FEBS Letters 579 (2005) 4692–4700 FEBS 29837

GABA, progesterone and zona pellucida activation of PLA_2 and regulation by MEK-ERK1/2 during acrosomal exocytosis in guinea pig spermatozoa

Wen-Ying Chen[a], Ya Ni[a], Yong-Miao Pan[b], Qi-Xian Shi[a,*], Yu-Ying Yuan[a], Ai-Jun Chen[a], Li-Zhen Mao[a], Shu-Qing Yu[a], Eduardo R.S. Roldan[c,*]

[a] *Zhejiang Academy of Medical Science, 310013 Hangzhou, China*
[b] *Department of OB/GYN, School of Medicine, Zhejiang Universtity, 310006 Hangzhou, China*
[c] *Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid, Spain*

Received 24 May 2005; revised 22 June 2005; accepted 28 June 2005

Available online 10 August 2005

Edited by Angel Nebreda

Abstract **We investigated whether GABA activates phospholipase A_2 (PLA_2) during acrosomal exocytosis, and if the MEK-ERK1/2 pathway modulates PLA_2 activation initiated by GABA, progesterone or zona pellucida (ZP). In guinea pig spermatozoa prelabelled with [^{14}C]arachidonic acid or [^{14}C]choline chloride, GABA stimulated a decrease in phosphatidylcholine (PC), and release of arachidonic acid and lysoPC, during exocytosis. These lipid changes are indicative of PLA_2 activation and appear essential for exocytosis since inclusion of aristolochic acid (a PLA_2 inhibitor) abrogated them, along with exocytosis. GABA activation of PLA_2 seems to be mediated, at least in part, by diacylglycerol (DAG) and protein kinase C since inclusion of the DAG kinase inhibitor R59022 enhanced PLA_2 activity and exocytosis stimulated by GABA, whereas exposure to staurosporine decreased both. GABA-, progesterone- and ZP-induced release of arachidonic acid and exocytosis were prevented by U0126 and PD98059 (MEK inhibitors). Taken together, our results suggest that PLA_2 plays a fundamental role in agonist-stimulated exocytosis and that MEK-ERK1/2 are involved in PLA_2 regulation during this process.**

Keywords: GABA; Progesterone; Zona pellucida; Exocytosis; PLA_2; MAP kinase

1. Introduction

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous systems (CNS), although GABAergic systems are also found in various peripheral tissues, including the female reproductive organs. GABA specific receptor sites and a GABA uptake system are present in the female genital tract of the rat and rabbit [1], and the human uterus, oviduct and ovary [2]. In addition, high concentrations of GABA exist in seminal plasma [3,4]. This evidence suggests that GABA may exert a regulatory role in mammalian sperm function [5].

GABA binding sites have been reported in sperm membranes [6–8] and the possible presence of a GABA receptor/Cl^- channel complex has been postulated in spermatozoa based on the detection of a $GABA_A$ receptor subunit in human sperm cells [9]. Further studies have revealed various $GABA_A$ receptor subunits [10–12], along with a $GABA_B$ receptor [13,14] in rat spermatozoa.

The physiological significance of GABA role(s) in spermatozoa has recently received considerable attention. GABA can mimic and potentiate the action of progesterone in inducing capacitation of ram [15], guinea pig and human [16] spermatozoa. GABA is also capable of inducing acrosomal exocytosis of mouse [17,18], rat [14] and human [19] spermatozoa, and inhibitors of GABA receptors block initiation of acrosomal exocytosis [9,17,18].

Stimulation of acrosomal exocytosis with zona pellucida (ZP) or progesterone leads to activation of phospholipases and subsequent production of lipid messengers and active metabolites. Among the phospholipases activated, phosphoinositide- and phosphatidylcholine (PC)-specific phospholipases C play a crucial role in the generation of diglycerides that are essential for downstream events [17,20]. In addition, progesterone and ZP trigger activation of phospholipase A_2 (PLA_2) and release of fatty acids and lysophospholipids important for membrane fusion [21,22]. GABA can stimulate activation of phospholipases C [17,23] but it is not known whether it triggers activation of PLA_2. In addition, there is still little information regarding mechanisms regulating PLA_2 activation in spermatozoa; evidence suggests that both DAG-PKC and cAMP-PKA pathways may be involved [22,24].

One possible mechanism regulating PLA_2 activation in sperm cells involves a Mitogen Activated Protein (MAP) kinase pathway, particularly that involving the highly conserved extracellular-signal regulated kinase (ERK1/2) module [24]. This ERK module is also involved in functions other than activation of transcription factors (reviewed in [25–27]) and is

*Corresponding authors. Fax: +34 91 564 5078.
E-mail addresses: qxshi@mail.hz.zj.cn (Q.-X. Shi), roldane@mncn.csic.es (E.R.S. Roldan).

Abbreviations: ATA, aristolochic acid; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GABA, γ-aminobutyric acid; LCa^{2+}-MCM, low-calcium minimal capacitation medium; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase (ERK) kinase; PC, phosphatidylcholine; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; ZP, zona pellucida

0014-5793/$30.00
doi:10.1016/j.febslet.2005.06.090

known to act in the regulation of PLA_2 in somatic cells [28–31]. Components of the MAP kinase kinase (MEK)-ERK1/2 pathway have been identified in mammalian spermatozoa [32,35]. However, evidence is not unanimous in favour of its role in acrosomal exocytosis, with some studies failing to detect evidence [33,34], and others supporting it [35–37].

The present study was designed to (a) characterize sperm PLA_2 activation in response to GABA and analyze whether its activation is necessary for GABA-induced exocytosis, (b) examine if GABA-induced activation of PLA_2 involves regulation by the DAG-PKC pathway, and (c) explore whether the MEK-ERK1/2 kinase pathway regulates PLA_2 activation triggered by GABA and compare this response with that elicited by progesterone or ZP.

