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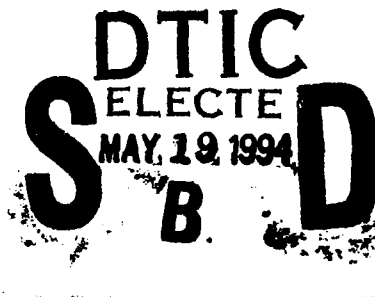
TITLE: MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXIN
TETANUS TOXIN

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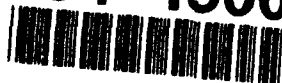


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13. ABSTRACT (Maximum 200 words) The goal of this project was to identify molecular mechanisms of action of Clostridial; neurotoxins so that effective therapeutic agents could be developed. In several experimental series the effects of Clostridial neurotoxins on protein kinase C and cGMP phosphodiesterase activities was examined in cultured neural cells, the PC12 cell line. In precise experiments it was shown that these enzyme systems are not targets for the toxins. Further studies showed that these cells expressed different modes or pathways of neurosecretion of neurotransmitter. Importantly, a series of focused experiments revealed that only one mode was inhibited by Clostridial neurotoxins. In most recent studies, antibodies against different botulinum neurotoxin serotypes were used to identify homologues of botulinum toxins in neural cells. Several specific proteins have indeed been identified in PC12 cells. These results have significance with respect to the development of effective drugs that will specifically block the action of exogenous toxin, while sparing the activities of endogenous homologues.				
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INTRODUCTION

Tetanus toxin, the enterotoxin produced by the bacterium *Clostridium tetani*, is one of the most potent neurotoxins known (minimal lethal dose of toxin in mice, 2 ng/kg body weight). This toxin shares many common properties with botulinum toxin, a group of neurotoxic substances also produced by Clostridial bacteria. These toxins have a common bacterial origin, similar molecular structures, and most likely the same mechanism of toxic action at the subcellular level (for recent reviews see Simpson, 1990; Habermann and Dreyer, 1986). The most striking feature in the action of these toxins, beside their potency, is that their site of action is the presynaptic nerve terminal where they inhibit neurosecretion without causing cell death. Thus studies on the mechanism of action of the Clostridial neurotoxins should not only provide methods to prevent or reverse the toxic sequelae of these lethal bacterial infections but will also provide valuable insight into the molecular events that underlie the neurosecretion process.

It has been recognized for some time that the effects of Clostridial neurotoxins are specific for neural cells, which is due, in part, to the specific recognition of these toxins by such tissues. Evidence gathered by the Principal Investigator, and others, supported the notion that the specific high affinity receptors for tetanus toxin were polysialo-gangliosides (22,30,32). However, there has also been evidence to suggest that protein plays some role in the high affinity binding site (8,21). Thus the precise nature of the tetanus toxin receptor remains to be characterized and more work is needed to assess the physiological importance of gangliosides as binding molecules.

It is now clear that the initial binding step of the Clostridial toxins is nontoxic. In fact tetanus is like several other microbial toxins that participate in a complex multi-step intoxication pathway (18). Various steps in the pathway have been studied in neural tissues. (3,7,28).

Recently, the principal investigator, utilizing an established preparation of tetanus toxin-sensitive PC12 cells, clearly identified a rapid, temperature-dependent internalization step following toxin binding to the surface (24). Further, there was a clear lag phase which followed internalization, revealing that other intracellular events, such as processing of the toxin and expression of some enzymatic activity, are obligatory events in the pathway (24). When this project was initiated there was little information on the toxin processing events, the compartments in which they occur, or on the enzymatic activity or substrates of the Clostridial neurotoxins. However, as described below, new developments over the past two years have provided new insights. As a result, such new information has changed the focus of some of the work in this project.

One important development over the past three years has been the identification of a new neurotransmitter/neuromodulator system, the nitric oxide (NO)/NO synthase pathway (for reviews see (6,15,17)). It now appears that NO is formed in a variety of neurosecretory cells and plays an important role in regulating cell function (6). NO is found to stimulate neural guanylate cyclase leading to the accumulation of cGMP. Since the Principal Investigator has previously shown that tetanus toxin interferes with cGMP production (23), an important question arises from these new developments. Do Clostridial neurotoxins somehow interfere with NO production by altering NO synthase activity in neural cells? Thus a series of experiments were performed with PC12 cells to examine the diversity of expression of NO synthase and its regulation in PC12 cells.

A major development in Clostridial neurotoxin research has been the identification of toxin-associated enzymatic activities which appear to underlie the neurotoxic action of tetanus and botulinum toxins (for recent review see (19)). As molecular cloning experiments revealed the amino acid sequences for Clostridial toxins, it was striking to observe a region of conserved amino acid sequence that was homologous to zinc-containing endoproteases (26). These results were followed by the discovery that tetanus toxin, BoNT/B, BoNT/D and BoNT/F were indeed

highly selective Zn^{2+} -dependent endopeptidases (25,27). These toxins were found to specifically cleave a synaptic vesicle associated protein, synaptobrevin. In contrast, BoNT/A had no proteolytic activity on this protein but it was recently found to be an active protease that specifically cleaved another synaptic protein, SNAP-25 (4). Therefore it appears that the Clostridial neurotoxins have evolved as proteases that are targeted toward specific components on the synaptic vesicle release machinery.

These studies have raised many interesting questions. Are SNAP-25 and synaptobrevin the only targets for these proteases? Will inhibitors of proteolytic activity prove to be effective therapeutic agents for BoNT infections? Finally, the discovery of a family of toxic zinc-dependent proteases raises the possibility that these toxins are homologous to endogenous proteases that play a key role in the regulation of synaptic function. Thus in a final series of new experiments that were initiated toward the end of this funded project, the Principal Investigator has begun to address these important issues.

RESULTS

Experimental Series 1 -- Do Clostridial neurotoxins alter protein kinase C activity in cultured neuronal cells ? In this phase of the research plan we have examined the hypothesis that the action of Clostridial neurotoxins is causally related to a decrease in protein kinase C (PKC) activity. This part of the project has been stimulated by the growing awareness that PKC is involved in secretion in a variety of cells (1,20), and from recent observations that tetanus toxin reduces PKC activity in neural tissues of intoxicated mice (9).

PC12 cells were cultured on multiwell dishes in the presence of NGF for 8-10 days by methods previously described by the PI (24,32). The cells were then be incubated for 16 hr with tetanus toxin in concentrations from 10 nM to 1 μ M. Following these incubations, the cells were homogenized and the cytosol and particulate fractions were separated. PKC was solubilized from the membrane fraction with Nonident NP-40 and then resolved by DEAE ion exchange chromatography. These methods have been previously described (31). The PKC activity was then be measured in extracts of the soluble and particulate fractions of cell homogenates by previously described methods (2,11,13,31). The PKC activity was assessed by the ability of the fractions to stimulate the phosphorylation of histone (Type III) *in vitro* and were calculated as the Ca^{2+} -phospholipid stimulated nmol [^{32}P]PO₄ incorporated /min/mg protein. The results are shown in Table 1.

TABLE 1
Effects of Tetanus Toxin on Protein Kinase C activity in PC12 Cells

Culture Conditions	PKC Activity (nmol 32 P/min/mg protein)	
	Cytosol	Particulate
SPARSE	1.4 ± 0.2	0.3 ± 0.08
+NGF	2.4 ± 0.15	0.8 ± 0.18
+NGF, + Tetanus Tox.	2.1 ± 0.18	0.9 ± 0.20

As shown in Table 1, when PC12 cells were incubated with 100 nM tetanus toxin for 16 hr, there was no effect of the toxin on the steady state levels of PKC in the cultures. There was no change in the distribution of the enzyme between the soluble and particulate fractions, and the specific activities were nearly identical under the two incubation conditions. It is important to note that we have previously demonstrated that such tetanus toxin incubations result in 80% inhibition of neurotransmitter release in PC12 cells. Thus, these results argue against an important role for PKC in the Clostridial toxin intoxication process.

These results are not consistent with previous studies in which intrathecal injection of tetanus toxin into spinal cord of mouse resulted in a significant decrease in the levels of PKC in this multicellular tissue (9). There are a number of potential reasons for the discrepancy, certainly not the least of which is the difference in the systems used. Thus, tetanus toxin infections may lower PKC activity in non-neuronal cells in the preparation. Such events would not be detected in the homogeneous population of neuronal cells in PC12 cultures.

Experimental Series 2 -- Do Clostridial Neurotoxins Modify cyclic nucleotide phosphodiesterase

activities in whole cell homogenates from PC12 cells? Previous work from our laboratory has

suggested that Clostridial neurotoxins act by increasing cGMP phosphodiesterase (PDE) activity in neural cells (23,24). Thus initial studies were performed to see if increases in cGMP PDE activity could be observed in whole cell homogenates of tetanus toxin-treated PC12 cells. PDE activity was determined in the homogenates using a combined two-step procedure as described previously (10). The reaction was initiated by addition of the enzyme preparation to an incubation mixture containing, in a final volume of 300 μ l, 10nM [3 H]cAMP or [3 H]cGMP, 1 μ M cAMP or cGMP, 1mM MgCl, 0.1mM EGTA, 0.2% soybean trypsin inhibitor, and 0.2mg/ml BSA in 50mM BES buffer, pH7.4. The hydrolysis of cAMP or cGMP catalyzed by PDE was usually allowed to proceed for 60 min at 30°C. Following the termination of the hydrolytic reaction 5'-nucleotidase from snake venom was used to convert 5'-nucleotide product derived from cAMP or cGMP hydrolysis to the corresponding nucleoside. The conversion was complete for 10-20 min at 30°C. The final products, [3 H]adenosine or [3 H]guanosine, were separated from the unreacted substrate by ion exchange chromatography using DEAE-Sephadex A25. As shown in Figure 1, significant levels of Mg^{2+} -dependent cGMP PDE activity was observed in whole cell extracts from NGF-treated PC12 cells.

