Rab3B is essential for GnRH-induced gonadotrophin release from anterior pituitary cells

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Abstract
Gonadotrophin-releasing hormone (GnRH) induces the release of gonadotrophins via an increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]. Rab3B, a member of the small GTP-binding protein Rab family, is known to be involved in Ca\(^{2+}\)-regulated exocytosis in pituitary cells. However, it is not known whether Rab3B functions in the physiological process regulated by GnRH in gonadotrophs. In this study using antisense oligonucleotide against Rab3B (AS-Rab3B) we determined that Rab3B is involved in GnRH-induced gonadotrophin release. Rab3B immunopositive cells were reduced in 24% of pituitary cells by AS-Rab3B. This treatment did not affect the population of gonadotrophs or the intracellular contents of gonadotrophins. However, AS-Rab3B significantly inhibited the total amount of basal and GnRH-induced gonadotrophin released from pituitary cells. These results show that Rab3B is involved in basal and GnRH-induced gonadotrophin release but not the storage of gonadotrophins. Next, the changes in [Ca\(^{2+}\)] and exocytosis in gonadotrophs treated with AS-Rab3B were compared among Rab3B-positive and -negative cells. The change in [Ca\(^{2+}\)] was not different in the two groups, but exocytosis was significantly inhibited in Rab3B-negative cells. These results suggest that Rab3B is essential for GnRH-regulated exocytosis downstream of cytosolic Ca\(^{2+}\) in gonadotrophs.


Introduction
Regulated exocytosis of hormones, enzymes, and neurotransmitters is thought to require specialized regulators through several steps of intracellular membrane traffic. In recent years, small GTP-binding proteins of the Rab family have emerged as major candidates for these regulators. In constitutive exocytosis in yeast, Rab proteins play functional roles in the trafficking steps of secretory vesicles (Pfeffer 1992, Takai et al. 1992, Ferro-Novick & Novick 1993, Simons & Zerial 1993, Zerial & Stenmark 1993, Nuoffer & Balch 1994). In mammalian cells, Rab3A has been demonstrated to be localized in neurons and neuroendocrine cells (Mizoguchi et al. 1989, 1990, Fischer et al. 1990, Holz et al. 1994). Particularly in neurons, Rab3A is mainly localized in synaptic vesicles, and is thought to have functional roles in the targeting or fusion of synaptic vesicles with the plasma membrane in cooperation with Rabphilin-3A, a putative target protein of Rab3A (Kishida et al. 1993, Fischer et al. 1994, Inagaki et al. 1994, Takai et al. 1994).

Recently, the involvement of Rab proteins in Ca\(^{2+}\)-regulated exocytosis in anterior pituitary cells was reported (Davidson et al. 1993, Perez et al. 1994). Both Rab3A and Rab3B were expressed, but the levels of the former were less than those of the latter in pituitary cells (Redecker et al. 1995, Stettler et al. 1995). Lledo et al. (1993) showed that suppression of expression of Rab3B by microinjection of antisense oligonucleotides against Rab3B (AS-Rab3B) inhibited Ca\(^{2+}\)-regulated exocytosis in rat anterior pituitary cells. The anterior pituitary contains various types of cells. Various hypothalamic release hormones induce Ca\(^{2+}\)-regulated exocytosis in pituitary cells. It has not yet been clarified in which types of pituitary cells Rab3B is expressed and functions or which release hormone is involved in the Rab3B-related mechanism of regulated exocytosis.

In the present study, first we determined the effects of suppression of Rab3B expression on the basal and gonadotrophin-releasing hormone (GnRH)-induced gonadotrophin release after introduction of AS-Rab3B into pituitary cells. Next, we determined the effects of suppression of Rab3B expression on GnRH-induced Ca\(^{2+}\) mobilization and exocytosis in single gonadotrophs. Our results indicated that Rab3B is essential for basal and GnRH-induced gonadotrophin release in gonadotrophs and that Rab3B functions as an intracellular signal of GnRH downstream of Ca\(^{2+}\) mobilization.
Materials and Methods

