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γ-Amino butyric acid (GABA) release in the ciliated protozoon *Paramecium* occurs by neuronal-like exocytosis

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SUMMARY

***Paramecium primaurelia* expresses a significant amount of γ-amino butyric acid (GABA). Paramecia possess both glutamate decarboxylase (GAD)-like and vesicular GABA transporter (vGAT)-like proteins, indicating the ability to synthesize GABA from glutamate and to transport GABA into vesicles. Using antibodies raised against mammalian GAD and vGAT, bands with an apparent molecular weight of about 67 kDa and 57 kDa were detected. The presence of these bands indicated a similarity between the proteins in *Paramecium* and in mammals. VAMP, syntaxin and SNAP, putative proteins of the release machinery that form the so-called SNARE complex, are present in *Paramecium*. Most VAMP, syntaxin and SNAP fluorescence is localized in spots that vary in size and density and are primarily distributed near the plasma membrane. Antibodies raised against mammal VAMP-3, sintaxin-1 or SNAP-25 revealed protein immunoblot bands having molecular weights consistent with those observed in mammals. Moreover, *P. primaurelia* spontaneously releases GABA into the environment, and this neurotransmitter release significantly increases after membrane depolarization. The depolarization-induced GABA release was strongly reduced not only in the absence of extracellular Ca^{2+} but also by pre-incubation with bafilomycin A1 or with botulinum toxin C1 serotype. It can be concluded that GABA occurs in *Paramecium*, where it is probably stored in vesicles capable of fusion with the cell membrane; accordingly, GABA can be released from *Paramecium* by stimulus-induced, neuronal-like exocytotic mechanisms.**

Key words: GABA exocytosis, membrane depolarization, SNARE proteins, ciliated protozoa.

INTRODUCTION

Eukaryotic cells release molecules by fusing secretory vesicles with the plasma membrane, and, in fact, all cell types appear to have a constitutive pathway for secretion. Some specialized cells, such as neurons, also maintain a separate reservoir of vesicles that secrete their content only in response to specific stimuli, a phenomenon called regulated exocytosis (Kelly, 1985).

Vesicular exocytosis is the physiological mechanism of neurotransmitter release from axon terminals. Classical exocytosis occurs when the terminal plasma membrane depolarizes and voltage-sensitive calcium channels (VSCCs) open. This influx of Ca^{2+} causes previously docked vesicles to undergo Ca^{2+}-dependent fusion and to release their messenger content directly into the extracellular space (Schweizer et al., 1995; Südhof, 1995). There is convincing evidence that exocytosis involves the assembly of core complexes from the so-called SNARE proteins. Core complexes are composed of the plasma membrane target proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) and different isoforms of the synaptic vesicle protein VAMP (vesicle-associated membrane protein) (Schweizer et al., 1995; Südhof, 1995). The core complex formation allows vesicles to dock at the nerve terminal plasma membrane, the first step of regulated exocytosis. Disassembly of the SNARE complex also involves other proteins that prime vesicles and make them competent for exocytosis (Fasshauer et al., 1997; Fukuda et al., 2000; Katz and Brennwald, 2000; Weber et al., 1998). These proteins include homohexameric ATPase, NSF and its adaptor protein α-soluble NSF attachment protein (α-SNAP). Additional proteins, such as the Ca^{2+} sensor synaptotagmin, facilitate fusion with the plasma membrane.

In the motile, ciliated, protozoon *Paramecium*, constitutive and stimulated exocytosis of dense-core vesicles (trichocysts) is a thoroughly studied process (Plattner et al., 1991; Vayssié et al., 2000). Trichocysts are defensive organelles that are secreted through a pathway of regulated exocytosis in response to extracellular stimulation (Plattner, 2002; Plattner and Kissmehl, 2003; Vayssié et al., 2000). Evidence originating from biochemical, electrophysiological and calcium-imaging experiments shows that a transient subcortical increase of intracellular free Ca^{2+} is necessary for exocytotic membrane fusion in *Paramecium*. This process involves a stimulus-dependent influx of Ca^{2+} from the external medium and a stimulus-dependent release of Ca^{2+} from internal stores (Erxleben et al., 1997; Kerboeuf and Cohen, 1990; Klauke and Plattner, 1997; Stelly et al., 1995).

We have previously reported that $GABA_A$ and $GABA_B$ receptors are expressed in *Paramecium*, where they regulate motor behavior and membrane potential (Bucci et al., 2005; Ramoino et al., 2003). In the present study, we investigated the presence and release of γ-amino butyric acid (GABA), the natural agonist of the above

receptors. We demonstrated that this amino acid and the machinery for its synthesis, storage and release are present in *Paramecium*. In addition, we showed that GABA is released into the environment in both a spontaneous and stimulus-dependent manner. This release may exert GABA's autocrine regulatory functions. Finally, we found that GABA release depends on the presence of extracellular calcium and is blocked by bafilomycin and botulinum toxin C1 serotype (BoNT/C1).

MATERIALS AND METHODS

Cell and culture conditions

Experiments were carried out on *Paramecium primaurelia* Sonneborn, stock 90, cultured at 25°C in lettuce medium (pH 6.9) inoculated with *Enterobacter aerogenes* (Sonneborn, 1970). Cells were harvested in the late log phase of growth.

