Covalent Modification of Synapsin I by a Tetanus Toxin-activated Transglutaminase*

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The synapsins are neuronal phosphoproteins that bind to small synaptic vesicles and to actin filaments and are believed to play a regulatory role in neurotransmitter release. Here we show that synapsin I is covalently modified with remarkable affinity and selectivity by the enzyme transglutaminase. Transglutaminase catalyzes the formation of covalent bonds between protein glutamine residues and primary amines and has been found recently to be potently activated by tetanus toxin, a diphtheria clostridial protein that selectively blocks neurotransmitter secretion. We also report the presence of two species of immunoreactive transglutaminases in nerve endings, one cytosolic and one located on synaptic vesicles; they are potently activated by tetanus toxin and, when activated, covalently modify synaptic vesicle-bound synapsin I. These results suggest a role for transglutaminase in the control of neurotransmitter secretion and provide evidence for synapsin I being a molecular target of tetanus toxin.

The transglutaminases (TGases) are a family of calcium and GTP-dependent enzymes abundant in numerous tissues, including brain, which catalyze the formation of covalent bonds between protein-bound glutamine residues and primary amines, resulting in protein-protein cross-linking or in amine incorporation into proteins (1, 2). Purified TGase has recently been shown to be activated by tetanus toxin (TT) (3), a potent and selective protein blocker of neurotransmitter secretion (4).

The observation that TGase is usually quiescent within the cell but that it can be potently activated by calcium and TT suggests that this enzyme can be involved in signal transduction in the brain and, more particularly, in the regulation of neurotransmitter release. These considerations prompted the search for neural-specific substrates of TGase that may mediate the biological effects of the enzyme on neurotransmitter release as well as the characterization of TGase activity localized at the nerve terminal level. We have recently observed that an isoform of synapsin I (an abundant synaptic vesicle phosphoprotein) contains a 11-amino acid sequence (from position 658 to 668) within the COOH terminus, showing strong homology to reactive glutamine-containing sites in known TGase protein substrates (3). Synapsin I is a neuronal phosphoprotein, which is specifically associated with the cytoplasmic side of small synaptic vesicles and interacts with actin filaments in a phosphorylation-dependent manner (5, 6). The observed correlation between phosphorylation of synapsin I and stimulation of neurotransmitter release, as well as the inhibition of spontaneous and stimulated exocytosis observed after the microinjection of dephosphosynapsin I into the squid giant axon of the goldfish Mauthner neuron suggest that synapsin I is involved in the short term regulation of neurotransmitter release (5). Recent data have shown that synapsin I is indeed able to reversibly anchor synaptic vesicles to the F-actin-based cytoskeleton of the nerve terminal, thereby regulating their availability for exocytosis (5-7).

In this paper we demonstrate that synapsin I is indeed an excellent substrate for TGase and is covalently modified by the enzyme activated by calcium and TT. Moreover, two forms of catalytically active brain TGase, one associated with synaptic vesicles and one present in the nerve terminal cytosol, have been identified, and their activation by TT has been investigated.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (150–250 g body weight) were obtained from Charles River (Calco, Italy). Controlled glass pore beads (CPG; GLY 03000B) were obtained from Electro-Nucleonics Inc. (Fairfield, NJ). Sephadex G-150 was from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade and were obtained from standard commercial suppliers.

Protein Purification—TT was purified from a Clostridium tetani culture broth as previously described (3). Partially pure commercial guinea pig liver TGase (Sigma) was further purified as described (3). Synapsin I (a mixture of the Ia and Ib isoforms) was purified from bovine brain as described (8). COOH-terminal fragments from synapsin Ia and Ib were prepared by cleavage with 2-nitro-5-thiocyanoenzoic acid as described (9).

Purification of Synaptic Vesicles—Synaptic vesicles were purified from rat forebrain either through the step of sucrose density gradient centrifugation or further purified by chromatography on CPG beads as described (10).

Assay of TGase Activity by Spermide Incorporation—To assay spermide incorporation, purified synapsin I was incubated for 2 min at 37 °C (initial velocity conditions) in TGase assay buffer containing 1 mM calcium and saturating concentrations of 3H-labeled spermide (200 µM, 2 µCi/sample) in the presence of purified guinea pig liver TGase (10 µg/ml), and processed as described (3). In some cases, TGase-induced incorporation of dansylcadaverine into vesicular proteins was evaluated. Synaptic vesicles were processed as above, except that the incubation was carried out in the presence of 2 mM dansylcadaverine. After gel electrophoresis, the fluorescent bands were visualized by exposure to UV light.

