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Epidermal growth factor stimulates hydrolysis of phosphatidylinositol 4,5-bisphosphate, generation of diacylglycerol and exocytosis in mouse spermatozoa

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Abstract Mouse spermatozoa stimulated with epidermal growth factor (EGF) or zona pellucida (ZP) experienced phosphatidylinositol 4,5-bisphosphate hydrolysis, diacylglycerol (DAG) generation and acrosomal exocytosis. The agonists showed additive effects but the action of EGF is likely to be mediated by a distinct receptor because maximal stimulation achieved with EGF was enhanced further by ZP. Generation of DAG and exocytosis stimulated by EGF were inhibited by tyrphostin A48, indicating that tyrosine kinase activity mediates EGF action. On the other hand, pertussis toxin did not affect the EGF-induced formation of DAG or exocytosis, ruling out the involvement of sperm G_i-like proteins. These results indicate that EGF could be an important co-factor in the initiation of exocytosis in spermatozoa.

Key words: Exocytosis; Epidermal growth factor; Zona pellucida; Diacylglycerol; Spermatozoa

1. Introduction

Mammalian spermatozoa undergo exocytosis of the acrosomal granule in response to specific stimuli derived from the oocyte vestments [1]. It is generally believed that zona pellucida (ZP), the acellular oocyte coat, is the inducer of acrosomal exocytosis [1], with one of its glycoproteins (ZP3 in the mouse) being responsible for its exocytosis-inducing activity [2]. However, recent work has suggested that a series of molecules present in the cumulus-oocyte complex or in the oviductal ampulla (the site of fertilization) could also induce exocytosis or may act as co-factors during the initiation or completion of this process [3–5].

Knowledge on signal transduction during acrosomal exocytosis is still fragmentary and the identity of receptor(s) mediating the action of ZP is still unclear. One candidate for the mouse ZP3 receptor is a 95-kDa protein with tyrosine kinase activity [6], and which becomes phosphorylated upon stimulation with ZP [7], thus resembling growth factor receptors [8]. Stimulation with ZP also triggers the activation of a pertussis toxin (PTX)-sensitive G-protein (G_i class) [9] and one possible mechanism activated by these G-proteins is channel-mediated Ca^{2+} entry [10]. Other targets for these transducing mechanisms are presently unknown.

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Abbreviations: ZP, zona pellucida; PTX, pertussis toxin; PIC, phosphoinositidase C; DAG, 1,2-diacylglycerol; EGF, epidermal growth factor; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

Studies using a model system in which exocytosis is triggered with Ca^{2+} ionophores have revealed that Ca^{2+} entry leads to an early and essential phosphoinositidase C (PIC)-mediated polyphosphoinositide hydrolysis [11], and a rapid phospholipase C-mediated breakdown of phosphatidylcholine [12], both contributing to generate 1,2-diacylglycerol (DAG) [13]. The major second messenger role of DAG appears to be the stimulation of phospholipase A_2 [14] which, in turn, would generate metabolites that directly or indirectly bring about membrane fusion [15,16]. Although this latter work has highlighted the apparent central role of DAG in events downstream of Ca^{2+} entry, the links between these and events upstream of Ca^{2+} influx are not well understood.

Recent studies have revealed that epidermal growth factor (EGF) can induce [17] or enhance [18] acrosomal exocytosis, which raises the possibility that this growth factor may have a role in sperm activation at fertilization. An EGF receptor has been identified in spermatozoa from various species [17,19,20] and, moreover, it has been found that the receptor becomes phosphorylated upon exposure to EGF [19]. However, the generation of second messengers elicited by EGF has not been investigated. Here we report that stimulation of capacitated mouse spermatozoa with EGF leads to generation of second messenger DAG and acrosomal exocytosis by a signalling cascade involving protein tyrosine kinase.

