

JPET #179762

## I. Title page

**Title:** Inhibition of Rab1 GTPase and endoplasmic reticulum-to-Golgi trafficking underlies statin's toxicity in rat skeletal myofibers

**Author affiliation:** Kazuho Sakamoto, Ikuo Wada, and Junko Kimura

Department of Pharmacology (K.S. and J.K.), School of Medicine and Department of Cell Science (I.W.), Institute of Biomedical Sciences, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan

JPET #179762

## II. Running title page

**a) Running title:** Statin inhibits myofiber Rab1 and ER-Golgi traffic

**b) Corresponding author:** Kazuho Sakamoto, Ph.D.

Address: Department of Pharmacology, School of Medicine, Fukushima Medical University, Hikarigaoka 1, Fukushima 960-1295, Japan. TEL: +81-27-547-1152, FAX: +81-27-548-0575, E-mail: [kazuho@fmu.ac.jp](mailto:kazuho@fmu.ac.jp)

**c) Manuscript informations:**

Text pages: 30

Figures: 8

References: 40

Abstract: 238 words

Introduction 492 words

Discussion 1250 words

**d) List of nonstandard abbreviations:**

Arf, ADP ribosylation factor; BIG, brefeldin A-inhibited guanine nucleotide-exchange protein; CBB, Coomassie brilliant blue; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FDB, flexor digitorum brevis; ER, endoplasmic reticulum, FPP, farnesylpyrophosphate; GRP, glucose-regulated protein; GGPP, geranylgeranylpyrophosphate; NP-40, nonyl phenoxypolyethoxyethanol; PBS, phosphate buffered saline; RIPA buffer, radio immuno-precipitation assay buffer; SR, sarcoplasmic reticulum; UPR, unfolded protein response

**e) Section:** Toxicology

JPET #179762

### III. Abstract

HMG-CoA reductase inhibitor statins are used for hypercholesterolemia. However, statins have adverse effects on skeletal muscles with unknown mechanism. We reported recently that fluvastatin induced vacuolation and cell death in rat skeletal myofibers by depleting geranylgeranylpyrophosphate (GGPP) and suppressing small GTPases, particularly Rab [Sakamoto et al., FASEB J (2007) 21, 4087-4094]. Rab1 is one of the most susceptible Rab isoforms to GGPP depletion and is essential for endoplasmic reticulum (ER)-to-Golgi trafficking. Here, we explored whether Rab1 and ER-to-Golgi vesicle trafficking were affected by statins in cultured single myofibers isolated from flexor digitorum brevis muscles of adult rats. Western blot analysis revealed that Rab1A protein resided predominantly in membrane but not in cytosol in control myofibers, while it was opposite in fluvastatin-treated myofibers, indicating that fluvastatin inhibited Rab1A translocation from cytosol to membrane. GGPP supplementation prevented the effect of fluvastatin on Rab1A translocation. Brefeldin A, a specific suppressor of ER-to-Golgi trafficking, induced vacuolation and cell death in myofibers in a manner similar to that of fluvastatin. Although ER-to-Golgi traffic suppression induces unfolded protein response (UPR) and cell death in some cell types, neither fluvastatin nor brefeldin A up-regulated UPR in myofibers. Immunofluorescence study revealed that the distribution of an ER marker, calnexin, was restricted to the region around nucleus with fluvastatin, suggesting the inhibition of ER membrane traffic by fluvastatin. We conclude that suppression of Rab1 GTPase and subsequent inhibition of ER-to-Golgi traffic are involved in statin-induced skeletal myotoxicity.

JPET #179762

#### **IV. Body of Manuscript**

##### **Introduction**

Statins, or 3-hydroxymethyl-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, are commonly prescribed drugs for hypercholesterolemia (Endo, 1992). However, statins have adverse effects on skeletal muscles, ranging from slight myalgia to severe rhabdomyolysis (Arora et al., 2006). Among skeletal muscles, statins selectively injure fast-twitch glycolytic type II skeletal muscles (Waclawik et al., 1993; Westwood et al., 2005). Vacuolation of myofibers is a typical morphological feature of statin-induced myotoxicity (Waclawik et al., 1993; Westwood et al., 2005). Since millions of people in the world are taking statins everyday (Dobson, 2008), it is an urgent task to elucidate the mechanism of the adverse effect of statins for its prevention and treatment.

A primary target of statins is an inhibition of mevalonate synthesis. Mevalonate is a precursor not only for cholesterol but also for isoprenoids such as geranylgeranylpyrophosphate (GGPP) or farnesylpyrophosphate (FPP). Prenylation with GGPP or FPP are essential for activation of small GTPases, including Rab, Ras, Rac, Raf, and Rho families (Zhang and Casey, 1996). By suppression of mevalonate synthesis, statins could deplete GGPP or FPP to the extent of preventing small GTPases from prenylation. Accumulating evidence suggests that depletion of isoprenoids, particularly GGPP is critical for statin-induced myopathy (Flint et al., 1997; Sakamoto et al., 2007), while depletions of cholesterol and ubiquinone are not responsible (Mullen et al., 2010).

JPET #179762

Intracellular vesicle trafficking is essential for cell survival. When proteins are newly synthesized in endoplasmic reticulum (ER), they are packed in small vesicles and transported to Golgi apparatus via ER-Golgi intermediate compartment (Marie et al., 2008). The proteins are matured in Golgi apparatus and redistributed to their destinations in the cell (Emr et al., 2009). Rab GTPases regulate intracellular vesicle trafficking events, such as vesicle budding, delivery, tethering, and fusion. More than 60 isoforms of Rab GTPases have been found in mammalian cells. Each Rab isoform has distinct target membranes (reviewed in Stenmark et al., 2009). Although it is not clear how it could determine specific membrane targeting, GGPP has a role in hydrophobicity and membrane association of Rab.

Recently, we found that statins, including fluvastatin, pravastatin and simvastatin, induce vacuolation and cell death in primary cultured rat skeletal myofibers by inhibiting Rab GTPases (Sakamoto et al, 2007). However, it is unknown which Rab isoforms and vesicle trafficking pathways are affected by statins. In this study, we focused on Rab1 and ER-to-Golgi trafficking, because Rab1 is responsible for ER-to-Golgi trafficking, which is the most fundamental intracellular membrane trafficking system (Stenmark, 2009). Furthermore, Rab1B is the most susceptible to GGPP depletion among Rab subfamily investigated with a low concentration (200 nM) of simvastatin in mouse N2a neuroblastoma cells (Ostrowski et al, 2007). Rab1 has two isoforms, Rab1A and Rab1B. While Rab1A is dominant in human skeletal muscles (Bao et al., 1998), both Rab1A and Rab1B are expressed in rat skeletal muscles with 92% amino acid sequence similarity (Vielh et al., 1989). In this study, we analyzed the effects of fluvastatin on Rab1A distribution and ER-to-Golgi trafficking in rat myofibers.

JPET #179762

## Methods

### Isolation of myofibers

All the protocols used here met the NIH animal experiment guidelines and was approved by the Animal Research Committee of Fukushima Medical University. The isolation of single skeletal myofibers was performed as described previously (Sakamoto et al. 2007, 2008; Tanaka et al., 2010) with some modifications. Male Wistar rats (8-16 weeks old) were anesthetized with ether and exsanguinated. The total number of rats used in this study was 16. The body weight of the rats ranged from 160-210 g. For anesthesia, an airtight glass container (2 L in volume) was filled with evaporated ether (from about 5 ml liquid ether absorbed in cotton), in which a rat was placed. Flexor digitorum brevis (FDB) muscles were extracted from both soles and the connective tissue was removed from the muscles with scissors under a binocular microscope (Nikon, Tokyo, Japan). FDB muscles were cleaned and incubated at 37 °C for 2.5-3.0 hours in Ringer's solution (2.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 137 mM NaCl, 8.1 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose) containing 0.3 % collagenase (Wako, Tokyo, Japan; lot# CER1568) and 0.001 % penicillin-G (Banyu Pharmaceutical, Tokyo, Japan) and 0.001 % streptomycin (Meiji Seika, Tokyo, Japan). After incubation, the muscles were washed three times with Ringer's solution, and the fibers were dispersed in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS), 0.001 % penicillin-G and 0.001 % streptomycin using a fire-polished wide-mouth Pasteur's pipette.

### Culture of myofibers

JPET #179762

Cultured myofibers were separated from fibroblasts by the following way. To minimize the number of fibroblasts, which would otherwise eventually overgrow myofibers during culture (Sakamoto et al., 2007 and 2009), the preparation was allowed to settle for 10 minutes. The sediments were transferred to a 60-mm dish and cultured overnight. By then, almost all fibroblasts had adhered to the bottom of the dish, while myofibers were still floating. After gentle agitation, supernatants were harvested and cultured in DMEM containing 10 % FBS, 0.001 % penicillin-G, 0.001 % streptomycin, and 10  $\mu$ M cytarabine.

#### Immunofluorescence

The myofibers cultured for 4 days were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline for 1 hr at 4 °C. After permeabilization with 0.05% saponin (Sigma) in IF buffer (100 mM NaCl, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, 10 mM PIPES-Na, pH7.2) for 30 seconds on ice, the cells were incubated with anti-calnexin antibody (Hatsuzawa et al., 2006) in IF buffer containing 10% BlockAce (DS Pharma Biomedical Co., Osaka, Japan) for 20 min on ice. The cells that were rinsed with 10% BlockAce/IF buffer were further treated with the secondary antibody labeled with Alexa555 (Invitrogen, CA, USA) for 20 min on ice followed by three-times rinse with 10% BlockAce/IF buffer. The confocal images of the fluorescence were recorded by LSM510meta using x20 PlanApo objective as described (Hatsuzawa et al., 2006).

#### Cell staining

Cell viability was examined using 0.2 % trypan blue/PBS. Trypan blue/ phosphate-buffered saline (PBS) (Invitrogen, CA, USA) was added to the myofibers for 5 min.

JPET #179762

Then the cells were washed 3 times with Ringer's solution and the number of trypan blue-stained cells and non-stained cells were counted.

