Effects of Tetanus Toxin on Catecholamine Release from Intact and Digitonin-Permeabilized Chromaffin Cells

Mary A. Bittner and Ronald W. Holz

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

Abstract: Tetanus exotoxin inhibited Ca^{2+}-dependent catecholamine secretion in a dose-dependent manner in digitonin-permeabilized chromaffin cells. The inhibition was specific for tetanus exotoxin and the B fragment of tetanus toxin; the C fragment had no effect. Inhibition required the introduction of toxin into the cell, and was not seen when intact cells were preincubated with the toxin or toxin fragments. The degree of inhibition was related to the length of preincubation with toxin, as well as the concentration of toxin used. A short preincubation with toxin was sufficient to inhibit secretion, and the continued presence of toxin in the incubation medium was not required during the incubation with Ca^{2+}. The inhibition of secretion by tetanus toxin or the B fragment was not overcome with increasing Ca^{2+} concentrations. Tetanus toxin also inhibited catecholamine secretion enhanced by phorbol ester-induced activation of protein kinase C. Thus, the toxin or a proteolytic fragment of the toxin can enter digitonin-permeabilized cells to interact with a component of the Ca^{2+}-dependent exocytotic pathway to inhibit secretion. **Key Words:** Tetanus toxin—Chromaffin cell—Catecholamine—Exocytosis—Digitonin-permeabilized—Botulinum toxin. **Bittner M. A. and Holz R. W.** Effects of tetanus toxin on catecholamine release from intact and digitonin-permeabilized chromaffin cells. *J. Neurochem.* **51,** 451–456 (1988).

Tetanus toxin inhibits secretion at central inhibitory synapses (Mellanby, 1984) and at the neuromuscular junction (Ambache et al., 1948; Kaeser and Saner, 1969; Mellanby and Thompson, 1972). Tetanus exotoxin is a 150-kilodalton (kDa) protein that consists of a heavy chain (100 kDa) and a light chain (50 kDa) held together by a disulfide bond. The toxin undergoes proteolytic cleavage by papain, resulting in a 45-kDa fragment from the C-terminal of the heavy chain called the C fragment. The remainder of the toxin molecule is termed the B fragment. Both fragments lack the toxicity of the intact toxin in vivo. The tissue binding site appears to be localized on the C-terminal of the heavy chain, since the C fragment (1) shares binding sites with whole toxin in a number of preparations (Morris et al., 1980) and (2) antagonizes the action of exotoxin at the cholinergic neuromuscular junction (Simpson, 1985).

Although tetanus toxin inhibits the release of a number of neurotransmitters (Habermann and Dreyer, 1986), it has no effect on adrenergic neurons or on chromaffin cells (Knight et al., 1985). This lack of activity is a reflection of the inability of these cell types to bind and/or internalize the toxin. Recently, Neher and his colleagues (Penner et al., 1986) have demonstrated that tetanus toxin, when introduced into chromaffin cells via patch clamp pipet, was able to block Ca^{2+}-dependent catecholamine secretion as measured by an increase in capacitance. The B fragment but not the C fragment of tetanus toxin was also an effective inhibitor of exocytosis. The basis of the inhibition of secretion by either the intact toxin or the B fragment is unknown.

Incubation of chromaffin cells with low concentrations of the detergent digitonin renders the plasma membrane permeable to ions and to proteins without altering the integrity of the intracellular storage granules or of the secretory machinery (Dunn and Holz, 1983; Wilson and Kirshner, 1983; Holz and Senter, 1985). Secretion is directly stimulated by micromolar Ca^{2+} in the medium. In this study we have taken advantage of the permeability of digitonin-treated chromaffin cells to proteins to study the intracellular effects of tetanus toxin on Ca^{2+}-dependent secretion.

MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimal essential medium (GIBCO, Grand Island, NY, U.S.A.) containing 10% heat-inactivated fetal calf serum. Cells were usually cultured as monolayers in 6.4-mm diameter plastic culture wells (Costar, Cambridge, MA, U.S.A.) at a density of 500,000 cells/cm^2. For some experiments, suspended chromaffin cells were purified by differential plating, and then plated in collagen-coated plastic wells. Sterile calfskin collagen solution (0.1 ml/cm^2, 50 g/ml in 0.1% acetic acid) (Calbiochem, San Diego, CA, U.S.A.) was applied to each 6.4-mm diameter well. Cells were prepared and plated in the presence of 1.3 μg/ml Fungizone (Squibb, Princeton, NJ, U.S.A.). At least 12 h prior to the start of an experiment, the culture medium was replaced by medium without Fungizone. Experiments were performed 4–8 days after preparation of cultures.

