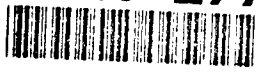

MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXIN, TETANUS TOXIN

ANNUAL REPORT

Terry B. Rogers, Ph.D

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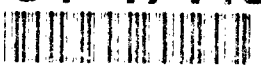

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During the early phase of this project we were successful in establishing a cultured cell model system, the PC12 pheochromocytoma cell line, to study the mechanism of action of tetanus toxin. further we have studied the characteristics of the intoxication pathway (Sandberg et al. 1989) and have found that it is analogous to that which has been characterized, to some extent, in vivo (Simpson, 1986; Habermann and Dreyer, 1986). The major thrust during the past year was to exploit this well characterized model system to gain insight into the molecular mechanism of action of tetanus toxin.

In the present study, we have utilized a preparation of permeabilized, NGF-differentiated, PC12 cells to examine the role of cGMP in neurotransmitter release. An important finding is that cGMP can stimulate neurotransmitter release from such cells in a Ca^{2+}-independent manner. Further, NGF-differentiated PC12 cells show two phases of vesicular neurotransmitter release that can be distinguished not only by their differential sensitivity to Ca^{2+}, but also in their sensitivity to cGMP.

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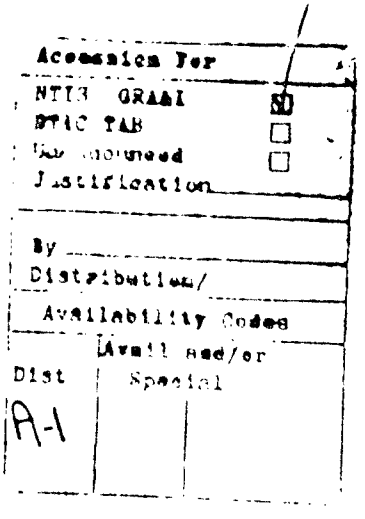
FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).



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Tetanus Toxin - Significance

Tetanus infections are no longer a serious health problem in developed countries because of effective immunization procedures. Therefore it is appropriate to ask why effort should be devoted to studying the mechanism of action of tetanus toxin. In the first place it is important to recognize that potent toxins produced by a variety of organisms have been valuable tools that have been used to probe the molecular features of the complex nervous system (Ceccarelli and Clementi, 1979). For example, the sodium channel and the nicotinic acetylcholine receptor have been well characterized as a result of the use of tetrodotoxin and α-bungarotoxin, respectively. Therefore one important reason to study tetanus action is that it may shed light on unknown molecular processes that occur in the brain.

The chemical communication of signals between neurons across the synaptic cleft, referred to as synaptic transmission, is mediated by neurotransmitter substances and is a crucial process in the nervous system. Yet, the molecular processes that underlie the neurotransmitter release mechanism in the presynaptic cell are not understood. Accordingly, it would be extremely valuable to have toxins that could be used as tools to probe this specific process.

Tetanus toxin, a protein produced by the bacterium *Clostridium tetani*, is an extremely potent neurotoxin (Simpson, 1986; Habermann and Dreyer, 1986). It is now well known that tetanus toxin inhibits neurotransmitter release from presynaptic terminals from a variety of neural preparations including neuromuscular junctions, primary cultured neurons, brain slices and synaptosomes (Schmitt *et al*.1981; Bergey *et al*.1983; Osborne and Bradford, 1973). Many laboratories have been active in trying to identify the mechanism by which tetanus brings about this inhibition. From such studies it is now clear that tetanus toxin does not: (1) cause cell death

or disrupt the ultrastructure of the presynaptic terminal (Mellanby and Green, 1981; Schwab and Thoenen, 1976); (2) alter the synthesis, storage or uptake of neurotransmitter (Collingridge *et al.*1980); (3) modify presynaptic action potentials or inward calcium currents (Dreyor *et al.*1983). Thus the current hypothesis for tetanus toxin action is that this toxin acts by perturbing the coupling of excitation to neurotransmitter secretion at a step that occurs downstream from Ca^{2+} entry into the neuron. Tetanus toxin is one member of a small class of unique neurotoxins that act at the presynaptic terminal on processes directly involved with neurotransmitter release. All of the evidence gathered to date strongly supports the idea that tetanus toxin is indeed a very valuable tool to study excitation-secretion coupling in the central nervous system.