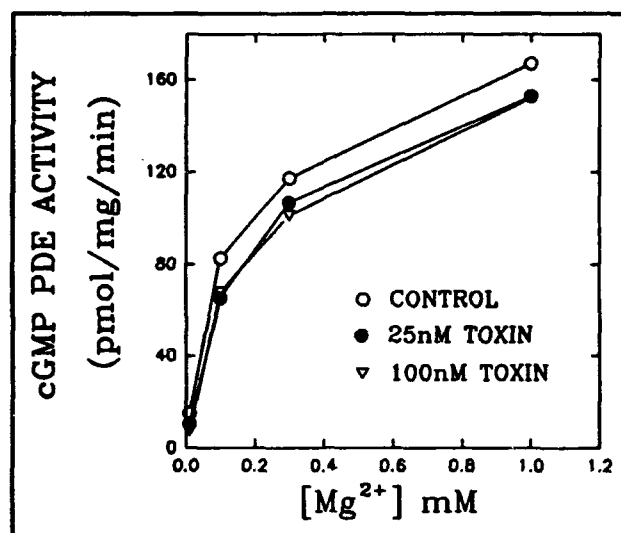
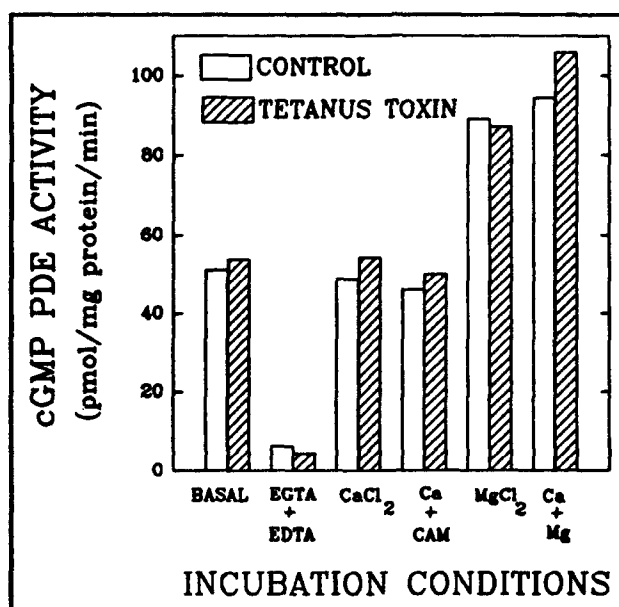


Figure 1. Effects of tetanus toxin on cGMP PDE activity in PC12 cells. NGF-treated cells were exposed to 100 nM tetanus toxin overnight. Whole cell extracts were prepared from control (O), toxin-treated (25 nM (●) or 100 nM (▽)) cells. The cGMP PDE activity as a function of Mg^{2+} concentration was determined.

Figure 1 also shows that tetanus toxin pretreatment has no effect on the resulting PDE activity in whole cell homogenates. However, it is now clear that the PDE activity in cells is a composite of many potential isoforms, each with distinct requirements for ions, such as Ca^{2+} , and other factors, such as calmodulin. Thus, the effects of tetanus toxin on cGMP PDE activities measured under different incubation conditions was assessed to determine if tetanus toxin was altering activity of one specific subtype of PDE.

Figure 2. Effects of tetanus toxin on cGMP PDE activity. PC12 cells were incubated with 100 nM tetanus toxin overnight. Extracts were prepared and were assayed for cGMP PDE activity under the conditions shown. The conditions were; EGTA+EDTA (1 mM); CaCl_2 (50 μM); CaCl_2 (50 μM), Calmodulin (20 nM); MgCl_2 ; MgCl_2 (2mM). The results are reported as the percent activity, compared to extracts from control, non-toxin treated cells.



As shown in Figure 2, there were no detectable effects of tetanus toxin on whole

cell extracts from PC12 cells under a variety of different ionic conditions. Thus, we failed to detect any effects of tetanus toxin on cyclic nucleotide PDE activity in whole cell homogenates. However, it is still possible that Clostridial neurotoxins alter PDE activity but that it could not be observed under the experimental conditions used. For example, the activation of PDE activity could be reversed during the time required for preparation and assay of homogenates. Further, it is clear that there are multiple forms of PDE in any cell, thus tetanus toxin might be altering the activity of one specific isoform. Such activation may go undetected in the whole cell homogenate assay. Accordingly, other experiments were performed in order to explore these hypotheses in

detail.

Experimental Series 3 -- Isolation and characterization of isoforms of cyclic nucleotide phosphodiesterase activity from PC12 cells.

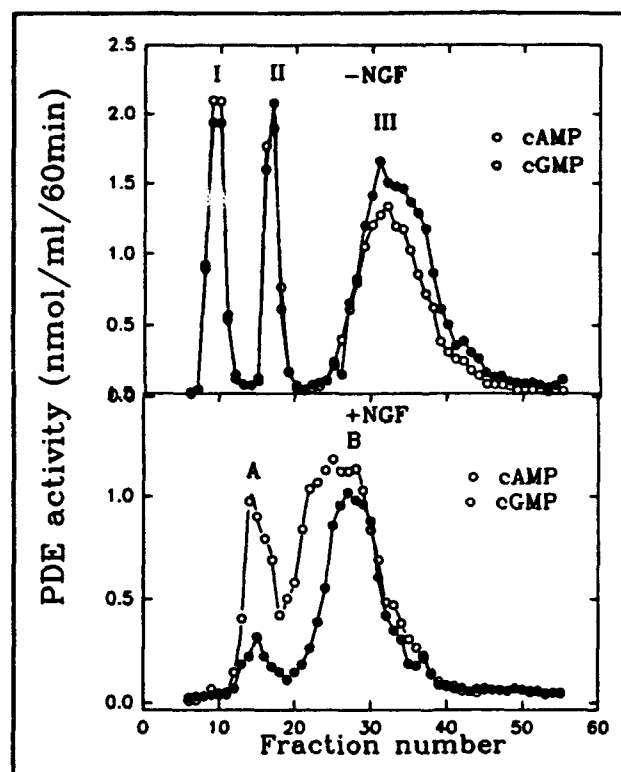
In order to understand cGMP metabolism in neural cells and the effects of Clostridial toxins on this system a detailed understanding of properties of the PDE isoforms present in PC12 cells is essential. Therefore in this experimental series, PDE isoforms were resolved from extracts of PC12 cells using ion exchange chromatography. PC12 cells were removed from culture dishes by incubating cells in a dissociation buffer (Ca^{2+} - and Mg^{2+} -free phosphate buffer consisting of 137mM NaCl, 5.2mM KCl, 1.7mM Na_2HPO_4 , 0.22mM EGTA, pH6.5, OSM340) for 5-10 min. The cells were collected by centrifugation and homogenized in 40mM Tris-HCl, pH8.0, containing 5mM MgCl_2 and 0.25mg/ml BSA. The homogenate was subsequently used as a whole cell homogenate preparation for the PDE assays or was separated into soluble and particulate fractions by centrifugation. The PDE isoforms were resolved in the cytosolic fraction using ion exchange chromatography methods adapted from those previously described by Dicou et al. (1982) and Bode et al. (1988,1989). In brief, the soluble fraction (10-12 mg of protein) was loaded onto a DEAE-cellulose DE52 column (bed volume of 15 ml) which was previously equilibrated with 20mM Tris-HCl, pH7.4. The column was washed with two bed volumes of 20mM Tris-HCl, 2mM MgCl_2 , pH7.4. PDE activity was eluted from the column with a linear gradient of 50-500mM NaCl in the same wash buffer. Fractions (1.5ml) were collected and stored at -80°C . The eluting PDE activity was assayed as described above. Pilot experiments revealed that the PDE activity in these fractions was stable for at least 1 month at -70°C .

Initial experiments in this series focused on resolving major PDE species from undifferentiated and NGF-treated PC12 cell cultures. Cells were grown in flasks and the cytosol prepared as described above. About 10-12 mg of cytosolic protein was applied to the DEAE columns and the PDE activity measured in the eluting fractions. The results from these studies

are shown in Figure 3.

Figure 3. Chromatographic separation of PDE isoforms from PC12 cell extracts. PC12 cells were grown in the presence (Panel B) or absence (Panel A) of NGF. Cytosolic protein (10-12 mg protein) was resolved on DEAE cellulose columns as described above. Each fraction (1.5 ml) was subsequently assayed for cAMP- and cGMP-PDE activity.

Ion exchange chromatographic methods resolved three peaks of PDE activity from the non-NGF-treated cells (Figure 3A). The peaks were designated I, II, and III, in the order of their elution by the NaCl gradient. The hydrolytic activities of these fractions toward 1 μ M cAMP or 1 μ M cGMP as substrates were



determined in all fractions. PDE activity in the three peaks exhibited no preference for either nucleotide. Figure 3B shows the chromatogram of PDE activity obtained from fractionation of cytosol obtained from NGF-treated cells. It was clear that there is a substantial difference in the profile of PDE activity in this differentiated system. The major differences can be summarized as follows. (1) Only two peaks, labelled A and B according to the order of elution from DEAE-cellulose column, rather than three peaks seen in Figure 3A, were resolved from the NGF-treated cells. (2) The positions of two peaks were shifted so that neither peaks could precisely coincide with any peak appearing with the non-differentiated PDE preparations. These chromatographic profiles were reproduced in three different preparations with identical results. (3) The PDE activity in Peak A appears to be very different from Peaks I and II in that the activity in Peak A showed a preference for cAMP as a substrate under the conditions used. Thus it is possible to resolve

the cyclic nucleotide PDE activity of PC12 cells into multiple distinct species by ion exchange chromatography. Taken together, these results support the idea that NGF treatment causes a significant change in the expression of PDE species in PC12 cells.

