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ORIGINAL ARTICLE

Transglutaminase participates in the blockade of neurotransmitter release by tetanus toxin: evidence for a novel biological function

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Abstract Inhibition of neuroexocytosis by tetanus neurotoxin (TeNT) involves VAMP-2/synaptobrevin-2 cleavage. However, deletion of the TeNT activity does not completely abolish its inhibitory action. TeNT is a potent activator of the cross-linking enzyme transglutaminase 2 (TGase 2) in vitro. The role of the latter mechanism in TeNT poisoning was investigated in isolated nerve terminals and intact neurons. TeNT-induced inhibition of glutamate release from rat cortical synaptosomes was associated with a simultaneous activation of neuronal transglutaminase (TGase) activity. The TeNT-induced blockade of neuroexocytosis was strongly attenuated by pretreatment of either live *Aplysia* neurons or isolated nerve terminals with specific TGase inhibitors or neutralizing antibodies. The same treatments completely abolished the residual blockade of neuroexocytosis of a non-proteolytic mutant of TeNT light chain. Electrophysiological studies indicated that TGase activation occurs at an early step of TeNT poisoning and contributes to the inhibition of transmitter release. Bioinformatics and biochemical analyses identified synapsin I and SNAP-25 as potential presynaptic TGase substrates in isolated nerve terminals, which are potentially involved in the inhibitory action of TeNT. The results suggest that neuronal TGase activity plays an important role in the regulation of neuroexocytosis and is one of the intracellular targets of TeNT in neurons.

Keywords Neuroexocytosis · Transglutaminase 2 · Tetanus toxin · VAMP/synaptobrevin-2 · Synapsin I · SNAP-25 · Melanoma · Proteomics

Abbreviations

BPA	5-Biotinamidopentylamine
DTT	Dithiotreitol
G_h	GTP-binding protein type *h*
HBS	HEPES buffered saline
MDC	Monodansylcadaverine
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecylsulfate

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SV	Synaptic vesicle
SNAP-25	Synaptosomal-associated protein M_r 25
SNARE	SNAP (soluble NSF attachment protein) receptors
SRS	Sequence retrieval system
SynI	Synapsin I
Syn-peptide	Rat synapsin $Ia^{658–668}$
TeNT	Tetanus neurotoxin
TeNT-LC	TeNT light chain
TGase	Transglutaminase
TRANSIT	TRANSglutaminase SITes
VAMP	Vesicle-associated membrane protein/synaptobrevin

Introduction

Tetanus neurotoxin (TeNT) inhibits neurotransmitter release with great potency and selectivity (Humeau et al. 2000; Rossetto et al. 2006). The secreted toxin consists of heavy and light chains linked by a disulfide bridge. TeNT light chain (TeNT-LC) is a Zn^{2+}-dependent metalloendoprotease that specifically binds to and cleaves at the Gln-Phe bond vesicle-associated membrane protein-2 (VAMP-2)/synaptobrevin-2 (Schiavo et al. 1992; Breidenbach and Brunger 2005) which participates in the priming/fusion of synaptic vesicles (SVs) with the presynaptic membrane (Südhof and Rothman 2009).

Although a direct cause-effect relationship exists between the cleavage of VAMP-2 and the blockade of neuroexocytosis by TeNT (Humeau et al. 2000; Rossetto et al. 2006), additional experimental evidence indicates that the TeNT-induced blockade of neurotransmitter release may also involve an alternative intracellular mechanism. Indeed, when mutated in the catalytic site at positions crucial for either Zn^{2+} binding (His^{233} and His^{237}) or cleavage of the Gln-Phe bond in VAMP-2 (Glu^{234}), TeNT-LC becomes inactive towards VAMP-2, but still able to partially inhibit evoked neurotransmission (Yamasaki et al. 1994; Li et al. 1994; Ashton et al. 1995). The residual inhibition of exocytosis can also explain why endopeptidase blockers, which abolish VAMP-2 cleavage in vitro, counteract only partially the inhibitory action of TeNT on neurotransmitter release (DePaiva et al. 1993; Ashton et al. 1995).

The observation that TeNT binds with high affinity and strongly activates GTP-binding protein type *h*/tissue transglutaminase type II (TGase 2) in vitro, suggests that such enzyme may participate in the intracellular action of TeNT (Facchiano and Luini 1992; Facchiano et al. 1993a, b). However, the precise contribution of TGase to the blockade of neurotransmission by TeNT has never been clarified and conflicting data exist in the literature (Coffield et al. 1994; Ashton et al. 1995; Gobbi et al. 1996).

TGase belongs to a large family of bifunctional and Ca^{2+}-dependent cross-linking enzymes (Fesus and Piacentini 2002; Lorand and Graham 2003) abundant in neurons and nerve endings (Facchiano and Luini 1992; Maggio et al. 2001) which has been implicated in secretory mechanisms (Pastuszko et al. 1986; Driscoll et al. 1997; Walther et al. 2003). Despite these effects, tissue TGase deficient mice did not exhibit any overt phenotype except for a reduced insulin secretion (De Laurenzi and Melino 2001; Bernassola et al. 2002), while transgenic mice overexpressing TGase experienced an increased neuronal death in response to excitotoxic insults (Tucholski et al. 2006).

To establish whether TGase is implicated in action of TeNT, we investigated whether TeNT activates transamidase activity in nerve terminals and, in turn, TGase cross-linking inhibition affects the TeNT-induced blockade of neurotransmission as well as the putative presynaptic substrates of TeNT-activated TGase. The results indicate that the cross-linking activity of TGase is activated by TeNT and participates in an early, non-proteolytic, step of TeNT intoxication by involving the presynaptic proteins synapsin I (SynI) and synaptosomal-associated protein-25 (SNAP-25).

Materials and methods

Materials

Tetanus toxin TeNT was purified as previously described (Facchiano and Luini 1992). For intracellular studies, TeNT was activated by pretreatment with dithiotreitol (DTT, 20 mM, 30 min at 37°C). The mRNAs encoding for either wild type (wt-mRNA) or protease-deficient (H233V-mRNA) TeNT-LC (Yamasaki et al. 1994) were prepared from linearized plasmids (originally donated by H. Niemann, Hannover, Germany). The mRNA concentration before intraneuronal injection was 0.4–0.6 μg/μl.

TGase inhibitors The TGase catalytic site blockers cystamine (Pierce Biotechnology, Rockford, IL, USA) and monodansylcadaverine (MDC; Sigma, Milano, Italy) were used. A more selective competitive inhibitor was designed based on the *consensus* sequence encompassing the reactive glutamines in excellent TGase substrates such as casein or SynIa (Facchiano and Luini 1992; Facchiano et al. 1993a). A 11-mer peptide corresponding to rat $SynIa^{658–668}$ (Syn-peptide) was synthesized and purified >98% by HPLC. In vitro, the Syn-peptide inhibited the TGase 2 cross-linking activity with an IC_{50} ~20 μM, whereas the corresponding scrambled peptide was virtually inactive in the 10–1,000 μM concentration range.

Anti-TGase 2 *antibodies* Two rabbit polyclonal antibodies were raised against repurified guinea pig liver TGase (Sigma, Milano, Italy). In Western blots of rat brain synaptosomes, both antibodies specifically recognized two TGase components, namely a cytosolic and a membrane-bound form also associated with SVs (Facchiano et al. 1993a). Both antibodies were effective inhibitors of the Ca^{2+}-dependent activity of both purified TGase 2 and endogenous synaptosomal TGase (96 ± 4 and 89 ± 8% inhibition, respectively).

