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Protein Kinase C and Clostridial Neurotoxins Affect Discrete and Related Steps in the Secretory Pathway

Mary A. Bittner[1,2] and Ronald W. Holz[1]

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SUMMARY

1. The effects on catecholamine secretion of activation of protein kinase C and clostridial neurotoxins were examined in digitonin-permeabilized bovine adrenal chromaffin cells.

2. The enhancement by phorbol esters increased only the initial rate of secretion; later rates were unaffected. This enhancement was present over a wide range of Ca^{2+} concentrations and was elicited at 18 as well as at 27°C.

3. Tetanus toxin inhibited both ATP-dependent and ATP-independent secretion, indicating that the tetanus toxin target is important during the final steps in the pathway.

4. Prior activation of protein kinase C by the phorbol ester 12-*O*-tetradecanoyl phorbol acetate rendered the primed state more sensitive to inhibition by tetanus toxin. The data indicate that a phosphorylated protein kinase C substrate is either identical to or closely associated with the tetanus toxin target protein at the final steps in the pathway.

5. The interaction between the effect of protein kinase activation and that of tetanus toxin suggests that protein kinase C activation does not stimulate a separate pathway of secretion but, rather, modulates the activity of the ongoing pathway.

6. The enhancement of secretion by protein kinase C is caused, at least in

[1] M6322 Medical Science Building I, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0626.
[2] To whom correspondence should be addressed.

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part, by a qualitative change in the characteristics of the primed state. This is indicated by the increased sensitivity of primed secretion to inhibition by tetanus toxin and a threefold increase in sensitivity of primed secretion to Ca^{2+}.

7. Because activation of protein kinase C does not increase the later rates of secretion that are limited by ATP-dependent priming reactions, it is unlikely that enhancement of the maximal rate of secretion by TPA is due to an increased amount of the primed state. Instead, protein kinase C activation may increase the efficacy with which Ca^{2+} stimulates secretion at all Ca^{2+} concentrations.

INTRODUCTION

In recent years permeabilized cell models have been developed and used to probe the molecular requirements for exocytosis. We have used digitonin-permeabilized bovine adrenal chromaffin cells to examine the sequence of events involved in exocytosis. We have found that secretion occurring immediately after the addition of Ca^{2+} differs in some respects from that seen some seconds or minutes later. The rapid initial rate and the slower later rates of secretion from permeabilized cells reflect different rate-limiting steps with distinct biochemical characteristics and temperature sensitivities (Bittner and Holz, 1992a,b). This has allowed us to propose a pathway for Ca^{2+}-dependent exocytosis (see Results). From previous studies (Knight and Baker, 1982; Dunn and Holz, 1983) it was known that ATP is required for optimal secretion in permeabilized chromaffin cells. A key finding was the discovery that ATP acts before Ca^{2+} in the secretory pathway (Holz *et al.*, 1989). A subsequent analysis of secretory rates as early as 5 sec and as late as 15 min after the introduction of Ca^{2+} to digitonin-permeabilized cells led to further insights concerning the sequence of events in the secretory pathway (Bittner and Holz, 1992a,b). The maximal early rates of secretion require 100–300 μM Ca^{2+}. This requirement for high Ca^{2+} had been suspected based upon theoretical calculations of Ca^{2+} concentrations next to the membrane during a secretory stimulus (Simon and Llinas, 1985; Augustine *et al.*, 1991). As secretion proceeds in digitonin-permeabilized cells, different processes become rate limiting. Initially, Ca^{2+} activates a temperature-insensitive step that has already been primed by ATP. Subsequently, secretion becomes limited by ATP-dependent and temperature-sensitive priming events which are stimulated by submicromolar to micromolar Ca^{2+}. The data indicate that there are at least two steps at which Ca^{2+} acts in the secretory pathway (Bittner and Holz, 1992a; Neher and Zucker, 1993).

The role played by protein kinase C in exocytosis has been extensively studied (Pocotte *et al.*, 1985; TerBush and Holz, 1986; TerBush *et al.*, 1988; Knight and Baker, 1983; Brocklehurst and Pollard, 1985; Burgoyne *et al.*, 1988; Holz and Bittner, 1993). In the chromaffin cell, phorbol esters such as 12-*O*-tetradecanoyl phorbol acetate (TPA) cause translocation of protein kinase C to the plasma membrane, phosphorylation of a number of proteins by the activated enzyme, and an increase in secretion of catecholamine (Pocotte *et al.*, 1985; Pocotte and Holz, 1986; TerBush and Holz, 1986; TerBush *et al.*, 1988). Specific

inhibitors of protein kinase C are able to inhibit the enhancement due to TPA while having little or no effect on Ca^{2+}-stimulated secretion in the absence of TPA (TerBush and Holz, 1990). Thus, protein kinase C appears to modulate the secretory process rather than to be an obligatory component of the pathway. The identity of the relevant protein kinase C substrate, its location in the cell, and its function in the secretory pathway remain a subject of much interest. Recently, Martin and his co-workers have identified a cytosolic protein, p145 (Walent *et al.*, 1992) whose phosphorylation may mediate the protein kinase C regulated enhancement of secretion in permeabilized PC12 cells (Nishizaki *et al.*, 1992). One or more of the 14-3-3 proteins have been reported either to activate protein kinase C (Isobe *et al.*, 1992) or to act in concert with activated protein kinase C to enhance secretion from permeabilized chromaffin cells (Morgan and Burgoyne, 1992).