A second important reason to study the action of tetanus toxin is that its mechanism of action is strikingly similar to that of another potent toxin, botulinum toxin, which is produced by another closely related gram positive bacterium, *Clostridium botulinum* (Simpson, 1986). In contrast to tetanus infections, immunization and protection against botulinum infections is very limited. Thus, an understanding of the action of tetanus should yield information which will lead to a therapeutic strategy for the treatment of the toxic sequelae of the very serious botulinum infections.

Results from the Principal Investigator's Laboratory

During the Past Year

During the initial phase of this research program, considerable effort was devoted to developing cultured cells systems that could be used as appropriate models in which to investigate the mechanism of action of tetanus toxin on neurotransmitter release (Staub *et al.*1986; Walton *et al.*1988; Sandberg *et al.*1989). We have established that pheochromocytoma cell line, PC12, when cultured with nerve growth factor (NGF) has a large concentration of high affinity tetanus toxin receptors (Walton *et al.*1988), and is a valid model system in which to study the mechanism of action of the Clostridial neurotoxins. Further, using this cell system in the second phase of the project, we have established that cGMP plays a role in the mechanism of toxic action in these cells (Sandberg *et al.*1989). During the past year we have continued to exploit this cell system and have extended these previous studies to a detailed examination of the role of cGMP in neurosecretion in NGF-treated PC12 cells. The rationale for this study was that by more clearly defining the role of cGMP a better understanding of the action of tetanus toxin will be derived.

It is well recognized that cGMP levels rise in nervous tissue in response to depolarizing stimuli (Nathanson, 1977; Goldberg and Haddox, 1977). In the previous annual report we described procedures that we have developed methods to permeabilize PC12 cells with a pore-forming exotoxin, α-toxin, obtained from *Staph. aureus*. This toxin has been utilized effectively to examine neurosecretion in several neural preparations (Ahnert-Hilger *et al.*1985; Thelestam and Blomqvist, 1988). The advantage of this approach is that in permeabilized cells one has direct access to the intracellular space to which one can apply probes in a controlled manner. Initial experiments with these cells demonstrated that both dopamine (DA) and acetylcholine (ACh) were secreted from such cells in a Ca^{2+}-dependent manner (Figure 1). The response was biphasic,

with half maximal effects observed at 0.6 μM and 20 μM free Ca^{2+}.

