H_C fragment (C-terminal portion of the heavy chain) of tetanus toxin activates protein kinase C isoforms and phosphoproteins involved in signal transduction

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A recent report [Gil, Chaib-Oukadour, Pelliccioni and Aguilera (2000) FEBS Lett. **481**, 177–182] describes activation of signal transduction pathways by tetanus toxin (TeTx), a Zn^{2+}-dependent endopeptidase synthesized by the *Clostridium tetani* bacillus, which is responsible for tetanus disease. In the present work, specific activation of protein kinase C (PKC) isoforms and of intracellular signal-transduction pathways, which include nerve-growth-factor (NGF) receptor trkA, phospholipase C(PLC)γ-1 and extracellular regulated kinases (ERKs) 1 and 2, by the recombinant C-terminal portion of the TeTx heavy chain (H_C-TeTx) is reported. The activation of PKC isoforms was assessed through their translocation from the soluble (cytosolic) compartment to the membranous compartment, showing that clear translocation of PKC-α, -β, -γ and -δ isoforms exists, whereas PKC-ϵ showed a slight decrease in its soluble fraction immunoreactivity. The PKC-ζ isoform showed no consistent response. Using immunoprecipitation assays against phosphotyrosine residues, time- and dose-dependent increases in tyrosine phosphorylation were observed in the trkA receptor, PLCγ-1 and ERK-1/2. The effects shown by the H_C-TeTx fragment on tyrosine phosphorylation were compared with the effects produced by NGF. The trkA and ERK-1/2 activation were corroborated using phospho-specific antibodies against trkA phosphorylated on Tyr^{490}, and antibodies against Thr/Tyr phosphorylated ERK-1/2. Moreover, PLCγ-1 phosphorylation was supported by its H_C-TeTx-induced translocation to the membranous compartment, an event related to PLCγ-1 activation. Since H_C-TeTx is the domain responsible for membrane binding and lacks catalytic activity, the activations described here must be exclusively triggered by the interaction of TeTx with a membrane component.

Key words: clostridial neurotoxins, rat brain, signal transduction, synaptosomes.

INTRODUCTION

Tetanus toxin (TeTx), one of the most lethal toxins known, is a protein with Zn^{2+}-dependent proteolytic activity and is produced by the bacilli *Clostridium tetani* under strictly anaerobic conditions [1]. TeTx is synthesized as a single polypeptide chain of 150 kDa and is subsequently activated by a bacterial endopeptidase to generate a dipeptide toxin. The toxigenic light chain (50 kDa) remains linked by a disulphide bridge to the binding domain of the targeting heavy chain (100 kDa). The heavy chain can be disrupted into two fragments by the protease papain, which correspond to its C-terminal part (H_C fragment, residues 864–1315 of TeTx; 50 kDa) and to its N-terminal part (50 kDa). The H_C domain is responsible for TeTx binding to the cell membrane [2]. Although the N-terminal of the H_C-TeTx is similar in structure to many lectins, deletion mutagenesis studies suggest that the C-terminus is essential for cell- and ganglioside-binding activity [3]. The main targets of TeTx are both the central and peripheral nervous systems (for a review see [4]), where inhibition of neurotransmitter release occurs through the selective cleavage of synaptobrevin II, a protein involved in synaptic vesicle docking and neurotransmitter release [5]. Other molecular mechanisms underlying TeTx toxicity have been proposed recently. TeTx can activate a Ca^{2+}-dependent, GTP-modulated transglutaminase [6], thus acting synergistically on neurotransmitter blocking. It has been demonstrated that TeTx can partially block neurotransmitter release by stimulating a transglutaminase activity, even when Zn^{2+}-dependent metalloprotease activity is abolished [7]. The first implication of TeTx in signal transduction pathways arose when TeTx-treated macrophages and spinal cord cells of mice manifesting generalized tetanus intoxication showed a diminution in protein kinase C (PKC) activity [8]. Later, our group described activation and subsequent down-regulation of PKC after intracerebral injection of TeTx into adult and neonatal rat brain [9]. PKC translocation appeared before the classic symptoms of tetanus neurotoxicity in the perinatal rat [10]. The increase in polyphosphoinositide hydrolysis in rat cerebral cortex preparations treated with TeTx indicates activation of phospholipases, an effect directly related to PKC activation [11]. This PKC activation is paralleled by an increase in 5-hydroxytryptamine levels [12], an effect which has recently been related to inhibition of the 5-hydroxytryptamine re-uptake produced by TeTx and by its H_C fragment [13]. These data indicate that TeTx is a powerful toxin that acts by different mechanisms which induce changes in relevant processes in the central nervous system.