#### Counting vacuoles per myofiber

Vacuoles, which appeared in each myofiber after statin treatment, were counted using a phase contrast microscope (CK40; Olympus, Tokyo). The magnification was  $\times 400$ . All vacuoles in each individual myofiber were counted by changing the focus. Five to 10 single myofibers were counted in every trial. To ensure objectivity, vacuoles were counted from all myofibers in each of random sights under the microscope. The stage of the microscope was moved irregularly to randomize the counting field.

#### Western blot

For the measurement of glucose-regulated protein 78 (GRP78), skeletal myofibers were incubated for 96 h with or without fluvastatin, brefeldin A, or tunicamycin and washed twice with PBS by centrifugation. Myofibers were harvested with radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% nonylphenoxypolyethoxylethanol (NP-40), 0.25% Na-deoxycholate, pH7.4) containing 1 % protease inhibitor cocktail set III solution (Merck chemicals, Darmstadt, Germany). Whole-cell lysates were sonicated and centrifuged at 500g for 5 min at 4 °C. The supernatants (12  $\mu$ g of protein) were subjected to Western blot analysis using anti-GRP78 rabbit polyclonal antibody (SPA-826; Assay designs, Ann Arbor, MI). For the quantification of Rab1A protein, myofibers were harvested in lysate solution (10 mM Tris-HCl and 10 mM EDTA) with 1% protease inhibitor cocktail set III solution and sonicated. Whole-cell lysates after removing nuclei were centrifuged at 800g for 10 min



JPET #179762

at 4 °C. The supernatant was collected and centrifuged at 100,000g for 20 min at 4 °C. The pellet was resuspended with RIPA buffer and used as crude membrane fraction. The supernatant was collected and used as cytosolic fraction. Membrane and cytosolic fractions were then subjected to Western blot analysis using anti-Rab1A rabbit polyclonal antibody (sc-311; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The intensities of immunoreactive protein bands were analyzed using Image-J software ver. 1.39 (National Institutes of Health, Bethesda, MA, USA). After the immunoblot, the membrane was treated in stripping buffer at 50°C for 30 minutes. In the experiment shown in Fig.1 and Fig.S1, we stained the blotted membrane with Coomassie Brilliant Blue (CBB) and digitized the contrasting density of each lane over the range from 20 to 250 kD. The CBB results were adopted as references. In Fig.6, re-probed membrane with GAPDH rabbit polyclonal antibody (sc-25778; Santa Cruz Biotechnology, Inc.) was used as a reference.

## Analysis

Each experiment was repeated 3 to 10 times. The data were expressed as the means  $\pm$  S.E.M. Statistical significance between two groups or among multiple groups was evaluated using Student's t-test, Scheffé's test after the F-test or one-way analysis of variance (ANOVA).

## Results

### Effect of Fluvastatin on Rab1A protein distribution in cultured myofibers

First, we examined whether fluvastatin inhibited Rab1A activation in cultured skeletal myofibers. To do so, we analyzed the distribution of Rab1A protein between

JPET #179762

membrane and cytosol of rat myofibers. It was expected that an active form of Rab1A was in the membrane, while an inactive form in the cytosol. After incubation with fluvastatin (1  $\mu$ M) for 4 days, myofibers were ultracentrifuged and fractionated into membrane and cytosol. Each fraction was subjected to the western blot by using antibody for Rab1A. In control myofibers, Rab1A was abundant in membrane (Fig.1A & C), but was scarce in cytosol (Fig.1B & D). In contrast, in Flv-treated myofibers, Rab1A signal was weak in membrane (Fig. 1A & C), but robust in cytosol (Fig.1B & D). Supplementation of GGPP, but not FPP, reversed the effect of fluvastatin (Fig. 1A-D).

The effect of fluvastatin was time-dependent. Even on day 1, Rab1A was recognizable in the cytosol of fluvastatin-treated myofibers (Fig.2A). With fluvastatin, the cytosolic fraction of Rab1A progressively increased, while the membrane fraction of Rab1A progressively decreased and the significant change was observed from 2 days of fluvastatin-treatment (Fig.2B). Without fluvastatin, this change was never observed (Fig.1A and Fig.2A). This indicated that fluvastatin inhibited translocation of Rab1A from cytosol to membrane by depleting GGPP and preventing geranylgeranylation of Rab1A. This is a direct evidence for statin-induced inhibition of membrane translocation of Rab1 in skeletal myofibers.

#### Effect of Brefeldin A on skeletal myofibers

Next, we attempted to knock down Rab1A expression in skeletal myofibers. However, floating cultured myofibers did not allow us to introduce small interference RNA intracellularly. Alternatively, we treated myofibers with brefeldin A, a specific inhibitor of ER-to-Golgi trafficking, which is a pathway mediated by Rab1. Brefeldin A is an

JPET #179762

agent that specifically binds and inhibits BIG (Brefeldin A-inhibited guanine nucleotide exchange protein), which is an activator of Arf1 (ADP ribosylation factor 1). Arf1 is also a small GTPase, which mediates ER-to-Golgi trafficking together with Rab1A. If brefeldin A inactivates BIG, Arf1 is inactivated, and as a result, ER-to-Golgi trafficking should be selectively inhibited.

Fig. 3A compares myofibers cultured for 4 days in the absence (control, Fig.3Aa) or presence of 1  $\mu$ M fluvastatin (Fig. 3Ab) or 30  $\mu$ M brefeldin A (Fig. 3Ac). Both fluvastatin (1  $\mu$ M) and brefeldin A (higher than 10  $\mu$ M) induced numerous vacuoles. The average number of vacuoles was increased by both drugs in a time- (Fig. 3B) and concentration-dependent manner (Fig. 3C). The EC<sub>50</sub> values of fluvastatin and brefeldin A for inducing vacuolation were 0.3  $\mu$ M and 18.5  $\mu$ M, respectively.

On day 6 with fluvastatin or brefeldin A, both agents increased the number of trypan blue-stained myofibers, while the control fibers were mostly unstained. Numerous blebs were observed on the surface of myofibers treated with fluvastatin as well as brefeldin A, which were stained with trypan blue, indicating cell death (Fig. 4A b and c). The concentration-viability relationships of the drugs were obtained on day 6 (Fig. 4B). The median lethal concentrations (LC<sub>50</sub>) of fluvastatin and brefeldin A were 0.3  $\mu$ M and 8.6  $\mu$ M, respectively (Fig. 4B). Since morphological changes induced by fluvastatin and brefeldin A were similar, we conclude that both fluvastatin and brefeldin A suppressed ER-to-Golgi trafficking, and induced vacuolation and cell death in skeletal myofibers.

Effects of GGPP or FPP supplementation on fluvastatin- or brefeldin A-induced

JPET #179762

vacuolation and cell death. Fluvastatin and brefeldin A induced similar morphological changes in myofibers. However, if brefeldin A could also inhibit HMG-CoA reductase, the effect should be prevented by GGPP but not by FPP in a manner similar to fluvastatin.

As we have shown previously (Sakamoto et al., 2007), fluvastatin at 10  $\mu$ M induced vacuolation and cell death in myofibers after 4 and 6 days of treatment, respectively (Fig. 5A and B). Supplementation of GGPP, but not FPP, prevented both of these effects of fluvastatin (Fig. 5A and B). The same protocol was performed with brefeldin A instead of fluvastatin (Fig.6). As for supplementation experiments, myofibers were pre-treated with 3  $\mu$ M GGPP or FPP for 1 h before adding 30  $\mu$ M brefeldin A. On day 4, the number of vacuoles induced by brefeldin A was  $115.7 \pm 32.6$  (n=4). Neither GGPP nor FPP suppressed brefeldin A-induced vacuolation (Fig.6A) and cell death (Fig.6B) in myofibers. This result indicated that molecular targets of fluvastatin and brefeldin A were distinct and assured that the effects of brefeldin A were not due to HMG-CoA reductase inhibition.

#### Effects of fluvastatin and brefeldin A on ER stress

Perturbations in ER homeostasis are termed 'ER stress' and lead to unfolded protein response (UPR) (Kaufman, 1999). Excessive UPR induces activation of caspases and consequently apoptosis (Kim et al, 2006). Suppression of ER-to-Golgi trafficking induces UPR followed by activation of caspases in some types of cells (Cooper et al., 2006, Carew et al., 2006, Citterio et al., 2008). Therefore, we investigated in myofibers whether fluvastatin or brefeldin A induce UPR, using GRP78 upregulation as an indicator of UPR.

JPET #179762

Myofibers were treated for 4 days with fluvastatin (1  $\mu$ M), brefeldin A (30  $\mu$ M), or tunicamycin (3  $\mu$ M). Tunicamycin is a well-documented inducer of UPR. GRP78 expression was analyzed by western blotting (Fig. 7A and B). Neither fluvastatin nor brefeldin A significantly up-regulated GRP78, while 3  $\mu$ M tunicamycin dramatically upregulated GRP78 protein in skeletal myofibers (Fig. 7A and B). Next, we compared the manners of cell death induced by these three drugs (Fig.7C). After 4 days of treatment, the numbers of trypan blue-positive myofibers were  $17.3\pm 1.5\%$  in control,  $85.8\pm 1.5\%$  with fluvastatin,  $78.8\pm 1.7\%$  with brefeldin A, and  $53.7\pm 2.9\%$  with tunicamycin (Fig.7C). There was little correlation between the expression levels of GRP78 protein and the percentage of cell death. This suggests that UPR was not a main factor of myofiber death induced by fluvastatin or brefeldin A.

#### Effect of fluvastatin on ER marker calnexin distribution in myofibers

To justify our conclusion that fluvastatin inhibits ER-Golgi transport, we performed an experiment that actually assayed ER-Golgi trafficking. Among a number of established and standard protocols to measure whether ER-Golgi trafficking is intact in cells, we employed an immunofluorescent method of calnexin as a marker of ER. Calnexin is an ER chaperone protein, which is located specifically in ER membrane and functions to maintain correct folding of glycoprotein (Ruddock & Molinari, 2006). We stained myofibers with an antibody against calnexin to highlight ER in myofibers. Fig. 8A shows that calnexin is distributed diffusely throughout the cytosol of a control myofiber. In contrast, in a myofiber treated with 1  $\mu$ M fluvastatin for 4 days, calnexin accumulated distinctly in the region around nucleus (Fig. 8B). Similar micrographs were obtained in 3 control myofibers and 4 fluvastatin-treated myofibers. This indicates

JPET #179762

that fluvastatin inhibited ER-Golgi membrane traffic and prevented calnexin distribution throughout the cytosol, confirming that fluvastatin disturbed intracellular ER membrane trafficking in myofibers.

