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Tandem mass spectrometry identifies proteins phosphorylated by cyclic AMP-dependent protein kinase when sea urchin sperm undergo the acrosome reaction

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Abstract

The exocytotic acrosome reaction (AR), which is required for fertilization, occurs when sea urchin sperm contact the egg jelly (EJ) layer. Among other physiological changes, increases in adenylyl cyclase activity, cAMP and cAMP-dependent protein kinase (PKA) activity occur coincident with the AR. By using inhibitors of PKA, a permeable analog of cAMP and the phosphodiesterase inhibitor IBMX, we show that PKA activity is required for AR induction by EJ. A minimum of six sperm proteins are phosphorylated by PKA upon exposure to EJ, as detected by a PKA substrate-specific antibody. The phosphorylation of these proteins and the percentage of acrosome reacted sperm can be regulated by PKA modulators. The fucose sulfate polymer (FSP), a major component of EJ, is the molecule that triggers sperm PKA activation. Extracellular Ca^{2+} is required for PKA activation. Six sperm proteins phosphorylated by PKA were identified by tandem mass spectrometry (MS/MS) utilizing the emerging sea urchin genome. Based on their identities and localizations in sperm head and flagellum, the putative functions of these proteins in sperm physiology and AR induction are discussed.

Keywords: Fertilization; Acrosome reaction; Tandem mass spectrometry; Cyclic nucleotides; Invertebrate reproduction

Introduction

For successful fertilization, sea urchin sperm must activate flagellar motility, swim to the egg, undergo the acrosome reaction (AR), bind to the egg vitelline layer, penetrate the vitelline layer and fuse with the egg plasma membrane (Vacquier, 1998). The AR of sea urchin sperm involves the exocytosis of the acrosomal vesicle and the polymerization of actin to form the acrosomal process. The EJ-induced AR depends on a net influx of Ca^{2+} and Na^{+} and a net efflux of K^{+} and H^{+} (Darszon et al., 2001, 2005; Neill and Vacquier, 2004). Among other changes, EJ increases sperm adenylyl cyclase activity 50-fold (Watkins et al., 1978), elevates cAMP levels over 100-fold and increases PKA activity 4- to 8-fold (Garbers et al., 1980; Garbers and Kopf, 1980).

In mammalian sperm, PKA induces the phosphorylation of several proteins required for capacitation, a series of changes that enable sperm to undergo the AR (Harrison, 2004; O'Flaherty et al., 2004; Visconti et al., 1995). PKA is also involved in the human sperm AR, induced by the egg zona pellucida (Bielfeld et al., 1994), progesterone (Harrison et al., 2000), follicular fluid and oviductal fluid (De Jonge et al., 1993) and the calcium ionophore A23187 (Lefievre et al., 2002). Gene deletion of the PKA catalytic subunit, Cα2, results in infertile male mice (Nolan et al.,

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2004). Cα2 is required for the enhancement of sperm motility by bicarbonate. However, whether sperm of the Cα2 knock out mice can undergo the AR remains unknown.

In both sea urchins and mammals, although PKA pathways seem to be generally important in sperm physiology, preparing the sperm for fertilization, few proteins have been identified as substrates for PKA phosphorylation (Bracho et al., 1998; Harrison, 2004; Porter et al., 1988; Tash and Bracho, 1999). Here we show that PKA-mediated phosphorylation of sea urchin sperm proteins is required for the EJ-induced AR and we use tandem mass spectrometry to identify six of these phosphoproteins.

Materials and methods

Materials

Alexa Fluor 488 phalloidin, Alexa Fluor 546 goat anti-rabbit IgG, fura-2 AM and Pluronic F-127 were from Molecular Probes. H-89 and myristoylated protein kinase A inhibitor peptide, 14–22 amide, were from Calbiochem. H-85 was from Seikagaku Corporation. cAMP-AM and protease inhibitor cocktail were from Sigma. Phospho-(Ser/Thr) PKA substrate antibody was from Cell Signaling Technology (Catalog number 9621). Speract was from Peninsula Laboratories. Anti-bindin antibody was prepared as described (Moy and Vacquier, 1979). All other reagents and chemicals were from Sigma. H-89 and H-85 were made as 10 mM stock solutions in DMSO and diluted into sperm suspensions so that the final DMSO concentration was 0.3%. The 0-μM control also contained 0.3% DMSO.