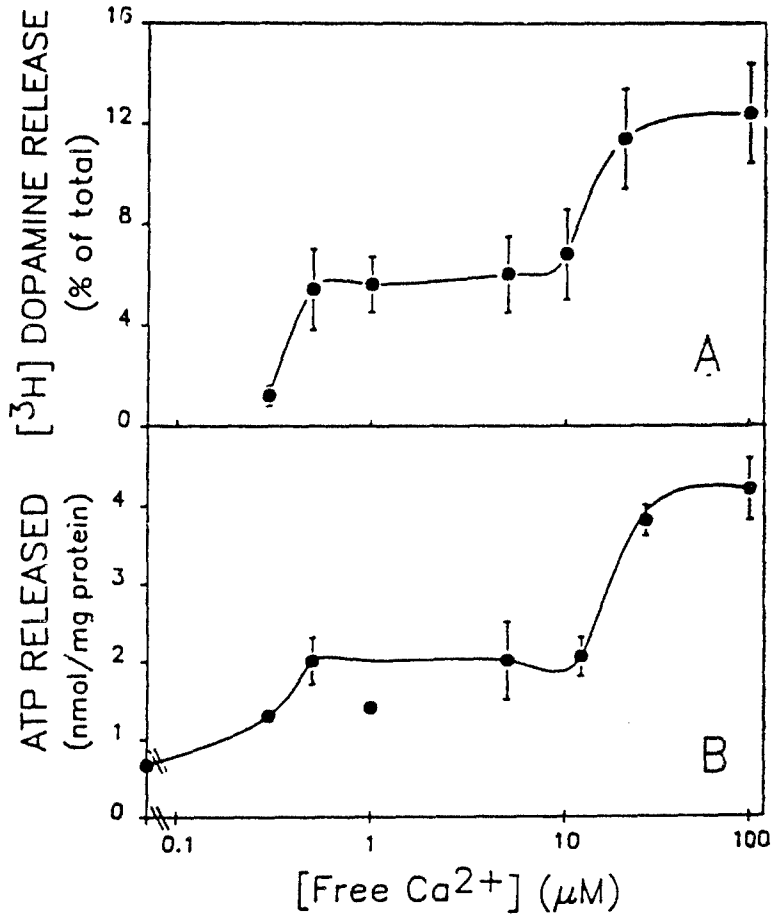

Figure 1. Ca^{2+}-dependent release of [^{3}H]DA and ATP from α-toxin-permeabilized cells. In Panel A, release of [^{3}H]DA was determined from prelabeled PC12 cells. Data are expressed as Ca^{2+}-dependent transmitter release after subtraction of values in the absence of Ca^{2+} (representing 5-12% of total). In panel B, ATP release was monitored in experiments similar to those in Panel A. ATP was determined using a luciferase assay. Ca^{2+}-independent release of ATP was 0.6 nmol/mg protein. Data points represent means ± SEM (n=9, Panel A; n=3, Panel B).

Experiments were performed to determine whether [^{3}H]DA release in both Ca^{2+}-dependent

phases was derived from transmitter stored in vesicles. Release of ATP, a nucleotide which has been shown to be co-localized with neurotransmitters in vesicles (Green and Rein, 1977), was used as an independent index of vesicular release. As shown in Figure 1B, the release of ATP from permeabilized cells showed a biphasic response to Ca^{2+} nearly identical to that for [^{3}H]DA.

There is accumulating evidence from this laboratory that tetanus toxin exhibits its effects by altering a step involved in cGMP metabolism. Such data suggests that cGMP may be an important signalling molecule in regulating neurosecretion in general. As an initial approach to examine this hypothesis, experiments were performed to examine the effects of cGMP on [^{3}H]DA release in permeabilized PC12 cells. As shown in Figure 2, cGMP did evoke the release of DA from such cells in a dose- and Ca^{2+}-dependent manner.

