Changes in intracellular calcium concentration in bovine oocytes following penetration by spermatozoa

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The pattern of changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in bovine oocytes after penetration by spermatozoa was determined. Dynamic video imaging, using Fura-2 as a probe for intracellular free calcium, showed that activation of oocytes by spermatozoa induced multiple transient increases in $[Ca^{2+}]_i$ with a spike interval of 24.2 ± 7.3 min, and that the early transient increases were propagated throughout the oocytes in the form of a wave. Calcium transients at fertilization are involved in the induction of cortical granule exocytosis and the resultant block to polyspermy. The hypothesis that the inhibition of Ca^{2+} release from inositol trisphosphate-sensitive stores would inhibit exocytosis and increase polyspermy was tested by injecting oocytes before fertilization with heparin, a potent inhibitor of inositol trisphosphate-activated Ca^{2+} release. There was no significant difference after fertilization in either $[Ca^{2+}]_i$ spikes or in polyspermy rates between control and experimental groups injected with low molecular mass heparin up to a final cytoplasmic concentration of $400 \mu\text{mol l}^{-1}$. We conclude that inositol trisphosphate-independent Ca^{2+} stores may be mobilized during the fertilization of bovine oocytes.

Introduction

At fertilization, spermatozoa not only deliver DNA to the oocyte to restore diploidy, but also trigger a series of intracellular processes essential to embryogenesis. Spermatozoa activate oocytes by inducing a marked and transient change in intracellular calcium concentration ($[Ca^{2+}]_i$) at an early stage of fertilization. In several mammalian species, sperm penetration produces transient, but periodic, Ca^{2+} increases that may last for several hours following the initial penetration (mice: Cuthbertson and Cobbold, 1985; Kline and Kline, 1992; hamsters: Miyazaki, 1990; pigs: Sun *et al.*, 1992). The biological significance of the changes in Ca^{2+} concentration is not fully understood. However, calcium ions are known to be involved in cortical granule exocytosis which leads to a block to polyspermy and in the control of cell cycle progression (Whitaker, 1989; Miyazaki, 1990; Ozil, 1990; Kline and Kline, 1992; Sun *et al.*, 1992).

The means by which sperm–oocyte interactions generate transient increases in $[Ca^{2+}]_i$ remain unresolved; evidence from sea urchin, hamster and mouse oocytes implicates the second messenger inositol trisphosphate (IP_3) in controlling Ca^{2+} release from IP_3 -sensitive intracellular stores (Miyazaki, 1990; Swann, 1990). However, it has been postulated that Ca^{2+} stores insensitive to IP_3 are also mobilized at fertilization. A number of studies in sea urchin (Rakow and Shen, 1990; Crossley *et al.*, 1991), *Xenopus* (Nuccitelli *et al.*, 1993) and hamster (Miyazaki *et al.*, 1993a) used heparin, a competitive inhibitor of IP_3 -

activated Ca^{2+} release to distinguish between IP_3 -dependent and -independent Ca^{2+} release.

The objective of the present study was to characterize the pattern of $[Ca^{2+}]_i$ increases in bovine oocytes after fertilization, and to examine the role of IP_3 -induced Ca^{2+} release in fertilization. Understanding the activation process in bovine oocytes is of fundamental importance for parthenogenetic studies, and is particularly relevant to the process of commercial embryo multiplication by nuclear transplantation. The amount of information presently available concerning Ca^{2+} transients during bovine fertilization is limited. Fissore *et al.* (1992) showed that spermatozoa activate bovine oocytes by inducing multiple Ca^{2+} spikes which are analogous to those of other species. In this study, we have further characterized the dynamic pattern of $[Ca^{2+}]_i$ changes at fertilization and observed that the early transient increases are propagated throughout the cells in the form of a wave. The results obtained from oocytes injected before fertilization with heparin, to block Ca^{2+} release from IP_3 -dependent stores, suggest that IP_3 -independent stores may be mobilized during fertilization of bovine oocytes.

