Molecular Human Reproduction vol.5 no.6 pp. 498–506, 1999

The neoglycoprotein mannose–bovine serum albumin, but not progesterone, activates T-type calcium channels in human spermatozoa*

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The neoglycoproteins α-D-mannose–bovine serum albumin (mannose–BSA) and *N*-acetyl-α-D-glucosamine–BSA (glucNAc–BSA) were shown to rapidly increase intracellular free calcium ($[Ca^{2+}]_i$) in human spermatozoa. The increase in $[Ca^{2+}]_i$ induced by these neoglycoproteins accounts for the known ability of these compounds to induce the acrosome reaction in human spermatozoa. Our data support the hypothesis that mannose–BSA, but not progesterone, activates T-type Ca^{2+} channels in human spermatozoa for the following reasons: (i) the capacity of mannose–BSA to increase $[Ca^{2+}]_i$ was inhibited by the specific T-type Ca^{2+} channel blocker mibefradil (IC_{50} = 10^{-6} mol/l) while progesterone was not inhibited by 10^{-5} M mibefradil; (ii) the effect of mannose–BSA to elevate $[Ca^{2+}]_i$ was inhibited more potently by Ni^{2+} (IC_{50} = 0.1 mmol/l) than Cd^{2+} (IC_{50} = 0.5 mmol/l), whereas the effect of progesterone to elevate $[Ca^{2+}]_i$ was inhibited equally by Ni^{2+} and Cd^{2+} (IC_{50} = 0.25 mmol/l); (iii) the effects of mannose–BSA and progesterone to increase $[Ca^{2+}]_i$ were greater than additive. These data support the idea that mannose–BSA and progesterone were activating distinct Ca^{2+} channels, one of which was a T-type Ca^{2+} channel activated by mannose–BSA whereas the Ca^{2+} channel that was activated by progesterone has yet to be defined at the molecular level.

Key words: calcium/mannose–BSA/progesterone/spermatozoa/T-type

Introduction

The nature of the Ca^{2+} channel(s) through which progesterone promotes Ca^{2+} influx into human spermatozoa remains undefined. The first studies showed that Ca^{2+} influx was blocked by the ions La^{3+} and Ni^{2+} and was relatively insensitive to L-type Ca^{2+} channel antagonists such as verapamil and diltiazem (Blackmore *et al*., 1990, 1991). These studies were subsequently confirmed by others, although one study showed that a supra-pharmacological 100 μmol/l concentration of verapamil was able to inhibit the action of progesterone (McLaughlin and Ford, 1994). The increase in $[Ca^{2+}]_i$ induced by progesterone occurred within seconds and reached a maximum effect after ~20 s, then declined towards basal levels during the next 1–2 min (Blackmore *et al*., 1990). The entire increase in $[Ca^{2+}]_i$, induced by progesterone, was abolished when the extracellular Ca^{2+} was removed by the addition of the Ca^{2+} chelator EGTA to the extracellular medium. There was evidence that internal Ca^{2+} stores were present in spermatozoa, since thapsigargin promoted Ca^{2+} influx into human spermatozoa, presumably by depleting intracellular Ca^{2+} stores, thus activating store operated Ca^{2+} channels in the plasma membrane (Blackmore, 1993a). The intracellular Ca^{2+} stores have been proposed to be either perinuclear (Blackmore, 1993a) or acrosomal in nature (Walensky and Snyder, 1995).

To study zona pellucida 3 (ZP3) induction of the acrosome reaction in human spermatozoa. it is necessary to have either: (i) solubilized human zona pellucida or (ii) recombinant ZP3 with the appropriate glycosylation. Both of these preparations are not currently available to most investigators. There are several studies that imply that several bovine serum albumin (BSA) neoglycoproteins are able to stimulate the acrosome reaction and that *N*-acetyl-α-D-glucosamine (glucNAc) and α-D-mannose may interact with the putative receptor for ZP3 in human spermatozoa (Brandelli *et al*., 1994, 1995, 1996; Brandelli, 1997). The following carbohydrates: *N*-acetylglucosamine, mannose, fucose and galactose have been shown to be involved in human sperm–zona pellucida binding (Miranda *et al*., 1997). In the mouse it has been shown that there are high-mannose/hybrid oligosaccharide chains in the ZP2 and ZP3 and that sperm surface α-D-mannosidase plays an important role during fertilization (Cornwall *et al*., 1991; Tulsiani *et al*., 1992). Mannose binding sites on human spermatozoa have been identified on the sperm head in the equatorial, pre- and post-equatorial areas (Chen *et al*., 1995; Youssef *et al*., 1996, 1997). It has also been shown that certain cases of male infertility may be related to altered mannose binding capacity and that the expression of mannose binding sites depends on capacitation (Benoff *et al*., 1993a,b, 1995, 1997a,b; Tesarik *et al*., 1991).

The induction of the acrosome reaction by glucNAc– or mannose–neoglycoproteins (glucNAc–BSA and mannose–BSA) has been shown to involve voltage dependent Ca^{2+} channels and a G_i-like guanine regulatory protein. The stimulation of the acrosome reaction induced by zona pellucida in

*Some of the material in this manuscript was presented during a talk given by Peter Blackmore at The First International Meeting on Rapid Responses of Steroid Hormones, held in Mannheim, Germany, September 18–20, 1998.

several other mammalian species also involves a G_i-like guanine regulatory protein (e.g. Ward *et al*., 1992). The effects of mannose–BSA and glucNAc–BSA to stimulate the acrosome reaction were blocked by pertussis toxin and could be mimicked by guanine nucleotides such as GppNHp, GTP-γ-S, mastoparan and AlF_4- (non-selective activator of G-proteins; e.g. Blackmore *et al*., 1985; Brandelli, 1997). Other studies (Brandelli *et al*., 1996) suggested that L-type Ca^{2+} channels were involved in the glucNAc–BSA and the mannose–BSA induction of the acrosome reaction, since nitrendipine and verapamil blocked the action of both neoglycoproteins.

