THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 19, Issue of July 5, pp. 13267-13271, 1992
Printed in U.S.A.

Tetanus Toxin Potently Stimulates Tissue Transglutaminase

A POSSIBLE MECHANISM OF NEUROTOXICITY*

(Received for publication, December 10, 1991)

Francesco Facchiano and Alberto Luini

From the Laboratory of Molecular Neurobiology, Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro (Chieti), Italy

The observation that tetanus toxin (TT) contains two sequences that show homology to known transglutaminase (TGase) substrate sites suggested that the toxin and TGase might interact. This prediction was confirmed by two pieces of evidence. First, TT potently stimulated the enzymatic activity of TGase. The effect was maximal at physiological (micromolar) concentrations of the endogenous TGase regulators calcium and GTP. Second, TT and TGase displayed marked variations of their intrinsic fluorescence properties when they were coincubated, indicating the occurrence of binding between them. TT-TGase binding and TGase activation occurred at similar concentrations of TT and are probably causally related. The activation of TGase, an enzyme present in nerve endings that, when activated, can irreversibly cross-link cellular proteins, might mediate the neurotoxic action of TT.

The clostridial tetanus and botulinum neurotoxins block exocytosis of neurotransmitters with great potency and selectivity (1). Understanding their mechanism of action might shed light on the still unresolved issue of the molecular mechanism of exocytosis. Moreover, it might suggest new ways to treat tetanus poisoning, still a significant cause of death in the third world.

Tetanus toxin (TT)[1] is a 150-kDa protein produced as a single chain molecule and then nicked to the biologically active dichain form by bacterial proteases (1). The heavy chain (100 kDa) and the light chain (50 kDa) of the dichain form are held together by both covalent (disulfide) and noncovalent bonds (1). The heavy chain is believed to contain the cellular binding site and to produce channels in the target membrane, through which the light chain would then be internalized. Once internalized, the light chain is believed to block an intracellular step of exocytosis by an as yet unknown mechanism (1-3). Since the secretory block is virtually irreversible and can be induced by very low toxin concentrations in a time- and temperature-dependent fashion, an enzymatic activity that would cause a progressive and irreversible modification of an indispensable component of the secretory machinery has been implicated in the toxin cellular effects (1). To find clues on the toxin's molecular mechanism, we have computer searched its primary structure for function-related domains (4). Two sequences showing homology with known substrate sites of the enzyme transglutaminase (TGase) (5, 6) were found. TGases are a family of calcium-dependent and GTP-modulated enzymes which catalyze the formation of covalent bonds between peptide-bound glutamine residues and primary amino groups, resulting in stable protein-protein cross-linking or in amine incorporation into proteins (7-12). TGases have been implicated in the regulation of exocytotic secretion of hormones and neurotransmitters (13-16) and are relatively abundant in nerve endings (13).

Prompted by the above considerations we have examined whether TT and TGase might interact. We find that TT stimulates TGase activity and that this effect is greatly increased in the presence of physiological concentrations of calcium and GTP. Such stimulation is compatible with TGase playing a role in the secretion-blocking action of TT.

MATERIALS AND METHODS

Trichloroacetic acid, casein *N,N*-dimethylated (DMC), spermidine trihydrochloride, guinea pig liver transglutaminase (EC 2.3.2.13), dithiothreitol (DTT), ATP disodium salt were from Sigma, and all the other nucleotides from Boehringer Manneim [^{3}H]spermidine was from Du Pont-New England Nuclear (specific activity, 21 Ci/mmol); anti-TT antiserum and Clostridium tetani culture broth were a kind gift from Dr. S. Peluso (ISI, S. Antimo, Naples, Italy); GT1b,(*N*-acetylneuraminyl-*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosyl-ceramide was donated by Fidia Research Laboratories (Abano Terme, Italy). Samples of pure TT and TT light chain were a generous gift of Dr. U. Weller (Buchheim Insitut für Pharmakologie der Justus-Liebig-Universität). All other reagents were of the highest available purity.