Antibodies

Monoclonal antibodies anti-sintaxin-1 and anti-SNAP25 as well as polyclonal rabbit anti-VAMP-3 (cellulobrevin) were purchased from SynapticSystem (Goettingen, Germany); the polyclonal guinea pig anti-vGAT (vesicular GABA transporter), rabbit anti-$GAD_{65/67}$ (glutamate decarboxylase) and rabbit anti-GABA were obtained from Chemicon International (Temecula, CA, USA); the monoclonal antibody anti-GABA was obtained from Sigma Chemical Co. (St Louis, MO, USA). The secondary antibodies anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 488, anti-guinea pig Alexa Fluor 488, anti-mouse Alexa Fluor 594 and anti-rabbit Alexa Fluor 594 were obtained from Molecular Probes, Invitrogen (Carlsbad, CA, USA).

Chemicals

Pre-stained molecular mass markers were obtained from Amersham (Buckinghamshire, England); BoNT/C1 was purchased from Wako (Osaka, Japan); remaining chemicals were purchased from Sigma unless otherwise specified in the text.

Immunolabeling

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) buffer (0.01 mol l^{-1}, pH 7.4) for 30 min, washed three times with PBS and incubated for 60 min with 3% bovine serum albumin (BSA) in PBS plus 1% Triton X-100. The blocking permeabilizing buffer was removed, and the cells were incubated overnight at 4°C with one or two of the following antibodies: mouse anti-GABA (1:1000), mouse anti-syntaxin-1 (1:100), mouse anti-SNAP25 (1:500), rabbit anti-VAMP3 (1:100), rabbit anti-GABA (1:400), rabbit anti-$GAD_{65/67}$ (1:400) or guinea pig anti-vGAT (1:2000). After three washes in 1% BSA in PBS plus 0.1% Triton X-100 for 10 min each, the appropriate secondary antibody conjugated to Alexa Fluor 488 (dilution 1:300) was applied for 1 h at 37°C. After extensive washing in PBS, cells were mounted in glycerol/buffer. In control experiments, the absence of cross-reactivity between the secondary antibodies was verified by omitting one of the primary antibodies during incubation. In addition, for every combination involving double labeling, singly labeled vesicles were always observed in the cells. The specificity of primary antibodies was tested by preabsorbion with the appropriate immunogen for GABA, GAD and vGAT (Chemicon) for 2 h at room temperature before processing *Paramecium* cells. No staining was detected in the negative control experiments.

Image acquisition and analysis

Images (1024×1024×8 bit) were acquired with a Leica TCS SP2 Confocal Scanner (Mannheim, Germany) equipped with an Ar (457–476–488–514 nm) 100 mW laser and a HeNe (543 nm) 1.5 mW laser using a one Airy disk unit pinhole diameter and an oil immersion HCX PL APO ×100/1.4 objective (Diaspro et al., 2006). Alexa Fluor 488 was excited with the 488 nm line of the Ar laser, and its fluorescence was collected in a spectral window of 500–530 nm. Alexa 594 fluorescence was collected using the 543 nm excitation wavelength and a 590–620 nm spectral window emission. Spectral windows were selected by the acousto-optic beam splitter of the Leica SP2 scanning head. Serial optical sections were taken through the cell at a *z*-step of 75 nm. The image acquisition through green and red channels was performed according to a sequential acquisition protocol to reject possible cross-talk artefacts.

The Leica Confocal Software programme was used for image acquisition, storage and analysis. Labeling experiments were repeated 4–5 times, and images are representative of observations of an average of 30 cells in each sample.

Western blot

Cells were centrifuged at 600 ***g*** to a density of 200×10^3 cells ml^{-1} and re-suspended in water containing a protease inhibitor cocktail. Samples were homogenized, sonicated and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. 100 µg of *Paramecium* protein and 10 µg of rat cerebral cortex protein per lane were used. The appropriate pre-stained molecular mass markers were run concomitantly. Transfer to nitrocellulose (NC) membranes was performed electrophoretically at 100 V for 75 min. After saturation with 5% non-fat dry milk, the NC membranes were incubated for 1 h with the following primary antibodies: mouse anti-syntaxin-1 (1:5000), mouse anti-SNAP25 (1:1000), rabbit anti-VAMP3 (1:10,000), rabbit anti-$GAD_{65/67}$ (1:1000) and guinea pig anti-vGAT (1:1000). The membrane was incubated with the appropriate horseradish peroxidase-conjugated (sheep) secondary antibody and coated using the ECL western blotting detection system for 1 min. The membrane was immediately exposed to Amersham autoradiography film at room temperature for various periods (from 5 s to 60 min) in a film cassette.

BoNT/C1 treatment of cell lysates

BoNT/C1 was activated by reduction in 200 mmol l^{-1} Tris-HCl (pH 8.0), 500 mmol l^{-1} NaCl and 50 µmol l^{-1} $ZnCl_2$ with 5 mmol l^{-1} dithiothreitol for 30 min at 37°C to release the light chain (Schilde et al., 2008). Crude cell lysates from *P. primaurelia* were incubated with BoNT/C1 (final concentration 500 nmol l^{-1}) for 1 h at 37°C. The protein was methanol precipitated and analyzed by SDS-PAGE. Syntaxin was detected on western blots with an anti-rat syntaxin-1 monoclonal antibody.

GABA release

Cells (100×10^3 ml^{-1}) were adapted in the adaptation solution ($CaCl_2$ 1 mmol l^{-1}, Hepes 1 mmol l^{-1}, pH 7.4) for 10 min and were then treated with 20, 30 or 40 mmol l^{-1} KCl (final concentration) for 10 min. After treatment, the free-cell medium was analyzed by high performance liquid chromatography (HPLC). GABA release was studied in standard or Ca^{2+}-free medium. In some experiments, *Paramecium* cells were pre-treated with bafilomycin (500 µmol l^{-1}; 60 min) or with BoNT/C1 (40 nmol l^{-1}; 24 h). The control release samples were cells that had been adapted in medium for 10 min without depolarization.