Other materials N1- and N8-(γ-glutamyl)spermidine and N1,N8-bis(γ-glutamyl)spermidine were synthesized as previously reported (Folk and Chung 1985). [^{3}H]-spermidine (15–30 μCi/mmol) and [^{3}H]-glutamate (40–80 μCi/mmol) were from Dupont NEN (Boston, MA, USA), immunopure 5-biotinamidopentylamine (BPA) was from Pierce and streptoavidin-Dynabeads were from Invitrogen (Milano, Italy).

Preparation of rat brain synaptosomes and their intoxication by TeNT

Brain synaptosomes were prepared from male Sprague-Dawley rats (150–250 g body weight) as described (Facchiano et al. 1993a). Synaptosomes were resuspended in HEPES buffered saline (HBS) consisting of (mM): HEPES, 10; K_2HPO_4, 1.2; $MgCl_2$, 1.0; EGTA, 0.1; D-glucose, 5; $CaCl_2$, 1.25; NaCl, 142; KCl, 2.4, pH 7.4). Intact synaptosomes were incubated with TeNT at 37°C, washed with HBS buffer, and used for either glutamate release or evaluation of TGase activity. Application of non-permeant compounds (reduced-TeNT, Syn-peptide, anti-TGase 2 antibodies) was performed by a previously described freeze–thaw technique (Nichols et al. 1989). For experiments of neurotransmitter release inhibition (see below), purified intact synaptosomes were loaded with [^{3}H]-glutamate and incubated with either vehicle or bichainal TeNT (10 nM) as described.

Glutamate release assays

Aliquots (30 μl, 5 mg/ml protein) of synaptosomes were incubated for 15 min at 37°C with 2 μCi of [^{3}H]-glutamate, loaded on prewetted 3 MM filter paper discs (8 mm diameter) in a superfusion apparatus and gently washed in HBS six times to remove free glutamate. In the release experiments, synaptosomes were incubated in HBS (3 × 2 min; 1 ml volume), followed by 3 × 2 min periods in depolarizing medium (HBS containing 95 mM NaCl/50 mM KCl) and by further 6 × 2 min periods in HBS for repolarization. All procedures were performed at 37°C. The radioactivity in the collected fractions and that remaining in the filters was determined by liquid scintillation counting. The rate of glutamate secretion was expressed as the radioactivity released per minute during each epoch divided by the total amount of radioactivity at the beginning of the superfusion. Glutamate release exhibited similar kinetics in both intact and permeabilized synaptosomes (Fig. 1 Supplementary data). [^{3}H]-glutamate release was assessed in three consecutive 2-min periods during the initial resting phase (wash), followed by three 2-min periods in depolarizing medium (high KCl) and three further 2-min periods in HBS for repolarization (wash). Statistical analysis was performed using the Tukey's multiple comparison test. TeNT-intoxicated synaptosomes displayed a significantly ($P < 0.001$) decreased KCl-evoked [3H]-glutamate release.

TGase activity assay

Cross-linking activity of TGase was determined by measuring either the incorporation of [^{3}H]-spermidine into substrate proteins or the formation of ε-(γ-glutamyl)lysine isodipeptides (Folk and Chung 1985). Intact synaptosomes (5 mg protein) were resuspended in 1 ml HBS, incubated with 120 μM [^{3}H]-spermidine (100 μCi) for 60 min at 37°C, and washed three times prior to intoxication with various amounts of TeNT for 70, 100 or 130 min. After intoxication, synaptosomes were washed with HBS containing 5 mM EGTA, and nerve terminal proteins were recovered by trichloroacetic acid (20% w/w) precipitation. Pellets were solubilized in 0.1 M NaOH, processed and digested as previously described (Facchiano et al. 2001; Beninati et al. 1993). ε-(γ-glutamyl)lysine was derivatized with o-phthalaldehyde and the resulting isodipeptide derivative was analyzed by reverse phase HPLC (Beninati et al. 1988). The identity of the ε-(γ-glutamyl)lysine isodipeptide was further established by quantitating lysine and glutamic acid produced upon acid hydrolysis. The in vitro activity assay for either purified or endogenous TGase was performed as previously described (Facchiano and Luini 1992). To evaluate the activity of TGase inhibitors, both synaptosomes and melanoma cells (a metastatic human cell line, SK-Mel 110) have been analyzed as above described.

Evaluation of TGase substrates

Synaptosomes (0.5 ml, 5 mg protein/ml) were resuspended in HBS, permeabilized by the freeze–thaw technique in the presence of 10 mM BPA and preactivated bichainal TeNT, washed three times, resuspended in HBS and incubated for 90 min at 37°C. After the incubation, synaptosomes were washed in homogenization buffer [10 mM Tris–HCl, 2 mM EDTA, 500 mM NaCl, 0.5% Triton X100, pH 7.4 and 0.5 μg/ml leupeptin, 2.0 μg/ml aprotinin, 2 μM pepstatin A, 0.5 mM 1,10-phenanthroline

and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and sonicated for 30 s on ice. After 20 min at 4°C, 0.8 mg of prewashed streptoavidin-Dynabeads was added. Samples were incubated for 25 min at room temperature with gentle rotation and washed six times by means of a magnetic particle concentrator. Pellets were resuspended in Laemmli sample buffer, boiled and separated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Biotinylated proteins were electrophoretically transferred to nitrocellulose membranes and visualized by incubation with streptavidin–alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate-p-toluidine and nitroblue tetrazolium chloride (GIBCO-BRL, Gaithersburg, MD, USA).

To achieve an optimal separation of large aggregates induced in cell lysates by the TGase cross-linking activity, samples were solubilized in 5% β-mercaptoethanol and 1.6% SDS, 60 mM Tris (final concentrations), pH 6.8, boiled for 10 min and immediately refrigerated in an ice cold bath. The resolving gel (14 × 16) was prepared with a continuous 2–18% polyacrylamide gradient (30/0.8% acrylamide/bis-acrylamide ratio) and the electrophoretic run was carried out at 4°C under constant voltage (2 h at 20 V, followed by 6–9 h at 100 V).

Analysis of evoked acetylcholine release at *Aplysia* synapses

Aplysia californica were purchased from the University of Florida (Miami, FL, USA). Electrophysiological experiments were performed at identified inhibitory cholinergic synapses in dissected buccal ganglia of *Aplysia* as extensively described previously (Schiavo et al. 1992; Ashton et al. 1995; Humeau et al. 2001, 2007). TeNT mRNA encoding wild-type or mutated TeNT-LC, anti-TGase 2 antibodies, Syn-peptide or non-permeant drugs were mixed with the vital dye Fast Green FCF (10% v/v; Sigma) and air-pressure injected intraneuronally as described (Humeau et al. 2001, 2007). The injected volume was in the range of 1% of that of the cell body, leading to a 100-fold dilution of the injected material. Intracellular administration of the used vehicles produced no changes in neurotransmitter release. Owing to solubility problems in the injection micropipette, the maximum intracellular concentration of the Syn-peptide that could be achieved was ~10 μM. To assess the presence of endogenous TGase in *Aplysia* nervous tissue, neurons and glial tissue were dissected out, homogenized by sonication in phosphate buffered saline and centrifuged at 14,000×*g* for 5 min at 4°C. Aliquots of the supernatant (50 μg protein) were subjected to SDS–PAGE and immunoblotting using the anti-TGase 2 antibody (1:2,000 dilution).

