell records from cells stimulated with 3 μM progesterone followed by application of 10 μM thapsigargin. In these two cells oscillations arrested at peak. (C) Records from two cells generating progesterone-induced oscillations which responded to 10 μM thapsigargin as in B. Subsequent application of 10 μM A23187 caused a further significant increase in fluorescence, showing that the 'stable' elevation of $[Ca^{2+}]_i$ was not an artefact due to saturation of the fluorescent probe. (D) Records from three cells in an experiment in which cells were exposed to a series of increasing doses of thapsigargin. In these cells 10 μM thapsigargin failed to inhibit $[Ca^{2+}]_i$ oscillations but raising the concentration to 30 μM caused $[Ca^{2+}]_i$ to stabilise at an elevated level.

settling at an elevated level. Oscillations failed to restart after removal of the drugs from the super-fusing media (data not shown).

As progesterone-induced oscillations persist in 'Ca^{2+}-free' sEBSS, we investigated whether, under these conditions, bis-phenol was equally effective. Application of 40 μM bis-phenol to cells oscillating in 'Ca^{2+}-free' sEBSS caused immediate arrest in all oscillating cells (81 cells; two experiments). Under these conditions, bis-phenol caused $[Ca^{2+}]_i$ to settle at a level similar to the trough of the oscillation cycle (Fig. 5F) rather than rising to levels close to the oscillation peak, as was typical when oscillating cells were arrested by bis-phenol in Ca^{2+}-containing salines (Fig. 5A,B,E).

To assess further the apparent lack of effect of thapsigargin on $[Ca^{2+}]_i$ signalling in human sperm we compared the potency of the action of thapsigargin on $[Ca^{2+}]_i$ oscillations with the well-established efficacy of the drug in inhibiting ATP-dependent microsomal Ca^{2+}-uptake and liberating stored Ca^{2+}. Inhibition of $[Ca^{2+}]_i$ oscillations (expressed as the percentage of cells in which oscillations persisted in the presence of thapsigargin; Fig. 6A) required far higher doses (two to three orders of magnitude) than inhibition of ATP-dependent Ca^{2+} uptake by rat cerebellar microsomes (Fig. 6A, ■) (Brown et al., 1994). Similarly, arrest of oscillations by thapsigargin required far higher doses than mobilisation of stored Ca^{2+} in intact cells (Fig. 6B) (Demaurex et al., 1992; Ely et al., 1991). In contrast, there was close correspondence between dose-dependence of the inhibition of sperm $[Ca^{2+}]_i$ oscillations by bis-phenol and the effects of this compound on the functioning of Ca^{2+} stores. Fig. 6C shows the dose-dependence of the effects of bis-phenol on human sperm $[Ca^{2+}]_i$ oscillations (□), ATP-dependent Ca^{2+}-uptake by rat cerebellar microsomes (■) (Brown et al., 1994), Ca^{2+}-dependent ATPase activity of pig cerebellar microsomes (▲) and thapsigargin resistant Ca^{2+}-ATPase activity of pig cerebellar microsomes (△). Fig. 6D shows that mobilisation by bis-phenol of Ca^{2+} in intact HL-60 cells (bathed in Ca^{2+}-free saline, ▲) had an almost identical dose-dependence to the inhibition of sperm $[Ca^{2+}]_i$ oscillations (△) (Brown et al., 1994).