Immediately before a secretion experiment, cells were incubated for 2 h in culture medium containing [3H]-norepinephrine and 0.5 mM ascorbate. Cultures were rinsed three times over 40 min with a physiological salt solution (CaPSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 15 mM HEPES (pH 7.4), and 0.5 mM ascorbate. The potassium glutamate solution (KGEP solution) that was used for secretion experiments with digitonin contained 139 mM potassium glutamate, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.6), 2 mM Mg ATP, 1 mM MgCl$_2$, 5% bovine serum albumin (BSA), and either 5 mM EGTA (without Ca^{2+}) or 5 mM EGTA and various amounts of CaCl$_2$ to yield buffered Ca^{2+} concentrations of 0–10 μM. Toxin or toxin fragments were diluted from a stock containing 0.5 M NaCl and 0.1 M phosphate buffer, pH 6.5. Control wells received an equal volume of 0.5 M NaCl and 0.1 M phosphate buffer, pH 6.5. At the end of an experiment, the incubation solution was removed, and the
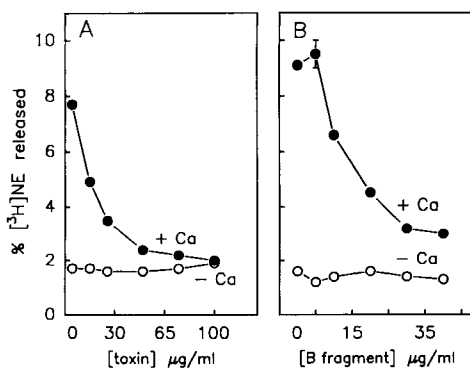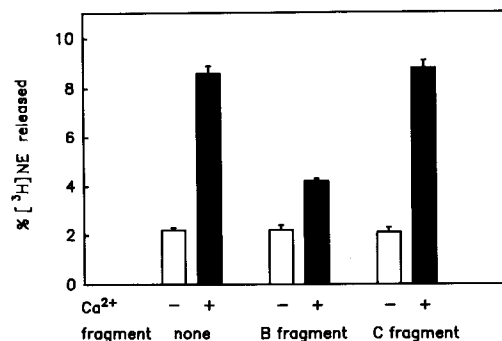

FIG. 2. Comparison of the effects of tetanus toxin B and C fragments on [3H]norepinephrine ([3H]NE) secretion. Cells were permeabilized in KGEP containing 20 μM digitonin, 1 mM MgCl$_2$, 2 mM MgATP, 5% BSA, and 27.5 μg/ml of the toxin fragments or an equivalent volume of buffer for 10 min in the absence of Ca^{2+}. This preincubation was removed, and the cells were incubated with or without Ca^{2+} in the continued presence of the toxin or buffer for 18 min, and the amount of [3H]norepinephrine released was determined. n = 3 wells/group.

cells were lysed with 1% Triton X-100. The radioactivity released into the incubation solutions and the radioactivity remaining in the cells were determined by liquid scintillation spectrometry. Experiments were performed at 25°C.

Data are expressed as means ± SEM unless otherwise indicated. Significance between groups was determined by analysis of variance or by Student's t test. Error bars smaller than symbols were omitted from figures.

Tetanus exotoxin (2,000 Lf/mg protein) and tetanus toxin B and C fragments (5,000 Lf/mg protein) were purchased from Calbiochem (San Diego, CA, U.S.A.), digitonin from Fluka Chemical (Hauppage, NY, U.S.A.), and l-[3H]norepinephrine (21.4 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). Botulinum toxin and other reagents were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

RESULTS

Inhibition of catecholamine secretion by toxin

Tetanus toxin inhibited Ca^{2+}-dependent secretion in digitonin-permeabilized chromaffin cells in a dose-related manner (Fig. 1). When cultured chromaffin cells were permeabilized in the presence of increasing concentrations of tetanus exotoxin (Fig. 1A), [3H]norepinephrine release was inhibited by 47% at 12.5 μg/ml and was virtually abolished at 100 μg/ml exotoxin. Figure 1B demonstrates that the inhibitory effects of the B fragment of tetanus toxin were similar to those of intact exotoxin.