Bioinformatic analysis

The search for transamidation sites in synaptic proteins was performed according to the following procedure: proteins specifically related to the synapse were selected from the UniProt database in the sequence retrieval system (SRS) environment by searching for protein description containing 'SYNAP' and selecting those with a molecular mass in the range of the protein bands of interest. Proteins obtained by this search were subjected to the SITEMATCHER searching tool, which compares a submitted sequence (query) with the TRANSIT (TRANSglutaminase SITes) database (Facchiano et al. 2003). The January 2009 release of TRANSIT, containing more than 150 known TGase substrates, was used for the analysis. Settings were fixed to find identity of at least 70% including the mandatory amino acid within a segment of at least five residues.

Results

TeNT blocks [^{3}H]-glutamate release and activates transglutamination in intact rat brain synaptosomes

Treatment of either intact or permeabilized synaptosomes with bichainal or activated TeNT virtually abolished the early, Ca^{2+}-dependent, phase of depolarization-evoked [^{3}H]-glutamate release (Fig. 1, Supplementary data), while the inhibition was not complete in the late phase which is also contributed by non-vesicular mechanisms (Ashton and Dolly 2000; McMahon et al. 1992). No effects of intoxication were observed on the basal [^{3}H]-glutamate efflux, which is attributable to non-vesicular leakage and/or TeNT-resistant spontaneous exocytosis (Fassio et al. 1999). Since we previously showed that TeNT activates TGase 2 in vitro (Facchiano and Luini 1992), we investigated the participation of endogenous synaptosomal TGase in the action of TeNT. TeNT increased the incorporation of spermidine into synaptosomal proteins in a time-dependent fashion, an effect that paralleled the inhibition of glutamate release (Fig. 1a). The isodipeptide levels in synaptosomes pre-treated with TeNT were measured and the correlation between activation of synaptosomal TGase and inhibition of [^{3}H]-glutamate release by TeNT was shown (Fig. 2 Supplementary Data). The basal levels of Spd-derivatives and glutamyl-lysine were reported in legend to Fig. 2 of Supplemental data. Analysis of the formation of ε-(γ-glutamyl)lysine isodipeptides also confirmed that the levels of the specific TGase products were dramatically increased as a function of the TeNT concentration (EC_{50} ~21 nM) and of extent of TeNT-induced inhibition of glutamate release (EC_{50} ~28.1 nM) (Fig. 1b). Since both the spermidine incorporation and formation of ε-(γ-glutamyl)lysine isodipeptides were highly correlated

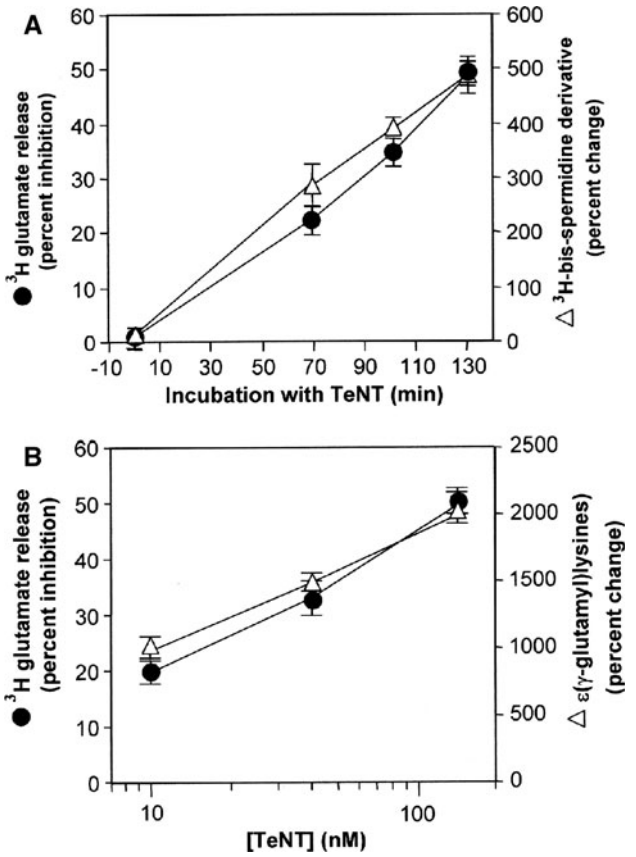

Fig. 1 Correlation between activation of synaptosomal TGase and inhibition of [^{3}H]-glutamate release by TeNT. **a** Synaptosomes were loaded with [^{3}H]-spermidine, washed and incubated in HBS with 20 nM TeNT for the specified times. Mono- and bis-(γ-glutamyl)-spermidine derivatives (*open symbols*) were measured as described in "Materials and methods" and expressed in percent of the basal levels determined before incubation with TeNT. Parallel samples incubated under the same conditions (except for the use of unlabeled spermidine) were loaded with [^{3}H]-glutamate during the last 15 min of incubation with TeNT, washed and challenged with HBS containing 50 mM KCl to evaluate the TeNT-dependent inhibition of glutamate release (*closed symbols*). The stimulation of [^{3}H]-glutamate release by high KCl–HBS was calculated as the percent increase in the release rate during the first 6 min of exposure to high KCl over the release rate during the preceding 6 min of incubation under resting conditions. **b** Synaptosomes were loaded with unlabeled spermidine and [^{3}H]-glutamate as described above, washed and incubated for 70 min at 37°C with increasing concentrations of bichainal TeNT. TeNT was washed out and [^{3}H]-glutamate release measured as described above (*closed symbols*). The concomitant activation of TGase was evaluated by measuring the levels of ε-(γ-glutamyl)lysine isodipeptides (*open symbols*) and expressed in percent of the basal levels determined before the incubation with TeNT. Data in **a**, **b** are mean values ± SD from at least three independent experiments

with the TeNT-induced blockade of glutamate release (Pearson's correlation coefficients were 0.983 ($n = 12$; $P < 0.01$) and 0.987 ($n = 12$; $P < 0.01$) for spermidine derivatives and (glutamyl)lysine dipeptides, respectively; see Supplementary data), further experiments were addressed to determine whether the two events were causally related.

Inhibition of TGase prevents TeNT-induced blockade of neurotransmitter release in synaptosomes

The catalytic site blocker cystamine, a pseudosubstrate peptide encompassing rat SynIa$^{658–668}$ sequence (Syn-peptide) or neutralizing anti-TGase 2 antibodies were used to inhibit TGase activity. The concentration of cystamine used was that able to achieve the maximal inhibiting effect without any detectable toxicity, as reported in other studies involving neuronal models (Dai et al. 2008) (see inset to Fig. 2a, d). Permeabilized synaptosomes were treated with reduced TeNT in the absence or presence of the specific TGase inhibitor and [^{3}H]-glutamate release was determined after a 70 min recovery period. TGase inhibitors were similarly effective in inhibiting the accumulation of transamidase products induced by TeNT (Fig. 2a) and had no effect on either basal or K^+-evoked glutamate release (Fig. 2b). Interestingly, the blocking activity of activated TeNT on the early phase of Ca^{2+}-dependent SV exocytosis was completely prevented by each of the TGase inhibitors, while the corresponding controls (vehicle, pre-immune serum or scrambled Syn-peptide, respectively) were totally ineffective (Fig. 2c). An intriguing effect of anti-TGase 2 antibody on TeNT intoxicated synaptosomes was observed at the longest time points of release (12 min, Fig. 2b). The possibility that TeNT activates TGase by increasing calcium concentration can be ruled out by evaluating the effects of TeNT induced TGase cross-linking activity in permeabilized synaptosomes (see Fig. 1 in Supplementary data). Further, the higher ability of TGase 2-Ab than cystamine to rescue the TeNT inhibition of [^{3}H] glutamate release is statistically significant (Fig. 2c, $P < 0.05$) and may reflect the possibility that this polyclonal antibody interfere with more than one enzymatic activity belonging to TGase 2 enzyme. Therefore, it is possible that cystamine rescues just one releasable pool of secretory vesicles, while the TGase 2-Ab might rescue a larger vesicles compartment.

