THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 41, Issue of October 10, pp. 26005–26008, 1997

Printed in U.S.A.

Regulated Secretion Is Impaired in AtT-20 Endocrine Cells Stably Transfected with Botulinum Neurotoxin Type A Light Chain*

(Received for publication, May 30, 1997, and in revised form, July 14, 1997)

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Botulinum neurotoxin type A (BoNT/A) inhibits neurotransmitter release by specific cleavage of SNAP-25, a synaptosome-associated protein also expressed in the ACTH secretory cell line AtT-20. Expression of light chain BoNT/A (L-BoNT/A) gene transfected into AtT-20 cells resulted in a cleaved form of SNAP-25 indistinguishable from that generated by bona fide BoNT/A. L-BoNT/A-transfected cells showed no difference in replication rate, viability, or phenotype, compared with control AtT-20 cells. In contrast, L-BoNT/A-transfected cells could not be induced to secrete ACTH upon stimulation by 8-bromo-cAMP or KCl. In addition, α-latrotoxin induced ACTH release from control cells, but not from L-BoNT/A-transfected cells. These experiments suggest an important role for SNAP-25 in regulated secretion from AtT-20 cells and underline the usefulness of this cell system as a tool for the study of the molecular mechanism of peptide hormone secretion.

There is increasing evidence indicating that regulated secretion is controlled by a limited set of proteins (1, 2), such as synaptobrevin, syntaxin, and SNAP-25,[1] which are also components of the docking and fusion machinery of synaptic vesicle exocytosis (3). These neuronal proteins form a complex also found in endocrine cells (4–7). Besides its essential role in synaptic vesicle exocytosis, SNAP-25 is involved in neurite elongation and synaptogenesis (8, 9) and is the molecular substrate of the botulinum neurotoxin types A and E (10, 11), which selectively block neurotransmitter release (12).

Like the other clostridial neurotoxins, botulinum neurotoxin type A (BoNT/A) is a dichain molecule formed by a light (M_r 50,000) and a heavy (M_r 100,000) chain linked by a single disulfide bond (13). The heavy chain is responsible for neuroselective recognition and binding of the toxin to ecto-acceptors at neuromuscular junctions and mediates the internalization of the active moiety of the toxin into nerve terminals (12–14). The light chain is a Zn^{2+}-dependent protease that specifically cleaves SNAP-25 (10). Such protease activity is considered to be the molecular basis of the neurotransmission blockade by BoNT/A (12). BoNT/A inhibits exocytosis in cells not displaying selective acceptors on their plasma membranes, provided the neurotoxin light chain is introduced in the cells bypassing the recognition and binding steps (*e.g.* by microinjection or by using permeabilizing agents, such as streptolysin-O or digitonin, or by electroporation) (6, 15–17).

Successful expression of clostridial neurotoxins has been achieved by transient transfection of the tetanus toxin gene into COS cells (18) and by microinjection of *Aplysia* neurons with tetanus toxin and BoNT/A mRNA (19). However, there have been no reports of permanent transfectants expressing the botulinum neurotoxins. The light chain of BoNT/A (L-BoNT/A) permanently transfected into nonneuronal cells should also be expressed and cleave SNAP-25. This approach, avoiding more aggressive permeabilizing methods, would also generate stably transfected cell lines useful in long term studies.

AtT-20 endocrine cells express high levels of SNAP-25 and secrete ACTH in response to stimulation with a variety of secretagogues. To study the role of SNAP-25 in regulated secretion, in this work we analyzed ACTH secretion from AtT-20 cells stably transfected with the L-BoNT/A gene and expressing the functional toxin.