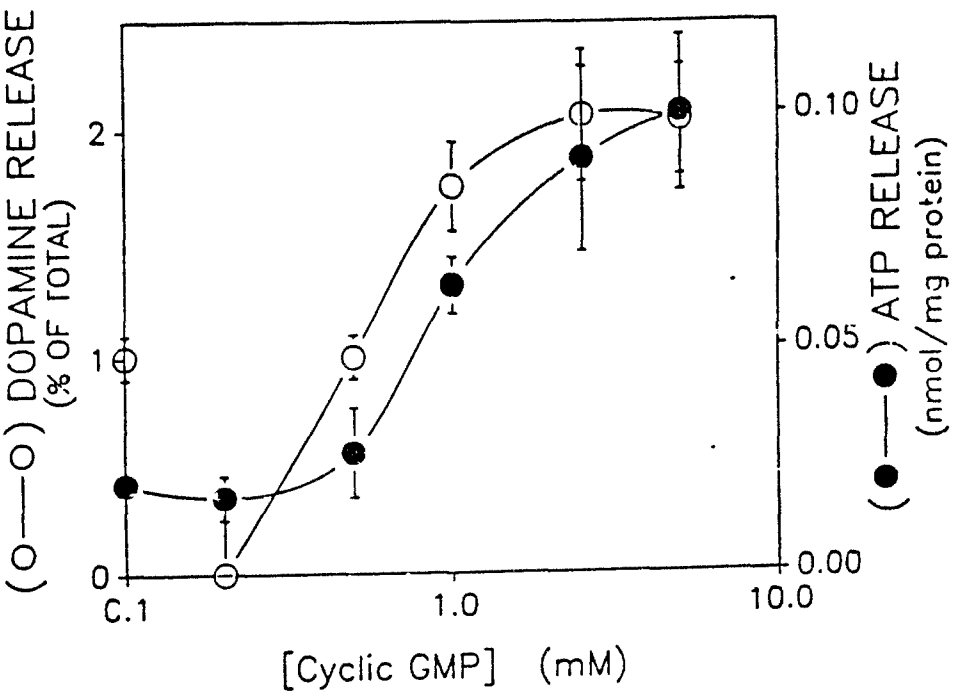

Figure 2. Dose-response curves for cGMP-mediated release of [^{3}H]DA and ATP from permeabilized PC12 cells. Cells were preincubated in [^{3}H]DA, washed and exposed to α-toxin (100 units/ml) prior to further incubation for 6 min in the same buffer containing the concentrations of cGMP shown. Release of radiolabel or ATP in the absence of nucleotide was subtracted from experimental values to show the specific increase due to cGMP.

The time course for the cGMP-evoked release of [^{3}H]DA is shown in Figure 3. After the cells were exposed to 1mM cGMP, there was a lag period of 1 min, after which, [^{3}H]DA release occurred, reaching maximal values by 3 min.

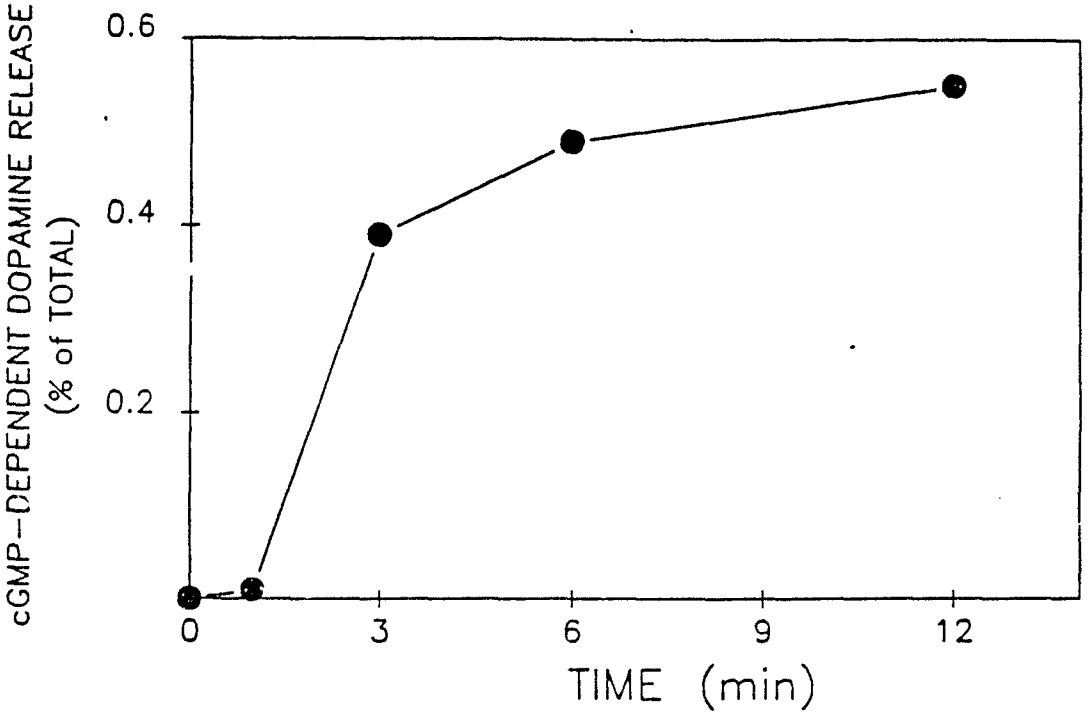

Figure 3. Time course of cGMP-mediated release of [^{3}H]DA. Cells, incubated in the presence of [^{3}H]DA were permeabilized with α-toxin in KG buffer (10mM EGTA). Permeabilization medium was removed and replaced with fresh buffer in the presence or absence of 1mM cGMP. At the times indicated this medium was removed and specific release due to nucleotide was determined at each time point. Values shown are from a single experiment (+/- SEM; n=3). Release in the absence of cGMP represented 2.3% of total label at 3 min.