The chromatographic results indicate that PC12 cells express distinctly different forms of PDE when cultured in the presence of NGF. This hypothesis was explored in more detail by the use of selective phosphodiesterase inhibitors. It is well recognized that different PDE isoforms display different sensitivities to synthetic inhibitors (33). There is considerable controversy over the precise selectivity of synthetic inhibitors of PDE isoforms isolated from diverse sources. Yet, the demonstration of the inhibitory potencies of selective inhibitors of PDE activity has formed part of the criteria by which isoenzymes from different sources are characterized and classified. Accordingly, we examined the susceptibilities of all peaks of PDE activity, isolated as shown in Figure 3, to a variety of isozyme-selective inhibitors. The dose-inhibition curves are displayed in Figures 4 and 5.

Figure 4. Effects of PDE inhibitors on PDE activity from nondifferentiated PC12 cells. The inhibitors used are displayed in the legend. The dose inhibition curves for PDE activity in peaks I, II, and III (Figure 3) are shown in Panels A, B, and C respectively.

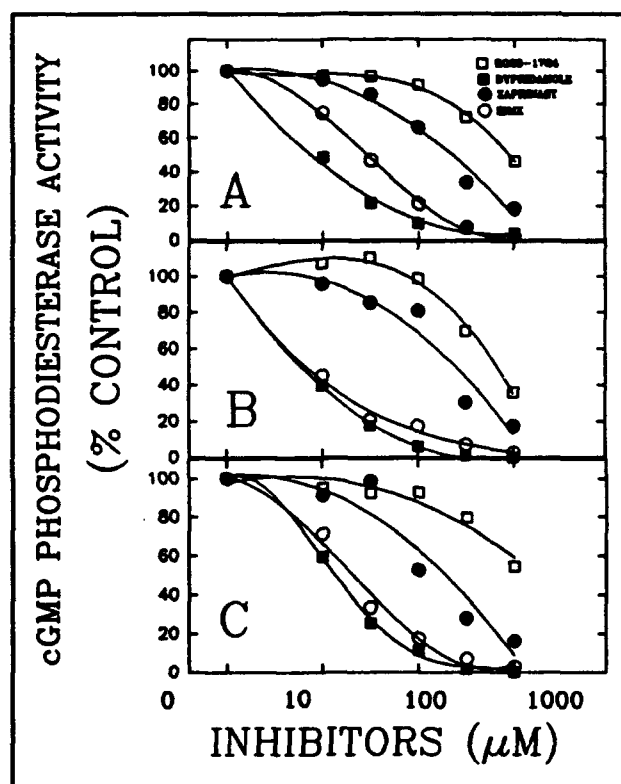
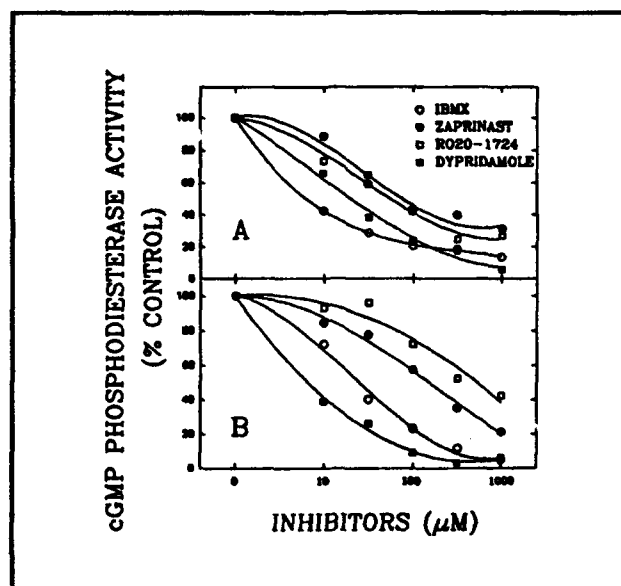


Figure 5. Effects of PDE inhibitors on PDE activity from NGF-differentiated PC12 cells. The inhibitors used are displayed in the legend. The dose inhibition curves for PDE activity in peaks A and B (Figure 3) are shown in Panels A and B, respectively.



PDE activity was measured by assessing the hydrolytic activity with 1 μM [^3H]cGMP as the substrate. The inhibition data from peaks I, II, and III from non-NGF-treated cells are plotted in Figure 4 and the data from peaks A and B

from NGF-treated cells are displayed in Figure 5. In general the PDE activities of all peaks could be inhibited by these PDE inhibitors in a dose-dependent manner. However, as shown in Figures 4 and 5, there were a number of differences in the inhibitory effects of the four selected inhibitors. In the non-NGF-treated cells, the rank order of inhibitory potency was identical in the three peaks; that is, dypridamole > IBMX > zaprinast > Ro20-1724. The first two inhibitors were much more potent, with IC_{50} 's in the range of 5-20 μ M range and zaprinast in the 100-300 μ M range. These data are summarized in Table 1. As shown in Figure 5, the pattern of inhibition in NGF-treated cells was clearly different. The rank order of potency for peaks A and B were IBMX > dypridamole > zaprinast > Ro20-1724 and dypridamole > IBMX > zaprinast >> Ro20-1724, respectively. Zaprinast was considerably more potent in inhibiting the NGF-cell PDE isoforms compared to those from non-differentiated cells. These data are summarized in Table 2 below.

TABLE 2
Sensitivity of PDE fractions to selective inhibitors

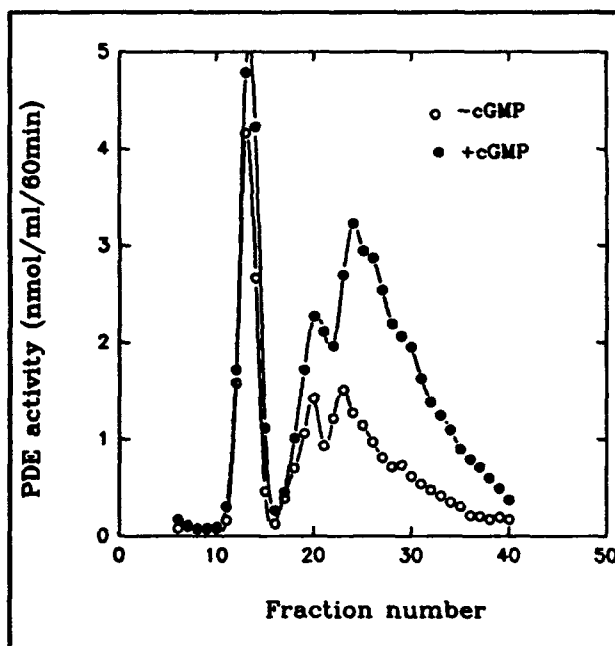
Inhibitor	-NGF Cultures			IC_{50} (μ M) +NGF Cultures	
	I	II	III	A	B
IBMX	20	8	18	7	23
DYPRIDAMOLE	9	5	14	18	7
ZAPRINAST	222	300	100	50	60
Ro20-1724	800	600	1000	60	500

The four PDE inhibitors selected for the present experiments have a range of specificities. IBMX is used widely as a non-selective inhibitor, whereas zaprinast, dypridamole, and Ro20-1724 are classified as selective inhibitors of PDE Type I, Type II, and Type III, respectively. Recent data

supports the view that dypridamole is a PDE Type II selective inhibitor, it is also reported as a potent PDE Type V inhibitor. The fact that dypridamole exerted potent inhibitory effect on all isozymes from both cells with or without NGF treatment suggests that PDE Type II, cGMP-stimulated form of PDE (cGMPs-PDE), and PDE Type V, a cGMP-binding form of PDE (cG-BPDE), are probably the major isoenzymes expressed in PC12 cells.

A recent report has documented the presence of Type II PDE in PC12 cells (34). A common characteristic of this form of PDE is that it has a cGMP-stimulated cAMP PDE activity. A series of experiments were performed in order to examine which of the PDE peaks may be related to the type II isoform. PDE activity was resolved from NGF-treated extracts by ion exchange chromatography, as described above, and the cGMP-stimulated cAMP PDE activity was measured in each fraction. The results are shown in Figure 6.

Figure 6 (file SCAMP) cGMP-stimulated PDE activity in fractions from NGF-treated PC12 cells. Extracts from PC12 cells were resolved on DEAE cellulose columns and the resulting PDE activity (toward 1 mM [3 H]cAMP) was determined in the presence or absence of 10 μ M cGMP as indicated.