EXPERIMENTAL PROCEDURES

Plasmid Construction—cDNA encoding L-BoNT/A was kindly provided by Dr. H. Niemann (Hannover, Germany). The entire L-BoNT/A coding sequence was amplified by polymerase chain reaction, using for 40 cycles the following parameters: denaturation 40 s at 95 °C; annealing, 40 s at 53 °C; and extension at 74 °C, 40 s. *Hin*dIII and *Xba*I restriction sites were included in the sense and antisense amplification primers, respectively. The polymerase chain reaction product was excised with *Hin*dIII and *Xba*I and cloned into the *Hin*dIII/*Xba*I-digested pRC/CMV vector (Invitrogen, San Diego, CA). The pRC/CMV vector contains the cytomegalovirus promoter and a neomycin resistance gene for selection of stably transfected cells in a medium containing the antibiotic G418 (Life Technologies, Inc.). Polymerase chain reaction products cloned in pRC/CMV were sequenced to confirm the presence of L-BoNT/A sequences. Special attention was given to the catalytic domain.

Primary Cell Cultures and Growth of GH_3 Cells—Pituitary cell cultures from male Sprague-Dawley rats (200–250 g) and rat GH_3 cells obtained from ICN Biomedicals (Thame, Oxfordshire, UK) were prepared and grown as described previously (7).

Growth and Transfection of AtT-20 Cells—An AtT-20 mouse anterior pituitary cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Cells were routinely grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 2 mM/liter glutamine (Life Technologies, Inc.), in a humidified atmosphere of 5% CO_2 and 95% air. AtT-20 cells were transfected with 2.5 μg of the DNA constructs (pRC/CMV-BoNT/A) or the parental vector (pRC/CMV) using Lipofectin ac-

* This work was supported by a grant from Comisión Interministerial de Ciencia y Tecnología, Spain. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a Comissió Interdepartmental de Recerca i Tecnologia predoctoral fellowship.

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[1] The abbreviations used are: SNAP-25, synaptosomal-associated 25-kDa protein; SNAP-23, SNAP 23-kDa protein; BoNT/A, botulinum neurotoxin type A; L-BoNT/A, light chain of BoNT/A; PAGE, polyacrylamide gel electrophoresis; Br, bromo; CMV, cytomegalovirus.

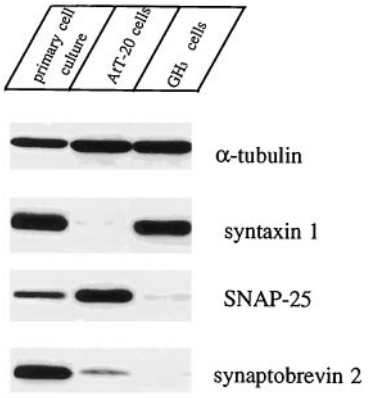

FIG. 1. **Immunoblot analysis of SNAREs proteins in anterior pituitary-derived cells.** Samples (12 μg of protein/lane) from anterior pituitary primary cell cultures, AtT-20 cells, and GH_3 cells were analyzed by immunoblotting for the presence of syntaxin 1, SNAP-25, synaptobrevin 2, and α-tubulin. Longer exposures of the films revealed the presence of syntaxin 1 in AtT-20 cells (see also Fig. 3).

cording to the manufacturer's instructions (Life Technologies, Inc.). After 48 h, stably transfected cells were selected by treatment with 0.5 mg/ml Geneticin (G418; Life Technologies, Inc.). Isolated individual clones were maintained in the presence of G418.

Cleavage of SNAP-25 by BoNT/A—BoNT/A was produced from a culture of *Clostridium botulinum* type A (NCTC no. 2916) as described previously (20). AtT-20 cell homogenates (50 μg of protein) were incubated with BoNT/A (100 nM final concentration) in 10 mM Hepes/KOH buffer, pH 7.2 containing 100 mM KCl, 40 mM NaCl, 10 mM dithiothreitol and 0.5% Triton X-100. Incubation proceeded for 1 h at 37 °C and was followed by fractionation in SDS-PAGE, 12.5% gel, and immunoblot analysis.