Other studies also support the existence of L-type Ca^{2+} channels in human spermatozoa (Benoff *et al*., 1994; Hershlag *et al*., 1995; Goodwin *et al*., 1997). It was proposed that the L-type Ca^{2+} channel blocker nifedipine, may act as a contraceptive by inhibiting sperm L-type Ca^{2+} channels (Hershlag *et al*., 1995). However, this would be a rather ineffective form of contraception, since men taking L-type Ca^{2+} channel antagonists are generally not infertile. When rats were given, prior to mating, nifedipine at a concentration 30-fold higher that the maximum recommended therapeutic dose there was a small reduction in fertility (Physicians' Desk Reference, 1998). Studies performed in rats using another specific L-type Ca^{2+} channel blocker, diltiazem, produced 'no intrinsic effect on fertility' (Physicians' Desk Reference, 1998). It would thus appear unlikely that L-type Ca^{2+} channel blockers would act as an effective contraceptive, especially in the light of recent data supporting the existence of T-type Ca^{2+} channels in spermatozoa and not L-type Ca^{2+} channels (see below).

There is now some evidence for the existence of T-type calcium channels in mouse spermatozoa and spermatocytes (Arnoult *et al*., 1996, 1997, 1998; Lievano *et al*., 1996; Santi *et al*., 1996, 1998). There is also preliminary evidence for T-type Ca^{2+} channels in human spermatozoa (Shiomi *et al*., 1996), and that progesterone may stimulate this channel. There are two classes of voltage-gated calcium channels based on their voltage characteristics. The high voltage group of calcium channels (L, N, P, Q and R-type) requires depolarization to approximately –20 mV with maximal activation occurring at >10 mV. The T-type of calcium channel is a low voltage type with a voltage threshold of ~–60mV, with maximal conduction occurring at –30 to –20 mV (Florman *et al*., 1998). This channel is more potently inhibited by Ni^{2+} than Cd^{2+}, is weakly inhibited by dihydropyridine Ca^{2+} channel antagonists, is not activated by dihydropyridine agonists, the various ω-conotoxins are without effect, and it is potently blocked by mibefradil (Ertel and Ertel, 1997). Pimozide and amiloride also inhibit T-type Ca^{2+} channels, although these compounds have several other actions (Arnoult *et al*., 1996).

In the present study we have investigated the possible involvement of T-type Ca^{2+} channels in the action of glucNAc–BSA and the mannose–BSA on human spermatozoa. We also decided to look more directly at the actions of glucNAc–BSA and the mannose–BSA by examining changes in $[Ca^{2+}]_i$ in spermatozoa rather than the acrosome reaction. We utilized the specific Ca^{2+} channel antagonist mibefradil and compared the ability of both Ni^{2+} and Cd^{2+} to inhibit the actions of mannose–BSA. At the same time we examined the action of progesterone to see if this agent activated T-type Ca^{2+} channels.

Materials and methods

Isolation of human spermatozoa

Human semen from healthy donors was collected by masturbation after 3 days of sexual abstinence, and allowed to liquefy at room temperature for ~30 min. Semen analysis was performed as described previously (Nassar *et al*., 1998), and indicated that the semen samples were normal. After liquefaction the semen was washed twice (500 *g* for 5 min each) with Biggers–Whitten–Wittingham (BWW) buffer (Biggers *et al*., 1971) containing 0.1% (w/v) BSA. The sperm pellet was re-suspended in 0.2 ml of BWW, mixed, and divided into two 15 ml centrifuge tubes. The sperm suspension was then overlaid with 0.5 ml of BWW buffer and incubated in 5% (v/v) CO_2 in air at 37°C for 60 min at an angle of 30°. The swim-up spermatozoa were collected and pooled and the volume was adjusted to give a final concentration of spermatozoa of ~1×10^7 spermatozoa/ml.

Measurement of $[Ca^{2+}]_i$ in human spermatozoa using fura-2

The swim-up spermatozoa were loaded with fura-2, as previously described (Blackmore *et al*., 1990), by incubating the spermatozoa with 4 μmol/l fura-2/AM for 1 h at 37°C. After centrifugation at 1500 *g* for 5 min, the spermatozoa were re-suspended in a HEPES-buffered salt solution containing 0.3% (w/v) bovine serum albumin (Thomas and Meizel, 1989; Blackmore *et al*., 1990) and kept in the dark to prevent photobleaching of the fura-2. Aliquots of sperm suspension (0.5 ml) were added to a 6×50 mm glass cuvette (Chrono-Log, Havertown, PA, USA) containing a magnetic stirring bar in a SPEX ARCM spectrofluorometer (SPEX Industries, Inc., Edison, NJ, USA). Aliquots (2.5 μl) of various agents were added to the sperm suspension in the cuvette in the fluorometer at the indicated times (see Figure legends for specific details). The spermatozoa were excited at 340 and 380 nm respectively and emission was measured at 505 nm. Data were collected for various times, depending on the experiment, with an integration time of 0.1 s and a time increment of 0.5 s (i.e. two data points per second were collected). To obtain the Ca^{2+} saturated fluorescence values of fura-2 at each wavelength, 0.01% (w/v) digitonin was added to the fura-2-loaded sperm suspension in the presence of 1.0 mmol/l Ca^{2+}. To obtain the Ca^{2+}-depleted fura-2 fluorescence values at each wavelength, 10 mmol/l EGTA was added to the digitonin-treated sperm suspension. Fluorescence values at each wavelength were corrected for autofluorescence of the cells by adding 2 mmol/l $MnCl_2$ in the presence of 20 μmol/l ionomycin to another aliquot of fura-2-loaded spermatozoa. The Mn^{2+} quenches the fura-2 fluorescence. The autofluorescence values were subtracted from the fura-2-loaded sperm cell fluorescence values obtained at both wavelengths. The level of $[Ca^{2+}]_i$ was calculated with the dm3000 SPEX software using a K_D of 224 nmol/l for the fura-2–Ca^{2+} complex.