Although the PDE specific activity in Peak A

was much larger, the activity was only minimally stimulated by cGMP. The region of Peak B has been resolved into two peaks as shown in Figure 6, with both being stimulated approximately two-fold by cGMP. Double reciprocal plots from these peaks revealed that the main effect of 1 μ M cGMP was to increase the V_{max} of the cAMP PDE activity from 9 to 24 μ M min^{-1} , with little effect on the K_m for cAMP, 14 μ M. These results are consistent with the typical Type II PDE

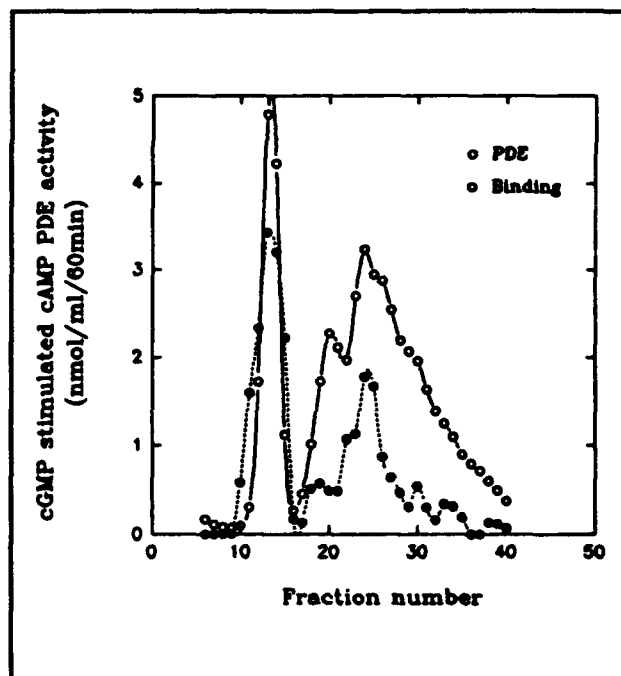
activity regulation by cGMP.

Another property of specific PDE isoforms is their ability to bind cGMP. Thus cGMP binding assays were performed in order to further distinguish and characterize PDE isoforms in PC12 cells. cGMP binding activity in isolated PDE fractions was measured in a total volume of 250 μ l in a buffer of 10 mM Na_2HPO_4 , 1 mM DTT, 1 mM EDTA, 0.5 mg/ml histone IIA and 0.2 μ M [^3H]cGMP, pH7.4 in the presence of 0.1 mM IBMX. The reaction was started by addition of the enzyme preparation and processed for 60 min at 4°C. Assay mixtures were then filtered on Millipore HA filters (pore size, 0.45 μ M). The reaction tubes were rinsed with 4 ml of a 10mM Na_2HPO_4 , pH7.4 and the filters were washed with 20ml of the same buffer. The radioactivities of the filters were counted in 5ml of scintillant. Nonspecific binding was estimated by performing the incubation without tissue or with tissue in the same assay mixtures at time zero. The specific binding activity was defined as the total amount of [^3H]cGMP bound minus the nonspecific binding component. The conditions employed for our binding assay were essentially derived from those described by Hamet et al. (1987) and Francis et al. (1988).

The cGMP binding activity of was determined in all of the PDE fractions and the results compared with cAMP PDE activity profiles. The results are shown in Figure 7.

Figure 7. [3 H]cGMP binding activity of PDE fractions. Extracts from NGF-treated PC12 cells were resolved by ion exchange chromatography. Each fraction was assayed for cGMP-stimulated cAMP PDE activity (○) as well as for [3 H]cGMP binding activity (●) as described above. The binding activity was reported as fmol [3 H]cGMP bound/ml of solution. The results are reported for each fraction from the column.

As shown in Figure 7 there is significant [3 H]cGMP binding activity associated with the two major peaks of PDE activity, Peaks A and B. Peak B, which had a significant level of cGMP-stimulated PDE activity bound cGMP to a level of 200 fmol/ml. This is consistent with



its designation as a Type II isoform. It is also clear that this large peak of activity is likely comprised of several distinct forms since there are areas of PDE activity that do not bind significant cGMP. Peak A, which did not show significant cGMP-stimulated PDE activity, did bind significant levels of cGMP, up to 400 fmol/ml. Thus it is not likely to be a Type II isoform, but may be related to the Type V isoform as recently reported. The sensitivity of this fraction to PDE inhibitors (Figure 3 and Table 2) is consistent with this view (33).

Taken together, the data to date demonstrate that PC12 cells express multiple isoforms of PDE, each with distinct biochemical properties. An important discovery during this work is the observation that the expression of isoforms is highly dependent upon the differentiation state of the cells. Thus, culturing of PC12 cells with NGF results in a pattern of PDE expression that is very different from that seen with non-differentiated cultures. These differences were identified by changes in the mobility of PDE activity in ion exchange chromatography as well as by their differential sensitivities to selective PDE inhibitors. PDE activities were also distinct in their ability

to be stimulated by cGMP and by their cGMP binding properties. Thus by many criteria, it is demonstrated that NGF treatment results in the expression of a different group of PDE isoforms.

From our previous studies we have hypothesized that Clostridial neurotoxins act by altering the activity of a zaprinast-sensitive PDE isoform in neural cells (23). Thus the data reported here are consistent with this with these previous studies in that there is a differential sensitivity of PC12 cells to tetanus toxin as a function of the differentiation state. Secretion in NGF-treated cells is sensitive to intoxication while neurotransmitter release in nondifferentiated cells is not sensitive to toxin treatment. Thus if PDE is a target for the toxins, then differential expression is a possible mechanism that underlies these results. Thus, a major goal for future studies will be to determine if any of the PDE isoforms that we have identified is modified by treatment with botulinum and tetanus toxins.

Experimental Series III -- Are there multiple neurotransmitter release mechanisms in PC12 cells?

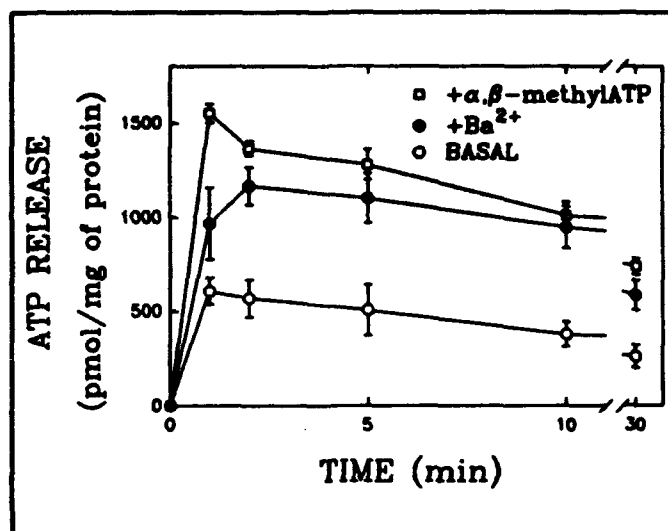
Do Clostridial neurotoxins display different sensitivities toward these different secretory pathways?

In previous studies by the Principal Investigator, PC12 cells have been developed as an effective model to study the action of the Clostridial neurotoxins (23,24). In the course of these studies a variety of results suggested that there were multiple "modes" of neurosecretion in these cultured cells. For example, only stimulus-evoked ACh secretion NGF-differentiated cells was sensitive to tetanus toxin (24). Also, ACh release was much more sensitive to the effects of Clostridial neurotoxins compared to dopamine release in the same cells. These results have raised several important questions. Is it possible to clearly define distinct modes or mechanisms of neurosecretion in PC12 cells? Can such information provide new insight into the mechanism of action of the Clostridial neurotoxins? Accordingly, this experimental series was developed to address these crucial questions.

In order to broadly examine neurosecretion in PC12 cells, we developed an ATP release assay. The rationale for this approach is that all secretory vesicles contain ATP, which is co-released along with neurotransmitters during a secretory event. Thus a sensitive assay for ATP release should serve as a useful index for all modes of neurotransmitter release. In contrast, assays that monitor [^3H]dopamine or [^3H]ACh release may be selective for certain "subpathways" of secretion.

The release of ATP from PC12 cells was developed as described previously (30). PC12 cells were cultured in 35mm multiwell plates and prior to experiments were washed twice at 37° C with washing buffer (Ca^{2+} -free Richelson's buffer consisting of 110 mM NaCl, 5.3 mM KCl, 2mM MgCl_2 , 25mM glucose, 70 mM sucrose and 2mM NaH_2PO_4 , pH 7.5, 340mOsm). The basal release of ATP was measured in cultures incubated in Richelson's buffer containing 2 mM CaCl_2 . In order to measure the evoked-release, the cells were exposed in parallel experiments to depolarizing buffer, that is Richelson's buffer supplemented with one of the following: 30 mM KCl (in this case, the NaCl concentration was reduced to maintain constant osmolarity); or 0.2 mM veratridine; or 2mM BaCl_2 (in this case, the Ba^{2+} replaced Ca^{2+}). In other experiments cells were exposed to Richelson's buffer including a given dose of agonists or antagonists of the purinergic receptors in order to identify the existence of the receptor-mediated secretion system in PC12 cells. The cells were incubated with these buffers for various times as indicated in the text. The supernatants were removed for ATP analysis. The total cellular ATP was assessed in each culture well by extracting the remaining intracellular ATP with 0.00125% SDS/0.5N NaOH. ATP was quantitated by the use of a chemiluminescence assay as previously described by the Principal Investigator (30).

Figure 8. Effects of secretagogues on ATP release from PC12 cells. PC12 cells were cultured in the presence of NGF as described above. Cells were then exposed to either control buffer (○), 2 mM Ba^{2+} (●) or 1 mM α,β methyl ATP (□).



As shown in Figure 8, as expected the secretagogue Ba^{2+} stimulated a rapid release of ATP at a level of 2-fold above basal levels. Similar results were obtained with 25 mM KCl (data not shown). Since

reports have indicated that ATP can stimulate neurotransmitter release in PC12 cells (29), the effects of α,β -methyl-ATP, an ATP receptor agonist, on ATP release was examined. It is important to note that pilot experiments revealed that this ATP analogue was neither detected in or interfered with the luciferin/luciferase chemiluminescent ATP assay. As shown in Figure 8 this ATP analogue also stimulated a rapid, within 2 min, ATP release. The effects of α,β -methylATP were dose-dependent with half maximal values observed at 50 μ M nucleotide. Maximal values were seen at 100 μ M (data not shown).