Protein Purification—Purification of TT from a Clostridium tetani culture broth was achieved through ultrafiltration (cutoff, 50 kDa), followed by a gel filtration step (Superose 12-HR 10/30, Pharmacia) and by an anionic exchange column (Mono Q HR 5/5, Pharmacia), using fast protein liquid chromatography equipment (Pharmacia). Commercial guinea pig liver transglutaminase (Sigma) was found 70-80% pure and was further purified (17) on a MONO Q HR 5/5 column (Pharmacia) with a linear gradient from 0 to 0.8 NaCl; the TGase containing fractions were pooled, dialyzed against 100 mM Tris, pH 7.4, 10 mM NaCl, and stored at −80 °C. Final purity (>95% for both TT and TGase) was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. Purified TT contained approximately 10^7 mouse lethal doses/mg toxin by subcutaneous injection into 20-g female mice. The enzymatic activity of TGase did not decay significantly during purification and was within values reported in the literature (11, 17).

TGase Enzymatic Assay—TGase activity was measured by evaluating the incorporation of ^{3}H-labeled spermidine into DMC at 37 °C

* This research was supported by Fidia S.p.A., by L' Agenzia per la Promozione e lo Sviluppo del Mezzogiorno (PS 3593/IND; 6168, PR2), and by the National Research Council (Convenzione Consiglio Nazionale delle Ricerche-Consorzio Mario Negri Sud). A preliminary account of part of the results (concerning the activity of TT as a TGase substrate) has been presented at the Fourth European Workshop on Bacterial Protein Toxins, July 3-6, 1989, Urbino, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: TT, tetanus toxin; TGase, transglutaminase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DMC, *N,N*-dimethylated casein; GT1b, (*N*-acetylneuraminyl-*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosyl-ceramide; DTT, dithiothreitol; the amino acid residues are presented in the standard IUPAC one-letter code.

in 12 mM Tris-HCl, pH 7.8, 14 mM DTT, 2 mM $MgCl_2$ (80 μl of final volume), according to published procedures (11, 17). The reaction was stopped by addition of 1 ml of cold trichloroacetic acid with 2 mM spermidine. After four washes, pellets were solubilized with 0.5 M NaOH. Other conditions (concentrations of calcium, GTP, DMC, and spermidine, temperatures, incubation times, etc.) were as specified in figure legends. Nonspecific incorporation was determined in each experiment in the absence of added calcium and with 5 mM EGTA; it never exceeded 5% of the maximal incorporation (with 3 mM calcium). All values presented in the figures represent specific incorporation (total minus nonspecific).

Fluorescence Quenching Experiments—TGase and TT were incubated either separately or together in 50 mM Tris, pH 7.6, 10 mM NaCl for 5 min at 25 °C. The emission spectra were then determined by a Perkin Elmer MPF-44B spectrofluorimeter equipped with a thermostatted cell.

RESULTS

Homology between TT and Protein Substrates of TGaseProtein sequences in the PIR 25 Data Bank were searched for homologies with TT by the GENEPRO software (release 4.1, Riverside Scientific). Two TT segments (190–200 and 818–828) were found to be homologous to sequences containing the reactive glutamine present in TGase substrate proteins such as casein, uteroglobin, crystallin, rat seminal vesicle sperm-binding protein (SV IV) (6), and lipocortin (18) (Fig. 1). This suggested that TT and TGase can interact. This prediction was subjected to experimental test.

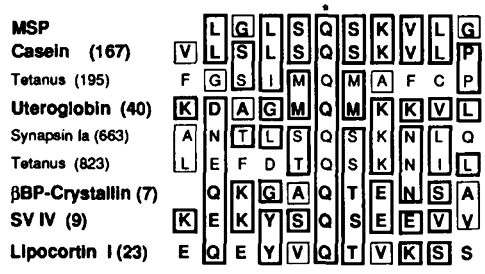

FIG. 1. **Amino acid alignment of homologous domains containing a glutamine residue acting as acyl donor in protein substrates of TGase.** All sequences in *bold type* are known TGase substrate sites. *Numbers* in *brackets* indicate the position of the reactive glutamine (marked by an *asterisk*) within the molecule. *Boxes* include identical or strongly conserved residues (*thick line*) or residues with similar chemical-physical properties (6, 33) (*thin line*) present in the same position in two or more of the sequences. All the sequences were also analyzed with the FAST-CHAMP computer program (34) to predict the local flexibility around the glutamine residues (35). The results suggest that they are all located within flexible regions which are probably sufficiently exposed to the external environment to play a role as substrate sites (data not shown).