The nucleotide specificity for evoked release of transmitter in the absence of Ca^{2+} was examined. Only analogues of cGMP were effective in evoking [^{3}H]DA release under the conditions used. In contrast, GMP and other cyclic nucleotides were not active in this system (data not

shown). Thus, taken together, these data suggest that cGMP can play a role in regulating neurosecretion from PC12 cells.

Putative sites of action of cGMP. A possible explanation for the action of cGMP on secretion is that it may release Ca^{2+} from intracellular stores. However, since 10 mM EGTA was used in the release buffer, it seemed unlikely that any released Ca^{2+} would not be buffered and could result in a Ca^{2+} transient sufficient to stimulate secretion. Experiments were performed to confirm this hypothesis. PC12 cells, permeabilized in the absence of Ca^{2+}, were treated with A23187 in order to release Ca^{2+} from intracellular stores. In the absence of EGTA this treatment resulted in increased release of transmitter (Figure 4). However, release observed in the presence of ionophore was reduced to control levels if the concentration of EGTA was greater than 1mM (Figure 4). These results argue against the possibility that cGMP-evoked [^{3}H]DA release, measured in the presence of 10mM EGTA, results from release of a cGMP-sensitive intracellular pool of Ca^{2+}.

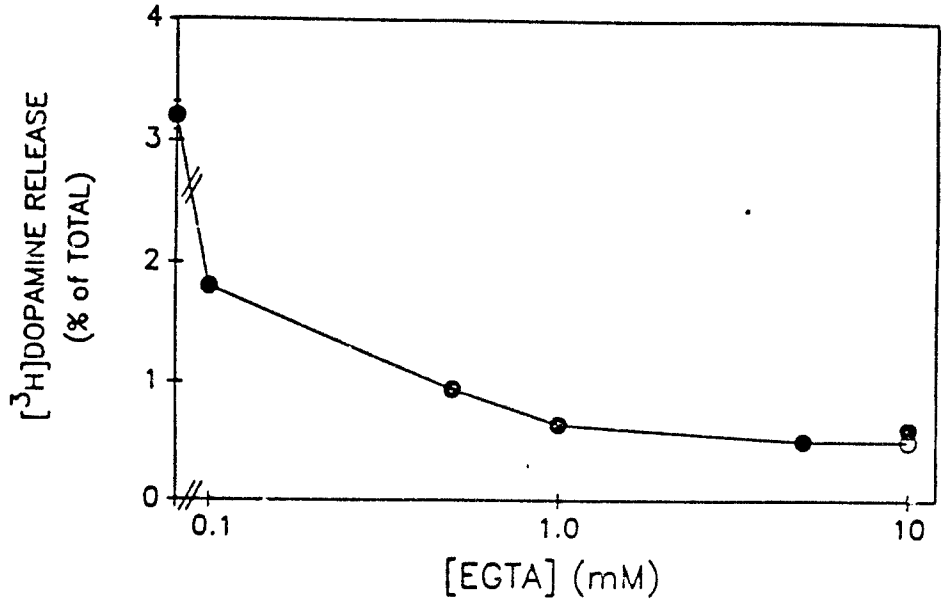

Figure 4. Effects of EGTA concentration on Ca^{2+} ionophore-induced release of [^{3}H]DA. PC12 cells were preincubated with [^{3}H]DA as detailed in the text. Subsequent washes were made in KG buffer (pH 7.4) containing 0-10mM EGTA (Mg^{2+} adjusted to maintain a free concentration of 2.8mM). After the cells were permeabilized with α-toxin (100 units/ml) in the appropriate EGTA buffer, they were incubated for 6 min at 37°C in the presence (▲) or absence (○) of A23187 (5 μM). Data points represent the mean of triplicate determinations from a single experiment.

The action of cGMP may involve a cGMP-dependent kinase. While the effects of cGMP did not require the presence of exogenous ATP (data not shown), there may be sufficient ATP still present in permeabilized PC12 cells to maintain phosphorylation-mediated events. This hypothesis was supported by results from experiments in which ATP levels were measured in permeabilized and intact cells and found to be 44 and 97 nmol/mg of protein respectively. Thus in order to further explore this hypothesis, the effects of a non-hydrolyzable analogue of ATP on cGMP-evoked [^{3}H]DA release was examined. Addition of AMPPNP (Yount *et al* 1971) completely inhibited any increase in secretion due to cGMP (Figure 5).

