A Role for cGMP During Tetanus Toxin Blockade of Acetylcholine Release in the Rat Pheochromocytoma (PC12) Cell Line

Kathryn Sandberg,^a Cathy J. Berry, Erica Eugster, and Terry B. Rogers

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

In order to identify the specific molecular mechanisms involved in neurosecretion, we investigated the mechanism of action of tetanus toxin, a potent presynaptic neurotoxin, in the rat adrenal pheochromocytoma PC12 cell line. It has recently been reported that tetanus toxin is a potent inhibitor of the release of depolarization-evoked ³H-acetylcholine (ACh) from nerve growth factor-differentiated PC12 cells (Sandberg et al., 1989a). In PC12 cells, as in many neural tissue preparations, cGMP accumulation in intact cells increased 6- to 17-fold when stimulated with veratridine (200 μ M), carbachol (1 mM), Ba²⁺ (2 mM), or K⁺ (30 mM). Preincubation of the cells with tetanus toxin inhibits this accumulation by greater than 95%. The toxin dose-inhibition curves for ³H-ACh release and cGMP accumulation are similar, with half-maximal doses of tetanus toxin seen at approximately 5 nM. The time courses for the development of the effects of tetanus on ³H-ACh release and on cGMP accumulation were also similar. Protocols which elevated intracellular cGMP levels reversed the action of the toxin. For example, evoked ACh release was restored in intoxicated PC12 cells by a 15 min exposure to 100 μ M 8-bromo-cGMP. The half-maximal dose was observed at 50 μ M nucleotide. Examination of the nucleotide specificity revealed that only cyclic guanine analogs were effective in reversing the effects of tetanus toxin. These results suggested that the inhibition of depolarization-evoked cGMP accumulation is causally related to the action of tetanus toxin on neurosecretion. This hypothesis was supported by the observation that phosphodiesterase inhibitors were able to restore depolarization-evoked cGMP accumulation and ³H-ACh release in tetanus-treated PC12 cultures. Zaprinast, an inhibitor of cGMP-degrading phosphodiesterases, completely reversed the effects of tetanus toxin on these parameters, while 3-methyl-1-isobutyl methylxanthine was partially effective. Taken together, these results support the view that the underlying mechanism of action of tetanus toxin is related to alterations in cGMP metabolism in PC12 cells. Tetanus toxin should prove to be a valuable probe in identifying this process.

Chemical communication of signals across the synaptic cleft is a crucial process in the nervous system. However, the molecular mechanisms that underlie neurotransmitter release from the presynaptic terminal are poorly understood. It would be valuable to have specific biochemical probes that could "dissect" the excitation-secretion process into discrete steps, thus allowing identification of the molecular events occurring in neurotransmission.

Tetanus toxin, a protein produced by the bacterium *Clostridium tetani*, is an extremely potent neurotoxin (for reviews, see Habermann and Dreyer, 1986; Simpson, 1986). It is well documented that tetanus toxin inhibits neurotransmitter release from presynaptic terminals in many neuronal preparations, including the neuromuscular junction, primary cultured neurons, brain slices, and synaptosomes (Schmitt et al., 1981; Bergey et al., 1983; Osborne and Bradford, 1973). Although the mechanism of action of tetanus is unknown, it is clear that the toxin does not (1) cause cell death or disrupt the ultrastructure of the presynaptic terminal (Schwab and Thoenen, 1976; Mellanby and Green, 1981); (2) affect the synthesis, storage, or uptake of neurotransmitter (Collingridge et al., 1980); or (3) alter presynaptic calcium currents (Dreyer et al., 1983). These results suggest that the action of tetanus toxin involves direct perturbations of the excitation-secretion process. Taken together, the accumulating evidence indicates that tetanus toxin is a valuable probe for studying mechanisms of neurosecretion.

Recent results from our laboratory have demonstrated that the rat pheochromocytoma cell line, PC12, is sensitive to tetanus toxin (Walton et al., 1988; Sandberg et al., 1989a). The toxin binds to high-affinity receptors on cultures of NGF-treated PC12 cells (Walton et al., 1988). Further, subnanomolar doses of tetanus toxin inhibit stimulus-evoked ACh release from these cultures (Sandberg et al., 1989a). The toxin was rapidly internalized ($t_{1/2}$ = 4 min), and after a lag period, which is inversely related to the toxin concentration, evoked ACh release is inhibited (Sandberg et al., 1989a). These findings support the 3-step model proposed for tetanus toxin action in nervous tissues (Schmitt et al., 1981; Simpson, 1986) and reveal that the PC12 cell line is a valid model system for studying the molecular mechanism of action of tetanus toxin.

The present report extends these previous studies to an examination of the biochemical effects of tetanus toxin on PC12 cells. The major results are that tetanus toxin attenuates stimulus-evoked elevation of cGMP levels in a dose- and time-dependent fashion that parallels the inhibition of ACh release. Further, the inhibitory effects of tetanus can be rapidly reversed by application of either cGMP analogs or inhibitors of cGMP-degrading phosphodiesterases. These results provide new insight

Received Mar. 13, 1989; revised June 12, 1989; accepted June 13, 1989.

This work was supported by a contract from the U.S. Army Medical Research and Development Command (DAMD-17-86-C-6160).

Correspondence should be addressed to Dr. Terry B. Rogers, Department of Biological Chemistry, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, MD 21201.

^a Present address: Bldg. 10, Rm. 8C-407, NICHD, 9000 Rockville Pike, Bethesda, MD 20892.

Copyright © 1989 Society for Neuroscience 0270-6474/89/113946-09\$02.00/0

into the mechanism of action of tetanus and support the view that tetanus toxin acts by interfering with cGMP metabolism. A preliminary report of this study has previously been published (Sandberg et al., 1989b).