Activity of TT as a TGase Substrate—As shown in Fig. 2, purified TT behaved as a weak TGase substrate in the spermidine incorporation assay. The apparent K_m and the V_{max} of the reaction were 0.45 μM and 0.5 nmol/min/mg enzyme (spermidine incorporated into TT, at saturating spermidine), respectively (Fig. 2*B*). The ganglioside GT1b, previously suggested to be a membrane receptor for TT and known to induce marked conformational changes in the toxin molecule (19), inhibited spermidine incorporation in TT by more than 80%. By contrast, the ganglioside did not affect spermidine incorporation into DMC, a known substrate of TGase (5, 6) (Fig. 2*C*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the reaction mixture showed high molecular weight aggregates labeled with [^{3}H]spermidine, likely due to the formation of TGase-induced cross-links among TT molecules (not shown).

Fluorescence Studies of the TT-TGase Interaction—Both TT and TGase possess tryptophan residues (13 in TT and 10 in TGase) and emit light at 339 and 337 nm (emission peaks for TT and TGase, respectively) upon excitation at 295 nm (Fig. 3*A*). Tryptophan fluorescence is generally sensitive to protein conformational changes such as those resulting from protein-protein binding (20, 21). Intrinsic fluorescence was thus used to reveal interactions between TT and TGase. At the low concentrations of TT and TGase (10^{-9} o 10^{-7} M) used for these studies, the fluorescence intensities of both proteins were linearly proportional to solute concentrations (not shown). In TT-TGase mixtures, such proportionality is expected to be maintained in the absence of interactions between the two molecules; deviations from linearity would indicate the occurrence of interactions (21). Indeed, when TT and TGase where mixed, the resulting fluorescence was markedly lower than that predicted from separate measures on the pure proteins (Fig. 3*A*). This quenching effect was complete within seconds. The *inset* in *panel B*, Fig. 3, shows the degree of quenching throughout the emission spectrum obtained by adding different amounts of TGase to a solution containing a fixed concentration of TT. In Fig. 3*B*, the quenching at 335 nm (emission peak) is plotted as a function of the TGase concentration. In principle, since it can be assumed that the

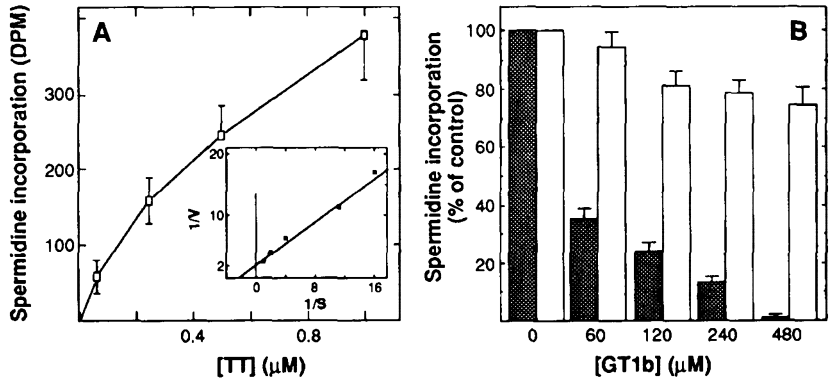

FIG. 2. **TGase-catalyzed spermidine incorporation into TT: effect of gangliosides.** *Panel A*, TT (at the specified concentrations) was first preincubated for 5 min at 25 °C in the fluorescence assay buffer (see "Materials and Methods"); it was then diluted 1:6 into the TGase assay mixture (see "Materials and Methods") containing the following reagents: 100 μM spermidine, 3 mM calcium, 0.08 μM TGase (final concentrations), and 2 μCi/sample of [^{3}H]spermidine. The enzymatic reaction was then allowed to proceed at 37 °C for 10 min. *Inset*, double-reciprocal plot of the same data; *V* is nmol of incorporated spermidine/min/mg of enzyme. *Panel B*, inhibitory effect of the specified concentrations of GT1b on spermidine incorporation into TT (3 μM, *filled bars*) and dimethylcasein (30 μM, *empty bars*). Other conditions were as in *panel A*. All experiments were repeated at least three times with similar results. Data are means ± S.D. of triplicate determinations.