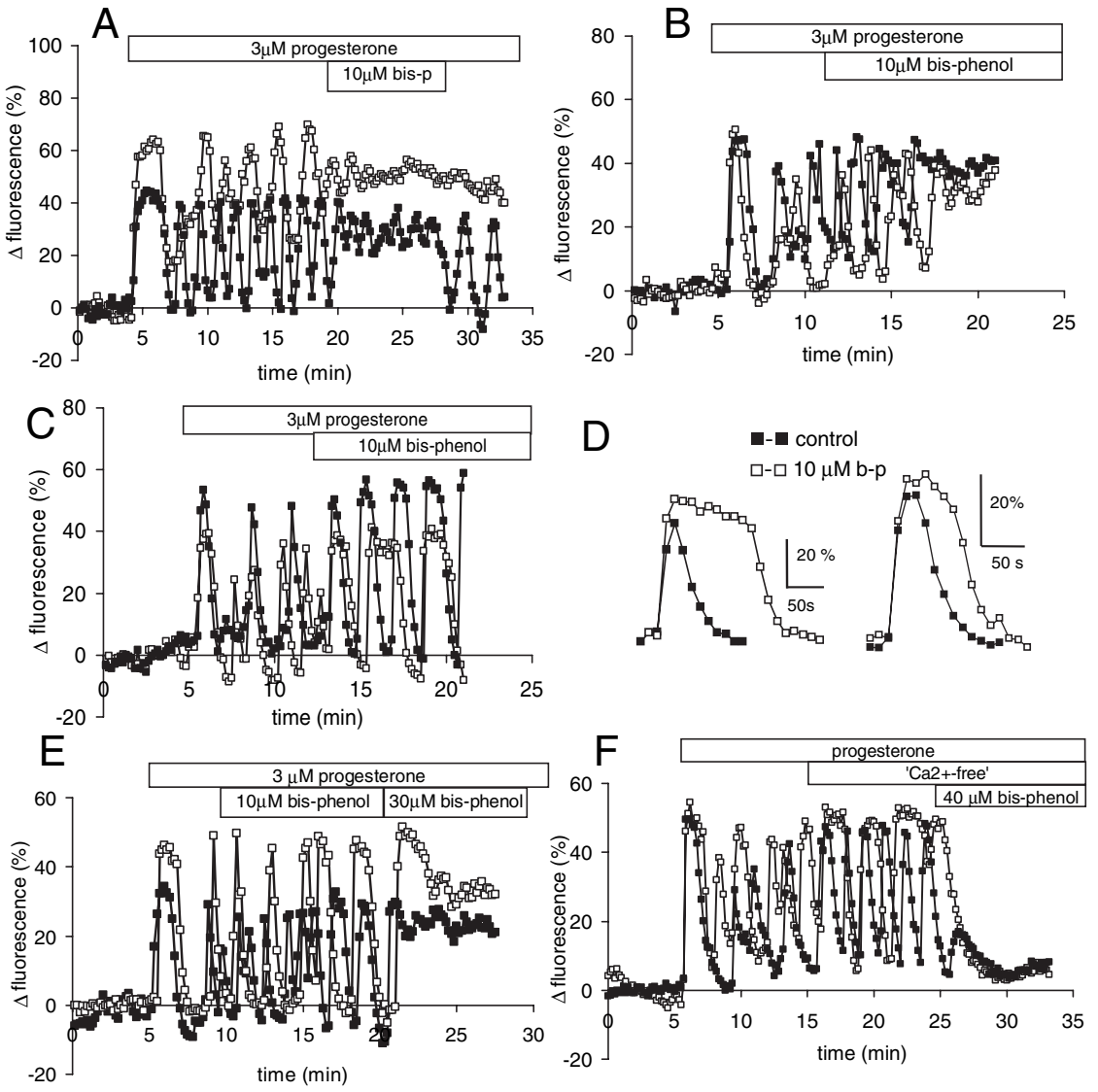

Fig. 5. Bis-phenol inhibits $[Ca^{2+}]_i$ oscillations. Application of 10 μM bis-phenol (bar above traces) to cells in which $[Ca^{2+}]_i$ oscillations were already established in response to progesterone caused some cells to arrest immediately (A, records from two cells) or gradually (B, records from two cells). Activity in some cells recovered upon washout of bis-phenol (shown by bar above A). (C) In half of the cells in which oscillations persisted in the presence of 10 μM bis-phenol there was a significant prolongation of $[Ca^{2+}]_i$ peaks (records from two cells from same experiment as A and B). (D) Mean oscillation transients (mathematical averages of three oscillation cycles synchronised to peak $[Ca^{2+}]_i$) from two cells. Transients generated before (■) and during (□) superfusion with 10 μM bis-phenol (b-p) have been overlaid to show the change in kinetics. Application of the drug extends the $[Ca^{2+}]_i$, peak, apparently revealing two phases of Ca^{2+} clearance. (E) Application of 30 μM bis-phenol to cells in which oscillations persist in the presence of 10 μM bis-phenol results in immediate arrest at or near peak $[Ca^{2+}]_i$. Records from two cells are shown. (F) Application of bis-phenol (40 μM) caused immediate arrest of $[Ca^{2+}]_i$ oscillations in cells superfused with 'Ca^{2+}-free' sEBSS (no added Ca^{2+}). Records from two cells are shown. Under these conditions, upon cessation of oscillation $[Ca^{2+}]_i$ settled at a level at or below that occurring during oscillation troughs (compare to panels A, B and E).

