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Abstract

Testicular Sertoli cells highly express dynamin 2 and amphiphysin 1. Here we demonstrate that dynamin 2 is implicated in phosphatidylserine (PS)-dependent phagocytosis in Sertoli cells. Immunofluorescence and dual-live imaging revealed that dynamin 2 and amphiphysin 1 accumulate simultaneously at ruffles. These proteins are specifically bound *in vitro*. Over-expression of dominant negative dynamin 2 (K44A) inhibits liposome-uptake and leads to the mis-localization of amphiphysin 1. Thus, the cooperative function of dynamin 2 and amphiphysin 1 in PS-dependent phagocytosis is strongly suggested.

KEYWORDS: dynamin, amphiphysin, phagocytosis, testis

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Original Article

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Testicular Sertoli cells highly express dynamin 2 and amphiphysin 1. Here we demonstrate that dynamin 2 is implicated in phosphatidylserine (PS)-dependent phagocytosis in Sertoli cells. Immunofluorescence and dual-live imaging revealed that dynamin 2 and amphiphysin 1 accumulate simultaneously at ruffles. These proteins are specifically bound *in vitro*. Over-expression of dominant negative dynamin 2 (K44A) inhibits liposome-uptake and leads to the mis-localization of amphiphysin 1. Thus, the cooperative function of dynamin 2 and amphiphysin 1 in PS-dependent phagocytosis is strongly suggested.

Key words: dynamin, amphiphysin, phagocytosis, testis

The family of dynamin GTPase is composed of 3 isoforms, dynamin 1–3 [1, 2]. All dynamins contain 4 functional domains, the N-terminal GTPase domain, the pleckstrin homology (PH) domain, the GTPase effector domain, and the C-terminal proline/arginine-rich domain (PRD). While the PH domain serves as binding motif for phosphatidylinositol 4, 5-bisphosphate (PIP_2), PRD mediates interaction with various SH3 domain-containing proteins such as amphiphysin 1. In neurons, dynamin 1 and amphiphysin 1 cooperatively function in the fission process of clathrin-mediated endocytosis [3].

Recent studies indicate that dynamins are implicated in the formation of a wide variety of actin-based cellular events, including the formation of growth cones [4], podosomes [5], invadopodia [6], lamellipodia and dorsal membrane ruffles [7, 8], and phagocytosis [9]. Ubiquitously expressed dynamin 2 directly interacts with cortactin, a F-actin binding protein, which in turn modulates cell shape and clathrin-mediated endocytosis [8, 10].

In testis, Sertoli cells participate in the maturation of germ cells and the release of sperm [11]. Sertoli cells have high phagocytic activity, by which the cells eliminate residual cytoplasm of spermatids [12, 13] or apoptotic germ cells [14]. Phagocytosis in Sertoli cells is initiated by the recognition of phosphatidylserine (PS) exposed at the germ cell surface by PS receptors [14, 15].

Sertoli cells express high levels of dynamin 2, dynamin 3, and amphiphysin 1 [16, 17]. Expression of amphiphysin 1 and dynamin 2 in testis coincidentally increase with the onset of spermatogenesis [16]. In addition, both proteins accumulate at tubulobulbar complexes, transiently formed structures in Sertoli cells prior to sperm release [18]. We have recently reported that amphiphysin 1 participates in actin polymerization during phagocytosis in Sertoli cells

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[19]. In response to stimulation with PS liposomes, Sertoli cells form membrane ruffles, where amphiphysin 1 is concentrated. The ruffle formation and phagocytosis, but not clathrin-mediated endocytosis, were found to be abolished in an amphiphysin 1 siRNA-treated Sertoli cell line, or Sertoli cells from amphiphysin 1 (−/−) mice. Interestingly, the expression of dynamin 2 was decreased by approximately 30% in amphiphysin 1 siRNA-treated cells. These results implied that dynamin 2 cooperates with amphiphysin 1 in Sertoli cells.

In the present study, we explored the possibility that dynamins function in Sertoli cell function. Here we demonstrate that dynamin 2 is implicated, at least in part, in PS-dependent phagocytosis with amphiphysin 1.