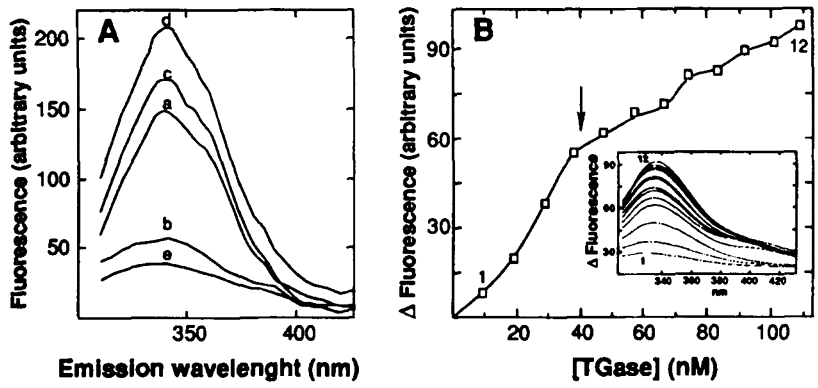

FIG. 3. **Fluorescence studies of the interaction between TT and TGase.** *Panel A*, emission spectra (with excitation at 295 nm) of: 10^{-8} M TT (*a*), 10^{-8} M TGase (*b*), a mixture containing both proteins (*c*), the arithmetic sum of a plus b (*d*), and the arithmetic difference between *d* and *c* (called differential plot) (*e*). All plots are blank-subtracted. The differential plot (*e*) represents the fluorescence quenching caused by TT-TGase interaction. TT and TGase were diluted from concentrated stock solutions prepared in the fluorescence assay buffer (see "Materials and Methods") and then incubated for 5 min at 25 °C before the measurement. Identical results were obtained with shorter (seconds) or longer (2 h at 4 °C) incubation times. *Panel B*, differential fluorescence values at 337 nm as a function of TGase concentration. These values were derived from differential plots (shown in the *inset*) generated by mixing TGase at the specified (final) concentrations with 40 nM TT. For example, the lowest value in the diagram (labeled *1*) derives from the lowest plot of the *inset* (also labeled as *1*), etc. It might be noted that a small blue shift (3 nm) of the differential plots was observed upon increasing the concentration of TGase. The *arrow* indicates the point of equimolarity between TT and TGase. The experiment was repeated three times with similar results.

