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BOTULINUM TOXIN A INHIBITS ACETYLCHOLINE RELEASE FROM CULTURED NEURONS IN VITRO

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Summary

Clostridium botulinum type toxin A (BoTx) blocks stimulus-induced acetylcholine (ACh) release from presynaptic nerve terminals at peripheral neuromuscular junctions. However, the detailed mechanism of this effect remains elusive. One obstacle in solving this problem is the lack of a suitable in vitro homogenous cholinergic neuronal model system. We studied the clonal pheochromocytoma PC12 cell line to establish such a model. PC12 cells were differentiated in culture by treatment with 50 ng/ml nerve growth factor (NGF) for 4 days to enhance cellular ACh synthesis and release properties. Stimulation of these cells with high K^+ (80 m*M*) in the perfusion medium markedly increased calcium-dependent [^{3}H]ACh release compared to undifferentiated cells. Stimulated [^{3}H]ACh release was totally inhibited by pretreatment of cells with 2 n*M* BoTx for 2 h. BoTx inhibition of [^{3}H]ACh release was time- and concentration-dependent. A 50% inhibition was obtained after 2 h incubation with a low (0.02 n*M*) toxin concentration. The time required for 2 n*M* BoTx to cause a measurable inhibition (18%) of stimulated [^{3}H]ACh release was 30 min. Botulinum toxin inhibition of stimulated ACh release was prevented by toxin antiserum and heat treatment, suggesting the specificity of the toxin effect. Our results show that by differentiation with NGF, PC12 cells can be shifted from an insensitive to a sensitive state with respect to BoTx inhibition of stimulated ACh release. This cell line, therefore, may serve as a valuable in vitro cholinergic model system to study the mechanism of action of BoTx.

Key words: PC12 cells; nerve growth factor; botulinum toxin A; acetylcholine.

Introduction

Botulinum toxin type A (BoTx), a protein toxin produced by the microorganism *Clostridium botulinum*, is an extremely potent neurotoxin. It causes fatal neuromuscular paralysis in most vertebrates in minute quantities (picomolar concentration) by blocking the Ca^{2+}-dependent release of the neurotransmitter acetylcholine (ACh) from presynaptic nerve terminals at neuromuscular junctions (Simpson, 1981; Dolly et al., 1987). The mechanism of internalization and action of BoTx at the cellular level has been proposed to involve the following steps: binding to cell surface receptor, translocation into the cytoplasm via endocytosis, and intracellular poisoning (Simpson, 1980). It has been reported that BoTx blocks presynaptic ACh release at peripheral neuromuscular junctions (Simpson, 1989), at the Torpedo electric organ (Dunant et al., 1987), and in synaptosomes (Marshal et al., 1988). Although these in vitro models are potentially useful in explaining some aspects of BoTx poisoning, a clonal neuronal cell culture system such as the PC12 cell line is more amenable to experimental manipulations for studying the specific biochemical and molecular events underlying this mechanism. Permeabilized adrenal chromaffin cells and the clonal rat pheochromocytoma PC12 cells have been used as models to study the action of BoTx (McInnes and Dolly, 1990; Lomneth et al., 1991) on the catecholamine release mechanism. Inasmuch as PC12 cells as such do not demonstrate binding sites for BoTx, cells were permeabilized either biochemically (e.g., by using digitonin) (McInnes and Dolly, 1990) or mechanically by a "cell cracking" technique (Lomneth et al., 1991) to bypass toxin binding to cell surface receptors and translocation across the membrane. In both of these permeabilization techniques, complete integrity of the intact cells cannot be maintained due to formation of pores (biochemical method) or tearing (physical method) of the plasma membrane. In a recent report, Shone and Melling (1992) have shown that exposure of nerve growth factor (NGF) (50 ng/ml) differentiated (14 days) PC12 cells to (0.3 μM) BoTx for 24 h reduced Ca^{2+}-mediated unstimulated release of [^{3}H]noradrenaline to less than 20% of untreated control over subsequent 4-day period. Although BoTx inhibition of catecholamine secretion has been demonstrated in these cells, the toxicologically relevant action of BoTx is to inhibit the stimulated release of ACh that leads to neuromuscular paralysis. The PC12 cell, which is an adrenergic as well as a cholinergic cell clone, can accumulate, synthesize, store, and release both neurotransmitters' catecholamine (primarily dopamine) and acetylcholine (Green and Tischler, 1983). We have made an interesting manipulation to render PC12 cells sensitive to BoTx without permeabilization. When a NGF obtained from rat salivary gland is added to PC12 cell culture medium, these cells show extensive neurite outgrowth and exhibit a marked enhancement of some biochemical properties (Green and Tischler, 1983). The present study demonstrates that BoTx inhibits K^+-stimulated ACh release from NGF differentiated PC12 cells without permeabilization. A preliminary report of this result has been presented (Ray and Middleton, 1992).