Materials and Methods

Maturation of oocytes in vitro

Bovine ovaries were obtained from a local abattoir and transported at 37°C in PBS. Intact, non-atretic follicles (3–8 mm in diameter) were dissected from the ovaries in PBS and opened to remove the entire cumulus–oocyte complex into Medium 199 (Gibco, Paisley).

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Groups of 30–50 cumulus-enclosed oocytes were washed and then transferred to 30 mm plastic dishes containing 2 ml Medium 199, supplemented with 20% heat-inactivated steer serum (Globe Pharmaceuticals, Esher, Surrey) and with additional granulosa cells from a freshly prepared non-atretic follicle. Maturation was performed non-statically at 38.5°C, in 5% CO₂ in air, for 23–24 h.

In vitro fertilization procedures

The procedures and media used for fertilization of bovine oocytes *in vitro* were essentially those of Parrish *et al.* (1988). Frozen-thawed bovine spermatozoa, obtained from a single bull ejaculate and stored under liquid N₂, were washed twice by centrifugation at 800 g for 5 min in a calcium-free modified Tyrode's medium (sperm-TALP) and then re-suspended at a concentration of 1×10^6 spermatozoa ml⁻¹ in calcium-containing TALP (fert-TALP). Drops (50 µl) were established under liquid paraffin oil (BDH, Lutterworth, Leicestershire). Oocytes were washed twice in low bicarbonate (2 mmol l⁻¹)-TALP buffered with Hepes (20 µmol l⁻¹).

Certain experiments required the oocytes to be totally denuded of cumulus cells before fertilization and this was carried out mechanically by gentle pipetting.

Measurement of intracellular calcium

Oocytes were labelled, either before or after insemination, with the calcium-sensitive fluorescent dye fura-2 acetoxymethylester (Fura-2; Sigma, Poole; 4 µmol l⁻¹) in fert-TALP for 30–90 min at 38.5°C. They were then gently washed three times in TALP.

The subsequent method and conditions for calcium measurement, together with dynamic video imaging procedures were as described by Neylon *et al.* (1990). Fura-2 fluorescence was measured using a Nikon inverted epifluorescence microscope. A rotating filter wheel and a shutter apparatus were used to alternate excitation wavelengths of 340 nm and 380 nm with a band width of 12 nm. Dose was attenuated with a series of neutral density filters. The time between measurements varied between 2 and 20 s, 240 ms being the exposure time for each datum point. Ratios (340 nm:380 nm) of 0.30 and 1.82 were estimated to correspond to [Ca²⁺]_i of 0 nmol l⁻¹ and 1000 nmol l⁻¹, respectively.

Microinjection of oocytes before insemination

The preparation and assembly of microinstruments and manipulation chamber were as described by Sun (1989). Injection pipettes were used with an inner diameter of 3–5 µm. Each oocyte was held with a holding pipette (overall diameter 120 µm) and injected with 10 pl (1% of oocytes volume) of either injection solution (as control) or low relative molecular mass heparin (*M_r* 3500; Sigma). The heparin was dissolved in the injection solution which contained the following: 0.9% NaCl; 0.01 mmol EDTA l⁻¹; 1.0 mmol Tris l⁻¹, pH 7.2. All manipulation procedures were performed in microdrops (5 µl) of Ca²⁺-free PBS covered by light liquid paraffin oil. After

injection, oocytes were washed in low-bicarbonate TALP before insemination.

Incubation with BAPTA-A/M

As a control for experiments using heparin, oocytes were incubated with BAPTA-A/M (Sigma), a highly specific calcium chelator. BAPTA-A/M contains a hydrophobic acetoxymethyl moiety which allows the uptake of BAPTA-A/M into the cell which is then cleaved by esterases, trapping BAPTA molecules within the cell.

Metaphase II oocytes (22 h maturation) were incubated for 1 h at 38.5°C, 5% CO₂ in BAPTA-A/M (25 µmol l⁻¹) in DMSO, washed several times and incubated for 1 h before fertilization. Control oocytes were similarly treated with DMSO.