Expression and localisation of Ca^{2+} store ATPases in human spermatozoa

To investigate expression in human sperm of SERCAs and of SPCA, we carried out western blot analysis on sperm lysates. In three separate experiments, using different samples for each experiment, we were unable to detect staining with the monoclonal pan-SERCA antibody Y1F4 (which recognises all SERCA types) (Tunwell et al., 1991; Hughes et al., 2000). However, positive control experiments showed strong staining, at the appropriate molecular weight, in rat cerebellar microsomes (Fig. 7A). With the same sperm lysates probed with Y1F4, we always obtained a robust band with a monoclonal antibody for tubulin (data not shown). Using a polyclonal antibody directed against SPCA1, which recognises a surface site believed to lie in the nucleotide binding domain (Wootton et al., 2004), we not only saw a heavy staining in the positive control (rat cerebellum), but also obtained a clear band of the appropriate molecular weight, in lanes containing human sperm proteins (Fig. 7B). The experiment was carried out on three separate sperm lysates.

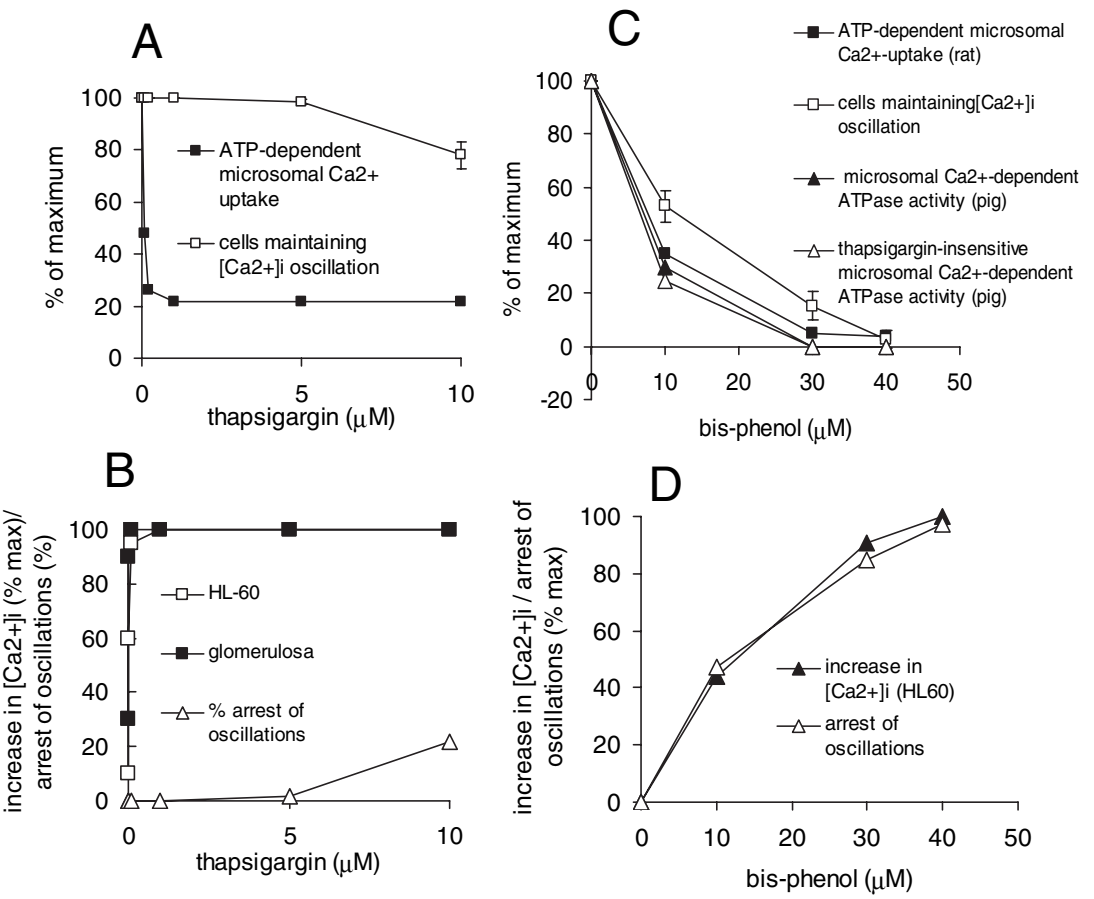

Fig. 6. Comparison of the potencies of thapsigargin and bis-phenol in their effects on $[Ca^{2+}]_i$ oscillations in human spermatozoa, Ca^{2+} store ATPase activity and mobilisation of stored Ca^{2+}. (A) Dose-dependence of the percentage inhibition by thapsigargin of ATP-dependent Ca^{2+} uptake by rat cerebellar microsomes (■) and arrest of progesterone-induced $[Ca^{2+}]_i$ oscillations in human spermatozoa (□). Results are the mean±s.e.m. percentage of cells continuing to oscillate after application of thapsigargin; three to six experiments were performed. (B) Dose-dependence (expressed as a percentage of the maximum) of thapsigargin-induced elevation of $[Ca^{2+}]_i$ in intact HL-60 cells (□) (Demaurex et al., 1992) and adrenal glomerulosa cells (■) (Ely et al., 1991) and dose-dependence of the percentage of oscillating cells in which thapsigargin caused arrest (△). (C) Dose-dependence of effects of inhibition by bis-phenol of ATP-dependent Ca^{2+} uptake by rat cerebellar microsomes (■), Ca^{2+} ATPase activity in pig cerebellar microsomes (whole, ▲; or in the presence of 1 μM thapsigargin, △) and arrest by bis-phenol of progesterone-induced $[Ca^{2+}]_i$ oscillations in human spermatozoa (□; mean±s.e.m. percentage of cells continuing to oscillate after application of bis-phenol). (D) Dose-dependence of the mobilisation of Ca^{2+} by bis-phenol in HL-60 cells (in EGTA-buffered Ca^{2+}-free saline; ▲) (Brown et al., 1994) and arrest of progesterone-induced $[Ca^{2+}]_i$ oscillations in human spermatozoa by bis-phenol (△).

Immunolocalisation studies, using the same antibodies, were carried out on preparations of human spermatozoa. In incubations with antibody YIF4 we were unable to detect localised binding anywhere on the sperm (Fig. 7C; compare to secondary antibody-only control, Fig. 7E). Using the antibody for SPCA1 we detected clear binding in all cells in a region at the anterior midpiece, often extending into the rear of the head (Fig. 7D). In control incubations without the primary antibody we detected only very low levels of fluorescence, similar to those seen with Y1F4 (Fig. 7F).

Manganese clearance from sperm cytoplasm

SPCAs differ from SERCAs in their much greater ability to transport Mn^{2+} into intracellular organelles (Wuytack et al., 2003). If human spermatozoa use SPCAs for at least part of their intracellular Ca^{2+} buffering, Mn^{2+} quench of Fura-2 fluorescence may be partly reversible upon accumulation of the ion into a store by the SPCA. We induced Mn^{2+} uptake (and consequent quenching of Fura-2) by treatment of cells with progesterone in Mn^{2+}-containing medium and then terminated Mn^{2+} influx with excess La^{3+}, to see whether there was observable recovery from quench owing to intracellular uptake of Mn^{2+}.

Stimulation with progesterone in the presence of 1 mM Mn^{2+} caused a transient acceleration of quench consistent with the known initial transient phase of progesterone-induced Ca^{2+}-influx. When 1 mM $LaCl_3$ was then added there was a small, rapid increase in fluorescence (Fig. 8, inset), followed by a second slower rise that occurred over a period of approximately 20 seconds (Fig. 8). Subsequent addition of bis-phenol caused reversal of quench recovery over a period of ~25 seconds. The amplitude of the effect of bis-phenol was consistent with abolition of the slow component (but not the fast component) of the fluorescence increase (Fig. 8). Similar effects on Mn^{2+} quench were seen in three experiments.