These results indicate that the inhibition of endogenous TGase significantly impairs the blocking action of TeNT on neurotransmitter release. On the other hand, the possibility that Syn-peptide or other inhibitors used in this study might interfere with more than one enzymatic activity of TGase cannot be definitely ruled out.

To rule out the possibility that TGase inhibition affects the proteolytic cleavage of VAMP by TeNT, activated TeNT was added to lysed synaptosomes in the presence or absence of the inhibitory Syn-peptide, and the cleavage of VAMP was determined by immunoblotting (Ashton et al. 1995). The inhibitory peptide at a concentration effective in inhibiting TGase (300 μM; >80% activity inhibition) did not show any toxic activity on cell viability and proliferation and was totally ineffective in modifying the TeNT-induced proteolysis of VAMP by TeNT (see Fig. 3

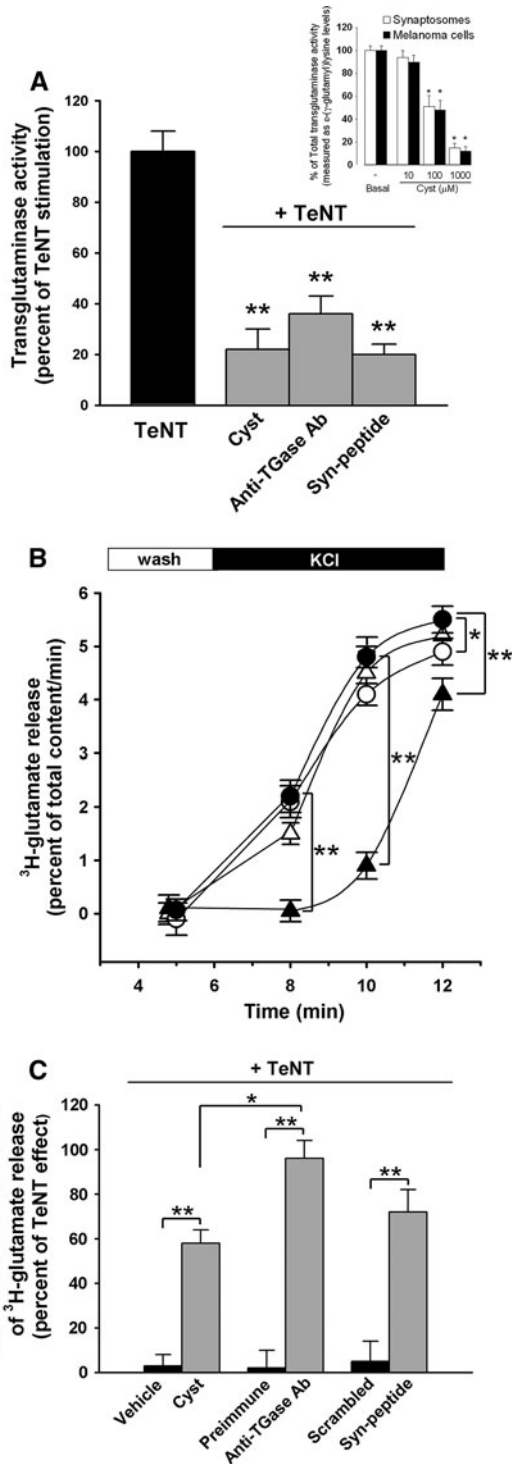

Fig. 2 Activation of TGase and blockade of evoked glutamate release by TeNT in synaptosomes are prevented by TGase inhibitors or anti-TGase 2 antibodies. Synaptosomes were transiently permeabilized by the freeze–thaw technique in the presence of either vehicle or reduced TeNT (40 nM) and/or the indicated TGase inhibitor and loaded with either [^{3}H]-spermidine or [^{3}H]-glutamate after a 70-min recovery period as described in Fig. 1. Cystamine (Cyst, 1 mM), Syn-peptide (300 μM) or anti-TGase 2 antibodies (Ab; 1:100 dilution) were used to specifically inhibit endogenous TGase activity. As controls, vehicle, scrambled Syn-peptide (300 μM) or pre-immune serum (pre-immune, 1:100 dilution) were used, respectively. **a** TGase activation by TeNT in the absence (*black bar*) or presence (*gray bars*) of the various inhibitors was determined by measuring the incorporation of [^{3}H]-spermidine in synaptosomal proteins and expressed in percent of the TGase activation by TeNT applied alone. In this series of experiments, TeNT-induced activation of TGase was 695% ± 62 (mean ± SD) of the activity present in untreated synaptosomes. *Inset* the dose-dependent effect of cystamine as inhibitor of cross-linking activity of TGase, measured as described in synaptosomes and human melanoma cells. **b** Synaptosomes were loaded with vehicle (*open symbols*) or TeNT (*closed symbols*) in the presence of either pre-immune serum (*triangles*) or anti-TGase 2 antibodies (*circles*) before measuring K^+-evoked [^{3}H]-glutamate release. The rate of [^{3}H]-glutamate release was measured as described in "Materials and methods". The effect of anti-TGase 2 antibody on TeNT was highly significant at all time points 8, 10 and 12 min (**$P < 0.01$). Another less significant difference (*$P < 0.05$) was that observed by comparing vehicle +Ab versus TeNT +Ab at the longest time points, 12 min. Additional studies are required to further investigate this effect. C: The rescue of [^{3}H]-glutamate release inhibition by TeNT was assessed in the presence of the various inhibitors (*gray bars*) or of their respective controls (*black bars*) and calculated as described in the legend to Fig. 1. Data are means ± SD from four to six independent experiments. Statistical analysis was carried out using either the Dunnett's (**a**, **b**) or the Tukey's (**c**) multiple comparison test (**$P < 0.01$, *$P < 0.05$)

and 4 in Supplementary data). To rule out the possibility that 1 mM cystamine might be toxic to our neurosecretory system, it should be taken into account that synaptosomes treated with cystamine 1 mM were able to actively uptake radiolabeled glutamate and secrete it upon K+ induced hyperpolarization, as well as the neurosecretory block induced by TeNT was partially rescued. Further, in other neuronal models it has been reported that 1 mM cystamine has no effect of on neuron cell viability (Dai et al. 2008).

Inhibition of TGase delays the blockade of acetylcholine release induced by TeNT at *Aplysia* synapses

In immunoblotting experiments, anti-TGase 2 antibodies, but not the pre-immune serum, stained two major bands of ~90 and 40 kDa in preparations of *Aplysia* ganglia (Fig. 3a) consistent with earlier observations (Ambron and Kremzner 1982). The 40 kDa band was weakly detectable also in the glial/connective tissue surrounding the ganglia. Presently, we cannot define whether both immunoreactive bands represent two distinct enzymes or whether the lighter is a degradation product derived from the heavier.

