Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate

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Summary

Microinjection of inositol 1,4,5-trisphosphate into sheep and hamster oocytes induces secretion of cortical granules in a dose-dependent manner. In the sheep, this effect is strongly pH-dependent with minimal exocytosis taking place at pH 6.8 but a full cortical reaction occurring at pH 8.0. Exocytosis in the hamster is also affected by the pH of the external medium but to a lesser extent. Injection of GTPγS also induces exocytosis in both species but is more effective in the hamster. It is suggested that inositol metabolism stimulated by sperm-egg interaction with a GTP-binding protein may be part of the mechanism leading to cortical granule exocytosis and that this may be modulated by the external pH.

Key words: sheep, hamster, oocyte, cortical reaction, inositol trisphosphate, pH.

Introduction

It has long been recognized that the exocytosis of cortical granules (the cortical reaction) and the resulting modification of the properties of the oocyte plasma membrane, and the overlying zona pellucida, are key events associated with the prevention of polyspermy (Austin, 1956; Barros & Yanagimachi, 1971; Gwatkin et al. 1973; Schuel, 1978; Gulyas, 1980; Guraya, 1983). An important question that remains to be answered is the nature of the signals induced in response to the fusion between the fertilizing spermatozoan and the plasma membrane of the oocyte. A wide range of treatments, both physical and chemical, are capable of inducing cortical granule loss. These include electrical stimulation (Gulyas, 1980; Zamboni et al. 1976), the use of ionophores (Steinhardt et al. 1974; Gwatkin et al. 1976), and the direct injection of calcium into the oocyte (Fulton & Whittingham, 1978).

However, none of these studies on mammalian oocytes has given an indication of the likely nature of the putative transducing messenger molecules. As in other exocytotic events, it seems clear that the regulation of calcium is an important feature and Miyazaki et al. (1986; Miyazaki, 1988) have recently demonstrated that in golden hamster oocytes, following fusion with a spermatozoan, there is a periodic increase in free calcium, which spreads from the point of contact. A similar calcium wave has been demonstrated in eggs of Xenopus (Busa et al. 1985; Kubota et al. 1987) and sea urchins (Swann & Whitaker, 1986). In these species, the understanding of the nature of the transducing secondary messengers has progressed more rapidly than in mammals. Various workers have demonstrated that inositol 1,4,5-trisphosphate when injected into sea urchin and Xenopus eggs will cause an elevation of the vitelline envelope (Whitaker & Irvine, 1984; Busa et al. 1985; Irvine & Moor, 1986; Slack et al. 1986; Turner et al. 1986). In addition, Miyazaki (1988) has shown that microinjection of inositol 1,4,5-trisphosphate (Ino(1,4,5)P3) will induce calcium transients in the hamster. It is an attractive hypothesis, therefore, that injection of Ino(1,4,5)P3 will result in the activation of the cortical reaction in mammalian oocytes. We report here results, consistent with this suggestion, from experiments using sheep and golden hamster oocytes.

Materials and methods

Oocytes

Mature hamster oocytes were collected from the oviducts of superovulated animals injected with 30 units of pregnant mare’s serum gonadotrophin and human chorionic gonadotrophin. The surrounding cumulus cells were removed by treatment with 0.05% hyaluronidase for 1–2 min followed by three washes in BWV medium (Biggers et al. 1971) at the appropriate pH (see below), at 37°C. Oocytes were maintained at 37°C in an atmosphere of 5% CO2 in air until use. Sheep oocytes were obtained and matured in culture as described by Staimiller & Moor (1984). Such in vitro matured oocytes have, following fertilization, full developmental capacity.