## **Discussion**

In the present study, we demonstrated that fluvastatin inhibited the translocation of Rab1A from cytosol to membrane, and this inhibition was prevented by supplementation of GGPP, but not FPP. This indicates that fluvastatin inhibited HMG-CoA reductase and mevalonate synthesis, depleted GGPP, and as a result, prevented geranylgeranylation of Rab1A. Rab1 is isoprenylated only by GGPP, but not by FPP, for its activation (Khosravi-Far et al., 1991). Therefore, fluvastatin consequently inhibited membrane association of Rab1A and thereby inactivated Rab1A.

Our present results suggest that inhibition of Rab1A is responsible for and precedes statin-induced vacuolation and cell death in skeletal muscles. As shown in Fig.1, the loss of Rab1 localization in membrane occurs on day 4 of 1  $\mu$ M fluvastatin treatment, by which time vacuolation is already well advanced but cell death is not (Fig.3). However, as demonstrated in Fig.2, as early as on day 1 and 2 fluvastatin inhibited translocation of Rab1, while vacuolation was not yet significant (Fig.3). This indicates that inhibition of Rab1 translocation precedes and causes the subsequent vacuolation and even later loss of viability in myofibers.

Using transmitted electron microscopy, we previously demonstrated that statins induced vacuolation and ER expansion in myofibers (Sakamoto et al., 2007). Similar ER

JPET #179762

expansion was also observed in skeletal muscle biopsy samples from statin-administered rodents (Waclawik et al., 1993; Westwood et al., 2005). Vacuolation was also observed in Ypt1 deficient yeast *Saccharomyces cerevisiae* (Becker et al., 1991). Rab1 is an ortholog of Ypt1, which regulates ER-to-Golgi trafficking in yeast (Segev et al., 1988). Abnormal intracellular vesicle accumulation was reported to derive from ER (Becker et al., 1991). Brefeldin A is an inhibitor of ER-to-Golgi traffic, which dilated ER in chronic lymphocytic leukemia cells (Carew et al., 2006). Similar morphological changes among statin treatment, Rab1/Ypt1 deficiency, and brefeldin A treatment support our conclusion that statin-induced vacuolation in myofibers is due to the inhibition of Rab1A.

Our conclusion was further supported by our calnexin result. Treatment of 1  $\mu$ M fluvastatin for 4 days, which did not yet cause loss of cell viability, dramatically reduced calnexin distribution throughout a myofiber, but instead accumulated calnexin around the nucleus. We previously reported that a higher concentration of 10  $\mu$ M fluvastatin induced significant loss of viability only after 5 days, but not after 4 days of treatment (see Fig.1D of Sakamoto et al., 2007). In the present study, myofibers were treated with 1  $\mu$ M fluvastatin for 4 days. Therefore, it is clear that the loss of calnexin distribution occurs prior to the loss of viability. Thus we conclude that the effect on calnexin distribution is a cause of general loss of cell viability. Calnexin is a chaperon protein distinctly localized in ER membrane. If ER membrane traffic is inhibited, calnexin remains in the perinuclear area, where it is originally synthesized by gene translation. Rab1A is an important molecule for the maintenance of intracellular tubular network structure (Marie et al., 2009). Therefore, our immunofluorescence data of

JPET #179762

calnexin directly and strongly support our conclusion that Fluvastatin inactivated Rab1A and disturbed ER membrane traffic, and this must be a cause of vacuolation and subsequent cell death in myofibers.

There seems to be a discrepancy in the reported relationships of Rab1 inhibition and cell death between yeasts and mammalian cells. Gene disruption of Ypt1 was lethal for yeasts (Segev and Botstein, 1987). However, gene knock down of Rab1B or dominant negative Rab1A did not impair viability of mammalian cells (Filipeanu et al., 2006; Sklan et al., 2007). This discrepancy may be due to the different Rab1 isoforms expressed in each cell type. Two isoforms of Rab1 (Rab1A and Rab1B) have been found in mammalian cells, while only one isoform in yeasts (Vielh et al., 1989). In mammalian cells, one isoform might have compensated for another, but this was not possible in yeast. Since statins inhibit both Rab1A and Rab1B (Ostrowski et al., 2007), the toxicity of statins may be more prominent than that of gene interference of Rab1 in mammalian cells.

There is a possibility that reduced GGPP by statins affected not only Rab1 but also various other small GTPases, including Rac, RhoA and RhoB (Greenwood et al., 2006; Maeda et al., 2005). However, Ostrowski et al. (2007) reported that reduced GGPP by statins inhibited a selective subset of small GTPases. Simvastatin at a low concentration (200 nM) decreased the membrane association of Rac1 and Rab1, but not that of Rab4 and Rab5. On the other hand, a higher concentration (10  $\mu$ M) of simvastatin decreased membrane-associated Rac1, RhoA, Rab1, Rab4, Rab5, and Rab6. Thus they concluded that Rab1 was more susceptible to GGPP depletion than other small GTPases they



JPET #179762

investigated (Ostrowski et al. 2007). Why is there such a difference in sensitivity to GGPP depletion among small GTPases? One possibility is the different turnover rates among different GTPases, including Rab isoforms. Because Rab1 is involved in the most fundamental ER-to-Golgi membrane trafficking, its turnover may be more rapid than that of other Rab isoforms and thus more susceptible to GGPP depletion. Another possibility is that there are intrinsic factors required for geranylgeranylation, which distinguish different Rab isoforms.

Suppression of Rab1 attenuates translocation to membrane of various proteins, including angiotensin II receptor type 1 (Wu et al., 2003), cystic fibrosis transmembrane conductance regulator (CFTR) (Yoo et al., 2002), and  $\beta$ -amyloid precursor protein ( $\beta$ APP) (Dugan et al., 1995). Interestingly, statins suppressed translocation of CFTR (Shen et al., 1995) and  $\beta$ APP (Ostrowski et al., 2007). This also implies that suppression of Rab1 is involved in the effect of statins.

If Rab1 inhibition is the main cause of statin toxicity, why does it preferentially appear in skeletal muscles? We found that this is because organic anion transporting polypeptides (Oatp/OATPs) for statins, Oatp2b1 and Oatp1a4, are expressed in rat skeletal myofibers, but not in fibroblasts or L6 cells (Sakamoto et al., 2008), so that influx of statins is accelerated in myofibers. Because of the transporters, median lethal concentration of fluvastatin is 30 times lower in myofibers than in fibroblasts isolated from skeletal muscles or L6 myoblasts (Sakamoto et al., 2008). More recently, Knauer et al. (2010) confirmed the expression and function of OATP2B1 (human organic anion transporting polypeptide 2B1) as a statin uptake transporter in human skeletal muscles.

JPET #179762

In the present study, neither fluvastatin nor brefeldin A induced UPR in skeletal myofibers. It is surprising and interesting, because not only statins but also brefeldin A induced UPR in other types of cells including chronic lymphocytic leukemia cells, and human hepatocytic carcinoma (HepG2) cells, or in *Caenorhabditis elegans* (Carew et al., 2006, Citterio et al., 2008, Morck et al., 2009). The mechanism to sense abnormality in ER-to-Golgi trafficking and induce UPR may be different between skeletal myofibers and other types of cells.

Our in vitro study clearly demonstrated that GGPP supplementation attenuated fluvastatin-induced vacuolation and cell death in skeletal myofibers. Therefore, statin-induced myotoxicity could be prevented by administering GGPP or geranylgeraniol, which would be converted to GGPP in the cells. Since GGPP is not a precursor of cholesterol, it should not interfere the cholesterol-lowering action of statins. If GGPP or geranylgeraniol administration is safe therapeutically, GGPP could be a candidate for the prevention and treatment of statin-induced myotoxicity.

Taken together, we conclude that by inhibiting HMG-CoA reductase, fluvastatin decreases geranylgeranyl pyrophosphate, and as a result, suppresses Rab1 GTPase activation. Since Rab1 mediates ER-to-Golgi trafficking, statins disturb ER-to-Golgi vesicular trafficking and induce vacuolation and cell death in skeletal myofibers. We propose that this is at least a part of the mechanism for statin-induced skeletal myotoxicity.

JPET #179762

### **Acknowledgement**

We thank Ms. Sanae Sato, and Dr. Tomoyuki Ono for their technical help. We also thank Dr. Midori S. Yatabe for reading the manuscript.

### **Authorship contributions**

Participated in research design: Sakamoto and Kimura.

Conducted experiments: Sakamoto and Wada.

Performed data analysis: Sakamoto and Wada.

Wrote or contributed to the writing of the manuscript: Sakamoto, Wada, and Kimura.

Other: Sakamoto and Kimura acquired funding for the research.

JPET #179762

## V. References

Arora R, Liebo M, and Maldonado F (2006) Statin-induced myopathy: the two faces of Janus. *J Cardiovasc Pharmacol Ther* **11**: 105-112.

Bao S, Zhu J, and Garvey WT (1998) Cloning of Rab GTPases expressed in human skeletal muscle: studies in insulin-resistant subjects. *Horm Metab Res* **30**: 656-662.

Becker J, Tan TJ, Trepte HH, and Gallwitz D (1991) Mutational analysis of the putative effector domain of the GTP-binding Ypt1 protein in yeast suggests specific regulation by a novel GAP activity. *EMBO J* **10**: 785-792.

Carew JS, Nawrocki ST, Krupnik YV, Dunner K Jr, McConkey DJ, Keating MJ, and Huang P (2006) Targeting endoplasmic reticulum protein transport: a novel strategy to kill malignant B cells and overcome fludarabine resistance in CLL. *Blood* **107**: 222-231.