2. Materials and methods

2.1. Materials

[^{32}P]P_i (carrier-free; 10 mCi/ml) was from Amersham International (Amersham, Bucks., UK). Mouse EGF was kindly provided by Dr. K.D. Brown of this Institute. Pertussis toxin was from RBI-Semat (St. Albans, Herts, UK). 1,2-Dioleoyl-*sn*-glycerol, chlortetracycline, and bovine serum albumin (Fraction V) were from Sigma Chemical Co. (Poole, Dorset, UK). Tyrphostin A48 was from LC Laboratories (Nottingham, UK). Thin layer chromatography plates were from E. Merck (Darmstadt, Germany). All the chemicals used were of analytical grade (BDH, Poole, Dorset, UK). Organic solvents were from BDH or Fisons (Loughborogh, Leics, UK).

2.2. Media

The medium used for incubation of spermatozoa was a modified Tyrode's solution (mT-B25) with 5.56 mM glucose and 4 mg bovine serum albumin/ml [21]; it was maintained in equilibrium with 5% CO_2 in air (pH 7.4 at 37°C; measured osmolality: 295 mOsm/kg). A HEPES-buffered medium (mT-H) was also used. It contained 20 mM HEPES in place of the $NaHCO_3$, and the concentration of NaCl was ajusted to give the same final osmolality. The pH was adjusted to 7.4 with NaOH, and was used under air.

2.3. Preparation of ZP

Zonae pellucidae were collected and solubilized as described previously [7,9]. Briefly, ovaries were isolated from outbred female mice

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(21–23 days old) of the TO strain and were homogenized in the HB buffer solution [22]. The homogenate was then loaded onto a two-step gradient of Percoll/HB buffer (10% and 20%, v/v), each with 0.2% (w/v) poly(vinylalcohol), and centrifuged for 10 min at $800 \times g_{max}$ at room temperature. The layer with ZP was recovered and washed twice with HB buffer; ZP were stored at −80°C until use. When required, ZP were solubilized by heating at 60°C for 1 h.

2.4. Preparation and treatment of spermatozoa

Spermatozoa were obtained from the caudae epididymides and vasa deferentia of outbred male TO mice (> 40 g) and were incubated in mT-B25 in culture dishes under liquid paraffin for 1.5 h at 37°C (gas phase: 5% CO_2 in air); sperm concentrations were $3–7 \times 10^6$ cells/ml and motility >75%. Agonists (EGF and/or ZP) were then added and incubations continued for 15 min under 5% CO_2 in air. The effects of putative inhibitors were examined by adding them (or their solvents as controls) 15 min prior to the addition of agonists. At the end of incubations, cells were stained with chlortetracycline and categorized into 3 groups [23]: 'F' pattern (uncapacitated), 'B' pattern (capacitated, acrosome-intact), and 'AR' pattern (cells that have undergone acrosomal exocytosis).

To study changes in sperm lipids, cells incubated in mT-B25 in plastic tubes (30 × 115 mm) for 1.5 h at 37°C under 5% CO_2/air (sperm concentration ~1×10^7 cells/ml) were diluted 1:1 (v/v) with mT-H and centrifuged at $600 \times g_{max}$ for 10 min at room temperature. The supernatant was removed and cells were resuspended in mT-H (concentration: ~5×10^7 cells/ml). Cell stimulation was carried out by incubation with the agonists at 37°C under air. When ^{32}P-labelled cells were required, spermatozoa were incubated in mT-B25 with 150 μCi [^{32}P]P_i/ml under 5% CO_2 in air for 1.5 h, and then washed and resuspended in mT-H medium (concentration: ~2×10^7 cells/ml). To test the effect of putative inhibitors, spermatozoa were preincubated in mT-B25 for 1 h, washed as described, and were resuspended in mT-H medium. Inhibitors were added and spermatozoa were incubated for 15 min under air before exposure to agonists.