Materials and Methods

Materials. NGF was obtained either as a gift from Dr. Gordon Guroff, National Institutes of Health, Bethesda, MD, or was purchased from Collaborative Research Product (Bedford, MA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from GIBCO (Grand Island, NY). Donor horse serum was obtained from Quality Biological (Gaithersburg, MD). Penicillin and streptomycin solution, choline kinase, and all other common reagents were from Sigma Chemical Co. (St. Louis, MO). Acetyl[methyl-^{3}H]choline chloride (84.3 Ci/mmol) was obtained from Amersham/Searle, Arlington Heights, IL). Choline chloride [methyl-^{3}H] (87.8 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). Botulinum toxin A was obtained from Sigma. Intraperitoneal mouse MLD (minimal lethal dose) of this toxin was 28 pg/mouse in 48 h. The amount of toxin that killed all mice in 48 h was taken as one MLD. This value is comparable to that reported (Ashton and Dolly, 1988) in the literature. Antiserum against BoTx (1 IU neutralizes 10^4 mouse LD_{50} of BoTx) was a gift from Dr. John Middlebrook of USAMRIID, Fort Detrick, MD.

Cell culture. Stock monolayer cultures of PC12 cells were maintained in 75-cm^2 tissue culture flasks in DMEM containing 6% (vol/vol) fetal bovine serum, 6% (vol/vol) donor horse serum, 100 μg streptomycin/ml, and 100 U penicillin/ml as described (Fink and Guroff, 1990). The cells were incubated at 37° C in a humidified atmosphere of 90% air:10% CO_2. Confluent stock cultures were routinely subcultured at 7-day intervals at a density ratio of 1:5.

Acetylcholine release measurements. ACh release was measured according to a published method (McGee et al., 1978) with some modification. Confluent monolayer cultures in 75-cm^2 flasks were incubated with 20 ml of isosmotic (340 mOsM/liter) medium A (116 m*M* NaCl, 1.8 m*M* $CaCl_2$, 5.4 m*M* KCl, 0.81 m*M* $MgCl_2$, 1.0 m*M* NaH_2PO_4, 25 m*M* glucose, and 25 m*M* HEPES, adjusted to pH 7.4 with NaOH and to 340 mOsM/liter with NaCl) supplemented with 10 μ*M* [^{3}H]choline chloride (0.2 μCi/ml) for 1 h at 37° C. After incubation, the cells were washed 3 times using 10 ml of medium B (medium A containing 30 μ*M* eserine sulfate to inhibit acetylcholinesterase) to remove excess [^{3}H]choline chloride. The detached cells were then centrifuged at 200 $\times g$ for 5 min to obtain a pellet which was suspended in 1 ml of medium B. Cells (2×10^6/ml) were then incubated with a desired concentration of BoTx (0.002 to 2 n*M*) in 1 ml of medium B containing 1 mg/ml bovine serum albumin at 37° C for desired times. After incubation with BoTx, the cells were centrifuged to remove excess toxin, resuspended in 1 ml medium B, and placed in a perfusion chamber as follows. Cells were loaded onto round glass microfiber filters (2.4 cm diameter GF/B) placed in a custom-designed perfusion chamber constructed from a 30-ml plastic syringe with inflow and outflow tubing connected to a reservoir and manual fraction collector, respectively. The filter was supported by a Teflon wire mesh screen. This wire mesh prevented the filters from being forced through the system. The filters and the screen were fixed between two O-rings and held in place by a Teflon fitting tube. Both the reservoirs of different perfusion media and the perfusion chamber were lowered into a 37° C water bath. The rate of perfusion was controlled by means of a two-speed proportioning pump (Bran and Luebbe, New York) at 1 ml/min, and the volume of solution bathing the cells inside the perfusion chamber was maintained at $\approx$1 ml. The cells were prewashed with medium B for 20 min to wash out radioactivity and to obtain a stable baseline [^{3}H]ACh release. Fractions were collected at 2-min intervals (2 ml/tube). Eight minutes after starting collection of fractions, the cells were exposed to medium B containing 80 m*M* KCl (NaCl replaced by KCl) for 8 min, followed by return to normal medium B as described above. The amount of [^{3}H]ACh in each fraction was assayed by the modified choline kinase method in which [^{3}H]choline was converted to [^{3}H]phosphorylcholine and [^{3}H]ACh was then separated from [^{3}H]phosphorylcholine by 0.5% tetraphenylboron/acetonitrile extraction. Briefly, a 0.25-ml aliquot of perfusate was treated for 15 min at 37° C with 2.5 mU of choline kinase in the presence of 10 m*M* ATP and 10 m*M* $MgCl_2$, which catalyzes the conversion of the [^{3}H]choline to [^{3}H]phosphorylcholine. After incubation, 1 ml of 0.5% of tetraphenylboron in acetonitrile was added to each fraction which was vortexed for 1 min at top speed. Econofluor scintillation (for non-aqueous counting) fluid (3 ml) was then added to each sample, which was again vortexed vigorously for 20 s. Control [^{3}H]ACh (84.3 Ci/mmol) samples were run with and without choline kinase along with the experimental samples for comparison.