Citterio C, Vichi A, Pacheco-Rodriguez G, Aponte AM, Moss J, and Vaughan M (2008) Unfolded protein response and cell death after depletion of brefeldin A-inhibited guanine nucleotide-exchange protein GBF1. *Proc Natl Acad Sci U S A* **105**: 2877-2882.

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet JC, Bonini NM, and Lindquist S (2006)  $\alpha$ -synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. *Science* **313**: 324-328.

JPET #179762

Dobson R (2008) Atorvastatin advertising misled over benefits for women, study claims. *BMJ* **337**: a2209.

Dugan JM, deWit C, McConlogue L, and Maltese WA (1995) The Ras-related GTP-binding protein, Rab1B, regulates early steps in exocytic transport and processing of beta-amyloid precursor protein. *J Biol Chem* **270**: 10982-10989.

Emr S, Glick BS, Linstedt AD, Lippincott-Schwartz J, Luini A, Malhotra V, Marsh BJ, Nakano A, Pfeffer SR, Rabouille C, Rothman JE, Warren G, and Wieland FT (2009) Through the Golgi--taking stock in a new era. *J Cell Biol* **187**: 449-453.

Endo, A (1992) The discovery and development of HMG-CoA reductase inhibitors. *J Lipid Res* **33**: 1569-1582.

Filipeanu CM, Zhou F, Fugetta EK, and Wu G (2006) Differential regulation of the cell-surface targeting and function of  $\beta$ - and  $\alpha$ 1-adrenergic receptors by Rab1 GTPase in cardiac myocytes. *Mol Pharmacol* **69**: 1571-1578.

Flint OP, Masters BA, Gregg RE, and Durham SK (1997) HMG CoA reductase inhibitor-induced myotoxicity: pravastatin and lovastatin inhibit the geranylgeranylation of low-molecular-weight proteins in neonatal rat muscle cell culture. *Toxicol Appl Pharmacol* **145**: 99-110.

JPET #179762

Greenwood J, Steinman L, and Zamvil SS (2006) Statin therapy and autoimmune disease: from protein prenylation to immunomodulation. *Nat Rev Immunol* **6**: 358-370.

Hatsuzawa K, Tamura T, Hashimoto H, Hashimoto H, Yokoya S, Miura M, Nagaya H, and Wada I (2006) Involvement of syntaxin 18, an endoplasmic reticulum (ER)-localized SNARE protein, in ER-mediated phagocytosis. *Mol Biol Cell* **17**:3964-3977.

Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* **13**: 1211-1233.

Khosravi-Far R, Lutz RJ, Cox AD, Conroy L, Bourne JR, Sinensky M, Balch WE, Buss JE, and Der CJ (1991) Isoprenoid modification of rab proteins terminating in CC or CXC motifs. *Proc Natl Acad Sci U S A* **88**: 6264-6268.

Kim R, Emi M, Tanabe K, and Murakami S (2006) Role of the unfolded protein response in cell death. *Apoptosis* **11**: 5-13.

Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BL, Kim RB, and Tirona RG (2010) Human Skeletal Muscle Drug Transporters Determine Local Exposure and Toxicity of Statins. *Circ Res* **106**: 297-306.

Maeda S, Matsuoka I, Iwamoto T, Kurose H, and Kimura J (2005) Down-regulation of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger by fluvastatin in rat cardiomyoblast H9c2 cells: involvement of

JPET #179762

RhoB in Na<sup>+</sup>/Ca<sup>2+</sup> exchanger mRNA stability. *Mol Pharmacol* **68**: 414-420.

Marie M, Sannerud R, Avsnes Dale H, and Saraste J (2008) Take the 'A' train: on fast tracks to the cell surface. *Cell Mol Life Sci* **65**: 2859-2874.

Marie M, Dale HA, Sannerud R, and Saraste J (2009) The function of the intermediate compartment in pre-Golgi trafficking involves its stable connection with the centrosome. *Mol Biol Cell* **20**: 4458-4470.

Morck C, Olsen L, Kurth C, Persson A, Storm NJ, Svensson E, Jansson JO, Hellqvist M, Enejder A, Faergeman NJ, and Pilon M (2009) Statins inhibit protein lipidation and induce the unfolded protein response in the non-sterol producing nematode *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **106**: 18285-18290.

Mullen PJ, Lüscher B, Scharnagl H, Krahenbuhl S, and Brecht K (2010) Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy. *Biochem Pharmacol* **79**: 1200-1209.

Ostrowski SM, Wilkinson BL, Golde TE, and Landreth G (2007) Statins reduce amyloid-beta production through inhibition of protein isoprenylation. *J Biol Chem* **282**: 26832-26844.

Ruddock LW and Molinari M (2006) N-glycan processing in ER quality control. *J Cell Sci* **119**: 4373-4380.

JPET #179762

Sakamoto K, Honda T, Yokoya S, Waguri S, and Kimura J (2007) Rab-small GTPases are involved in fluvastatin and pravastatin-induced vacuolation in rat skeletal myofibers. *FASEB J* **21**: 4087-4094.

Sakamoto K, Mikami H, and Kimura J (2008) Involvement of organic anion transporting polypeptides in the toxicity of hydrophilic pravastatin and lipophilic fluvastatin in rat skeletal myofibres. *Br J Pharmacol* **154**: 1482-1490.

Sakamoto K, Owada Y, Shikama Y, Wada I, Waguri S, Iwamoto T, and Kimura J (2009) Involvement of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in migration and contraction of rat cultured tendon fibroblasts. *J Physiol* **587**: 5345-5359.

Segev N and Botstein D (1987) The ras-like yeast YPT1 gene is itself essential for growth, sporulation, and starvation response. *Mol Cell Biol* **7**: 2367-2377.

Segev N, Mulholland J, and Botstein D (1988) The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell* **52**: 915-924.

Shen BQ, Widdicombe JH, and Mrsny RJ (1995) Effects of lovastatin on trafficking of cystic fibrosis transmembrane conductance regulator in human tracheal epithelium. *J Biol Chem* **270**: 25102-25106.



JPET #179762

Sklan EH, Serrano RL, Einav S, Pfeffer SR, Lambright DG, and Glenn JS (2007) TBC1D20 is a Rab1 GTPase-activating protein that mediates hepatitis C virus replication. *J Biol Chem* **282**: 36354-36361.

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* **10**: 513-525.

Tanaka S, Sakamoto K, Yamamoto M, Mizuno A, Ono T, Waguri S, and Kimura J (2010) Mechanism of statin-induced contractile dysfunction in rat cultured skeletal myofibers. *J Pharmacol Sci* **114**:454-463.

Vielh E, Touchot N, Zahraoui A, and Tavitian A (1989) Nucleotide sequence of a rat cDNA: rab1B, encoding a rab1-YPT related protein. *Nucleic Acids Res* **17**: 1770.

Waclawik AJ, Lindal S, and Engel AG (1993) Experimental lovastatin myopathy. *J Neuropathol Exp Neurol* **52**: 542-549

Westwood FR, Bigley A, Randall K, Marsden AM, and Scott RC (2005) Statin-induced muscle necrosis in the rat: distribution, development, and fibre selectivity. *Toxicol Pathol* **33**: 246-257.

Wu G, Zhao G, and He Y (2003) Distinct pathways for the trafficking of angiotensin II and adrenergic receptors from the endoplasmic reticulum to the cell surface: Rab1-independent transport of a G protein-coupled receptor. *J Biol Chem* **278**: 47062-47069.

JPET #179762

Yoo JS, Moyer BD, Bannykh S, Yoo HM, Riordan JR, and Balch WE (2002) Non-conventional trafficking of the cystic fibrosis transmembrane conductance regulator through the early secretory pathway. *J Biol Chem* **277**: 11401-11409.

Zhang FL and Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem* **65**: 241-269.

JPET #179762

### III. Footnotes:

a) This study was supported by grants-in-aid for Young Scientists (B) to KS (No.20790210 and No.22790257) and for Scientific Research (C) to JK (No. 21590288) from Japan Society for the Promotion of Science, and in part from the Smoking Research Foundation (KI18003) to JK. b) A part of this study was presented in the 83<sup>rd</sup> Annual Meeting of the Japanese Pharmacological Society. c) Reprint request to Kazuho Sakamoto; Department of Pharmacology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima, Fukushima 960-1295, Japan; [kazuho@fmu.ac.jp](mailto:kazuho@fmu.ac.jp).

JPET #179762

#### IV. Legends for figures

Fig.1 Effects of fluvastatin (Flv) on Rab1A protein distribution in myofibers. After 4 days with 1  $\mu$ M Flv, myofibers were ultracentrifuged. (A) Membrane fraction. Western blot with Rab1A-specific antibody detected Ra1A signal strongly in control but weak with Flv. GGPP supplementation with Flv restored Rab1A protein but FPP did not. Coomassie brilliant blue (CBB) staining of gels served as internal control. (B) Cytosol fraction. Rab1A signal was weak in control but robust with Flv. GGPP, but not FPP, reversed the effect of Flv. Coumassie brilliant blue staining of gel (C) Quantification of the results presented in A. (D) Quantified results of B. \* $p < 0.05$ , \*\* $p < 0.01$ . Scheffé's test was used as post ad-hoc test.

Fig. 2 Time dependent changes of Rab1A distribution by fluvastatin (Flv) in myofibers. After culturing for 1 or 2 days with 1  $\mu$ M Flv, myofibers were ultracentrifuged as shown in Fig.1 (day 4). (A) Western blot of Rab1A from membrane (above) and cytosol (below) fractions. At day 1, while Rab1A signals of membrane were similar between control and Flv, but that of cytosol was increased with Flv. At day 2, Rab1A with Flv was reduced in membrane, but increased in cytosol. Coomassie brilliant blue (CBB) staining of gels served as internal control. (B) Quantified results of A. The density of Rab1A signal was divided by that of CBB, and normalized to each control. \* $p < 0.05$ , \*\* $p < 0.01$  vs. Control (100%). Student's t-test was used.