Tetanus and botulinum neurotoxins can inhibit exocytosis in permeabilized chromaffin or PC12 cells (Bittner *et al.*, 1989a,b; Ahnert-Hilger *et al.*, 1989a,b; Mochida *et al.*, 1989; Lazarovici *et al.*, 1989; Ahnert-Hilger *et al.*, 1992) or when injected via patch pipette (Penner *et al.*, 1986). A major finding of these studies was that the ability of clostridial neurotoxins to inhibit secretion is confined to the light-chain region of these proteins. Unlike the situation in *Aplysia* (Maisey *et al.*, 1988; Poulain *et al.*, 1988), the heavy chain is not necessary for inhibition when the toxin is allowed to enter the permeabilized cell. In permeabilized cells, inhibition by the toxin light chains occurs very rapidly, is not reversible over the time course of an experiment (15–20 min), and is not reversed by increasing the Ca^{2+} concentration. In addition, there appear to be functional differences between botulinum type A or type B and tetanus toxin (Bittner *et al.*, 1989a; Marxen *et al.*, 1991; Ahnert-Hilger and Weller, 1993), suggesting that botulinum type A may have a different molecular target.

There is mounting evidence that the clostridial neurotoxins are Zn^{2+}-proteases. The toxins share a common structural motif with a class of metalloendopeptidases, HExxH, which comprises the Zn^{2+} binding site for these enzymes (Jongeneel *et al.*, 1989; Kurazono *et al.*, 1992; Schiavo *et al.*, 1992b). Chelation of the Zn^{2+} blocks poisoning *in vivo* (Bhattacharyya and Sugiyama, 1989) and release of [^{3}H]norepinephrine (NE) from permeabilized chromaffin cells (Sanders and Habermann, 1992). Treatment of brain synaptic vesicles with botulinum type F, botulinum type B, and tetanus neurotoxins *in vitro* results in the cleavage of an integral membrane protein, synaptobrevin-2 (VAMP-2) (Schiavo *et al.*, 1992a, 1993; Link *et al.*, 1992). Botulinum type F also cleaves synaptobrevin-1 (Schiavo *et al.*, 1993). A synaptobrevin homologue, cellubrevin, is also proteolysed by tetanus toxin light chain *in vitro* (McMahon *et al.*, 1993). Botulinum type A has recently been reported to cleave selectively the synaptic protein SNAP-25 (Blasi *et al.*, 1993).

Our work complements the recent progress being made in identifying possible candidates for the target proteins for protein kinase C and tetanus toxin. In this paper we do not identify the individual proteins involved but, rather, analyze how they function and interact in the secretory pathway. Evidence is presented which indicates that a phosphorylated protein kinase C substrate is

identical to or closely associated with the tetanus toxin target during the final steps of the pathway.

MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimal essential medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum. Cells were usually cultured as monolayers in 6.4-mm-diameter collagen-coated plastic culture wells (Coster, Cambridge, MA) at a density of 500,000 cells/cm^2. Sterile Vitrogen 100 bovine dermal collagen solution (32 μg/ml in 0.01 HCl) (Celtrix Laboratories, Palo Alto, CA) was applied to each 6.4-mm-diameter well (5 $\mu g/cm^2$) and allowed to air-dry before the cells were plated. Cells were prepared and plated in the presence of 1.3 μg/ml Fungizone (Squibb, Princeton, NJ). At least 12 hr prior to the start of an experiment, the culture medium was replaced by medium without Fungizone. Experiments were performed 4–8 days after the preparation of the cultures.