Results

The effects of BoTx on depolarization evoked release of [^{3}H]ACh into the culture medium of PC12 cells grown with or without 50 ng/ml NGF for 4 days is shown in Fig. 1. Incubation of untreated PC12 cells with BoTx (2 n*M*) for 2 h at 37° C before KCl stimulation had no effect on [^{3}H]ACh release (Fig. 1 *A*). Treatment of cells with 50 ng/ml NGF for 4 days caused marked morphologic differentiation as indicated by extensive neurite extension (data not shown). These differentiated cells exhibited enhanced basal (fivefold) as well as high K^+ (80 m*M*)-stimulated release (6 times basal) of [^{3}H]ACh compared to cells that were not treated with NGF (Fig. 1 *B*). Stimulated [^{3}H]ACh release from NGF-treated cells was Ca^{2+}-dependent (data not shown). Differentiated PC12 cells also became sensitive to BoTx intoxication (Fig. 1 *B*). Inhibition of stimulated [^{3}H]ACh release by BoTx was both time- and concentration-dependent. The effects of different BoTx concentrations on K^+-stimulated [^{3}H]ACh release is shown in Fig. 1 *B*. Treatment of cells with 0.02 n*M* BoTx for 2 h inhibited release by $\approx$50%, whereas release was almost completely inhibited by treatment of cells with 2 n*M* BoTx for 2 h (Fig. 1 *B*). The time-dependence of 2 n*M* BoTx inhibition of K^+-stimulated [^{3}H]ACh release is shown in Fig. 2. The inhibition was detectable when cells were treated with toxin for 30 min, before high K^+-stimulated [^{3}H]ACh release (Fig. 2 *upper left*). To obtain a complete inhibition, cells had to be treated with toxin for 2 to 3 h (Fig. 2 *lower left*). These results showed that NGF-treated PC12 cells shifted from an insensitive to a sensitive state to demonstrate a concentration- and time-dependent response of BoTx inhibition of K^+-stimulated [^{3}H]ACh release. Differentiation of cells for more than 4 days did not produce any measurable change in their stimulated [^{3}H]ACh release property (data not shown). No significant effect of BoTx on the unstimulated release was observed. The specificity of the BoTx effect on stimulated [^{3}H]ACh release was demonstrated by the experiments shown in Fig. 3. When BoTx was preincubated at 37° C with a 10-fold excess of horse anti-BoTx antiserum, or was heated in a boiling water bath for 5 min, the toxin was ineffective in inhibiting stimulated [^{3}H]ACh release.