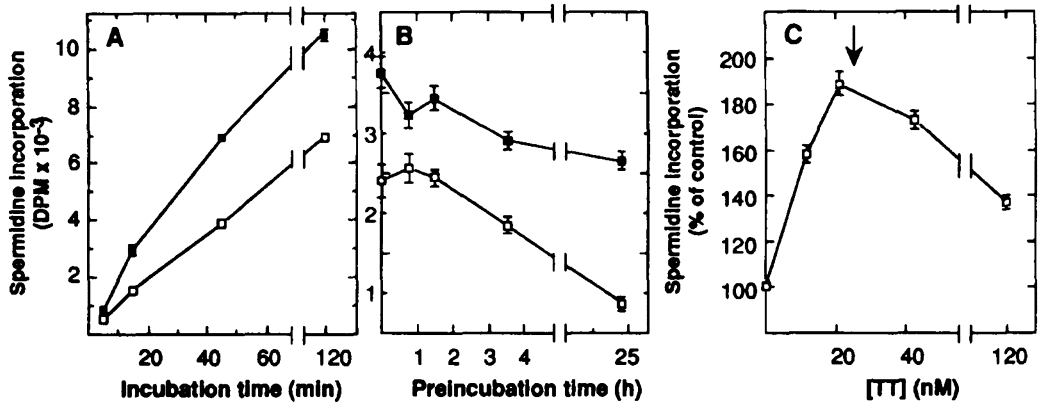

FIG. 4. **Effects of TT on TGase activity.** *Panel A*, time course of TGase-catalyzed spermidine incorporation into DMC in the presence (*filled squares*) or absence (*empty squares*) of TT. TGase (240 nM) was first preincubated (with or without 240 nM TT) for 5 min at 25 °C in the fluorescence assay buffer (see "Materials and Methods"); it was then diluted 1:6 into the TGase assay mixture (see "Materials and Methods") containing the following reagents: 125 μM spermidine, 0.5 μCi/sample [^{3}H]spermidine, 20 μM DMC, and 2 mM calcium (final concentrations). The enzymatic reaction was then allowed to proceed at 37 °C for the specified times. *Panel B*, dependence of TGase activity on the time of preincubation with TT. Preincubations were carried out as in *panel A*, but at 4 °C, for the specified times, with (*filled squares*) or without (*empty squares*) TT. The enzymatic reaction was then started as described in *panel A* and allowed to proceed for 20 min at 37 °C. *Panel C*, stimulation of TGase by TT; concentration-dependence. TGase (150 nM) and TT (at the specified concentrations) were preincubated for 5 min; the reaction was then started by diluting 1:6 in the TGase assay buffer and allowed to proceed for 20 min, as described in *panel A*. The *arrow* indicates the point of TT-TGase equimolarity. The experiment was repeated three times using different concentrations of TGase. The peak stimulation was always obtained near TT-TGase equimolarity and was followed by partial inhibition at higher TT concentrations. All other experiments (*panels A* and *B*) were also repeated at least three times with similar results. Data are means ± S.D. of triplicate determinations.

degree of quenching at each point of the curve is a function of the TT-TGase binding, the resulting curve should be analyzed using binding models. Unfortunately, a straightforward application of such models is precluded by the fact that fluorescent yield and stoichiometry of the TT-TGase complex are unknown and therefore the concentration of the complex cannot be determined from the present data. A simple visual inspection of the binding curve, however, suggests the existence of at least two modes of interaction, one of which would appear to saturate at near equimolar concentrations of TT and TGase (see legend to Fig. 3).

Stimulatory Effect of TT on TGase Activity—The effect of TT on TGase activity was studied using spermidine and DMC as TGase substrates under optimal conditions for TGase activity according to Achyuthan and Greenberg (11) (1 mM calcium, 2 mM $MgCl_2$, DTT, and no GTP, see "Materials and Methods"). Fig. 4*A* shows that the toxin significantly accelerated the reaction rate. The spermidine incorporation into TT itself is negligible under these experimental conditions (compare legends to Figs. 2 and 4). The maximal stimulatory effect of the toxin (ranging between 40 and 110% over controls) was observed at nearly equimolar concentrations of TT and TGase. Higher TT concentrations were partially inhibitory (Fig. 4*C*). A kinetic analysis of the data indicated that the V_{max} of the reaction was not changed by TT, while the K_m for spermidine (at saturating DMC concentrations) was decreased from 12 to 4.5 μM, and the K_m for DMC (at saturating spermidine) from 3.3 to 0.9 μM.

It has been reported that TGase can undergo thermal deactivation (22). Fig. 4*B* shows that TT largely prevented

such deactivation. This protective effect is quite separated from the activatory effect of the toxin, since the latter is evident already after 5 min of coincubation, while deactivation becomes significant only after 2 h.

Specificity of the TT Stimulatory Effect—To control for nonspecific "protein effects" in the activation of TGase by the toxin, we carried out both the preincubation and the TGase assay in the presence of serum proteins and at elevated ionic strength. As shown in Fig. 5*A*, the effect of TT persisted, and was actually increased, under these conditions. Furthermore, boiling or treating TT with specific antibodies largely inhibited the toxin-induced stimulation of TGase. Finally, the light chain of TT was at least as effective as the whole toxin in the TGase assay. The light chain is believed to be responsible for the intracellular blocking effects of the toxin on the secretory machinery (1–3).

Effects of TT on TGase Prepared from Nerve Endings—As neurons are the natural targets of TT, we examined the effects of the toxin on the TGase activity contained in nerve endings. TT (40 nM) had a significant potentiating action (450 ± 70% over controls) on the TGase activity (measured using DMC and spermidine as substrates) of lysed rat brain synaptosomes (13).

Role of Calcium and GTP—All of the above experiments were carried out using standard assay conditions for TGase activity, which include the presence of millimoles of calcium and the absence of nucleotides. As shown in Fig. 6*A*, however, TGase (an intracellular enzyme) can be activated by micromolar concentrations of free calcium. Such activation is potently counteracted by physiological concentrations of GTP (see Fig. 6, *A* and *B*, *inset*), as reported previously. This is believed to result in TGase being silent under resting cellular conditions (10). Other nucleotides tested (ATP and CTP) were inactive. We thus examined whether TT might display more potent effects on TGase activity under conditions where the enzyme is inhibited by GTP. Indeed, the effect of TT was much stronger, with the toxin inducing a 12-fold stimulation of TGase activity (Fig. 6*B*).