Assessment of cytology

Groups of oocytes at various stages after insemination were fixed for a minimum of 24 h in ethanol:acetic acid (3:1, v:v), stained with aceto-orcein (BDH) and assessed for sperm penetration and nuclear morphology using phase contrast microscopy.

Results

Optimization of Fura-2 labelling of bovine oocytes

Preliminary experiments involved labelling freshly matured bovine oocytes with Fura-2 (4 µmol l⁻¹) for 60–90 min in the same manner as that used successfully for pig oocytes (Sun *et al.*, 1992). However, bovine oocytes appeared to be more sensitive than were pig oocytes and, although sperm entry was not inhibited, the intracellular level of calcium became markedly and permanently increased 5 h after treatment. Furthermore, the presence of cumulus cells around bovine oocytes was found to interfere with the measurement of [Ca²⁺]_i. The required Fura-2 labelling conditions finally defined by experimentation for the measure of free Ca²⁺ in bovine oocytes used mechanically denuded oocytes incubated with 4 µmol Fura-2 l⁻¹ for 30 min immediately before image analysis.

Fertilization and monospermy rates of control, denuded and Fura-2-labelled denuded oocytes are given (Fig. 1). Fertilization rates, as judged by pronuclear formation, were not affected by denuding oocytes before insemination or by incubation with Fura-2. Polyspermy rates were very low (about 10%), indicating that observed changes in [Ca²⁺]_i (see next section) are the result of penetration by a single spermatozoon. Experiments in which groups of oocytes were fixed and stained at intervals of 1 h showed that the majority were penetrated 4–6 h after insemination.

Measurement of [Ca²⁺]_i changes in oocytes following fertilization

Groups of oocytes were labelled with Fura-2 at different times (0–6 h) after insemination and the changes in their [Ca²⁺]_i concentrations were examined by dynamic imaging.

Fig. 1. Overall fertilization (▨) and monospermy (■) rates of control (cumulus-enclosed) bovine oocytes compared with cumulus-free and Fura-2-treated oocytes. Duration of Fura-2 treatment was 60 min.

Fig. 2. An example of the pattern of the first two transient increases in $[Ca^{2+}]_i$ in a bovine oocyte undergoing fertilization.

Individual oocytes were examined for the presence of spiking and, if spiking was detected, were studied for 2–3 h. Certain oocytes failed to show transients, probably owing to the cessation of their period of calcium change.

The results show that, before sperm penetration, the oocyte basal $[Ca^{2+}]_i$ remained consistently low (in the range 30–140 nmol l⁻¹). Transient $[Ca^{2+}]_i$ increases were observed only in oocytes penetrated by spermatozoa; an example of the pattern of changes is shown (Fig. 2).

Analysis of the dynamic pattern of individual oocytes following activation by spermatozoa from a homogeneous population is summarized (Table 1). During the first 9 h after insemination (about 3–5 h after sperm penetration), the number of calcium spikes per oocyte observed was 2.8 ± 1.6 (mean $\pm$ SD, $n = 12$ oocytes). The range was from 1 to 5 spikes. Among the twelve oocytes characterized, the percentage showing 1, 2, 3, 4 or 5 spikes was 33.3, 16.7, 8.3, 16.7 and 25.0, respectively. Before each spike, there was always a gradual

increase in basal $[Ca^{2+}]_i$; a spike was initiated only when this reached a threshold value of between 150 and 200 nmol l⁻¹. The interval between spikes was 24.3 ± 7.3 min ($n = 12$ oocytes). The peak $[Ca^{2+}]_i$ achieved was 487.3 ± 141.0 nmol l⁻¹ ($n = 12$ oocytes), but there was a large variation in the peak $[Ca^{2+}]_i$ observed in oocytes of different experimental groups. It appeared that the first Ca^{2+} spike achieved tended to be slightly greater in amplitude than subsequent ones. The time taken for an oocyte to achieve a peak $[Ca^{2+}]_i$ from a threshold value was 66.1 ± 20.8 s ($n = 12$); this was similar to the time taken to then decline to threshold values.