2.5. Lipid analyses

To measure changes in ^{32}P-labelled phosphoinositides, sperm lipids were extracted 5 min after stimulation, and were separated and quantified as reported previously [15]. Briefly, after extraction, lipids were separated by t.l.c. using the solvent system chloroform/methanol/water/concentrated NH_3 (38:40:5:5, v/v) [24]. Lipid spots were made visible by autoradiography, identified by using the autoradiographs as templates, scraped off, and the radioactivity in each was determined by liquid scintillation counting.

For quantification of DAG mass, reactions were stopped by adding chloroform/methanol (1:2, v/v) 2.5 min after stimulation, and lipids were then extracted according to Bligh and Dyer [25]. Neutral lipids were separated by t.l.c. using the solvent system chloroform/methanol/acetic acid (98:2:1, v/v) [12,26]. 1,2-Diacylglycerol was quantified by Coomassie blue staining [27] and densitometry, using 1,2-dioleoyl-*sn*-glycerol to construct standard curves for each plate [13,28].

2.6. Statistics

Results are means ± S.E.M. Percentages of cells undergoing exocytosis, and DAG mass, were transformed to arcsin $\sqrt{(\%\ \text{'AR' cells} \div 100)}$, or $\log_{10}$ (μg/10^9 spermatozoa), respectively, and data were analysed using Student's *t*-test. Values of $P < 0.05$ were regarded as statistically significant.

3. Results

Mouse spermatozoa capacitated for 1.5 h in mT-B25 medium and stimulated with EGF for 15 min underwent acrosomal exocytosis, as revealed by the increase in the proportion of cells exhibiting an 'AR' pattern (Fig. 1A); the effect was concentration-dependent and was maximal at 100 ng/ml. EGF did not impair sperm motility. Spermatozoa capacitated in a similar way and exposed to 1 ZP/μl for 15 min also underwent exocytosis (Fig. 1A). Exposure of capacitated spermatozoa to half-maximal concentrations of both agonists (0.1 ng EGF/ml and 0.5 ZP/μl) revealed additive effects (Fig. 1b). However, since the effect seen with maximal concentrations of EGF (100 ng/ml) was enhanced further by inclusion of 0.5 ZP/μl (Fig. 1B) or 1 ZP/μl (Fig. 1C), it is likely that both agonists act on separate receptors.

When mouse spermatozoa were capacitated in mT-B25 medium in the presence of 150 μCi [^{32}P]P_i/ml to label the phosphoinositides, washed, resuspended in mT-H medium and stimulated with 100 ng EGF/ml for 5 min, breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) was revealed (Fig. 2A); spermatozoa stimulated with 1 ZP/μl for 5 min also experienced breakdown of PtdIns(4,5)P_2. In parallel experiments, unlabelled spermatozoa were similarly treated and DAG mass measured after 2.5 min (when DAG levels are maximal; data not shown). Stimulation with 100 ng EGF/ml resulted in a considerable generation of DAG and similar results were obtained after stimulation with 1 ZP/μl (Fig. 2B). These results confirm that hydrolysis of PtdIns(4,5)P_2 by PIC is taking place (cf. [12]) after EGF and ZP stimulation and, since the mass of polyphosphoinositides in sperm membranes is small [29,30], suggest that hydrolysis of phosphatidylcholine by phospholipase C contributes substantially to the DAG pool.

To investigate if the action of EGF is related to protein tyrosine phosphorylation, the effects of tyrphostin A48 were examined. Capacitated mouse spermatozoa were exposed to