Discussion

In agreement with previous studies, we found that 10 μM thapsigargin induced a large sustained $[Ca^{2+}]_i$ response in human spermatozoa both in populations as reported previously (Blackmore, 1993; Bonaccorsi et al., 1995; Williams and Ford, 2003) and also in 35% of imaged cells. A small proportion of these cells also generated $[Ca^{2+}]_i$ transients upon treatment. At 1 μM thapsigargin, a dose well in excess of that required for SERCA inhibition, a small elevation of $[Ca^{2+}]_i$ was caused in

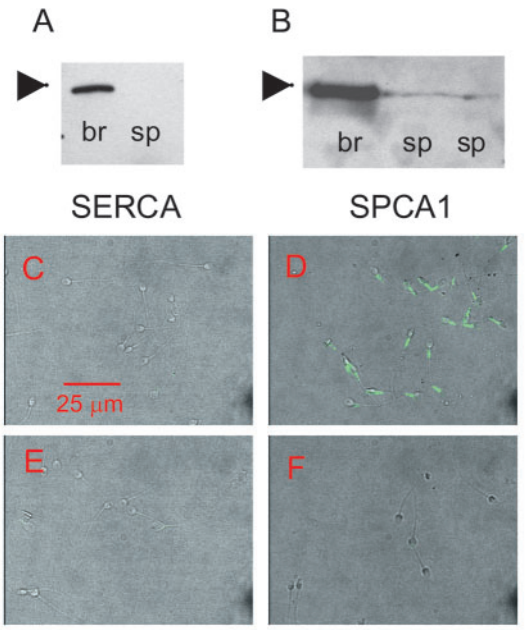

Fig. 7. Expression and localisation of Ca^{2+} store ATPases in human spermatozoa. Western blotting was used to detect SERCAs (A) and SPCA1 (B) in rat brain (br) and sperm (sp) lysates. A robust signal, at the appropriate molecular weight, was obtained with the SERCA antibody (Y1F4) in brain lysates but no staining was detected with sperm (three experiments). In contrast, using the same lysates, we detected SPCA1 both in rat brain and in sperm. Though the intensity of the band was considerably lighter in sperm than in brain, the band was found consistently. The blot has two sperm protein lanes to establish that the band was not due to 'bleed' from the brain lysate lane. (C-F) In-situ localisation of SERCA and SPCA1 in human spermatozoa. Pictures show overlays of fluorescence and phase-contrast images. All fluorescent images were obtained and processed using identical procedures. Immunolocalisation using antibody Y1F4 showed no significant staining (C) whereas the antibody directed against SPCA1 (D) localised clearly to the rear head and midpiece. Incubation with the secondary antibody alone gave no significant labelling in either case (E and F). Scale bar in C applies to C-F.