Materials and Methods

Cell culture. Three-week-old male Wistar rats purchased from Shimizu Laboratory Supplies Co (Kyoto, Japan), were maintained in clean conditions with free access to food and water. Sertoli cells were isolated from 3-week-old rats, plated in mono-layers, and cultured at 32℃ under 5% CO_2 as previously described [14]. Ser-W3 cells, a rat Sertoli cell line, were cultured with DMEM containing 10% fetal bovine serum at 37℃ under 5% CO_2 [19].

cDNA constructs and transfection. Complementary DNAs encoding full-length rat dynamin 2 and dynamin 2 K44A were prepared by PCR amplification using specific primers [7]. Full-length dynamin 2 and dynamin 2 K44A were subcloned into the plasmid pcDNA 4/V5-His A vector as EcoRI-XhoI fragments. The plasmids for full-length rat dynamin 2-pEGFP and dynamin 2 K44A-pEGFP were donated by Dr. McNiven (Mayo Clinic, Rochester, MN, USA). The pmCherry N1 plasmid coding for mCherry was a kind gift from Dr. Tsien (HHMI-UCSD, San Diego, CA, USA). Full-length rat dynamin 2 was subcloned into the pmCherry-N1 vector as a Hind III-EcoRI fragment. The nucleotide sequences of the constructs were verified using DNA sequence analysis. Sertoli cells were transfected using a Lipofectamine 2000 transfection system (Invitrogen, Carlsbad, CA, USA). The efficiency of the transfection, which was determined by the expression of green fluorescence protein (GFP), was approximately 90% in Ser-W3 cells. Transfected cells were subjected to analysis 24 h after the transfection.

Preparation for liposomes and assay of liposome uptake. For PS-stimulation, small unilamellar liposomes containing 70% phosphatidylcholine (PC) and 30% PS were prepared as described previously [14]. For the liposome uptake assay, large multilamellar liposomes containing 0.1% N-(lissamine rhodamine B sulfonyl)-L-α-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL, USA) were prepared by vortexing. Liposomes at the indicated concentrations were added to culture cells in serum-free DMEM. After incubation at 32℃ or 37℃ for the indicated times, the cells were extensively washed 5 times with PBS containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} [PBS (+)].

Immunoprecipitation. Ser-W3 cells were washed with ice-cold PBS (+), scraped, and resuspended in 2 ml of SME (0.2 M sucrose, 20 mM Mops, 1 mM EDTA supplemented with 1 mM PMSF, aprotinin, leupeptin, pepstatin, antipain at 5 μg/ml, pH 7.4). The cells were homogenized for 10 strokes in a glass-Teflon homogenizer. The particulate fraction was isolated by low- and high-speed centrifugation, and the resultant post-nuclear supernatant was centrifuged at 100,000 g for 30 min at 4℃. Two milligrams of membrane fractions were cross-linked with 0.5mM dithiobis(succinimidylpropionate) (DSP) (Pierce Biotechnology, Rockford, IL, USA) for 1 h at 4℃ [20]. Excess DSP was quenched by incubation with 50 mM Tris for 15 min at 4℃. Samples were solubilized with 1% TX-100 and centrifuged at 20,000 g for 30 min at 4℃. Immunoprecipitation for amphiphysin 1 was carried out as described previously [10] using monoclonal antibodies, mab8, and mab14 (kindly donated by Dr. De Camilli, Yale University, New Haven, CT, USA). The collected beads were washed thoroughly and extracted with sample buffer. Western blotting was performed using anti-dynamin 2 polyclonal antibodies (Santacruz Biotechnologies, Santa Cruz, CA, USA) or mab8. A GST pull-down assay was performed using full-length or the SH3 domain of amphiphysin 1 fused to GST, as described [8, 21].