Video imaging of $[Ca^{2+}]_i$ changes of an oocyte (Fig. 3) indicated that the transient rise is initiated at a single site within the oocyte and is then propagated throughout the cell in the form of a wave.

After video imaging, oocytes were returned to culture and fixed at approximately 16 h from the start of insemination. Oocytes exhibiting spikes, and the majority of those without, had undergone fertilization as evidenced by the presence of two pronuclei.

Antagonism of IP_3 -sensitive calcium release by heparin

In this part of the study we examined the possibility that the inhibition of Ca^{2+} release from IP_3 -sensitive stores in bovine oocytes would block exocytosis and consequently increase the rate of polyspermy. Denuded bovine oocytes were micro-injected immediately before fertilization with heparin, yielding a final cytoplasmic concentration of 100–400 μ mol l⁻¹. However, not only were fertilization rates consistently high (> 85%), but the proportions of monospermic and polyspermic eggs in control and heparin-injected groups were statistically indistinguishable (Table 2). Moreover, cytological assessment showed that the majority of polyspermic oocytes in control and treated groups were penetrated by only two spermatozoa and that none contained more than four spermatozoa.

In contrast, incubation with BAPTA-A/M induced a high level of polyspermy; the number of spermatozoa penetrating the oocyte ranged from two to seven. Oocytes remained at metaphase II stage and the second polar body was not extruded.

Video imaging showed that during the first 9 h after insemination four of the twenty heparin-injected oocytes examined showed 1–2 $[Ca^{2+}]_i$ spikes.

Discussion

The study reported here established that in bovine oocytes sperm penetration causes the generation of multiple transient increases in intracellular calcium. Although the general pattern of $[Ca^{2+}]_i$ transients is similar to that of other species (see Sun *et al.*, 1992 for references), bovine oocytes exhibit a characteristic pattern both with respect to spike amplitude and spiking interval. In mice and hamsters, the first increase in $[Ca^{2+}]_i$ occurs immediately after insemination (Miyazaki *et al.*, 1989; Kline and Kline, 1992), whereas in cows several hours elapse before the first transient. Two factors contribute to this delay. First, the time required for sperm capacitation is longer in cattle than in

Table 1. Dynamic characteristics of changes in intracellular calcium concentration $[Ca^{2+}]_i$ in response to penetration of bovine oocytes by spermatozoa

Experiment	Number of eggs examined	Number of eggs showing spike(s)	Egg number	Ca ²⁺ spike number	Basal $[Ca^{2+}]_i$ (nmol l ⁻¹)	Time to peak $[Ca^{2+}]_i$ (s)	Peak $[Ca^{2+}]_i$ (nmol l ⁻¹)	Time to return to basal $[Ca^{2+}]_i$ (s)	Duration of peak (s)	Intervals between Ca ²⁺ spikes (min)
A	8	2	1	1	66	100	759	108	208	—
			2	1	59	82	460	100	182	—
			1	1	55	47	444	67	114	20.3
				2	55	36	366	57	93	17.7
B	7	4		3	51	43	320	58	101	
			2	1	37	38	456	56	94	26.2
				2	39	34	338	67	101	15.7
				3	56	58	311	73	131	17.8
C	6	1		4	55	63	287	82	145	
			3	1	31	37	370	70	107	—
			4	1	39	51	263	75	126	—
			1	1	118	121	685	117	238	30.3
D	7	4		2	118	115	728	114	229	
			1	1	76	55	480	60	115	25.4
				2	94	79	503	89	168	18.2
				3	92	91	547	91	182	23.8
E	7	1		4	90	66	444	103	169	21.8
				5	137	76	456	—	—	23.8
			2	1	78	76	511	76	152	27.5
				2	122	82	464	78	160	—
Total	35	12		3	94	39	299	41	80	22.5
				4	102	56	440	67	123	19.3
				5	220	60	433	—	—	22.5
			3	1	137	59	744	75	134	29.5
Mean ± SD				2	192	57	622	81	138	18.4
				3	165	69	460	85	154	49.6
				4	122	79	460	—	—	18.4
			4	1	62	77	610	65	142	31.1
Total	35	12		2	55	71	472	47	118	26.0
				3	76	77	450	69	146	—
				4	66	61	397	129	190	22.1
			5	5	52	79	480	—	—	—
Mean ± SD			1	1	43	58	755	69	127	22.4
			2	2	43	56	754	71	128	—
			34	34	34	34	34	30	30	20
			2.83 ± 1.62	82.24 ± 44.96	66.12 ± 20.82	487.29 ± 141.04	78.0 ± 20.51	143.17 ± 38.76	24.28 ± 7.27