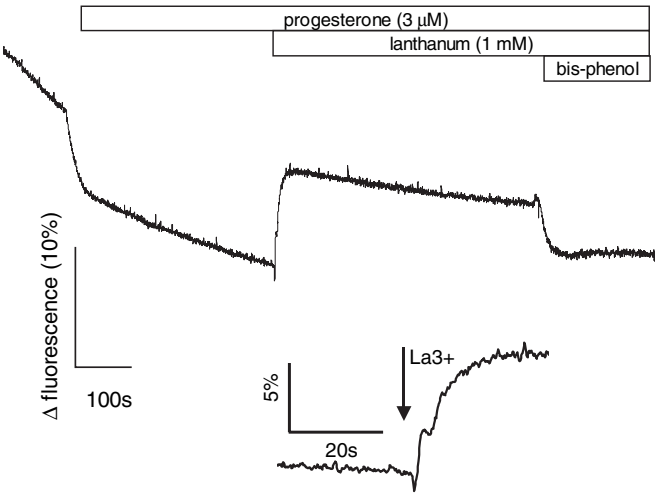

Fig. 8. Intracellular manganese clearance in human spermatozoa. One of three repeat experiments in which Fura-2-loaded human spermatozoa were suspended in sEBBSS containing 1 mM $MnCl_2$ and stimulated with progesterone. Measurement of fluorescence at 360 nm (isobestic point for Fura-2) shows rapid quenching of fluorescence due to the initial rapid progesterone-induced Mn^{2+} influx followed by a slower quench due to the subsequent slower influx. Subsequent addition of 1 mM La^{3+} (to block Mn^{2+} influx) not only prevented further quench but resulted in partial recovery of fluorescence. The initial fast phase (see inset showing detail of response upon La^{3+} application) is probably, at least in part, artefactual (see text). A subsequent slower phase appears to reflect removal of Mn^{2+} from the cytoplasm, consistent with activity of a Mn^{2+} transporting pump (such as SPCA1) in spermatozoa. Subsequent application of 40 μM bis-phenol caused a rapid, partial reversal of the fluorescence recovery with an amplitude corresponding to the slow phase.

some experiments and discernibly raised $[Ca^{2+}]_i$ was seen in only a small minority of imaged cells. CPA did not raise $[Ca^{2+}]_i$ in population or single cell experiments. In contrast, bis-phenol, which inhibits both SERCA and non-SERCA store Ca^{2+} ATPases (Brown et al., 1994) caused a dose-dependent response in population measurements of $[Ca^{2+}]_i$ comprising a 1-2 minute transient followed by a sustained elevation of $[Ca^{2+}]_i$. Consistent with this, a mixture of sustained and transient responses were seen in imaged cells treated with bis-phenol with 70-85% of cells responding. Reduction of $[Ca^{2+}]_o$ to <5 μM did not inhibit this effect. The dose-dependence of this action of bis-phenol is closely consistent with its ability to inhibit microsomal Ca^{2+} ATPase activity and mobilise stored Ca^{2+} (Brown et al., 1994). The more potent effects of this compound that have been reported previously (Sokolove et al., 1986) are seen only with highly purified, de-lipidated Ca^{2+}ATPase (Brown et al., 1994).

It is known that micromolar concentrations of thapsigargin can act non-specifically, for example in the blockade of L-type and vanilloid Ca^{2+} channels (Nelson et al., 1994; Toth et al., 2002). Nevertheless, the data reported here, particularly the occurrence of transient $[Ca^{2+}]_i$ responses in cells exposed to thapsigargin, are consistent with existence of a thapsigargin-sensitive Ca^{2+} store in human spermatozoa (Rossato et al., 2001; Williams and Ford, 2003). Furthermore, previous findings that sites on intracellular membranes are labelled by BODIPY-thapsigargin (Rossato et al., 2001) suggest that thapsigargin does 'recognise' a Ca^{2+} ATPase in human sperm. However, the high doses that are required to observe any effects and the complete failure of CPA to mobilise Ca^{2+} cannot be reconciled with the characteristics of somatic cell SERCAs. On the basis of these data we find it impossible to conclude that SERCAs of the type characterised in somatic cells contribute significantly to buffering of resting $[Ca^{2+}]_i$ in human spermatozoa, and suggest that at high, non-SERCA-specific doses, thapsigargin mobilises stored Ca^{2+} in sperm by acting at another target, which is also sensitive to bis-phenol (see below). A thapsigargin-insensitive, ionomycin-releasable component of intracellular Ca^{2+}-storage has been identified in rat spermatids (Berrios et al., 1998) and recently a discrete, thapsigargin-insensitive component of Ca^{2+} storage, which is liberated only at micromolar doses of the drug, has been described in rat brain (Watson et al., 2003).