Sources of materials

Mibefradil was a gift from Roche Pharmaceuticals, Switzerland. Progesterone, albumin, bovine *p*-aminophenyl-α-D-mannopyranoside (mannose–BSA), albumin, bovine *p*-aminophenyl-*N*-acetyl-β-D-glucosaminide (glucNAc–BSA), BSA, sodium pyruvate, sodium lactate, EGTA were from Sigma Chemical Co., St Louis, MO, USA. The mannose–BSA contained 26 moles of α-D-mannose per mole of albumin and the glucNAc–BSA contained 16 moles of glucNAc/mol of albumin. Fura-2/AM was from Molecular Probes, Inc., Eugene, OR, USA.

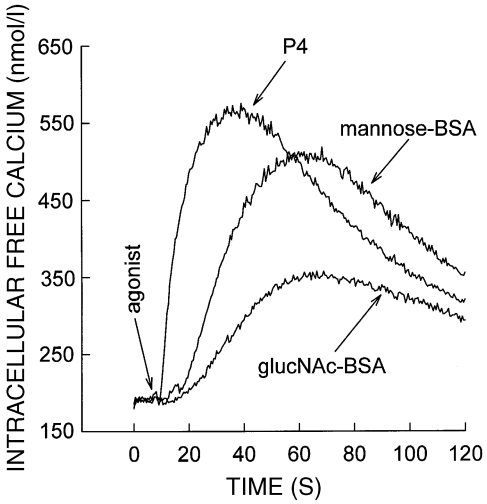

Figure 1. Effect of 1 μmol/l progesterone (P4), mannose–bovine serum albumin (BSA) (20 μmol/l based on mannose) and glucNAc–BSA (20 μmol/l based on *N*-acetyl-glucosamine) on $[Ca^{2+}]_i$. At 10 s P4, mannose–BSA or glucNAc–BSA were added to fura-2-loaded spermatozoa (200-fold dilution of stock solutions). Each agent produced a rapid increase in $[Ca^{2+}]_i$ with P4 producing the fastest rise in $[Ca^{2+}]_i$. The effects of mannose–BSA and glucNAc–BSA at these maximally effective concentrations were not as effective as P4 at increasing $[Ca^{2+}]_i$. Typical representative traces from one experiment are shown.

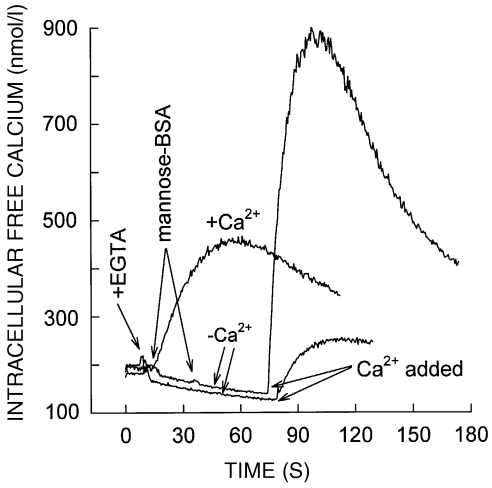

Figure 2. Effect of removing extracellular Ca^{2+} on the ability of mannose–bovine serum albumin (BSA) to increase $[Ca^{2+}]_i$. Mannose–BSA was added to spermatozoa in the presence of extracellular Ca^{2+} at 10 s or to spermatozoa that had been treated with the Ca^{2+} chelator EGTA at 40 s. Ca^{2+} was added back at ~70 s to the EGTA–mannose–BSA-treated spermatozoa, a rapid increase in $[Ca^{2+}]_i$ was seen. Ca^{2+} was also added back to control EGTA-treated spermatozoa at 80 s. Representative tracings of three experiments are shown.

Results

Effect of progesterone and mannose–BSA on $[Ca^{2+}]_i$ in human spermatozoa

The data in Figure 1 show the effects of adding progesterone, mannose–BSA or glucNAc–BSA to fura-2-loaded human spermatozoa. The amount of progesterone added was a maximally effective concentration of 1 μmol/l (Blackmore *et al*., 1990). The amount of mannose–BSA was equivalent to 20 μmol/l α-D-mannose; this was a maximally effective concentration at increasing $[Ca^{2+}]_i$ in spermatozoa, based on dose–response experiments (data not shown). The amount of glucNAc–BSA was also equivalent to 20 μmol/l glucNAc and was a maximally effective concentration at elevating $[Ca^{2+}]_i$ (data not shown). A concentration of 2 μmol/l mannose–BSA produced an effect on $[Ca^{2+}]_i$ ~20% of that observed with 20 μmol/l mannose–BSA, whereas 0.2 μmol/l mannose–BSA did not produce any detectable effect on $[Ca^{2+}]_i$, while increasing mannose–BSA up to 40 μmol/l did not elicit any greater effect than that observed with 20 μmol/l mannose–BSA (data not shown).

Progesterone, glucNAc–BSA and mannose–BSA produced rapid increases in $[Ca^{2+}]_i$ which occurred without apparent delay, although the rate of $[Ca^{2+}]_i$ rise observed with mannose–BSA was slightly slower than that seen with progesterone, and glucNAc–BSA was even slower than the mannose–BSA effect (Figure 1). The slower rise in $[Ca^{2+}]_i$ induced by glucNAc–BSA and mannose–BSA when compared to free progesterone may be related to the steric hindrance of the BSA in these preparations. The increase in $[Ca^{2+}]_i$ induced by progesterone is also slower when it is conjugated to BSA (Blackmore *et al*., 1991). Progesterone produced a peak Ca^{2+} response after ~20 s (Figure 1), whereas mannose–BSA and glucNAc–BSA took ~50 s to reach a maximum level (Figure 1). The $[Ca^{2+}]_i$ level then declined towards baseline values with all three stimuli. Since mannose–BSA consistently gave a larger response than glucNAc–BSA we utilized mannose–BSA in the rest of this study, although in a limited number of experiments using glucNAc–BSA, similar data to that seen with mannose–BSA was obtained.