To study the kinetics of the effect of TGase inhibition on the action of TeNT, experiments were carried out in identified *Aplysia* neurons treated with the membrane-permeant inhibitors MDC or cystamine. Because MDC and cystamine may have side effects on membrane excitability and neurotransmission when applied at high concentrations, low concentrations (10–25 μM MDC and 100 μM

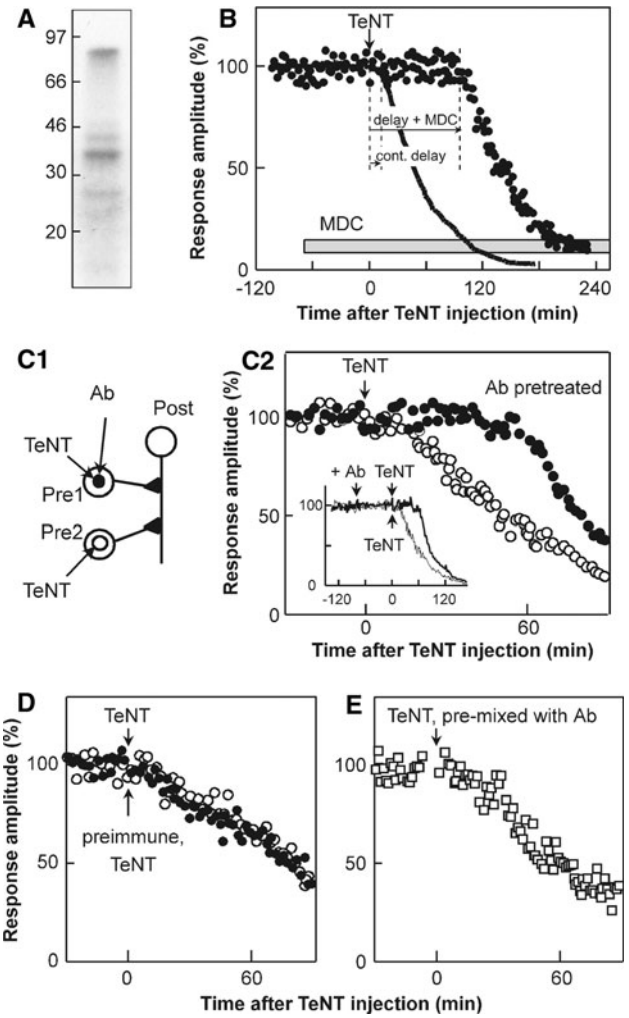

Fig. 3 Inhibition of endogenous TGase in identified *Aplysia* synapses by MDC delays the TeNT-induced blockade of acetylcholine release. **a** Immunobotting with anti-TGase 2 antibodies of an extract of *Aplysia* nerve tissue. Two major immunoreactive bands of ~90 and 40 kDa are present. **b** Representative experiment from a series of 3. Acetylcholine release was evoked at the buccal ganglion. The evoked postsynaptic response was recorded and its amplitude was expressed in percent of the mean value before any treatment. Buccal ganglion was superfused with the TGase inhibitor MDC (25 μM) as denoted by the hatched horizontal bar. TeNT (10 nM final) was intraneuronally injected at time 0 (*arrow*). A representative time-course of TeNT-induced blockade of acetylcholine release performed in the absence of MDC is shown (*solid line*). The delay in the appearance of the TeNT effect under either experimental condition is indicated by a *horizontal arrow*. **c–e** Anti-TGase 2 antibodies delay the TeNT-induced inhibition of acetylcholine release at identified *Aplysia* synapses. **c1** Schematic representation of the configuration used for antibody microinjection experiments. Acetylcholine release from two presynaptic neurons afferent to the same postsynaptic cell was simultaneously determined. **c2** One of the two presynaptic neurons was pressure injected (*arrow in inset*) with anti-TGase 2 antibodies (Ab); acetylcholine release from this neuron is denoted by *filled symbols* and, in the inset, by a *thick solid line*. The second neuron served as a control (*open symbols*, *thin solid line in inset*). Subsequently, both presynaptic neurons were injected with TeNT (10 nM final intrasomatic concentration). **d** Same experiment as in C2, but with injection of IgG from pre-immune serum. **e** The anti-TGase 2 antibodies were preincubated with TeNT for 30 min before intraneuronal injection of the mixture (TeNT ~10 nM). Note that, in contrast with c2 (**d**, **e**), the delay in the action of TeNT was not modified

cystamine, respectively) which achieved a partial (~50–70%) inhibition of TGase in vitro and in vivo (Karpuj et al. 2002) were used. Under these conditions, the treatment with either inhibitor did not significantly perturb cholinergic neurotransmission for at least 4 h (data not shown, $n = 3$ for each condition).

Under control conditions, the intraneuronal injection of TeNT (final intrasomatic concentration ~10 nM) induced a potent inhibition of neurotransmitter release (average $t_{½}$ of inhibition = 57 min) beginning after an average delay of ~12 min after injection and achieving a virtually complete blockade of acetylcholine release within 120–180 min (Fig. 3b), consistent with previous observations (Schiavo et al. 1992; Ashton et al. 1995; Poulain et al. 1996). However, when reduced TeNT was injected 60 min after pretreatment of the neuron with either MDC (Figs. 3b, 4) or cystamine (Fig. 4), the blocking action of the neurotoxin was delayed over sevenfold (Fig. 4a), in the absence of changes in the initial rate and the extent of blockade (Fig. 4b).

In order to inhibit endogenous TGase more specifically, neutralizing anti-TGase 2 antibodies or the non-permeant Syn-peptide were injected into the presynaptic neuron. While the anti-TGase 2 antibodies or the Syn-peptide (at a concentration of ~10 μM approaching the intraterminal concentration of endogenous SynI) had no effect on evoked acetylcholine release under control conditions, they significantly impaired the blocking action of TeNT (Figs. 3c, 4). After injection of the anti-TGase 2 antibodies, no change in evoked acetylcholine release was detected for at least 4 h (data not shown). However, when activated TeNT was microinjected 60 min later into the same neuron, the inhibitory action of TeNT was greatly delayed, although the rate and the extent of inhibition were not modified (Figs. 3c, 4). Both the injection of the corresponding pre-immune serum (Fig. 3d) or of a mixture of anti-TGase 2 antibody and TeNT which had been pre-incubated at 4°C for 30 min (Fig. 3e) were completely ineffective on the TeNT-induced inhibition of acetylcholine release. This indicates that the anti-TGase 2 antibodies did not directly interfere with TeNT as previously suggested (Coffield et al. 1994) and that TGase 2 should be inhibited prior to the application of TeNT in order to antagonize the TeNT inhibitory action on neurotransmitter release.