2. Materials and methods

2.1. Reagents and incubation media

[1-^{14}C]Arachidonic acid (56 mCi/mM; toluene solution), and [methyl-^{14}C]choline chloride (55 mCi/mM) were purchased form Amersham Pharmacia Biotech, UK, Ltd (Little Chalfont, UK). Chemicals (reagent grade) and reagents were purchased from Sigma (St Louis, MO, USA) and Shanghai Chemical Reagents Co. (Shanghai, China). Percoll was obtained from Amersham Biosciences AB (Uppsala, Sweden). Organic solvents were of reagent grade and were obtained from Shanghai Chemical Reagents Co. Arachidonic acid, phospholipids and neutral lipids used as standards were purchased from Sigma. Staurosporine, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0124), and 2-amino-3-methoxyflavone (PD98059) were purchased from Calbiochem (La Jolla, CA, USA).

The medium used throughout this study was a low-Ca^{2+} minimal capacitation medium (LCa^{2+}-MCM) previously described [16]. Although no Ca^{2+} was added to this medium, the Ca^{2+} concentration was 23 μM when measured. This low-Ca^{2+} medium induces capacitation of guinea pig spermatozoa under in vitro conditions but does not support acrosomal exocytosis [21,38]. When required, 2 mM $CaCl_2$ were added.

2.2. Collection and preparation of spermatozoa

White and black retired male guinea pigs (750 ± 30 g body weight) were purchased from the Center for Experimental Animals, Zhejiang University, and housed in environmentally controlled rooms with 12-h light/dark cycles, and maintained at approximately 20 ± 2 °C. Food and water were provided ad libitum. Animals were killed with CO_2. The caudae epididymides and vasa deferentia were incised and their contents were milked into LCa^{2+}-MCM. Spermatozoa (final concentration: $2–3 \times 10^7$ cells/ml) were incubated for 1 h in a capped jar in a shaking water bath (Wagen, Japan Ferrotec, Hangzhou, China) and then incubated at 38.5 °C under 5% CO_2 in air. Sperm viability at this stage was 90–95% as estimated using a trypan blue exclusion test and phase contrast microscopy.

2.3. Capacitation and labelling of spermatozoa

Spermatozoa were labelled with 2 μCi [methyl-^{14}C]choline chloride/ml or 0.5 μCi [^{14}C]arachidonic acid/ml by incubating them for 5–6 h at 35.8 °C under 5% CO_2 in air. During this period, the viability of spermatozoa remained constant (85–90%) as estimated using the trypan blue exclusion test and phase contrast microscopy. Spermatozoa were washed through a Percoll gradient (30–55–85% Percoll in LCa^{2+}-MCM) by centrifugation for 18 min at $700 \times g$. After centrifugation, the supernatant was removed leaving in each tube about 0.3 ml of the infranatant (85% Percoll) in which the spermatozoa were loosely pelleted. The pellet was diluted 1:10 (v/v) with LCa^{2+}-MCM and centrifuged again at $400 \times g$ for 8 min. After centrifugation the supernatant was removed and spermatozoa were diluted in Ca^{2+}-containing MCM (final concentration: $2–3 \times 10^7$ cells/ml). At this stage, 85% viable cells were found.

2.4. Isolation and preparation of zona pellucida

Female guinea pigs (21–22 days old) of the White-with-Flower-spots strain were killed with CO_2 and the ovaries removed. The ZP were isolated as described previously [21] and were solubilized at 60 °C for 1 h before use.

2.5. Experimental design

Stocks solutions of progesterone (15 mM), staurosporine (10 mM), MEK inhibitors U0126 (1.3 mM), and its inactive control U0124 (1.3 mM), and PD98059 (5 mM) were prepared in DMSO. When

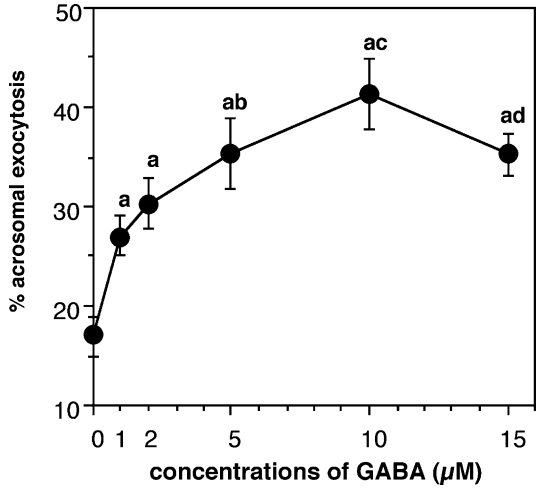

Fig. 1. Concentration-dependence of GABA-induced acrosomal exocytosis in guinea pig spermatozoa. Spermatozoa were preincubated at 38.5 °C under 5% CO_2 in air in LCa^{2+}-MCM for 6 h, washed, resuspended in similar medium with 2 mM Ca^{2+}, and exposed to GABA for 15 min. Acrosomal exocytosis was assessed by phase-contrast microscopy. Results are means ± S.E.M. ($n = 3$). (a) Significantly different from control ($P < 0.0002$); (b) significantly different from 2 μM or 10 μM GABA ($P < 0.04$); (c) significantly different from 5 μM or 15 μM GABA ($P < 0.02$); (d) significantly different from 10 μM GABA ($P = 0.01$).