The neurotrophin family comprises nerve growth factor (NGF), brain-derived neurotrophic factor and neurotrophins 3

Abbreviations used: TeTx, tetanus toxin; H_C-TeTx, C-terminal half of the TeTx heavy chain; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; PKC, protein kinase C; PLC, phospholipase C.

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and 4/5 [14], which exert their effects through the interaction with membrane receptors, so-called trk receptors. NGF binds specifically to the trkA receptor, whereas brain-derived neurotrophic factor and neurotrophins 4/5 bind to trkB and neurotrophin 3 to trkC (for a review see [15]). In addition, neurotrophins can bind non-specifically to a 75 kDa membrane receptor ($p75^{NTR}$), but NGF seems to be uniquely able to activate it [16]. Binding of neurotrophin to its trk receptor activates the protein kinase intrinsic to the receptor, leading to autophosphorylation of tyrosine residues of the receptor, which allows recognition of the receptor by several intracellular signalling proteins that contain Src homology domains, such as phospholipase C (PLC)γ-1, Shc proteins and the p85 PtdIns(1,4,5)P_3 kinase subunit (for a review see [17]). Such interactions lead, by means of a kinase cascade, to the activation of the extracellular regulated kinases (ERKs), a family of protein serine/threonine kinases of which the best characterized members are ERK-1 (p44) and ERK-2 (p42), on threonine and tyrosine residues [18]. Once activated, ERK phosphorylates and activates other protein kinases, in addition to a set of effector proteins, which includes cytoskeletal proteins and transcription factors [19]. ERK activity can also be enhanced by direct action of PKC on Raf kinase [20]. PKC is an extended family of protein Ser/Thr kinases, especially abundant in the nervous system, and is composed of homologous isoforms which can be divided into three subfamilies, depending on their activation requirement. (1) Classical PKCs (α, βI, βII and γ isoforms); (2) novel PKCs (δ, ϵ, θ, and η isoforms) and (3) atypical PKCs (ζ and ι isoforms) [21]. Some physiological activators can cause the translocation of PKC activity from the cytosolic fraction to the membrane [22], initially resulting in activation followed by down-regulation, by means of a not yet well-known proteolytic pathway [23]. One relevant feature of trk receptors is the ability to communicate their signal in a retrograde manner from distal axons to neuron cell bodies. This communication is exerted through the endocytosis of activated receptors and formation of signalling endosomes [24]. Since both TeTx and its H_C fragment are internalized through an endocytic mechanism and are subsequently retroaxonally transported, the possibility of an interaction between the TeTx and trk receptors arises. This mechanism could be used by *Clostridium* in order to transport the TeTx to the central nervous system.

In a recent publication, the activation of several enzymes involved in signal transduction by the whole TeTx protein (trkA, PKC isoforms, ERK-1/2 and PLCγ-1) is described [25]. In the present study, the existence of a differential activation of PKC isoenzymes by the H_C fragment of TeTx, which has been described as being responsible for toxin binding to cellular membrane, is addressed. The analysis of several tyrosine-phosphorylated proteins reveals the stimulation of PLCγ-1 tyrosine-phosphorylation and translocation to the membrane, as well as trkA activation. The parallel activation of ERK-1/2 is a further observation that indicates the triggering of signal-transduction pathways by TeTx by means of its H_C fragment.

MATERIALS AND METHODS

Materials

Polyclonal antibodies against PKC isoforms α, γ and ζ and Protein A–agarose beads were purchased from Boehringer Mannheim Gmbh (Mannheim, Germany). A specific antibody against phosphotyrosine (Clone PY20) was obtained from Zymed Laboratories Inc. (San Francisco, CA, U.S.A.). Monoclonal antibodies against PLCγ-1 and polyclonal anti-trkA were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Phospho-p44/42 mitogen-activated protein kinase (MAPK) (ERK-1/2) E10 monoclonal antibody and phospho-specific trkA (Tyr^{490}) antibody were from New England BioLabs (Beverly, MA, U.S.A.). Monoclonal antibody against ERK-1 and anti-mouse and anti-rabbit secondary antibodies conjugated with horse-radish peroxidase and monoclonal antibodies against PKC isoforms β, δ and ϵ were from Transduction Laboratories (Lexington, KY, U.S.A.). Despite anti-ERK-1 antibody being described as specific for this kinase, it also detected ERK-2, although with less potency. NGF 2.5S was supplied by Alomone Labs (Jerusalem, Israel). All electrophoresis reagents were of analytical grade and were from Pharmacia Biotech (Uppsala, Sweden).