Microscopy. Immunofluorescence of Sertoli cells was performed as described [20] using mab3 (kindly donated by Dr. De Camilli, Yale University) or anti-dynamin 2 polyclonal antibodies (Santacruz Biotechnologies). The samples were examined using

an inverted microscope (IX-71, Olympus Optical Co., Ltd., Tokyo, Japan) equipped with a spinning disc confocal microscope system (CSU10, Yokogawa Electric Co., Kanazawa, Japan), a CoolSNAP-HQ camera (Roper Industries, Sarasota, FL, USA), and a Dual View (Roper Industries). The system was steered by Metamorph Software (Molecular Devices Corp., Downingtown, PA, USA). When necessary, images were further processed using Adobe Photoshop and Illustrator software. Live imaging was performed as described previously [19].

Results

Dynamin 2 is implicated in PS-stimulated ruffle formation. Dynamin 2, dynamin 3, and amphiphysin 1 are highly expressed in Ser-W3 cells, a rat Sertoli cell line. We have previously demonstrated that amphiphysin 1 is implicated in ruffle formation in PS-dependent phagocytosis by Sertoli cells [19]. As it was anticipated that dynamin 2 interacts with of amphiphysin 1 in the cells [9], we first determined localization of dynamin 2 in the PS-stimulated Sertoli cells. Cells with peripheral ruffles were characterized by the presence of thick peripheral actin filament accumulation [19]. Dynamin 2 accumulated at ruffles, which were actin filament-rich structures (Fig. 1A, arrowheads). Double immunofluorescence demonstrated that dynamin 2 partially colocalized with amphiphysin 1 at bright puncta throughout the cytoplasm. Notably, both proteins were highly enriched at ruffles (Fig. 1B, arrowheads; ref. [19]). In contrast, dynamin 3 did not co-localize with amphiphysin 1 at either ruffles in PS-stimulated Sertoli cells (data