Materials

Lipofectin was obtained from Gibco/BRL (Gaithersburg, MD, USA). GnRH was obtained from Peninsula Laboratories, Inc. (Belmont, CA, USA). Anti-rat luteinizing hormone (LH) rabbit polyclonal antibody and anti-rat follicle-stimulating hormone (FSH) rabbit polyclonal antibody were obtained from UCB-Bioproducts SA (Brussels, Belgium). Anti-Rab3B rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Biotinylated anti-rabbit IgG goat antibody, cell sorting grade fluorescein-labelled avidin (avidin DCS), and alkaline phosphatase avidin D were obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). Indo1-AM and TMA-DPH were obtained from Molecular Probes (Eugene, OR, USA). Trypsin, pancreatic, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was obtained from Cell Culture Laboratories (St Louis, MO, USA). Trypsin, pancreatic, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal calf serum (FCS) was obtained from Cell Culture Laboratories (Cleveland, OH, USA). pSV-β-galactosidase control vector was obtained from Promega Corporation (Madison, WI, USA).

Cell preparation

Female Wistar rats weighing 180–200 g were decapitated, and an anterior pituitary cell suspension was prepared as follows. The tissues were cut into fragments and incubated for 20 min at 37 °C with 0·2% trypsin in medium 199 (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 0·3% BSA. They were then incubated for 15 min at 37 °C with 0·25% pancreatin in the same medium. Cell aggregates were isolated by gentle pipetting in Ca2+-free phosphate-buffered saline (PBS). Non-dispersed fragments were separated by filtration through four layers of gauze cloth.

Synthesis and purification of oligonucleotides

AS-Rab3B (5′-AGTTACTGAGCCATCTCGGAT-3′) and sense oligonucleotide (5′-ACAGTTTACCGTGATGGAAGC-3′) against Rab3B (S-Rab3B) were synthesized on an automated DNA-RNA synthesizer (model 394, Perkin Elmer, Osaka, Japan). Crude oligonucleotides were purified using reversed-phase HPLC. AS-Rab3B, which is complementary to Rab3B DNA from −7 to +15, overlapped the translation initiation codon, while S-Rab3B corresponded to the DNA sequence located downstream from the initiation codon (+187 to +208) (Lledo et al. 1993).

Introduction of oligonucleotide into pituitary cells

The isolated cells were seeded at a density of 5 × 105 cells per well in 1 ml complete medium (medium 199 supplemented with 10% FCS) in Falcon 6-well plates, and cultured for 24 h at 37 °C. Then the cells were washed with medium 199 without serum. AS-Rab3B (3 mg) or S-Rab3B (3 mg) and lipofectin (6 mg) were diluted to 10 ml with distilled water, mixed, and incubated for 30 min at room temperature. A 20 µl aliquot of the mixture was added to each well, mixed, and the cells were incubated for 18 h at 37 °C. We confirmed in a preliminary study using an enzyme-linked immunosorbent assay for the detection of parallel-transfected β-galactosidase activity (Gibco/BRL) (Casadaban et al. 1983) that incubation for 18 h is necessary and sufficient for introduction of oligonucleotides into pituitary cells. After incubation, the cells were washed with fresh medium and cultured in 1 ml complete medium for 6 h or 72 h until experimentation. Lledo et al. (1993) showed that expression of Rab3B did not change 5–6 h after the introduction of AS-Rab3B into pituitary cells, but was suppressed after 72 h. Therefore, in the present study we prepared two groups of cells incubated for 6 h or 72 h in complete medium. To evaluate the efficiency of introduction of oligonucleotides, parallel transfection was performed using a pSV-β-galactosidase control vector (1 mg/ml) for each experiment. Cells expressing β-galactosidase were visualized by staining with 5-bromo-4-chloro-3-indolygalactopyranoside (X-gal). The treated cells were fixed directly with phosphate-buffered 0·2% glutaraldehyde and 2% formaldehyde for 5 min at 4 °C, washed, and then incubated at 37 °C with a buffered solution containing 0·1% X-gal. After 24 h, the cells were examined under phase-contrast microscopy. The cells actively expressing galactosidase were identified by their intense dark blue staining. We also confirmed by staining with trypan-blue that treatment with lipofectin, oligonucleotides, and plasmids at the above concentrations has no effect on the viability of pituitary cells.