Taken together these data suggest that neurosecretion can be stimulated either by depolarization, Ba^{2+} or KCl, or by activation of a nucleotide (purinergic) receptor, α,β -methylATP. This hypothesis was explored in more detail by the use of inhibitors of purinergic receptors.

Figure 9. Effects of purinergic receptor antagonists on ATP release from PC12 cells. PC12 cells (undifferentiated) were exposed to purinergic receptor agonists, α,β -methylATP (50 μ M) or β,γ -methylATP (50 μ M), as indicated in the figure. In parallel experiments evoked ATP release was measured in the presence of purinergic receptor antagonists, suramin (10 μ M) or Coomassie blue (10 μ M) as indicated. The results are reported as percent of basal release which was 610 pmole/mg of protein.

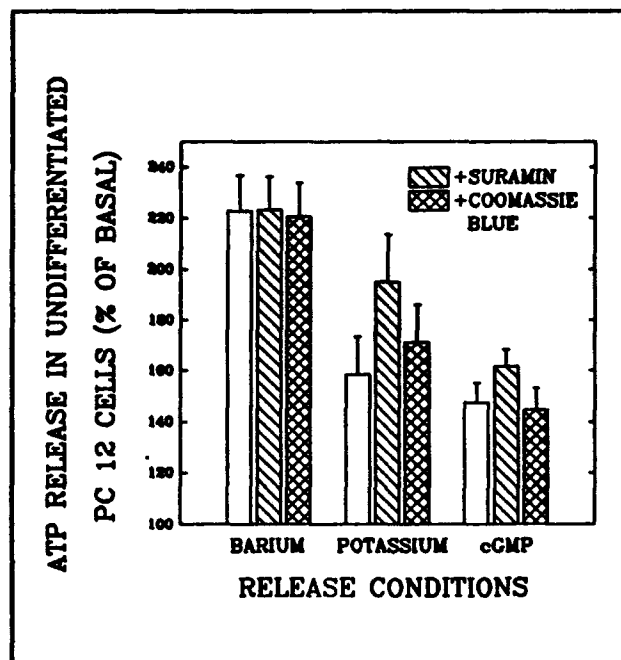
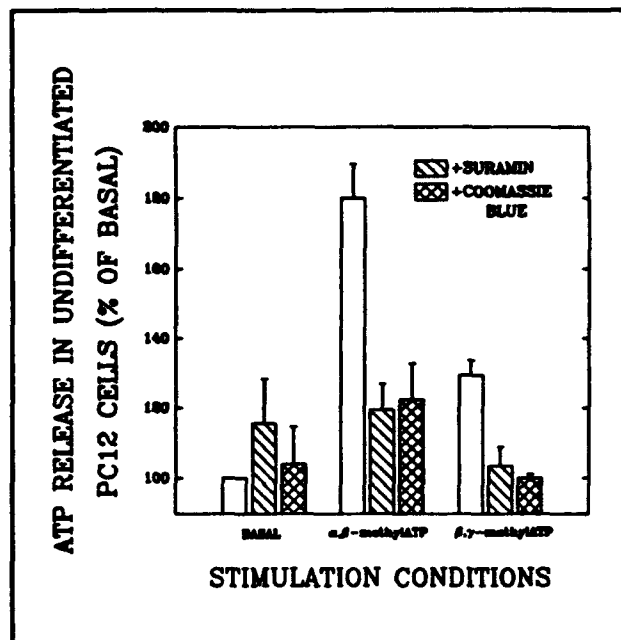
As shown in Figure 9, classic P_2 -purinergic antagonists, suramin and Coomassie blue, were effective in inhibiting nucleotide evoked release of ATP. Control experiments revealed that the effects of these antagonists were restricted to the evoked levels, as basal ATP

release was not blocked by these agents. Further these antagonists had no effect on ATP release evoked by other secretagogues, such as Ba^{2+} , KCl, or cGMP (Figure 10)

Figure 10. Purinergic P_2 receptor antagonists do not block ATP release evoked by Ba^{2+} , KCl (25 mM) or cGMP (1 mM). The methods were identical to those described in Figure 9.

Thus taken together, these results are consistent with the hypothesis that neurosecretion can occur through at least two mechanisms in PC12 cells. One mechanism is a classic voltage dependent release while the other is a receptor-operated mechanism, likely mediated by purinergic (P_2) receptors.

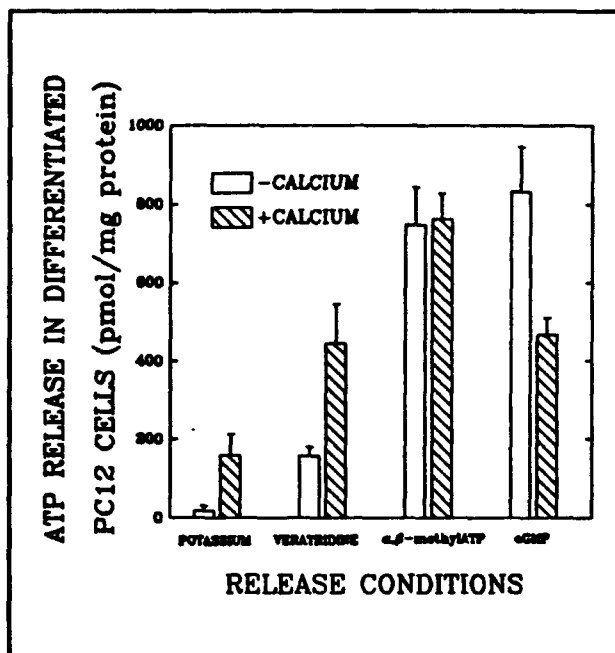
Differences in the release mechanisms



were further articulated in experiments in which the dependency of release on extracellular Ca^{2+} was assessed. Figure 11 shows that different release mechanisms display different requirements for extracellular Ca^{2+} .

Figure 11. Effects of extracellular Ca^{2+} on neurosecretion in PC12 cells. PC12 cells were cultured in the presence of NGF for 8 days. Cells were exposed to various secretagogues and the amount of ATP release was quantitated as describe above. Shown is the level of evoked ATP in the absence (open bars) and in the presence of 2 mM Ca^{2+} (hatched bars).

Thus nucleotide-evoked release of ATP was independent of extracellular Ca^{2+} . In contrast ATP secretion evoked by depolarizing secretagogues, such as KCl and veratridine, was highly dependent on Ca^{2+} , as expected. The same results were seen with nondifferentiated PC12 cells (data not shown).



These data further underscore that there are distinctly different neurosecretion mechanisms in PC12 cells.

Are all of these neurosecretion processes sensitive to Clostridial neurotoxin inhibition? In order to examine this issue PC12 cells, either nondifferentiated or NGF-treated, were incubated overnight with 100 nM tetanus toxin. The following day the cells were stimulated with various agents and the resulting ATP release was quantitated. The results are shown in Figures 12 and 13.

Figure 12. Effects of tetanus toxin on different modes of neurosecretion in PC12 cells. PC12 cell cultures (undifferentiated) were treated overnight with tetanus toxin (100 nM). The cells were then stimulated with various secretagogues and the resulting ATP release was quantitated. The results are presented as percent of basal release (in the absence of stimulator) for toxin treated (hatched bars) and control cells (open bars).

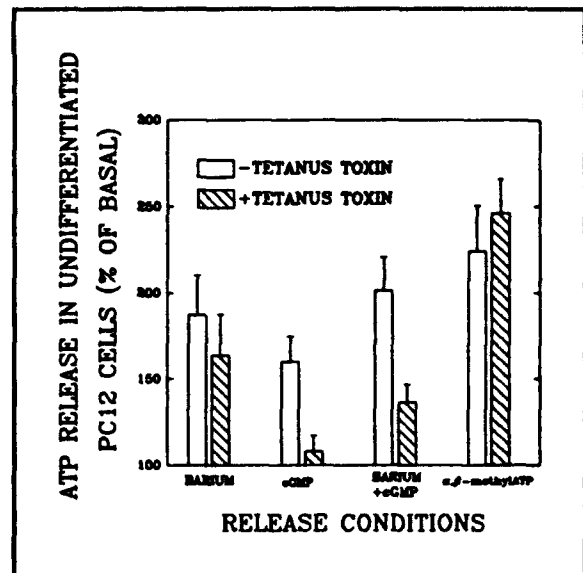
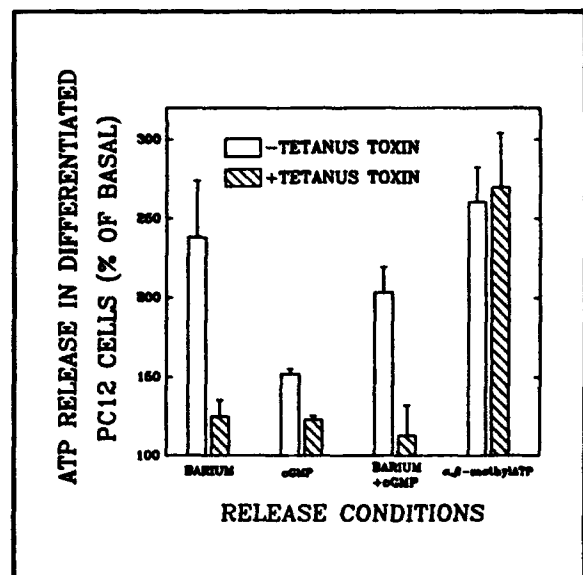


Figure 13. Effects of tetanus toxin on different modes of neurosecretion in NGF-differentiated PC12 cells. The protocol was essentially the same as that described in Figure 12 except that the cells were differentiated in the presence of NGF for eight days.