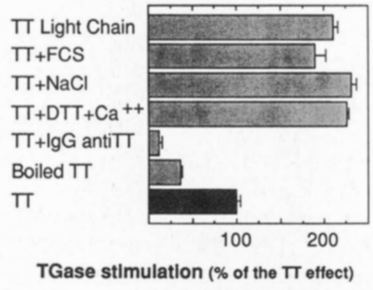

FIG. 5. **Specificity of the TGase activation by TT.** TGase (150 nM) was preincubated as described in the legend to Fig. 4 with the specified agents at the following concentrations: TT, 150 nM; TT light chain, 150 nM; boiled TT, 150 nM; antitoxin antiserum in excess of the neutralizing concentrations; fetal calf serum, 50%; calcium, 5 mM; DTT, 12 mM; NaCl, 70 mM. The reaction was then started as described in the legend to Fig. 4 and allowed to proceed at 37 °C for 20 min. *Bars* represent means ± S.D. of triplicate determinations. Each experiment was repeated at least three times with similar results.

DISCUSSION

The possibility of interactions between TT and TGase was suggested by the observation that peptides 190–200 and 818–828 of TT are homologous to the reactive glutamine-containing sites of a number of known TGase protein substrates (Fig. 1) and subsequently confirmed by two independent pieces of evidence, which indicated that TT binds to TGase and activates its enzymatic activity. That TT and TGase bind to each other and change conformation as a result (21) was indicated by the marked quenching of the intrinsic fluorescence of pure TT and TGase occurring upon coincubating the two proteins. It is unlikely that phenomena other than specific binding could account for the observed quenching since (*a*) the inner filter effect (23) is negligible under our experimental conditions, and (*b*) the quenching reaction reaches completion within a few seconds in the absence of calcium, an essential cofactor of TGase (see "Results"). This rules out enzymatic reactions between TT and TGase. In spite of the lack of a formal analysis of the binding curve, it seems reasonable to suggest that multiple modes of interaction between TT and TGase exist (Fig. 3*B*), one of which would saturate at equimolar concentrations of the two proteins. A second evidence for the TT-TGase interaction was the stimulation of TGase activity by the toxin. This effect also reached saturation at equimolarity between TT and TGase. It seems therefore likely that the two proteins bind with high affinity and binding and activation of the enzyme are related. The stimulation of TGase was a specific effect of the toxin and not a generic "protein effect" since: 1) the activation by TT persists in the presence of a high concentration of serum proteins, 2) it was abolished by antibodies against TT, 3) it is abolished by boiling the toxin. Furthermore, TGase activation was induced by the toxin light chain, a finding which is in line with the accepted notion that the light chain mediates the intracellular intoxication step (2, 3).

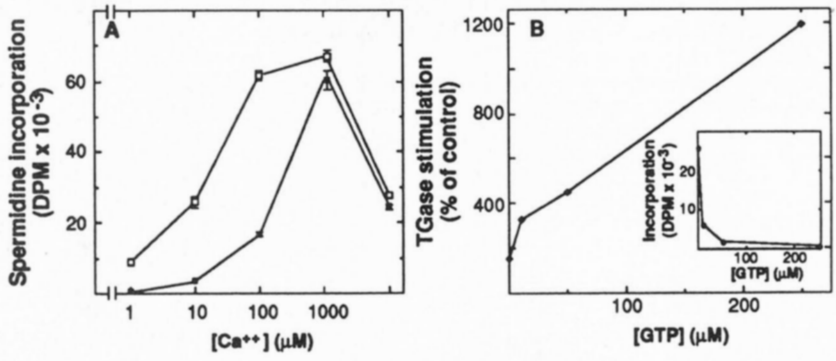

FIG. 6. **Effects of calcium and GTP on the TT-induced TGase activation.** *Panel A*, calcium dependence of TGase activity in the presence (*filled squares*) or in the absence (*empty squares*) of 200 μM GTP. [^{3}H]Spermidine was 5 μCi/sample; other experimental conditions were as in the legend to Fig. 4, *panel A*; the reaction time was 20 min. *Panel B*, stimulatory action of TT on TGase as a function of [GTP], in the presence of 10 μM calcium. *Inset*, effect of GTP on TGase activity. Other experimental conditions were as described in legend of Figs. 4 and 6, *panel A*. Data are means of triplicate determinations. Each experiment was repeated at least three times with similar results.