Measurement of the populations of gonadotrophin-containing cells and Rab3B-expressing cells

The oligonucleotide-treated cells were harvested using 0·02% EDTA and 0·25% trypsin. Harvested cells in suspension were washed, and the number of collected cells was counted using a Coulter cell counter. Then the cells were fixed with Zamboni’s fixative (4% paraformaldehyde and 15% saturated picric acid solution in phosphate buffer) for 24 h at 4 °C and treated with 0·3% Triton X-100 for 30 min. The fixed cells were preincubated for 10 min at room temperature in 10% normal goat serum (Nichirei, Tokyo, Japan). The cells were incubated with anti-rat LH or FSH rabbit antibodies at a final dilution of 1:1000 or rabbit antibody against Rab3B peptide at a final dilution of 1:200 in PBS containing 0·1% BSA overnight at 4 °C and washed twice with PBS. Then the cells were incubated with biotinylated anti-rabbit IgG goat antibody (1:250) overnight at 4 °C, washed twice with PBS and incubated
with fluorescein-labelled avidin (1:250) for 30 min at room temperature. The cells were resuspended in PBS. The population of fluorescence-labelled cells was analysed using a fluorescence-activated cell sorter (EPICS Elite, Coulter Electronics, Hialeah, FL, USA), and $1 \times 10^4$ cells were analysed in each preparation.

**Measurement of gonadotrophin release and content**

The oligonucleotide-treated cells were washed and then stimulated with or without various concentrations of GnRH for 30 min at 37°C. The medium was collected and the concentrations of LH and FSH were determined by double-antibody radioimmunoassay (RIA), using the materials and protocols provided by the NIDDK, NIH (Bethesda, MD, USA). The intra-assay variations for LH and FSH were less than 5%. For measurement of gonadotrophin content, the oligonucleotide-treated cells were washed and solubilized in medium 199 containing 0.3% Triton X-100 (Sigma) for 18 h at room temperature, and then the concentrations of LH and FSH in the collected medium were determined by RIA.

**Measurement of the population of gonadotroph-releasing cells**

The original cell immunoblot assay (Kendall & Hymer 1987) was used for the detection of LH-releasing cells. The oligonucleotide-treated cells were resuspended with Hanks’ balanced salt solution (HBSS) containing 0.3% BSA at a concentration of $2 \times 10^5$ cells/ml. Fifty-microlitre aliquots of the cell suspensions were gently dropped onto equilibrated polyvinylidene difluoride transfer membranes (Immobilon, Millipore Corporation, Bedford, MA, USA), and these were incubated for 30 min at 37°C with or without $10^{-7}$ M GnRH. After incubation, the membranes were carefully washed and immersed in Tris-buffered saline containing 10% BSA for 4 h at room temperature. LH bound to the transfer membranes was immunostained by incubation with the following solutions at room temperature: (1) anti-rat LH rabbit antibodies at a final dilution of 1:20,000 in Tris–buffered saline containing 3% BSA for 12 h; (2) biotinylated anti-rabbit IgG goat antibodies (1:250) in the same solution for 30 min; (3) alkaline phosphatase avidin D (1:500) for 30 min; and (4) the enzyme substrate nitro-blue tetrazolium (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (0.15 mg/ml) in Alkaline Phosphatase Color Development Reagent (GIBCO/BRL) for 3 min. Blots were then analysed and counted with a grey level image processor. We confirmed in a preliminary study that the anti-rat LH rabbit antibody used here can specifically detect LH around the basal and GnRH-induced secretory levels. On the other hand, we were not able to acquire complete specificity for the detection of FSH in the cell immunoblot assay, although we used several kinds of antibodies and several different sets of experimental conditions.

**Purification and identification of gonadotrophs**

Pituitary gonadotrophs were purified, as reported previously (Masumoto et al. 1991). Briefly, the oligonucleotide-treated pituitary cell aggregates were preincubated at 37°C in complete medium for 30 min. Then, for the labelling experiments, anti-rat LH rabbit antibodies were applied under the same conditions for 20 min. The cells were washed twice with HBSS. Biotinylated anti-rabbit goat IgG was applied for 40 min at room temperature, and then the cells were washed twice with HBSS. Fluorescein-labelled avidin was applied for the next 10 min at room temperature, and the cells were then washed twice with HBSS. Gonadotroph-enriched (85–90%) pituitary cells were then obtained with a fluorescence-activated cell sorter (EPICS Elite; Coulter Electronics).