HPLC

The amount of intracellular and released GABA was determined by HPLC. The analytical method involved pre-column derivatization with

o-phthalaldehyde followed by separation on a C_{18} reverse-phase chromatography column (Chrompack, Middleburg, The Netherlands; 10 mm × 4.6 mm, 3 μm; 30°C) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm) (Raiteri et al., 2000). The amount of GABA in the biological samples was determined by interpolating the peak area of the *Paramecium* sample using a curve obtained with different standard GABA solutions. Areas have been normalized using homoserine as an internal standard. The sensitivity of the assay amounted to about 0.1 pmol GABA.

Statistical analysis

Differences between means (±s.e.m.) were determined using the Student's *t*-test or analysis of variance (ANOVA) with Dunnett's or Newman–Keuls *post-hoc* tests (GraphPad Prism, GraphPad, San Diego, CA, USA). Tests were repeated on four different occasions over several weeks.

RESULTS

GABA localization in *Paramecium*

The localization and distribution of GABA in *P. primaurelia* were examined using a polyclonal antibody and laser confocal microscopy. Labeling was detected primarily at the cell surface and in the food vacuoles of cells in the late log phase of growth (Fig. 1A). Fig. 1B documents staining of dot-like structures located on the outlines of the regularly arranged surface fields (kinetics) that are characteristic of *Paramecium* cells.

The presence of the biosynthetic enzyme GAD and vGAT was determined by both immunofluorescence and immunoblotting. Using confocal microscopy, GAD- and vGAT-like immunoreactivity was detected at the cell surface and inside the cytoplasm (Fig. 1C,D). No immunostaining was observed in the negative controls when the primary antibody was omitted or when the primary antibody was pre-absorbed by GABA or the specific GAD or vGAT immunogen peptides.

Immunoblots showed two bands with an apparent molecular weight of about 67 kDa and 57 kDa, using antibodies raised against mammalian GAD and vGAT, respectively (Fig. 1E, lane 2). These molecular mass values were consistent with those obtained in synaptosomes prepared from rat cerebral cortex under the same experimental conditions (Fig. 1E, lane 1).

Proteins of the release machinery in *Paramecium*

Transmitter release at the nerve terminal involves assembly of the SNARE complex proteins. VAMPs, in conjunction with syntaxins and SNAP-25, are thought to play a role in docking synaptic vesicles to the presynaptic plasma membrane (Rothman and Wieland, 1996; Sudhof, 1995). Evidence from both confocal microscopy and immunoblot indicated the presence in *Paramecium* of putative VAMP, syntaxin and SNAP proteins, which were identified using antibodies raised against mammalian proteins (monoclonal anti-syntaxin-1 and anti-SNAP-25 antibodies and the polyclonal antibody anti-cellubrevin/VAMP3 that cross-reacts with both synaptobrevins VAMP1 and VAMP2). By confocal microscopy, immunoreactivity appeared localized as a punctate pattern on the cell surface (Fig. 2A–C). Negative controls, obtained by omitting the primary antibody, showed no immunostaining.

Western blots of proteins derived from *Paramecium* cells, performed using anti-syntaxin-1 antibody, revealed two bands with estimated molecular masses of about 32 kDa and 36 kDa (Fig. 2D, line 2). Bands with an apparent molecular mass of about 20 kDa and 25 kDa were detected using a broad-spectrum anti-VAMP antibody and an anti-SNAP-25 antibody, respectively (Fig. 2D, line 2). These values are consistent with those obtained for rat cerebral cortex synaptosomes (Fig. 2D, line 1).

Co-localization experiments

Double-labeling experiments in *Paramecium* not only demonstrated the relationship between GABA and the putative vGAT, which transports GABA into vesicles, but also showed the relationship between GABA and vesicular VAMP. GABA was stained with Alexa Fluor 594 (red fluorescence) whereas vGAT and VAMP were stained with Alexa Fluor 488 (green fluorescence). GABA was partially co-localized (yellow fluorescence) with vGAT (Fig. 3A) and VAMP (Fig. 3B), a co-localization that may occur at the