Previous studies have shown that progesterone elevated $[Ca^{2+}]_i$ in spermatozoa by promoting calcium influx exclusively (e.g. Blackmore *et al*., 1990). There are intracellular Ca^{2+} stores in spermatozoa since thapsigargin has been shown to promote mobilization of internal Ca^{2+} together with inducing Ca^{2+} influx (e.g. Blackmore, 1993a). However, progesterone does not appear to be able to utilize any of this internal Ca^{2+} (Blackmore *et al*., 1990). The data in Figure 2 show that mannose–BSA likewise elevated $[Ca^{2+}]_i$ exclusively by promoting Ca^{2+} influx, since the increase in $[Ca^{2+}]_i$ induced by mannose–BSA was totally abolished when extracellular Ca^{2+} was chelated with EGTA. When extracellular Ca^{2+} (1.0 mmol/l) was present in the medium, mannose–BSA rapidly elevated $[Ca^{2+}]_i$ similar to that seen in Figure 1. Ten seconds after 5 mmol/l EGTA was added the basal $[Ca^{2+}]_i$ concentration began slowly to decline (Figure 2), consistent with the EGTA sequestering Ca^{2+} from inside the cell as it is extruded out across the plasma membrane by the Ca^{2+}-ATPase pump. When mannose–BSA was added at 40 s the $[Ca^{2+}]_i$ did not increase, but still continued to decline (Figure 2). At 75 s 5 mmol/l calcium was added back to the mannose–BSA challenged spermatozoa in EGTA containing medium. The $[Ca^{2+}]_i$ rose more rapidly to a value approximately double that observed when mannose–BSA was added to spermatozoa in 1.0 mmol/l Ca^{2+}-containing medium (Figure 2). It appears that the mannose–BSA had activated the Ca^{2+} channel in the absence of extracellular Ca^{2+} and that when Ca^{2+} was added back it entered the cell more efficiently, possibly due to a lack of

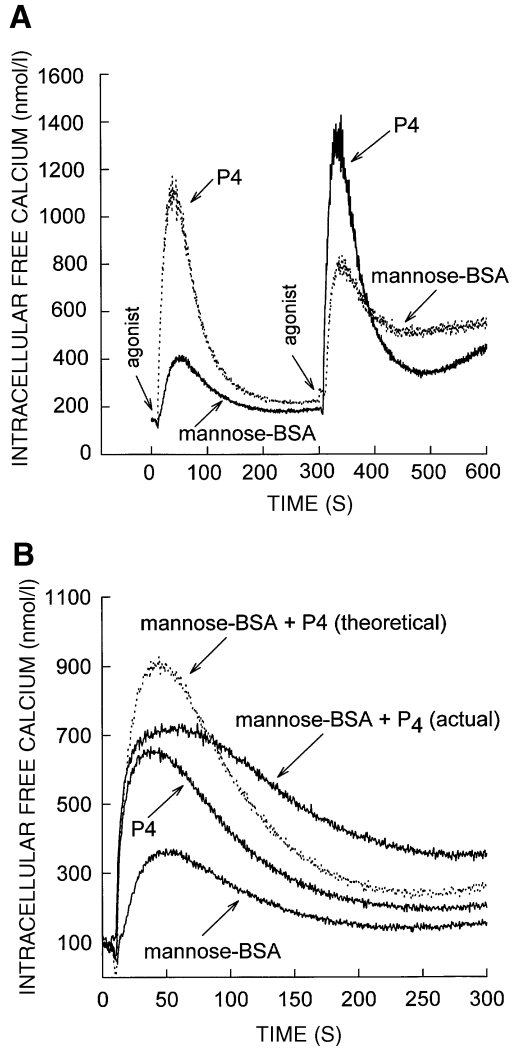

Figure 3. (**A**) The effects of sequential additions of progesterone (P4) and mannose–bovine serum albumin (BSA) to increase $[Ca^{2+}]_i$ in human spermatozoa. P4 (1 μmol/l) or mannose–BSA (20 μmol/l, based on mannose content) were added to separate aliquots of fura-2-loaded human spermatozoa at 10 s. At 300 s to the P4-stimulated spermatozoa was added mannose–BSA and to mannose–BSA-stimulated spermatozoa was added P4. Each of these effects seen at 300 s was slightly larger than each effect that was observed at 10 s. Representative tracings of three experiments are shown. (**B**) Effect of adding P4, mannose–BSA or P4 plus mannose–BSA simultaneously on $[Ca^{2+}]_i$ in human spermatozoa. P4 (1 μmol/l), mannose–BSA (20 μmol/l, based on mannose content) or P4 plus mannose–BSA were added to fura-2-loaded human spermatozoa at 10 s. Together with the actual experimental data is shown the theoretical trace of the individual effects of P4 and mannose–BSA added together. Representative tracings of three experiments are shown.

negative feedback of elevated $[Ca^{2+}]_i$ on the channel. In the control spermatozoa when Ca^{2+} was added back to EGTA-treated spermatozoa (without mannose–BSA treatment), the $[Ca^{2+}]_i$ slowly increased to a value slightly higher than the pre-EGTA treatment value (Figure 2).

