SNAP-25 is essential for cortical granule exocytosis in mouse eggs

YOSHIHIDE IKEBUCI, NOBUYUKI MASUMOTO, TETSU MATSUOKA, TAKESHI YOKOI, MASAIRO TAHARA, KEIICHI TASAKA, AKIRA MIYAKE, AND YUJI MURATA

Department of Obstetrics and Gynecology, Osaka University Medical School, Osaka 565, Japan

Ikebuchi, Yoshihide, Nobuyuki Masumoto, Tetsu Matsuoka, Takeshi Yokoi, Masahiro Tahara, Keiichi Tasaika, Akira Miyake, and Yuji Murata. SNAP-25 is essential for cortical granule exocytosis in mouse eggs. Am. J. Physiol. Cell Physiol. 274 (Cell Physiol. 43): C1496–C1500, 1998.—Synaptosome-associated protein of 25 kDa (SNAP-25) has been shown to play an important role in Ca2+-dependent exocytosis in neurons and endocrine cells. During fertilization, sperm-egg fusion induces cytosolic Ca2+ mobilization and subsequently Ca2+-dependent cortical granule (CG) exocytosis in eggs. However, it is not yet clear whether SNAP-25 is involved in this process. In this study, we determined the expression and function of SNAP-25 in mouse eggs. mRNA and SNAP-25 were detected in metaphase II (MII) mouse eggs by RT-PCR and immunoblot analysis, respectively. Next, to determine the function of SNAP-25, we evaluated the change in CG exocytosis with a membrane dye, tetramethylammonium-1,6-diphenyl-1,3,5-hexatriene, after microinjection of a botulinum neurotoxin A (BoNT/A), which selectively cleaves SNAP-25 in MII eggs. Sperm-induced CG exocytosis was significantly inhibited in the BoNT/A-treated eggs. The inhibition was attenuated by coinjection of SNAP-25. These results suggest that SNAP-25 may be involved in Ca2+-dependent CG exocytosis during fertilization in mouse eggs.

CORTICAL GRANULE (CG) exocytosis in mammals is one of the most important steps in fertilization after sperm-egg fusion. The CG content, which is released from eggs in response to the elevation of intracellular free Ca2+ at fertilization (24), plays a role in preventing polyspermy (9, 12, 29). Intracellular Ca2+ mobilization in eggs induced by sperm is known to be essential for CG exocytosis (13, 26). However, little is known about the intracellular signaling mechanism in CG exocytosis downstream of Ca2+ mobilization.

In synaptic cells, a model for docking and fusion of vesicles with the plasma membrane in exocytosis has been established (25). This docking and fusion process is mediated by the general fusion machinery, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) system (19). This SNARE system has been identified in the mammalian brain (2, 3, 18). The SNARE proteins are composed of several proteins, vesicle-associated membrane protein in the synaptic vesicle membrane, synaptosome-associated protein of 25 kDa (SNAP-25), and syntaxin in the presynaptic plasma membrane. Many reports have suggested that these SNARE proteins mediate the docking and fusion of synaptic vesicles to the plasma membrane (16, 22). In neurons and neuroendocrine cells, SNAP-25 is specifically cleaved at a site nine amino acid residues from the COOH-terminal by botulinum neurotoxin A (BoNT/A) (5, 6), which results in the inhibition of exocytosis. Additional proof for the involvement of SNAP-25 in the fusion process came from studies using this toxin (4, 20).

There have been no reports of SNARE proteins playing a role during fertilization in mouse eggs. In this study, we determined whether SNAP-25, one of the SNARE proteins, is involved in CG exocytosis. First, we investigated the expression of SNAP-25 mRNA and protein in mouse metaphase II (MII) eggs. The function of SNAP-25 in sperm-induced CG exocytosis was determined with the microinjection of BoNT/A into eggs. We demonstrate here that SNAP-25 is involved in Ca2+-dependent CG exocytosis during fertilization in mouse eggs.