JPET #179762

Fig.3 Effect of fluvastatin (Flv) and brefeldin A (BFA) on vacuolation of myofibers. (A) Myofibers cultured for 4 days, (a): control. (b): with 1  $\mu$ M Flv, (c):with 30  $\mu$ M BFA. (B) Relationships between time and number of vacuoles with control, Flv and BFA. Data points were averaged from 5 rats. Number of vacuoles were counted in each myofiber and averaged for each rat. (C) Relationships between average number of vacuoles and concentrations of Flv or BFA.

Fig.4 Effect of 6-day treatment with fluvastatin (Flv) or brefeldin A (BFA) on myofiber death.

(A) Images of myofibers. (a) control, (b): Flv 1  $\mu$ M, (c): BFA 30  $\mu$ M. (B) Concentration-viability relationships of Flv and BFA.

Fig.5 Effect of GGPP and FPP supplementation on fluvastatin (Flv)-induced vacuolation and death of myofibers

(A) Quantified vacuoles in myofibers treated for 4 days with control, 1  $\mu$ M Flv alone, 1  $\mu$ M Flv with GGPP and 1  $\mu$ M Flv with FPP. (B) Quantified myofiber deaths induced on day 6 by control, Flv alone, Flv with GGPP, Flv with FPP. Significant differences were indicated with \* $p < 0.05$ , \*\* $p < 0.01$ . Scheffé's test was used as post ad-hoc test.

Fig.6 Effect of GGPP or FPP supplementation on BFA-induced vacuolation and death of myofibers

(A) Quantified vacuoles in myofibers treated for 4 days with control, 30  $\mu$ M BFA alone, BFA with GGPP and BFA with FPP. (B) Quantified myofiber deaths induced on day 6 by control, BFA alone, BFA with GGPP, BFA with FPP. Significant difference were

JPET #179762

indicated with \* $p < 0.05$ , \*\* $p < 0.01$ . Scheffé's test was used as post ad-hoc test.

Fig.7 Effects of Flv , BFA and tunicamycin on ER stress

A. Western blot using GRP78-specific antibody. Myofibers were treated for 4 days with 1  $\mu$ M Flv, 30  $\mu$ M BFA, and 3  $\mu$ M tunicamycin (Tum). GAPDH served as internal standard. (B) Quantified result of data represented in A. \* $p < 0.05$ , \*\* $p < 0.01$ . (C) Quantified data of myofiber death under the conditions described above. \* $p < 0.05$ , \*\* $p < 0.01$ . Scheffé's test was used as post ad-hoc test.

Fig. 8 Effect of fluvastatin on calnexin distribution in myofibers

Immunofluorescent images of single myofibers cultured for 4 days under control condition without (A) and with (B) 1  $\mu$ M fluvastatin (Flv). Myofibers were fixed and stained with an antibody against an ER marker, calnexin.