Materials and Methods

Materials. Tetanus toxin was purchased from the Department of Public Health, Commonwealth of Massachusetts (N. Jamaica Plains, MA) or was kindly supplied to us by Dr. R. O. Thomson of Wellcome Research Laboratories (Beckenham, England). The toxin was stored lyophilized or frozen in PBS, 0.25% BSA at -70°C . DMEM, fetal calf serum and horse serum were purchased from Hazelton Dutchland (Denver, PA). Bio-Rad protein assay dye was purchased from Bio-Rad (Rockville Centre, NY). Toluene Pure Solv and PermaBlend (PPO 98%; POPOP, 2%) were purchased from Packard Instrument Co., Inc. (Downers Grove, IL). Collagen used for coating the culture dishes was obtained from Flow Laboratories, Inc. (McLean, VA). Horse tetanus antitoxin was purchased from Sclavo Labs (Wayne, NJ). Sodium tetraphenylboron was purchased from Aldrich Chem. Co. (Milwaukee, WI). cAMP, cGMP, 8-Br-cGMP, 8-Br-cAMP, $N^6,2'$ -dibutyl- α -cGMP, $N^6,2'$ -dibutyl- α -cAMP, 8-Br-guanosine, 8-Br-inosine cyclic monophosphate, O^2 -monobutyl- α -cGMP, N^2 -monobutyl- α -cGMP, O^2 -monobutyl- α -cAMP, N^2 -monobutyl- α -cAMP, dexamethasone, alumina oxide, choline kinase, choline, veratridine, IBMX, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). ^3H -Ch and cGMP radioimmunoassay kits were purchased from New England Nuclear (Boston, MA). The following tissue culture plasticware was used: 75 cm^2 Corning tissue culture flasks, 35 mm Nunc multiwell plates, and 35 mm Nunc petri dishes.

PC12 cell cultures. PC12 cells were obtained from Dr. Lee Eiden (NIH, Bethesda, MD). PC12 cells were cultured on collagen-coated tissue cultureware as previously described (Sandberg et al., 1989a). In brief, PC12 cells were maintained by subculturing weekly at 5×10^5 cells per 75 cm^2 flask. The medium, DMEM, was supplemented with 7.5% fetal calf serum and 7.5% horse serum. The PC12 medium was exchanged every 2–3 d.

cGMP determinations. Unless otherwise specified, PC12 cells were grown in the presence of NGF for at least 8 d; 16–18 hr before experiments, tetanus toxin was added to the cultures in DMEM containing 1% fetal calf serum and NGF (100 ng/ml). At the end of this incubation, the PC12 cells were washed by replacing the media with 1 ml DMEM. Unless otherwise described, the wash media was supplemented with 100 μM IBMX. After a 2 min incubation at 37°C , this wash media was removed and ACh release buffer was added. The release buffer was: 110 mM NaCl, 5.5 mM KCl, 2 mM Na_2PO_4 , 1.8 mM CaCl_2 , 1 mM MgCl_2 , 25 mM glucose, 70 mM sucrose, 340 mOsm, pH 7.4. In order to measure evoked release, the cells were exposed to depolarizing buffer [release buffer supplemented with one of the following: 30 mM KCl (in this case, the NaCl concentration was reduced to maintain constant osmolality); 1 mM carbachol; 200 μM veratridine; or 2 mM barium chloride (in this case, the barium chloride replaced the calcium chloride)]. The cells were incubated with these buffers for various times as indicated in the text. The incubations were terminated by aspirating the buffers and rapidly adding 750 μl of ice-cold 6% TCA to each well. The wells were scraped with a rubber policeman, and the suspensions were centrifuged for 5 min at $12,000 \times g$. The pellets were used to quantitate protein from each well by resuspending each in 0.5 ml of 0.00125% SDS, 0.5 N NaOH and analyzing the samples in a BioRad protein assay using BSA as a standard. cGMP in the TCA-soluble fraction was measured using a radioimmunoassay method. It was found that further fractionation of the samples improved the precision of the assay. TCA extracts were applied to columns containing 2 ml of Dowex AG50-X4 (H^+ form) which were previously equilibrated with H_2O . The columns were washed with 3 ml of 0.1 N HCl followed by 2 ml of distilled water. cGMP was eluted in the following 1.5 ml of distilled water. The cGMP fractions were lyophilized, and the lyophilisate was dissolved in 50 mM Na acetate, pH 6, before radioimmunoassay. cGMP levels were measured using New England Nuclear radioimmunoassay kits and were corrected for recovery, which was assessed by adding ^3H -cGMP to the samples at the onset of the experiment. The recovery was typically between 70–85% and the cGMP levels were normalized to protein from each cell culture well.

In several experiments described in Results, both ^3H -ACh release and intracellular cGMP levels were quantitated from the same culture well of PC12 cells. It was possible to obtain both of these values from the

same cells by first performing the ^3H -ACh release protocol using ^3H -Ch-prelabeled cells as described below. Immediately after the release, buffer was removed (in this case, release buffer was supplemented with 30 μM eserine) from the culture dish for ^3H -ACh quantitation, 750 μl of 6% TCA was added to the dish and cGMP levels were assayed as described above.

ACh release. After treatment of PC12 cells with the specified release buffers (see above for details), ^3H -ACh release was measured from cells that had been prelabeled with ^3H -Ch as previously described (Sandberg et al., 1989a). In brief, the ^3H -ACh released was separated from any free ^3H -Ch by first phosphorylating the free ^3H -Ch with choline kinase. ^3H -ACh was separated from the ^3H -phosphoryl-Ch and quantitated by 2 methods. In the first method, ^3H -ACh was extracted with Na tetraphenylboron into an organic phase containing scintillant. Because elevated K^+ interfered with this extraction procedure, a second method was developed so that K^+ -stimulated ^3H -ACh release could be measured. In this approach, the release media was incubated with choline kinase, and the reaction solution (1 ml) was added to ion-exchange columns containing 8 ml of Bio Rad AG1-X8 (formate form) equilibrated with H_2O . The columns were washed with 1 ml of 50 mM Tris-HCl, pH 8, and then the ^3H -ACh was eluted with 2 ml of the same buffer and counted using scintillation methods. Pilot experiments revealed that the ^3H -ACh was well resolved from ^3H -phosphoryl-Ch under these conditions.