When bis-phenol or high-dose thapsigargin were applied after progesterone (during the sustained phase of progesterone-induced, Ca^{2+} influx) the pattern of response (proportion of

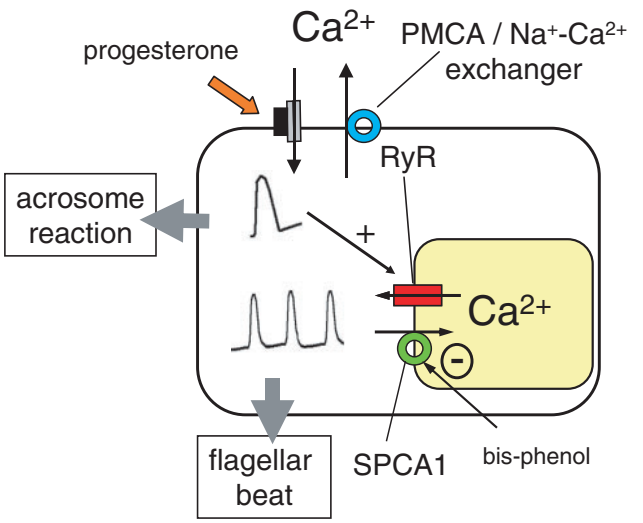

Fig. 9. Tentative model for intracellular Ca^{2+} storage and generation of $[Ca^{2+}]_i$ signals and cellular responses in progesterone-stimulated human spermatozoa. The initial $[Ca^{2+}]_i$ transient that occurs immediately upon application of progesterone is mediated primarily by Ca^{2+}-influx and induces acrosome reaction in a proportion of cells (grey arrow). Ca^{2+} is removed from the cytoplasm primarily by pumping at the plasma membrane (PMCA and Na^+-Ca^{2+} exchanger, blue). Acrosome reaction may involve mobilisation of Ca^{2+} stored in the acrosome itself (De Blas et al., 2002; Herrick et al., 2005). Sustained elevation of $[Ca^{2+}]_i$, which follows the transient (or can be induced by a progesterone concentration gradient to simulate approach to the oocyte, a procedure that induces $[Ca^{2+}]_i$ oscillations without a preceding $[Ca^{2+}]_i$ transient) (Harper et al., 2004), acts on RyRs (red) on a Ca^{2+} store in the caudal part of the head or midpiece, causing Ca^{2+}-induced Ca^{2+} release. Cyclic release and re-uptake occur as a result of refilling of this store by bis-phenol-sensitive SPCA1 (green), generating slow $[Ca^{2+}]_i$ oscillations at the base of the flagellum and causing alternation of flagellar beat pattern (grey arrow).

cells and response amplitude) was essentially similar to that in non-pretreated cells. It thus appears most unlikely that emptying of the bis-phenol (and high-dose thapsigargin)-sensitive store in human sperm is responsible for the sustained phase of progesterone-induced Ca^{2+} influx. However, induction of $[Ca^{2+}]_i$ transients by thapsigargin or bis-phenol was greatly reduced in cells previously stimulated with progesterone, suggesting that stores may be already at least part-mobilised under these conditions.

Mobilisation of Ca^{2+} stores plays an important role in sperm function in various species (Butler et al., 1999; O'Toole et al., 2000; De Blas et al., 2002). However, the only clear example of a $[Ca^{2+}]_i$ signal in intact spermatozoa that is believed to be generated primarily by store mobilisation is the large $[Ca^{2+}]_i$ oscillations induced in human spermatozoa by progesterone (Harper et al., 2004; Kirkman-Brown et al., 2004). Strikingly, thapsigargin was no more potent in disrupting $[Ca^{2+}]_i$ oscillations than in modulating resting $[Ca^{2+}]_i$, being effective only at doses $\geqslant$5 μM. Very high doses of thapsigargin (30 μM) were able to block oscillations in the majority of cells, consistent with a non-specific action of this compound on Ca^{2+} homeostasis in human spermatozoa. In contrast, bis-phenol induced clear dose-dependent arrest of $[Ca^{2+}]_i$ oscillations over the concentration range at which it blocks microsomal Ca^{2+}-ATPase activity and mobilises stored Ca^{2+} in intact cells (Fig. 6C,D). At 10 μM, bis-phenol was significantly more effective than 10 μM thapsigargin in this respect ($P<0.01$; unpaired *t*-test on arcsine-transformed data), despite the much lower potency of this compound in inhibiting microsomal ATPase activity and Ca^{2+} uptake (complete inhibition at 30-40 μM) compared to thapsigargin (complete inhibition at 50-100 nM) (Brown et al., 1994). When oscillations persisted in the presence of 10 μM bis-phenol they were often prolonged and slightly larger. In some cells there was a marked 'plateau' at the start of the decay phase (Fig. 5D). However, as the response of the dye is non-linear (and in some cells the dye may be approaching saturation) this transition from slow to faster decay is probably exaggerated. We conclude that the observed actions of bis-phenol on oscillations reflect primarily inhibition of store Ca^{2+} ATPases and that this store does not depend upon SERCAs (such that their inhibition fails to prevent store filling), but does possess a non-SERCA pump which is sensitive to bis-phenol and very high doses of thapsigargin.