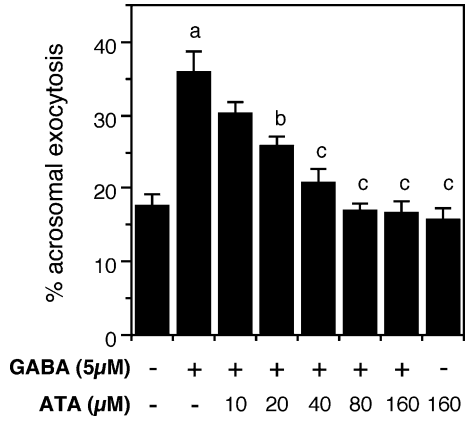

Fig. 2. Effect of the PLA_2 inhibitor aristolochic acid (ATA) on acrosomal exocytosis induced by GABA in guinea pig spermatozoa. Spermatozoa were capacitated in LCa^{2+}-MCM medium for 6 h washed, resuspended in MCM with 2 mM Ca^{2+}, exposed to various concentrations of ATA for 5 min, and then treated with 5 μm GABA for 15 min before examination for the occurrence of acrosomal exocytosis. Results are means ± S.E.M. from three experiments. Letters above bars indicate statistically significant differences: (a) different from control ($P < 0.01$); (b) different from GABA 5 μM ($P < 0.05$); (c) different from GABA 5 μM ($P < 0.01$).

diluted, final concentrations of DMSO were 1% (v/v) or lower and they did not affect sperm motility or acrosomal integrity. Stocks of aristolochic acid (ATA, sodium salt, 10 mM) were made up weekly in triple-distilled water and were kept at −20 °C. GABA stock (10 mM) was prepared daily in 0.16 M NaCl and further diluted in the same solvent.

For experiments, spermatozoa preincubated for 6 h (i.e. capacitated) were resuspended in MCM containing 2 mM Ca^{2+} (final concentration: $2–3 \times 10^7$ cells/ml) and exposed to test reagents (or their solvents as controls) for 5–15 min at 38.5 °C under 5% CO_2/air. Spermatozoa were then treated with GABA (1–15 μM), progesterone (5 or 10 μM) or ZP (1 ZP/μl), incubated for another 15 min under similar conditions and then lipids were extracted and analyzed. To test effects on acrosomal exocytosis, unlabelled capacitated spermatozoa were first exposed to reagents (or their solvents as controls), were then treated with GABA (5 μM), progesterone (5 or 10 μM) or ZP (1 ZP/μl)(or left untreated, as control) and examined using phase contrast microscopy. A total of at least 600 spermatozoa were counted to assess acrosomal exocytosis in each sample.

2.6. Lipid analyses in labelled spermatozoa

For quantification of arachidonic acid, incubations of spermatozoa prelabelled with [^{14}C]arachidonic acid were terminated by the addition of chilled chloroform/methanol (1:2, v/v) and lipids were extracted and analyzed as described previously [21]. To measure changes in lysoPC and PC, incubations of spermatozoa prelabelled with [^{14}C]choline chloride were terminated and lipids extracted as described [21].

2.7. Statistical analyses

Data are expressed as means ± S.E.M. For statistical analyses, data were transformed [$\log_{10}$ for lipid levels and arcsin $\sqrt{}$(percent of acrosome-reacted cells ÷ 100) for exocytosis] and comparisons were made with one-way ANOVAs and Fisher's post-hoc tests. Values of $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Stimulation of acrosomal exocytosis by GABA

To examine the effect of GABA on acrosomal exocytosis, spermatozoa were preincubated in LCa^{2+}-MCM for 6 h, washed and then resuspended in MCM with 2 mM Ca^{2+}. Spermatozoa were stimulated without or with 1–15 μM GABA for 15 min. The stimulatory effect was biphasic, with a significant, concentration-dependent increase in the proportion of spermatozoa undergoing acrosomal exocytosis with GABA concentrations of up to 10 μM (Fig. 1). Further

