Tyrosine Phosphorylation Modulates the Activity of Clostridial Neurotoxins*

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Antonio V. Ferrer-Montiel, Jau Me M. Canavesil, Bibhu R. DasGupta, Michael C. Wilsoni, and Mauricio Montalii

*From the Department of Biology, University of California San Diego, La Jolla, California 92036-0366, and the University of Wisconsin, Madison, Wisconsin 53706, and the Department of Neuropharmacology, Scripps Research Institute, La Jolla, California 92037

Clostridial neurotoxins' metalloprotease domain selectively cleaves proteins implicated in the process of synaptic vesicle fusion with the plasma membrane and, accordingly, blocks neurotransmitter release into the synaptic cleft. Here we investigate the potential modulation of these neurotoxins by intracellular cascades triggered by environmental signals, which in turn may alter its activity on target substrates. We report that the nonreceptor tyrosine kinase Src phosphorylates botulinum neurotoxins A, B, and E and tetanus neurotoxin. Protein tyrosine phosphorylation of serotypes A and E dramatically increases both their catalytic activity and thermal stability, while dephosphorylation reverses the effect. This suggests that the biologically significant form of the neurotoxins inside neurons is phosphorylated. Indeed, in PC12 cells in which tyrosine kinases such as Src and PYK2 are highly abundant, stimulation by membrane depolarization in presence of extracellular calcium induces rapid and selective tyrosine phosphorylation of internalized light chain, the metalloprotease domain, of botulinum toxin A. These findings provide a conceptual framework to connect intracellular signaling pathways involving tyrosine kinases, G-proteins, phosphoinositides, and calcium with the action of botulinum neurotoxins in abrogating vesicle fusion and neurosecretion.

Clostridial neurotoxins produced by Clostridium botulinum and Clostridium tetani are some of the most potent neurotoxic agents known to man and the causative agents of botulism and tetanus (1, 2). Paradoxically, botulinum neurotoxin (BoTx)1 A is also an effective therapeutic agent for certain neuromuscular disorders associated with muscle spasms such as strabismus and dystonias (3). BoTxA and its related serotypes produce flaccid paralysis in skeletal muscles by blocking exocytotic acetylcholine release at the neuromuscular junction (1, 2).

Botulinum neurotoxins are soluble proteins, produced by the bacteria as a single chain of M, 150,000 (1, 2). The holotoxin undergoes proteolytic cleavage, yielding a fully active dichain polypeptide composed of a heavy chain (HC, M, 100,000) and a light chain (LC, M, 50,000) linked by a disulfide bond. The LCs of these neurotoxins are Zn2+ metalloproteases that selectively cleave proteins involved in targeting to and fusion with the plasma membrane of presynaptic vesicles, thereby blocking neurotransmitter release into the synaptic cleft (4–12).

The long term paralytic effects exerted by these neurotoxins, from several days in cell culture up to several weeks even months in animals and humans (1, 2, 13, 14), suggest important differences between the in vivo and in vitro forms of the toxin implying potential modulation of the neurotoxins by intracellular regulatory processes, such as protein phosphorylation. It is noteworthy that neurons and neuroendocrine cells have relatively high levels of tyrosine kinases (15–18), which are thought to play a key role in neuronal differentiation (15–17).

This paper reports that dostridal neurotoxins are substrates of protein-tyrosine kinases and that tyrosine phosphorylation produces a prominent augmentation of their proteolytic activity and thermal stability, and suggests that the phosphorylated form of these neurotoxins may be the physiological active species within cells. Demonstration of intracellular tyrosine phosphorylation of the neurotoxin by selective stimulation of neuroendocrine cells underscores that tyrosine phosphorylation may indeed be a missing link in the biology of dostridal neurotoxins.