Discussion

This study shows that BoTx inhibits 80 m*M* K^+-stimulated [^{3}H]-ACh release from differentiated PC12 cells in a concentration- and time-dependent manner. About 50% inhibition of 80 m*M* KCl-induced [^{3}H]ACh release was obtained at 0.02 n*M* BoTx. Neuromuscular paralysis has been reported in neuromuscular junction preparations due to cholinergic blockade with 0.2 n*M* BoTx within 1 to 2 h (Black and Dolly, 1986; Maisey et al., 1988). The half maximal Ca^{2+}-dependent release of noradrenaline from digitonin-permeabilized PC12 cells or chromaffin cells has been reported to occur at $\approx$2 n*M* BoTx (McInnes and Dolly, 1990; Bittner et al., 1989). Ashton and Dolly (1988) also provided some evidence that BoTx causes 50% inhibition of high K^+-evoked ACh release from synaptosomes at 10 n*M* concentration. In the present study, the inhibition was almost complete at 2 n*M* toxin concentration, suggesting that the in vitro PC12 cell model may be more sensitive. However, experiments with permeabilized cells have demonstrated that toxin can act within a relatively short time (minutes) when introduced directly into the cell (McInnes and Dolly, 1990; Lomneth et al.,

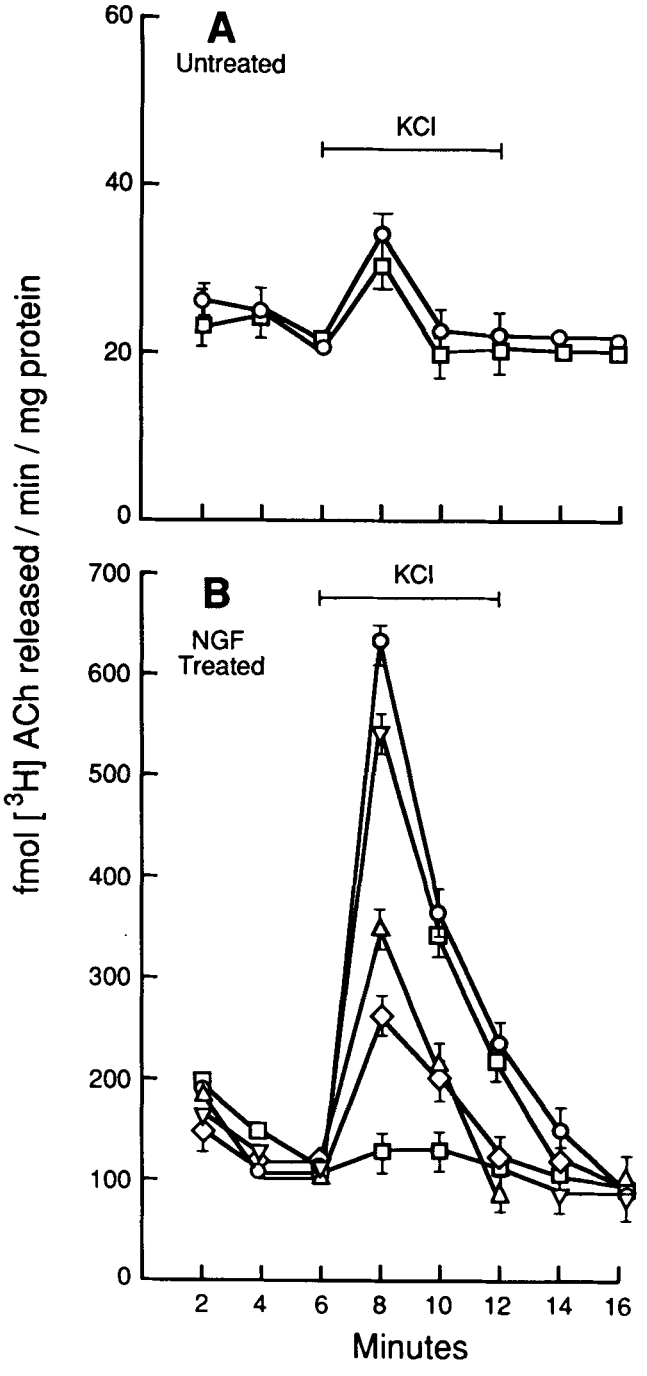

FIG. 1. Effect of BoTx on KCl-stimulated [^{3}H]ACh release from PC12 cells cultured without (*A*) or with (*B*) NGF (50 ng/ml) for 4 days. Cells were incubated with 10 μM [^{3}H]choline chloride for 1 h at 37° C to label intracellular ACh. Cells were then washed and incubated without or with BoTx at indicated concentrations for 2 h at 37° C. Cells were then perfused, 2-min fractions were collected for 8 min before and during 8-min K^+ stimulation, and [^{3}H]ACh release was measured as described in Materials and Methods. Toxin concentrations used were (○) no toxin; (▽) 0.002 n*M*; (△) 0.02 n*M*; (◇) 0.2 n*M*; (□) 2 n*M*. Each point represents the mean ± SEM of results obtained from three separate cultures.