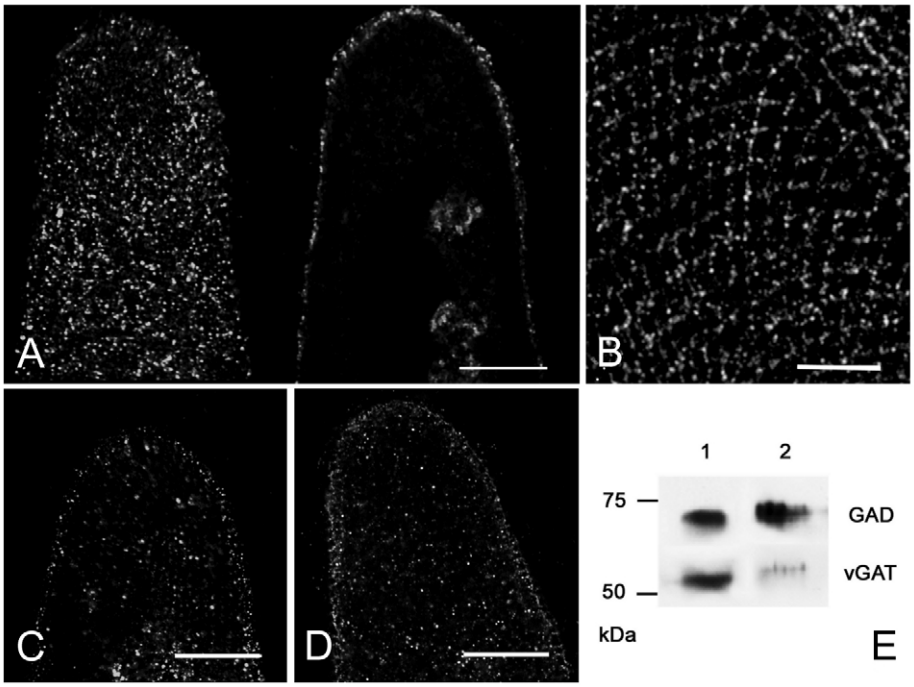

Fig. 1. (A,B) Immunofluorescence localization and distribution of γ-amino butyric acid (GABA) in *Paramecium primaurelia*. Punctate label was detected over the entire cell surface (A, left) and in food vacuoles inside the cytoplasm (A, right) of cells stained with a polyclonal antibody against GABA. Dot-like structures are stained on the outlines of the regularly arranged surface fields (kinetics) characteristic of *Paramecium* cells (B). GABA synthesis enzyme glutamate decarboxylase (GAD)-like (C) and vesicular GABA transporter (vGAT)-like (D) immunofluorescence were detected on the cell membrane and inside the cytoplasm. Bars, 20 μm (A,C,D) or 5 μm (B). (E) Proteins from *P. primaurelia* (line 2) and rat cerebral cortex synaptosomes (line 1) were subjected to SDS-PAGE and western blotting. Protein bands with estimated molecular masses of about 57 kDa and 67 kDa were detected when anti-vGAT and anti-GAD raised against mammal proteins were used. The position of the molecular mass marker is shown on the left.

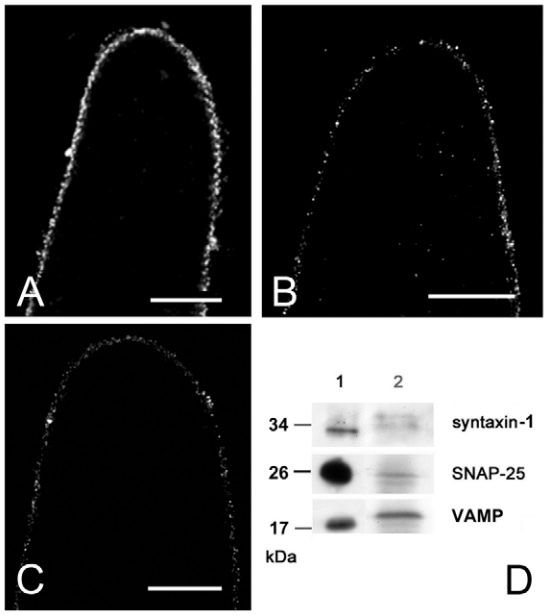

Fig. 2. Localization and distribution of vesicle-associated membrane protein (VAMP)- (A), syntaxin- (B) and SNAP-like (C) immunofluorescence in *Paramecium primaurelia* detected using antibodies raised against mammal proteins. The label was detected over the entire cell membrane using cells in the late log phase of growth. Bars, 20 μm. (D) Proteins from *P. primaurelia* (line 2) and rat cerebral cortex synaptosomes (line 1) were subjected to SDS-PAGE and western blotting. Bands with apparent molecular mass of 25 kDa and 20 kDa were detected using the SNAP-25 and VAMP-3 antibodies, respectively, and two bands with estimated molecular masses of 32 kDa and 36 kDa were detected using the syntaxin-1 antiserum. The position of the molecular mass marker is shown on the left.

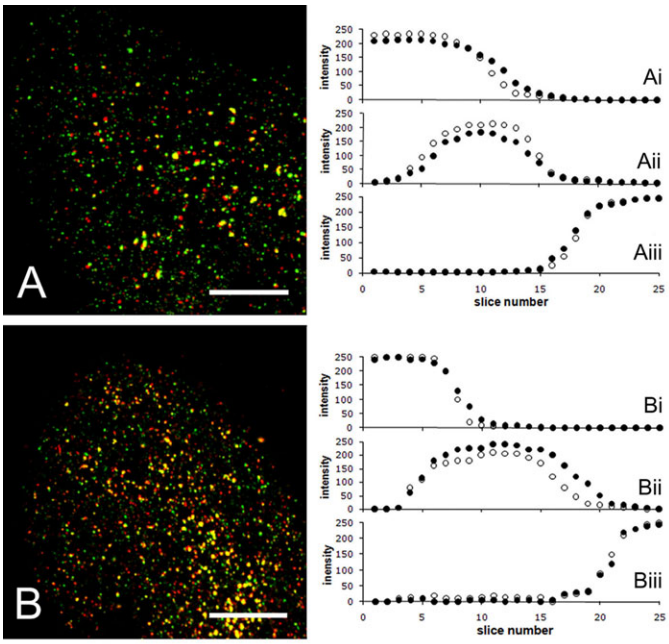

Fig. 3. (Left) Co-localization of γ-amino butyric acid (GABA) and the vesicular GABA transporter (vGAT)-like (A), as well as co-localization of GABA and vesicle-associated membrane protein (VAMP)-like (B), in *Paramecium primaurelia* cells labeled with an antibody against GABA, conjugated with Alexa 594 (red), and antibodies against mammal vGAT or VAMP-3, conjugated with Alexa Fluor 488 (green). Co-localization is shown as yellow fluorescence. (Right) *z*-stack profiles of fluorescence intensity in double-labeled vesicles; data represent the fluorescence-intensity distribution along the *z*-axis of three yellow-labeled vesicles selected from different optical planes (Ai, Bi, top; Aii, Bii, middle; Aiii, Biii, bottom) (green, ○; red, ●). Bars, 10 μm.

vesicular level. Co-localization along the *z*-axes was demonstrated by the similarity of the green and red *z*-profiles of the fluorescence intensity of three double-stained vesicles collected at the top (Fig. 3Ai,Bi), middle (Fig. 3Aii,Bii) and bottom (Fig. 3Aiii,Biii) planes of a 2-μm thick *z*-stack.