EXPERIMENTAL PROCEDURES

Phosphorylation of Clostridial Neurotoxins—Botulinum toxins were purified as described (19). The tyrosine phosphorylation reaction (final volume of 20–40 l) contained 20 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 20 mM Hepes (pH 7.4), 3–6 units of Src kinase (Upstate Biotechnology, Inc.), 0.1 mM ATP, and 4 Ci of [γ-32P]ATP (3,000 Ci/mmol). Non-phosphorylated toxin refers to samples in which Src kinase was omitted. Reactions proceeded at 30 °C for the indicated times and were terminated by addition of SDS-PAGE sample buffer or 200 mM peptide A (Peninsula Laboratories), a specific inhibitor of Src. PKC (20 ng Upstate Biotechnology, Inc.) phosphorylation buffer was: 20 mM Hepes, pH 7.4, 0.1 mM CaCl2, 10 mM MgCl2, 0.25 mM l-ω-phosphatidylserine, and 1 mM dithiothreitol. Phosphorylation by PKA (4 units; Sigma) was as described (20). Phosphorylated samples subjected to SDS-PAGE on 12% gels were stained with Coomassie Blue R-250, destained, dried, and exposed to Kodak X-Omat AR x-ray film. Stoichiometry of phosphorylation was obtained as described (20) using 10% gels to minimize the contribution of Src autoc phosphorylation.

Dephosphorylation of Botulinum Neurotoxin A—Dephosphorylation reactions (15 l) contained 100 nM tyrosine-phosphorylated BoTxA and 50 ng of PTP-1B-agarose conjugate (Upstate Biotechnology, Inc.). Reactions proceeded at 37 °C for indicated times and terminated by centrifugation at 14,000 rpm, 1 min. The extent of BoTxA dephosphorylation was assessed by SDS-PAGE and autoradiography.

In Vivo Cleavage of SNAP-25—In vitro translation of the cDNA clone coding for SNAP-25 from mouse brain (21) in the presence of [3S]methionyl associated protein of 25 kDa; PTP-1B, protein-tyrosine phosphatase 1B; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PKC, protein kinase C; PKA, cAMP-dependent protein kinase.
Tyrosine phosphorylation of clostridial neurotoxins. a, Src phosphorylates the HC and LC of BoTxA, BoTxB, BoTxE, and TeTx. The autoradiograms display incorporation of $^{32}$P into both LC (M$_r$ = 50,000) and HC (M$_r$ = 100,000). Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phospho-tyrosine mAb (clone 4G10; Upstate Biotechnology, Inc.) (4 phospho-terminal) and analyzed by immunoblot or used for immunoprecipitation. Tyrosine-phosphorylated proteins were precipitated with anti-BoTxA HC mAb 6, and an anti-SNAP-25 mAb (Sternberger Monoclonals). Bands were visualized using the ECL system.

The extent of phosphorylation was time dependent, with an apparent half-maximum after ~15 min (Fig. 2, a and c). At saturation, the stoichiometry of phosphorylation was ~1.0 mol of $^{32}$P/mol of BoTxA chain for both the HC and the LC, suggesting a predominant phosphorylation site in each chain. The reaction was reversible as demonstrated by dephosphorylation in presence of PTP-1B and of the augmentation of BoTxA protease activity by protein tyrosine phosphatase activity (Fig. 2, d). Neurotoxin activity was quantified using the ECL system. For immunoprecipitation analyses, cells were incubated with 100 nM BoTxA, 3 units) at 30°C for the indicated time periods, as described in Fig. 1. The middle band (M$_r$ = 55,000) corresponds to autophosphorylated Src. b, tyrosine-phosphorylated BoTxA is enzymatically dephosphorylated by PTP-1B. Tyrosine-phosphorylated BoTxA was incubated with PTP-1B and dephosphorylated by BoTxA as function of phosphorylation time by Src. c, tyrosine phosphorylation of BoTxA is accompanied by an increase in the extent of tyrosine phosphorylation, and, consequently, an increase in protease activity. Fluorograms display the mobility shift of both LC and HC (Fig. 1, d). Neurotoxin activity was quantified using the ECL system. For immunoprecipitation analyses, cells were incubated with 100 nM BoTxA (prepared in PBS) in RPMI 1640 for 12 h at 37°C, 5% CO$_2$.