Immediately before a secretion experiment, cells were incubated for 3 hr in culture medium containing [^{3}H]norepinephrine and 0.5 m*M* ascorbate. Cultures were rinsed for at least 30 min in fresh culture medium without [^{3}H]norepinephrine or with a physiological salt solution (CaPSS) containing 145 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.6 m*M* glucose, 15 m*M* HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (pH 7.4), 0.5 m*M* ascorbate, and 0.5% bovine serum albumin (BSA). The potassium glutamate solution (KGEP solution) that was used for secretion experiments with digitonin contained 139 m*M* potassium glutamate, 20 m*M* PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.6; 2 m*M* MgATP (unless otherwise specified), 0.5% BSA, and either 5 m*M* [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) without Ca^{2+} or 5 m*M* EGTA and various amounts of $CaCl_2$ to yield buffered Ca^{2+} concentrations of 0.3–30 μM. In some experiments, 5 m*M* nitrilotriacetic acid (NTA) (KGENP solution) was used to extend the range of buffered Ca^{2+} concentrations to 1 m*M*. In the past, we have used the constants from Portzehl *et al.* (1964) and Bjerrum *et al.* (1957) to calculate free Ca^{2+} in EGTA solutions. However, in a recent study (Bittner and Holz, 1992a), Ca^{2+} was measured with a Ca^{2+}-sensitive electrode, and we found that free Ca^{2+} concentrations calculated using the constants of Martell and Smith (1974) adjusted for ionic strength and hydrogen ion activities corresponded more closely to the values determined empirically (±20%). We have therefore converted to using the Martell and Smith constants to calculate Ca^{2+} concentrations. The Ca^{2+} concentrations calculated with the Martell and Smith constants are 75–100% greater than those used in earlier papers from this laboratory.

Botulinum toxin types A and B were diluted from stock solutions containing 20 m*M* Na_2HPO_4 and 50 m*M* NaCl, pH 7.5. Control wells received an equal volume of phosphate buffer with or without dithiothreitol as appropriate. Tetanus toxin was diluted from a stock containing 0.5 *M* NaCl and 0.1 *M* phosphate

buffer, pH 6.5. Control wells received an equal volume of 0.5 *M* NaCl and 0.1 *M* phosphate buffer, pH 6.5.

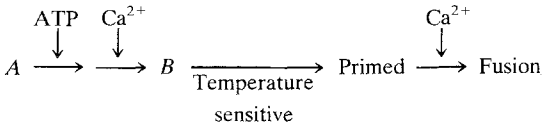
At the end of an experiment, the incubation solution was removed, and the cells were lysed with 1% Triton X-100. The radioactivity released into the incubation solutions and the radioactivity remaining in the cells were determined by liquid scintillation spectrometry. Unless otherwise indicated, experiments were performed at 27°C.

Ca^{2+}-dependent secretion is defined as the difference between secretion in the presence of Ca^{2+}-EGTA buffer and secretion in the absence of Ca^{2+}. Data are expressed as mean ± standard error of the mean unless otherwise indicated. Significance between groups was determined by analysis of variance or by Student's *t* test. Error bars smaller than symbols were omitted from figures.

Digitonin was purchased from Fluka Chemical Corp. (Hauppage, NY), l-[^{3}H]norepinephrine (21.4 Ci/mmol) from New England Nuclear (Boston, MA), reduced streptolysin-O from Wellcome Reagents Ltd. (Beckenham, England), tetanus toxin from Calbiochem, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

The secretory response measured in permeabilized cells is a composite of a number of different processes which become rate-limiting at different times after the addition of Ca^{2+}. Secretion immediately after the addition of Ca^{2+} depends entirely upon the previous action of ATP (while the cells are intact or during the permeabilization step) to prime the cells to secrete. In the absence of MgATP, the absolute amount of the primed state declines rapidly and must be regenerated for secretion to occur. The later phase of secretion is much slower and is limited by the rates of the steps in the priming process, which include an additional Ca^{2+}-dependent step and a temperature-sensitive step, as well as the MgATP-dependent step.



Protein Kinase C Activation Enhances the Initial Rate of Ca^{2+}-Dependent Secretion. We and others have previously demonstrated that activation of protein kinase C by phorbol esters or diacylglycerol enhanced Ca^{2+}-dependent secretion from permeabilized chromaffin cells (Pocotte *et al.*, 1985; TerBush and Holz, 1986; TerBush *et al.*, 1988; Knight and Baker, 1983; Brocklehurst and Pollard, 1985; Burgoyne *et al.*, 1988). We have now demonstrated that the enhancement of the rate of secretion occurred within the first 6 min of a Ca^{2+}

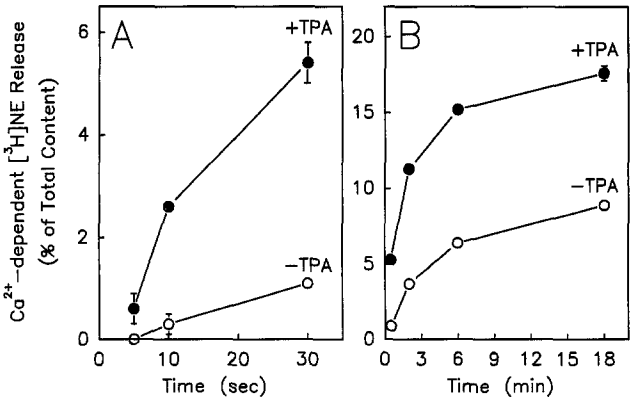


Fig. 1. Time course of secretion after phorbol ester treatment. Cultured chromaffin cells were labeled with [^{3}H] norepinephrine and incubated with or without 100 n*M* TPA in CaPSS for 30 min. Cells were incubated for 4 min in KGEP without Ca^{2+} with 20 μM digitonin, 2 m*M* MgATP, and 1 m*M* $MgCl_2$. Cells were then incubated in KGEP without digitonin, with MgATP and $MgCl_2$, and with or without 1.9 μM Ca^{2+} for the indicated times. Secretion in the absence of Ca^{2+} was 1.0–1.7% (±TPA) at 5 sec and 1.3–2.0% (±TPA) at 30 sec. A and B depict separate experiments. $n = 3$ wells/group.