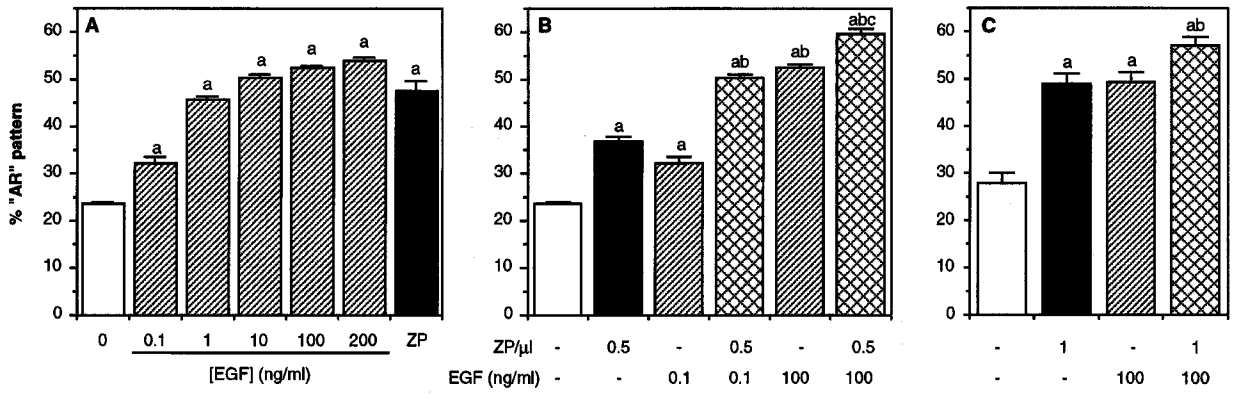

Fig. 1. Effect of EGF and/or ZP on mouse sperm acrosomal exocytosis. Capacitated spermatozoa were exposed to agonists for 15 min, and were then stained with chlortetracycline and examined for exocytosis (% 'AR' pattern). (A) Effect of different concentrations of EGF or 1 ZP/μl ($n = 3$); a: different from control ($P < 0.01$). (B) Effect of simultaneous exposure to 0.1 ng EGF/ml and 0.5 ZP/μl, or 100 ng EGF/ml and 0.5 ZP/μl ($n = 3$); a: different from control ($P \leq 0.001$); b: different from 0.5 ZP/μl or 0.1 ng EGF/ml ($P \leq 0.004$); c, different from 0.5 ZP/μl or 100 ng EGF/ml. (C). Effect of exposure to 100 ng EGF/ml and 1 ZP/μl ($n = 4$); a: different from control ($P \leq 0.003$); b: different from 1 ZP/μl or 100 ng EGF/ml ($P \leq 0.04$).

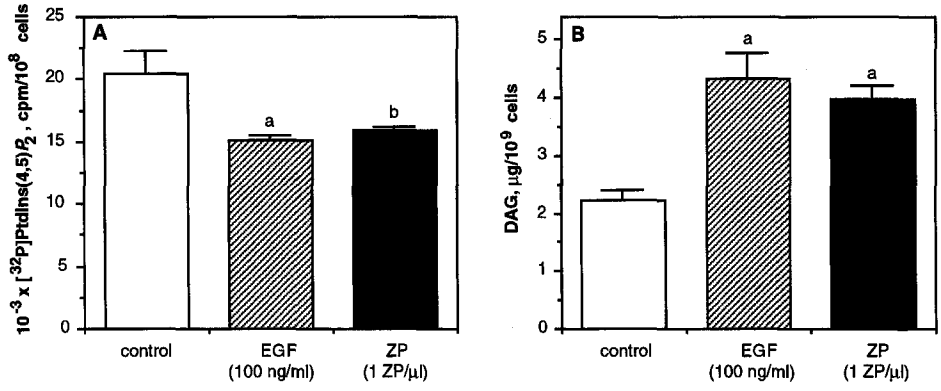

Fig. 2. Effect of EGF and ZP on PtdIns(4,5)P_2 hydrolysis and DAG generation in mouse spermatozoa. (A) Spermatozoa capacitated in mT-B25 for 1.5 h in the presence of 150 μCi [^{32}P]P_i/ml were washed, resuspended in mT-H medium and stimulated with EGF (100 ng/ml) or 1 ZP/μl for 5 min. Lipids were extracted, separated and radioactivity in each spot counted (n = 4); a,b: different from control ($P < 0.01$ and $P = 0.02$, respectively). (B) Spermatozoa capacitated for 1.5 h were washed, resuspended, and stimulated with EGF (100 ng/ml) or 1 ZP/μl for 2.5 min. Lipids were extracted, separated and DAG mass was quantified by Coomassie blue staining and densitometry (n = 3); a: different from control ($P < 0.05$).