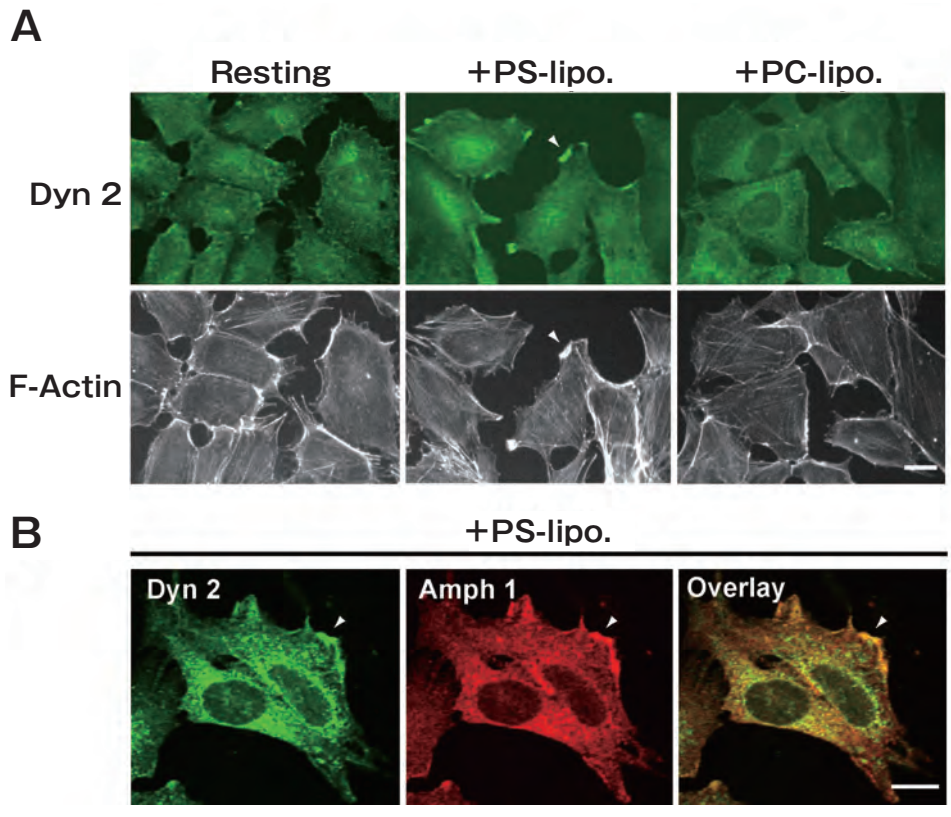

Fig. 1 Both dynamin 2 and amphiphysin 1 accumulate at membrane ruffles.
A, Dynamin 2 accumulated at ruffles in PS-stimulated Sertoli cells. Ser-W3 cells treated with 0.25 mM PS- or PC-liposomes were stained with anti dynamin 2 antibodies and Rhodamine-phalloidin (F-Actin). Note that dynamin 2 is concentrated at actin-rich ruffles, (arrowheads). Bar: 10 μm. **B**, Immunofluorescent localization of dynamin 2 and amphiphysin 1 in PS-stimulated Sertoli cells. Ser-W3 cells treated with 0.25 mM PS-liposomes were stained with anti dynamin 2 antibodies and monoclonal anti amphiphysin 1 antibodies. Note that both proteins are concentrated at ruffles, (arrowheads). Bar: 10 μm.

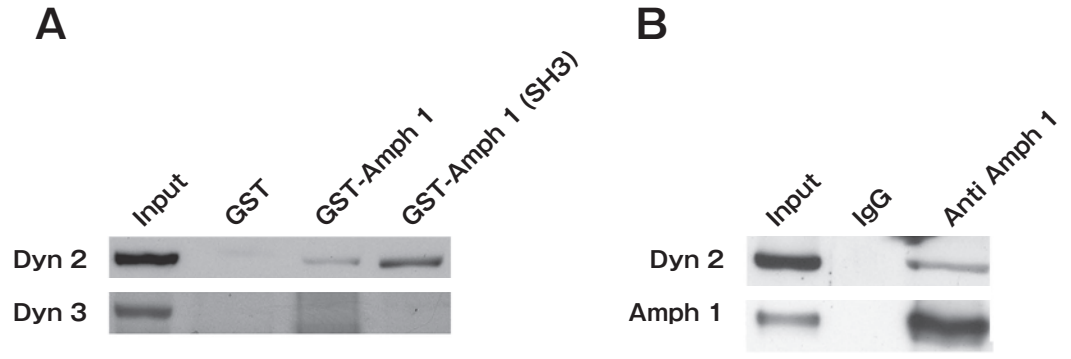

Fig. 2 Dynamin 2 but not dynamin 3 directly binds to amphiphysin 1 via its SH3 domain.
A, Pull-down assay demonstrating that both GST-amphiphysin 1 and GST-amph 1 (SH3) bind to dynamin 2 (upper lane) but not to dynamin 3 (lower lane). Four hundreds micrograms of GST-fusion proteins bound to glutathione beads was incubated with 1mg of Ser-W3 cell lysates. Dynamin 2 or dynamin 3 bound to the resin beads were analyzed by Western blotting. Twenty micrograms of untreated lysates was loaded as starting material (Input); **B**, Amphiphysin 1 is cross-linked with dynamin 2 in membrane of PS-stimulated Ser-W3 cells. Two milligrams of DSP treated membranes of PS-stimulated cells was subjected to immunoprecipitation, as described in Materials and Methods. Twenty micrograms of untreated membranes was loaded as starting material (Input).

not shown) or in seminiferous tubules [18].

We next determined the interactions between dynamins and amphiphysin 1 by GST pull-down assay. Consistent with the co-localization of dynamin 2 with amphiphysin 1 (Fig. 1), dynamin 2 specifically bound to both full-length and the SH3 domain of amphiphysin 1 (Fig. 2A, right 2 lanes). The association of endogenous amphiphysin 1 and dynamin 2 in Sertoli cells was confirmed by cross-linking with DSP (Fig. 2B, right lane). It was therefore suggested that dynamin 2 might also function with amphiphysin 1 in the formation of ruffles.

Dynamin 2 may be involved in PS-dependent phagocytosis together with amphiphysin 1. We next examined the dynamics of dynamin 2 during PS-dependent phagocytosis. For this purpose, Ser-W3 cells expressing dynamin 2-GFP were stimulated with rhodamine-labeled PS-liposomes and observed by time-lapse. Dynamin 2-GFP began to accumulate at the phagocytic sites soon after the PS stimulation, and then soon later the accumulation disappeared (Fig. 3A, arrowhead). A similar transient accumulation of dynamin at ruffles was observed, as revealed by dual-live imaging of dynamin 2-mCherry and GFP-amphiphysin 1. Importantly, amphiphysin 1 acted synchronously with dynamin 2 during ruffle formation (Fig. 3B).