stimulus and was most apparent during the first 30 sec (Fig. 1). The degree of enhancement was ninefold at 10 sec and fivefold at 30 sec (Fig. 1A). Activation of protein kinase C had no effect on the secretory rate between 6 and 18 min (Fig. 1B).

We have reported that the maximal early rates of secretion require 100–300 μM Ca^{2+} (Bittner and Holz, 1992a). In the experiments shown in Fig. 1, the free Ca^{2+} concentration was 1.9 μM. Thus, one possible mechanism for the enhancement of the initial rate of secretion would be via an increase in sensitivity to Ca^{2+}. In Fig. 2, we examine the enhancement due to TPA 10 sec after adding

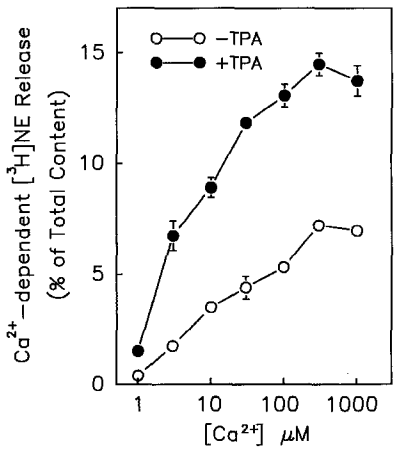


Fig. 2. Enhancement of initial rate of secretion by phorbol ester treatment at various Ca^{2+} concentrations. Cultured chromaffin cells were labeled with [^{3}H]norepinephrine and incubated with or without 100 n*M* TPA in CaPSS for 30 min. Cells were incubated for 4 min in KGENP without Ca^{2+} with streptolysin-O (SLO) (4 U/ml) and 2 m*M* MgATP. Cells were then incubated in KGENP without SLO, with MgATP, and with or without the indicated free Ca^{2+} concentrations for 10 sec. $n = 3$ wells/group.

a broad range of Ca^{2+} concentrations. Preincubation with TPA markedly increased secretion during the first 10 sec at all Ca^{2+} concentrations. Maximal secretion from cells treated with or without TPA was elicited by 300 μM Ca^{2+}. The Ca^{2+} concentration which elicited half-maximal secretion was shifted from 11 μM (−TPA) to 4 μM (+TPA).

The cells are unable to maintain the maximal rates elicited during the first few seconds of a high Ca^{2+} stimulus (Bittner and Holz, 1992a). In the experiment shown in Fig. 2, the rate of secretion stimulated by 3 μM Ca^{2+} during the first 10 sec was 4-fold greater and during the 10- to 30-sec interval (data not shown) was 2.2-fold greater in the TPA-treated cells. For untreated cells, the rate of secretion stimulated by 100 μM Ca^{2+} was 31.9% min^{-1} during the 0- to 10-sec interval and 17.9% min^{-1} during the 10- to 30-sec interval (data not shown). The rates for TPA-treated cells were 78.3 and 28.3% min^{-1}, respectively. As discussed in our earlier paper (Bittner and Holz, 1992a), it is unlikely that leakage of soluble proteins is responsible for the rapid decline in the secretory rates over a 30-sec interval.

Because the effects of protein kinase C activation are greatest at the earliest times during a Ca^{2+} stimulus, it seemed likely that activation of protein kinase C enhances already primed secretion. We had previously demonstrated that secretion that is already primed is little affected by decreasing the temperature from 27 to 18°C, whereas the pathway by which secretion is primed by ATP is almost completely inhibited by the drop in temperature. If protein kinase C activation enhances primed secretion, then its effects should be manifest at 18 as well as at 27°C. Indeed, the effects of TPA at 18°C (Fig. 3) were similar to those at 27°C (Figs. 1 and 2). TPA treatment increased the initial rates of secretion (Fig. 3A) while having little effect on the rates after 30 sec (Fig. 3B). The rate of