The inhibition of Ca^{2+}-dependent secretion was due to a specific effect of the active exotoxin or B fragment, since it did not occur in cells treated with the inactive C fragment of the toxin (Fig. 2). Treatment with 27.5 μg/ml of toxin B fragment inhibited secretion by 69%, whereas the same concentration of C fragment had no effect.

Role of permeabilization in permitting toxin actions

In contrast to the marked inhibition of secretion seen when permeabilized cells were preincubated

FIG. 1. Effect of tetanus exotoxin (A) or tetanus toxin B fragment (B) on Ca^{2+}-dependent secretion in digitonin-permeabilized chromaffin cells. Cells were permeabilized for 12 min in KGEP containing 20 μM digitonin, 1 mM MgCl$_2$, 2 mM MgATP, 5% BSA, and the indicated concentrations of toxin in the absence of Ca^{2+}. The solution was replaced with KGEP with or without 10 μM Ca^{2+}, and the cells were incubated for an additional 15 min. In A, tetanus exotoxin was present during the 12-min preincubation only; in B, tetanus toxin B fragment was present throughout the second incubation as well. After 15 min, the percentage of [3H]-norepinephrine ([3H]NE) released into the medium was determined. n = 3 wells/group.

with either exotoxin or B fragment, little or no effect on secretion was seen when intact cells were incubated with the toxins. Preincubation of nonpermeabilized cells for 15 min with 125 μg/ml exotoxin or 50 μg/ml B fragment had no effect on secretion stimulated by the nicotinic agonist dimethylphenylpiperazinium, 2.2 mM Ba^{2+}, or elevated K$^+$ (data not shown). When intact cells were incubated with 80 μg/ml of B fragment or 125 μg/ml exotoxin for 15 min, secretion induced by 10 μM Ca^{2+} from subsequently permeabilized cells was also unaltered. When chromaffin cells were treated with either 30 μg/ml B fragment or 25 or 75 μg/ml exotoxin in culture medium for up to 45 h and subsequently stimulated with dimethylphenylpiperazinium or 2.2 mM Ba^{2+}, or depolarized by 56 mM K$^+$, secretion was unaltered. Similarly, when chromaffin cells were incubated with the same concentrations of the toxins for 22 or 45 h, secretion induced by 10 μM Ca^{2+} in subsequently permeabilized cells was no different from that of vehicle-treated cells. The data demonstrate that both the exotoxin and tetanus toxin B fragment must gain access to the cell interior to inhibit catecholamine release.

The effect of preincubating permeabilized cells with increasing amounts of tetanus toxin B fragment for various times is shown in Fig. 3. Ca^{2+}-dependent secretion in the absence of toxin decreased with increasing length of permeabilization (Dunn and Holz, 1983). For any given toxin concentration, increasing the period of exposure to toxin increased the degree of inhibition seen. Thus, addition of toxin B fragment (30–80 μg/ml) simultaneously with Ca^{2+} had no effect on secretion (data not shown). If cells were preincubated for 6 min with 20 μg/ml toxin, the inhibition was 24%, whereas increasing the preincubation time to 12 min gave a 54% inhibition. Similarly, in-

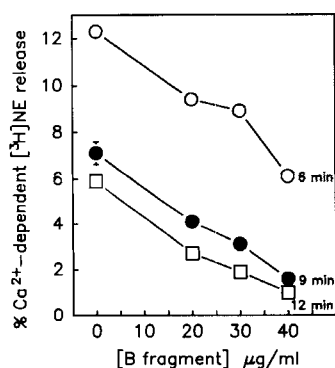**TABLE 1.** *Effects of various preincubation times on the ability of tetanus toxin B fragment to inhibit secretion*

Group	Preincubations 1	Preincubations 2	Ca^{2+}-dependent [^{3}H]norepinephrine secretion
1	−	−	10.5 ± 0.4
2	−	+	8.2 ± 0.4[a]
3	+	−	5.3 ± 0.4[b]
4	+	+	4.9 ± 0.4[b]