Fig. 3. Pseudo-coloured Ca^{2+} images from the rising phase of the initial $[Ca^{2+}]_i$ transient in a bovine oocyte undergoing fertilization. Frame interval: 2.2 s.

other species studied. Second, the time for sperm penetration in mouse and hamster oocytes has been reduced by the use of zona-free oocytes. In addition to these technical differences, clear species-related spiking patterns were observed. Thus, after penetration bovine oocytes show a much longer spiking interval than did either mouse or hamster oocytes (Miyazaki *et al.*, 1989; Kline and Kline, 1992). However, spiking intervals in bovine oocytes are reasonably similar to those of pig

oocytes (Sun *et al.*, 1992). The differences in the spiking interval may reflect the varying efficiency with which the second messenger systems regulate $[Ca^{2+}]_i$ changes in oocytes of different species. In hamsters, the amplitude of the first two $[Ca^{2+}]_i$ transients is much greater than in succeeding transients. Furthermore, the first two transients are initiated at the site of sperm-oocyte binding and propagate from there in the form of a wave (Miyazaki *et al.*, 1989). Our results suggest that a

Table 2. The rates of fertilization, monospermy and polyspermy in control and heparin-injected^a groups of bovine oocytes and control and BAPTA-A/M-incubated groups of bovine oocytes

Groups		Oocytes fertilized/total (%)	Number of fertilized oocytes that were monospermic (%)
Control oocytes	Denuded	71/78 (91)	63 (89)
	Injected	37/42 (88)	34 (92)
Heparin-injected oocytes	100 $\mu\text{mol l}^{-1}$	25/30 (83)	22 (88)
	200 $\mu\text{mol l}^{-1}$	41/48 (85)	36 (88)
	400 $\mu\text{mol l}^{-1}$	34/41 (83)	32 (94)
Control oocytes	DMSO 0.4%	44/72 (61)	44 (100)
BAPTA-A/M-treated oocytes	26 $\mu\text{mol l}^{-1}$	77/83 (93)	23 (30)

^aInjection volume was 10 pl, equivalent to 1% of the volume of a typical bovine oocyte. Oocytes were fixed 12–15 h after insemination.

similar situation occurs in bovine oocytes. Video imaging revealed that the calcium wavefront commences at a single site and then travels across the whole oocyte. Owing to the presence of numerous spermatozoa surrounding the bovine oocyte after *in vitro* fertilization, we were unable to confirm unequivocally that the wave is propagated from the site of sperm–oocyte fusion. The wavefront travels at a velocity of approximately $2 \mu\text{m s}^{-1}$; the entire oocyte is traversed in approximately 1 min. This velocity is slower than the rates in *Xenopus* ($10 \mu\text{m s}^{-1}$; Delisle, 1991; $7.5 \mu\text{m s}^{-1}$; Nuccitelli *et al.*, 1993) and other aquatic species (Jaffe, 1985) and much slower than in hamsters (16 – $28 \mu\text{m s}^{-1}$, Miyazaki *et al.*, 1993b).