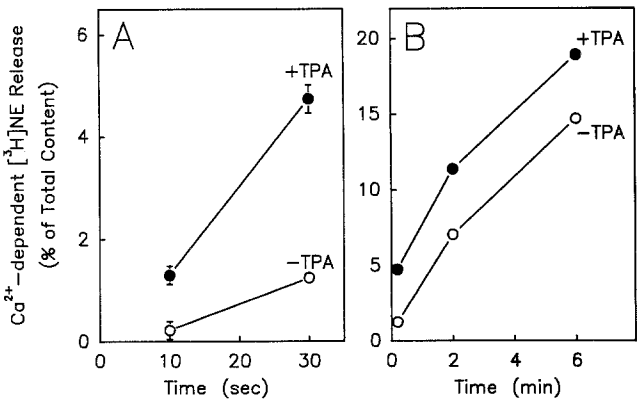


Fig. 3. Effect of phorbol ester treatment at 18°C. Cultured chromaffin cells were labeled with [^{3}H]norepinephrine and incubated with or without 100 nM TPA in CaPSS for 30 min at 18°C. Cells were incubated for 4 min in KGEP without Ca^{2+} with 20 μM digitonin and 2 mM MgATP. Cells were then incubated in KGEP without digitonin, with MgATP, and with or without 30 μM Ca^{2+} for the indicated times at 18°C. $n = 3$ wells/group.

secretion was increased by 6-fold during the first 10 sec and by 3.33-fold during the next 20 sec.

There are two ways in which protein kinase C activation could enhance primed secretion. First, protein kinase C could increase the absolute amount of the primed state, either by stimulating one or more of the steps required for priming or by stabilizing already primed vesicles. Alternatively, phosphorylation by protein kinase C might increase the rate of the final fusion step(s) acting on primed vesicles. An examination of the effects of the clostridial neurotoxins distinguishes between these possibilities and suggests that the latter possibility is correct.

Effects of Clostridial Neurotoxins on ATP-Dependent and ATP-Independent Secretion. We and others had previously reported that tetanus toxin was able to inhibit secretion completely (Bittner and Holz, 1988). This suggests that the target of tetanus toxin is likely to be a protein required for both ATP-dependent and ATP-independent secretion. In contrast, the maximal inhibition produced by botulinum type A neurotoxin did not exceed 65% (Bittner *et al.*, 1989a), suggesting that either the toxin itself or its target may act at an early step in the pathway. Accordingly, we compared the sensitivity to these toxins of the ATP-dependent and -independent components of secretion. Both components of secretion were indeed inhibited by tetanus toxin (Fig. 4A). Inspection of the dose–effect curves reveals that ATP-dependent secretion was more sensitive to inhibition than ATP-independent secretion. ATP-dependent secretion (the difference between secretion in the presence and that in the absence of MgATP)

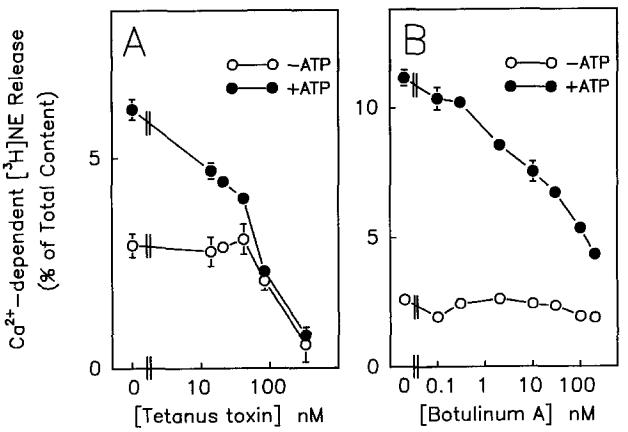


Fig. 4. Inhibition of ATP-dependent and ATP-independent secretion by clostridial neurotoxins. Cultured chromaffin cells were labeled with [^{3}H]norepinephrine. Cells were incubated for 4 min in KGEP without Ca^{2+} with 20 μM digitonin, with or without 2 mM MgATP, and with or without the indicated concentrations of tetanus (A) or botulinum type A (B) toxins. The incubation solution contained 2 mM (B) or 4 mM (A) dithiothreitol and 1 nN free Zn^{2+}. Cells were then incubated in KGEP without digitonin or MgATP but with 0.35 mM $MgCl_2$ and with or without 30 μM Ca^{2+} for 2 min. A and B depict separate experiments. $n = 3$ wells/group.