Guanylate cyclase assay. PC12 cells were removed from the culture dishes by gentle scraping with a rubber policeman and then collected by centrifugation. The cells were homogenized in 0.25 M sucrose, 20 mM Tris-HCl, 10 mM mercaptoethanol, 1 mM EDTA, pH 7.4, in a Teflon glass homogenizer. Soluble and particulate fractions were obtained by centrifuging the homogenate at $100,000 \times g$. Guanylate cyclase activity was assessed by quantitating the conversion of α - ^{32}P -GTP to ^{32}P -cGMP *in vitro*. The final reaction mixture contained 60 nmol α - ^{32}P -GTP (0.02 $\mu\text{Ci}/\text{nmol}$), 0.3 μmol MnCl_2 , 30 μmol Tris-HCl, pH 7.7, and 15 μg protein (soluble fraction) or 50 μg protein (particulate fraction) in a total volume of 150 μl . After the mixture was incubated for 15 min at 30°C with constant shaking, the reaction was stopped by heating for 2 min in a boiling water bath. The ^{32}P -cGMP was isolated by ion-exchange chromatography. The reaction mixture was eluted through an Alumina column (1 ml of resin equilibrated with H_2O) with 5 ml Tris-HCl. The eluate flowed directly onto a second column filled with Dowex AG-1 (1.5 ml) that had been pretreated with 5 ml 1 N formic acid and then 10 ml H_2O . Then the column was washed sequentially with 4 ml 50 mM Tris-HCl, pH 7.4, 10 ml of 50 mM formic acid, 3 ml 0.2 M ammonium formate in 4 N formic acid, and finally with 10 ml of the ammonium formate solution. Pilot experiments demonstrated that 75–85% of the cGMP was recovered in this final fraction. This assay was found to be linear with respect to tissue and time (20 min). All assays were performed within this linear range.

Results

cGMP accumulation in response to depolarizing stimuli

It is well documented that intracellular cGMP levels rise when neuronal tissues are depolarized (Nathanson, 1977; Ferrendelli et al., 1978; Study et al., 1978). When PC12 cells were depolarized with veratridine, potassium, carbachol, or barium, cGMP levels were increased 7- to 12-fold (Fig. 1). These time-course studies reveal that there is a transient response of the cells to most of the depolarizing conditions; cGMP rapidly increases, reaching a maximum within 10–30 sec, which is followed by a declining phase in which the cyclic nucleotide decreases to control values. Barium evoked a slower and more sustained cGMP response. The reason for this difference is not clear.

The declining phase in the cGMP response may be due to its degradation by phosphodiesterases. This is supported by results from experiments in which IBMX was added. As shown in Figure 1 (inset), brief preincubation (2 min) with IBMX significantly decreased the rate of the decline in cGMP levels after depolarization. Although maximal levels of cGMP were not altered under these short incubation conditions, this maximal level was stabilized. This plateau of cGMP was more easily

Figure 1. Time course of stimulus-induced cGMP levels in PC12 cells. Shown are the cGMP levels when the cells were exposed to buffer (○) or buffer supplemented with 200 μ M veratridine (■), 1 mM carbachol (Δ), 2 mM BaCl_2 ($\diamond$), or 30 mM KCl (●). *Inset*, Time course for cultures treated with carbachol. Cultures were treated in an identical manner except for a 2 min preincubation with 100 μ M IBMX. These results are the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

measurable and sample-to-sample variability was greatly diminished. Thus, this brief IBMX treatment was included in the experiments described below.

Muscarinic receptor activation leads to the production of cGMP in several cell systems (Goldberg and Haddox, 1977; Snider et al., 1984). It is possible that the increases in cGMP observed in PC12 cells result directly from depolarization or indirectly from ACh release and subsequent stimulation of muscarinic receptors present on PC12 cells (Green and Tischler, 1982). To distinguish between these alternative mechanisms, cells were depolarized with potassium in the presence of a cholinergic antagonist, atropine. As shown in Table 1, atropine had no effect on the cGMP response. Thus, as previously reported by Snider et al. (1984) for N1E-115 cells, cGMP elevation is a response to depolarization rather than to the action of ACh released upon depolarization.

Evoked ^3H -ACh release and cGMP accumulation were measured in the same cultures following depolarization by a variety of secretagogues. There was some variability in cGMP levels

Table 2. Effect of tetanus toxin on cGMP levels

Incubation conditions	Intracellular cGMP levels (fmol $\times 10^{-1}$ /mg protein)		% Control
	Control	Toxin	
Basal	11 $\pm$ 0.7	12 $\pm$ 0.6	—
Veratridine	42 $\pm$ 1.5	24 $\pm$ 0.5	39
Carbachol	48 $\pm$ 2.7	25 $\pm$ 0.7	35
Barium	85 $\pm$ 5.0	27 $\pm$ 0.6	20
Potassium	117 $\pm$ 7.8	51 $\pm$ 3.1	37

PC12 cell cultures were incubated without (Control) or with 10 nM tetanus toxin for 16 hr at 37°C. At the end of this incubation, the cells were washed and then stimulated for 2 min at 37°C with either 200 μ M veratridine, 1 mM carbachol, 2 mM BaCl_2 , or 30 mM KCl as indicated. At the end of this incubation, intracellular levels of cGMP were measured. The values are the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

and the magnitude of ACh release between cultures. Accordingly, it was advantageous to develop methods which permitted measurement of both parameters in the same well. As shown in Figure 2, there was a correlation between the 2 parameters in response to the depolarizing stimuli. Because of the methods that had to be employed, there was an inherent difference in the timing of these determinations: ACh release is the total release over 2 min, while cGMP levels were measured at 2 min. Therefore, these results provide qualitative evidence that the 2 effects are related.