A48 for 15 min before agonist addition, and DAG mass and % 'AR' cells (indicative of exocytosis) were quantified. The ability of EGF (100 ng/ml) to induce either formation of DAG or exocytosis was prevented by A48 in a concentration-dependent manner (Fig. 3A,B). The concentration of A48 (12.5 μM) that completely blocked DAG generation and exocytosis induced by EGF also blocked the formation of DAG and exocytosis elicited by ZP (data not shown); A48 (or its DMSO solvent) did not affect sperm motility or cell integrity (data not shown).

To test if EGF action is related to activation of sperm G_i-like proteins [9], the effect of pertussis toxin (PTX) was examined. Concentrations of up to 1 μg PTX/ml did not affect the ability of EGF (100 ng/ml) to stimulate DAG formation (Fig. 3A) or exocytosis (Fig. 3B), although at 1 μg/ml it did inhibit the ZP-induced generation of DAG (93% inhibition) and exocytosis (87% inhibition) (not shown). PTX alone (up to 1 μg/ml) did not affect cell motility or integrity, and did not induce exocytosis (data not shown).

4. Discussion

The results of this study clearly demonstrate that EGF induces generation of DAG and acrosomal exocytosis in mouse spermatozoa and that protein tyrosine kinase activation is likely to be a fundamental event in the signalling pathway evoked by this agonist.

The action of EGF does not appear to be mediated by a receptor targeted by ZP/ZP3 but by a separate receptor. Previous work [6,7] has hinted similarities between a sperm 95-kDa protein, presumed to be a ZP3 receptor, and growth factor receptors, because the former has intrinsic tyrosine kinase activity and may be autophosphorylated upon stimulation with ZP. However, this 95-kDa protein has recently been found to be a unique hexokinase that is phosphorylated on tyrosine residues [31]. On the other hand, recent studies have shown the existence of a distinct 170-kDa EGF receptor in spermatozoa from various species, including the mouse [17,19,20], and that this receptor is phosphorylated upon exposure to EGF [19]. The results presented here agree with the idea that EGF acts on a distinct receptor, because cell responses evoked with maximal concentrations of EGF were enhanced further by stimulation with ZP.

Growth factor actions on somatic cells are transduced by receptors with tyrosine kinase activity [8]. Our results indicate that tyrosine kinase activity of the sperm EGF receptor is essential for the activation of downstream processes, such as DAG generation and subsequent exocytosis, because treatment with tyrphostin A48, a tyrosine kinase inhibitor, blocked EGF-induced responses.

On the other hand, EGF receptors appear to be coupled to PTX-sensitive G_i proteins in some cells [32]. We have therefore examined whether sperm PTX-sensitive G_i proteins [9] are involved in EGF action. We found that PTX was unable to interfere with DAG formation or exocytosis triggered by EGF. Failure to reveal PTX inhibition of acrosomal exocytosis triggered by progesterone [33], another agonist of acrosomal exocytosis [3], has been criticised on the grounds that evidence for sufficient inactivation of G-proteins had not been demonstrated [34]. Although direct inactivation of G_i proteins was not examined in our study, we believe our results are meaningful because in parallel experiments, using identical conditions, PTX was found to inhibit significantly the formation of DAG and exocytosis evoked by ZP (cf. [35], for exocytosis).

Stimulation of spermatozoa with EGF resulted in hydrolysis of PtdIns(4,5)P_2. It is therefore possible that the sperm EGF receptor is coupled to a PIC. No information exists on the possible classes of PICs present in spermatozoa but, based on information of EGF-activated pathways in somatic cells [36], it is possible that a sperm PIC-γ1 is being activated. It is interesting to mention in this context that sperm PIC activation and PtdIns(4,5)P_2 hydrolysis appear to take place only after Ca^{2+} entry [11,37,38]. Thus, it would be important to clarify whether the EGF-induced PtdIns(4,5)P_2 breakdown observed in this study is due to a PIC directly coupled to the EGF receptor or whether this receptor and its tyrosine kinase activity are linked to an early Ca^{2+} entry preceding and leading to PIC activation.