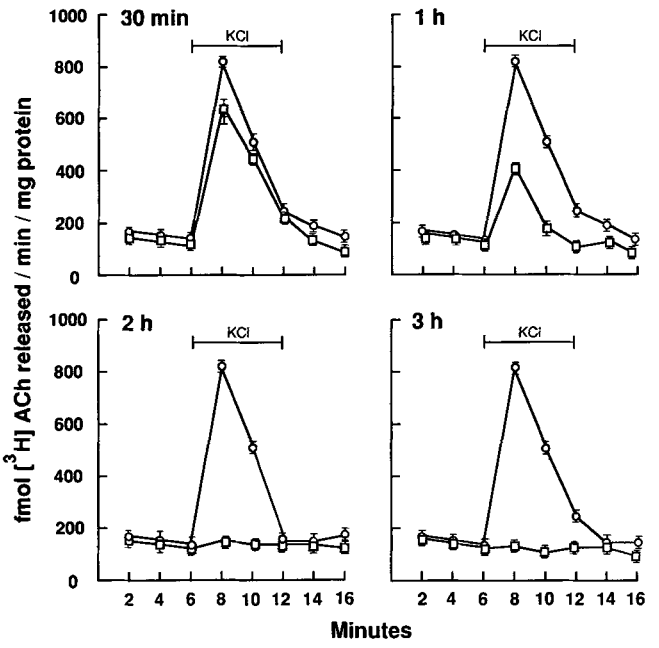

FIG. 2. Time course of BoTx effect on K^+-stimulated [^{3}H]ACh release from NGF-treated PC12 cells. Cells were preincubated with 10 μM [^{3}H]-choline chloride for 1 h at 37° C to label intracellular ACh. Cells were then washed and incubated without and with 2 n*M* BoTx at 37° C for the indicated times. Two-minute fractions were collected for 8 min before and during stimulation with 80 m*M* KCl as described in Materials and Methods. Fractions were then collected, and release of [^{3}H]ACh due to 80 m*M* KCl was measured as described in Methods. Each point is the mean ± SEM of results obtained from experiments using three separate cultures. (○) Cells without BoTx; (□) with BoTx.

1991). In our study, the time required for BoTx to cause a measurable inhibition (18%) in the PC12 cell model was 30 min. In the report by Shone and Melling (1992), a prolonged exposure (24 h) to a higher concentration (0.3 μM) of BoTx was required to inhibit the Ca^{2+}-dependent, unstimulated release of [^{3}H]noradrenaline. Experiments with NGF-treated PC12 cells and dibutyryl cAMP-treated NG108-15 cells have demonstrated that the inhibition of ACh release by tetanus toxin is seen after a lag period of 30 min (Sandberg et al., 1989 and Wellhoner and Neville, 1987). This implies that this lag period may be required for binding and internalization of toxin before reaching its intracellular target. In neuromuscular junction preparations, time to paralysis is a function of toxin dose. The lower the dose, the slower the paralysis and vice versa at doses between 10^{-8} to 10^{-9} *M* (Lomneth et al., 1991). To demonstrate that the results obtained were specific for BoTx, we showed that the inhibitory effect of BoTx was prevented by toxin antiserum and heat treatment. We attempted to use PC12 cells treated with 1 m*M* dibutyryl cAMP (Bt_2cAMP) to study the effect of BoTx on [^{3}H]ACh release. Bt_2cAMP-treated PC12 cells exhibited less morphologic differentiation in terms of neurite outgrowth when compared to NGF-differentiated cells. The response of Bt_2cAMP-treated cells was inferior to that of NGF-treated cells in terms of high K^+-stimulated [^{3}H]ACh release and its inhibition by BoTx. Green and Tischler (1983) and Richter-Landsberg and Jastorff (1986) also reported that cAMP, cAMP analogues, phosphodiester-