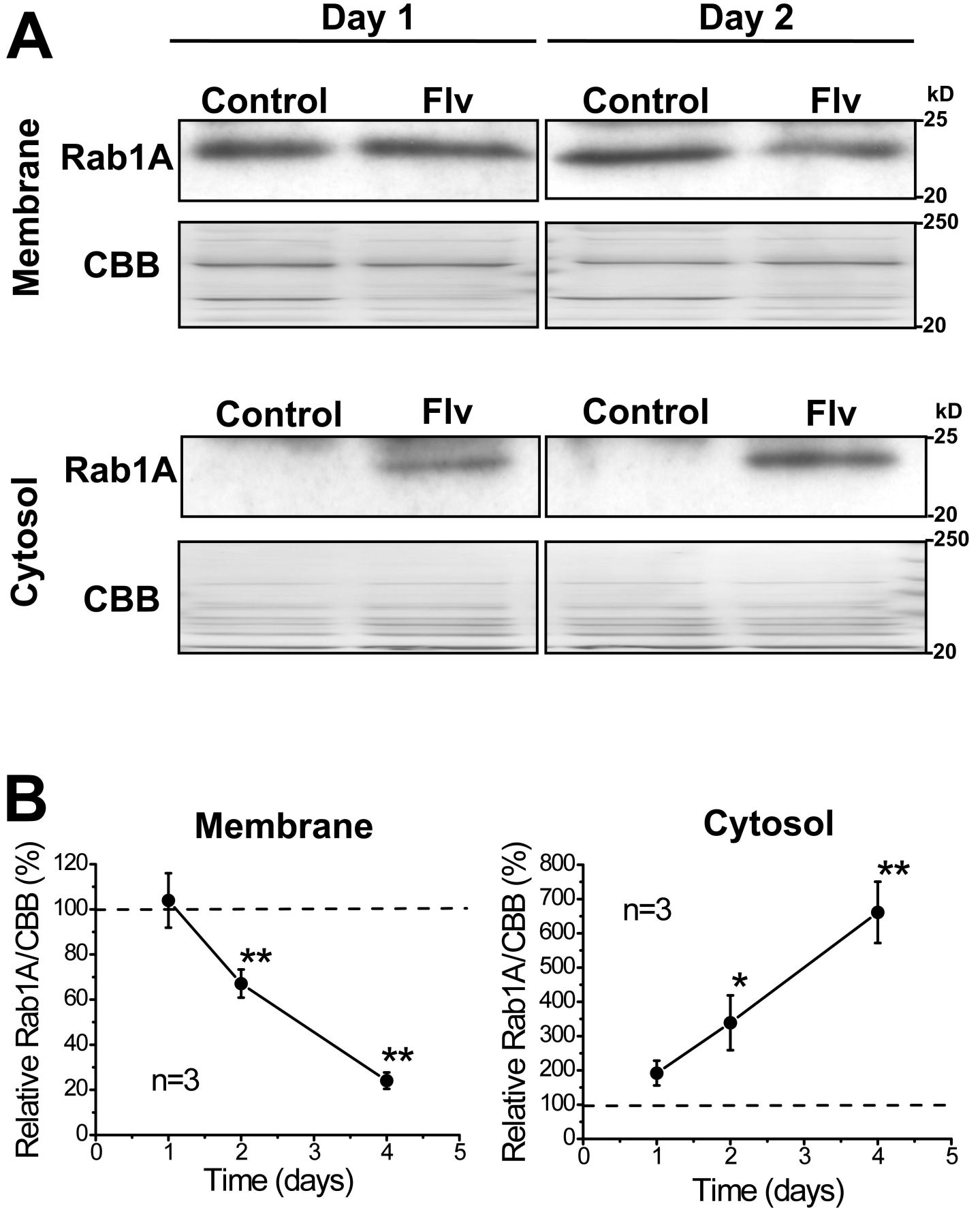


Fig. 2



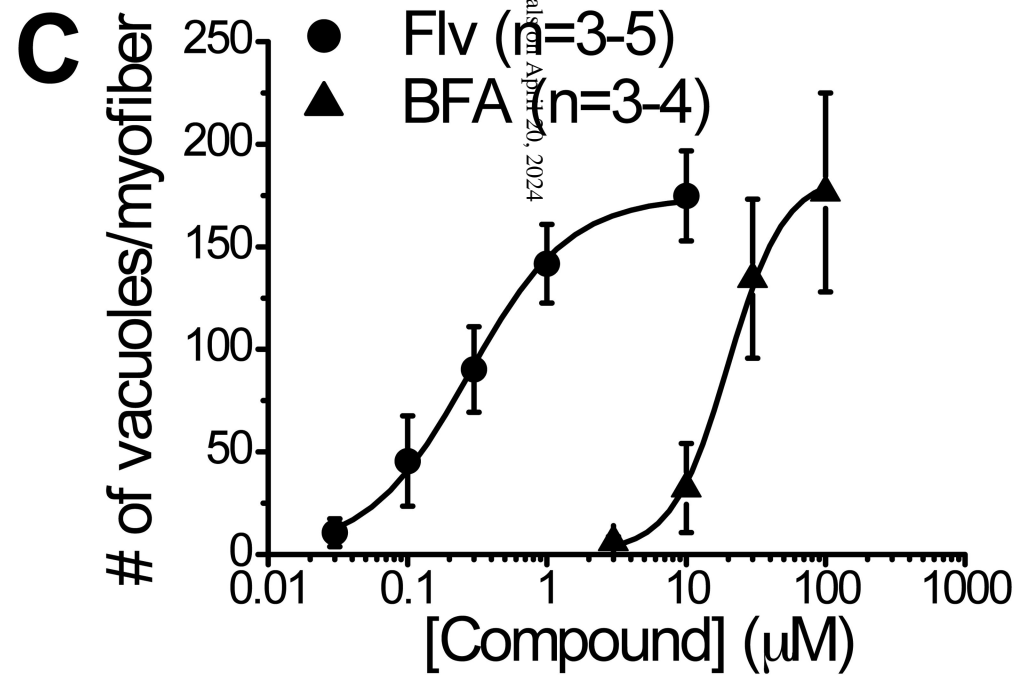
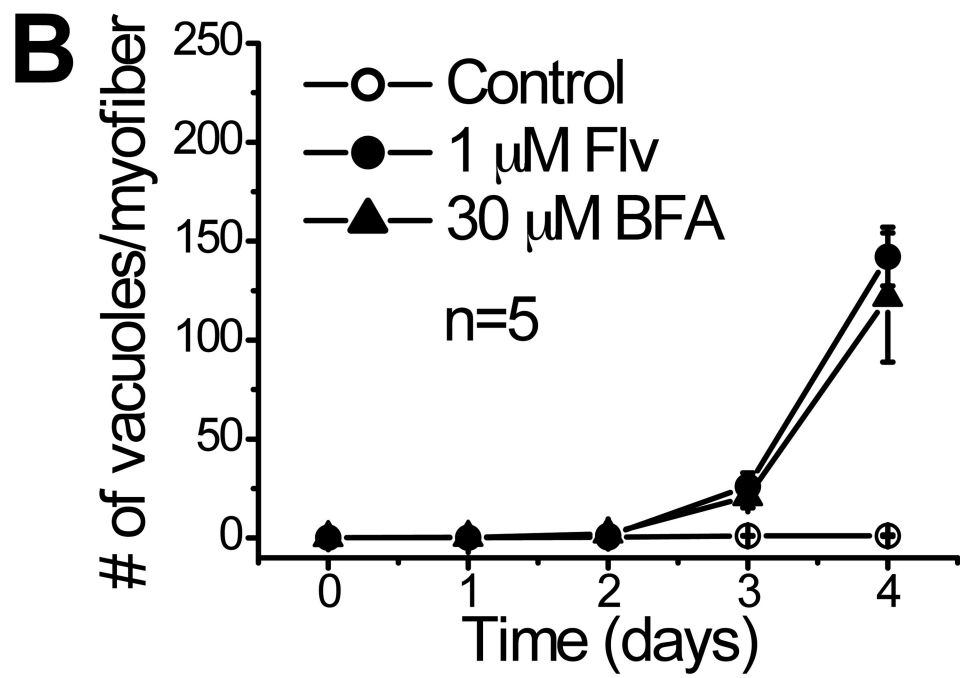
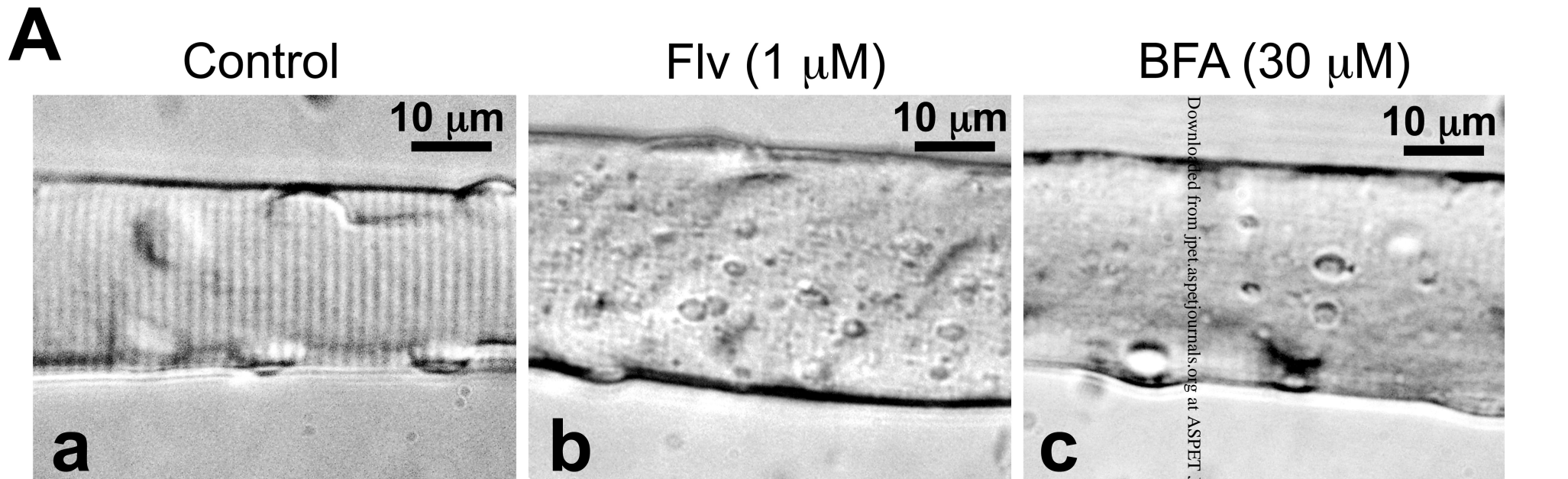
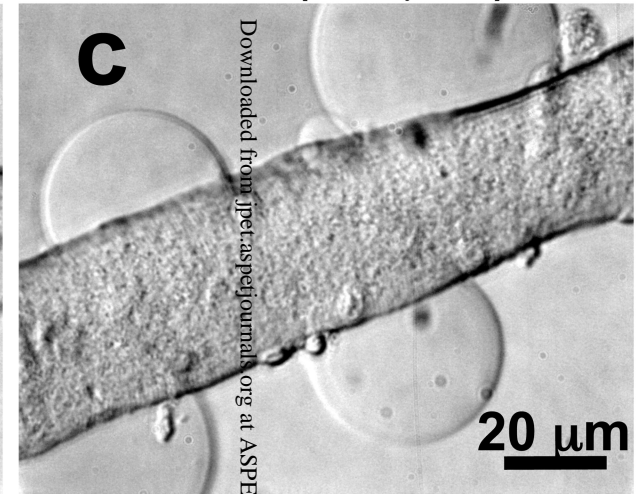
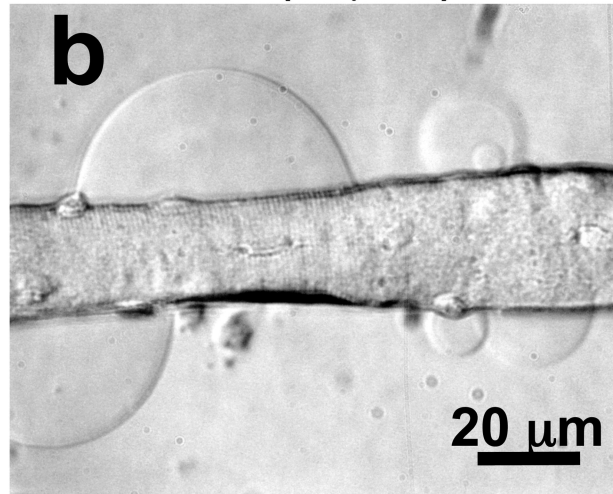
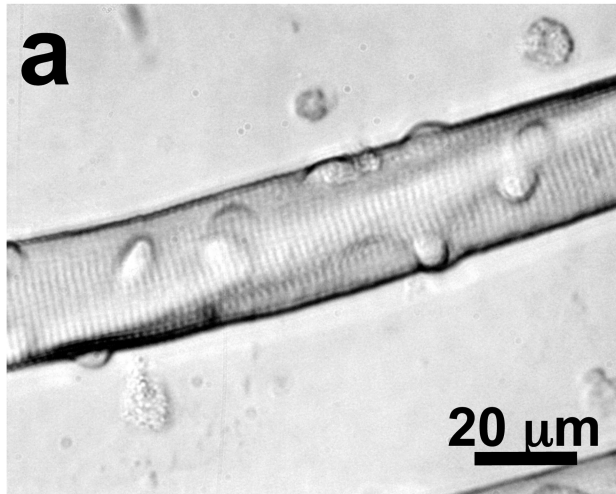


Fig. 3

**A**

Control

Flv (1  $\mu\text{M}$ )BFA (30  $\mu\text{M}$ )