Western Blot and Immunoprecipitation Analysis—For immunoblots, protein bands were electro transferred onto nitrocellulose membranes and blocked with 3% bovine serum albumin (23). Proteins were detected using the ECL system. For immunoprecipitation analyses, cells were stimulated with PBS supplemented with indicated additives, washed with cold PBS, lysed with cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM ß-glycerophosphate, 1 mM sodium bisulfite, 1 mM sodium selenite, and 1 mM sodium selenite). Immunoprecipitates were then subjected to gel electrophoresis and transferred to nitrocellulose membranes. Bands were visualized using the ECL system.

RESULTS AND DISCUSSION

Protein tyrosine phosphorylation is a prominent covalent modification of the diphtheria 50-kDa clostridial neurotoxins. As shown in Fig. 1a, both the 50-kDa LC and 100-kDa HC of botulinum neurotoxin serotypes A, B, and E, and the structurally homologous tetanus neurotoxin (TeTx) were found to be specifically phosphorylated by the tyrosine kinase Src. Incubation of the neurotoxins in presence of Src and $[\gamma-32P]$ATP for 60 min resulted in the conspicuous incorporation of $^{32}$P on both the HC and the LC of BoTxA, BoTxE, and TeTx. Immunoblotting with anti-phosphotyrosine specific mAbs displays the occurrence of phosphoryrosines according to the banding pattern that is characteristic of these neurotoxins (Fig. 1b). BoTxA was not effectively phosphorylated by protein-serine/threonine kinases such as PKA, whereas phosphorylation, especially of the HC, was obtained by PKC (Fig. 1c). The extent of tyrosine phosphorylation was time dependent, with an apparent half-maximum after ~15 min (Fig. 2, a and c). At
lanes the timedependence of proteolysis in the right section (bottom) of SNAP-25 substrate showed that the proteolytic activity decayed substantially in a time- and temperature-dependent manner. Remarkably, phosphorylated neurotoxin exhibited a pronounced stabilization, evidenced by the delayed inactivation of its proteolytic activity at the temperatures studied. After exposure to 37°C for 10 h, phosphorylated neurotoxin exhibited ≈60% protease activity, whereas unphosphorylated neurotoxin was virtually inactive in ≈2 h. Protection of protease activity provides evidence of thermal stabilization consequent to the covalent modification of the toxin by tyrosine phosphorylation.

These results raised the intriguing question of whether tyrosine phosphorylation could restore the activity of thermally inactivated neurotoxin. To examine this issue, BoTxA was first incubated at 37°C for 6 h to ensure ≈90% inactivation (Fig. 3b). Neurotoxin was then separated in two samples, one of which was phosphorylated by Src for 60 min at 30°C, prior to the assay of proteolytic activity. As shown in Fig. 3c, tyrosine phosphorylation reconstituted the protease activity of previously inactivated neurotoxin whereby phosphorylated toxin reached maximal activity at lower [BoTxA] and shorter reaction times than the unmodified neurotoxin. Tyrosine phosphorylation, therefore, appears to regenerate the catalytic activity of the LC, presumably by promoting the proper refolding and hence active conformation of the protease, or by activating the small amount of folded enzyme that remains after thermal inactivation.