TT also protected TGase from thermal deactivation (22). It might be argued that this effect might mask, or mimic, the true activatory action of TT on the enzyme. However, the deactivation of TGase and therefore the TT protection become significant only after long (>2 h) incubations of the two proteins (see Fig. 4*B*), while the stimulation of TGase was observed after very short preincubation times with TT.

What might be the role of the TGase stimulation by TT in tetanus toxicity in neurons? In resting cells, probably as a result of the interplay between calcium and GTP, TGase is virtually silent (10). This fits with the observation that although "physiological" free calcium concentrations (between 0.1 and 10 μM) can activate the enzyme, physiological concentrations of GTP (10–200 μM), can nearly completely inhibit such activation (see Fig. 6*A* and Ref. 11). The inhibition by GTP, however, can be overcome by raising free calcium above 10 μM (see Fig. 6*A*). Activation of TGase by elevated cytosolic calcium has been proposed to result in inhibition of release in neurons, and thus to serve as a negative feedback mechanism to prevent excessive release of neurotransmitters induced by prolonged calcium entry (13). A decrease of neurotransmitter release following sustained release stimulation ("fatigue") has indeed been reported (24, 25). We propose that TT might exploit and amplify this safeguard inhibitory mechanism and induce block of exocytosis even at physiological intracellular calcium levels.

The TGase stimulation hypothesis is in line with a number of observations: 1) the action of TT is greatly accelerated by nerve stimulation and the consequent influx of the TGase activator calcium (1); 2) TT exerts its maximal TGase-activating action (up to 1200%) precisely at physiological concentrations of calcium and GTP (see Fig. 6*B*); 3) millimolar ammonium chloride and methylamine, both agents which can inhibit TGase (13), have been reported to inhibit tetanus and botulinum toxin-induced secretory paralysis; their effect has been attributed to the lysosomotropic properties of these compounds (26), but it might in fact be at least in part mediated by TGase inhibition; 4) brain TGase is stimulated by TT, and nerve endings have been reported to have a rather high content of the enzyme (only 6–10-fold lower than that of liver, the richest source in the body) (this paper and Ref. 13); 5) a few molecules of TT are sufficient to intoxicate a nerve terminal (1); given the high apparent affinity of TT for TGase, it is likely that a few molecules/terminal would activate the enzyme; moreover, since the action of TGase is nearly irreversible, even few activated TGase molecules, given sufficient time, would produce effects. Finally, it is of interest to note that, since TGase is a GTP-binding protein, the ability to interact with a protein of this family is a feature TT has in common with other bacterial toxins (27) although most such toxins act by ADP-ribosylating their targets.

Regarding the mechanism by which the activation of TGase would inhibit transmitter release, it is interesting to note that several proteins putatively involved in secretion such as PLA2 (28) and the cytoskeletal proteins actin, lipocortin I, and spectrin are substrates of TGase (18, 29, 30). Possibly related to these observations, Marxen and Bigalke (31) have recently reported that TT and the related neurotoxin botulinum toxin prevent secretagogue-induced actin disassembly in chromaffin cells, an event believed to be necessary for secretion. Furthermore, synapsin I, an abundant vesicular protein crucially involved in exocytosis (32), contains a clear consensus sequence common to TGase substrates.

In summary, our working hypothesis is that the tetanus toxin light chain, once internalized into nerve endings, binds to TGase and allows it to escape the inhibitory control by GTP. The activated TGase then irreversibly modifies a crucial component(s) of the secretory machinery.

Acknowledgments—We are grateful to Drs. Vincenzo Guardabasso and Nicola Rosato for valuable discussions and to Dr. Gianni Colonna for help in fluorescence measurements.