To evaluate if progesterone and mannose–BSA were acting on the same Ca^{2+} influx process, experiments were performed in which progesterone and mannose–BSA were added sequentially (Figure 3A) or simultaneously (Figure 3B). Progesterone and mannose–BSA both produced rapid and transient effects on $[Ca^{2+}]_i$ (Figure 3A), similar to that observed in Figure 1. At 300 s progesterone was added to the mannose–BSA-stimulated spermatozoa and mannose–BSA was added to the progesterone-stimulated spermatozoa. The effect of progesterone to increase $[Ca^{2+}]_i$, after a pre-stimulation with mannose–BSA, was slightly more rapid and the peak response was higher by ~200 nmol/l (Figure 3A). The effect of mannose–BSA to increase $[Ca^{2+}]_i$ after a progesterone pre-stimulation was also potentiated with the rate of rise in $[Ca^{2+}]_i$ being faster and the peak response was higher by ~400 nmol/l (Figure 3A). The effect of the second stimulus was not as transient as the first stimulus. If, instead of adding mannose–BSA to progesterone-treated spermatozoa, an equivalent amount of progesterone was added a second time, no further increase in $[Ca^{2+}]_i$ was observed (data not shown). This result indicates that a saturating concentration of progesterone was being used and that adding more progesterone could not elicit any further response. The same was also true for mannose–BSA added a second time after an initial challenge with mannose–BSA, i.e. there was no further increase in $[Ca^{2+}]_i$ (data not shown).

Another approach to confirm that progesterone and mannose–BSA are activating distinct calcium channels in the sperm plasma membrane was to add both stimuli, at maximally effective concentrations at the same time. If each stimulus was acting on a distinct and separate Ca^{2+} channel then the effects should be additive. The data in Figure 3B show the results from such an experiment. Progesterone and mannose–BSA by themselves produced a rapid and transient rise in $[Ca^{2+}]_i$ similar to that seen in Figures 1 and 3A. When both agonists were added together the effect was additive, if one considers the area under the curves. The peak area was 102% of the theoretical value if both individual traces were added together. The peak area plus the background area was 115% of the theoretical value if both individual traces were added together (data generated with SPEX dm3000 software). The kinetics of the actual $[Ca^{2+}]_i$ increase was different, however, with the peak response at ~40 s being less than the theoretical value, but the sustained response between 100 and 300 s being greater than the theoretical value.

Effect of Ni^{2+} and Cd^{2+} on the ability of progesterone and mannose–BSA to increase $[Ca^{2+}]_i$ in human spermatozoa

T-type calcium channels can be distinguished from other voltage-gated Ca^{2+} channels since they are more sensitive to inhibition by Ni^{2+} than Cd^{2+} (e.g. Ertel and Ertel, 1997). The data presented in Figure 3A and B support the notion that there are at least two types of Ca^{2+} channel present in human spermatozoa and that they can be activated independently of each other. We therefore examined the ability of both Ni^{2+} and Cd^{2+} to block the actions of progesterone and mannose–BSA to increase $[Ca^{2+}]_i$ in human spermatozoa. The data in Figure 4 shows a typical experiment in which either 0.1 mmol/l Ni^{2+} or 0.1 mmol/l Cd^{2+} was added to spermatozoa that were stimulated with 1.0 μmol/l progesterone. Both ions, at this concentration, produced a similar degree of inhibition of the

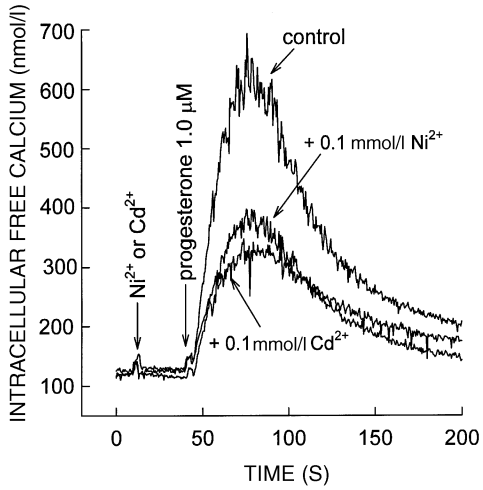

Figure 4. Effect of 0.1 mmol/l Ni^{2+} and 0.1 mmol/l Cd^{2+} on the ability of progesterone to increase $[Ca^{2+}]_i$ in human spermatozoa. At 10 s either 0.1 mmol/l Ni^{2+} or 0.1 mmol/l Cd^{2+} was added, 30 s later 0.1 µmol/l progesterone was added. These concentrations of Ni^{2+} and Cd^{2+} produced ~ the same 50% inhibition of the effect of progesterone to increase $[Ca^{2+}]_i$. A representative experiment is shown.

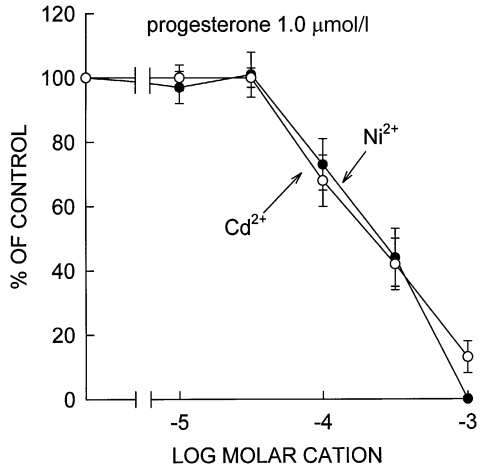

Figure 5. Dose–response of Ni^{2+} (closed circles) and Cd^{2+} (open circles) to inhibit the effect of 1 µmol/l progesterone to increase $[Ca^{2+}]_i$ in human spermatozoa. Various concentrations of Ni^{2+} and Cd^{2+} were added to the sperm suspension 30 s before progesterone was added (see Figure 4 for details). The inhibitory effect of each ion was measured by comparing the change in $[Ca^{2+}]_i$ which was the difference between $[Ca^{2+}]_i$ measured just prior to the addition of progesterone and the peak response observed ~30–40 s later. Each value is the mean ± SEM from six separate sperm preparations.

increase in $[Ca^{2+}]_i$. The data in Figure 5 show the complete dose–response of Ni^{2+} and Cd^{2+} to inhibit the ability of progesterone to increase $[Ca^{2+}]_i$. Both divalent cations showed identical dose–response relationships to inhibit progesterone. The IC_{50} was ~0.25 mmol/l for both ions, suggesting that progesterone does not activate T-type calcium channels in human spermatozoa.