KCl-induced GABA release

We used KCl depolarization (from 0 mmol l^{-1} up to 40 mmol l^{-1} KCl; 10 min exposure) in the presence of Ca^{2+} to induce GABA release in *Paramecium*. The amino acid was spontaneously released into the environment (control condition, 0 mmol l^{-1} KCl) but the amount of released amino acid increased after K^+ depolarization in a concentration-dependent manner (Fig. 4A). The GABA released in the 10-min fraction collected before the onset of K^+ stimulation amounted to 32±3.6 pmol 10^4 cells^{-1}. The K^+-evoked overflow was 65±7.0, 120±7.8 and 160±10.3 pmol 10^4 cells^{-1} at 20, 30 and 40 mmol l^{-1} KCl, respectively. In these sets of experiments, the total GABA content in the paramecia amounted to about 2450±6.0 pmol 10^4 cells^{-1}.

A lack of extracellular Ca^{2+} abolished the depolarization-induced GABA release (Fig. 4B), suggesting the involvement of regulated exocytosis. The involvement of vesicular GABA in amino acid release is supported by experiments with bafilomycin-A1, which was expected to prevent the accumulation of GABA into vesicles (Moriyama and Futai, 1990; Roseth et al., 1995). Cells were first incubated with bafilomycin-A1 (0.5 μmol l^{-1}, 60 min) and then exposed to 30 mmol l^{-1} KCl. Pre-incubation with bafilomycin A1 greatly reduced the depolarization-evoked GABA release (Fig. 4B). As revealed by examination of GABA-labeled vesicle abundance using confocal microscopy, there was a substantial decrease in the number of labeled vesicles in *Paramecium* cells exposed to bafilomycin A1 (Fig. 5A).

We also tested the ability of BoNT/C1, which hampers neurotransmitter exocytosis by cleaving syntaxin-1 and SNAP-25 in mammals (Foran et al., 1996; Schiavo et al., 1995), to block GABA release. Incubation of cells for 24 h in the presence of the activated toxin (40 nmol l^{-1}) significantly inhibited GABA release (Fig. 4B). Activity of botulinum toxin was demonstrated in western blot experiments by directly measuring the amount of *Paramecium* syntaxin present after exposure of cell extracts to the botulinum toxin. Syntaxin, detected with the anti-rat syntaxin-1 monoclonal antibody used above, was totally cleaved by BoNT/C1 (Fig. 5B).

To identify putative cleavage sites for BoNT/C1 in the *Paramecium* syntaxin (PtSyx) sequences, a multiple sequence alignment was performed using the *Rattus norvegicus* syntaxin as consensus sequences (Fig. 6). Sequences were aligned using the ClustalW program in the Molecular Evolutionary Genetics Analysis (MEGA) software package (Kumar et al., 2004) (http://www.megasoftware.net/). Domain predictions (SNARE and transmembrane domains) were determined by PROSITE and TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Our analysis showed that some *Paramecium* syntaxin homologs (PtSyx 3-1/3-2), as well as some ciliate-specific syntaxins (PtSyx4-1 and PtSyx 11-1), possess a single Lys–Ala peptide bond in the same

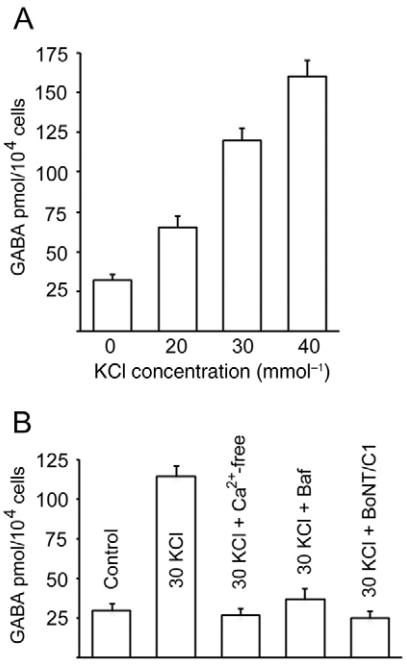

Fig. 4. Release of γ-amino butyric acid (GABA) induced by KCl-depolarization in *Paramecium primaurelia*. (A) Cells (100×10^3 ml^{-1}) were exposed to different KCl concentrations (0–40 mmol l^{-1}) for 10 min. GABA released into the supernatant was analyzed by high performance liquid chromatography (HPLC). The releases evoked by 20, 30 and 40 mmol l^{-1} KCl were significantly different from the control release (P<0.01 at least, N=4; one-way ANOVA plus Dunnett's test) and from each other (P<0.001, N=4; one-way ANOVA plus Newman–Keuls test). (B) Effect of bafilomycin A1 (Baf, 0.5 μmol l^{-1}; 60 min), botulinum toxin C1 serotype (BoNT/C1, 40 nmol l^{-1}; 24 h) or omission of Ca^{2+} from the cell medium. GABA release in Ca^{2+}-free medium and after bafilomycin A1 or BoNT/C1 pre-treatment was significantly different from the release evoked by 30 mmol l^{-1} KCl (P<0.001, one-way ANOVA plus Dunnett's test). Control cells were exposed to a KCl-free medium.