The results presented here are similar to those obtained by Fissore *et al.* (1992) using Fura-2-injected bovine oocytes. The penetration rates reported by Fissore *et al.* were low, a maximum of 56% by 16 h. Furthermore, the wide variation in parameters, particularly interspike interval (15–51 min) may have been, at least partially, due to the heterogeneous source of spermatozoa used. In contrast, the present work used a homogeneous sperm source and a non-invasive method of Fura-2 labelling.

The deleterious effects of ultraviolet upon oocytes during calcium measurements were minimized. The narrow band width (12 nm) used meant that the wavelengths used (340 and 380 nm) are well-removed from the more harmful wavelengths (254 nm). The use of a variably rotating filter wheel and shutter apparatus minimized the exposure time of oocytes to UV, and this dose was further attenuated by neutral-density filters. The majority of oocytes, when returned to culture after analysis, showed the presence of normal male and female pronuclei.

The absence of spiking in several oocytes examined may not be significant. A likely explanation is that, when a single oocyte is studied for 2–3 h, the period of calcium increases in other oocytes may well have passed unstudied.

It is now well established that an increase in intracellular Ca^{2+} at fertilization is essential for oocyte activation (Whitaker and Patel, 1990; Kline and Kline, 1992; Whitaker and Swann, 1993). However, the mechanism by which spermatozoa generate transient Ca^{2+} increases during oocyte activation remains unclear. It is widely acknowledged that IP_3 plays an essential second messenger role in regulating Ca^{2+} changes at fertilization and that injection of IP_3 into mammalian oocytes induces $[\text{Ca}^{2+}]_i$ increases and cortical granule exocytosis (see Swann,

1990 for references). However, opinions differ as to whether IP_3 is the sole means by which Ca^{2+} release (and the resultant block to polyspermy) is induced at fertilization.

The high affinity of heparin for the IP_3 receptor (Ghosh *et al.*, 1988) has been used to distinguish calcium release from IP_3 -regulated and IP_3 -independent stores. Injection of heparin into sea urchin oocytes failed to inhibit fertilization or the production of calcium spikes (Rakow and Shen, 1990; Crossley *et al.*, 1991). However, the oocytes were polyspermic. Similar results were obtained for hamster (Miyazaki *et al.*, 1993a) and *Xenopus* (Nuccitelli *et al.*, 1993) oocytes.

The results of the experiments reported here support the concept of calcium release from multiple stores, since fertilization and the block to polyspermy were unaffected by intracellular heparin concentrations of 100 – $400 \mu\text{mol l}^{-1}$. These concentrations are considerably in excess of those used by other workers, and would appear to be adequate to compete with IP_3 generated at fertilization (less than $10 \mu\text{mol l}^{-1}$ (Ciapa and Whitaker, 1986; Rakow and Shen, 1990)). The existence of calcium transients in heparin-injected oocytes (one or two spikes found in four of 20 oocytes) suggests that some calcium release is independent of IP_3 but the results are not conclusive.

Introduction of BAPTA into oocytes has been shown to inhibit all sperm-induced calcium transients, resulting in the inhibition of the resumption of meiosis together with polyspermy (Kline and Kline, 1992; Crossley *et al.*, 1991). The present study obtained similar results in bovine oocytes.

These results support the concept that sperm activation may initiate calcium release from intracellular stores in bovine oocytes without the involvement of IP_3 , and that sufficient calcium is released to at least block polyspermy. This concept is not in agreement with that of Miyazaki *et al.* (1992), who used a monoclonal antibody to IP_3 receptors present in hamster oocytes. At high concentrations the antibody could block sperm-induced $[\text{Ca}^{2+}]_i$ increases, suggesting the sole involvement of IP_3 receptors in calcium release, and contradicting results obtained using heparin injections. Further work is required to clarify the mechanism of $[\text{Ca}^{2+}]_i$ increases in cattle and other species.

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