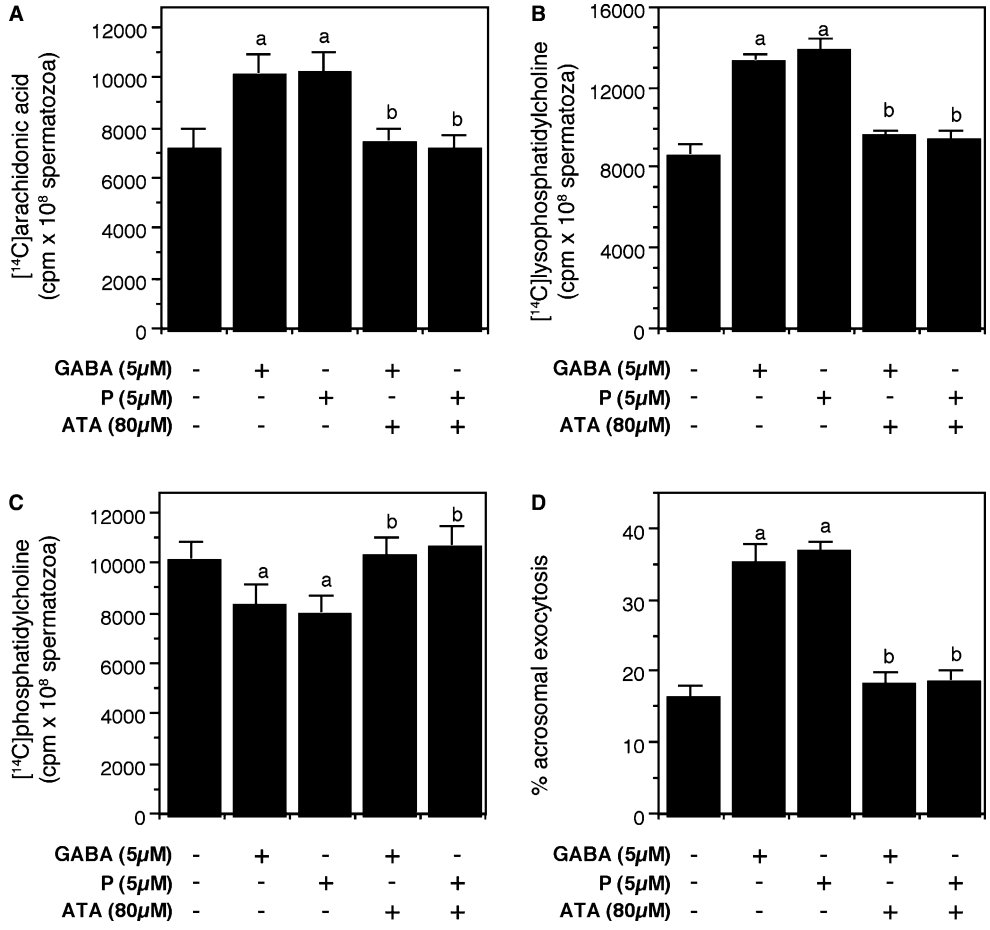

Fig. 3. Effect of the PLA_2 inhibitor ATA on changes in (A) arachidonic acid, (B) lysoPC, (C) PC, and (D) acrosomal exocytosis stimulated by GABA or progesterone in guinea pig spermatozoa. Spermatozoa were capacitated and labelled with [^{14}C]arachidonic acid or [^{14}C]choline chloride for 5 h, washed and resuspended in medium with 2 mM Ca^{2+}, and incubated with or without 80 μM ATA for 5 min. They were then stimulated with 5 μM GABA or 5 μM progesterone for 15 min before lipid extraction and analysis. Unlabelled spermatozoa were similarly treated and assessed for the occurrence of acrosomal exocytosis. Results are the means ± S.E.M. of three to five different experiments. For each graph, letters above bars indicate statistically significant differences: (a) significantly different from control ($P < 0.01$); (b) significantly different from GABA or progesterone ($P < 0.005$).

increases in GABA concentrations resulted in significantly lower responses.

3.2. ATA inhibits GABA- and progesterone-induced PLA_2 activity and acrosomal exocytosis

To test whether GABA-stimulated acrosomal exocytosis involves PLA_2 activation, capacitated guinea pig spermatozoa were pre-exposed to ATA, an effective inhibitor of sperm PLA_2 [21,39], before stimulation. Inclusion of ATA significantly inhibited GABA-induced acrosomal exocytosis in a concentration-dependent fashion (Fig. 2). This compound, when used alone, did not affect sperm motility or integrity.

We explored further if GABA-triggered responses involve PLA_2 activation by quantifying changes in PC, arachidonic acid and lysoPC. To this end, prelabelled, capacitated spermatozoa were stimulated with GABA in the presence of Ca^{2+} and, after 15 min of additional incubation, lipids were extracted and analyzed. After stimulation with GABA, levels of arachidonic acid and lysoPC were significantly higher than those seen in controls (Fig. 3A and B). In parallel samples, a decrease in PC was observed after GABA stimulation (Fig. 3C). The lipid changes induced by GABA were accompanied by acrosomal exocytosis in a large proportion of cells (Fig. 3D). Inclusion of 80 μM ATA abrogated all these responses (Fig. 3A–D), further confirming the involvement of PLA_2.

We compared responses to GABA with those seen after exposure to progesterone. After progesterone (5 μM) stimulation, levels of arachidonic acid and lysoPC increased, and PC levels decreased (Fig. 3A–C) and a large proportion of sperm cells experienced acrosomal exocytosis (Fig. 3D). As seen with GABA, inclusion of 80 μM ATA inhibited the responses triggered by progesterone (Fig. 3A–D).

3.3. GABA-induced activation of PLA_2 is modulated by the DAG-PKC pathway

To test whether PLA_2 activation stimulated by GABA involves any role for DAG, we used the DAG kinase inhibitor R59022 and examined if in its presence, which results in an increase in endogenous levels of DAG [21,40], there were changes in PLA_2 activation and exocytosis. Inclusion of R59022 (2 μM) before GABA resulted in an enhancement of arachidonic acid release (indicative of PLA_2 activation) and of acrosomal exocytosis (Fig. 4) suggesting a role for DAG in these events.

To test for PKC involvement, prelabelled capacitated spermatozoa were exposed to staurosporine (1 μM) before GABA stimulation. Staurosporine blocked GABA-induced activation of PLA_2 and acrosomal exocytosis (Fig. 5) thus suggesting that