Microinjection

Microinjection was made centrally into the oocyte cytoplasm using micropipettes backfilled with oil and the volume injected was calibrated by measurement of an oil droplet expelled into
the medium. Approximately 10 oocytes per treatment were used. For sheep eggs 21 pl were injected, which represents some 4% of the cytoplasmic volume (oocyte diameter, 100 \mu m) and for hamster oocytes some 16 pl were injected, representing 3-2% of oocyte volume. Calculations of intracellular concentration ([I]) assume an even diffusion of the injected compounds, which were injected in a carrier medium consisting of 100\mu m-EGTA and 10 mM-Hepes at pH 7.0. The following compounds were injected: inositol 1,4,5-trisphosphate (Ins\((1,4,5)P_3\)), inositol 1,4-bisphosphate (Ins\((1,4)P_2\)), guanosine 5'-[\gamma-thio]trisphosphate (GTP\(\gamma\)S) and guanosine 5'-[\beta-thio]diphosphate (GDP\(\beta\)S) (Fluka, Buchs, Switzerland).

**External solutions**

For experiments with sheep oocytes, medium M199 containing 10% foetal calf serum was employed, this being the medium in which maturation was carried out. This was equilibrated with 5% CO\(_2\) in air. Adjustments to the pH were made by adding the appropriate quantity of bicarbonate with 50 mM-Hepes buffer. In experiments with hamster oocytes BWW medium was used (Biggersef et al. 1971), which comprised 94-6 mM-NaCl, 4-8 mM-KCl, 1-7 mM-CaCl\(_2\), 1-2 mM-MgSO\(_4\), 1-2 mM-KH\(_2\)PO\(_4\), 22 mM-sodium lactate, 0-5 mM-sodium pyruvate, 5-6 mM-glucose, 25-1 mM-NaHCO\(_3\).

**Controls**

Controls for exocytosis were as follows: non-injected oocytes, oocytes pricked with an injection pipette but not injected, oocytes injected with the carrier medium and oocytes injected with Ins\((1,4)P_2\) (a compound that does not mobilize intracellular Ca\(^{2+}\); Berrierde & Irvine, 1984) and oocytes treated with 10 \mu M-ionophore A23187 at pH values of 6-8 and 8-0.

**Quantification**

Following injection, the oocytes were maintained under CO\(_2\) in air at 37°C for a further 1 h. They were then fixed in 2% glutaraldehyde in cacodylate buffer (pH 7-2) and postfixed in 1% OsO\(_4\). During dehydration they were kept for a minimum of 8 h in 70% ethanol containing 1% uranyl acetate. This step was found to be essential for visualisation of the cortical granules at the level of the light microscope and also provided enhanced contrast at the level of the electron microscope (Figs 1 and 3). The eggs were embedded in Epon, and 1 \mu m sections were cut and lightly stained with 1% Toluidine Blue in 1% borax. Sections were taken near the centre of the oocytes and all granules lying close to the plasma membrane were counted using a \(\times 63\) oil immersion objective at a total magnification of \(\times 1260\). The fine structure of several oocytes was also examined. These observations confirmed that only cortical granules were being scored. Numbers of granules are presented as the number per 10 \mu m of overlying plasma membrane.

**Results**

**Distribution of granules**

All the oocytes used were mature, possessing the first polar body and having progressed to metaphase II. The cortical granules were closely aligned with the plasma membrane. In sheep oocytes the granules measured some 200 nm in diameter while those in the hamster were noticeably larger with a mean diameter of 220 nm. In the sheep the granules occurred as discontinuous groups and were present throughout the peripheral cytoplasm. In the hamster, however, they were more uniformly distributed, with the noticeable exception of the region underlying the metaphase plate where they were infrequent. In this regard, therefore, they have a distribution similar to that observed for the mouse (Nicosia et al. 1977).

**Controls**

In the sheep, either non-injected oocytes or those injected with 1 mM-Ins\((1,4,5)P_3\) at pH 8-0. Few granules remain within the cytoplasm.

**pH effect**

In the sheep, initial experiments to determine whether Ins\((1,4,5)P_3\) could stimulate the cortical reaction met with limited success even at very high concentrations (data not shown). These were carried out at a pH routinely used to mature oocytes (pH 7-4). Since a variety of cellular interactions concerned with Ca\(^{2+}\) homeostasis are modulated by extracellular pH (Blackmore et al. 1984; Altin & Bygrave, 1987; Garty et al. 1987) and in \textit{vitro} fertilization of domestic species takes place more readily at an elevated pH (Cheng, 1985), it was considered that this was likely to be a pertinent factor.
in the stimulation of the cortical reaction in sheep oocytes.