**Measurement of $[Ca^{2+}]_{i}$ and exocytosis in gonadotrophs**

Purified gonadotrophs were plated on glass coverslips which were sealed under a 1·0 cm hole in the bottom of 35 mm culture dishes and coated with poly-d-lysine. The cells were maintained at 37°C in an atmosphere of 95% air:5% CO₂ in complete medium and used for experiments after 24–48 h. Intracellular free calcium concentration ([Ca²⁺]i) was monitored with a digital imaging fluorescence microscope using the Ca²⁺-sensitive fluorescent dye indo1-AM as described previously (Masumoto et al. 1991). Briefly, gonadotrophs were incubated at 37°C for 60 min in medium 199 containing 5 µM indo1-AM. The cells were then washed with PBS and placed on the microscope stage. [Ca²⁺]i in single cells was measured at 5-s intervals with a digital imaging microscope system (ACAS Ultima; Meridian, USA). The ratio of the intensities of fluorescent emission at 450 nm and 510 nm with excitation at 350 nm was determined as [Ca²⁺]i. The fluorescent excitation beam was targeted on the cells, and then the emission fluorescence was recorded by digital imaging and analysed with a computer.

For measurement of exocytosis, the gonadotrophs were incubated with a final concentration of 1 µM TMA-DPH and the 430 nm fluorescence of single cells at 360 nm excitation was measured (Masumoto et al. 1993, 1995). Cells were intermittently irradiated for 10–50 ms every 1 s to prevent damage caused by ultraviolet rays. A preliminary study showed that the TMA-DPH fluorescence of gonadotrophs had a peak intensity with emission at 430 nm and excitation at 350–360 nm. The changes in fluorescence intensity in individual cells were analysed by a fluorescence microscope system with computer analysis (ACAS Ultima; Meridian). The increase in fluorescence represents the sum total of exocytosis.

After the [Ca²⁺]i and exocytosis experiments, Rab3B-positive cells were identified by immunofluorescence analysis as described above using cells which were fixed with Zamboni’s fixative (4% paraformaldehyde and 15%
Rab3B protein was inhibited.

medium for 72 h. Arrows indicate cells in which expression of
c) or AS-Rab3B (b and d) and then incubated in complete
pituitary cells. The used cell had been treated with S-Rab3B (a and
digital images (c and d) of Rab3B immunofluorescence in anterior
(1998)
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saturated picric acid solution in phosphate buffer) at 4 °C
for 24 h and treated with 0.3% Triton X-100 for 30 min.

Analysis of data

Representative data from three replicated experiments are
shown as the mean values plus S.E.M. The homoscedastic-
tiy of the data was analysed by Bartlett’s test. The
significance of differences was assessed by the Kruskal-
Wallis test or analysis of variance, followed by Scheffe’s
multiple comparison, and a P value of less than 0.01 was
considered to be significant.

Results

Inhibition of expression of Rab3B protein in pituitary cells by
AS-Rab3B

Pituitary cells treated with oligonucleotides were fixed 6 h
or 72 h after incubation in complete medium. Cells
expressing β-galactosidase, which was parallel-transfected
with AS-Rab3B or S-Rab3B, comprised approximately
20–25% of pituitary cells (data not shown). The cells
treated with AS-Rab3B or S-Rab3B were immunostained
with a polyclonal antibody against Rab3B (Fig. 1). While
almost all S-Rab3B-treated cells were positively stained,
some AS-Rab3B-treated cells were not labelled with
Rab3B antibody (Fig. 1). The populations of Rab3B-
positive cells and Rab3B-negative cells in cultures treated
with lipofectin alone as a control, with lipofectin and
S-Rab3B, or with lipofectin and AS-Rab3B were analysed
by flow cytometry. The percentages of Rab3B-positive
cells were 94.6 ± 1.7 and 70.8 ± 2.8 (means ± S.E.M.) 72 h
after the treatment with S-Rab3B and AS-Rab3B respec-
tively (Table 1). The reduction induced by AS-Rab3B was
significant (P<0.01). On the other hand, there was no
difference between these two groups 6 h after treatment
(Table 1).