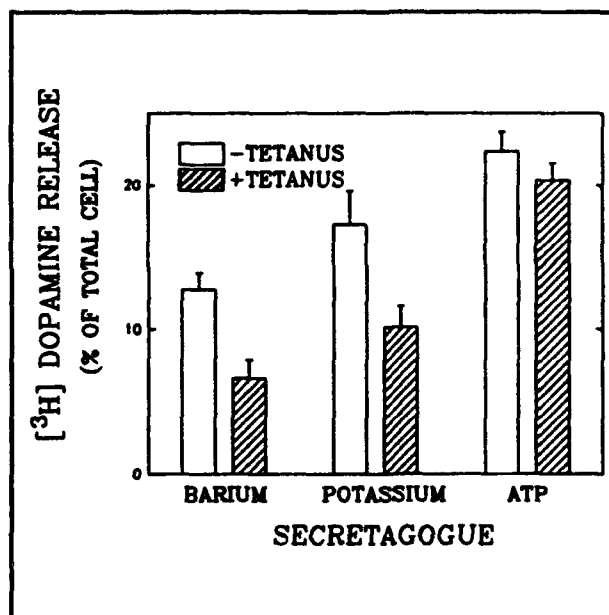
The results in Figure 12 with undifferentiated cells PC12 cells reveal that ATP secretion supported by the P_2 purinergic agonist, α, β -methylATP, is insensitive to tetanus toxin. In contrast cGMP evoked release is inhibited by >85%. In these cultures Ba^{2+} -evoked release was only minimally

inhibited. As shown in Figure 13, in NGF-differentiated cells the P_2 agonist-evoked release was still insensitive to tetanus toxin, while the Ba^{2+} -evoked release had become tetanus toxin sensitive, as expected from our previous work (24). Thus taken together, these results provide the most compelling information on different modes of secretion in PC12 cells. Further, it is clear that

culturing the cells in the presence of growth factors favors the expression of distinct release modes. The results in Figure 14 demonstrate that it is possible to observe different release modes with [^3H]DA as well as with ATP release.

Figure 14. Effects of tetanus toxin on [^3H]dopamine release from NGF-differentiated PC12 cells. NGF-differentiated cultures were incubated overnight with tetanus toxin. Cultures were prelabeled with [^3H]DA and were then stimulated with either Ba^{2+} (2 mM), KCl (20 mM), or ATP (50 μM) for 2 min. The resulting release of neurotransmitter into the medium was quantitated.

Thus Ba^{2+} -evoked dopamine release was partially inhibited by tetanus toxin while the nucleotide-evoked release was insensitive to toxin in the same cells. However it is important to note that toxin was more effective in inhibiting Ba^{2+} -evoked ATP release compared to Ba^{2+} -evoked [^3H]DA release in the same cells.



These studies have raised many intriguing questions. In particular, what molecular differences underlie these different modes of release? What accounts for the differential susceptibilities of these modes toward Clostridial neurotoxins? Is the BoNT-sensitive vesicle protein, synaptobrevin, uniquely involved in a secretion "mode"? The results in this experimental series further underscore the utility of PC12 cultures in Clostridial neurotoxin studies.

Experimental Series IV -- Do Clostridial neurotoxins alter the activity of nitric oxide synthase in neural cells? As described in Introduction, a very exciting development over the past few years is the identification of nitric oxide (NO) as a messenger molecule in the CNS (6,15,17). Thus considerable effort has been devoted to understanding the mechanisms of NO production in neural cells. It is now known that NO is derived from the reaction of the conversion of arginine

to citrulline a reaction catalyzed by the enzyme NO synthase. One important role for NO is the stimulation of soluble guanylate cyclase, resulting in the accumulation of cGMP in cells (15). Interestingly, previous studies by the Principal Investigator have shown that tetanus toxin inhibits the stimulus-evoked cGMP accumulation in PC12 cells (23,24). Is there a relation between toxin infection and NO synthase activity? Do Clostridial neurotoxins inhibit NO synthase (NOS)? In this experimental series we first determined whether or not PC12 cells express NOS. Then the particular isoforms present were identified. In the final phase of this series we examined the effects of tetanus toxin on NOS activity in intact cells.

NOS activity in *in vitro* experiments was assessed by measuring the conversion of [^3H]arginine to [^3H]citrulline in extract of PC12 cells. Briefly, incubations were initiated by the addition of whole cell homogenates from cultured cells to 1 mM NADPH, 0.45 mM Ca^{2+} and [^3H]arginine to a final volume of 125 μl . The reactions were quenched by the addition of 4 ml of 20 mM HEPES, 2 mM EDTA, pH 5.5. The mixtures were applied to 0.5 ml columns of Dowex 50 WX (Na^+ form). The [^3H]citrulline was quantitated as the radioactivity eluting from the columns.

Initial experiments revealed that non differentiated PC12 cells did not express significant NOS activity that could be measured. Accordingly, experiments were focused on NGF-differentiated PC12 cells. As shown in Figure 15, substantial levels of NOS were observed in both the particulate and cytosolic fractions.

Figure 15. Distribution of NO synthase activity in PC12 cells. PC12 cells were grown in the presence of NGF for 8 days. Cells were homogenized and NO synthase activity was assessed using the [3 H]arginine assay described above. Shown are the specific activities in the whole cell homogenate, cytosol, and particulate fraction. These reactions were performed in the absence (open bars) or presence of calmodulin (hatched bars).

The important NOS isoform in brain is the calmodulin-dependent species (5). Therefore it was

unexpected to see that the majority of NOS

activity expressed in response to NGF

treatment was calmodulin independent (see

Fig. 15). These results suggest that the

major species of NOS that is induced in PC12

cells in response to growth factors is

analogous to the Ca^{2+} -independent species

that has been cloned from macrophages (16).

The properties of PC12 NOS were

examined in more detail prior to examining the

effects of tetanus toxin on this enzyme form.

As shown in Fig. 16, the NOS activity in the

cytosolic fraction from PC12 cells was sensitive

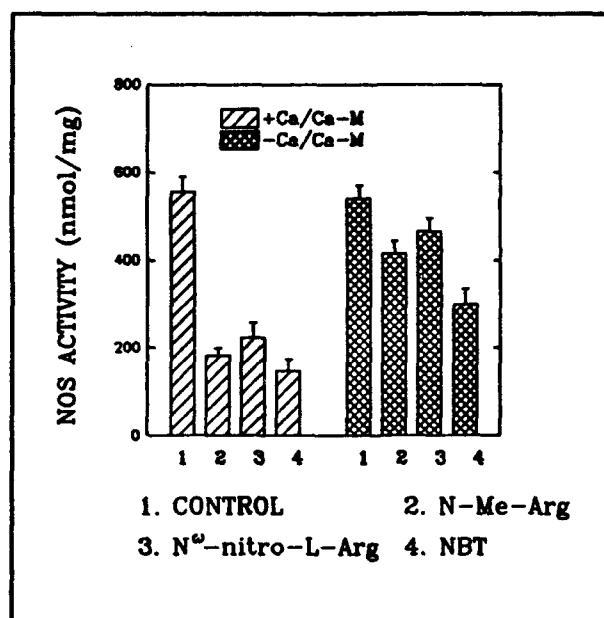
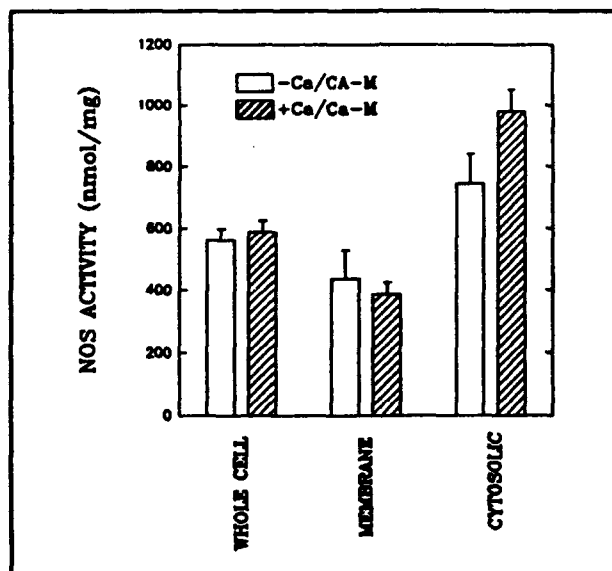
to several classic inhibitors of the enzyme,

although the activity was not activated by the

classic activator of the brain isoform,

calmodulin.

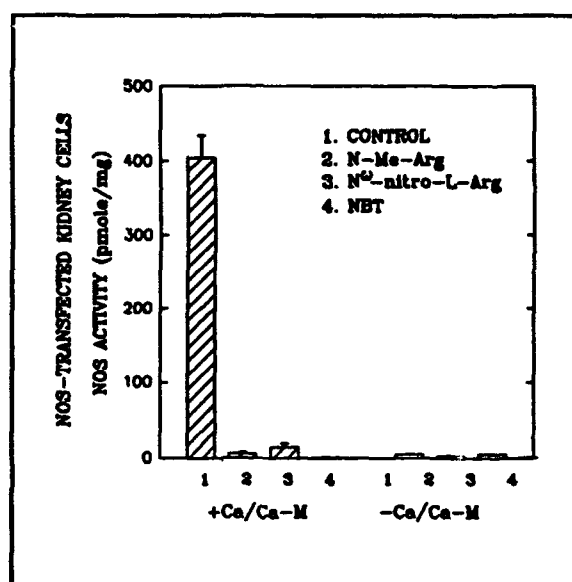
Figure 16. Effects of NO synthase inhibitors on enzyme activity in extracts from PC12 cells. Homogenates were made from PC12 cells and the cytosolic fraction was assayed using the [3 H]arginine method described above. Shown are the effects of inhibitors in the absence (open bars) or the presence of calmodulin (hatched bars).