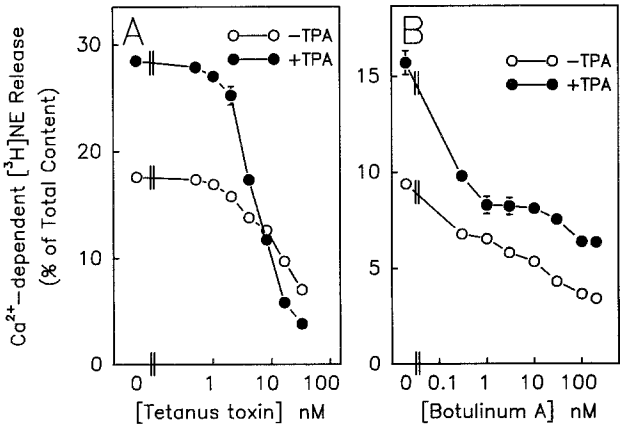


Fig. 5. Inhibition of the phorbol ester-induced enhancement of secretion by clostridial neurotoxins. Cultured chromaffin cells were labeled with [^{3}H]norepinephrine and incubated with or without 100 n*M* TPA in CaPSS for 30 min. Cells were incubated for 4 min in KGEP without Ca^{2+} with 20 μM digitonin and 2 m*M* MgATP and with or without the indicated concentrations of tetanus (A) or botulinum type A (B) toxins. The incubation solution contained 2 m*M* (B) or 4 m*M* (A) dithiothreitol and 1 n*N* free Zn^{2+}. Cells were then incubated in KGEP without digitonin and with 2 m*M* MgATP and with or without 30 μM Ca^{2+} for 4 min. A and B depict separate experiments. $n = 3$ wells/group.

was inhibited by 70% at 40 n*M* tetanus toxin, a concentration which had no effect on ATP-independent secretion.

In contrast, only the ATP-dependent component was substantially inhibited by botulinum type A neurotoxin (Fig. 4B). In other experiments, increasing the toxin concentration gave no further inhibition (data not shown).

Effects of Clostridial Neurotoxins on the TPA-Induced Enhancement of Secretion. We had previously reported that both tetanus toxin (Bittner and Holz, 1988) and botulinum type A neurotoxin (Bittner *et al.,* 1989a) are able to inhibit secretion in cells preincubated with phorbol esters. When this inhibition was examined more closely, we found differences in the effects of the two neurotoxins (Fig. 5). Tetanus toxin was remarkably effective at inhibiting the TPA-induced enhancement of secretion. At higher concentrations, tetanus toxin reduced the total amount of secretion in the presence of TPA to a level significantly *below* that seen in the absence of TPA. In contrast, botulinum type A neurotoxin partially inhibited secretion whether or not TPA was present and was unable to abolish the enhancement due to TPA.

Although phosphorylation by protein kinase C requires ATP, prior treatment of cells with TPA enhances Ca^{2+}-dependent secretion *in the absence of ATP* if Ca^{2+} is added soon after the cells are permeabilized (Fig. 6A; no tetanus toxin). The phosphorylation by protein kinase C probably occurs during the incubation of the intact cells with TPA before permeabilization. Indeed, in contrast to the effectiveness of longer incubations with the pseudosubstrate inhibitor

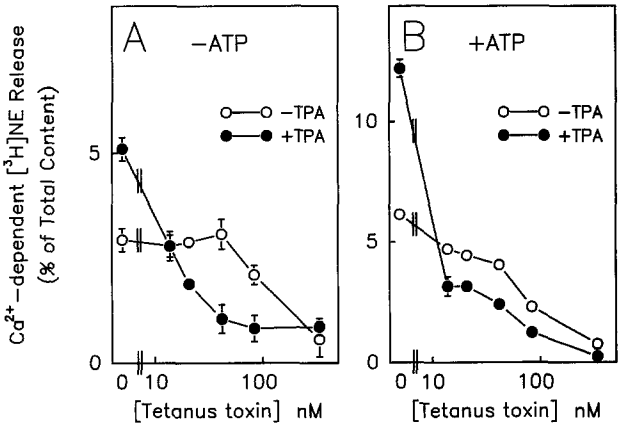


Fig. 6. Inhibition of the TPA-induced enhancement of ATP-dependent and ATP-independent secretion by tetanus toxin. Cultured chromaffin cells were labeled with [^{3}H]norepinephrine and incubated with or without 100 n*M* TPA in CaPSS for 30 min. Cells were incubated for 4 min in KGEP without Ca^{2+} with 20 μM digitonin, with (B) or without (A), 2 m*M* MgATP and with or without the indicated concentrations of tetanus toxin. The incubation solution contained 2 m*M* dithiothreitol and 1 n*N* free Zn^{2+}. Cells were then incubated in KGEP without digitonin or MgATP but with 0.35 m*M* $MgCl_2$ and with or without 30 μM Ca^{2+} for 2 min. $n = 3$ wells/group.

PKC(19-31) (TerBush and Holz, 1990), permeabilization for 4 min in the presence of the inhibitor had little effect on the subsequent enhancement of secretion in cells pretreated with TPA (data not shown). Thus, we were able to investigate the ability of the clostridial neurotoxins to inhibit the TPA-induced enhancement of secretion in the presence and absence of ATP (Fig. 6). Tetanus toxin (13.3 n*M*) completely abolished the TPA-induced increment for ATP-independent secretion (Fig. 6A). Tetanus toxin (40 n*M*) inhibited ATP-independent secretion in the presence of TPA by 80%, while having no effect on secretion in the absence of TPA. Similarly, the TPA-induced increment in the presence of ATP was also abolished by 13.3 n*M* toxin (Fig. 6B). The experiments confirm the observation in Fig. 5A that pretreatment with TPA increases the sensitivity of the secretory pathway to inhibition by tetanus toxin. We have already seen that tetanus toxin is more potent at inhibiting ATP-dependent secretion rather than the already primed ATP-independent secretion (Fig. 4A). It appears that a primary effect of the phosphorylated protein kinase C substrate is to increase the sensitivity of the ATP-independent component to inhibition by tetanus toxin.