It has been reported that expression of the dynamin 2 dominant negative mutant (K44A) results in a decrease in phagocytosis by inhibiting extension of the plasma membrane [9], and contraction of the cells [22]. We confirmed in Sertoli cells that liposome uptake is inhibited by expression of dynamin K44A mutant, and the expression-induced contraction of cell shape (Fig. 4). Under these conditions, the dynamin 2 K44A mutant led to mis-localization of amphiphysin 1, which appeared as large dots, and inhibited formation of ruffles in PS-stimulated rat cultured Sertoli cells (Fig. 3C upper right). These results suggest cooperative functions of dynamin 2 with amphiphysin 1 in PS-dependent phagocytosis.

Discussion

We have demonstrated in the present study that dynamin 2 but not dynamin 3 associates with amphiphysin 1, and that dynamin 2 may be implicated in PS-dependent phagocytosis in Sertoli cells.

Sertoli cells highly express dynamin 2, dynamin 3, and amphiphysin 1 [16–19]. However, the physiological function of these proteins in the cell remains to be elucidated. We have recently reported that amphiphysin 1 stimulates actin assembly and participates in both ruffle formation and phagocytosis in Sertoli cells. Interestingly, RNAi for amphiphysin 1 in cells decreases expression of dynamin 2 to up to 30% of control [19]. These observations strongly suggest that dynamin 2 functions in phagocytosis together with