In order that any pH effect might not be potentially confounded by the use of a suboptimal concentration of injected Ins(1,4,5)P$_3$, a high level of Ins(1,4,5)P$_3$ (1 mM) was used, representing a final intracellular concentration of 40 µM. In this species there was a clear correlation between the number of cortical granules remaining in eggs that had been injected with Ins(1,4,5)P$_3$ and the pH of the culture medium (Fig. 2). At pH 6-8 there was no difference from the controls, while at pH 8-0 most of the cortical granules had been lost. At intermediate pH values there was considerable variability between oocytes. The results based on light microscopy were confirmed by examination with the electron microscope. At pH 6-8 it was immediately evident that there were numerous granules located adjacent to the plasma membrane in a manner similar to the unfertilized cell (Fig. 3A). At pH 8-0 very few granules could be detected. However, it was clear that remains of the granules (the so-called cortical granule exudate) were present within the perivitelline space (Fig. 3B,C). These had an appearance identical to that observed following in vitro fertilization (Sananthanan & Trounson, 1982; Cran & Cheng, 1986). At pH 8-0, without injection, the plasma membrane in a manner similar to that observed following alkalinization of sea urchin eggs after treatment with NiCl$_2$ at high pH (Charbonneau & Webb, 1987).

In the hamster, also, there was a clear effect of pH on exocytosis (Fig. 2A). For this species Ins(1,4,5)P$_3$ was injected at a pipette concentration of 10 µM at pH values of 6-8, 7-4 and 8-0. As in the sheep there was negligible exocytosis at pH 6-8 while the number present at the higher pH values was markedly reduced.

Effect of concentration of Ins(1,4,5)P$_3$

Pipette concentrations of Ins(1,4,5)P$_3$ ranging from 1 µM ([32 nM]) to 200 µM ([6-4 µM]) were injected into hamster oocytes at pH 7-4 and from 1 µM ([40 nM]) to 1 mM ([40 µM]) were injected into sheep oocytes at pH 8-0. In both species microinjection of 1 µM was ineffective, whereas the higher concentrations caused a marked loss of cortical granules (Fig. 4). In both species the highest concentration used brought the numbers of granules down to some 10% of control levels. In addition, 10 µM ([0-32 µM], hamster), ([0-4 µM], sheep) resulted in more than a halving of the granule content.

Injection of GTPyS

This compound has been shown to cause repeated Ca$^{2+}$ transients in hamster oocytes (Miyazaki, 1988). We therefore investigated its effects upon the cortical reaction for comparative purposes.

Pipette concentrations of 0-2 µM ([6-4 nM]), 1 µM ([32 µM]) and 10 mM ([320 µM]) were injected into hamster oocytes (Fig. 5). In addition, 10 mM-GDPyS was injected into a further set of oocytes. GDPyS was completely without effect on cortical granule exocytosis as was an internal concentration of 6-4 µM-GTPyS. Internal concentrations of 32 and 320 µM, however, reduced the number of granules to about 25% of control levels. In the sheep, microinjection of 10 mM-GDPyS was also without effect. However, GTPyS was not as efficacious at stimulating exocytosis as in the hamster, the number of granules being reduced to only about 60% of control levels following injection of 10 mM-GTPyS into oocytes, which were maintained in culture medium at pH 8-0.

Discussion

In the present study it has been shown that both Ins(1,4,5)P$_3$ and GTPyS will induce the cortical reaction in hamster and sheep oocytes. In both species a pipette concentration of 10 µM-Ins(1,4,5)P$_3$ ([350 nM and 420 nM]) induced the reaction while the lower levels tested, 1 µM ([35 nM and 42 nM]), were ineffective. There is a large body of evidence implicating a central role for Ca$^{2+}$ in the cortical reaction, and Miyazaki (1988) has recently provided evidence that central microinjection of Ins(1,4,5)P$_3$ into hamster oocytes will provoke a release of Ca$^{2+}$ throughout the cell. In his study a pipette concentration of 80 nM ([2 nM]) induced the Ca$^{2+}$ release (Miyazaki, 1988). This is clearly considerably less than that required to initiate the cortical reaction in the present study. There are several possible explanations for the apparent disparity.