These data provide more evidence that the predominant isoform of NOS was indeed related to the species identified in macrophages (5). This conclusion was further supported from results in positive control experiments with kidney cells that had been transfected to overexpress the brain calmodulin-dependent isoform. As shown in Figure 17, the enzyme activity in homogenates from such cells is strictly dependent on Ca^{2+} /calmodulin under conditions used above with PC12 cell extracts.

Figure 17. Effects of NO synthase inhibitors and calmodulin on NOS in homogenates from kidney cells overexpressing the brain isoform of NOS. The methods are identical to those in Figure 16.

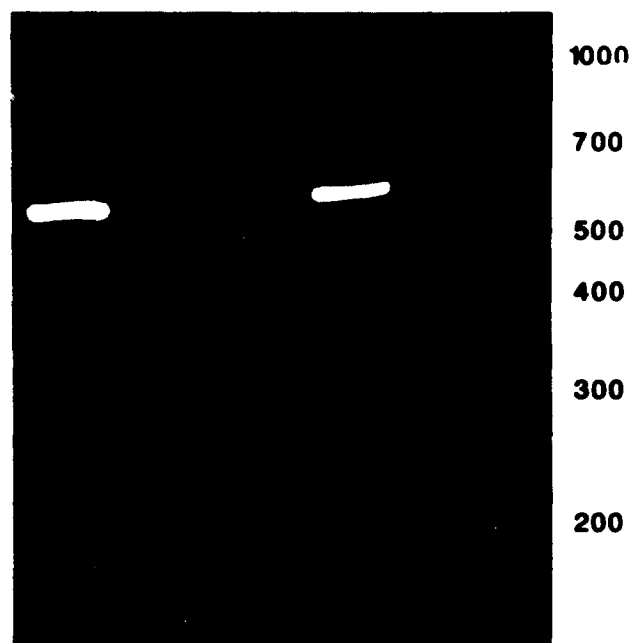
However the results from Figure 15 suggest that there may be multiple NO synthase isoforms that are expressed in PC12 cells. In order to examine the effects of Clostridial neurotoxins on NOS activity it is essential to characterize the diversity of NOS expression in these cultured cells. Is the brain, calmodulin-dependent species present? In order to address this question we assessed mRNA expression using a PCR based method that would specifically amplify mRNA for the brain



NOS isoform (5). Total RNA was prepared from PC12 cell cultures according to methods previously used by the Principal Investigator (12). The mRNA was amplified using a reverse transcriptase-PCR method using primers specific for brain NOS (5). As shown in Figure 18, as a positive control a DNA product of the PCR reaction of the expected size (5) was obtained when brain RNA was used as a template. The identity of this species was further confirmed as brain

NOS transcript by the use of restriction mapping

Figure 18. PCR amplification of brain NOS transcripts in PC12 cell RNA. Total RNA was extracted from cultures of PC12 cells and was converted into the corresponding cDNA using reverse transcriptase as described previously by the Principal Investigator (12). The cDNA was subjected to PCR methods using primers designed to specifically amplify brain NOS transcript (5). The DNA products were resolved on acrylamide gels and stained with ethidium bromide. Shown is the stained gel of reaction products from brain RNA (lane 1), PC12 cell nondifferentiated (lane 2), and NGF-differentiated PC12 cells (lane 3).



BRAIN -NGF +NGF
PC12 CELLS

Thus as shown in Figure 18, NGF induces the expression of the brain isoform of NOS in PC12 cells, a form that is absent in nondifferentiated cultures. Thus PC12 cells

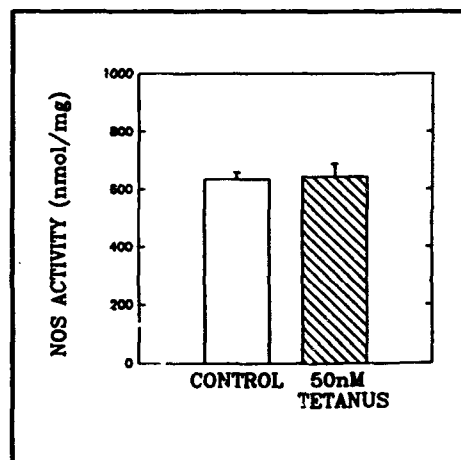
express multiple forms of NOS following treatment with growth factors. One isoform is certainly that found in brain, while another calmodulin-independent isozyme, is similar, if not identical to that cloned from macrophages.

In the next series of experiments the effects of tetanus toxin on NOS activity was examined. NGF-differentiated cultures were incubated for 12 hr with 50 nM tetanus toxin. Homogenates were prepared and the levels of NOS were assessed using the in vitro assay.

Figure 19. Effects of tetanus toxin on NO synthase activity in cultures of NGF-differentiated PC12 cells.

As shown in Figure 19, treatment of PC12 cells under conditions that block neurotransmitter release (23,24), had no effect on NOS activity.

Conclusion -- Through the use of classical biochemical techniques combined molecular biological approaches, we have demonstrated that PC12 cells express multiple forms



of nitric oxide synthase. Further, an important finding was that undifferentiated PC12 cells had not detectable NOS activity or transcript. However NGF-treatment resulted in the expression of several NOS isozymes. Thus an appealing hypothesis might be that it is the expression of NOS that accounts for the NGF-evoked sensitivity of PC12 cells to tetanus toxin. However, an important conclusion from our work is that this enzyme is not a target for Clostridial neurotoxins.

Experimental Series V. Are endogenous homologues of Clostridial neurotoxins present in neural cells? In the past two years new results from several laboratories have provided fresh insight into the molecular mechanism of action of the Clostridial neurotoxins. First, the cloning of several BoNT's cDNA's rapidly led to the realization that the Clostridial toxins are in fact a family of homologous zinc-dependent endoproteases (19,26). A remarkable feature of the proteolytic activity of these toxins resides is their selectivity for target proteins known to be specifically associated with synaptic vesicles. Thus BoNT/B BoNT/E, and tetanus toxin cleave synaptobrevin at the same exact peptide bond, while BoNT/F cleaves the same protein at another distinct site (25,27). In contrast BoNT/A is inactive against synaptobrevin, but specifically hydrolyses a peptide bond in SNAP-25, another synaptic vesicle protein (4). In all of these studies a wide variety of neural proteins were screened for their ability to be cleaved by BoNT's, yet only these two substrates have been identified to date. These important results raise many new questions. Are there other substrates for these neurotoxic proteases? Is it possible to develop mechanism-based selective inhibitors of these toxic enzymes that will allow for therapeutic intervention in Clostridial infections? Have these toxins evolved to mimic the action of endogenous neural proteases? Thus, is there a family of endogenous neural zinc-dependent proteases that is homologous to the family of Clostridial neurotoxins?

It is clear that if selective, nontoxic inhibitors are to be developed it is crucial to have information on endogenous homologues of BoNT's. Since recent results open the possibility that there are analogues that are structurally and functionally similar to BoNT, the Principal Investigator has initiated a project designed to identify such species in cultured cells of neural origin. The approach selected is an immunological one. The rationale was that such analogues should share some structural homology to BoNT's and that this homology should be identified as BoNT-like immunoreactivity when using BoNT polyclonal antisera as probes. Several considerations have provided a focus for this experimental strategy. Since there are shared sequences of homology between the different serotypes of BoNT there may be shared antigenic epitopes as well. Thus some endogenous BoNT homologues might cross react with polyclonal antisera prepared with different BoNT's. Conversely, there may be some epitopes that react uniquely with a particular BoNT antiserum.

Thus with these considerations in mind, we devised a strategy that included the use of horse polyclonal antisera made against BoNT toxoids from different serotypes. These antisera, as well as the others used in this series were kindly provided by Dr. John Middlebrook, USARIID, Fort Detrick, Frederick, MD. As a source of neural tissue we have utilized cultured PC12 cells for several important reasons. This preparation represents a homogenous population of cells with a well differentiated neurotransmitter release system. Second the neurosecretory mechanism in these cells is sensitive to BoNT (14). Finally it is possible to manipulate the neurochemical properties of these cells by the use of growth factors such as NGF. For example the PI has shown that NGF-differentiated cells become more sensitive to Clostridial neurotoxins (24). Further, as described in previous sections of this report, it is possible to regulate a variety of neurochemical systems, including nitric oxide synthase and cAMP phosphodiesterase, by culturing the cells in the presence of NGF. Thus this system provides a powerful opportunity for

control. It may be possible to favorably upregulate the expression of putative endogenous BoNT's by altering the growth conditions of the cultures.

Initial experiments were performed with high serum titer horse antiBoNT's. As shown in Figure 20 an immunoreactive band of MW $\approx$ 40 kDa was labeled with antiBoNT/A that is not seen with preimmune serum or antiserum against BoNT/B.