Gametes

Sea urchin (*Strongylocentrotus purpuratus*) gametes were spawned by injection of 0.5 M KCl into adults. Undiluted semen was collected and kept on ice for no longer than 24 h. Egg jelly (EJ), fucose sulfate polymer (FSP) and EJ sialoglycans (SG) were prepared and quantified as described (Hirohashi and Vacquier, 2002).

Acrosome reaction assay

Undiluted semen was diluted 1:100 in ASW (486 mM NaCl, 10 mM $CaCl_2$, 10 mM KCl, 27 mM $MgCl_2$, 29 mM $MgSO_4$, 2.5 mM $NaHCO_3$ and 10 mM HEPES, adjusted to pH 8.0 with 1 N NaOH). Sperm suspensions were mixed with various reagents before exposure to EJ (15°C) for 2 min. Fifty-microliter sperm suspensions were fixed for 30 min by adding 750 μl of 3% paraformaldehyde in ASW. A recently published method to score AR (Biermann et al., 2004) was slightly modified as follows. Fixed sperm were washed twice by resuspension in 500 μl of PBS followed by a 2-min centrifugation at 5000 × *g*. The sperm pellet was then stained with 0.4 units Alexa Fluor 488 phalloidin in 100 μl 1 mg/ml BSA in PBS for 2 h in the dark. The cells were washed three times with 1 ml PBS and resuspended in 70% glycerol in PBS. Acrosome reacted sperm were then scored by fluorescence microscopy.

Immunoblots

For whole sperm protein preparation, sperm suspensions were precipitated with acetone (80% final concentration), sedimented by centrifugation for 5 min at 21,000 × *g* and the pellet dissolved in 10% SDS. Isolation of sperm heads from flagella was done as previously described (Vacquier and Hirohashi, 2004). Sperm proteins were separated on 4–15% SDS/PAGE precast gels (BioRad) and transferred to PVDF. The PVDF membranes were probed with the phospho-(Ser/Thr) PKA substrate antibody, detected with an HRP-conjugated goat anti-rabbit secondary antibody (following the manufacturer's instructions) and developed with SuperSignal West Dura Extended Duration Substrate (Pierce).

Measurement of intracellular Ca^{2+}

Sperm were loaded with fura-2 AM and intracellular Ca^{2+} was measured as previously described (Darszon et al., 2004; Su and Vacquier, 2002). Briefly, undiluted semen was diluted 5-fold in ASW and incubated with 12 μM fura-2 AM overnight on ice in the dark. Free fura-2 AM was removed by centrifugation at 1000 × *g* for 10 min at 4°C. The cell pellets were washed twice with ASW. Ca^{2+} measurements were at 15°C under constant stirring in a FluoroMax-2 fluorometer with excitation at 340 and 380 nm and emission at 500 nm.

Identification of proteins by MS/MS

For immunoprecipitation, EJ-treated sperm were extracted for 30 min in RIPA buffer (1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium vanadate, 100 nM okadaic acid, 10 μg/ml aprotinin, 1 mM benzamidine and 1:100 dilution of protease inhibitor cocktail). The RIPA-solubilized proteins were obtained after centrifugation at 26,000 × *g* for 90 min. Two hundred microliters supernatant (2 mg/ml) was precleared by incubation with 2 μl of normal rabbit IgG (100 μg/ml) and 20 μl of 50% Protein-A Sepharose CL4B beads (Amersham Biosciences) for 2 h. The precleared supernatants were incubated overnight with the phospho-(Ser/Thr) PKA substrate antibody at a 1:100 dilution of the commercial stock. Twenty microliters of 50% Protein-A Sepharose CL4B beads was added to the antibody-supernatant mixture and incubated for 2 h. Precipitated immunocomplexes, bound to the Protein-A