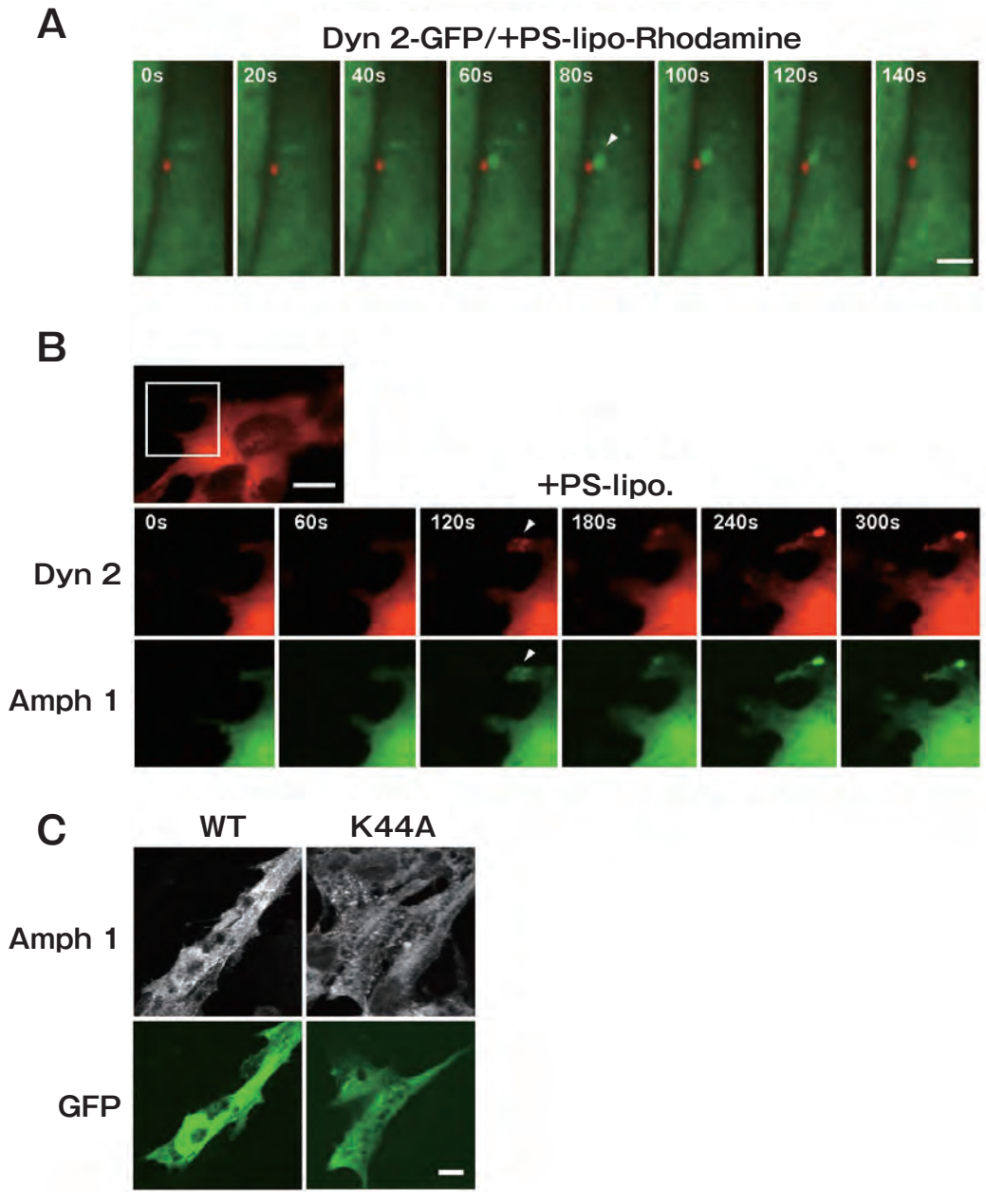

Fig. 3 Dynamin 2 may cooperate with amphiphysin 1 in PS-dependent phagocytosis.
A, Dual-live imaging of dynamin 2-GFP (green) and rhodamine-labeled PS-liposome (red) in Ser-W3 cells. Cells expressing dynamin 2-GFP were stimulated with rhodamine-PS-liposomes, and images were taken every 20 sec. Note that dynamin 2-GFP transiently accumulated at the phagocytic site. Bar: 5 μm. **B**, Dual-live imaging of dynamin 2-mCherry (red) and GFP-amphiphysin 1 (green) in PS-stimulated Ser-W3 cells. Images of area surrounded by a rectangle in the top panel were taken every 60 sec. Both GFP and mCherry fluorescence simultaneously appear at ruffles (arrowheads). The scale bar represents 10 μm in upper panels, 5 μm in lower panels; **C**, Mutant dynamin 2 (K44A) induces mis-localization of amphiphysin 1 in PS-stimulated Sertoli cells. Wild-type or mutant dynamin were co-transfected with EGFP (dynamin : EGFP = 10 : 1 in cDNA ratio) in primary cultured rat Sertoli cells. The cells were stimulated with PS-liposomes, as described in Fig. 1A, and stained for amphiphysin 1. Note that immuno-reactivity for amphiphysin1 is represented as large dots in dynamin K44A expressing cells (upper right), which can be identified by GFP expression (lower right). Bar: 10 μm.