Other Procedures—Protein concentrations were determined by the method of Bradford (11). Statistical analysis was carried out as described (12). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (13). TGase activity was hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** The abbreviations used are: TGase, transglutaminase; TT, tetanus toxin; DMC, N,N'-dimethylated casein; CPG, controlled glass pore; PAGE, polyacrylamide gel electrophoresis; dansyl, 5-dimethylaminoazophenyl-1-sulfonyl.
immunoreactivity was probed in subcellular fractions by SDS-PAGE and immunoblotting with a polyclonal antibody against guinea pig liver TGase followed by 125I-protein A overlay and autoradiography or standard immunoperoxidase staining. In experiments to verify the specificity of immunostaining, identical samples were exposed either to the antibody or to antibody pretreated with excess purified TGase for 60 min at room temperature.

RESULTS AND DISCUSSION

The TGase substrate properties of synapsin I were evaluated by assaying the incorporation of spermidine into purified bovine brain protein (a mixture of synapsins Ia and Ib) in the presence of purified liver TGase (3). The Ia-Ib mixture was indeed an excellent substrate of TGase, exhibiting a $K_m$ of 3.2 $\mu$M and a $V_{max}$ of 40 nmol/mg enzyme/min (Fig. 1a); in fact, it was more active than dimethylcasein (DMC), one of the best TGase substrates known to date (Fig. 1b). In order to ascertain whether both synapsin I isoforms are substrates for TGase, we took advantage of the fact that synapsins Ia and Ib are structurally identical with the exception of a short COOH-terminal domain where the putative consensus sequence for TGase is present in the Ia but not in the Ib isoform (14). By analyzing the $[^{3}H]$spermidine incorporation into the COOH-terminal fragments, obtained from synapsin Ia and Ib by 2-nitro-5-thiocyanobenzoic acid cleavage, we found that the COOH-terminal fragment of synapsin Ia is indeed a much more active substrate than the corresponding fragment of synapsin Ib (Fig. 2a). Interestingly, the dissimilar sensitivity to TGase is the only difference observed to date between the two synapsin I isoforms. Besides cross-linking reactive glutamines to polyamines, TGase can also induce the formation of intra- or inter-molecular bonds between glutamines and lysines of protein substrates (1, 2). Indeed, upon exposure to calcium-activated TGase in the absence of spermidine, covalent synapsin I oligomers of high molecular weight, most of which could not enter the separating gel, were rapidly generated concomitantly with the disappearance of the monomers (Fig. 2b). Both Ia and Ib monomers disappeared, indicating that synapsin Ib, despite the lack of the glutamine-containing consensus sequence and its low activity in the spermidine incorporation assay (see Fig. 2a), can take part in the cross-linking reaction, probably through the participation of lysine residues. The selectivity of action of TGase on synapsin I relative to other synaptic proteins was tested by using purified synaptic vesicles as substrate. TGase did not modify the SDS-PAGE protein pattern of these membranes, with the exception of an 80-kDa doublet (corresponding to synapsins Ia and Ib) and of two bands at 38 and 105 kDa, which were markedly attenuated by TGase (Fig. 3a). Immunostaining with an antisynapsin I antibody confirmed that both synapsins Ia and Ib monomers become cross-linked and move as large aggregates to the top of the separating gel (Fig. 3b). A smaller amount of aggregate was sometimes seen in samples incubated in the absence of added liver TGase, possibly due to the activity of the endogenous enzyme (see text below and Fig. 4). When the reaction was run in the presence of an excess of dansylcadav-
Fig. 4. Rat brain subcellular distribution of TGase immunoreactivity (lower panel) and enzymatic activity (upper panel). Subcellular fractions were prepared as described under "Experimental Procedures" and in Ref. 10. H, homogenate; S1, postnuclear supernatant; S2, postmitochondrial supernatant; P3, microsomes; S3, soluble; L, mitochondrial pellet after hypotonic lysis; LP1, low speed pellet of L; LS1, supernatant of LP1; LP2, crude synaptic vesicle fraction; LS2, supernatant of LP2; FT, flow-through from a controlled pore glass column; CPG-V, pure synaptic vesicles. The TGase activity of each fraction (50 μg of protein/ml) was assayed by spermidine incorporation into 16 μM DMC in the presence of 1 mM calcium under TGase assay conditions (3). Data are means ± S.D. of triplicates, repeated in two separate experiments. TGase immunoreactivity was probed in each fraction by SDS-PAGE and immunoblotting with a polyclonal antibody against guinea pig liver TGase followed by 125I-protein A overlay and autoradiography.