To investigate the biological significance of these in vitro modifications of BoTxAs, we examined whether the neurosecretory PC12 cells would effectively phosphorylate internalized BoTxA at tyrosine residues. PC12 cells express high levels of Src, which can be exacerbated by exposure to NGF (24, 25). PC12 cells were therefore incubated with 100 nM BoTxA for 12 h before lysis, and cell extracts were probed with an anti-BoTxA LC mAb and an anti-SNAP-25 mAb to assess neurotoxin internalization and cytosolic translocation of its LC. As shown in Fig. 4a, BoTxA was internalized (top) and the LC translocated into the cytosol where it cleaved SNAP-25 (middle). In NGF-differentiated PC12 cells exposed to BoTxA, a faint band comigrating at the position expected for BoTxA LC was labeled with the anti-phosphotyrosine mAb, suggesting that BoTxA LC is tyrosine-phosphorylated within these cells. PC12 cells exhibit Ca2+ influx, which can be exacerbated by NGF (26); moreover, it is interesting that PYK2, a protein-tyrosine kinase abundant in PC12 cells, is activated by stimuli that elevate the intracellular Ca2+ level (18), and Ca2+ influx mimics the action of NGF on neurite growth (15–17). To examine whether induction of Ca2+ influx would enhance phosphorylation of internalized BoTxA, PC12 cells were incubated with 100 nM BoTxA, lysed, and tyrosine-phosphorylated proteins immunoprecipitated with the anti-phosphotyrosine mAb followed by SDS-PAGE. As shown in Fig. 4a (bottom), immunoblots probed with monoclonal antibodies (mAbs) against tyrosine-phosphorylated proteins revealed a phospho-protein band at the position of the LC of BoTxA, indicating that tyrosine phosphorylation is induced in PC12 cells by exposure to BoTxA.

The modulation of protease activity produced by protein tyrosine phosphorylation is accompanied by an increase in thermal stability of the neurotoxins (Fig. 3b). Incubation of phosphorylated (P) and unphosphorylated (U) forms of the neurotoxins at 22°C and 37°C prior to the addition of the SNAP-25 substrate showed that the proteolytic activity decayed substantially in a time- and temperature-dependent manner. Remarkably, phosphorylated neurotoxin exhibited a pronounced stabilization, evidenced by the delayed inactivation of its proteolytic activity at the temperatures studied. After exposure to 37°C for 10 h, phosphorylated neurotoxin exhibited ≈60% protease activity, whereas unphosphorylated neurotoxin was virtually inactive in ≈2 h. Protection of protease activity provides evidence of thermal stabilization consequent to the covalent modification of the toxin by tyrosine phosphorylation.

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with an anti-BoTxA LC mAb revealed a prominent band corresponding to the LC only in cells treated with BoTxA after depolarization with 75 mM KCl leading to the elevation of [Ca\(^{2+}\)]. Omission of Ca\(^{2+}\) markedly diminished the extent of LC tyrosine phosphorylation (Fig. 4b). Together, these results indicate that BoTxA internalized by NGF-differentiated PC12 cells undergoes strong phosphorylation by protein-tyrosine kinases. Additional studies are needed to assess the physiological significance of the in vivo phosphorylation of clostridial neurotoxins.

These new findings that clostridial neurotoxins are substrates of protein-tyrosine kinases, and that tyrosine phosphorylation results in prominent augmentation of BoTx proteolytic activity and thermal stability, suggest that the phosphorylated form of these neurotoxins may be the physiologically active species within neurosecretory cells. Indeed, demonstration of intracellular tyrosine phosphorylation of BoTxA by selective stimulation of PC12 cells provides strong support to this idea. To our knowledge, the results show for the first time that a family of bacterial neurotoxins is regulated by protein tyrosine phosphorylation. This previously unrecognized modification of BoTxA may therefore provide a mechanism to explain why the in vivo neuroparalytic effects of a single dose of BoTxA can persist for several days and can account for documented discrepancies between in vitro and in vivo effects of BoTx (1–3, 13, 14). These findings also raise the possibility that the role played by the tyrosine phosphorylation of BoTxA and E, which disable the vesicular fusion process required for neurotransmission, may be subject to regulation by intracellular signaling pathways that ultimately affect vesicular traffic. These cascades involve tyrosine kinases/phosphatases, G-proteins, phosphoinositides, and calcium (15–18, 27–31). The “calcium connection” is significant, as it is known that tyrosine kinase activity is modulated by increases in [Ca\(^{2+}\)], which may be evoked by extracellular stimuli and, notably, that tyrosine kinases such as Src and PYK2 are highly abundant in brain and neuroendocrine cells (4, 15–18). The potentiation of BoTxA and E protease activity by tyrosine phosphorylation suggests that a focus on intracellular modulation of tyrosine kinases and phosphatases as a new means of intervention in botulinum neurotoxicity deserves serious consideration.

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