The data in Figure 6 show a typical experiment in which either 0.3 mmol/l Ni^{2+} or 0.3 mmol/l Cd^{2+} was added to spermatozoa that were stimulated with mannose–BSA. At this 0.3 mmol/l concentration of cation, Ni^{2+} produced a large

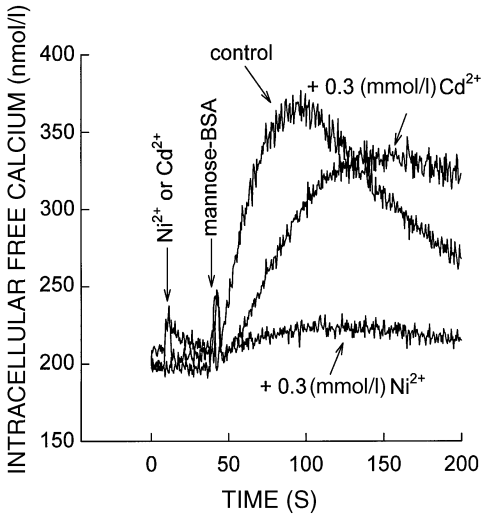

Figure 6. Effect of 0.3 mmol/l Ni^{2+} and 0.3 mmol/l Cd^{2+} on the ability of mannose–bovine serum albumin (BSA) to increase $[Ca^{2+}]_i$ in human spermatozoa. At 10 s either 0.3 mmol/l Ni^{2+} or 0.3 mmol/l Cd^{2+} was added at 10 s; 30 s later 20 µmol/l mannose–BSA was added. At these concentrations of metal ion, Ni^{2+} produced an almost complete inhibition of the mannose–BSA effect on $[Ca^{2+}]_i$, whereas Cd^{2+} produced a small inhibition of the maximum effect although the rate of $[Ca^{2+}]_i$ increase was reduced. A representative experiment is shown.

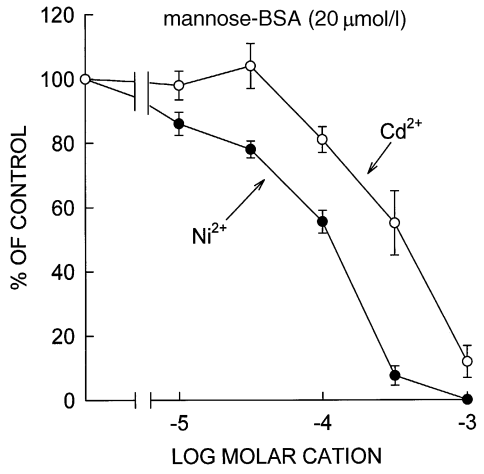

Figure 7. Dose–response of Ni^{2+} (closed circles) and Cd^{2+} (open circles) to inhibit the effect of 20 µmol/l mannose–bovine serum albumin (BSA) to increase $[Ca^{2+}]_i$ in human spermatozoa. Various concentrations of Ni^{2+} and Cd^{2+} were added to the sperm suspension 30 s before mannose–BSA was added (see Figure 6 for details). The inhibitory effect of each ion was measured by comparing the change in $[Ca^{2+}]_i$ which was the difference in $[Ca^{2+}]_i$ measured just prior to the addition of mannose–BSA to the peak response observed ~50–60 s later. Each value is the mean ± SEM from eight separate sperm preparations.

inhibition, whereas Cd^{2+} produced a small inhibition of the response (Figure 6). The complete dose–response of Ni^{2+} and Cd^{2+} to inhibit the ability of mannose–BSA to elevate $[Ca^{2+}]_i$ is shown in Figure 7. The effect of Ni^{2+} to inhibit mannose–BSA-induced elevation of $[Ca^{2+}]_i$ was ~ one-half a log unit more potent than Cd^{2+} at inhibiting the increase.

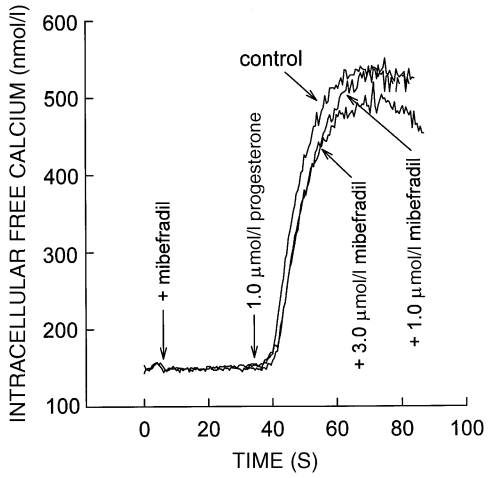

Figure 8. Effect of adding either 1.0 or 3.0 µmol/l mibefradil on the ability of progesterone to increase $[Ca^{2+}]_i$ in human spermatozoa. Mibefradil was added to the sperm preparation at 5 s. At 35 s 1 µmol/l progesterone was added; both concentrations of mibefradil had little effect on the ability of progesterone to increase $[Ca^{2+}]_i$. A representative experiment is shown.

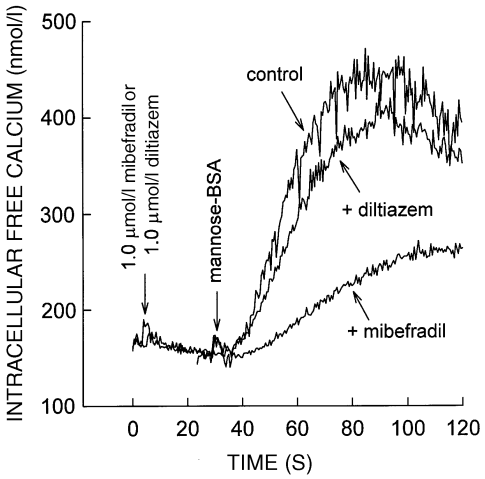

Figure 9. Effect of adding either 1.0 µmol/l diltiazem or 1.0 µmol/l mibefradil on the ability of mannose–bovine serum albumin (BSA) to increase $[Ca^{2+}]_i$ in human spermatozoa. Diltiazem produced a small inhibitory effect whereas mibefradil produced a much larger inhibition, of both the rate of increase and the magnitude, of the increase in $[Ca^{2+}]_i$. A representative experiment is shown.