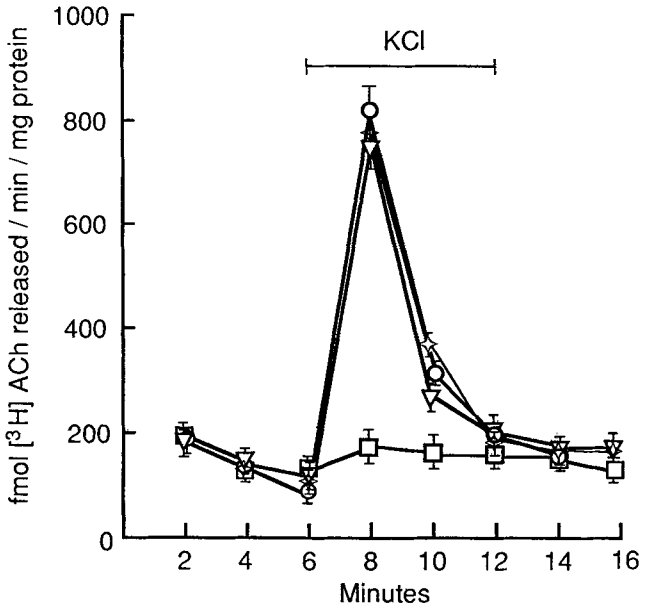

FIG. 3. Specificity of BoTx action on K^+-stimulated [^{3}H]ACh release from PC12 cells. Cells were pretreated with 10 μM [^{3}H]choline chloride to label intracellular ACh as described in Materials and Methods. Aliquots of washed cell suspension were first exposed to 10-fold excess of horse BoTx antiserum at 37° C for 5 min or BoTx previously heated at 100° C for 5 min. These cells were then pretreated with 2 nM BoTx for 2 h at 37° C before stimulation with 80 mM KCl. Release of [^{3}H]ACh from cells was measured as described in Materials and Methods. Control cells were treated with equivalent amounts of buffered salt solutions instead of antiserum or BoTx. (○) Cells without BoTx; (□) with BoTx and (▽) BoTx and antiserum; (*) BoTx previously heated for 5 min at 100° C. Results are the mean ± SEM of three separate cultures.

ase inhibitors, and forskolin were not as effective as NGF in inducing PC12 cell differentiation. For these reasons, all of our experiments were conducted using NGF-differentiated cells. Several explanations are possible for the fact that NGF-differentiated PC12 cells become sensitive to BoTx inhibition of stimulated [^{3}H]ACh release. Although the expression of recognition site is an attracting proposition, we do not have any experimental data to substantiate the claim. The presence of a tetanus toxin binding protein was demonstrated in differentiated PC12 cells, and this protein was absent in undifferentiated cells (Schiavo et al., 1991). Other possibilities include induction of a toxin transport system or toxin-sensitive intracellular targets as a result of NGF-differentiation of these cells. Inasmuch as the main effect of BoTx is on peripheral cholinergic mechanisms, NGF-differentiated PC12 cells can be used as an in vitro model to study the mechanism of neurotransmitter release using BoTx as a valuable probe. Recently, attempts have been made to use BoTx therapeutically as a specific localized muscle relaxant to treat human muscle disorders (Gansel et al., 1987; Scott et al., 1973; Schantz and Johnson, 1992; Hambleton, 1992). To be successful in these attempts, a clear understanding of the mechanism of action of BoTx is essential. The establishment of an in vitro cell culture model as described in this report should be useful in the attempt to develop BoTx as a therapeutic agent. We have used this model to demonstrate that arachidonic acid is involved in BoTx inhibition of K^+-stimulated [^{3}H]ACh release in NGF-differentiated PC12 cells (Ray et al., 1992).

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REFERENCES

Ashton, A. C.; Dolly, J. O. Characterization of the inhibitory action of botulinum neurotoxin type A on the release of several transmitters from rat cerebrocortical synaptosomes. J. Neurochem. 50:1808–1816; 1988.

Bittner, M. A.; DasGupta, B. R.; Holz, R. W. Isolated light chains of botulinum neurotoxin inhibit exocytosis: studies in digitonin-permeabilized cells. J. Biol. Chem. 264:10354–10360; 1989.

Black, J. D.; Dolly, J. O. Interaction of ^{125}I-labelled botulinum neurotoxins with nerve terminals. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell Biol. 103:521–534; 1986.