MATERIALS AND METHODS

Preparation of MII eggs and spermatozoa. MII eggs were obtained from CD1 female mice (6–8 wk old, Charles River, Kanagawa, Japan) primed with 5 IU of pregnant mare serum gonadotropin and 5 IU of human chorionic gonadotropin (HCG) 48 h later. MII eggs were collected from the ampulla of oviducts (14–16 h after HCG injection) and placed into the collection medium. Modified human tubal fluid (101.6 mM NaCl, 4.69 mM KCl, 2.04 mM CaCl2, 0.20 mM MgSO4, 0.37 mM KH2PO4, 4 mM NaHCO3, 21.0 mM HEPES, 2.78 mM glucose, 0.33 mM pyruvic acid, 21.4 mM sodium lactate, 0.05 mg/ml streptomycin, 100 IU/ml penicillin G, and 0.01 mg/ml phenol red) was used as the collection medium. In some experiments, the zona pellucidae were removed by brief exposure (~1 min) to acid Tyrode solution (pH 2.5). The eggs were then drawn into and expelled from a small-bore pipette to ensure removal of the zona pellucidae. After each procedure, the eggs were thoroughly washed in human tubal fluid (HTF; 101.6 mM NaCl, 4.69 mM KCl, 2.04 mM CaCl2, 0.20 mM MgSO4, 0.37 mM KH2PO4, 25.0 mM NaHCO3, 2.78 mM glucose, 0.33 mM pyruvic acid, 21.4 mM sodium lactate, 0.05 mg/ml streptomycin, 100 IU/ml penicillin G, and 0.01 mg/ml phenol red). MII eggs were cultured in 100-µl drops of HTF under mineral oil on petri dishes and incubated in an atmosphere of 5% CO2-95% air at 37°C.

Spermatozoa were obtained from CD1 male mice (Charles River) with proven fertility. Spermatozoa were released from the caudal epididymis into the HTF. After incubation for 10 min in an atmosphere of 5% CO2-95% air at 37°C, the sperm concentration was adjusted to 1–2 × 106 spermatozoa/ml. The sperm suspension was overlaid with mineral oil and further incubated for 3 h.

mRNA extraction and RT-PCR. MII eggs were collected, immediately frozen in a liquid nitrogen, and stored at −80°C until mRNA was extracted by the oligo(dT)-cellulose method (Micro mRNA Purification Kit, Pharmacia Biotech). The mRNA isolated from the eggs was then reverse transcribed into cDNA. The SNAP-25 oligonucleotide primers were synthesized on a model 394 DNA/RNA synthesizer (Applied
As a positive control for SNAP-25, mouse brain was used. 

Chemiluminescence (Amersham International, Amersham, Bucks, U.K.) was performed using a Microslab gel (30 × 30 × 1 mm). Samples were run through the stacking and separating gels at 10 mA/gel. The proteins were electrophotically transferred onto a nitrocellulose membrane sheet, and the transferred proteins were immunoblotted using mouse monoclonal antibodies against SNAP-25 (Wako Pure Chemical Industries, Osaka, Japan). Immunoreactive bands were detected by using goat anti-mouse whole antibody conjugated to horseradish peroxidase, followed by detection with enhanced chemiluminescence (Amersham International, Amersham, UK). As a positive control for SNAP-25, mouse brain was prepared. The filters were exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY).

Labeling of CGs with Lens culinaris agglutinin. To determine the effect of microinjection on spontaneous CG exocytosis, CGs were evaluated by Lens culinaris agglutinin (LCA) staining. The MII eggs injected with BoNT/A (2 µM) or non-treated were fixed with Zamboni’s fixative (0.2% picric acid and 2% paraformaldehyde). These fixed eggs were washed in PBS and incubated for 15 min in 10 mg/ml FITC-conjugated LCA (E-Y Laboratories, San Mateo, CA) with 0.03% Triton X. The eggs were excited at 488 nm by the argon laser in the confocal laser microscope (Zeiss LSM 410), and confocal optical images of FITC-LCA were analyzed. The confocal parameters of scan rate, aperture, gain, black level, and frames accumulated were the same for all samples.

Measurement of CG exocytosis in living eggs. As previously described (14, 26), exocytosis in living eggs was measured using membrane-impermeable membrane probe tetramethylammonium-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH, Molecular Probes, Eugene, OR). Zona-free eggs were plated in a 90-µl drop of HTF on glass coverslips sealing a 1.0-cm hole in the bottom of 35-mm culture dishes coated with poly-l-lysine. Drops of HTF containing eggs were overlain with mineral oil, and 10 µl of 50 µM TMA-DPH was added into the drop at 37°C, to a final concentration of 5 µM. The 430-nm emission fluorescence of single eggs at an excitation wavelength of 350 nm was measured, and changes in the fluorescence intensity of individual eggs were analyzed with a digital imaging microscopy system (Zeiss LSM 410). The percentage increase of fluorescence represents the total amount of exocytosis. Eggs were intermittently irradiated for 1 s every 60 s to minimize ultraviolet damage. Spermatozoa or a Ca²⁺ ionophore (A-23187, 10 µM) was applied to the treated eggs, and CG exocytosis was observed.