Tetanus toxin blocks depolarization-induced cGMP accumulation

When PC12 cells were treated with 10 nM tetanus toxin, there was a marked reduction in the depolarization-induced increase in cGMP regardless of the stimulus (Table 2). In contrast, no detectable effect of toxin was seen on the basal levels of cGMP under these experimental conditions. Control experiments revealed that these effects were specific to tetanus toxin. Heat-inactivated toxin, toxin preincubated with toxin antibody, or tetanus toxoid were all ineffective at reducing the accumulation of cGMP in response to depolarizing stimuli (data not shown).

The inhibitory effects of tetanus toxin on cGMP accumulation were dose dependent (Fig. 3). Figure 3 also shows that this dose-response relation is very similar to that observed for the effects of tetanus toxin on the inhibition of evoked ^3H -ACh release from these same cells. In these experiments, the half-maximal dose of tetanus toxin for inhibition of evoked ^3H -ACh release was 5 nM, while the half-maximal effect of toxin on cGMP accumulation was observed at 4.5 nM.

We have previously reported that the time course for the development of inhibitory effects of 10 nM tetanus toxin on ACh release has a characteristic 1 hr lag phase (Sandberg et al., 1989a). As shown in Figure 4, the time course for the appearance of the inhibitory effects of tetanus toxin on cGMP accumulation and ACh release are similar. Both responses display a lag phase followed by the rapid onset of inhibition. The half-maximal effects on cGMP accumulation and ACh release occur at approximately 2 and 3 hr, respectively. Taken together, these results suggest that the attenuation of cGMP levels and ^3H -ACh release in PC12 cells are related.

Effect of cell differentiation

We have previously demonstrated that the differentiation state of PC12 cultures was a critical factor in the development of

Table 1. Effect of atropine on potassium-induced cGMP accumulation

Incubation conditions	Intracellular cGMP levels (fmol $\times 10^{-1}$ /mg protein)		% Control
	Control	Atropine	
Basal	12 $\pm$ 0.4	14 $\pm$ 0.4	117
30 mM KCl	165 $\pm$ 12	159 $\pm$ 14	96

Prior to depolarization, the cells were incubated with 1 μ M atropine for 15 min as indicated. Following this preincubation, the cells were either stimulated with 30 mM KCl at 37°C for 1 min or left in normal release buffer. cGMP levels were then determined. Results are the means of 2–3 experiments, each performed in sextuplicate $\pm$ SEM.

Figure 2. Effect of various stimuli on stimulus-evoked cGMP levels and evoked ACh release in PC12 cultures. The cells were depolarized with various stimuli for 2 min at 37°C. The incubations were terminated and ³H-ACh release and intracellular cGMP levels were determined from the same culture wells. *A*, cGMP levels are shown for cells stimulated with 200 μ M veratridine (open bar), 1 mM carbachol (stippled bar), 2 mM BaCl₂ (hatched bar), or 30 mM KCl (solid bar). *B*, Levels of evoked release of ³H-ACh from the same cultures. The designations of the histogram are the same as described in *A*. These results are the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

sensitivity of ACh release to tetanus toxin (Sandberg et al., 1989a). Experiments were performed to determine if toxin-mediated inhibition of evoked cGMP levels was related to differentiation as well. The cells were cultured under conditions known to stimulate distinct forms of differentiation as previously described (Walton et al., 1988; Sandberg et al., 1989a): nondifferentiated, low density for 7 d (SPARSE); glucocorticoid treatment, in the presence of dexamethasone for 14 d (DEX); NGF for 14 d (NGF); autodifferentiated, high density for 7 d (DENSE). As shown in Figure 5, tetanus toxin blocked barium-evoked cGMP elevation and ³H-ACh release only in cultures treated with NGF. It is interesting to observe that evoked cGMP levels varied with the differentiation state. This was not the direct result of guanylate cyclase expression since we have previously reported that the soluble and particulate enzyme activities are comparable in the different cultures (Eugster et al., 1986).

Figure 3. Dose-response curve of tetanus toxin action on K⁺-stimulated ³H-ACh release and cGMP levels. The cells were preincubated for 3 hr with increasing doses of tetanus toxin at 37°C. At the end of the incubation period ³H-ACh release and cGMP levels, in response to stimulation with 30 mM K⁺, were measured from the same culture wells. Shown are the release of ³H-ACh (●) and cGMP accumulation (○) after 2 min incubations expressed as a percentage of the maximal value in control cultures that were not exposed to toxin. These results are the means of 2–3 experiments each performed in sextuplicate.

Detailed examination of the development of toxin sensitivity in NGF-treated cultures revealed that cells became sensitive to tetanus toxin only after culturing in NGF for at least 8 d (Fig. 6). We previously reported that day 6 NGF-treated cultures were poor in releasing ACh in response to veratridine (Sandberg et al., 1989a). Likewise, day 6 cultures were poor at releasing ³H-ACh (Fig. 6*A*) and accumulating cGMP in response to barium (Fig. 6*B*). The characteristics of ³H-ACh release and cGMP accumulation in day 3 cells were similar to those observed in densely grown cells. This may reflect the fact that day 3 cells retain some characteristics of dense cells since day 0 cells were subcultured from confluent flasks. In summary, it is clear that the differentiation state of the cells determines the sensitivity of PC12 cultures to tetanus toxin when assessed either at the

Figure 4. Time course of tetanus toxin action on K⁺-stimulated ³H-ACh release and cGMP levels. ³H-Ch-prelabeled PC12 cells were incubated with 10 nM tetanus toxin at 37°C. At various times the cultures were removed from the incubator and the K⁺-evoked release of ³H-ACh (○) and cGMP accumulation (●) levels were measured in the same culture wells. These results are the means of 2–3 experiments, each performed in sextuplicate.