Effect of the specific T-type calcium channel blocker mibefradil on the ability of progesterone and mannose–BSA to increase $[Ca^{2+}]_i$

Previous studies showed that L-type calcium channel blockers such as verapamil and diltiazem were very weak antagonists of the progesterone induced increase in $[Ca^{2+}]_i$ in spermatozoa (e.g. Blackmore *et al*., 1990). Since there was evidence for the existence of T-type calcium channels in spermatozoa from a variety of studies, we examined the effects of mibefradil on progesterone- and mannose–BSA-induced increases in $[Ca^{2+}]_i$. The data in Figure 8 show a typical experiment in which two concentrations of mibefradil were added to spermatozoa that were challenged with progesterone. Both concentrations of mibefradil tested in this experiment had no effect on the ability of progesterone to increase $[Ca^{2+}]_i$. In some experiments mibefradil slightly potentiated the effect of progesterone to increase $[Ca^{2+}]_i$. When spermatozoa were treated with 1.0 µmol/l mibefradil and then stimulated with mannose–BSA, there was an ~50% inhibition of the increase in $[Ca^{2+}]_i$ (Figure 9). In the same experiment 1.0 µmol/l diltiazem (L-type calcium channel blocker) produced a small inhibition of the mannose–BSA-induced rise in $[Ca^{2+}]_i$. This result is compatible with mannose–BSA stimulating T-type, not L-type, calcium channels in human spermatozoa.

The data in Figure 10 shows that mibefradil in the concentration range (0.1–10 µmol/l) did not inhibit the action of progesterone. The effect of mannose–BSA was, however, potently blocked by mibefradil with a 40% inhibition being seen with 0.1 µmol/l mibefradil and 90% inhibition being observed with 10 µmol/l mibefradil. These data are entirely consistent with those of Figures 5 and 7 showing that mannose–BSA was acting to activate T-type calcium channels and that progesterone stimulated Ca^{2+} influx via a non T-type calcium channel. The molecular nature of this progesterone activated channel has not yet been defined.

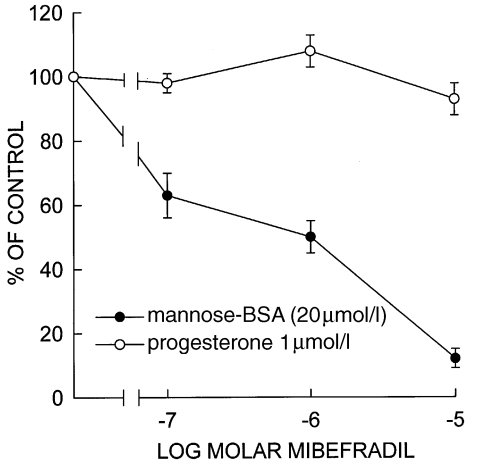

Figure 10. Dose–response of miferadil on the actions of progesterone (open circles) and mannose–bovine serum albumin (BSA) (closed circles) to increase $[Ca^{2+}]_i$ in human spermatozoa. The protocol used to generate these data was generated from experiments shown in Figures 8 and 9. Mibefradil over the concentration range of 10^{-7} to 10^{-5} mol/l did not have any effect on the ability of progesterone to increase $[Ca^{2+}]_i$ whereas the action of mannose–BSA to elevate $[Ca^{2+}]_i$ was dose-dependently inhibited. The data shown were the mean ± SEM from four separate experiments.

Activation of T-type calcium channels in human spermatozoa by depolarizing concentrations of KCl

A simple method used to activate voltage-dependent Ca^{2+} channels is to add a depolarizing concentration of K^+ to the tissue or cells in question. When 60 mmol/l KCl was added to fura-2-loaded spermatozoa there was a small slow rise in $[Ca^{2+}]_i$ compatible with the presence of potential or voltage-dependent Ca^{2+} channels (Figure 11). This increase was partially blocked by mibefradil (T-type Ca^{2+} channel blocker) but not by diltiazem (L-type Ca^{2+} channel blocker) (Figure 11).

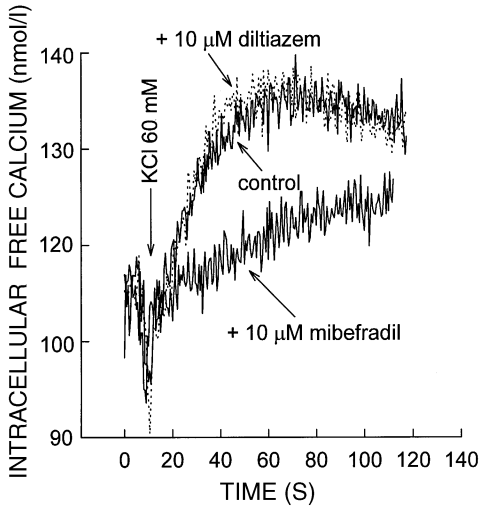

Figure 11. Effect of 10 μmol/l diltiazem and 10 μmol/l mibefradil on the ability of 60 mmol/l KCl to increase $[Ca^{2+}]_i$ in human spermatozoa. Approximately 10 s before data collection was initiated (zero time), either 10 μmol/l diltiazem or 10 μmol/l mibefradil was added to the sperm suspension. At 10 s 60 mmol/l KCl was added. This concentration of KCl produced an immediate but small (~20nmol/l) increase in $[Ca^{2+}]_i$. Diltiazem at this concentration (dotted line) had no effect on the KCl-induced increase in $[Ca^{2+}]_i$, whereas mibefradil produced a substantial inhibition of the increase in $[Ca^{2+}]_i$, both in rate of rise and magnitude of effect. This result is compatible with the presence of T-type Ca^{2+} channels in spermatozoa. A representative experiment of three is shown.