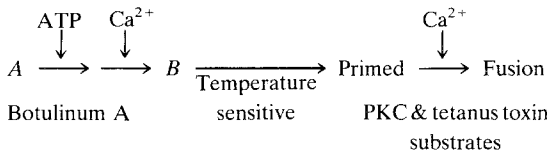
Tetanus toxin does not inhibit activated protein kinase C (Considine *et al.*, 1990). The data in Fig. 6A suggest that tetanus toxin is not acting by *preventing* the phosphorylation of the protein kinase C substrate. Since both the permeabilization with toxin and the stimulation of secretion with Ca^{2+} were done in the absence of ATP, the phosphorylation by protein kinase C probably occurred during the preincubation with phorbol ester, *before the addition of tetanus toxin.*

Furthermore, simply preventing the phosphorylation of the protein kinase C substrate would not result in secretion in the TPA-treated cells being *less* than that in the untreated cells.

DISCUSSION

In two earlier studies (Bittner and Holz, 1992a,b), we reported that the early rates of Ca^{2+}-dependent secretion from permeabilized cells were rapid and did not require the presence of ATP. The initial rate of secretion appeared to represent secretion from a pool of granules that was primed to undergo exocytosis. A distinguishing characteristic of this primed secretion was its ability to be triggered equally well at 18 and 27°C. The later rates of secretion were much slower, required ATP, and were strongly inhibited by reducing the temperature from 27 to 18°C. Evidence was presented that this component of secretion involved several relatively slow steps necessary to prime secretory granules and preceded the final steps triggered by Ca^{2+}. Based on these data, we postulated a model for the sequence of events which lead up to Ca^{2+}-dependent exocytosis. Important elements of this model have been independently confirmed using other techniques (Hay and Martin, 1992; Neher and Zucker, 1993; Heinemann *et al.*, 1993).

In this paper we demonstrate that protein kinase C activation specifically enhances the ability of cells to undergo primed secretion and sensitizes the secretory pathway to the inhibitory effects of tetanus toxin.



Activation of Protein Kinase C Enhances Primed Secretion. The following results indicate that activation of protein kinase C specifically enhances primed secretion. (1) Pretreatment of the cells with TPA enhances only the *initial* rates of secretion; the later rates are unaffected (Figs. 1 and 3). The enhancement is greatest during the first 10 sec following the addition of Ca^{2+}. In our previous work, the rapid initial rate of secretion was associated with an already primed state of the pathway. The increase in initial rate is not simply due to an increased sensitivity to Ca^{2+}, since it was observed at free Ca^{2+} concentrations as high as 1 m*M* (Fig. 2). (2) Enhancement of Ca^{2+}-dependent secretion due to prior activation of protein kinase C occurs in the absence of ATP if Ca^{2+} is added soon after the cells are permeabilized (Fig. 6). Primed secretion is ATP-independent (Holz *et al.*, 1989; Bittner and Holz, 1992a). (3) The enhancement of secretion induced by pretreatment of cells with TPA occurred at 18 as well as at 27°C. Only primed secretion occurs at 18°C (Bittner and Holz, 1992b).

Based upon these data, protein kinase C may be either increasing the amount of the primed state or accelerating the rate at which already primed granules can be released. The effects on secretion of the combination of activated protein kinase C and tetanus toxin (see below) suggest that the latter possibility is correct.

Activation of protein kinase C enhanced initial rates of secretion many fold. Rates approaching 90% min^{-1} were observed during the first 10 sec of the Ca^{2+} stimulus. This corresponds to exocytosis of approximately 450 granules/sec in a cell with 30,000 chromaffin granules (Phillips, 1982).

Tetanus Toxin Inhibits Both ATP-Dependent and -Independent Secretion. We examined the sensitivity of the ATP-dependent and ATP-independent steps in the secretory pathway to various toxins. When permeabilized cells were incubated with tetanus toxin before the addition of Ca^{2+}, we found that ATP-dependent secretion was more susceptible than ATP-independent secretion to inhibition by tetanus toxin (Fig. 4A). What can be inferred from these data? First, ATP-independent secretion *is* inhibited by the toxin, albeit at rather high toxin concentrations. This suggests that the target for tetanus toxin plays a role in the final step(s) of the pathway. If this target is bound to or has been modified by the toxin, then the final steps of primed secretion are prevented. However, the toxin is more potent at blocking ATP-dependent secretion, which suggests that the toxin can *interact* with its target **before** the whole sequence of priming is completed. The difference in sensitivity suggests that early in the priming process, tetanus toxin has free access to its target, but once the secretory machinery becomes fully primed, access to the target is much more restricted. Alternatively, the target may undergo a conformational change by which it loses affinity for the toxin.