Immunoblot Analysis—Immunoblot analysis was performed as described previously (7). 12.5% SDS-PAGE gels were electroblotted to nitrocellulose membranes. The membranes were blocked in a solution consisting of 5% non-fat milk powder in TBS (140 mM NaCl, 10 mM Tris/HCl, pH 7.4, and 0.1% Tween 20) for 1 h at room temperature. The blocked membranes were individually incubated with antibodies, in blocking buffer, at the indicated concentrations: anti-α-tubulin, 1/1000 (Sigma); anti-SNAP-25, 1/1000 (SMI 81, Sternberger Monoclonals Inc., Baltimore, MD); anti-Rab3A, 1/2000 (21); anti-syntaxin 1, 1/2000 (HPC1) (22); anti-synaptobrevin 2, 1/2000 (23). After several washes with blocking solution, membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody, and peroxidase activity was detected using the enhanced chemiluminescent method (ECL, Amersham Corp.).

Immunoprecipitation—AtT-20 cells were collected by centrifugation, washed once with 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and lysed by resuspending the cells in the same buffer containing 1% Nonidet 40 and 1 mM phenylmethylsulfonyl fluoride. After 30 min on ice, nonsoluble material was removed by centrifugation for 10 min at 14,000 $\times$ *g*. Anti-SNAP-25 monoclonal antibody (SMI-81) was added to the supernatant and incubated for 4 h at room temperature. Protein G-Sepharose beads (Pharmacia Biotech, Sweden) were then added, and the mixture was incubated for 90 min at room temperature. The Sepharose beads were then rinsed twice in the same buffer, twice in 50 mM Tris/HCl, pH 7.4, 350 mM NaCl, and 1% Nonidet 40, and twice with 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, without detergent and then processed for SDS-PAGE and immunoblot analysis.

Immunocytochemistry—L-BoNT/A-transfected and control mock-transfected cells were plated onto poly-D-lysine-coated glass coverslips in 24-multiwell plates and grown in 2 mM 8-Br-cAMP for 4 days to induce the appearance of large and extended cellular processes (24). Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. Following further rinsing, cells were preincubated in phosphate-buffered saline containing 5% fetal calf serum and 0.1% Triton X-100. Incubations with primary antibodies against α-tubulin, SNAP-25, Rab3A, and ACTH (Dako, Glostrup, Denmark) were done overnight at 4 °C in phosphate-buffered saline containing 0.1% Triton X-100 and 0.2% gelatin. The immunocytochemical detection of antibodies was done using the Vectastain ABC kit (Vector Laboratories, UK).

ACTH Release—Cells were plated in 35-mm tissue culture dishes coated with poly-D-lysine to minimize cell loss and grown for 5–6 days