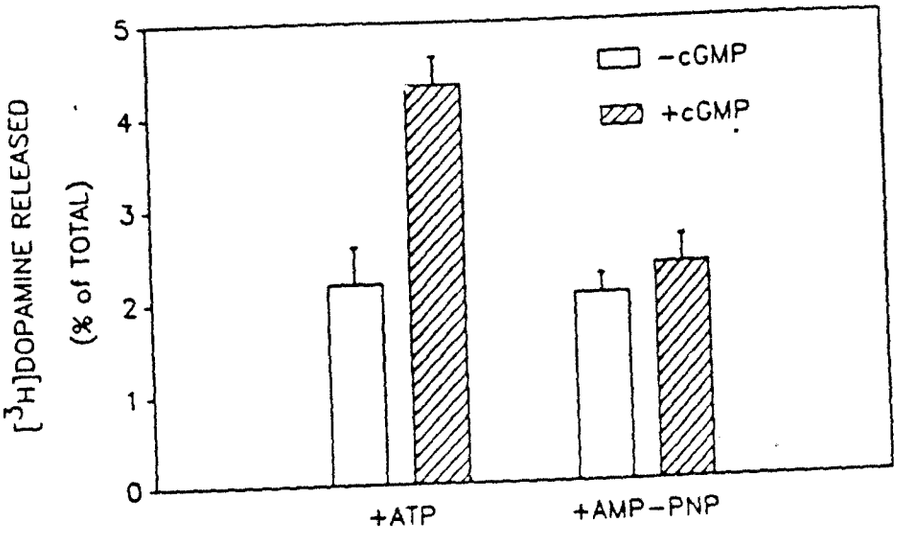

Figure 5. Effects of ATP analogue, AMPPNP, on cGMP-induced [^{3}H]DA release. [^{3}H]DA-prelabeled cells were permeabilized in the presence (shaded bars) or absence (open bars) of 1mM cGMP and specific release of [^{3}H]DA was quantitated. All buffers were supplemented with 1mM ATP (control) or 1mM AMP-PNP.

These results suggest that hydrolysis of ATP is important in mediating the effects of cGMP. Thus, these data suggest the importance of phosphorylation-mediated events, through the activation of a cGMP-activated kinase for example, in the stimulation of secretion by cGMP.

CONCLUSIONS

During the early phase of this project we were successful in establishing a cultured cell model system, the PC12 pheochromocytoma cell line, to study the mechanism of action of tetanus toxin. Further we have studied the characteristics of the intoxication pathway (Sandberg *et al.*1989) and have found that it is analogous to that which has been characterized, to some extent, *in vivo* (Simpson, 1986; Habermann and Dreyer, 1986). The major thrust during the past year was to exploit this well characterized model system to gain insight into the molecular mechanism of action of tetanus toxin.

In the present study, we have utilized a preparation of permeabilized, NGF-differentiated, PC12 cells to examine the role of cGMP in neurotransmitter release. An important finding is that cGMP can stimulate neurotransmitter release from such cells in a Ca^{2+}-independent manner. Further, NGF-differentiated PC12 cells show two phases of vesicular neurotransmitter release that can be distinguished not only by their differential sensitivity to Ca^{2+}, but also in their sensitivity to cGMP.

Permeabilized NGF-treated PC12 cells retain their ability to release catecholamines in response to Ca^{2+}. The Ca^{2+} dose-response curve for release of catecholamines revealed two phases of neurotransmitter release which is similar to that reported for non-differentiated PC12 cells (Ahnert-Hilger *et al.*1985). Two series of experiments indicated that both the high and low affinity Ca^{2+}-dependent release originated from a vesicular pool(s); firstly, preincubation with the plant alkaloid reserpine, which significantly reduces the level of transmitter within vesicles (Kittner *et al.*1987), inhibited Ca^{2+}-dependent secretion from both phases. Second, the release of ATP, which is stored in secretory vesicles with transmitter and co-released upon stimulation (Green and Rein, 1977), exhibits a similar biphasic response to Ca^{2+} (Figure 1B). Thus, although the

biological significance of these two phases of transmitter release remain to be defined, they arise from pools of secretory vesicles.