Expression and purification of H_C-TeTx

Escherichia coli M15 cells were induced to express a pQE3 (Qiagen, Chatsworth, CA, U.S.A.)-derived expression vector containing the cDNA for a H_C-TeTx–His_6 fusion protein by addition of 0.4 mM isopropyl β-D-thiogalactoside. Cells were pelleted, after 4 h, by centrifugation at 6000 g for 10 min, resuspended in 50 mM NaH_2PO_4/300 mM NaCl (pH 8), and sonicated with a probe sonicator, on ice, for two 60 s periods. The suspension was centrifuged at 15000 g for 20 min, and the clear supernatant was applied to a Ni-NTA–agarose precast column (Qiagen). Protein purification was carried out according to the manufacturer's instructions. Briefly, after a washing step with 50 mM NaH_2PO_4/300 mM $CaCl_2$/10% (v/v) glycerol (pH 6), the attached protein was eluted with a 50–300 mM imidazole gradient. Fractions (1 ml) were collected, separated by SDS/PAGE and the gels were stained with Coomassie Blue. Those fractions containing purified H_C-TeTx were concentrated by centrifugation using ultrafree-MC filtration tubes with a molecular mass cut-off of 10000 (Millipore, Bedford, MA, U.S.A.), and stored in aliquots at −80 °C.

Preparation of synaptosomes from rat brain

All experiments were performed with a crude synaptosomal fraction (P_2) prepared from Sprague–Dawley, 4-to-6-week-old rat brains, as described previously [26] with slight modifications. The whole brain was homogenized in 40 vol. (wt/vol.) of phosphate buffer (pH 7.4) supplemented with 0.32 M sucrose. Homogenization was performed with 12 strokes (900 rev./min) of a Potter homogenizer with a Teflon pestle (0.1–0.15 mm clearance). The homogenate was centrifuged at 1000 g for 5 min at 4 °C. The resultant supernatant was centrifuged at 12000 g for 20 min. The crude synaptosomal pellet obtained from one brain was gently resuspended in 7 ml of Krebs–Ringer bicarbonate buffer containing 125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 10 mM glucose, gassed before use with a mixture of O_2/CO_2 (19:1) for 20 min, and the pH was adjusted to 7.4.

Subcellular fractionation

Where subcellular fractionation was required, after each treatment the synaptosomes were collected by centrifugation and resuspended in 0.5 ml of homogenization buffer [20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 1 mM Na_3VO_4, 50 mM NaF and 2 mM PMSF containing 10 μg/ml leupeptin and 25 μg/ml aprotinin] and disrupted by sonication in a sonic dismembrator (Dynatech). The homogenate was centrifuged at 100000 g for 1 h to separate the soluble fraction (cytosolic compartment) from the particulate fraction (membranous compartment). The precipitated fraction

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(pellet) was resuspended to the original volume in homogenization buffer supplemented with Triton X-100 (0.3 % final concentration), and subsequently sonicated and kept for 1 h at 4 °C. The extract was centrifuged for 1 h at 100000 ***g*** and the resulting supernatant was considered the particulate fraction (membranous compartment).