Cells were permeabilized for 3 min in KGEP solution containing 20 μM digitonin and 5 mg/ml BSA. Cells were then preincubated for 3 min with (+) or without (−) 50 μg/ml tetanus toxin B fragment in KGEP containing BSA. The solution was removed, and cells preincubated for an additional 3 min with or without B fragment (50 μg/ml) in KGEP with BSA. Again, solutions were removed and cells were incubated for 15 min in KGEP in the presence or absence of 10 μM Ca^{2+}. n = 3 wells/group. A two-factor (treatment by Ca^{2+} level) analysis of variance was completed. This was followed by multiple comparisons for the differences in means by treatments with Ca^{2+} present versus Ca^{2+} absent by the Bonferroni method (Neter and Wasserman, 1974).
[a] $p < 0.01$ compared with Groups 1, 3, and 4.
[b] $p < 0.01$ compared with Groups 1 and 2.

creasing the concentration of toxin permitted a higher degree of inhibition after a shorter preincubation. A 50% inhibition of secretion resulted from a 12-min preincubation with 20 μg/ml toxin or a 6-min preincubation with 40 μg/ml.

The data demonstrated that a preincubation with toxin was necessary for inhibition of secretion, but it was unclear whether this was simply a reflection of the time required for the toxin to enter the cell, or whether the toxin might require some time to exert its effects. The experiment shown in Table 1 was designed to distinguish between these possibilities. A 3-min preincubation of permeabilized cells in the absence of toxin followed by a 3-min preincubation with tetanus toxin B fragment immediately prior to incubation with Ca^{2+} caused a slight (21%) inhibition of secretion, whereas a 3-min preincubation with B fragment followed by an additional 3-min preincubation in the absence of toxin inhibited Ca^{2+}-dependent secretion by 50%. There was no difference between this latter group and cells that were exposed to toxin during both 3-min preincubations. Thus, although time is undoubtedly necessary for toxin to enter the cell, it is clear that additional time is required for the toxin to exert its effects.

Time course of secretion

After a 12-min permeabilization with 30 μg/ml B fragment, there was between 71 and 74% inhibition at all times measured (3–21 min) (Fig. 4). Ca^{2+}-dependent secretion was virtually complete by 9 min in both control and toxin-treated cells. Hence, the extent but not the time course of secretion was altered by the toxin.

FIG. 3. Time course of preincubation with tetanus toxin B fragment. Cells were permeabilized in KGEP containing 20 μM digitonin, 1 mM MgCl$_2$, 2 mM MgATP, 5% BSA, and the indicated concentration of tetanus toxin B fragment or buffer for 6, 9, or 12 min. After the removal of the preincubation, cells were incubated in KGEP for 15 min without toxin in the presence or absence of 10 μM Ca^{2+}. n = 3 wells/group. [^{3}H]NE, [^{3}H]norepinephrine.
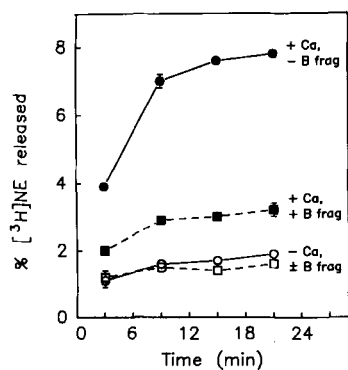
FIG. 4. Effect of preincubation with tetanus toxin on the time course of Ca^{2+}-dependent secretion. Cells were permeabilized for 12 min in KGEP containing 20 μM digitonin, 1 mM $MgCl_2$, 2 mM MgATP, 5% BSA, and 30 μg/ml tetanus toxin B fragment or the appropriate amount of buffer. Cells were then incubated without toxin for 3, 9, 15, or 21 min in the presence or absence of 10 μM Ca^{2+}, and the amount of [^{3}H]norepinephrine ([^{3}H]NE) released was determined. n = 3 wells/group.

Ca^{2+} dose–response curve and pH effects

Increasing the Ca^{2+} concentration was not able to overcome the inhibition of secretion induced by tetanus toxin B fragment (40 μg/ml) (Fig. 5) or by tetanus exotoxin (125 μg/ml) (data not shown). Thus, the major effect of the toxin is to inhibit the maximal extent of secretion rather than the sensitivity of the secretory pathway to Ca^{2+}.

The preceding experiments were all performed using KGEP buffer at pH 6.6. Secretion induced by 1 μM Ca^{2+} was inhibited identically at pH 6.6 and pH 7.0 (data not shown).