An important goal of the present study was to verify the hypothesis, presented elsewhere (Sandberg *et al.*1989), that cGMP may play a role as a signalling molecule in secretion. Several results presented here support the conclusion that cGMP is involved in this process; under nominally Ca^{2+}-free conditions (pCa>9), cGMP stimulates transmitter release in a time-dependent manner; the co-release of ATP indicates that cGMP-evoked release of DA was derived from vesicular pools; the magnitude of cGMP-evoked release in Ca^{2+}-free medium is similar to that evoked by excitatory concentrations of free Ca^{2+} (1-10μM). However, while the effects of cGMP were dose-dependent and highly specific (i.e. DA release was seen only for cyclic analogues of guanine nucleotides), it is not clear whether the nucleotide stimulates secretion from the same population of vesicles as Ca^{2+}.

Dose-response studies revealed that, under the conditions used, half maximal doses of cGMP were in the range of 500μM. These levels may be higher than expected in a physiological context. However, several results indicate that the apparent potency of cGMP is reduced due to two factors; a lack of complete permeability of the plasma membrane to cGMP and degradation of the nucleotide.

Permeabilization of cells with saponin (which produces larger pores than α-toxin (Ahnert-Hilger and Gratzl, 1988), increased the potency of cGMP by 40%. Furthermore, when cells were incubated with [^{3}H]cGMP, 60% of the cell-associated nucleotide was degraded within 3 min. Inclusion of phosphodiesterase inhibitors partially reversed this degradation and increased the apparent potency of cGMP. Thus, while it is difficult to accurately estimate the effective concentration of intracellular cGMP in these experiments, it is clear that cGMP is significantly more potent than estimated by the half-maximal concentration of the dose-response relation.

While many of the experiments were performed in the absence of Ca^{2+}, under physiological conditions Ca^{2+} would be present. Therefore it was important to determine if there were interactions between Ca^{2+} and cGMP on secretion. Transmitter release induced by cGMP was observed only in Ca^{2+}-free buffers. While a small increase in release was observed if cGMP was present during incubations with low Ca^{2+} concentrations (<1μM, data not shown), cGMP effects were not additive with release induced by 10μM free Ca^{2+}. Although this effect could be explained by a cGMP-mediated release of Ca^{2+} from intracellular stores this is unlikely, since, in the presence of 10mM EGTA, intracellular Ca^{2+} levels are effectively buffered (Figure 4). Furthermore, in contrast to its stimulatory action under conditions of low free Ca^{2+}, cGMP was actually inhibitory to release induced by 100μM free Ca^{2+}. It was also clear that AMPPNP inhibited the action of cGMP suggesting that hydrolyzable ATP is required for the action of the nucleotide. These results suggest that a cGMP-dependent kinase may be an important mediator of the response. Further experiments are needed to clarify this issue.

Thus, in summary, cGMP was found to stimulate the rapid release of neurotransmitter from permeabilized PC12 cells under essentially Ca^{2+}-free conditions. Further, in the presence of Ca^{2+}, cGMP regulated one phase or mode of Ca^{2+}-dependent release. These observations provide new insight on the importance of cGMP in regulating the molecular events that are triggered by depolarization and that lead to neurotransmitter release. It will be important in future studies to examine the effects of tetanus and botulinum toxins on the process.

BIBLIOGRAPHY OF PUBLISHED WORK

1. Sandberg, K., Berry, C.J., and Rogers, T.B. (1989) A Role for cGMP During Tetanus Toxin Blockade of Acetylcholine Release in the Rat Pheochromocytoma (PC12) Cell Line. *J. Neurosci. 9*, 3946-3954.

2. Evans, D.M., Grandin, A.B., Lokuta, A., and Rogers, T.B. (1990) Cyclic GMP Regulates Catecholamine Secretion from Permeabilized PC12 Cells. *J. Neurochem.*, revised manuscript submitted.