Immunoprecipitation and immunoblotting analysis

The synaptosome suspension was diluted to a final protein concentration of 1 mg/ml, and divided into the necessary aliquots. After treatment, synaptosomes were collected by centrifugation and the reaction medium was removed. Next, 1 ml of homogenization buffer supplemented with 0.3 % Triton X-100 was added and synaptosomes were disrupted by sonication (three times for 10 s each). For immunoprecipitation, 1 mg of total protein was incubated in the presence of 4 μg of antibody overnight at 4 °C, with gently rocking. The immunocomplex was then recovered by adding 50 μl of washed Protein A–agarose bead slurry (25 μl of packed beads) with gentle rocking for 2 h at room temperature. The agarose beads were collected by centrifugation in a microcentrifuge and the supernatant was removed. The beads were washed three times with ice-cold PBS and resuspended in 100 μl of 2 × reducing sample buffer [62 mM Tris/HCl, 0.1 M sucrose, 2 % (v/w) SDS, 2 mM EDTA, 50 mM dithiothreitol, 0.02 % Bromophenol Blue] and boiled for 2 min. The agarose beads recovered by centrifugation and 15 μl of each sample were subjected to SDS/PAGE. The separated proteins were transferred on to a PVDF membrane (Millipore) using a Mini TransBlot Cell II (Bio-Rad, Hercules, CA, U.S.A.) at 100 V for 1 h. The blotting buffer used contained 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20 % (v/v) methanol (pH 8.3). The membrane filters were blocked for 1 h with PBS supplemented with 0.1 % (v/v) Tween 20 and 5 % (w/v) defatted powdered milk. The membranes were then incubated overnight with the appropriate antibody diluted in blocking buffer, and then for 1 h with a secondary antibody conjugated with horseradish peroxidase diluted in blocking buffer. Several washes with PBS/0.1 % Tween 20 were performed between each of the steps. The Western blots were developed using SuperSignal West Pico chemilunininiscent substrate from Pierce (Rockford, IL, U.S.A.) and exposed to ECL® films (Amersham). Computer-assisted analysis of the bands was performed using a GS700 system (Bio-Rad), and data were processed with a Molecular Analyst image program (Bio-Rad) using a Dell workstation. Repeated scans were taken for correction of film non-linearity.

RESULTS

H_C-TeTx induces differential translocation of PKC isoforms

The classical PKC isoforms (PKC-α, -β and -γ), as well as PKC-δ, were detected in rat brain synaptosomes, and had an apparent molecular mass of 80 kDa, whereas the apparent molecular mass of PKC-ϵ was 110 kDa and of PKC-ζ 68 kDa. The treatment of synaptosomes with 100 nM PMA (results not shown) induced a typical response in PKC isoforms, as described previously [25], proving the reliability of the system. The incubation of synaptosomes with increasing concentrations of H_C-TeTx for 15 min induced changes in the distribution of PKC isoforms (Figure 1). Sustained translocation was observed in PKC-β, -γ and -δ isoforms, without significant loss of total immunoreactivity. The H_C-TeTx fragment induced, at the highest concentration tested (100 nM), a slight translocation of the PKC-α isoform (a 27.6 % loss in the soluble fraction and a 21 % increase in the particulate fraction). A decrease in PKC-ϵ immunoreactivity in the soluble fraction after treatment with the H_C-TeTx fragment at 100 nM was also observed, whereas no significant effect was seen at any concentration with PKC-ζ. In order to determine whether down-regulation exerted by H_C-TeTx exists, time-course experiments were performed. At a fixed H_C-TeTx concentration of 100 nM, only PKC-β showed clear down-regulation between 10 and 30 min, whereas neither PKC-γ nor