REFERENCES

1. Simpson, L. L. (1986) *Annu. Rev. Pharmacol. Toxicol.* **26,** 427–453
2. Ahnert-Hilger, G., Weller, U., Dauzenroth, M. E., Habermann, E., and Gratzl, M. (1989) *FEBS Lett.* **242,** 245–248
3. Bittner, M. A., Habig, W. H., and Holz, R. W. (1989) *J. Neurochem.* **53,** 966–968
4. Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., Hudel, M., Habermann, E., and Niemann H. (1986) *EMBO J.* **5,** 2495–2502
5. Gorman, J. J., and Folk, J. E. (1981) *J. Biol. Chem.* **256,** 2712–2715
6. Metafora, S., Facchiano, F., Facchiano, A., Esposito, C., Peluso, G., and Porta, R. (1987) *J. Protein Chem.* **6,** 353–359
7. Folk, J. E., and Chung ,S., I. (1973) *Adv. Enzymol.* **38,** 109–191
8. Folk, J. E. (1980) *Annu. Rev. Biochem.* **49,** 517–531
9. Lorand, L., and Conrad, S. M. (1984) *Mol. Cell. Biochem.* **58,** 9–35
10. Bergamini, C. M., Signorini, M., and Poltronieri, L. (1987) *Biochim. Biophys. Acta* **916,** 149–151
11. Achyuthan, K. E., and Greenberg, C. S. (1987) *J. Biol. Chem.* **262,** 1901–1906
12. Lee, K. N., Birckbichler, P. J., and Patterson, M. K. (1989) *Biochem. Biophys. Res. Commun.* **162,** 1370–1375
13. Pastuszko, A., Wilson, D. F., and Erecinska, M. (1986) *J. Neurochem.* **46,** 499–508
14. Bungay, P. J., Potter, J. M., and Griffin, M. (1984) *Biochem. J.* **219,** 819–827
15. Sener, A., Dunlop, M. E., Gomis, R., Mathias, P. C. F., Malaisse-Lagae, F., and Malaisse W. J. (1985) *Endocrinology* **117,** 237–242
16. Sener, A., Gomis, R., Billaudel, B., Malaisse, W. J. (1985) *Biochem. Pharmacol.* **34,** 2495–2499
17. Hand, D., Bungay, P. J., Elliot, B. M., and Griffin, M. (1985) *Biosci. Rep.* **5,** 1079–1086
18. Ando, Y., Imamura, S., Owada, M. K., and Kannagi, R. (1991) *J. Biol. Chem.* **266,** 1101–1108
19. Lazarovici, P., Yanai, P., and Yavin, E. (1987) *J. Biol. Chem.* **262,** 2645–2651
20. Chen, R. F., and Edelhoch, H. (1976) *Biochemical Fluorescence: Concepts*, Vol. 2, pp. 410–439, Marcel Dekker Inc., New York
21. Pesce, A. J., Rosen, C. G., and Pasby, T. L. (1971) *Fluorescence Spectroscopy*, pp. 203–240, Marcel Dekker Inc., New York
22. Nury, S., Meunier, J. C., and Mouranche, A. (1989) *Eur. J. Biochem.* **180,** 161–166
23. Udenfriend, S. (1962) in *Fluorescence Assay in Biology and Medicine* (Kaplan, N. O., and Scheraga, H. A., eds) pp. 108–110, Academic Press, New York
24. Israël, M., Meunier, F. M., Morel, N., and Lesbats B. (1987) *J. Neurochem.* **49,** 975–982
25. Katz, B., and Milady, R. (1969) *J. Physiol.* **203,** 459–487
26. Simpson, L. L. (1983) *J. Pharmacol. Exp. Ther.* **225,** 546–552
27. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* **348,** 125–132
28. Cordella-Miele, E., Miele, L., and Mukherjee, A. B. (1990) *J. Biol. Chem.* **265,** 17180–17188
29. Takashi, R. (1988) *Biochemistry* **27,** 938–943
30. Simon, M., and Green, H. (1984) *Cell,* **36,** 827–834
31. Marxen, P., and Bigalke, H. (1991) *Neuroreport* **2,** 33–36
32. De Camilli, P., and Jahn, R. (1990) *Annu. Rev. Physiol.* **52,** 625–645
33. Dayhoff M. O. and Hunt L. T. (1972) in *Atlas of Protein Sequence and Structure 5* (Dayhoff M. O., ed), National Biomedical Research Foundation, Washington, D. C.
34. Facchiano, A., Facchiano, A., Facchiano, F., Ragone, R., and Colonna, G. (1989) *Comput. Appl. Biosci.* **5,** 299–303
35. Ragone, R., Facchiano, F., Facchiano, A., Facchiano A. M., and Colonna, G. (1989) *Protein Eng.* **2,** 497–504