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 20, 2024

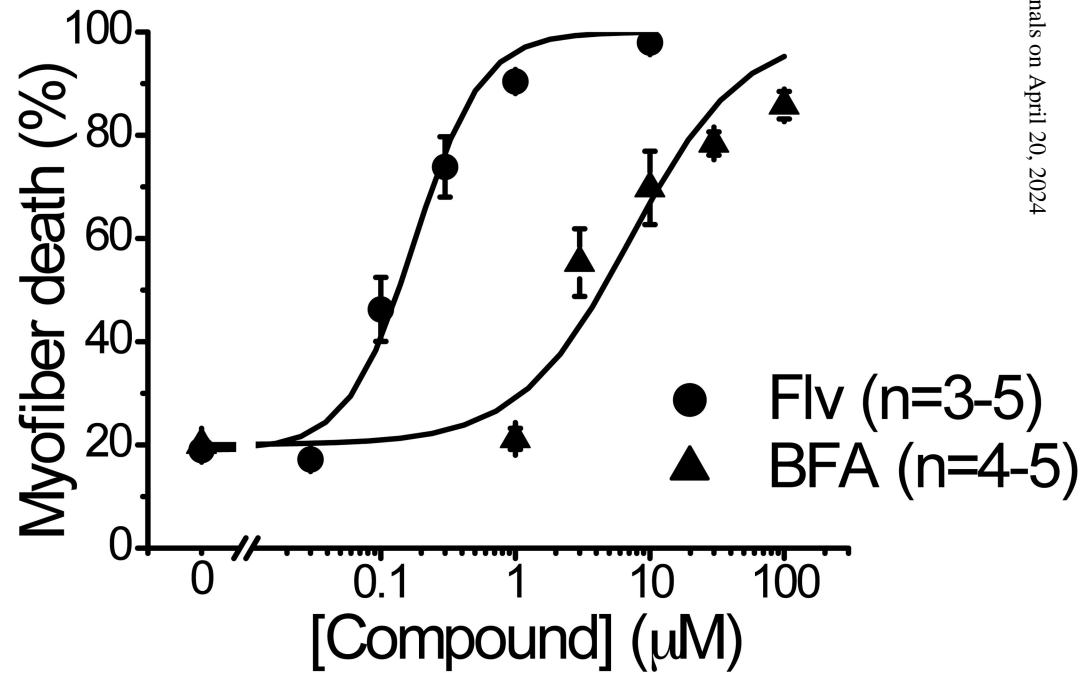
**B**

Fig. 4

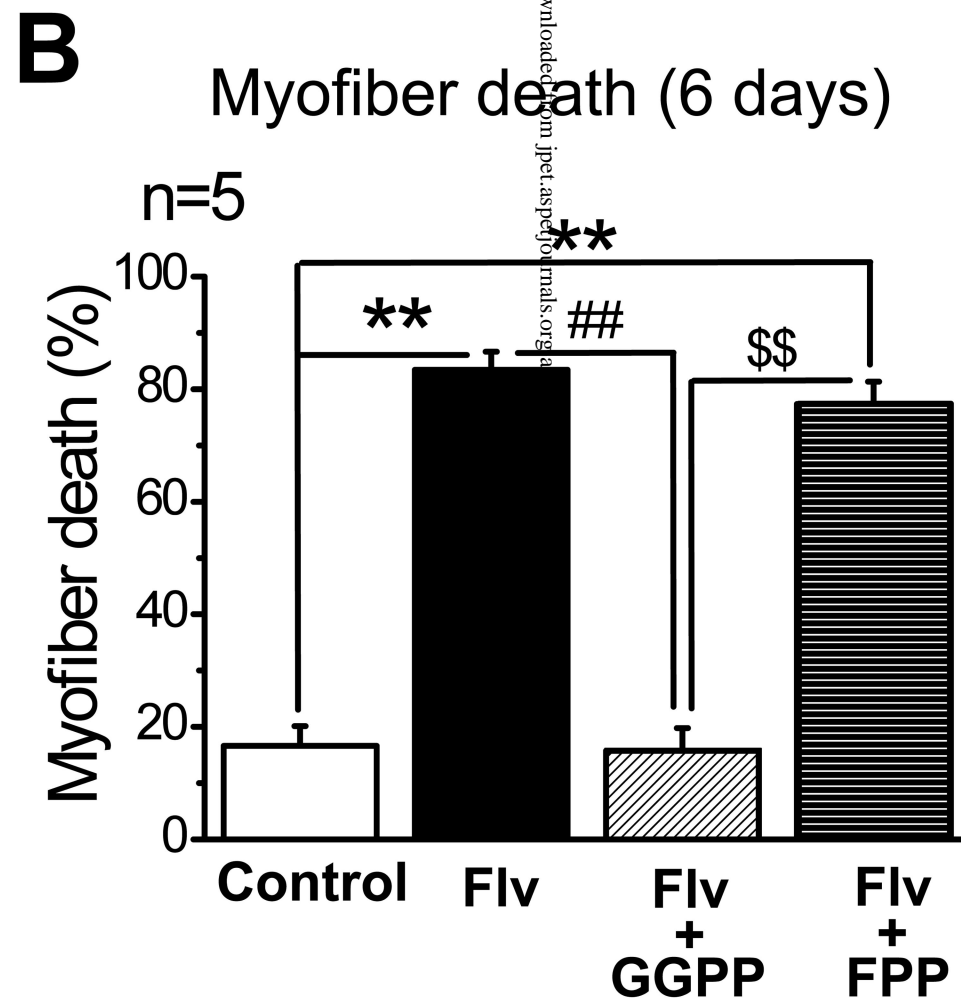
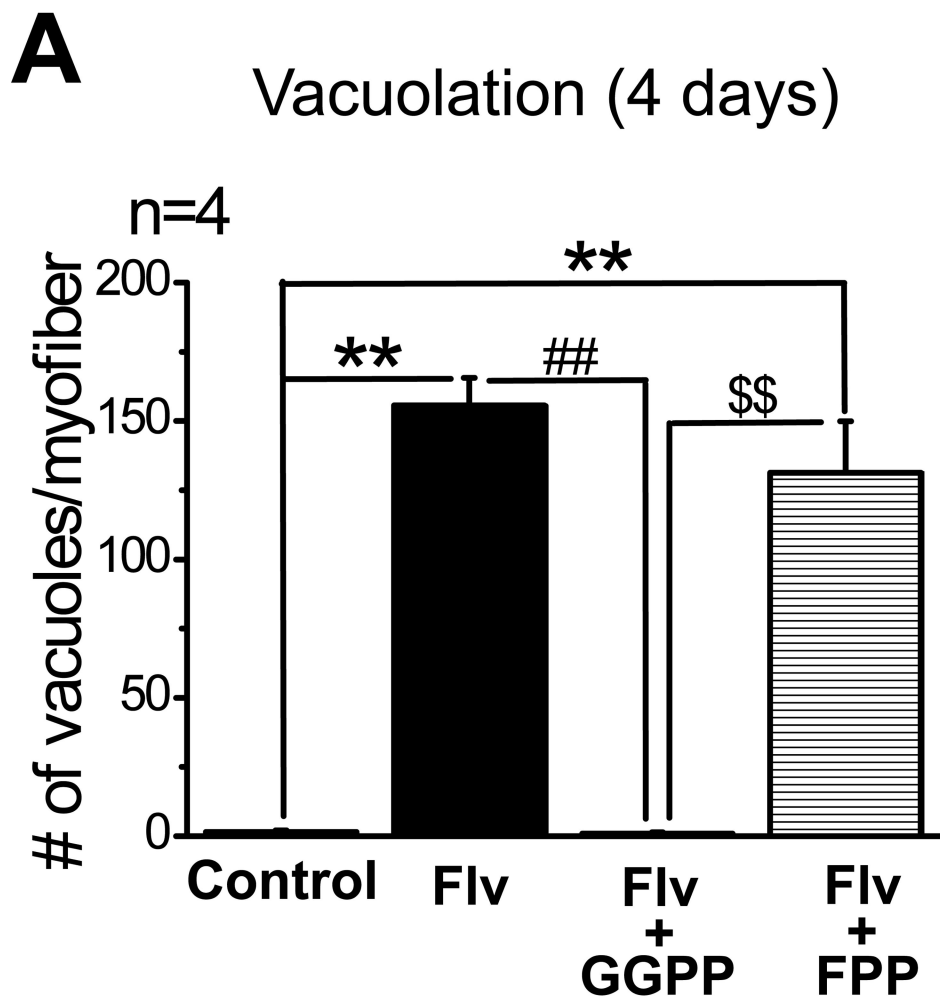


Fig. 5

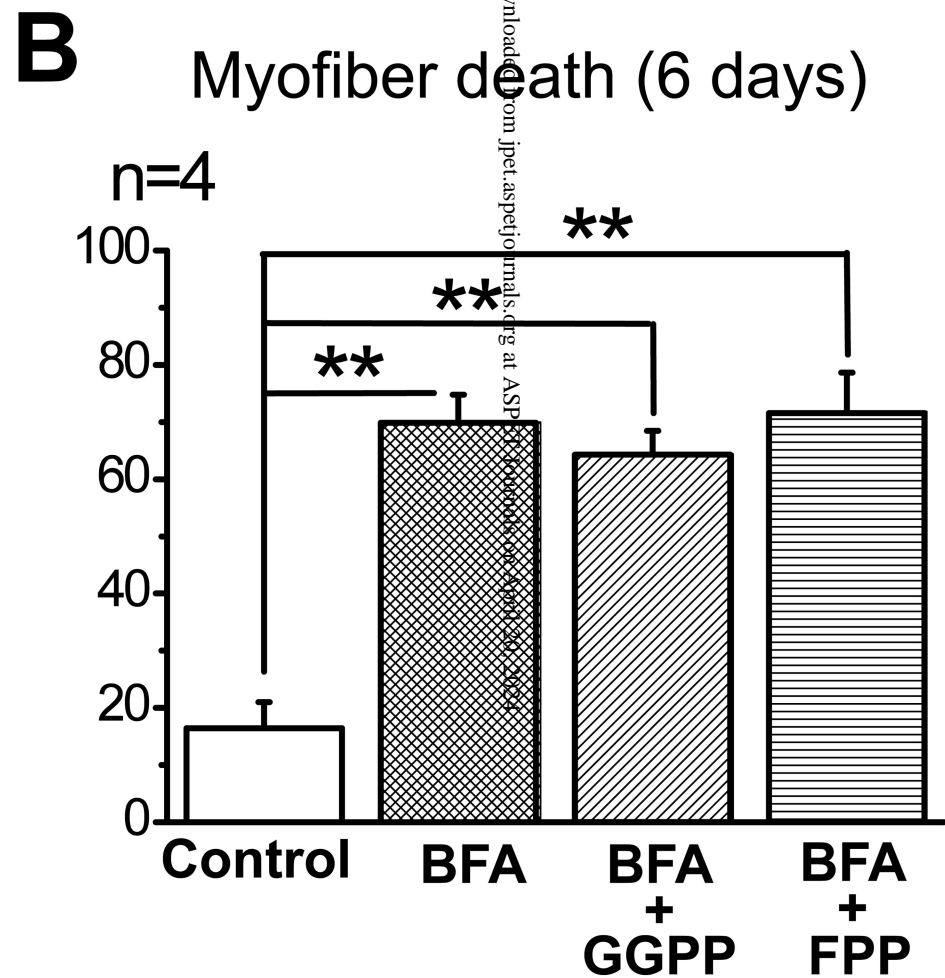
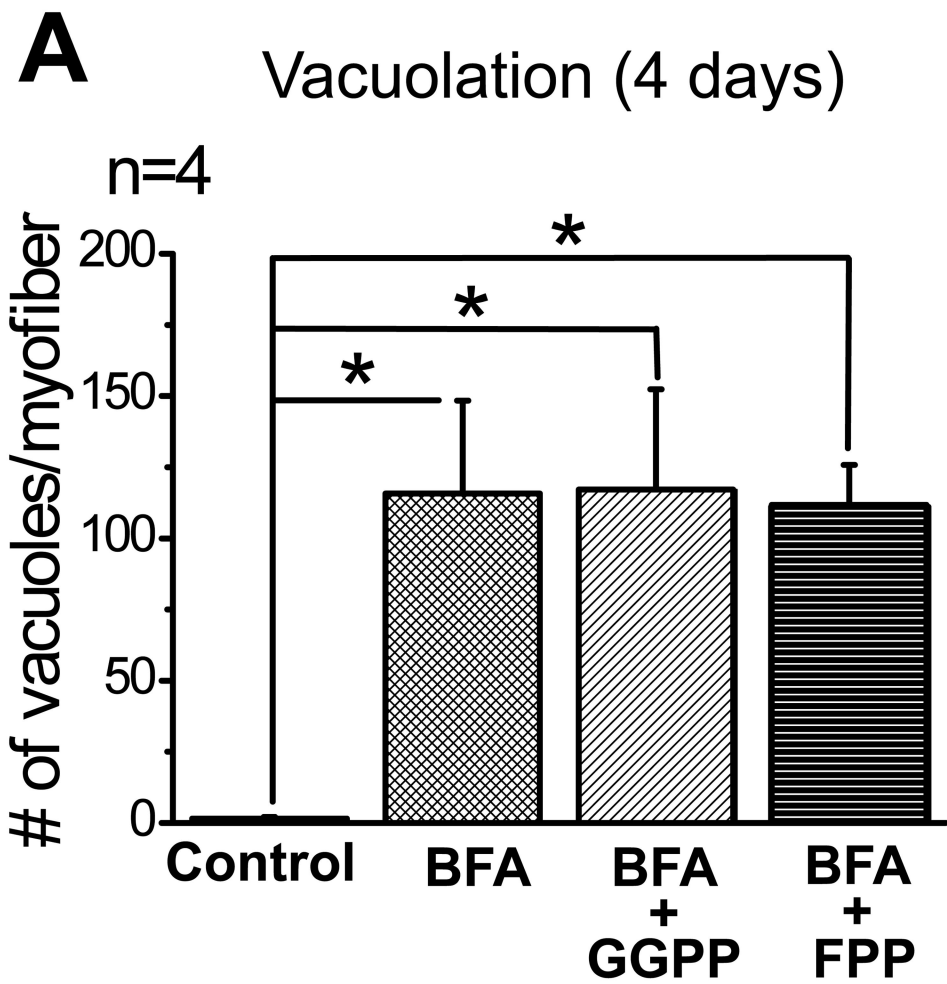
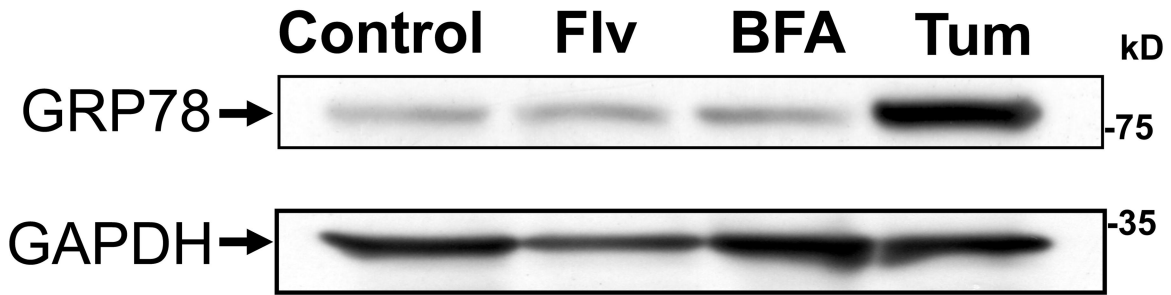
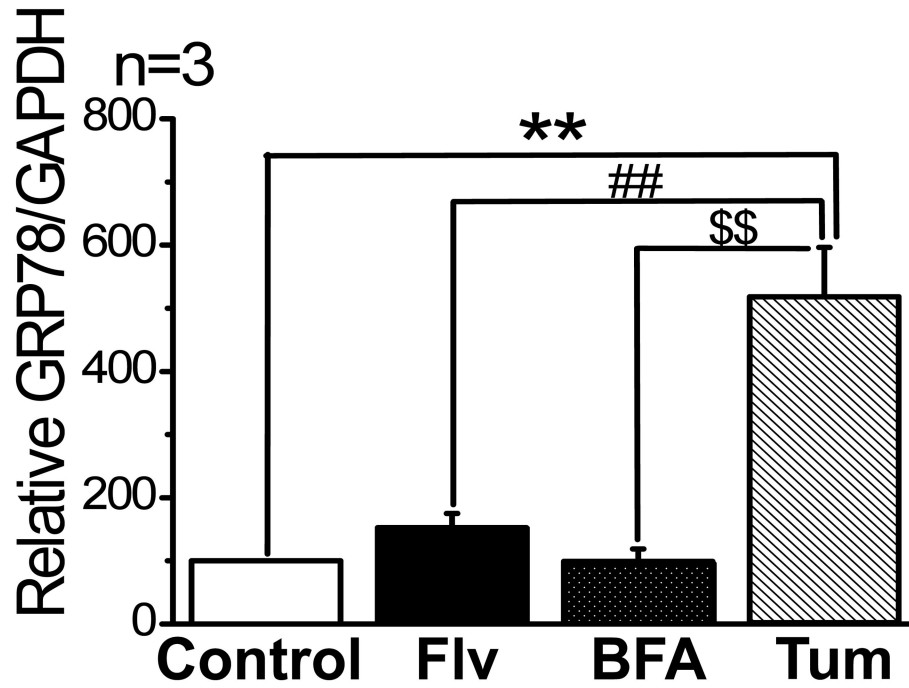