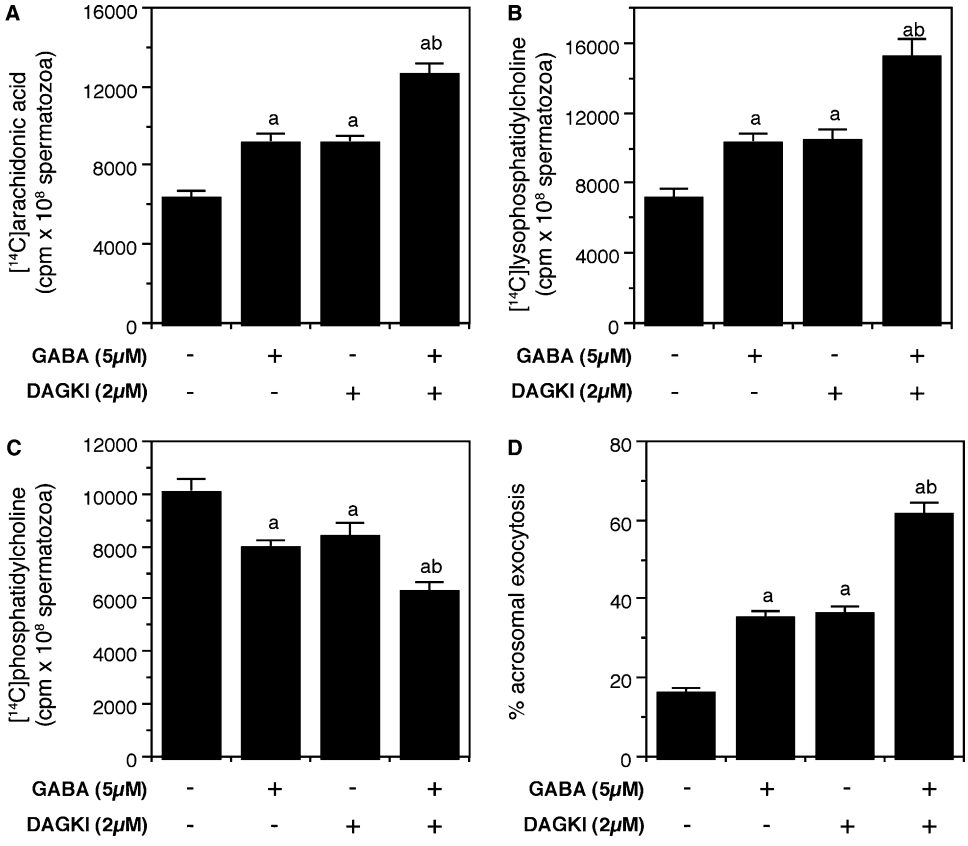

Fig. 4. Effect of the DAG kinase inhibitor R59022 on GABA-induced changes in arachidonic acid, lysoPC, PC and acrosomal exocytosis. Spermatozoa were labelled with [^{14}C]arachidonic acid for 5 h in LCa^{2+}-MCM, washed and resuspended in MCM with Ca^{2+}, or they were left unlabelled and treated similarly. The DAG kinase inhibitor (DAGKI) R59022 (2 μM) was added 5 min before GABA (5 μM) and incubation was extended for 15 min before lipid extraction and quantification (A, arachidonic acid; B, lysoPC; C, PC) and assessment of acrosomal exocytosis (D). Results are the means ± S.E.M. of six (arachidonic acid) or three (lysoPC, PC, acrosomal exocytosis) experiments. (a) Significantly different from control ($P < 0.01$ to $P < 0.0001$); (b) significantly different from GABA or DAGKI alone ($P < 0.002$).

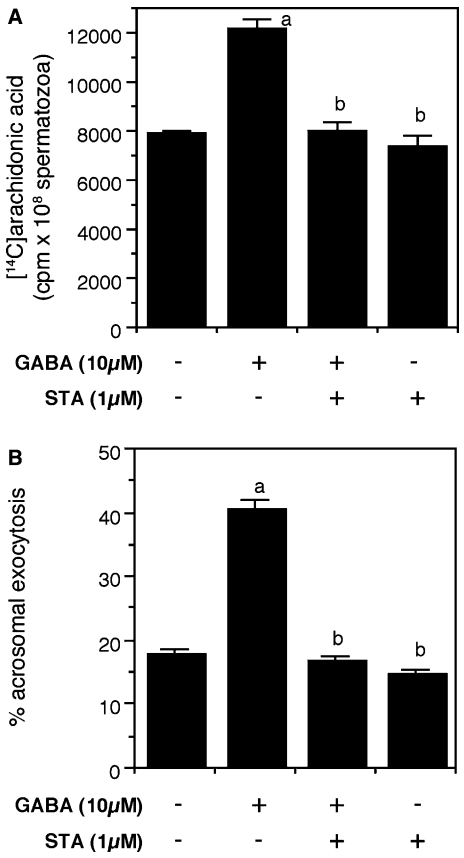

Fig. 5. Effect of staurosporine on GABA-induced arachidonic acid release and acrosomal exocytosis. Spermatozoa prelabelled with [^{14}C]arachidonic acid for 5 h in LCa^{2+}-MCM, were washed and resuspended in MCM with Ca^{2+}, or they were left unlabelled and treated similarly. Staurosporine (STA) (1 μM) was added 5 min before stimulation with GABA (10 μM) and sperm cells were further incubated for 15 min before lipid extraction and quantification (A) and assessment of acrosomal exocytosis (B). Results are the means ± S.E.M. of three different experiments. (a) Significantly different from control ($P < 0.0001$); (b) significantly different from GABA ($P < 0.0001$).

PKC is involved in the regulation of these events. Staurosporine, at the low concentration used in this study, may be acting on PKC and the effect is similar to that seen with chelerythrine chloride (another, more specific PKC inhibitor) on PLA_2 and exocytosis stimulated by progesterone or ZP [22].

3.4. GABA-, progesterone- and ZP-induced activation of PLA_2 is regulated by the MEK-ERK1/2 pathway

In a previous study, we found that progesterone- and ZP-induced PLA_2 activation is regulated by PKA and PKC [22]. Here, we tested in two series of experiments whether PLA_2 activation and exocytosis induced by GABA, progesterone or ZP is regulated by the MEK-ERK1/2 pathway.

In a first series of experiments, capacitated spermatozoa were exposed to various concentrations of the MEK inhibitors U0126 (0.065–1.3 μM) or PD98059 (10–100 μM) for 15 min and were then treated with 5 μM GABA, 10 μM progesterone or 1 ZP/μl for 15 min before assessment of acrosomal exocytosis. Results showed that U0126 or PD98059 caused a concentration-dependent inhibition of agonist-induced acrosomal exocytosis (data not shown). The maximal effects were seen with 0.5 μM U0126 or 50 μM PD98059, and these concentrations are similar to those found to inhibit MEK in somatic [41–43] and sperm cells [35–37]. These concentrations of inhibitors did not affect sperm motility or acrosomal integrity (data not shown). As control, we tested the effect of U0124, an inactive analogue of U0126, on acrosomal exocytosis induced by GABA, progesterone or ZP. It was found that inclusion of U0124 did not inhibit agonist-induced acrosomal exocytosis (data not shown).