Effect of toxin on the enhancement of secretion by phorbol esters

In chromaffin cells, incubation of intact cells with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates protein kinase C and markedly enhances the secretory response to micromolar Ca^{2+} in subsequently permeabilized cells (Pocotte et al., 1985; Lee and Holz, 1986; TerBush and Holz, 1986).
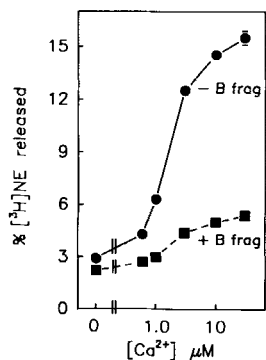

FIG. 5. Effect of preincubation with tetanus toxin B fragment on the Ca^{2+} dose–response curve for secretion. Cells were permeabilized for 6 min in KGEP containing 20 μM digitonin, 1 mM $MgCl_2$, 2 mM MgATP, 5% BSA, and 40 μg/ml tetanus toxin B fragment or an equivalent amount of buffer. After removal of the preincubation, cells were incubated for 15 min with the indicated Ca^{2+} concentrations in the absence of toxin, and the amount of [^{3}H]norepinephrine ([^{3}H]NE) released was determined. n = 3 wells/group.
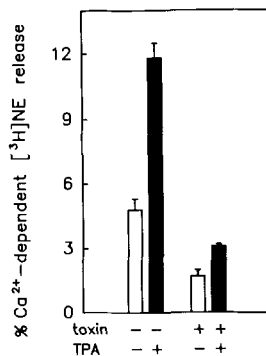

FIG. 6. Effect of preincubation with tetanus exotoxin on the phorbol ester-induced enhancement of secretion. Cells were incubated for 30 min in CaPSS containing 100 nM TPA or CaPSS only. Cells were permeabilized in KGEP with 20 μM digitonin in the presence or absence of 100 nM TPA and with or without 125 μg/ml tetanus exotoxin for 6 min. Cells were then incubated with or without 1 μM Ca^{2+}, in the absence of TPA or exotoxin, for 15 min, and the amount of [^{3}H]norepinephrine ([^{3}H]NE) released was determined. n = 3 wells/group.

When cells that had been preincubated with 100 nM TPA were permeabilized in the presence of 125 μg/ml tetanus exotoxin, the enhancement of secretion due to TPA was also inhibited (Fig. 6). Thus, it is possible that the toxin inhibits at a common site both the Ca^{2+}-dependent secretion that occurs in the absence of TPA-induced activation of protein kinase C and Ca^{2+}-dependent secretion enhanced by TPA-induced activation of protein kinase C.

Effect of botulinum A toxin

Preincubation of intact chromaffin cells with 1 μg/ml botulinum A toxin for 45 h had no effect on secretion stimulated by dimethylphenylpiperazinium, 56 mM K^+, or 2.2 mM Ba^{2+}, nor did it inhibit Ca^{2+}-dependent secretion from subsequently permeabilized cells (data not shown). In a single experiment, chromaffin cells were permeabilized for 12 min in the presence of 20 μg/ml botulinum A toxin, and were stimulated to secrete by 10 μM Ca^{2+} in the absence of the toxin. In this experiment, botulinum A toxin inhibited Ca^{2+}-dependent [^{3}H]norepinephrine release by 68%.

DISCUSSION

Although the toxic properties of the clostridial neurotoxins have been studied for almost a century, relatively little is known about the specific mechanisms whereby these toxins exert their effects. By analogy with other toxins that are better characterized, a three-step model has been proposed (Schmitt et al., 1981; Simpson, 1986). The first step is binding to the cell membrane, followed by internalization of the toxin, and finally interaction of the possibly modified toxin with some intracellular target molecule. The identity of this putative target is at present unknown.

Preincubation of intact chromaffin cells with tetanus toxin had no effect on nicotinic agonist-, elevated K^+-, or Ba^{2+}-induced secretion from intact cells or on secretion induced by micromolar concentrations of Ca^{2+} in subsequently permeabilized cells. However, when cells were first permeabilized with digitonin to

bypass the binding and internalization steps, tetanus toxin was indeed an effective inhibitor of Ca^{2+}-dependent secretion. The inhibition required time to develop, was dependent on the concentration of toxin, and occurred at physiological concentrations of Ca^{2+} (0.1–30 μM). Our work confirms the results of Neher and his colleagues who used patch clamp techniques (Penner et al., 1986) to demonstrate that secretion in chromaffin cells can be inhibited by tetanus toxin (or botulinum A toxin) if it gains access to the cell interior. We also confirm that the active portion of the tetanus toxin molecule is the B rather than the C fragment.