First, it is possible that a strain difference exists between animals, conferring differing degrees of sensitivity. We cannot rule out this possibility, but it is unlikely because in our hands the concentrations of GTPyS required to stimulate the cortical reaction were very similar to those required to initiate Ca$^{2+}$ mobilization in the experiments of Miyazaki (1988). This observation raises the possibility that GTPyS might poten-
Fig. 3. A. Electron micrograph of a sheep oocyte following injection of 1 mM-Ins(1,4,5)P$_3$ at pH 6.8. Numerous cortical granules (g) are present below the plasma membrane. ×5500. B. After injection at pH 8.0 few granules remain within the cytoplasm. Remains of their contents can be detected in the perivitelline space (arrows). ×16000. C. High-power view of the periphery of a sheep oocyte injected at pH 8.0. Cortical granule exudate (c) is present adjacent to the plasma membrane. ×30000.

potentially act directly on exocytosis as has been observed by Cockcroft & Gomperts (1985) in mast cells.

A more likely explanation follows from the fact that it has been shown that both the fertilizing spermatozoan and the introduction of GTPyS will elicit an initial hyperpolarization response (HR) followed by a regular series of further HRs (Miyazaki, 1988). While Ins(1,4,5)P$_3$ stimulates an initial large HR, only a very high dose (240 μM) will introduce further repetitive HRs and even then there is a considerable time lag, and the size of the repetitive HRs is less than that found following the previous two treatments. Following injection of 10 μM-Ins(1,4,5)P$_3$, exocytosis of cortical granules in hamster oocytes is not immediate, but takes place over a matter of several minutes (unpublished results). In order, therefore, to obtain such a continued secretion it seems likely that the continued presence of calcium at the periphery of the oocyte is necessary. Thus it may be that Ins(1,4,5)P$_3$ needs to be present for a time exceeding that of the initial Ca$^{2+}$ burst (Miyazaki, 1988) in order to cause a full cortical reaction. *In vitro,* it seems likely that several pulses of Ca$^{2+}$ may substitute for this prolonged presence of Ins(1,4,5)P$_3$ and sperm may themselves effect pulsatile production of Ins(1,4,5)P$_3$.

It is interesting to note that sheep oocytes were less sensitive to GTPyS than those of hamsters. We have no explanation for this difference, which may be a reflection of the conditions used or the operation of a somewhat different mechanism in this species.

The effect of pH on the cortical reaction has interesting implications for oocyte fertilization *in vivo.* Reliable estimates of the pH of the fluid within fallopian tubes are
difficult to obtain. However, original studies indicate that it is likely to be significantly above neutrality (Hamner & Fox, 1969). Furthermore, in vitro fertilization in the hamster (Bavister, 1969) and sheep (Cheng, 1985) is more efficient at an alkaline pH. In sheep and hamsters we found that the action of Ins(1,4,5)P$_3$ was ineffective at neutral pH, the most consistent exocytosis being observed in the region of pH 8-0. While the effect of a change in external pH on the pH of the peripheral cytoplasm of mammalian oocytes is not known, in most cells (Moolenaar, 1986) when inositol turnover is stimulated an alkalization of the cytoplasm occurs, due to the stimulation of Na$^+$/H$^+$ exchange caused by protein kinase, which is in turn regulated by diacylglycerol.

In conclusion, it has been demonstrated that, in addition to stimulating Ca$^{2+}$ release within mammalian oocytes, Ins(1,4,5)P$_3$ induces the exocytosis of cortical granules and that this event is modulated by external pH.

References


of the periodic increase in the intracellular calcium at fertilization of golden hamster eggs. Devl Biol. 18, 259–267.


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