Synapsin I Is Selectively Modified by Transglutaminase

activity by spermidine incorporation into DMC confirmed that catalytically active enzyme is indeed associated with nerve terminal-derived fractions such as synaptosomal cytosol and synaptic vesicles (Fig. 4, upper panel). Synaptic TGases could be activated by calcium in a dose-dependent fashion (EC_{50} = 4.9 μM for the TGase in synaptosomal cytosol; under the same experimental conditions the EC_{50} of purified guinea pig liver TGase was 16 μM). The concentration of TGase in intact nerve terminals was estimated to range between 50 and 100 μg/ml by semiquantitative immunoblotting and assays of enzymatic activity using purified liver TGase as a standard. This amount of enzyme would be sufficient, if fully activated, to rapidly and completely modify synapsin I. Presumably, synaptic TGase is normally kept in check by endogenous inhibitors such as GTP and zinc (2, 18), the latter of which is actively taken up in nerve terminals and concentrated in synaptic vesicles (19). The regulation of nerve ending TGases is an interesting topic in itself and is currently being investigated in our laboratory.

We have recently reported that purified liver TGase is potently activated by TT (3). The effect of TT on the vesicular and cytosolic synaptic TGases, which are likely to be the most relevant for modulation of synapsin I function, was examined. TT markedly increased the TGase activity of synaptic vesicles, both in the presence and in the absence of synaptosomal cytosol, as assessed by [3H]spermidine incorporation into trichloroacetic acid-precipitable endogenous protein substrates (Fig. 5).

The enzymatic activity was dependent on the presence of calcium (3500 cpm incorporated versus 250 cpm in the presence of 20 mM EGTA). The TT dose-response relationship was biphasic, with a lower plateau at concentrations similar to those found to inhibit exocytosis in microinjected Aplysia neurons (20). From 30 to 40% of the spermidine-labeled material was immunoprecipitable by an antisynapsin I antibody, indicating that the substrate specificity of brain TGase is similar to that of the liver enzyme, at least with regard to synapsin I. The TGase from synaptic cytosol was also stimulated by TT, as assessed by using DMC as substrate. The degree of stimulation was, however, lower with respect to that observed for synaptic vesicle-associated TGase (not shown). The mechanism of brain TGase activation by TT is still to be determined. It is likely to involve high affinity specific

FIG. 5. Activation of endogenous TGase by TT. The enzyme was assayed by spermidine incorporation into the proteins of sucrose gradient-purified synaptic vesicles (11) (300 μg/ml), incubated for 30 min in TGase assay buffer (3) containing 500 μM calcium. Vesicles were preexposed to TT, 10 mM dithiothreitol, and 10 mM NaCl for 2 min before addition of the other reagents. Neither DMC nor exogenous TGase were present. Comparable levels of Tt-stimulated activity were observed in experiments in which synaptic cytosol (700 μg of protein/ml, final concentration) was added to the reaction mixture containing synaptic vesicles. Data are means ± S.D. of triplicates, repeated in 4 separate experiments.
binding between the two proteins (3), possibly followed by a proteolytic event, as suggested by the recent observation that TT contains the "signature" motif of metalloendopeptidases and is inhibited by blockers of these enzymes (20-23).

One substrate for the proteolytic activity of TT has been recently identified within nerve terminals as the synaptic vesicle protein synaptobrevin 2 (23). Since TGase is present in nerve terminals and at least some TGase isoforms are known to undergo proteolytic activation (18), they might represent an additional target for the toxin and induce a covalent modification of another synaptic vesicle-specific protein, synapsin I.

How would TGase-induced covalent synapsin I modification(s) affect secretion? Synapsin I coats secretory vesicles and links them to the actin-based cytoskeleton in a phosphorylation-dependent manner, thus probably regulating vesicle availability for exocytosis (5-7). The formation of synapsin I-containing oligomers or the incorporation of cytosolic polyamines induced by TGase could inhibit the phosphorylation-dependent modulatory activity of synapsin I, thereby preventing the release of synaptic vesicle from the cytoskeleton. In this respect, the recent report that TT inhibits the depolarization-stimulated synapsin I phosphorylation and redistribution from the vesicle surface into the cytosol supports a role for synapsin I in the neuroapalytic action of TT (24). Synapsin I is a neuron-specific protein (5, 25). Its excellent TGase substrate properties make it a likely major target of this enzyme in nerve endings and might be one of the causes of the sensitivity of neurons to TT. Non-neuronal secretory cells, where synapsin I is absent or scarce, are generally insensitive to TT. Well studied exceptions are chromaffin cells; however, actin and fodrin, cytoskeletal proteins reported to regulate secretion in chromaffin cells (26, 27) are substrates, albeit weaker than synapsin I, of TGase (28, 29). In addition, chromaffin cells have been shown to express other isoforms of the synapsin family, namely synapsin IIa and IIb (30). The possibility that these isoforms are also substrates for TGase represents an interesting topic for future research.

TGase is normally silent in the cell but can be activated by large cytosolic calcium increases. One regulatory role of TGase might be to prevent excessive release of neurotransmitters induced by abnormally prolonged calcium entry (16). “Fatigue” of neurotransmitter release following sustained stimulation has indeed been reported (31). TT might parasitize and amplify this safeguard inhibitory mechanism to induce block of exocytosis even under resting conditions.

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