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

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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

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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

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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

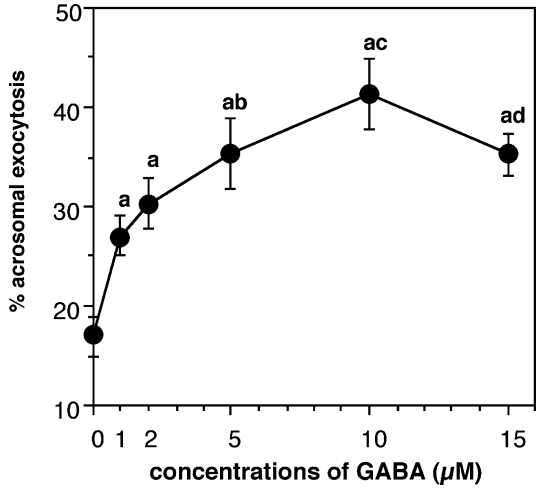

Fig. 1. Concentration-dependence of GABA-induced acrosomal exocytosis in guinea pig spermatozoa. Spermatozoa were preincubated at 38.5 °C under 5% CO_2 in air in LCa^{2+}-MCM for 6 h, washed, resuspended in similar medium with 2 mM Ca^{2+}, and exposed to GABA for 15 min. Acrosomal exocytosis was assessed 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 ($P = 0.01$).

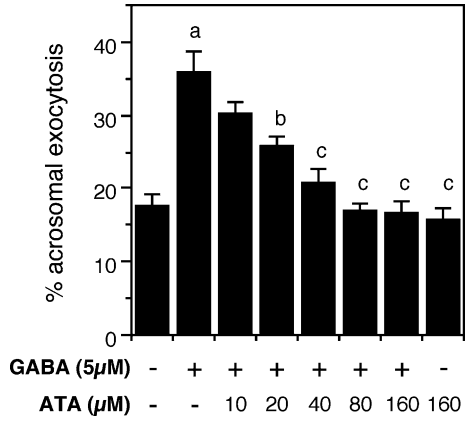

Fig. 2. Effect of the PLA_2 inhibitor aristolochic acid (ATA) on acrosomal exocytosis induced by GABA in guinea pig spermatozoa. Spermatozoa were capacitated in LCa^{2+}-MCM medium for 6 h washed, resuspended in MCM with 2 mM Ca^{2+}, exposed to various concentrations of ATA for 5 min, and then treated 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 ($P < 0.01$).