Effects of AS-Rab3B on the population of
gonadotrophin-containing cells and the intracellular content of
gonadotrophins in pituitary cells

Seventy-two hours after treatment with lipofectin alone as
a control, with lipofectin and S-Rab3B, or with lipofectin
and AS-Rab3B, the numbers of pituitary cells and the
populations of LH- and FSH-containing cells were ana-
ysed by flow cytometry. The total numbers of pituitary
cells were not different in the three groups (data not
shown). The percentages of LH-containing cells and
FSH-containing cells were not different in the three
groups (Table 1). The total intracellular contents of
gonadotrophins (LH and FSH) 72 h after treatment were
measured. The total amounts of LH and FSH in cells
treated with AS-Rab3B were similar to those in cells
treated with S-Rab3B or lipofectin alone as a control
(Table 1).

Effects of AS-Rab3B on basal and GnRH-induced
gonadotrophin release

We determined the basal and GnRH-induced release of
gonadotrophins in anterior pituitary cells 6 h or 72 h after
treatment with lipofectin alone as a control, with lipofectin

![Figure 1 Phase-contrast images (a and b) and corresponding digital images (c and d) of Rab3B immunofluorescence in anterior pituitary cells. The used cell had been treated with S-Rab3B (a and c) or AS-Rab3B (b and d) and then incubated in complete medium for 72 h. Arrows indicate cells in which expression of Rab3B protein was inhibited.](image-url)
and S-Rab3B, or with lipofectin and AS-Rab3B. Six hours after treatment, basal and GnRH-induced release of LH and FSH were similar in the three groups (Fig. 2). However, 72 h after treatment, concentrations of LH and FSH in the media of the AS-Rab3B-treated group were lower than those of the S-Rab3B-treated group. AS-Rab3B reduced both the basal and GnRH-induced LH and FSH release by approximately 20% in total (Fig. 2).

Effects of AS-Rab3B on the population of LH-releasing cells

To determine the population of LH-releasing cells after treatment with lipofectin alone as a control, with lipofectin and S-Rab3B, or with lipofectin and AS-Rab3B, we identified LH released from single pituitary cells by a cell immunobloting assay (Fig. 3a). Six hours after treatment, the populations of LH-releasing cells were almost the same in all three groups both with and without GnRH stimulation (Fig. 3b). However, 72 h after treatment with AS-Rab3B, the numbers of LH-releasing cells were significantly reduced by approximately 20% relative to those treated with S-Rab3B and lipofectin alone both with and without GnRH stimulation (Fig. 3b).

Effects of AS-Rab3B on GnRH-induced Ca2+ mobilization and exocytosis in single gonadotrophs

Seventy-two hours after treatment with AS-Rab3B, gonadotrophs were sorted from the pituitary cells with a cell sorter and concentrated to 90%. Rab3B-positive and Rab3B-negative cells were identified by immunohistochemical analysis with an antibody against Rab3B after experiments on [Ca2+], and exocytosis. Rab3B-negative cells comprised 20–30% of gonadotrophs. GnRH-induced Ca2+ mobilization and exocytosis in Rab3B-negative cells and Rab3B-positive cells were evaluated (Fig. 4). There was no difference between the
Discussion