Figure 20. Identification of BoNT-like immunoreactivity in PC12 cells using Western blot methods. PC12 cells were grown in the presence of NGF for 8 days. Cells were homogenized and total particulate and cytosolic fractions were prepared. Cytosolic fractions (60 μ g of protein) were resolved on SDS gel acrylamide electrophoresis (8% acrylamide) and transferred to nitrocellulose filters. After blocking the filter with 5% nonfat powdered milk filters were incubated with horse polyclonal antisera (1:1000), antiBoNT/A, antiBoNT/B, antiBoNT/E, as indicated, for 2 hr at room temperature. Blots were then incubated with the second antibody, goat antihorse IgG conjugated to peroxidase (1:2,000 dilution) for 2 hr. The blots were then developed using a chemiluminescence protocol (Amersham).



There was a band of MW $\approx$ 90 kDa which was occasionally visible, but was difficult to detect consistently because of the high level of nonspecific labeling in this range. This

background labeling was seen in lane 1, preimmune serum, and to some extent with the 2° antibody control (lane 5). (Note: this problem will be resolved by the use of antisera from other species as described below). Thus under these conditions the most reproducible labeling was seen with the 40 kDa band. The subcellular distribution of this band was examined as shown in Figure 21. A higher percentage acrylamide (12% compared to 8%) was used to better resolve this band from background labeling.

Figure 21. Subcellular distribution of 40 kDa immunoreactive species in PC12 cells. Subcellular fractions from PC12 cells were prepared as described in Figure 20. Proteins in these fractions were resolved by SDS gel electrophoresis as in Fig. 20 except that 12% acrylamide was present in the separating gel. Blots were developed with either preimmune horse serum or horse antiBoNT/A as indicated.

As shown, the majority of the 40 kDa band is found in the cytosol, with minor levels associated with the particulate fraction. Since these membranes have not been extensively washed it is not clear how significant this interaction is at present. These results reveal that it should be possible to further enrich this 40 kDa protein from

cytosolic extracts. Accordingly, the cytosolic fraction was subjected to DEAE ion exchange chromatography and the elution profile of this BoNT/A-like immunoreactivity was followed by Western blot analysis.

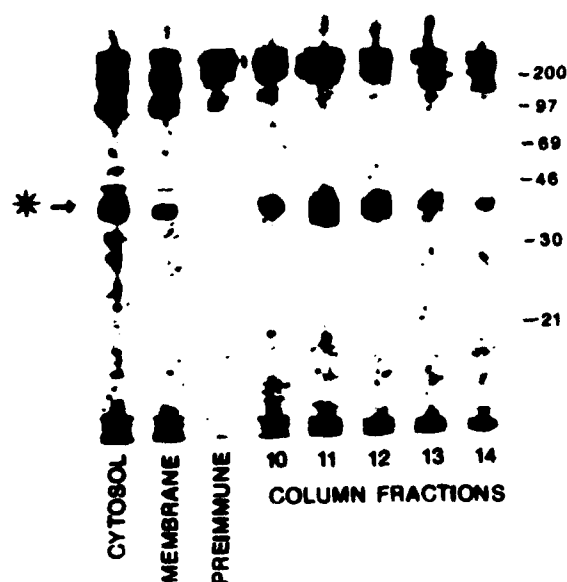
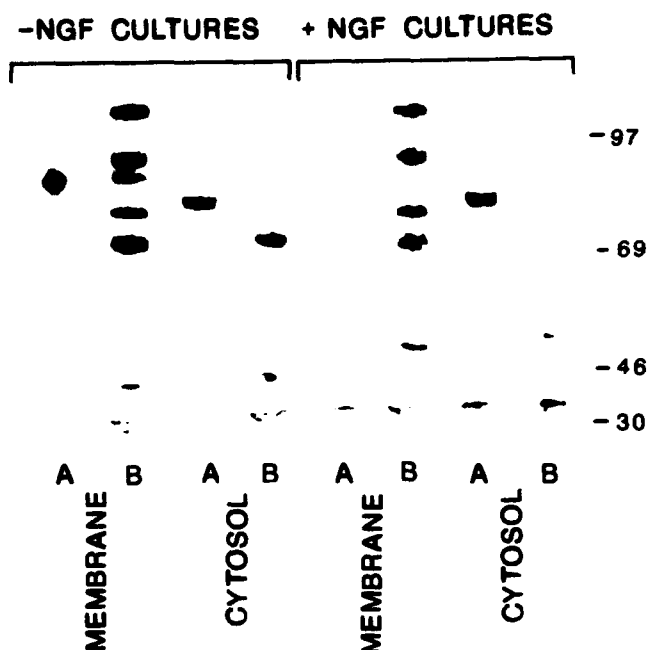


Figure 22. Fractionation of 40 kDa antiBoNT/A-like immunoreactivity by ion exchange chromatography. A cytosolic fraction from NGF-cultured PC12 cells (12 mg of protein) was resolved by ion exchange chromatography by methods similar to those used in Figure 3. The fraction was loaded onto a DEAE cellulose (DE 52) column, bed volume 15 ml, previously equilibrated with 20mM Tris-HCl, pH7.4. The column was washed with two bed volumes of 20mM Tris-HCl, 2mM MgCl₂, pH7.4. Protein was then eluted from the column with a linear gradient of 50-500mM NaCl in the same wash buffer. Fractions (1.5ml) were collected and stored at -80°C. The fractions (20 µl) were resolved by SDS gel electrophoresis and assayed for antiBoNT/A-like activity using Western blot analysis. Shown is the peak of antiBoNT/A-like reactivity that eluted around fraction number 11.



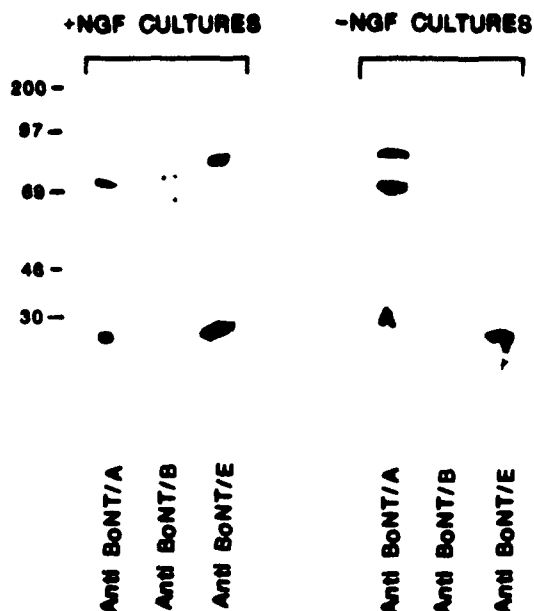
As shown in Figure 22, the 40 kDa protein eluted around a maximum at fraction 11 from the column. These data reveal that it is

possible to enrich the preparation in the 40 kDa protein, a valuable process toward the ultimate purification of this protein.

Although the results with horse antiBoNT/A are very promising, several problems were also observed. Most notably, there was considerable background in the blots in the MW range of 50-150 kDa. Are there other immunoreactive species in this molecular weight range that were not identified with these horse antisera probes? And more important, because of this background labeling it did not appear likely that these antisera would be useful in immunoprecipitation protocols to isolate specific proteins of interest. These concerns have motivated us to examine antibodies raised in other species in order to identify more useful probes. Accordingly, rabbit polyclonal antiBoNT/A and antiBoNT/B were assessed in Western blots. The results are shown in Figure 23.

Figure 23. Use of rabbit polyclonal antisera as probes for BoNT homologues in PC12 cells. Subcellular fractions from PC12 cell cultures (50 µg of protein) were resolved by SDS gel electrophoresis and developed using Western blot methods. The first antibody was either rabbit antiBoNT/A or antiBoNT/B (1:1000 dilution) as indicated. The blots were developed by chemiluminescence methods.

As shown, the rabbit antisera reacted in Western blots with low background. In addition several immunoreactive bands were clearly visible. Rabbit antiBoNT/A recognized a band of MW~80 kDa in cytosolic fractions of undifferentiated or NGF-treated PC12 cells (lanes 3 and 7). The immunoreactive band in



the membrane fraction shown in lane 1 was not a reproducible signal. Five immunoreactive bands (MW range 70-110 kDa) were seen with AntiBoNT/B probe and PC12 membrane fractions, while one band MW~75 kDa was seen in cytosol (lane 4). Clearly, rabbit polyclonal antiBoNT antisera are useful probes for identifying BoNT immunoreactivity in the MW range of 70-150 kDa.

Chicken polyclonal antisera were also examined as probes for BoNT homologues. As shown in Figure 24 these antisera recognized several specific proteins in the PC12 cell extracts.

Figure 24. Use of chicken polyclonal antisera as probes for BoNT homologues in neural cells. Subcellular fractions from PC12 cell cultures (60 μ g of protein) were resolved by SDS gel electrophoresis and developed using Western blot methods. The first antibody was chick antiBoNT/A, antiBoNT/B, or antiBoNT/E (1:1000 dilution) as indicated. The second antibody was goat anti-chick IgG (1:1000). The blots were developed by chemiluminescence. methods.

Chick antiBoNT/A recognized two bands in both membrane fractions from both undifferentiated and NGF-differentiated cells of MW $\approx$ 81 and 65 kDa.

Conclusions -- Polyclonal antibodies prepared in horse, rabbit and chick against botulinum toxoids recognize several specific proteins in extracts from PC12 cells. The major species appear to be localized at MW $\approx$ 80 kDa in both rabbit and chick Ab's. Further, different BoNT serotypes have identified different proteins. These preliminary results reveal that an immunological approach offers a promising method that will contribute toward the ultimate identification and characterization of endogenous homologues of BoNT's in neural cells.

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