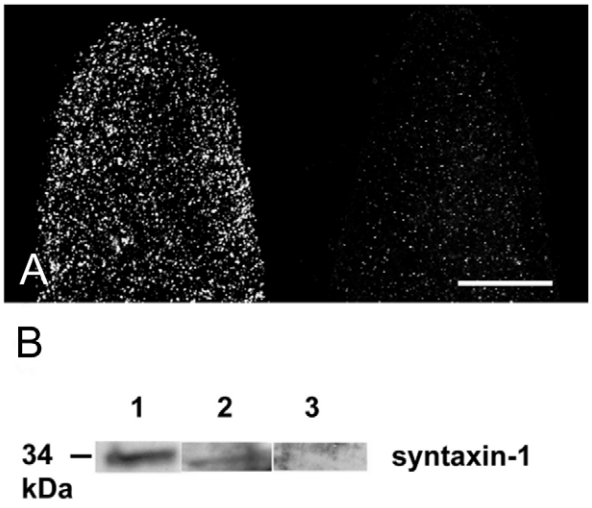

Fig. 5. (A) Distribution of GABA-labeled vesicles in control (left) and in bafilomycyn A1-treated cells of *Paramecium primaurelia* (right). Cells exposed to 0.5 μmol l^{-1} bafilomycin A1 for 60 min showed a substantial decrease in the number of labeled vesicles. Bar, 20 μm. (B) Treatment of cell lysate with activated botulinum toxin C1 (BoNT/C1). Proteins from *P. primaurelia* (lines 2 and 3) and rat cerebral cortex synaptosomes (line 1) were subjected to SDS-PAGE and western blotting. Activity of botulinum toxin was demonstrated by the cleavage of syntaxin-1 (line 3).

location as rat syntaxins. The classical functional domains described in mammal syntaxins, such as the single transmembrane domain at the C-terminus and the coiled-coil region before the transmembrane domain (SNARE domain), are both present in all *Paramecium* syntaxins (PtSyxs).

DISCUSSION

In this study, the presence of GABA synthesis and transport machinery in the ciliated protozoan *P. primaurelia* was demonstrated by western blotting and confocal microscopy. GAD is the main enzyme involved in the synthesis of GABA. Two GAD isoforms in the human brain, GAD_{65} and GAD_{67}, have well-characterized molecular masses (Erlander and Tobin, 1991). GAD_{65} and GAD_{67} are present as homodimers or heterodimers in both soluble GAD (sGAD) and membrane-associated GAD (mGAD) pools (Sheikh and Martin, 1996). GABA synthesized by mGAD is preferentially transported into the vesicles by the vGAT, a 10-transmembrane protein (McIntire et al., 1997). The vGAT forms a protein complex with GAD on the vesicle and ensures an efficient transition between the synthesis of GABA and its packaging into the synaptic vesicle (Jin et al., 2003). In *P. primaurelia* cells in the late log phase of growth, both GAD-like and vGAT-like immunoreactivity was detected on the cell surface and inside the cytoplasm. Furthermore, immunoblot analysis showed that the molecular masses of GAD and vGAT proteins derived from *Paramecium* cells were consistent with corresponding proteins in mammalian cells. In a further indication of the above proteins in *Paramecium*, we not only detected GABA immunoreactivity but also found a co-localization of GABA with the vesicular transporter vGAT. This co-localization suggests that the amino acid is packaged into vesicles, an assumption supported by the blockade of GABA accumulation and release mediated by bafilomycyn A1 (see below).

GABA is the principal inhibitory neurotransmitter of the mammalian central nervous system, a function shared by a number of invertebrate systems. Immunocytochemical studies have localized GABA throughout the echinoderm nervous and muscular systems (Florey et al., 1975; Newman and Thorndyke, 1994), suggesting that GABA may play a role in modulating motor activities (Newman and Thorndyke, 1994). GABA has been detected in various digestive structures of *Asterias* and acts as a classic inhibitory neurotransmitter in both the holothurian cloaca (Hill, 1970) and body wall longitudinal muscles, where it causes muscle relaxation, suppresses spontaneous rhythmicity and inhibits cholinergic contractions (Devlin and Schlosser, 1999). The GABA system is involved in coordinating certain bilateral central pattern generator systems related to feeding and locomotion in the gastropod mollusk *Aplysia californica* (Diaz-Rios et al., 1999), and it controls foraging, regulates defecation and causes muscle relaxation during locomotion in the nematode *Caenorhabditis elegans* (White et al., 1986). Furthermore, GABA receptors modulate feeding response in the cnidarian *Hydra vulgaris*, one of the most primitive organisms bearing a neuronal system (Concas et al., 1998). These receptors are also found in sponges (Ramoino et al., 2007). In these organisms, which lack both nerves and muscles, GABA receptors are involved in the regulation of body contraction (Ellwanger and Nickel, 2006; Ellwanger et al., 2007).