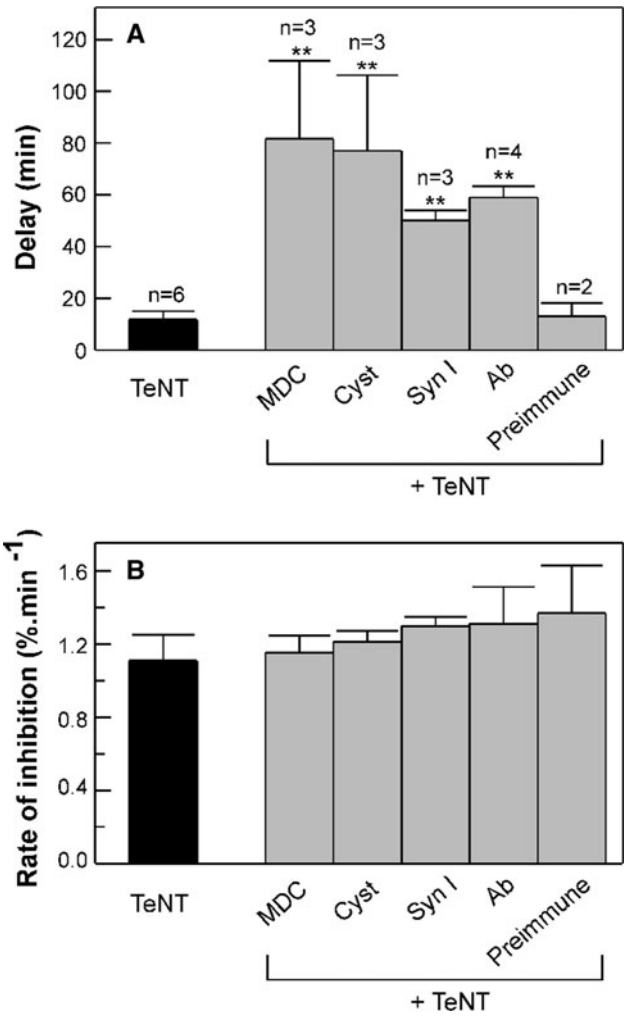

Fig. 4 TGase inhibitors delay the inhibition of acetylcholine release induced by TeNT at *Aplysia* synapses without affecting the rate of inhibition. The experiments were performed as described in the legend to Fig. 3. TGase inhibitors were either extracellularly applied (MDC, 25 μM; Cyst, 100 μM,) or intracellularly injected (Syn-peptide, 10 μM; anti-TGase 2 antibodies, Ab). **a** Average delays in the onset of the TeNT action induced by the various inhibitors. **b** Average rates of TeNT-induced release blockade (see "Material and methods" for calculation) in the absence or the presence of the various inhibitors. Data are mean values ± SD from three to six independent experiments. $^{**}P < 0.01$ with respect to TeNT alone, Dunnett's multiple comparison test

Inhibition of TGase antagonizes the protease-independent blockade of neurotransmitter release by TeNT

TeNT blocks exocytosis by both proteolytic and non-proteolytic mechanisms, as previously reported (Ashton et al. 1995). To determine whether the non-proteolytic action is affected by TGase inhibition, the activity of a mutant light chain of TeNT devoid of proteolytic activity H233V TeNT-LC; Yamasaki et al. 1994) on acetylcholine release was examined in *Aplysia* neurons which had been pre-treated with either vehicle, cystamine or anti-TGase antibodies. When presynaptic neurons were microinjected with the TeNT-LC mutant mRNA, acetylcholine release was inhibited by ~50%, with a $t_{½}$ of the blockade of 106 ± 14 min and a delay of 22 ± 2 min, presumably due to intraneuronal synthesis and transport of TeNT-LC ($n = 5$; Fig. 5a). When presynaptic neurons were superfused with cystamine (100 μM; Fig. 5a) or preinjected with anti-TGase 2 antibodies (Fig. 5b) 1 h before mRNA injection, the inhibition of acetylcholine release induced by the H233V mutant was prevented for the entire duration of the experiments. In contrast, neither cystamine (Fig. 5c) nor anti-TGase 2 antibodies were able to prevent the inhibition of release induced by injection of wild type TeNT-LC mRNA, although they caused a delay in the onset of inhibition that was comparable to that observed after injection of activated wild type TeNT (see Figs. 3, 4). The complete rescue of the blockade of release caused by the TeNT-LC mutant by TGase inhibitors indicates that the protease-independent activity of TeNT occurs primarily through a TGase-dependent mechanism.

Identification of TGase substrates in nerve terminals

The transamidating activity present in nerve terminals is likely to occur through the covalent modification of nerve terminal targets. SynI, an abundant nerve terminal protein involved in the regulation of SV availability for exocytosis (Baldelli et al. 2006), is an excellent substrate for TGase 2 and is heavily cross-linked in vitro by TGase 2 (Facchiano et al. 1993a). Thus, we investigated whether SynI is modified by activated TGase 2 under TeNT intoxication. Indeed, TeNT-intoxicated synaptosomes, previously loaded with ^{14}C-labeled spermidine, displayed a significantly increased amount of ^{14}C-labeled cross-linked products of high molecular mass that are likely to result from an increased transamidation (Fig. 6a). Additional experiments carried out using the TGase substrate BPA to precipitate the individual transamidation products isolated four major bands of ~80, 44, 34 and 24 kDa from synaptosomes incubated under basal conditions, of which the recovery of the 80 and 24 kDa bands was significantly increased under TeNT intoxication (Fig. 6b).

In order to identify the BPA-labeled bands, potential candidate proteins related to synapses were first selected from the UniProt database in SRS environment and the resulting sequences were subsequently analyzed for putative *consensus* transamidation sequences by comparing the query sequences to the TRANSIT site database described elsewhere (Facchiano et al. 2003) using the SITEMAT-CHER algorithm. While it was not possible to conclusively identify the 44 and 34 kDa bands, we identified two