Dolly, J. O.; Ashton, A. C.; Evans, D. M., et al. Molecular action of botulinum neurotoxins role: role of acceptors in targeting to cholinergic nerves and in the inhibition of the release of several transmitters. In: Dowdall, M. J.; Hawthorne, J. A., eds. Cellular and molecular basis of cholinergic function. Chichester, UK: Ellis Horwood; 1987:517–533.

Dudant, Y.; Esquerda, J. E.; Loctin, F., et al. Botulinum toxin inhibits quantal acetylcholine release and energy metabolism in the torpedo electric organ. J. Physiol. 385:677–692; 1987.

Fink, D. W.; Guroff, G. Nerve growth factor stimulation of arachidonic acid release from PC12 cells: independence from phosphoinositide turnover. J. Neurochem. 55:1716–1726; 1990.

Gansel, M.; Penner, R.; Dreyer, F. Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor terminals. Pfluegers Arch. Eur. J. Physiol. 409:404–416; 1987.

Green, L. A.; Tischler, A. PC12 pheochromocytoma cultures in neurobiological research. Adv. Cell. Neurobiol. 3:373–414; 1987.

Hambleton, P. Clostridium botulinum toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use. J. Neurol. 239:16–20; 1992.

Lomneth, R.; Martin, T. F. J.; DasGupta, B. R. Botulinum neurotoxin light chain inhibits norepinephrine secretion in PC12 cells at an intracellular membranous or cytoskeletal site. J. Neurochem. 57:1413–1421; 1991.

Maisey, E. A.; Wadsworth, J. D. F.; Poulain, B., et al. Involvement of the constituent chains of botulinum neurotoxins A and B in the blockade of neurotransmitter release. Eur. J. Biochem. 177:683–691; 1988.

McGee, R.; Simpson, P.; Christian, C., et al. Regulation of acetylcholine release from neuroblastoma x glioma hybrid cells. Proc. Natl. Acad. Sci. USA 75:1314–1318; 1978.

McInnes, C.; Dolly, J. O. Ca^{2+}-dependent noradrenaline release from permeabilized PC12 cells is blocked by botulinum neurotoxin A or its light chain. FEBS Lett. 261:323–326; 1990.

Ray, P.; Middleton, W. The role of arachidonic acid in stimulated acetylcholine release from PC12 cells. Pharmacologist 34:187; 1992.

Ray, P.; Berman, J. D.; Schuster, B. G., et al. A novel insight into the mechanism of action of botulinum toxin in a cholinergic neuronal cell culture model. Soc. Neurosci. 18:790; 1992.

Richter-Landsberg, C.; Jastorff, B. The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. J. Cell Biol. 102:821–829; 1986.

Sandberg, K.; Berry, C. J.; Rogers, T. B. Studies on the intoxication pathway of tetanus toxin in the rat pheochromocytoma (PC12) cell line. J. Biol. Chem. 264:5679–5686; 1989.

Schantz, E. J.; Johnson, E. A. Properties and use of botulinum toxin and

other microbial neurotoxins in medicine. Microbiol. Rev. 56:80–99; 1992.

Schiavo, G.; Ferrari, G.; Rossetto, O., et al. Specific cross-linking of tetanus toxin to a protein of NGF-differentiated PC12 cells. FEBS Lett. 290:227–230; 1991.

Scott, A. B.; Rosenbaum, A. L.; Collins, C. C. Pharmacological weakening of extraocular muscles. Invest. Ophthalmol. 12:924–927; 1973.

Shone, C. C.; Melling, J. Inhibition of calcium-dependent release of noradrenaline from PC12 cells by botulinum type-A neurotoxin—long-term effects of the neurotoxin on intact cells. Eur. J. Biochem. 207:1009–1016; 1992.

Simpson, L. L. Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. J. Pharmacol. Exp. Ther. 212:16–21; 1980.

Simpson, L. L. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol. Rev. 33:155–188; 1981.

Simpson, L. L. In botulinum neurotoxin and tetanus toxin. In: Simpson, L. L., ed. San Diego, CA: Academic Press; 1989:153–178.

Wellhoner, H. H.; Neville, D. M., Jr. Tetanus toxin binds with high affinity to neuroblastoma x glioma hybrid cells NG108-15 and impairs their stimulated acetylcholine release. J. Biol. Chem. 262:17374–17378; 1987.