Figure 5. Effect of tetanus toxin on Ba^{2+} -evoked ^3H -ACh release and cGMP levels in PC12 cells grown under various differentiation conditions. Ba^{2+} -evoked cGMP accumulation (*A*) or ^3H -ACh release (*B*) were measured. Evoked ^3H -ACh release and cGMP levels were measured in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 16–18 hr preincubations at 37°C) from PC12 cells grown under a variety of conditions: 14 d at 5×10^4 cells/10 cm^2 , in the presence of 1×10^{-6} M dexamethasone (DEX); 14 d at 5×10^4 cells/10 cm^2 , in the presence of 100 ng/ml nerve growth factor (NGF); 7 d, at high density (5×10^5 cells/10 cm^2) (DENSE); or at low density (5×10^4 cells/10 cm^2) (SPARSE). The results are the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

functional or biochemical level. These results provide further circumstantial evidence for a close relation between the inhibitory effects of toxin on both ACh release and cGMP elevation.

Reversibility of tetanus toxin action by cGMP analogs

If the effects of tetanus toxin on neurotransmitter release are causally related to the observed inhibition in stimulus-evoked cGMP accumulation, then it should be possible to reverse the blockade of neurotransmitter release by increasing cGMP levels in the toxin-treated PC12 cells. Accordingly, toxin-treated cells

Figure 6. Effect of tetanus toxin on Ba^{2+} -evoked ^3H -ACh release and cGMP levels from PC12 cells as a function of days in NGF. Evoked ^3H -ACh release (*B*) and cGMP levels (*A*) were measured as a function of culture days in NGF (100 ng/ml) in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 16–18 hr incubation at 37°C). The results are the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

were challenged with 100 μM 8Br-cGMP during the last 15 min of a 4 hr toxin incubation, before assaying for neurotransmitter release. Under these conditions, the evoked release of ^3H -ACh was restored to 65% of control (toxin free) levels (Fig. 7). In contrast, 8Br-cGMP had no significant effect on either spontaneous or evoked ACh release in control PC12 cells (Fig. 7). Control experiments demonstrated that the observed reversal of toxin action cannot be explained by increased cell lysis. The amount of lactate dehydrogenase released into the media in the presence of 8Br-cGMP was nearly identical to that released in control cultures (data not shown). The effect of this cGMP analog was dose-dependent (Fig. 7, inset). In 15 min preincubations, significant effects were seen at 10 μM , and the half-maximal effect was observed at approximately 50 μM .

The specificity of the reversal effect was characterized as shown in Figure 8. All cGMP analogs that were tested were found to be effective in reversing the toxin-mediated inhibition of ^3H -

Figure 7. 8Br-cGMP reverses the action of tetanus toxin on PC12 cells. Spontaneous (open bars) and veratridine-evoked (solid bars) ^3H -ACh release was measured in ^3H -Ch-prelabeled PC12 cells. Both spontaneous and evoked ^3H -ACh release were measured following 4 different preincubation conditions: **CONTROL**, no additions; **8 Br-cGMP**, 15 min incubation at 37°C with $100\ \mu\text{M}$ 8-Br-cGMP; **TOXIN**, 4 hr incubation at 37°C with $4\ \text{nM}$ tetanus toxin; **8 Br-cGMP + TOXIN**, after a 3.75 hr incubation at 37°C with $4\ \text{nM}$ tetanus toxin, the cells were incubated with $100\ \mu\text{M}$ 8-Br-cGMP for 15 min. The results are the means of 6 experiments each performed in triplicate ($\pm\text{SEM}$). **Inset**, Dose-response curve for the ability of 8-Br-cGMP to reverse the inhibition of ^3H -ACh release induced by tetanus toxin in PC12 cells. PC12 cells were cultured in the presence of NGF and ^3H -ACh release was measured as described in Experimental procedures. During the last 15 min of the $4\ \text{nM}$ tetanus toxin 4 hr incubation, increasing concentrations of 8-Br-cGMP were added to the PC12 cells. The results are the means of 6 experiments, each performed in triplicate ($\pm\text{SEM}$).

ACh release (Fig. 8). In contrast, analogs of cAMP and other cyclic nucleotides, as well as acyclic analogs of guanine phosphates, at concentrations as high as $1\ \text{mM}$, were not effective in reversing the action of tetanus toxin (Fig. 8). Control experiments indicated that the binding of ^{125}I -tetanus toxin to these cells was not affected by cyclic guanine nucleotides (data not shown). These findings demonstrate that the ability to reverse the blockade of neurotransmitter release induced by tetanus toxin is specific to cGMP analogs.

Effects of toxin on cGMP metabolism

The 2 simplest explanations for the finding that tetanus toxin prevents the depolarization-induced increase in cGMP are that (1) tetanus toxin inhibits the production of cGMP in response to depolarizing stimuli by interfering with guanylate cyclase activity, as suggested by Smith and Middlebrook (1985); or (2) tetanus toxin stimulates the breakdown of cGMP by activating a phosphodiesterase. We examined the effects of tetanus toxin treatment on guanylate cyclase activity in soluble and particulate fractions as well as whole-cell homogenates from PC12 cells. As shown in Table 3, when cells were treated with toxin under conditions in which ACh release is inhibited, there was no detectable change in enzyme activity in any of these subcellular fractions. Thus, under the conditions used, we could not find experimental evidence to support the first hypothesis.