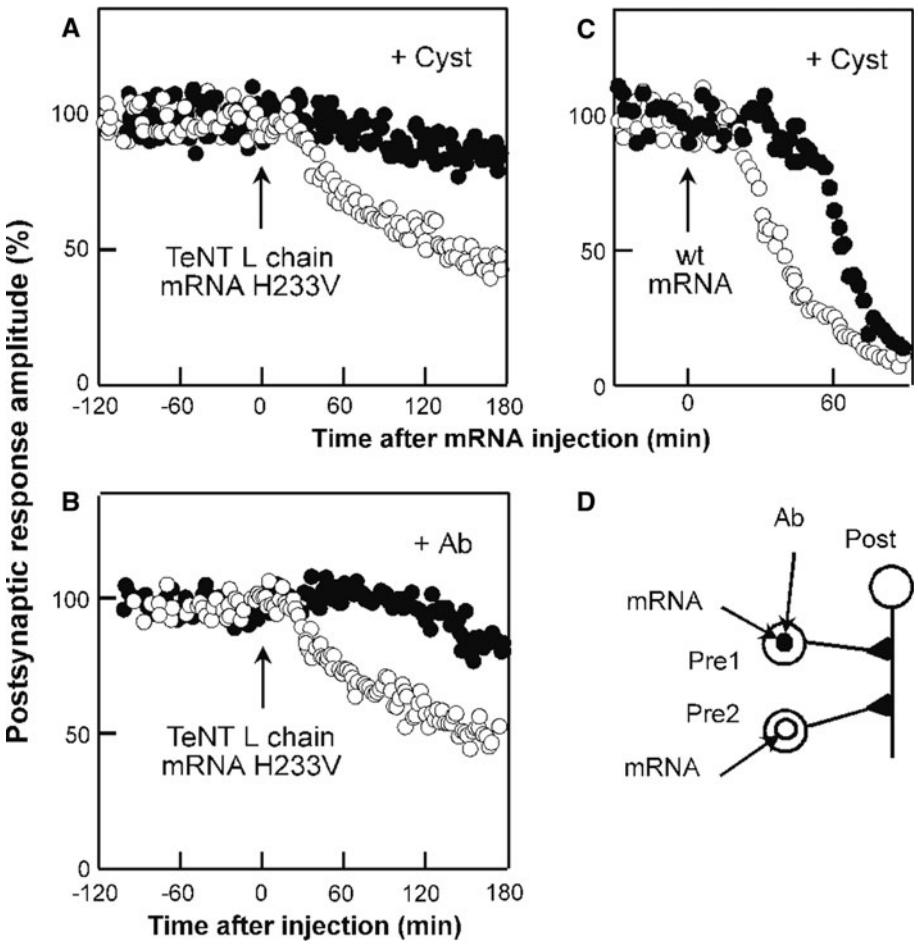

Fig. 5 Effect of TGase inhibitors on the inhibition of acetylcholine release induced by protease-deficient mutants of TeNT-LC in *Aplysia* neurons. Experiments were carried out as described in the legend to Fig. 3, except that the mRNA was injected instead of the protein toxin. Representative experiments from series of 3 to 5 are shown. Messenger RNA (~0.5 μg/μl in the injection micropipette) encoding for either wild type (**c**; $n = 3$) or protease-deficient (**a, b**) TeNT-LC was injected into presynaptic neurons in the absence (*filled symbols*) or the presence (*open symbols*) of cystamine (Cyst, 100 μM; **a, c**) or anti-TGase 2 antibodies (injected 1 h prior to mRNA injection; **b** The controls shown in **a**, **c** come from distinct sets of experiments, since cystamine is membrane permeant. **d** Schematic representation of the experimental configuration used for Ab and mRNA injection

presynaptic TGase substrates with a molecular mass compatible with the ~80 and 24 kDa bands, namely SynIa, previously shown to contain a 11-amino acid sequence in the COOH terminal E domain highly similar to reactive glutamine-containing sites (Facchiano and Luini 1992), and SNAP-25, a presynaptic SNARE [SNAP (soluble NSF attachment protein) receptors] found to be a potentially good amine donor TGase substrate (Fig. 6c). Indeed, in Western blotting, the BPA-purified 80 and 24 kDa bands were specifically recognized by anti-SynI and anti-SNAP-25 antibodies, respectively and their immunoreactivity was virtually abolished when the respective antibody was previously pre-adsorbed with an excess of purified SynI or SNAP-25 (Fig. 6b).

The involvement of SynI and SNAP-25 as intracellular targets of the cross-linking activity of TeNT-activated TGase was tested by assaying extracts of TeNT-intoxicated synaptosomes with anti-SynI and anti-SNAP-25 antibodies. The results showed that in intoxicated synaptosomes, but not in control synaptosomes, a high (>250 kDa) molecular mass protein complex immunoreactive for both SynI and SNAP-25 proteins was present (Fig. 6d). Although in some experiments, a faint SynI and SNAP-25-positive band of ~110–115 kDa (likely to correspond to a SynI/SNAP-25 heterodimer) could be observed, in most cases SynI and SNAP-25 immunoreactivities were exclusively confined to the monomeric proteins and to the very large molecular mass complex(es).

Discussion

The discovery of the proteolytic activity of TeNT specifically directed against VAMP-2 and its closely related isoforms provided the first molecular explanation for the inhibition of exocytosis by TeNT (Schiavo et al. 1992; Humeau et al. 2000; Rossetto et al. 2006). However, based on the initial demonstration that TeNT is a TGase 2 activator in vitro (Facchiano and Luini 1992), here we demonstrate that, in isolated nerve terminals and live neurons, the inhibition of neurotransmitter release by TeNT also involves an early step mediated by the intraneuronal activation of a TGase cross-linking activity, likely due, at least in part, to TGase 2.

We show that TeNT stimulates the TGase cross-linking activity and that this orphan TeNT activity is causally related to the inhibitory action of the toxin on