Discussion

This study demonstrates that human spermatozoa contain T-type Ca^{2+} channels and that agents that can mimic ZP3 induction of the acrosome reaction, such as the neoglycoprotein mannose–BSA, are capable of activating these Ca^{2+} channels. The ability of progesterone to increase $[Ca^{2+}]_i$ in human spermatozoa was not mediated by T-type channels and is at odds with the other studies (Shiomi *et al*., 1996), although these studies only utilized Ni^{2+} and amiloride to define the channel. Our conclusion was based upon: (i), the sensitivity to inhibition by Ni^{2+} and Cd^{2+} and (ii), to inhibition by the selective T-type Ca^{2+} channel blocker mibefradil. Based on these data there appear to be at least two calcium (cation) channels in human spermatozoa. This concept has been proposed by Florman (Florman *et al*., 1998). In their model, ZP3 in the zona pellucida interacts with a cell surface receptor on the spermatozoa to produce two separate intracellular signals. One pathway involves activating a cation channel via a pertussis toxin-insensitive and voltage-insensitive mechanism. This channel has properties similar to the one activated by progesterone, (Blackmore 1998), but it may not be the same Ca^{2+} channel. Evidence that this progesterone-activated Ca^{2+} channel is voltage-insensitive comes from experiments utilizing a variety of L-type Ca^{2+} channel blockers (e.g. verapamil, diltiazem and nifedipine (Blackmore *et al*., 1990) and T-type Ca^{2+} channel antagonists such as mibefradil, the antagonist used in the present study. The activation of this channel by progesterone was also not blocked by treatment of the spermatozoa with pertussis-toxin (e.g. Blackmore, 1993b; Tesarik *et al*., 1993). The channel also appears to be non-selective since progesterone also stimulated the influx of Na^+ (Foresta *et al*., 1993).

The nature of the receptor on spermatozoa that is activated by mannose–BSA has yet to be defined; however, it shares some similarities with the receptor that ZP3 activates. This receptor(s) on spermatozoa has yet to be conclusively characterized, although there are many candidates containing protein and carbohydrate ranging in molecular weight from 15 to 105 kDa (e.g. Benoff, 1997; McLeskey *et al*., 1998). Many researchers agree that there are several sperm ligands that form a complex which is involved in the binding to ZP3 (e.g. Kopf *et al*., 1995; Fisher *et al*., 1998).

The conductance of Ca^{2+} through the voltage-insensitive cation channel activated by ZP3 (Florman *et al*., 1998) would then produce a depolarization, since spermatozoa maintain an inwardly negative membrane potential. The result of this membrane depolarization would be to activate the low voltage-activated T-type Ca^{2+} channel. The second pathway that is activated by ZP3 involves an alkalinization of the intracellular pH. The ZP3 apparently activates a pertussis toxin-sensitive pH regulator, that most likely involves a guanine-nucleotide regulatory protein of the G_i class. The increases in $[Ca^{2+}]_i$ and pH are proposed to synergistically produce a prolonged elevation of $[Ca^{2+}]_i$. It was also proposed that Ca^{2+} was released from intracellular calcium stores. These Ca^{2+} stores could be either located in the acrosome or nucleus (Blackmore, 1993a). The sustained increase in $[Ca^{2+}]_i$ would then promote the acrosome reaction. The evidence for mobilization of intracellular Ca^{2+} by agonists in human spermatozoa is sparse. It appears that progesterone is able to cause an increase in inositol-1,4,5-P_3 following Ca^{2+} influx (Roldan and Harrison, 1989; Thomas and Meizel, 1989). This result would imply that the $[Ca^{2+}]_i$ activates a Ca^{2+}-sensitive form of phospholipase C that results in the hydrolysis of phosphatidyl 4,5-bisphosphate to yield diacylglycerol and inositol-1,4,5-P_3. Consistent with this result is the fact that the Ca^{2+} ionophore also stimulates the production of inositol-1,4,5-P_3 (Thomas and Meizel, 1989). The phospholipase C inhibitor U-73122 does not attenuate the progesterone-induced increase in $[Ca^{2+}]_i$, but instead potentiates the increase in $[Ca^{2+}]_i$ (data not shown). These results imply that the increase in $[Ca^{2+}]_i$ induced by progesterone and mannose–BSA (Figure 2), does not involve the generation of inositol-1,4,5-P_3 and subsequent mobilization of intracellular Ca^{2+}. It would thus seem that the contribution of intracellular mobilization of Ca^{2+} contributes only a minor role to the overall increase in $[Ca^{2+}]_i$ induced by progesterone and mannose–BSA.

Some of the data presented in this study are consistent with some previously published ideas (Florman *et al*., 1998). The data in Figure 3 show that the action of mannose–BSA to increase $[Ca^{2+}]_i$ was potentiated by prior activation of the pertussis toxin-insensitive Ca^{2+} channels that are activated by progesterone. Perhaps the Ca^{2+} influx induced by progesterone caused a depolarization that permitted mannose–BSA to activate more effectively the T-type Ca^{2+} channels, resulting in a more rapid and a more prolonged rise in $[Ca^{2+}]_i$. Conversely stimulation of T-type Ca^{2+} channels by mannose–BSA potenti-

ates the ability of progesterone to increase $[Ca^{2+}]_i$ through pertussis toxin-insensitive Ca^{2+} channels. The mechanism by which this occurs is not known, although it may involve alterations in membrane potential or intracellular pH (Florman, 1998). In conclusion it appears that the neoglycoprotein mannose–BSA is an activator of T-type Ca^{2+} channels in human spermatozoa, and is a convenient ligand to probe the mechanism of ZP3-induced elevations of $[Ca^{2+}]_i$ in human spermatozoa.

Acknowledgements

The technical assistance of Ms Patricia G.Loose is greatly appreciated. Special thanks to Paul Ratz, PhD and Gustavo Doncel, MD for critical reading of the manuscript.

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Received on November 27, 1998; accepted on February 24, 1999