In this study the reduction of Rab3B-positive cells was accompanied by inhibition of gonadotrophin release 72 h after treatment with AS-Rab3B, while neither was observed 6 h after treatment. This suggested that incubation for 72 h after treatment is necessary for the inhibition of expression of Rab3B protein by AS-Rab3B. Moreover, we found that the number of the cells which showed LH release was decreased by AS-Rab3B although the number of LH-containing cells was not affected. The total content of intracellular gonadotrophins in pituitary cells was also not changed by AS-Rab3B. These observations indicated that treatment with AS-Rab3B induced cells to synthesize and store but not release gonadotrophins. AS-Rab3B inhibited Rab3B-positive cells in 20–25% of pituitary cells, resulting in a 20% inhibition of gonadotrophin release into the medium and a 20% reduction in the number of gonadotrophin-releasing cells. AS-Rab3B did not affect the number of pituitary cells or the population of gonadotrophs, suggesting that Rab3B is not involved in the proliferation or differentiation of gonadotrophs. AS-Rab3B did not affect the intracellular contents of gonadotrophins, suggesting that Rab3B is not involved in the storage of gonadotrophins. These results suggest that Rab3B protein is essential for the exocytosis of gonadotrophins. Moreover, to establish the specificity of the role of Rab3B in exocytosis, we examined GnRH-induced Ca²⁺ mobilization and exocytosis in single gonadotrophs. Ca²⁺ mobilization was similar in Rab3B-positive and -negative cells, but exocytosis was significantly inhibited in the latter. These results suggest that Rab3B is not involved in GnRH-induced Ca²⁺ mobilization but is involved downstream of Ca²⁺ mobilization in GnRH-regulated exocytosis in gonadotrophs. Moreover, Rab3B was involved not only in GnRH-induced release but also in basal gonadotrophin release. Little is known about the mechanism by which basal release is regulated. It is known that spontaneous Ca²⁺ oscillation is observed in gonadotrophs (Masumoto et al. 1991). If spontaneous Ca²⁺ oscillation is involved in basal release, the role of Rab3B may be similar in both basal and GnRH-induced gonadotrophin release.

In neurons, a model for docking and fusion of vesicles with the plasma membrane in exocytosis has been established. This docking and fusion process is mediated by the general fusion machinery, named the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) system (Rothman 1994) and the Rab3A system. The SNARE system was first identified in the brain. SNAREs are composed of several proteins: vesicle-associated membrane protein (VAMP) in the synaptic vesicle membrane, and synaptosome-associated protein 25 kDa (SNAP-25) and syntaxin in the presynaptic plasma membrane (Pevsner et al. 1994). Many reports (Blasi et al. 1993, Sollier et al. 1993a, b, DeBello et al. 1995) have suggested that these SNARE proteins may mediate the docking and fusion of synaptic vesicles to the plasma membrane. Recently, these SNARE proteins have been reported to be expressed not only in neurons but also in...
pituitary cells (Oho et al. 1995, Aguado et al. 1996, Jacobsson & Meister 1996). We also reported that SNAP-25 is involved in prolactin release in GH4C1 pituitary cells (Masumoto et al. 1997). On the other hand, Rab3A, which is a small GTP-binding protein, is involved in Ca\(^{2+}\)-dependent exocytosis particularly in neurotransmitter release (Nuoffer & Balch 1994). Rab3A functions with a putative target protein of Rab3A, Rabphilin-3A, which has a Rab3A-binding N-terminal domain and a Ca\(^{2+}\)-binding C-terminal domain (Shirataki et al. 1992, 1993, Yamaguchi et al. 1993). Intracellular Ca\(^{2+}\) mobilization may regulate Rab3A via Rabphilin-3A. It has been reported that in pituitary cells Rab3B is expressed at higher levels than Rab3A (Redecker et al. 1995, Stettler et al. 1995) and positively regulates Ca\(^{2+}\)-regulated exocytosis (Lledo et al. 1993). In this study, we also found that Rab3B positively regulates GnRH-regulated gonadotrophin release. On the other hand, Rab3A is known to negatively regulate exocytosis in neuroendocrine cells (Holz et al. 1994, Johannes et al. 1994, Weber et al. 1996). The differences in the functions of Rab3A and Rab3B are not clear. Rabphilin-3A can bind not only to Rab3A but also to Rab3B (Weber et al. 1996). Rabphilin-3A positively regulates exocytosis in neuroendocrine cells (Chung et al. 1995, Komuro et al. 1996) and cortical granule exocytosis in eggs (Masumoto et al. 1996). However, we found that Rabphilin-3A is not expressed in pituitary cells (not shown). The molecular mechanism of Rab3B action in pituitary hormone secretion has not yet been clarified, and future studies should address its mechanism of action including the interaction with the SNARE system.

References


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