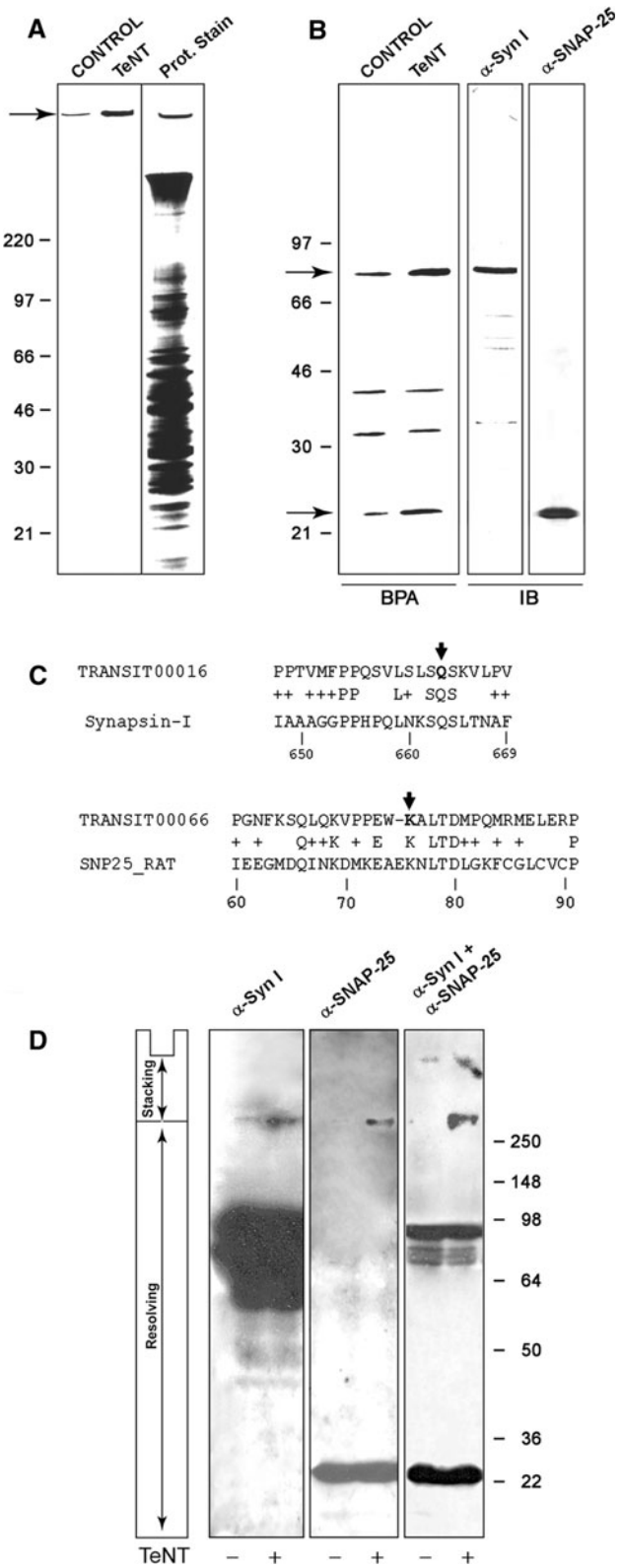

Fig. 6 Modification of presynaptic proteins by TeNT-activated TGase in intact synaptosomes**. a** Treatment of synaptosomes with TeNT induces the formation of covalent high molecular mass complexes. Synaptosomes were loaded with [^{14}C]-spermidine, exposed to bichainal TeNT (20 nM) at 37°C for 120 min and subjected to SDS–PAGE analysis (200 µg protein/lane). A representative example of three independent experiments is shown. While the overall pattern of synaptosomal proteins did not show any significant change upon TeNT treatment, a markedly higher level of [^{14}C]-labeled protein aggregates focused at the beginning of the stacking gel (*arrow*) was present in TeNT-intoxicated samples with respect to control synaptosomes. **b** Labeling of individual TGase substrates in synaptosomes. Synaptosomes were permeabilized by the freeze–thaw procedure to entrap BPA in the presence or absence of reduced TeNT (20 nM), washed, incubated at 37°C for 120 min and solubilised. Extracts were incubated with streptavidin-Dynabeads to purify TGase-modified proteins, subjected to SDS–PAGE, blotted and developed with alkaline phosphatase-conjugated streptavidin. The *arrows* indicate the two bands of ~80 and 24 kDa (corresponding to the molecular mass of SynI and SNAP-25) whose recovery was reproducibly increased by TeNT treatment. The same blots were stripped and re-hybridized with anti-SynI and anti-SNAP-25 polyclonal antibodies (*right lanes*, IB). **c** Identification of putative *consensus* transamidation sequences in the primary structure of SynI and SNAP-25 using the TRANSIT site database. The alignments show the similarity between the query sequences (rat synapsin I and SNAP-25, respectively) and the known TGase substrates β-casein and fibrinogen. The bold Q and the K residues (*arrow*) are the reactive residues glutamine in β-casein and lysine in human fibrinogen. The original alignments found by SITEMATCHER, detecting identical residues, were further improved to highlight similarities. **d** Large protein complexes generated in TeNT-treated synaptosomes are immunoreactive for both SynI and SNAP-25. Extracts of TeNT-intoxicated synaptosomes were separated by high resolution SDS–PAGE and subjected to immunoblotting with anti-SynI, anti-SNAP-25 and anti-SynI+anti-SNAP-25 antibodies. The scheme on the *left* indicates the edges of the stacking and resolving gels. Note that a large protein complex of high (>250 kDa) molecular mass immunoreactive for both SynI and SNAP-25 was generated only in synaptosomes treated with TeNT

neurosecretion. Indeed, treatments killing TGase activity strongly delayed the neurosecretory block induced by TeNT and virtually abolished the inhibitory effect of non-proteolytic mutants of TeNT.

Further, since several protein bands immunoreactive for anti-TGase 2 antibody were reported in synaptosomes (Facchiano et al. 1993a, b), probably reflecting the presence in nerve terminals of more than one TGase, the present data may be explained by an effect on a transglutaminase activity belonging to one or more members of this family of enzymes. As well as since some of the inhibitors used, e.g. MDC and Syn-peptide, are competitive primary amine substrates, the inhibition of cross-linking due to other TGases cannot be ruled out**.**

TGase is a multifunctional protein comprised of α and β subunits. The α subunit is a bifunctional enzyme that, depending on its binding to the β subunit, GDP/GTP or Ca^{2+}, can switch a from GTPase activating phospholipase Cδ1 to a transglutaminase in a mutually exclusive fashion (Mhaouty-Kodja 2004). The activation by TeNT of cross-linking activity of TGase 2 (Facchiano and Luini 1992) and the high correlation between TeNT-induced inhibition of

neurotransmitter release and formation of glutamyl-peptide indicate switching on of the transglutaminase activity of TGase, and therefore, the switching off of the GTPase activity. When unbound to GDP/GTP and in presence of Ca^{2+}, TGase 2 can cross-link its substrates, induce amine incorporation, deamidation, or act as an isopeptidase and known substrates include RhoA GTPase and several cytoskeletal proteins (Fesus and Piacentini 2002). The two additional TGase substrates SynI and SNAP-25, whose covalent modification by TGase was found to be increased by TeNT, are major nerve terminal proteins implicated in pre- and post-docking stages of neurotransmitter release.

Modification of SynI and/or SNAP-25 by TGase can account for by a decrease in neurotransmitter release. SynI regulates SV trafficking by clustering SVs and tethering them to the actin cytoskeleton in a phosphorylation-dependent fashion and by participating in post-docking steps of exocytosis (Humeau et al. 2001; Baldelli et al. 2006). It is therefore possible that, when the endogenous TGase is stimulated by TeNT, SynI is covalently modified in such a way that the SV availability for release is reduced and neurosecretion diminished. Such a possibility is supported by the following observations: (1) the depolarization-stimulated phosphorylation and redistribution of SynI is altered after the action of TeNT (Presek et al. 1992), (2) the blocking action of TeNT is attenuated when SVs are dissociated from the cytoskeleton by prior disassembly of microfilaments (Ashton and Dolly 1997) and (3) the amplitude of post-tetanic potentiation, a plasticity paradigm which involves SynI in *Aplysia* synapses, is highly reduced after TeNT treatment (Humeau et al. 2001, 2007).

SNAP-25 is a plasma membrane protein involved in the formation of the SNARE core complex that drives SV exocytosis (Breidenbach and Brunger 2005; Südhof and Rothman 2009). Hence, perturbing SNAP-25 availability for SNARE-complex formation is likely to inhibit exocytosis. Since SNAP-25 mediates the Ca^{2+}-dependent interactions between synaptotagmin and the SNARE complex (Gerona et al. 2000), it is possible that TeNT-activated TGase, by structurally modifying SNAP-25, reduces its ability to interact with synaptotagmin, thereby decreasing the efficiency of Ca^{2+} to trigger release.

An alternative possibility is that activation of TGase impacts the structural amenability of VAMP-2 for cleavage by TeNT-LC. Indeed, VAMP-2 cannot be cleaved when engaged into the SNARE complex (Hayashi et al. 1994) or bound to lipids or Ca^{2+}-calmodulin (Quetglas et al. 2002; de Haro et al. 2004). As TeNT can access VAMP-2 only during a defined 'physiological window' (Humeau et al. 2000) TGase activation may modulate this access via the modification of proteins involved in regulation of the SV cycle.

The relative contribution of proteolytic and TGase-mediated mechanisms may vary among synapses due to heterogeneity in the distribution of synaptic proteins (Atwood and Karunanithi 2002). A participation of TGase activation in the blockade of secretion by TeNT has been ruled out at the mouse neuromuscular junction and in NG108 cells (Coffield et al. 1994), and non-proteolytic TeNT mutants were ineffective in inhibiting neurotransmission at the mouse hemidiaphragm (Li et al. 1994) or neurohypophysial nerve endings (Dayanithi et al. 1994). In other cells, both proteolytic and non-proteolytic effects are likely to coexist. Indeed, expression of TeNT-insensitive VAMP-2 mutant in HIT-T15 cells is associated with an only partial rescue of insulin secretion after TeNT inhibition (Regazzi et al. 1996) and protease-deficient TeNT-LC mutants can partially inhibit evoked transmitter release at cortical synaptosomes and *Aplysia* synapses (Ashton et al. 1995; this paper). These observations suggest that the variable importance of proteolytic and non-proteolytic mechanisms of TeNT may depend on differential expression of endogenous members of the family of TGases and of toxin-sensitive/toxin-insensitive VAMP isoforms.

In conclusion we demonstrated that, in addition to the proteolytic cleavage of VAMP, an alternative mechanism for TeNT action exists, involving the activation of TGase in nerve endings supporting a novel TGase involvement in cellular secretion. This accounts for an early phase in the inhibitory action of TeNT and for the entire effect of non-proteolytic TeNT mutants, and implicates at least two putative presynaptic TGase targets, SynI and SNAP-25. The existence of additional synaptic targets, as well as the contributions of the multiple activities of TGase in neuronal physiology, remains to be investigated.

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