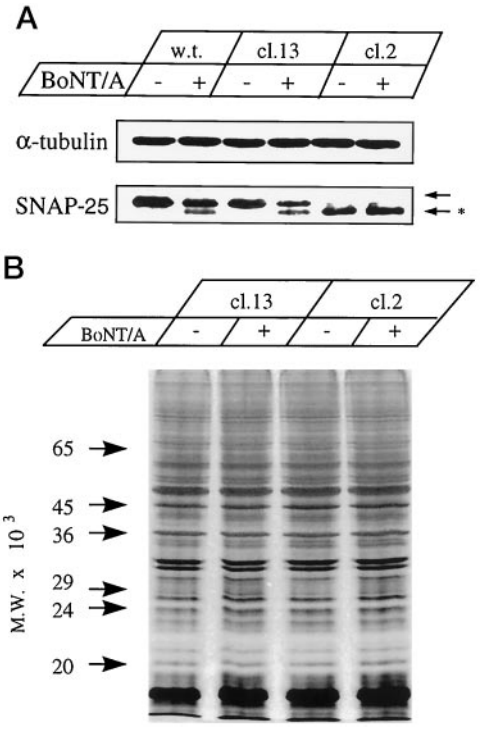

FIG. 2. *A*, immunoblot analysis of SNAP-25 and α-tubulin in samples from wild type (*w.t.*), control (*cl.13*), and L-BoNT/A transfected (*cl.2*) cells, which were previously incubated in the presence or absence of BoNT/A (100 nM final concentration). Note the different SNAP-25 electrophoretic mobility (*arrows*), corresponding to intact and degraded (*asterisk*) SNAP-25 forms. *B*, silver-stained SDS-PAGE analysis from control (*cl.13*) and L-BoNT/A-transfected (*cl.2*) cells, which were previously incubated in the presence or absence of BoNT/A (100 nM final concentration). No differences in protein bands between control and L-BoNT/A-transfected cells were observed.

to near confluence. Before the experiments, cells were rinsed twice with serum-free Dulbecco's modified Eagle's medium and then incubated at 37 °C in 5% CO_2 in 1 ml of serum-free Dulbecco's modified Eagle's medium containing the appropriate test substances. At the indicated times after incubation, conditioned medium was collected from each dish, centrifuged at 14,000 $\times$ *g* for 5 min, and stored at −20 °C until assayed for ACTH content. All tests were done at least in quadruple in two independent experiments. ACTH was determined by a two-site immunoradiometric assay (Allegro IRMA, Nichols Institute, San Juan Capistrano, CA), using synthetic human $ACTH_{1-39}$ as the standard (25). All test were done in duplicate. 5 mM 8-Br-cAMP (Sigma), high (55 mM KCl) extracellular potassium concentration, and 3 nM α-latrotoxin (Alomone, Israel) were used to stimulate secretion. Before use in AtT-20 cells the excitatory capacity of α-latrotoxin was tested by measuring glutamate release in rat brain synaptosomes (26). The secretion data presented are the mean ± S.E. determined by a two-tailed, unpaired *t* test.

RESULTS

Cleavage of SNAP-25 in L-BoNT/A-transfected Cells—The pituitary-derived cell line AtT-20 selected for these transfection studies contained considerably higher levels of SNAP-25 than other anterior pituitary cell types (Fig. 1).

Geneticin-resistant AtT-20 cells transfected with the L-BoNT/A gene were tested for expression of L-BoNT/A protease activity by immunoblot analysis of SNAP-25 to show the appearance of the L-BoNT/A-specific SNAP-25 proteolytic fragment. Of the antibiotic-resistant clones having variable capacity to cleave SNAP-25 (data not shown) a clone (*cl.2*) showing complete L-BoNT/A specific cleavage of SNAP-25 was chosen for further studies (Fig. 2*A*).

The cleaving activity on SNAP-25 in the L-BoNT/A transfectants was indistinguishable from that of bona fide BoNT/A. Incubation of AtT-20 cell homogenates from either control

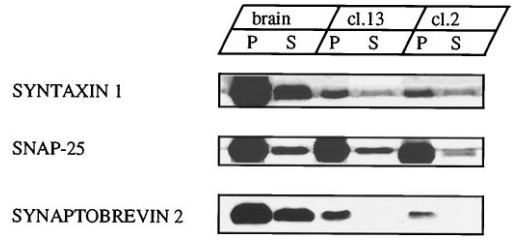

FIG. 3. **Immunoprecipitation of SNAP-25-containing protein complexes from Nonidet 40 extracts of brain, control (*cl.13*) and L-BoNT/A-transfected (*cl.2*) cells were analyzed by immunoblot for the presence of syntaxin 1, SNAP-25, and synaptobrevin 2.** Cleaved SNAP-25 in L-BoNT/A-transfected cells (*cl.2*) is able to form protein complexes with syntaxin 1 and synaptobrevin 2. Note the cleaved form of SNAP-25 in L-BoNT/A-transfected cells. *P*, pellet; *S*, supernatant.

clones (*cl.13*), transfected with parental vector only, or wild type cells with exogenous BoNT/A, resulted in the appearance of the cleaved form of SNAP-25 having identical size to that of SNAP-25 fragments derived from the L-BoNT/A-transfected cells (cl.2) (Fig. 2*A*). Furthermore, no additional protein bands were detected when cell extracts from L-BoNT/A-transfected clones were incubated with the exogenous BoNT/A (Fig. 2*A*). Expression of L-BoNT/A had no detectable effect on the protein composition of the transfected cells as revealed by Coomassie Blue or silver staining of SDS-PAGE gels of cell lysates from control and L-BoNT/A-transfected cells (Fig. 2*B*).