The mass of DAG generated after EGF stimulation exceeds that potentially arising via PtdIns(4,5)P_2 breakdown, so it is

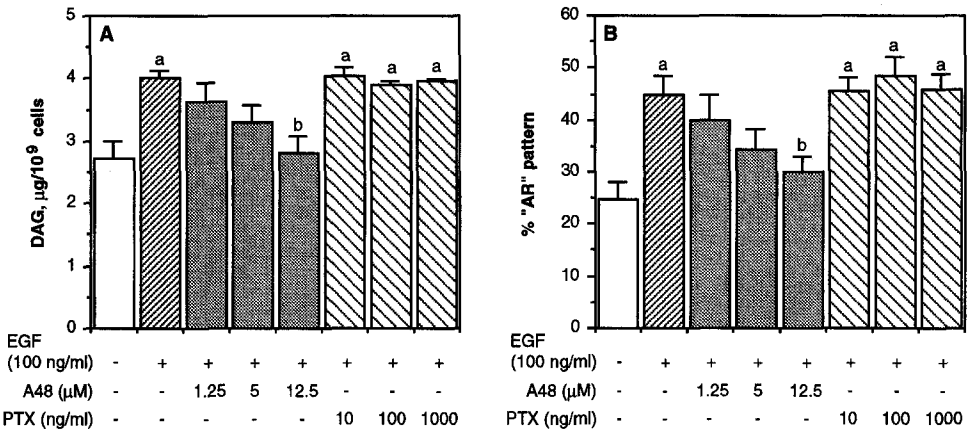

Fig. 3. Effect of tyrphostin A48 and pertussis toxin (PTX) on DAG generation and acrosomal exocytosis triggered by EGF in mouse spermatozoa. (A) DAG generation. Spermatozoa were capacitated in mT-B25 for 1 h, washed, resuspended in mT-H medium and exposed to A48 or PTX for 15 min before addition of EGF (100 ng/ml). Lipids were extracted 2.5 min after EGF, separated, and DAG quantified by Coomassie blue staining and densitometry ($n = 3$); a: different from control ($P < 0.05$); b: different from EGF ($P < 0.05$). (B) Acrosomal exocytosis. Spermatozoa were capacitated for 1.5 h in mT-B25; A48 or PTX were added for the last 15 min of capacitation. Cells were then stimulated with EGF for 15 min, stained with chlortetracycline and examined for exocytosis (% 'AR' pattern) ($n = 3$); a: different from control ($P < 0.05$); b: different from EGF ($P < 0.05$).

most likely that a phosphatidylcholine-specific phospholipase C is being activated in parallel to PIC or, perhaps, by PtdIns(4,5)P_2-derived DAG (cf. [12]). Activation of a phosphatidylcholine-phospholipase C in the absence of PIC-mediated PtdIns(4,5)P_2 breakdown has been found in several somatic cell systems after EGF stimulation ([39] and references therein), but it is not known whether such phospholipase C is directly coupled to the EGF receptor or whether there are other kinases involved in a phosphorylation cascade [40]. Therefore, the mechanism regulating the activation of sperm phospholipase C after EGF stimulation deserves further attention.

In conclusion, it is likely that EGF plays a significant role in the initiation of acrosomal exocytosis under physiological conditions. This growth factor is present in the oviduct [41,42] and could be present in the oocyte itself [20]. Concentrations of EGF even lower than those present in the oviduct [43] were able to enhance acrosomal exocytosis stimulated by ZP, the agonist believed to be the main inducer of exocytosis in this cell [1,34]. Thus, EGF could be an important co-factor during sperm activation at fertilization.

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