In a second series of experiments the involvement of MEK-ERK1/2 in the regulation of PLA_2 activation and acrosomal exocytosis was further examined. Prelabelled and capacitated spermatozoa were exposed to U0126 or PD98059 for 15 min and were then stimulated with GABA (5 μM), progesterone (10 μM) or ZP (1/μl) and changes in arachidonic acid (as indicative of PLA_2 activation), and occurrence of exocytosis, were examined. Exposure of prelabelled and capacitated spermatozoa to 0.5 μM U0126 resulted in a complete inhibition of arachidonic acid release (Fig. 6A–C) and exocytosis (Fig. 6D–F) triggered by any of the agonists. Similarly, spermatozoa exposed to PD98059 (range 10–100 μM) showed a concentration-dependent inhibition of arachidonic acid release (Fig. 7A–C) and exocytosis (Fig. 7D–F) when they were treated with GABA, progesterone or ZP.

4. Discussion

The results of this study strongly suggest that GABA stimulates sperm PLA_2 activation and that this event is important for acrosomal exocytosis, a phenomenon resembling the effect of progesterone on sperm cells. Furthermore, PLA_2 activation appears to be modulated by the MEK-ERK1/2 kinase pathway when spermatozoa are stimulated to undergo exocytosis with GABA, progesterone or ZP.

Treatment of precapacitated guinea pig spermatozoa with GABA resulted in a concentration-dependent increase of exocytosis. The optimal concentration of GABA inducing acrosomal exocytosis in guinea pig spermatozoa was 10 μM. At lower (<10 μM) or higher concentration (>10 μM), GABA was less effective. This result is in agreement with earlier observations of GABA-stimulated acrosomal exocytosis of precapacitated mouse [18], human [19], and rat [14] spermatozoa. The optimal concentration of GABA in initiating acrosomal exocytosis varies slightly between species but in all cases the response is biphasic with higher concentrations producing lesser stimulation. Both $GABA_A$ [9,11,12] and $GABA_B$ [13] receptors have been identified in spermatozoa. Studies in rat sperm cells suggest that activation of the $GABA_B$ receptor could inhibit the $GABA_A$ receptor [14], which may result in GABA having this paradoxical effect. It is thus possible that GABA at high concentration (>10 μM in guinea pig spermatozoa) may also activate the $GABA_B$ receptor, thereby nullifying the stimulatory activity on the $GABA_A$ receptor, and thus leading to a decrease in acrosomal exocytosis. This could explain results of studies on human spermatozoa that have shown little or no effect of very low or high GABA concentrations on intracellular calcium rise or exocytosis [9,44,45]. On the other hand, it is also possible that differences in sperm capacitation status could be related to differences in responses to GABA [19].