The cleaved form of SNAP-25 in L-BoNT/A-transfected cells retained the capacity to form protein complexes with synaptobrevin 2 and syntaxin 1. Immunoprecipitation experiments followed by immunoblot analysis show that anti-SNAP-25 monoclonal antibodies recognized and immunoprecipitated with equal efficiency both the intact and the L-BoNT/A-cleaved forms of SNAP-25 (Fig. 3).

L-BoNT/A Expression Does Not Affect Viability and Morphology of AtT-20 Cells—L-BoNT/A-transfected cells displayed no apparent difference in viability, replication rate, or phenotype compared with wild type or control transfected cells. In addition, no differences in morphology were detected between L-BoNT/A-transfected cells exhibiting complete cleavage of SNAP-25 and control cells, showing an AtT-20 characteristic pattern of processes (Fig. 4). α-Tubulin also showed similar intracellular distribution in wild type, control cells, and L-BoNT/A-transfected cells (Fig. 4). In addition, in L-BoNT/A-transfected cells, secretory granule (ACTH), synaptic-like microvesicle (Rab3A) markers, and SNAP-25 showed the same distribution pattern as that of control cells (Fig. 4).

Stimulated Secretion of ACTH Release—We examined whether L-BoNT/A transfected cells retained the capacity to release hormone upon appropriate stimulation. Basal release of ACTH (27) was not significantly different in L-BoNT/A-transfected and control cells after a 15-min ($p > 0.5$) (Fig. 5*A*) or a 60-min release period ($p > 0.5$) (Fig. 5*B*). In contrast, when cells were stimulated for 15 min with a high (55 mM) concentration of extracellular potassium, a significant increase was observed in ACTH released from control cells compared with L-BoNT/A-transfected cells ($p < 0.03$) (Fig. 5*A*).

Stimulation with 8-Br-cAMP and α-latrotoxin for 60 min also failed to induce ACTH secretion from L-BoNT/A transfectants (Fig. 5*B*). An induced increase of cytosolic calcium by a 5 mM concentration of the cAMP analog 8-Br-cAMP (28) resulted in the release to the medium of ACTH from control cells ($p < 0.01$) but failed to stimulate release of the hormone from L-BoNT/A-transfected cells ($p > 0.5$). In a similar manner, treatment with α-latrotoxin (29) at 3 nM concentration resulted in the release of ACTH from control cells ($p < 0.05$) but not from L-BoNT/A-transfected cells ($p > 0.5$). Furthermore, simultaneous use of 8-Br-cAMP and α-latrotoxin resulted in even higher increases in released ACTH from control cells ($p < 0.01$) but again failed to stimulate hormone release from L-BoNT/A-transfected cells over basal levels ($p > 0.5$) (Fig. 5*B*).