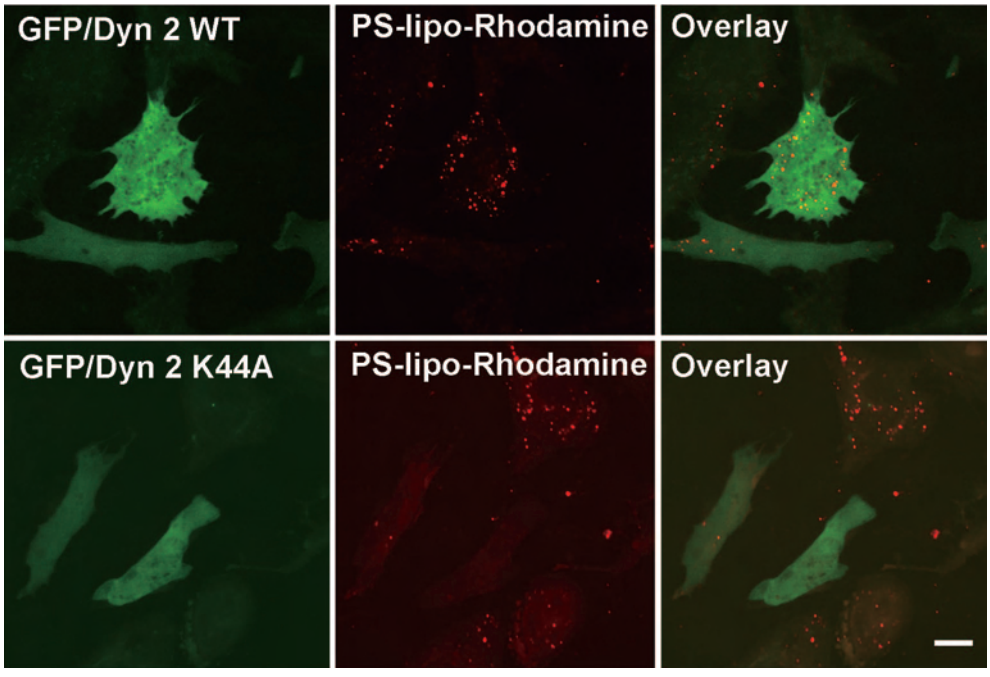

Fig. 4 Uptake of PS-liposomes is inhibited by over-expression of dynamin 2 K44A.
PS-dependent liposome uptake by Ser-W3 cells expressing wild-type dynamin 2 (upper panels) or K44A mutant (lower panels). Wild-type (WT) or K44A mutant dynamin 2 (K44A) were transiently co-transfected with EGFP (dynamin : EGFP = 10 : 1 in cDNA ratio) in Ser-W3 cells. After 24 h transfection, the cells were incubated at 37°C for 120 min with 0.25 mM rhodamine-labeled PS-liposomes. Note that cells expressing the mutant showed a small cell shape and could not uptake the liposomes. Bar: 10 μm.

amphiphysin 1.

Dynamin 2 in the present study accumulated at ruffles together with amphiphysin 1, as revealed by double immunofluorescence and live-imaging (Figs. 1 and 3). The association between dynamin 2 and amphiphysin 1 was mediated via the SH3 domain of amphiphysin 1, as shown by pull-down and cross-linking experiments. The interaction between dynamin 2 and amphiphysin 1 might be rather weak and/or tentative in Sertoli cells, because the association could not be detected without a cross-linker (data not shown). This result is consistent with transient accumulation of these proteins at ruffles (Fig. 3B). Over-expression of dynamin 2 K44A in Sertoli cells resulted in not only the inhibition of PS-dependent liposome uptake (Fig. 4), but also mis-localization of amphiphysin 1 (Fig. 3C). The immunoreactivity of amphiphysin 1 in dynamin 2 K44A-expressed cells appeared as distinct puncta compared to that in dynamin 2 WT-expressed cells (Fig. 3C). These results suggest that dynamin 2 may participate in PS-dependent phagocytosis together with amphiphysin 1. Dynamin 3 is unlikely to be involved in Sertoli cell phagocytosis because dynamin 3 neither binds to amphiphysin 1 (Fig. 2A) nor localizes at ruffles (data not shown). In addition, dynamin 3 does not co-localize with amphiphysin 1 in seminiferous tubules [18]. The proline arginine-rich domain (PRD) of dynamin 3 has < 60% of identity with that of dynamin 2. Thus, it may be possible that dynamin 3 PRD has different characteristics, and therefore it is possible that the functions of dynamin 3 are different than those of dynamin 2.

Dynamin 2 is involved in actin dynamics during ruffle formation in fibroblasts [10, 23, 24] and in phagocytosis in macrophages [9]. Dynamin 2 at the plasma membrane associates with cortactin, which specifically binds to F-actin [10, 29]. Furthermore, dynamin 2 interacts with Rac1 and regulates the localization with Rac1, and both proteins are implicated in the formation of ruffles [25]. Thus, dynamin 2 as well as amphiphysin 1 may cooperate with these molecules that regulate actin dynamics.

In conclusion, we have demonstrated that dynamin

2 associates with amphiphysin 1, and participates in PS-dependent phagocytosis in Sertoli cells.

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