The effects of tetanus toxin have been studied at the cholinergic neuromuscular junction. At the rat soleus neuromuscular junction, blockade of stimulated acetylcholine release by tetanus toxin could be partially overcome by manipulations that raised the intracellular Ca^{2+} (Bevan and Wendon, 1984). The authors suggested that the maximum number of quanta releasable by nerve stimulation was reduced. These data are consistent with the effects of tetanus toxin in chromaffin cells. We found that the maximal secretory response elicited by Ca^{2+} was reduced. The more direct analysis possible in the permeabilized chromaffin cells demonstrated that the fractional inhibition due to toxin was virtually identical over a wide range of Ca^{2+} concentrations (Fig. 5).

The effects of botulinum D toxin are similar to those of tetanus toxin observed in our experiments. When cultured chromaffin cells were preincubated for 48 h with 50 μg/ml botulinum D toxin, and subsequently rendered permeable by brief exposure to intense electric fields, Ca^{2+}-dependent catecholamine release was virtually abolished (Knight, 1987). Pretreatment of intact cells with lower concentrations of botulinum D toxin (5 μg/ml) for 48 h before electrical permeabilization resulted in a partial inhibition of secretion that could not be overcome by Ca^{2+}.

Dreyer and Schmitt (1983) measured the amplitude distribution of miniature end-plate potentials in the mouse hemidiaphragm both before and after tetanus toxin treatment, and showed that the toxin inhibited normal amplitude potentials without inhibiting smaller amplitude potentials. They suggest that in the motor neuron there may be either two mechanisms for the spontaneous release of secretory vesicles or two categories of vesicles, only one of which is susceptible to inhibition by tetanus toxin. However, Bevan and Wendon (1984) observed that tetanus toxin had little or no effect on either the frequency or the amplitude of spontaneous miniature end-plate potentials in the rat soleus preparation. When permeabilized chromaffin cells were preincubated with tetanus toxin B fragment for 12 min before the addition of Ca^{2+}, secretion was inhibited to the same extent throughout the entire time course of the experiment (Fig. 4). Furthermore, a sufficiently high concentration of toxin totally abolished Ca^{2+}-dependent secre-

tion (Fig. 1A). Thus, there is neither a temporally distinguishable mechanism of secretion nor a special pool of granules that is sensitive to the toxin.

Incubation of permeabilized chromaffin cells with toxin inhibited secretion subsequently induced by micromolar Ca^{2+} in the absence of toxin. The degree of inhibition increased with the length of incubation with toxin (Fig. 3). One possible cause of the time-dependent inhibition is the time required for toxin entry into the permeabilized cell. The enzyme lactate dehydrogenase exits the digitonin-permeabilized cell with a half-life of 10–15 min (Dunn and Holz, 1983). Since the molecular weights of tetanus exotoxin (150 kDa) and the B fragment (95 kDa) are similar to that of lactate dehydrogenase (134 kDa), one might expect the kinetics of their entry into the cell to be similar. Experiments in vivo and in cell culture (Bergey et al., 1983) have demonstrated a latency period before tetanus toxin exhibits its activity. Because of the relatively long times involved for binding and internalization, it is uncertain whether the inhibitory effect of the toxin requires time to develop once toxin has entered the cell. In the present experiments, there is a time-dependent step after toxin entry which is necessary for inhibition, since pretreatment with toxin immediately prior to the addition of Ca^{2+} was relatively ineffective, whereas an identical toxin treatment followed by an additional 3-min preincubation before the addition of Ca^{2+} resulted in a 50% inhibition (Table 1).

The data indicate that the toxin interacts with a component of the secretory pathway before the introduction of Ca^{2+}. It has been suggested that tetanus toxin may enzymatically modify a target involved in exocytosis, analogous to the ADP-ribosylating activity of cholera, pertussis, or diphtheria toxins. While such an enzymatic mechanism of action might be expected to result in a long-lived effect, to date no enzymatic activities have been associated with the tetanus toxin molecule.