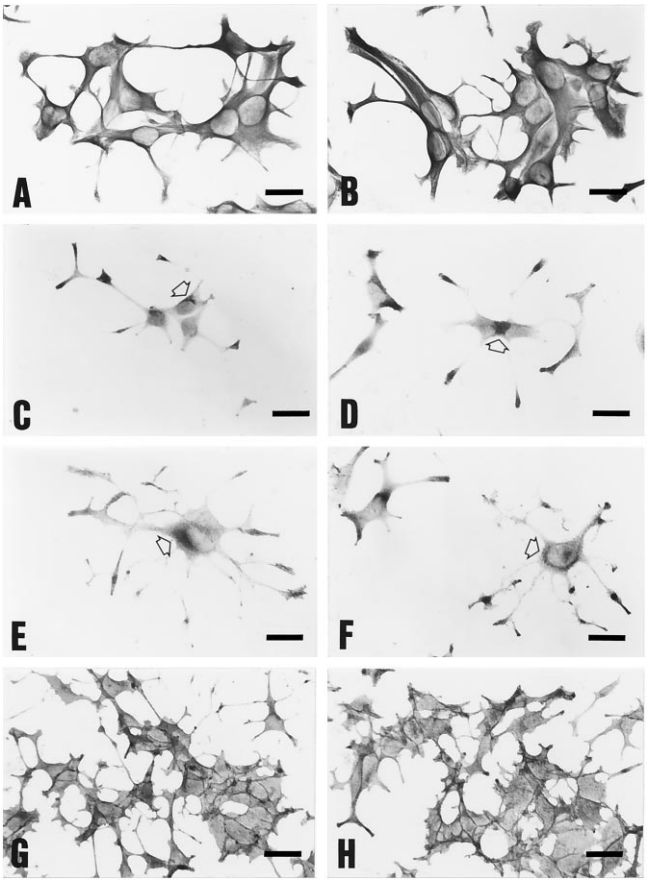

FIG. 4. **Immunocytochemical location of α-tubulin (*A* and *B*), ACTH (*C* and *D*), Rab3A (*E* and *F*), and SNAP-25 (*G* and *H*) in control (*A*, *C*, *E*, and *G*) and L-BoNT/A-transfected AtT-20 (*B*, *D*, *F*, and *H*) cells.** *A* and *B*, α-tubulin immunoreactivity is occupying the cytoplasmatic areas. *C–F*, secretory granule and synaptic-like microvesicles markers (ACTH and Rab3A) are restricted to the tips of the AtT-20 processes and to their Golgi regions. *G* and *H*, SNAP-25 immunoreactivity is located mainly at the plasma membrane. *Arrows* indicate the Golgi region. *Scale bars*: *A–F*, 20 μm; *G* and *H*, 30 μm.

DISCUSSION

The L-BoNT/A gene, coding for a protease that specifically targets the carboxyl terminus of SNAP-25 (10, 12), was introduced into ACTH-secreting AtT-20 cells as a probe to investigate the role of SNAP-25 in regulated secretion.

L-BoNT/A-transfected cells but not mock-transfected control cells expressed an L-BoNT/A activity indistinguishable from that of bona fide BoNT/A. The extent of SNAP-25 processing in L-BoNT/A transfectants was variable, a provable consequence of different dosages of genome-integrated L-BoNT/A sequences.

The majority of cellular parameters we tested were unaffected by expression of L-BoNT/A. Furthermore, transfected cells expressing L-BoNT/A activity at the highest level showed no detectable changes in viability and growth rate when compared with control cells, in agreement with previous studies showing that BoNT/A toxin injected into muscles or added to neuronal cell cultures caused no physical or morphological damage to neurons (30–32).

The similar cellular distribution of SNAP-25 in control and transfected cells suggests that synthesis, distribution, and sorting of this protein was not affected by L-BoNT/A. In contrast to