In addition to inhibiting primed secretion, cleavage of the tetanus toxin substrate early in the priming process may prevent the ATP-dependent priming reactions from reaching completion.

The effects of botulinum type B are similar to those of tetanus toxin (data not shown). Again, its target is most sensitive to inhibition before ATP acts and then loses its sensitivity as the priming process proceeds.

While the manuscript for this paper was being prepared, a paper comparing the ability of tetanus and botulinum type A neurotoxins to inhibit secretion in SLO-permeabilized chromaffin and PC12 cells in the presence and absence of ATP was published (Ahnert-Hilger and Weller, 1993). The protocols used differed significantly from ours and did not isolate a strictly ATP-independent component, i.e., with both permeabilization and secretion steps performed in the absence of ATP. Nonetheless, their results are compatible with the results presented here. The authors also conclude that botulinum type A probably acts early in the secretory pathway, while tetanus and botulinum type B neurotoxins block a step which could be closely related to the final fusion event.

The Sensitivity to Tetanus Toxin of Secretion Enhanced by Activation of Protein Kinase C Indicates That the Protein Kinase C and Tetanus Toxin Substrates are Important for the Final Steps of Exocytosis. Activation of protein kinase C not only increases the rate of primed secretion but also renders the primed state much more sensitive to inhibition by tetanus toxin (Figs. 5 and 6).

Four nanomolar tetanus toxin inhibited the TPA-induced increment of secretion by 67% while inhibiting secretion in the absence of phorbol ester by only 20%. Remarkably, at higher concentrations of tetanus toxin (>10 n*M*), total secretion in TPA-treated cells was actually *less* than in untreated cells. Tetanus toxin must be able to distinguish between priming which occurs in the presence of activated protein kinase C and priming which occurs when the enzyme is not exogenously activated. Thus, protein kinase C cannot simply increase the amount of the primed state by enhancing its synthesis. Phosphorylation must in some way change the toxin target or increase access to it. This positions a protein kinase C substrate at or near the final fusion event. The toxin target may itself be phosphorylated by protein kinase C or the protein kinase C substrate may be closely associated with it.

The effect of protein kinase C activation to increase greatly the inhibition by tetanus toxin of *ATP-independent secretion* reinforces the conclusion that both protein kinase C and tetanus toxin alter the ability of the primed state to undergo exocytosis. Furthermore, the interaction between the effect of protein kinase activation and that of tetanus toxin indicates that protein kinase C activation does not stimulate a separate pathway of secretion but, rather, modulates the activity of the ongoing pathway.

In summary, the enhancement of secretion by protein kinase C activation is caused, at least in part, by a qualitative change in the characteristics of the primed state. This is indicated by both an increased sensitivity of primed secretion to inhibition by tetanus toxin and a threefold increase in sensitivity of primed secretion to Ca^{2+}. TPA also enhances the maximal initial rates of secretion at high Ca^{2+} concentrations. Because activation of protein kinase C does not increase the later rates of secretion that are limited by ATP-dependent priming reactions, it is unlikely that enhancement of the maximal rate of secretion by TPA is due to an increased *amount* of the primed state. Instead, protein kinase C activation may increase the *efficacy* with which Ca^{2+} stimulates secretion at all Ca^{2+} concentrations.

Botulinum Toxin Type A Specifically Inhibits ATP-Dependent Secretion. Botulinum type A neurotoxin differs from tetanus toxin and botulinum type B neurotoxin in that it has virtually no effect on ATP-independent secretion, only partially blocks ATP-dependent secretion, and only partially blocks the TPA-induced enhancement of secretion. Prior activation of protein kinase C does not significantly increase the sensitivity of the secretory pathway to botulinum-type A neurotoxin. This toxin probably inhibits the secretory pathway at a step prior to completion of priming. The simplest interpretation of the data is that botulinum type A neurotoxin and tetanus toxin act on different substrates. This conclusion is supported by the recent report that botulinum type A neurotoxin selectively cleaves the synaptic protein SNAP-25 (Blasi *et al.*, 1993), as well as by the differences in synaptic transmission at the neuromuscular junction after poisoning with botulinum type A neurotoxin and tetanus toxin (Gansel *et al.*, 1987; Dreyer and Schmitt, 1983; Dreyer *et al.*, 1987) and by other studies (Ahnert-Hilger and Weller, 1993; Schiavo *et al.*, 1992a; Marxen *et al.*, 1991; Simpson, 1988; Ashton and Dolly, 1991).

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