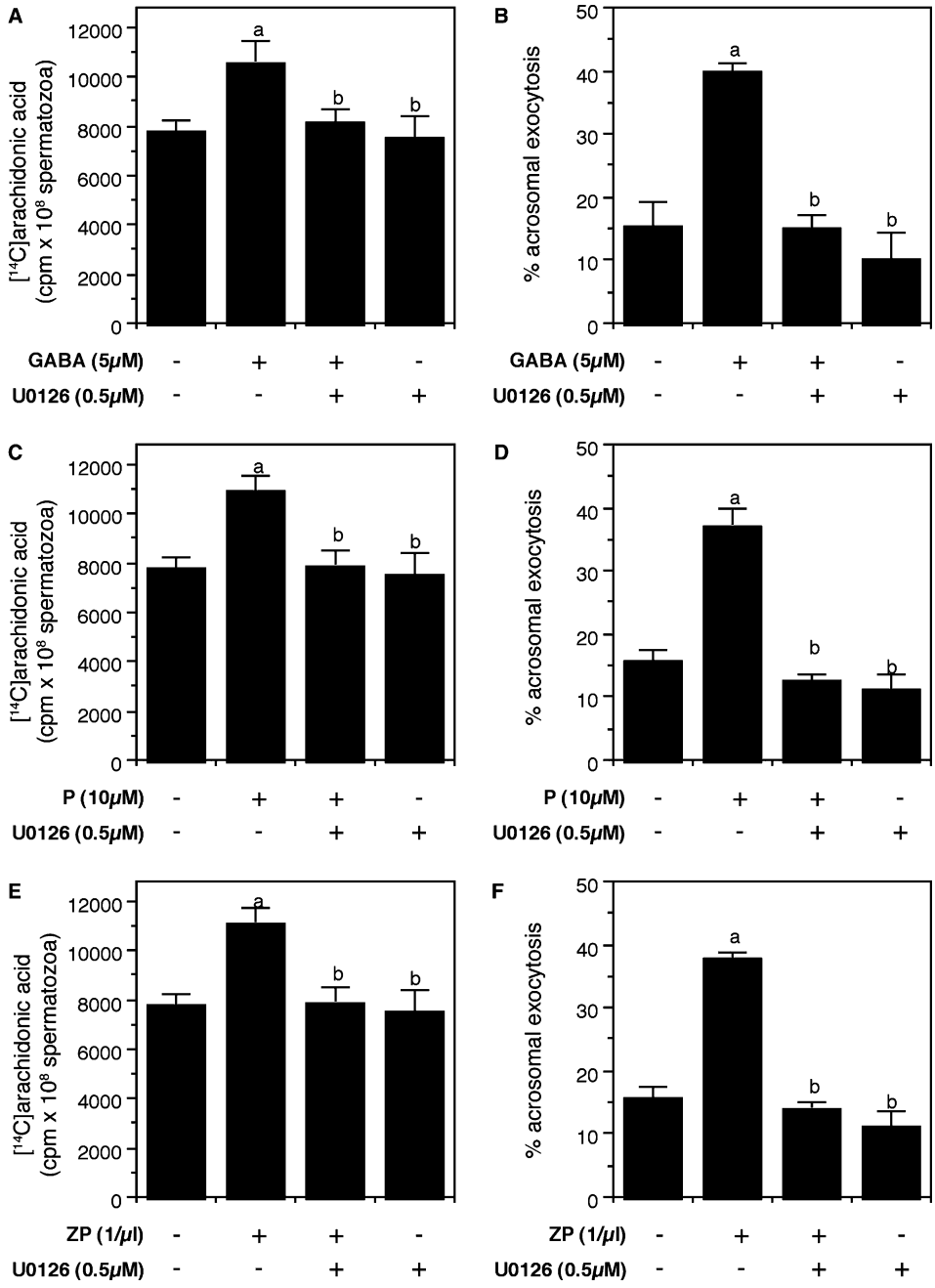

Fig. 6. Effect of U0126 on GABA-, progesterone (P)- or ZP-induced release of arachidonic acid and acrosomal exocytosis. Spermatozoa were labelled with [^{14}C]arachidonic acid for 5 h in LCa^{2+}-MCM, washed and resuspended in MCM with Ca^{2+}, or they were left unlabelled and treated similarly. U0126, a MEK inhibitor, was added to the medium 15 min before stimulation with 5 μm GABA (A, B), 10 μM progesterone (C, D) or 1 ZP/μl (E, F). Stimulation was extended for 15 min before lipid extraction and quantification (A, C, E) or assessment of acrosomal exocytosis (B, D, F). Results are the means ± S.E.M. of five different experiments. (a) Significantly different from control ($P < 0.0001$); (b) significantly different from GABA, progesterone or ZP ($P < 0.0002$).

Spermatozoa precapacitated in low-Ca^{2+} medium and simultaneously incubated with radioactive precursors [^{14}C]choline chloride or [^{14}C]arachidonic acid to label sperm phospholipids were used to characterize PLA_2 activation in response to GABA. Stimulation with this agonist led to an increase in arachidonic acid and lysoPC levels, and a parallel decrease in PC. These lipid changes are indicative of PLA_2 activity (demonstrating for the first time that GABA leads to activation of sperm PLA_2), and resemble changes observed when spermatozoa were stimulated with progesterone, as seen in this study, and earlier work [22,46], or ZP [21,22]. GABA-induced activation of PLA_2 seems essential for exocytosis because the PLA_2 inhibitor aristolochic acid blocked these lipid changes and acrosomal exocytosis. It has been postulated that, under natural conditions, GABA actions would be mimicked by progesterone, with the steroid acting both on $GABA_A$ receptors and progesterone receptors on the surface of the sperm membrane [17–19]. However, it is also possible that GABA itself could have a physiological role in acrosomal exocytosis (and other sperm functions) due to its presence in the