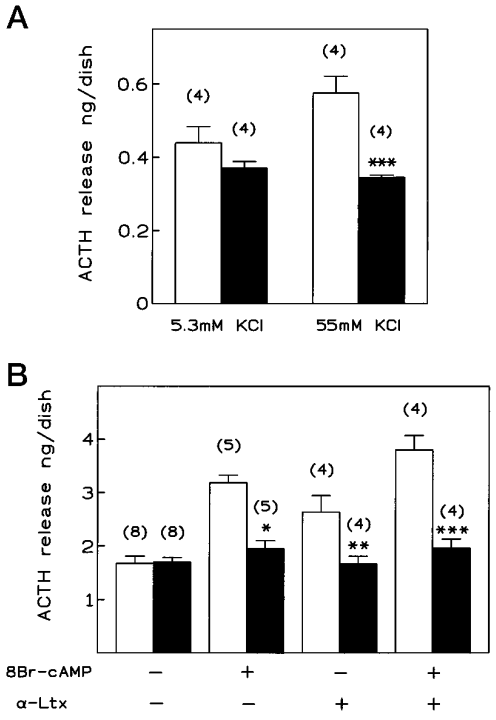

FIG. 5. **Effect of concentration of extracellular potassium (*A*) or 8-Br-cAMP and α-latrotoxin (*B, α-Ltx*) on ACTH release from control (*open bars*) and L-BoNT/A-transfected (*solid bars*) cells.** Cells were incubated with low (5.3 mM) or high (55 mM) potassium concentrations for 15 min (*A*) or with 5 mM 8-Br-cAMP and 3 nM α-latrotoxin for 60 min (*B*). ACTH released was determined by specific radioimmunometric assay. *Bars* represent the mean value of at least four determinations from one independent experiment, whereas *error bars* represent values ± S.E. *Asterisks* indicate differences from control: $^{*}p < 0.01$, $^{**}p < 0.05$), and $^{***}p < 0.03$.

the observation of others (9), showing that BoNT/A impairs neurite extension and formation of new synapses in neuronal primary cell cultures, our data show that cellular processes, containing secretory granules and synaptic-like microvesicles typical of AtT-20 cells (24, 33, 34), were unaffected by expression of L-BoNT/A. This suggests that SNAP-25 is not essential for the elongation of cellular processes and transport of secretory organelles in AtT-20 cells. A plausible means of reconciling our results with those reports (9) is the finding of a non-neuronal form of SNAP-25, SNAP-23, that could fulfill the same role of SNAP-25 in AtT-20 cells (see below) (35).

The stimulus-evoked hormone secretion of ACTH, but not basal secretion, was completely blocked in L-BoNT/A-transfected cells regardless of the stimulus used, 8-Br-cAMP, KCl, α-latrotoxin, or both together, confirming a significant role of SNAP-25 in regulated exocytosis (10). A similar inhibitory effect of BoNT/A has been shown on insulin secretion from permeabilized pancreas-derived cells (6, 17).

The present report shows that at low concentration α-latrotoxin, an inducer of neurotransmiter and neuropeptide release from nerve terminals (29, 36, 37), evokes ACTH secretion from AtT-20 cells over basal levels, suggesting the presence of an α-latrotoxin receptor in these endocrine cells. Our finding that α-latrotoxin was unable to restore ACTH secretion in L-BoNT/A-transfected AtT-20 cells is in contrast with data showing that this toxin can stimulate neurotransmiter release from BoNT/A-poisoned nerve terminals (38). These observations may suggest the existence of different mechanisms in neurons and endocrine cells for the α-latrotoxin-induced secretion.

One of the most remarkable features observed in L-BoNT/A-transfected AtT-20 cells was that the basal secretion level of hormone was not significantly affected. One possible explanation for this result is that SNAP-23, a SNAP-25 homologue lacking the peptide bond cleaved by L-BoNT/A (35), may also be responsible for constitutive and/or basal secretion level of ACTH.

As far as we know the present report is the first successful attempt to obtain a stably transfected secretory cell line expressing the light chain of botulinum neurotoxin. This feature is especially relevant since the main effect of these toxins, the impairment of the regulated exocytosis, becomes amenable to study in secretory non-neural cells for long periods of time.

Acknowledgments—We thank Drs. R. Jahn and C. Barnstable for the antibodies used in this report and Dr. H. Niemann for L-BoNT/A cDNA. We are thankful to Dr. J. Herreros for neurotoxin purification, to I. Gomez de Aranda for technical help, and to Jordi Ballabriga and Rafael Abost for statistics. We are grateful to Dr. C. Gundersen for helpful discussions.

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