In the next series of experiments, the potential importance of

Figure 8. Specificity of nucleotide analogs to reverse the action of tetanus toxin on PC12 cells. Various nucleotides were added to the PC12 cells during the last 15 min of the $4\ \text{nM}$ tetanus toxin 4 hr incubation at 37°C : 1, control, no nucleotides; 2, $100\ \mu\text{M}$ 8-Br-cGMP; 3, $1\ \text{mM}$ N^6 , 2'-O-dibutyryl-cGMP; 4, $1\ \text{mM}$ N^2 -monobutyryl-cGMP; 5, $1\ \text{mM}$ O^2 -monobutyryl-cGMP; 6, $100\ \mu\text{M}$ 8-Br-cAMP; 7, $1\ \text{mM}$ N^6 , 2'-OP-dibutyryl-cAMP; 8, $1\ \text{mM}$ N^2 -monobutyryl-cAMP; 9, $1\ \text{mM}$ O^2 -monobutyryl-cAMP; 10, $100\ \mu\text{M}$ 8-Br-inosine-cyclic monophosphate; 11, $1\ \text{mM}$ 8-Br-guanosine monophosphate; 12, $1\ \text{mM}$ 8-Br-guanosine. ^3H -ACh release is expressed as a percentage of the maximum evoked ^3H -ACh release in controls which were prepared in an identical manner except that toxin was omitted from the incubations.

the second hypothesis was examined. PC12 cells were incubated overnight with tetanus toxin and were then treated for 15 min with either of 2 phosphodiesterase inhibitors, IBMX or zaprinast. The latter is reported to be selective for cGMP-degrading phosphodiesterases (Weishaar et al., 1985; Lugnier et al., 1986). Treatment with IBMX or zaprinast had a small but reproducible effect on basal cGMP, increasing levels by 15 and 19%, respectively (data not shown). As shown in Figure 9A, these agents had a greater effect on stimulus-evoked cGMP elevation. IBMX and zaprinast increased these values by 50 and 115%, respectively, in control cultures. The important finding was that in

Table 3. Effect of tetanus toxin on guanylate cyclase activity in PC12 cells

Tissue preparation	Guanylate cyclase activity (pmol cGMP/min/mg protein)	
	Control	+Tetanus toxin
Total cell homogenate	232	244
Soluble fraction	408	392
Particulate fraction	224	197

PC12 cells were cultured as described in Materials and Methods. The cultures were treated with $10\ \text{nM}$ tetanus toxin in DMEM containing 0.25% BSA. At the end of the incubation, the cells were homogenized in $0.25\ \text{M}$ sucrose, $50\ \text{mM}$ Tris, $1\ \text{mM}$ EGTA, pH 7.4, at 0°C . This total cell homogenate was either used directly in the guanylate cyclase assay or was further fractionated into soluble and particulate fractions by centrifugation at $100,000 \times g$ for 1 hr. The fractions were assayed for guanylate cyclase activity. The results are the means of 3 experiments with a variation of 10% or less.

Figure 9. Reversibility of tetanus toxin action on PC12 cells by phosphodiesterase inhibitors. *A*, Evoked levels of cGMP were measured on cells preincubated (16–18 hr at 37°C) with (hatched bars) and without (open bars) 10 nM tetanus toxin. *B*, Levels of evoked ³H-ACh release measured with these same cells. Evoked cGMP levels and evoked ³H-ACh release levels are defined as total stimulated levels minus basal levels. Basal levels were measured in the presence of “control release buffer,” and stimulated levels were measured in the presence of “depolarizing release buffer” (control release buffer supplemented with 200 μM veratridine). Before cells were exposed to release buffers, they were preincubated for 15–20 min with the following buffers: *CONTROL*, DMEM alone; *IBMX*, DMEM supplemented with 100 μM IBMX; *ZAP*, DMEM supplemented with 100 nM zaprinast. These results are the means of 2–3 experiments, each performed in sextuplicate (±SEM).

toxin-treated cultures IBMX partially reversed, and zaprinast fully reversed, the inhibition of cGMP elevation (Fig. 9*A*). Evoked ³H-ACh release was measured from these same cells as shown in Figure 9*B*. Neither IBMX nor zaprinast had any effect on Ba²⁺-evoked ACh release from control cultures. However, IBMX and zaprinast restored ACh release levels by 16 and 100%, respectively, when compared with toxin-free cultures.

The experiments described in Figure 9 were performed using barium as a stimulant. However, zaprinast was also effective in reversing the effects of tetanus toxin when the cells were depolarized using other conditions (Table 4). The results demonstrate a correlation between the ability of phosphodiesterase inhibitors to restore cGMP levels and to restore evoked ACh release levels in tetanus toxin-poisoned cultures.

Discussion

Although the mechanism of action of tetanus toxin is unknown, it has been recognized for some time that the Clostridial neurotoxins, including tetanus toxin, are promising tools to study mechanisms of excitation–secretion coupling in neurons (Habermann and Dreyer, 1986; Simpson, 1986). In a previous report, we have shown that tetanus toxin is a potent inhibitor of ACh release from cultures of PC12 cells and is an excellent model system of toxin studies (Sandberg et al., 1989a). The major thrust of the current work was to exploit the PC12 cell–tetanus toxin system in order to identify the underlying molecular mechanism of tetanus toxin inhibition of neurotransmitter release. The major conclusions from this work are: (1) tetanus toxin inhibits stimulus-evoked cGMP levels in PC12 cells under conditions in which it blocks stimulus-evoked ACh release; (2) the inhibitory effects of tetanus on ACh release are rapidly reversed with cGMP analogs; and (3) a cGMP specific phosphodiesterase is a possible site of action for tetanus toxin since phosphodiesterase inhibitors restored stimulus-evoked ACh release and cGMP levels in a similar manner.

Our results demonstrate that intracellular cGMP levels increase when PC12 cells are depolarized. The evoked levels of cGMP peaked within 20–60 sec with various stimuli, which is consistent with the time course of neurotransmitter release under these conditions (Sandberg et al., 1989a). Further support for a link between neurosecretion and cGMP levels was provided by results from experiments where cGMP levels and ACh release were measured in the same cells. Although these parameters had to be measured over different time intervals in these experiments, the results clearly demonstrate a qualitative relation between evoked cGMP levels and ACh release under a variety of conditions of depolarization and cell growth (Figs. 3–6). It has been recognized for many years that cGMP levels in neural tissues increase in response to depolarizing stimuli (Nathanson, 1977; Ferrendelli et al., 1978; Study et al., 1978). However, the functional significance of this effect has not been elucidated. These initial results provided circumstantial evidence for a role of this cyclic nucleotide in neurosecretion.