In *P. primaurelia*, the unicellular organism chosen for this study, $GABA_B$ receptors modulate swimming behavior by inhibiting dihydropyridine-sensitive Ca^{2+} channels *via* G-proteins (Ramoino

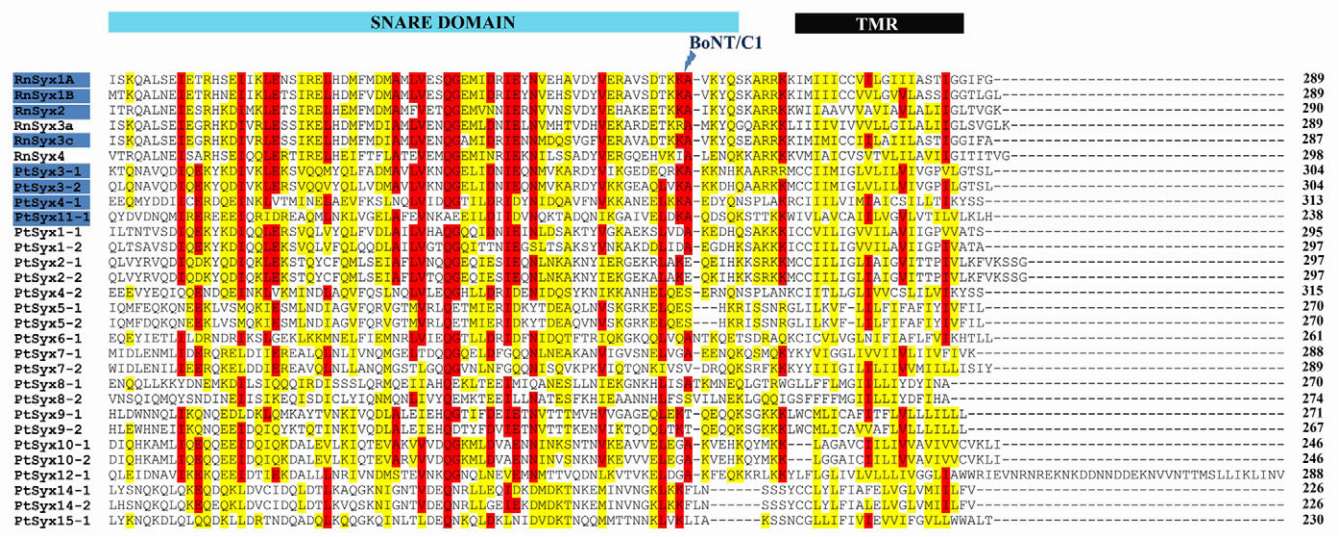

Fig. 6. Multiple sequence alignment of *Paramecium* (PtSyxs) and *Rattus* syntaxins (RnSyxs) showing SNARE and carboxy-terminal transmembrane domains (TMR). Accession numbers for *P. tetraurelia sequences* (PtSyxs) discussed are indicated in the paper of Kissmehl et al. (Kissmehl et al., 2007). Accession numbers for RnSyxs sequences are as follows: Syx1A, NM_053788; Syx1B, NM_12700; Syx2, NM_012748; Syx3a, NM_031124; Syx3c, EDM12895. The predicted TMR are between 17 and 22 amino acids long in the *Paramecium* and 21–22 in the *Rattus* syntaxins. Only the sequences corresponding to RnSyxs syntaxins localized to the plasma membrane (Syx1A, Syx1B, Syx2, Syx3a,c, Syx4) have been used. The botulinum toxin C1 (BoNT/C1) scissile bonds on RnSyxs are indicated. In the aligned region, sequences containing the cleavage sites for BoNT/C1 have the name highlighted in blue. Shading indicates identical (red) and similar (yellow) residues in >50% of sequences aligned. Residue numbers are indicated at the left.

et al., 2003). In addition, prolonged occupancy of $GABA_B$ receptors by baclofen decreases $GABA_B$ receptor function. This decrease is due not only to receptor internalization *via* a clathrin-dependent and -independent mechanism but also by the partial degradation *via* lysosomes (Ramoino et al., 2005; Ramoino et al., 2006). Conversely, $GABA_A$ receptor activation induces membrane depolarization caused by a gradient-dependent efflux of chloride ions through $GABA_A$-associated chloride channels (Bucci et al., 2005). $GABA_B$ receptors are also involved in *Paramecium* food uptake; administration of baclofen produces a dose-related increase in particle ingestion (P.R., A.D. and M.F., unpublished data). Furthermore, $GABA_B$ receptors are involved in cell–cell interactions (Delmonte Corrado et al., 2002). In a related function, $GABA_A$ receptors play a role in sexual processes involving mating-pair formation, and, in fact, the $GABA_A$ receptor antagonists bicuculline and picrotoxin both prevent cell pairing. In *Paramecium*, GABA modulates a number of GABA receptor-mediated, physiologically relevant activities, creating the possibility of autocrine and/or paracrine control through functional GABA release.