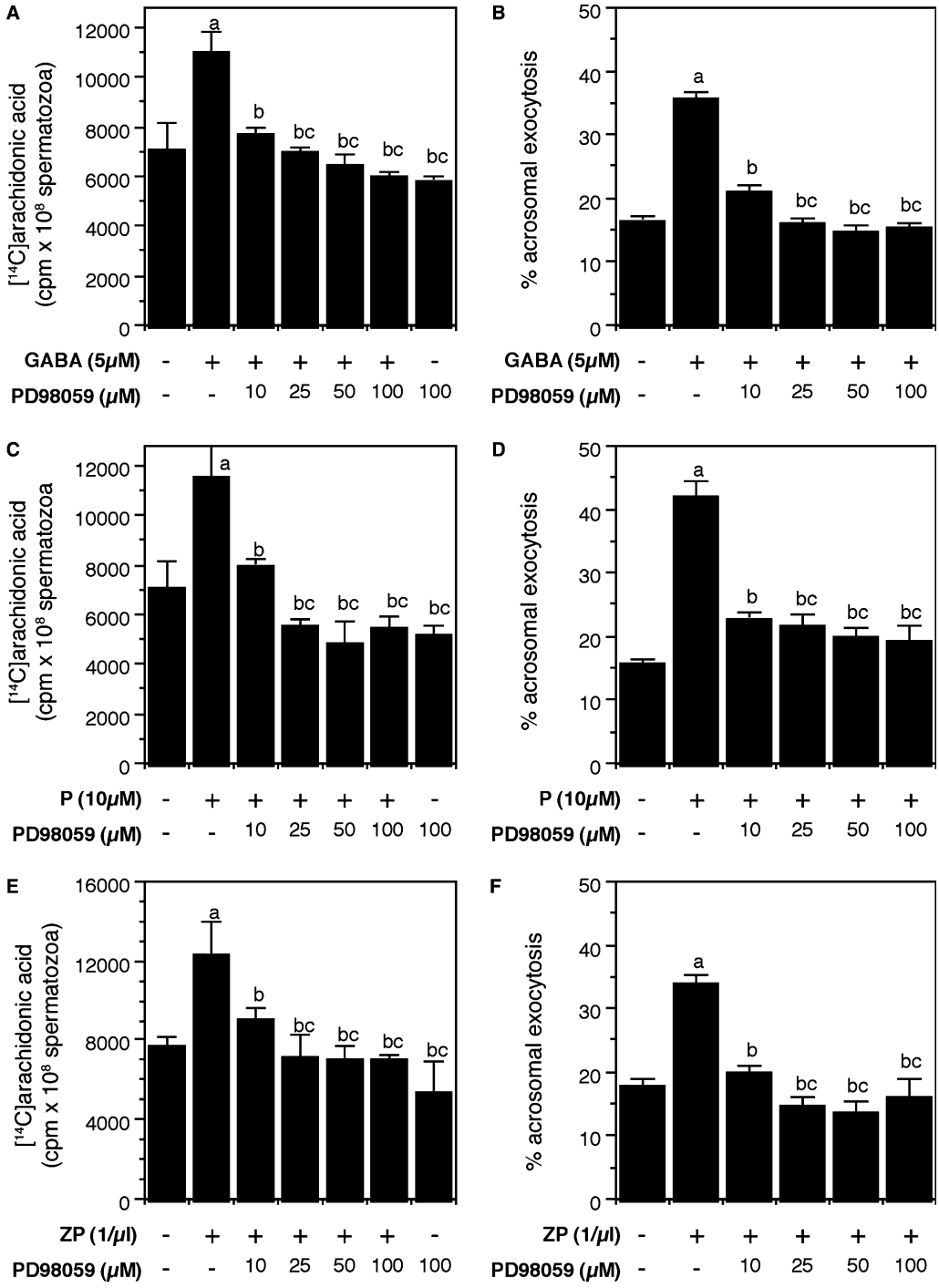

Fig. 7. Effect of PD98059 on GABA-, progesterone (P)- or ZP-induced release of arachidonic acid and acrosomal excytosis. Spermatozoa were capacitated and labelled with [^{14}C]arachidonic acid for 5 h in LCa^{2+} MCM, washed and resuspended in MCM with Ca^{2+}, or they were left unlabelled and treated similarly. Spermatozoa were exposed to various concentrations of PD98059 for 15 min and challenged with 5 μM GABA (A,B), 10 μM progesterone (C, D), or 1 ZP/μl (E, F) for 15 min before lipid extraction and separation (A, C, E) or assessment of acrosomal exocytosis (B, D, F). Results are the means ± S.E.M. of 3–5 different experiments. (a) Different from control ($P < 0.0001$); (b) significantly different from GABA, progesterone or ZP ($P < 0.0002$), (c) different from agonist *plus* 10 μM PD98059 ($P < 0.02$ to $P < 0.0001$).

female genital tract. Future studies should characterise this further.

Several mechanisms may regulate PLA_2 during acrosomal exocytosis, and differences may exist depending on the agonist triggering exocytosis in spermatozoa. Evidence for the involvement of various pathways has already been presented, namely: (a) G_i-protein mediated PLA_2 activation after ZP stimulation, but not after progesterone stimulation [21], (b) activation by PKA after stimulation with progesterone or ZP [22], (c) a direct modulation by DAG [21,47], and (d) regulation by PKC in sperm cells stimulated with progesterone or ZP [22]. The present study has revealed that the DAG-PKC pathway is also important for the modulation of PLA_2 activation triggered by GABA stimulation.

Other pathways may also be involved in PLA_2 regulation. Recent evidence indicates that ERKs (ERK1/ERK2) belonging to the family of MAP kinases are present in spermatozoa and could be involved in regulation of motility [33,34,48,49], capacitation [32–35,50], and acrosomal exocytosis [35,36]. However, the targets of the ERK1/2 kinase pathway in spermatozoa are not known. We explored if the MEK-ERK1/2 kinase pathway regulates PLA_2 activation by using two inhibitors of MEK-mediated ERK1/ERK2 activation (PD98059 [41,42]; U0126 [43]) and following changes in PLA_2-mediated arachidonic acid release and exocytosis. These inhibitors have been previously found to inhibit MEK-ERK1/2-induced phosphorylation and capacitation [33–35] in human spermatozoa. In addition, PD98059 and U0126 were found to inhibit lysoPC-induced acrosomal exocytosis in capacitated human spermatozoa [35,37] and ZP- or A23187-induced exocytosis, also in capacitated human spermatozoa [36]. However, another study could not detect evidence for inhibition of progesterone- or A23187-induced acrosomal exocytosis by PD98059 [33].

Our results showed that U0126 completely blocked exocytosis induced by GABA, progesterone or ZP, whereas its inactive analogue U0124 had no such effect. U0126 also inhibited arachidonic acid release triggered by these three agonists, suggesting that the MEK-ERK1/2 pathway may be necessary for PLA_2 activation. Similar results were obtained with PD98059, which inhibited both arachidonic acid release and acrosomal exocytosis stimulated by GABA, progesterone or ZP.

It is interesting the all three agonists of exocytosis (GABA, progesterone and ZP) stimulate activation of PLA_2 and that pathways leading to such activation involve the MEK-ERK1/2 pathway. However, these agonists may not elicit the same upstream signalling processes leading to activation of the MEK-ERK1/2 pathway since previous work has revealed that GABA and progesterone effects are transduced by tyrosine kinase(s) but not by G_i proteins, whereas ZP action is mediated by both tyrosine kinase(s) and G_i proteins [20].

Taken together, our results indicate that exocytosis initiated by physiological agonists involves PLA_2 activation, that this activation may be regulated by the MEK-ERK1/2 pathway, and that this MAP kinase-regulated PLA_2 activation is important for acrosomal exocytosis. Furthermore, our results suggest that GABA could play an important role in sperm function by triggering DAG-PKC- and MEK-ERK1/2-modulated PLA_2 activation during acrosomal exocytosis.

Acknowledgements: This investigation was supported by the National "973" project (G199905592) and the National Natural Science Foundation of China (No. 39870364). The authors are grateful to Professors Q.X. Shao, for assistance with liquid scintillation counting, and C.Y. Yan (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai) for help in acquiring radioactive isotopes.

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