The relationship between cGMP levels and neurotransmitter release was further underscored by the observation that tetanus toxin inhibited both processes. Under the experimental conditions used, there is a qualitative correlation between the toxin dose-inhibition curves and the time course for the development of the inhibitory responses of cGMP accumulation and ACh release in PC12 cells (Figs. 3, 4). We have previously reported that only NGF-treated PC12 cells were sensitive to tetanus toxin (Sandberg et al., 1989a). The same relation was found here for the toxin inhibition of evoked-cGMP accumulation. Although the subject of many studies, the molecular mechanisms of tetanus toxin have remained elusive. These results provide important new evidence for the underlying mechanism of action for this toxin and support the view that tetanus toxin interferes with cGMP metabolism in PC12 cells.

Substantial support for this relationship was obtained in experiments in which 8Br-cGMP and other cyclic guanine nucleotides were able to reverse the blockade of ACh release induced by tetanus toxin (Figs. 7, 8). These cyclic nucleotides were not secretagogues alone, since they had minimal effects on ACh release from control PC12 cells in the absence of other stimuli. However, their ability to rapidly reverse the inhibitory effects of tetanus toxin on toxin-treated cells was striking since the inhibitory effects of

Table 4. Reversal of tetanus toxin action by zaprinast

Incubation conditions	cGMP levels (fmol $\times 10^{-1}$ /mg protein)			Cultures treated with zaprinast (1 μ M)		
	Cultures without phosphodiesterase inhibitors					
	Control	Toxin	% Control	Control	Toxin	% Control
Carbachol (1 mM)	32 $\pm$ 1.5	7.8 $\pm$ 1.1	24	51 $\pm$ 3	55 $\pm$ 2	108
Barium (1 mM)	59 $\pm$ 4.2	19 $\pm$ 1.0	29	76 $\pm$ 4.5	70 $\pm$ 3.7	92
Potassium (30 mM)	125 $\pm$ 8.3	43 $\pm$ 3.2	34	178 $\pm$ 23	169 $\pm$ 12	95
	ACh release (dpm $\times 10^{-2}$ /mg protein)					
	Control					
	Control	Toxin	% Control	Control	Toxin	% Control
Carbachol (1 mM)	119 $\pm$ 10	40 $\pm$ 5	34	125 $\pm$ 15	115 $\pm$ 13	92
Barium (1 mM)	165 $\pm$ 18	61 $\pm$ 7	37	191 $\pm$ 25	166 $\pm$ 12	87
Potassium (30 mM)	212 $\pm$ 20	89 $\pm$ 7.4	42	247 $\pm$ 9	277 $\pm$ 32	112

Control PC12 cells or tetanus toxin-treated cultures (10 nM, 16 hr) were incubated in the absence of phosphodiesterase inhibitors or in the presence of 1 μ M zaprinast for 15–20 min. Following this preincubation, the cells were exposed to depolarizing stimuli as indicated above for 2 min at 37°C. Both 3 H-ACh release and cGMP levels were measured in the same culture. The results are reported as the means of 2–3 experiments, each performed in sextuplicate ($\pm$ SEM).

tetanus, once established, are stable for days in PC12 cells and *in vivo* (Simpson, 1986; Sandberg et al., 1989a). Only short-term incubations (on the order of minutes) with micromolar doses of these agents were required to reverse the tetanus toxin-induced inhibition of ACh release. Thus, not only do these results reveal a relation between toxin action and cGMP, but to our knowledge, this is the first demonstration of significant reversal of the effects of tetanus toxin once they have been established in neural cells.

Results reported here identify the metabolic pathway for cGMP as a potential site of action of tetanus toxin. Preliminary studies have revealed that guanylate cyclase activity is not inhibited in intoxicated PC12 cells. Although we cannot rigorously rule out a possible role for this enzyme in toxin action, all of the evidence reported here is consistent with the view that the degradation of cGMP is stimulated in toxin-treated cells. The phosphodiesterase inhibitors, IBMX and zaprinast, were effective in reversing the effects of tetanus toxin on both the inhibition of evoked cGMP accumulation and ACh release in a similar manner (Fig. 9). IBMX, a wide-spectrum, rather low-affinity phosphodiesterase inhibitor (Weishaar et al., 1985), partially restored cGMP levels and ACh release. Zaprinast has been reported to be specific for cGMP-degrading phosphodiesterases in a number of diverse tissues (Windquist et al., 1984; Weishaar et al., 1985; Luginer et al., 1986). Results reported here reveal that this agent was very effective in elevating cGMP levels in control PC12 cells as well. This compound completely restored the stimulus-evoked cGMP response and ACh release after it was applied for 15 min to intoxicated cells (Fig. 9, Table 3). While it is possible that the hydrophobic agents, 8Br-cGMP and zaprinast, act through nonspecific mechanisms, the observation that the effects of tetanus can be reversed by these 2 distinctly different chemicals that share the common property of elevating cGMP levels in PC12 cells strongly argues against nonspecific mechanisms.

A minimal interpretation of these results is that tetanus toxin acts directly or indirectly to increase the activity of cGMP phosphodiesterase in PC12 cells. Multiple forms of phosphodiesterases, which are differentially inhibited by various drugs, have been identified in many tissues (Beavo et al., 1982; Windquist et al., 1984; Weishaar et al., 1985). It is not clear which isozyme may be involved in tetanus toxin action. The fact that basal

cGMP levels are not significantly different in control and toxin-treated cells suggests that such an enzymatic activity may predominate during depolarization. The particular target enzyme remains to be identified, and zaprinast may prove to be a valuable probe for such studies.