In the present study, we demonstrated that *P. primaurelia* releases GABA into the medium and that acceleration of this release can be triggered by increasing the extracellular K^+ concentration to 30 mmol l^{-1}. Physiologically, the resting membrane potential in *Paramecium* is about –25 mV and displays random fluctuations of small amplitude (Majima, 1980; Moolenaar et al., 1976). Larger fluctuations generate a more extensive, spike-like depolarization that is accompanied by both the opening of Ca^{2+} channels localized in the ciliary membrane (Dunlap, 1977; Ogura and Takahashi, 1976) and by a simultaneous influx of Ca^{2+}. As a consequence of this depolarization and Ca^{2+} influx, a graded response is produced that reorients the cilia and reverses the swimming direction. In *Paramecium*, the intracellular K^+ concentration ranges between 17 mmol l^{-1} (Oertel et al., 1978) and 34 mmol l^{-1} (Ogura and Machemer, 1980). It thus seems reasonable to assume that the membrane depolarizes in the presence of 30 mmol l^{-1} of external K^+ and that its potential can reach values moderately below 0 mV. Therefore, we propose that 30 mmol l^{-1} KCl-induced depolarization provokes GABA release because it allows Ca^{2+} to enter the cell and trigger exocytosis of the neurotransmitter. Interestingly, exocytosis has already been reported to be triggered by Ca^{2+} influx in *Paramecium* (Erxleben et al., 1997; Klauke and Plattner, 1997). Further confirming the role of Ca^{2+} in exocytosis, the omission of Ca^{2+} from the medium abolished the stimulus-induced release of GABA under our experimental conditions. Moreover, depolarization-induced GABA release was inhibited by incubating *Paramecium* cells with bafilomicyn A1 or BoNT/C1 prior to the release experiments. Bafilomicyn A1 is a vesicle membrane V-type ATPase inhibitor (Bowman et al., 1988; Floor et al., 1990) that dissipates the proton gradient and prevents the vGAT-mediated accumulation of GABA into acidic vesicles (Moriyama and Futai, 1990; Roseth et al., 1995). BoNT/C1 is a *Clostridium* neurotoxin that cleaves specific amino-acid bonds of mammalian syntaxin-1 and SNAP-25, impeding SNARE complex formation and the consequent vesicle fusion to the cell membrane (Foran et al., 1996; Schiavo et al., 1995).

In *Paramecium*, V-ATPase is involved in osmoregulation, phagocytosis, endocytosis and dense-core secretory vesicle (tricocyst) biogenesis (Wassmer et al., 2005; Wassmer et al., 2006; Wassmer et al., 2009). It was found that the contractile vacuole complex V-ATPase in *Paramecium* is only sensitive to concanamycin and not to bafilomicyn (Fok et al., 1995). This lack of response to bafilomicyn even occurred when the *c* subunit of the enzyme included the Thr32, Phe136 and Tyr143 residues, which are reportedly involved in bafilomycin binding (Wassmer et al., 2005). The role of V-ATPase in tricocyst membrane fusion is not yet known (Wassmer et al., 2005; Wassmer et al., 2009). The V-ATPase was shown to be essential for the biogenesis of these secretory organelles (Wassmer et al., 2005), although they have been described as not acidic (Garreau de Loubresse et al., 1994;

Lumpert et al., 1992). Our release and confocal microscopy experiments, performed with bafilomycin A1-pretreated *Paramecium* cells, not only showed a significant reduction of GABA release induced by depolarization but also a less efficient storage of GABA in the vesicular compartment. This finding indicates that bafilomycin A1 prevents GABA uptake into vesicles in *Paramecium* and that the Ca^{2+}-dependent amino acid release is likely to be of vesicular origin.

In the release experiments, we used confocal microscopy to show that the release-machinery proteins syntaxin, SNAP and VAMP, which aggregate in mammals to form the SNARE complex, occur in this ciliated protozoon. Because GABA release is strongly reduced by BoNT/C1, these proteins probably also interact in *Paramecium* to form the SNARE complex. Co-localization of GABA and VAMP supports this view and suggests that GABA-containing vesicles can undergo docking and priming processes.

The presence of the SNARE-forming proteins syntaxin, VAMP and SNAP, involved in synaptic vesicle exocytosis, and of NSF, the SNARE-specific chaperone, has been recently demonstrated in *Paramecium* (Froissard et al., 2002; Kissmehl et al., 2002; Kissmehl et al., 2007; Schilde et al., 2006; Schilde et al., 2008). Seven subfamilies of synaptobrevins encoded by 12 genes, as well as at least 26 syntaxins grouped into 15 subfamilies, were identified (Kissmehl et al., 2007; Schilde et al., 2006). It is well known that, in mammals, syntaxins and SNAP25 are cleaved by BoNT/C1 at distinct sites close to the C-terminus. Specifically, the single Lys(K)–Ala(A) and Arg(R)–Ala(A) peptide bonds found in syntaxin1A/1B and SNAP25 are recognized by BoNT/C1 (Blasi et al., 1993; Schiavo et al., 1995). The sequence analysis that we performed suggests that this cleavage site for BoNT/C1 is conserved in *P. tetraurelia* syntaxin (Fig. 6). In addition, our experiments used crude cell lysates from *P. primaurelia* incubated with BoNT/C1 to show that the ciliated syntaxin is hydrolyzed by the botulinum toxin, explaining why the toxin also reduces GABA release.

To conclude, here we produced further evidence that a synaptic vesicle-like exocytotic pathway occurs in *P. primaurelia*. We also showed for the first time that the ciliated protozoon utilizes this process to release the messenger molecule GABA. In fact, our data suggest that (1) GABA newly synthesized by GAD is transported into acidic vesicles by vGAT, (2) that GABA-containing vesicles are competent for fusion to the plasma membrane upon depolarization, and (3) that GABA release involves a fusion-protein complex and occurs by means of a neuronal-like mechanism.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BoNT/C1	botulinum toxin C1
BSA	bovine serum albumin
GABA	γ-amino butyric acid
GAD	glutamate decarboxylase
HPLC	high performance liquid chromatography
mGAD	membrane-associated GAD
NC	nitrocellulose
PBS	phosphate buffered saline
sGAD	soluble GAD
VAMP	vesicle-associated membrane proteins
vGAT	vesicular GABA transporter
VSCC	voltage-sensitive calcium channels

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