PERSONNEL INVOLVED IN CONTRACT WORK

1. Terry B. Rogers, PhD -- Principal Investigator, 25% time
2. Andrea Grandin, MS -- Research Assistant, 100% time
3. David Evans, PhD -- Research Associate, 100% time
4. Shirley Gaa, BS - Research Assistant, 25% time

REFERENCES

Ahnert-Hilger, G., Bhakdi, S. and Gratzl, M. Minimal requirements for exocytosis. *J.Biol.Chem.* 260:12730-12734, 1985.

Ahnert-Hilger, G. and Gratzl, M. Controlled manipulation of the cell interior by pore forming proteins. *TIPS* 9:195-197, 1988.

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

Ceccarelli, B. and Clementi, F. Neurotoxins: tools in neurobiology. *Adv.Cytopharmacol.* 3:1979.

Collingridge, G.L., Collins, G.G.S., Davies, J., James, T.A., Neal, M.J. and Tongroach, P. Effect of tetanus toxin on transmitter release from substantia nigra and striatum in vitro. *J.Neurochem.* 34:540-547, 1980.

Dreyer, F., Mallart, A. and Brigant, J.L. Botulinum A toxin and tetanus toxin do not affect presynaptic membrane currents in mammalian motor nerve endings. *Brain Res.* 270:373-375, 1983.

Goldberg, N.D. and Haddox, M.K. Cyclic GMP metabolism and involvement in biological regulation. *Ann.Rev.Biochem.* 46:823-896, 1977.

Green, L.A. and Rein, G. Release, storage and uptake of catecholamines by a clonal cell line of

NGF responsive pheochromocytoma cells. *Brain Res.* 129:247-263, 1977.

Habermann, E. and Dreyer, F. Clostridial neurotoxins: handling and action at the cellular and molecular level. *Curr.Topics Microbiol.Immunol.* 129:93-179, 1985.

Kittner, B., Brautigam, M. and Herken, H. PC12 cells: a model system for studing drug effects on dopamine synthesis and release. *Arch.Int.Pharmacodyn.* 286:181-194, 1987.

Mellanby, J. and Green, J. How does tetanus toxin act?. *Neuroscience* 6:281-300, 1981.

Nathanson, J.A. Cyclic nucleotides and nervous system function. *Physiol.Revs.* 57:158-256, 1977.

Osborne, R.H. and Bradford, H.F. Tetanus toxin inhibits amino acid release from nerve endings in vitro. *Nature* 244:157-158, 1973.

Sandberg, K., Berry, C., Eugster, E. and Rogers, T. A role for cGMP during tetanus toxin blockade of acetylcholine releae in the rat pheochromocytoma (PC12) cell line. *J.Neurosci.* 9:3946-3954, 1989.

Sandberg, K., Berry, C. and 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.

Schmitt, A., Dreyer, F. and John, C. At least three sequential steps are involved in the tetanus toxin-induced block of neuromuscular transmission. *Nauyn-Scmeid.Arch.Pharmacol.* 317:326-330,

1981.

Schwab, M.E. and Thoenen, H. Electron microscopic evidence for a transsynaptic migration of tetanus toxin in spinal cord motoneurons: an autoradiographic and morphometric study. *Brain Res.* 105:213-224, 1976.

Simpson, L.L. Molecular pharmacology of botulinum toxin and tetanus toxin. *Ann.Rev.Pharmacol.Toxicol.* 26:427-454, 1986.

Staub, G.C., Walton, K.M., Schnaar, R.L., et al. Characterization of the binding and internalization of tetanus toxin in a neuroblastoma hybrid cell line. *J.Neurosci.* 6:1443-1451, 1986.

Thelestam, M. and Blomqvist, L. Staphylococcal alpha toxin - recent advances. *Toxicon* 26:51-65, 1988.

Walton, K.M., Sandberg, K., Rogers, T.B. and Schnaar, R.L. Complex ganglioside expression and tetanus toxin binding by PC12 pheochromocytoma cells. *J.Biol.Chem.* 263:2055-2063, 1988.

Yount, R.G., Babcock, D., Ballantyne, W. and Ojala, D. AMP-PNP, an ATP analoque containing a P-N-P linkage. *Biochemistry* 10:2484-2489, 1971.