Fig. 6

**A**



**B**



**C**

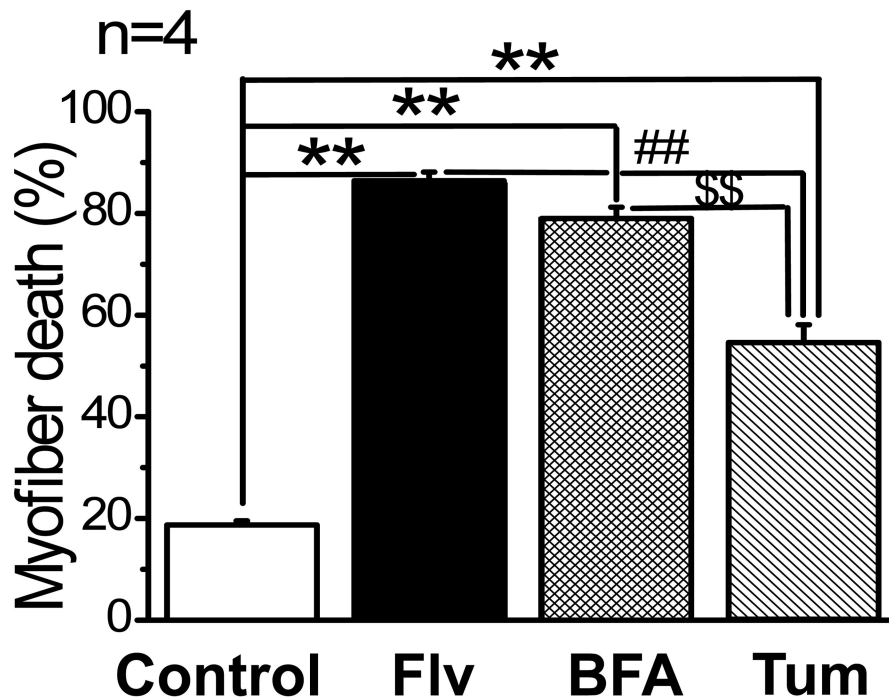


Fig. 7

Control (4 d)

Flv (4 d)

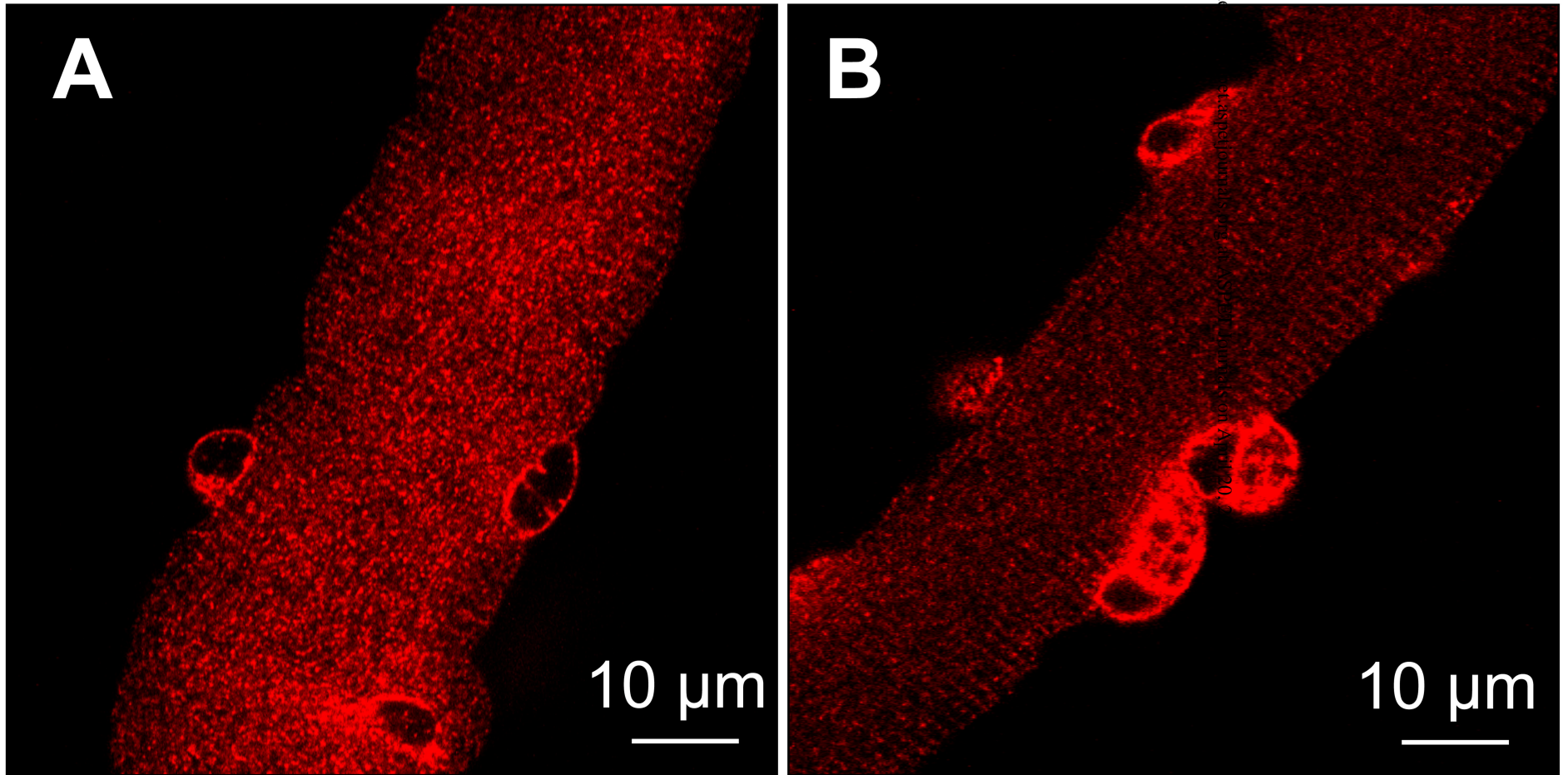


Fig. 8