Data analysis and statistics. The significance of differences was assessed by ANOVA, followed by Scheffé’s multiple comparison, and P < 0.01 was taken to be significant.

RESULTS

Expression of SNAP-25 mRNA in MII eggs. RT-PCR was performed to detect SNAP-25 gene transcripts in MII eggs. cDNA samples reverse transcribed from mRNA isolated from eggs were amplified using the primers described in MATERIALS AND METHODS. An amplified product of 200 bp was detected (Fig. 1), indicating that SNAP-25 mRNA was expressed in MII eggs. No amplified DNA fragments were observed when PCR was performed without prior reverse transcription (Fig. 1). This shows that the amplified fragments were generated from SNAP-25 mRNA and did not originate from genomic DNA.
Presence of SNAP-25 in MII eggs. We determined the presence of SNARE proteins in MII eggs by immunoblot analysis. Immunoreactive bands with anti-SNAP-25 antibodies were observed in mouse MII eggs as in mouse brains (Fig. 2), and the apparent molecular masses estimated from the molecular mass markers were 25 kDa for SNAP-25.

Cleavage of SNAP-25 in eggs by BoNT/A. To cleave SNAP-25 in MII eggs, we microinjected 5–10 pl BoNT/A (2 µM) into eggs. The proteins from the control and treated eggs were analyzed by immunoblotting. A reduction in the molecular mass of SNAP-25, by 1 kDa, was observed in the MII eggs treated with BoNT/A (Fig. 2). This indicates that SNAP-25 in MII eggs is sensitive to BoNT/A.

Distribution of CGs in BoNT/A-injected eggs. Before application of sperm, both control MII eggs and BoNT/A (2 µM)-injected MII eggs had similar CG distribution, with a CG-free domain occupying 40% of the cortex (Fig. 3). BoNT/A injection caused no change in the distribution of CGs.

Effects of BoNT/A on CG exocytosis at fertilization. To determine the function of SNAP-25 in eggs during fertilization, BoNT/A was microinjected into the egg cytoplasm. CG exocytosis was evaluated by the increase membrane surface area using the membrane dye TMA-DPH. In control eggs, insemination of sperm induced CG exocytosis (Fig. 4). However, CG exocytosis was significantly inhibited in eggs injected with BoNT/A (Fig. 4). CG exocytosis in eggs injected with BoNT/A (200 nM) was reduced to 50% of the control 30 min after insemination (Fig. 5). At a concentration of 2 µM BoNT/A, the exocytosis was decreased by 80% (Fig. 5), indicating that this inhibition was dependent on the concentration of BoNT/A (200 nM and 2 µM). CG exocytosis was not affected in eggs injected with heat-inactive BoNT/A (Fig. 5). Coinjection of BoNT/A and SNAP-25 that contained BoNT/A cleaves sites attenuated the inhibition of CG exocytosis by BoNT/A at 200 nM and 2 µM, respectively (Fig. 5).

Effects of BoNT/A on Ca$^{2+}$ ionophore-induced CG exocytosis. The Ca$^{2+}$ ionophore also induced CG exocytosis as much as sperm did. Next, to determine the...
function of SNAP-25 in Ca\(^{2+}\) ionophore-induced CG exocytosis, a Ca\(^{2+}\) ionophore was applied to MII eggs pretreated with BoNT/A. Ca\(^{2+}\) ionophore-induced CG exocytosis was significantly inhibited by BoNT/A (200 nM) (Fig. 6). On the other hand, sperm-induced cytosolic Ca\(^{2+}\) mobilization was not changed between control and BoNT/A injected eggs (not shown). These results suggest that BoNT/A specifically blocked a pathway downstream of Ca\(^{2+}\) mobilization.