In summary, we have shown that inhibition of secretion by tetanus toxin or its B fragment requires time to develop and is not overcome by increasing the Ca^{2+} concentration. The target of the toxin appears to be a necessary component of the Ca^{2+}-dependent exocytotic pathway. Thus, digitonin-permeabilized chromaffin cells are a useful model in which to study the biochemical basis for the action of tetanus toxin. Furthermore, the identification of the target of tetanus toxin in chromaffin cells may advance our understanding of the process of exocytosis.

REFERENCES

Ambache N., Morgan R. S., and Wright G. P. (1948) The action of tetanus toxin on the rabbit's iris. *J. Physiol. (Lond.)* **107,** 45–53.

Bergey G. K., Macdonald R. L., Habig W. H., Hardegree M. C., and Nelson P. G. (1983) Tetanus toxin: convulsant action on mouse spinal cord neurons in culture. *J. Neurosci.* **3**, 2310–2323.

Bevan S. and Wendon L. M. B. (1984) A study of the action of tetanus toxin at rat soleus neuromuscular junctions. *J. Physiol. (Lond.)* **348**, 1–17.

Dreyer F. and Schmitt A. (1983) Transmitter release in tetanus and botulinum A toxin-poisoned mammalian motor end-plates and its dependence on nerve stimulation and temperature. *Pflugers Arch.* **399**, 228–234.

Dunn L. A. and Holz R. W. (1983) Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* **258**, 4989–4993.

Habermann E. and Dreyer F. (1986) Clostridial neurotoxins: handling and action at the cellular and molecular level. *Curr. Top. Microbiol. Immunol.* **129**, 93–179.

Holz R. W. and Senter R. A. (1985) Plasma membrane and chromaffin granule characteristics in digitonin-treated chromaffin cells. *J. Neurochem.* **45**, 1548–1557.

Kaeser H. E. and Saner A. (1969) Tetanus toxin, a neuromuscular blocking agent. *Nature* **223**, 842.

Knight D. E. (1987) Calcium and diacylglycerol control of secretion. *Biosci. Rep.* **7**, 355–367.

Knight D. E., Tonge D. A., and Baker P. F. (1985) Inhibition of exocytosis in bovine adrenal medullary cells by botulinum toxin type D. *Nature* **317**, 719–721.

Lee S. A. and Holz R. W. (1986) Protein phosphorylation and secretion in digitonin-permeabilized adrenal chromaffin cells: effects of micromolar Ca^{2+}, phorbol esters and diacylglycerol. *J. Biol. Chem.* **261**, 17089–17098.

Mellanby J. (1984) Comparative activities of tetanus and botulinum toxins. *Neuroscience* **11**, 29–34.

Mellanby J. and Thompson P. A. (1972) The effect of tetanus toxin at the neuromuscular junction in the goldfish. *J. Physiol. (Lond.)* **224**, 407–419.

Morris N. P., Consiglio E., Kohn L. D., Habig W. H., Hardegree M. C., and Helting T. B. (1980) Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. *J. Biol. Chem.* **255**, 6071–6076.

Neter J. and Wasserman W. (1974) *Applied Linear Statistical Models Regression, Analysis of Variance and Experimental Design*, p. 480. Richard D. Irwin, Homewood, Illinois.

Penner R., Neher E., and Dreyer F. (1986) Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature* **324**, 76–78.

Pocotte S. L., Frye R. A., Senter R. A., TerBush D. R., Lee S. A., and Holz R. W. (1985) Effects of phorbol ester on catecholamine secretion and protein phosphorylation in adrenal medullary cell cultures. *Proc. Natl. Acad. Sci. USA* **82**, 930–934.

Schmitt A., Dreyer F., and John C. (1981) At least three sequential steps are involved in the tetanus toxin-induced block of neuromuscular transmission. *Naunyn Schmiedebergs Arch. Pharmacol.* **317**, 326–330.

Simpson L. L. (1985) Pharmacological experiments on the binding and internalization of the 50,000 dalton carboxyterminus of tetanus toxin at the cholinergic neuromuscular junction. *J. Pharmacol. Exp. Ther.* **234**, 100–105.

Simpson L. L. (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu. Rev. Pharmacol. Toxicol.* **26**, 427–453.

TerBush D. R. and Holz R. W. (1986) Effects of phorbol esters, diglyceride, and cholinergic agonists on the subcellular distribution of protein kinase C in intact or digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* **261**, 17099–17106.

Wilson S. P. and Kirshner N. (1983) Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells. *J. Biol. Chem.* **258**, 4994–5000.