The SPCA-type Ca^{2+}-ATPases were first identified in the yeast *Saccharomyces cerevisiae* and localised to the Golgi (Rudolph et al., 1989; Antebi et al., 1992). The Ca^{2+}-uptake activity of *Caenorhabditis elegans* SPCA (PMR1) and its mammalian homologue are insensitive to thapsigargin (Van Baelen et al., 2001; Missiaen et al., 2002) and have similar affinities for Ca^{2+} and Mn^{2+} (Van Baelen et al., 2001). We detected expression of SPCA1 in human sperm using western blot analysis. Experiments designed to demonstrate activity of this pump, by exploiting its ability to transport Mn^{2+}, suggest that it contributes to $[Ca^{2+}]_i$ buffering in human spermatozoa. In contrast, we were unable to detect expression of SERCAs (using the pan-SERCA antibody Y1F4) in the same sperm extracts in which SPCA1 was detected, although positive controls for SERCA staining and for transfer of proteins both gave clear bands. Y1F4 is an extremely robust antibody, which is known to provide a sensitive and reliable probe for all SERCA isoforms (Hughes et al., 2000; Wootton et al., 2004). This strongly suggests that SPCA1 is the primary intracellular ATPase expressed in human spermatozoa or that if other Ca^{2+} store ATPases are present they are not SERCAs as characterised in somatic cells. Immunolocalisation confirmed both the failure of Y1F4 to recognise a SERCA in human spermatozoa and also the presence of SPCA1, this ATPase being localised to an area behind the nucleus and in the midpiece. This localisation coincides both with the area in which the main $[Ca^{2+}]_i$ elevation appears to occur during oscillations and with the location of BODIPY-ryanodine binding (Harper et al., 2004). Ca^{2+} oscillations in human spermatozoa appear not to involve IP3 signalling, but are sensitive to manipulation of ryanodine receptors (Harper et al., 2004). We believe that $[Ca^{2+}]_i$ oscillations in human spermatozoa are not generated by the IP3-sensitive acrosomal Ca^{2+} store, but by Ca^{2+}-induced mobilisation of Ca^{2+} from a store situated behind the nucleus (possibly the redundant nuclear envelope) (Westbrook et al., 2001; Ho and Suarez, 2003) which is refilled primarily by SPCA1. It has been proposed that Ca^{2+} mobilised from this store regulates flagellar beating (Ho and Suarez, 2001; Ho and Suarez, 2003). We showed previously that $[Ca^{2+}]_i$ oscillations in human

spermatozoa do not induce acrosome reaction but regulate flagellar beat (Harper et al., 2004). Thus at least part of the action of progesterone on human spermatozoa is apparently to activate ryanodine receptors on a Ca^{2+} store at the head-midpiece junction, causing cyclic mobilisation of Ca^{2+} and change in flagellar beating (Fig. 9).

Our findings are controversial and further investigation is clearly required. However, the balance of data at present supports three conclusions: (1) human spermatozoa possess at least one (and probably two) $[Ca^{2+}]_i$ store(s) regulated by Ca^{2+} ATPases which contributes to $[Ca^{2+}]_i$ homeostasis and $[Ca^{2+}]_i$ signalling; (2) human (and probably other) spermatozoa express non-SERCA (SPCA) store Ca^{2+}-ATPases and these ATPases are important in regulating $[Ca^{2+}]_i$ and contributing to the generation of complex $[Ca^{2+}]_i$ signalling; (3) the idea that SERCAs of the type characterised in somatic cells contribute significantly to $[Ca^{2+}]_i$ buffering in mammalian sperm should be considered, at best, to be open to debate.

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