The results reported here provide compelling evidence that cGMP plays an essential role in neurotransmitter release. It is interesting to speculate on the functional role of cGMP in this process. Since neither 8Br-cGMP nor zaprinast stimulated ACh release directly, it is not likely that cGMP alone is the intracellular messenger that evokes secretion. The implication is that in order to elicit neurotransmitter release, cGMP-mediated events must work in concert with other events that are triggered by depolarization. At present the target for cGMP in neurosecretion is not known. However, tetanus toxin is a promising tool to identify this mechanism.

References

- Beavo, J. A., R. S. Hansen, S. A. Harrison, R. L. Hurwitz, T. J. Martins, and M. C. Mumbry (1982) Identification and properties of cyclic nucleotide phosphodiesterases. *Mol. Cell. Endocrinol.* 28: 387–405.
- Bergey, G. K., R. L. MacDonald, W. H. Habig, M. C. Hardegree, and P. G. Nelson (1983) Tetanus toxin convulsant action on mouse spinal cord neurons in culture. *J. Neurosci.* 3: 2310–2323.
- Collingridge, G. L., G. G. S. Collins, J. Davies, T. A. James, M. J. Neal, and P. Tongroach (1980) Effect of tetanus toxin on transmitter release from substantia nigra and striatum *in vitro*. *J. Neurochem.* 34: 540–547.
- Dreyer, F., A. Mallart, and J. L. Brigan (1983) Botulinum A toxin and tetanus toxin do not affect presynaptic membrane currents in mammalian motor nerve endings. *Brain Res.* 270: 373–375.
- Eugster, E., K. Sandberg, and T. B. Rogers (1986) The effect of differentiation on guanylate cyclase activity and cGMP levels in PC12 cells. *Soc. Neurosci. Abstr.* 14: 822.
- Ferrendelli, J. A., D. A. Kinscherf, and M. M. Chang (1978) Distribution and regulation of cyclic GMP in the central nervous system. *Adv. Cyclic Nucleotide Res.* 9: 453–464.
- Goldberg, N. D., and M. K. Haddox (1977) Cyclic GMP metabolism and involvement in biological regulation. *Annu. Rev. Biochem.* 46: 823–896.
- Green, L. A., and A. S. Tischler (1982) PC12 pheochromocytoma cultures in neurobiological research. *Adv. Cell. Neurobiol.* 3: 373–414.
- Habermann, E., and F. Dreyer (1986) Clostridial neurotoxins: Handling and action at the cellular and molecular level. *Curr. Topics Microbiol. Immunol.* 129: 93–179.
- Luginer, C., P. Schoeffter, A. LeBec, E. Strouthou, and J. C. Stoclet

- (1986) Selective inhibition of cyclic nucleotide phosphodiesterase of human, bovine and rat aorta. *Biochem. Pharmacol.* 35: 1743–1751.
- Mellanby, J., and J. Green (1981) How does tetanus toxin act? *Neuroscience* 6: 281–300.
- Nathanson, J. A. (1977) Cyclic nucleotides and nervous system function. *Physiol. Rev.* 57: 158–256.
- Osborne, R. H., and H. F. Bradford (1973) Tetanus toxin inhibits amino acid release from nerve endings *in vitro*. *Nature [New Biol.]* 244: 157–158.
- Sandberg, K., C. J. Berry, and T. B. Rogers (1989a) Studies on the intoxication pathway of tetanus toxin in the rat pheochromocytoma (PC12) cell line. *J. Biol. Chem.* 264: 5679–5686.
- Sandberg, K., C. J. Berry, E. Eugster, and T. B. Rogers (1989b) Studies on the molecular mechanism of action of tetanus toxin: A role for cGMP in the release of acetylcholine from PC12 cells. *Proceedings of the 8th International Conference on Tetanus* (Leningrad, Russia), B. Bizzini and G. Nistico, eds., Pitagora Press, Bologna, Italy (in press).
- Schmitt, A., F. Dreyer, and C. John (1981) At least three sequential steps are involved in the tetanus toxin-induced block of neuromuscular transmission. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 317: 326–330.
- Schwab, M. E., and H. Thoenen (1976) Electron microscopic evidence for a transsynaptic migration of tetanus toxin in spinal cord motoneurons: An autoradiographic and morphometric study. *Brain Res.* 105: 213–224.
- Simpson, L. L. (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu. Rev. Pharmacol. Toxicol.* 26: 427–454.
- Smith, L. A., and J. L. Middlebrook (1985) Botulinum and tetanus neurotoxins inhibit guanylate cyclase activity in synaptosomes and cultured nerve cells. *Toxicon* 23: 611a.
- Snider, R. M., M. McKinney, C. Forray, and E. Richelson (1984) Neurotransmitter receptors mediate cyclic GMP formation by involvement of arachidonic acid and lipoygenase. *Proc. Natl. Acad. Sci. USA* 81: 3905–3909.
- Study, R. E., X. O. Breakfield, T. Bartfai, and P. Greengard (1978) Voltage-sensitive calcium channels regulate guanosine 3',5'-cyclic monophosphate levels in neuroblastoma cells. *Proc. Natl. Acad. Sci. USA* 75: 6295–6299.
- Walton, K. M., K. Sandberg, T. B. Rogers, and R. L. Schnaar (1988) Complex ganglioside expression and tetanus toxin binding by PC12 pheochromocytoma cells. *J. Biol. Chem.* 263: 2055–2063.
- Weishaar, R. E., M. H. Cain, and J. A. Bristol (1985) A new generation of phosphodiesterase inhibitors: Multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J. Med. Chem.* 28: 537–545.
- Windquist, R. J., E. P. Faison, S. A. Waldman, K. Schwartz, F. Murad, and R. M. Rappoport (1984) Atrial natriuretic factor elicits an endothelial-independent relaxation and activates guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA* 81: 7661–7664.