**DISCUSSION**

It has recently been reported that SNAREs are expressed in not only synaptic cells but also endocrine cells (1, 11, 17) and that SNAP-25 is involved in Ca\(^{2+}\)-dependent exocytosis in pancreatic cells (20) and chromaffin cells (10). At fertilization, sperm also induces Ca\(^{2+}\)-dependent CG exocytosis in eggs. Thus, in the present study, we determined whether SNAP-25 plays a role in this process. We observed the expression of SNAP-25 mRNA in MII eggs by RT-PCR. Maternal mRNAs, which are degraded at the two-cell stage, support meiosis, fertilization, and the first cleavage in mice (7, 8). Moreover, we showed by immunoblot analysis that SNAP-25 are present in mouse MII eggs. This expression suggests that SNAP-25 may play an important role during the MII stage. We evaluated sperm or Ca\(^{2+}\)-ionophore-induced CG exocytosis to determine the function of SNAP-25 in MII eggs. We measured dynamic CG exocytosis in mouse eggs at fertilization using the membrane-labeling dye TMA-DPH. We previously established a method to measure the dynamics of exocytosis in living mouse eggs (26) and pituitary gonadotropes (15). We microinjected BoNT/A into eggs to inhibit the functions of SNAP-25. BoNT/A is known to specifically cleave the SNAP-25 at sites nine residues from the COOH-terminal (5, 6). The cleavage is confirmed by the reduction in its molecular mass as seen in the immunoblot using anti-SNAP-25 antibodies. We also showed that the microinjection of 2 µM BoNT/A resulted in the cleavage of SNAP-25 in MII eggs. In BoNT/A-injected eggs before application of sperm or ionophore, the distribution of CGs induced by sperm was similar to that in control eggs. Next, in BoNT/A (2 µM)-injected MII eggs, repetitive intracellular free Ca\(^{2+}\) mobilization induced by sperm was observed as in control MII eggs (data not shown). These results indicate that the injection of BoNT/A causes neither premature exocytosis nor cell damage.

Sadoul et al. (20) reported that BoNT/A cleavage of SNAP-25 resulted in the inhibition of Ca\(^{2+}\)-evoked insulin release in islet cells. We also found that Ca\(^{2+}\) ionophore or sperm-induced CG exocytosis in eggs was inhibited by BoNT/A, but not by heat-inactivated BoNT/A. However, there is a report suggesting that BoNT/A may cleave additional proteins besides SNAP-25. Bi et al. (4) showed that although SNAP-25 is not identified in sea urchin eggs, rescaling of plasma membrane by a vesicular mechanism is inhibited by BoNT/A. The inhibition may be due to cleavage of an isoform of SNAP-25 so far not detected with the available antibodies or to cleavage of an unrelated protein. Thus we have used SNAP-25 that had cleavage site by BoNT/A to block the effect of BoNT/A (4, 10, 23). The SNAP-25 attenuates the effect of BoNT/A, suggesting that BoNT/A may cleave SNAP-25 in eggs specifically. However, we cannot entirely exclude the possibility that BoNT/A may cleave another unidentified protein and that injected SNAP-25 simply reduce the ability of BoNT/A to cleave that protein.

The inhibition of Ca\(^{2+}\) ionophore-induced exocytosis by BoNT/A was similar to that of sperm-induced exocytosis. Intracellular Ca\(^{2+}\) mobilization induced by sperm in BoNT/A-injected eggs was the same as control. These results suggest that SNAP-25 is involved in the signal transduction pathway downstream of sperm-induced intracellular Ca\(^{2+}\) mobilization.

We previously reported that rabphilin-3A, a putative target protein of Rab3A (21), is involved in CG exocytosis in mouse eggs (14). Rab3A, which is a small GTP binding protein, is involved in Ca\(^{2+}\)-dependent exocytosis, particularly in neurotransmitter release (27). Rabphilin-3A has a Rab3A binding NH\(_2\)-terminal domain and a Ca\(^{2+}\) binding COOH-terminal domain (28). Intracellular Ca\(^{2+}\) mobilization is thought to regulate Rab3A via rabphilin-3A. In other words, the Rab-rabphilin system may play an important role in the signal transduction pathway downstream of sperm-induced intracellular Ca\(^{2+}\) mobilization. Also, Horikawa (10a) indicated that the action of Rab3A is mediated by its interaction with synaptic SNAREs in presynaptic terminals. Thus it is possible that Ca\(^{2+}\) exocytosis in eggs may occur in a similar way (Rab-rabphilin and SNAREs system) to exocytosis in synaptic cells.

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