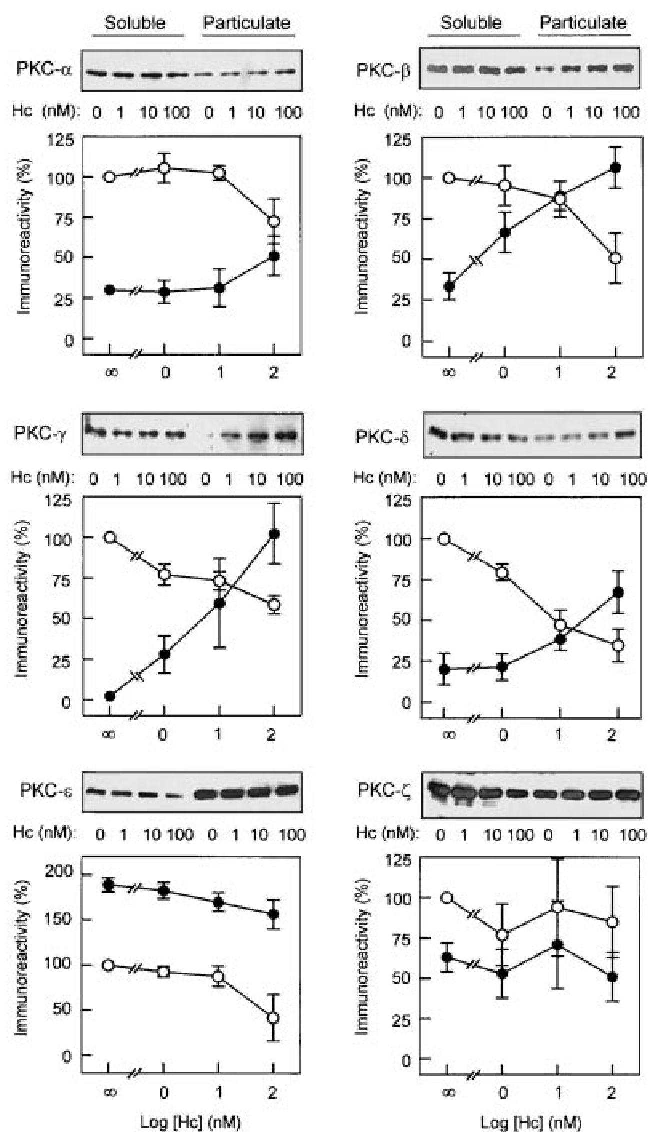

Figure 1 PKC isoform translocation induced by H_C-TeTx

Aliquots of 1 ml from a suspension of synaptosomes (2 mg/ml protein) were exposed to 1, 10 or 100 nM H_C-TeTx (Hc) for 15 min, and soluble and particulate fractions were prepared as described in the Materials and methods section. For SDS/PAGE, protein loading was 20 μg/lane for each fraction. Following SDS/PAGE and transfer of protein on to the PVDF membrane, immunoblotting was carried out and bands were detected by ECL®. The upper panels show representative blots obtained with each immunodetected isoform (PKC-α, -β, -γ, -δ, ϵ and -ζ), as is indicated on the left of each blot. The lower panels show quantification, by laser-scanning densitometry, of the concentration dependence of the immunoreactivity of the soluble (○) and particulate (●) fractions. Data were normalized to the soluble control absorbance arbitrary units, taking this value as 100%. The amounts of immunoreactive PKC isoform in the soluble or particulate fraction (expressed as a percentage of the soluble control value) were plotted against log H_C-TeTx concentration. Data from three separate experiments are expressed as means ± S.D.

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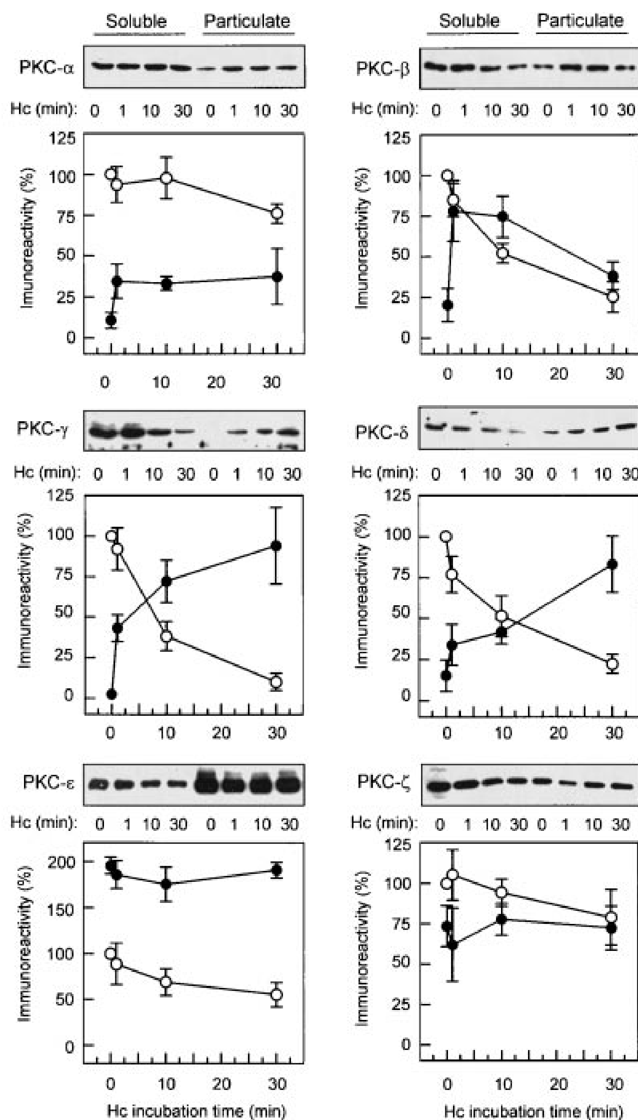

Figure 2 Time course of PKC isoform redistribution by H_C-TeTx

Synaptosomes were treated with vehicle, as a control, for 30 min, or with 100 nM H_C-TeTx (Hc) for 1, 10 or 30 min, at 30 °C. Representative blots of the results obtained for each PKC isoform are included at the top of each panel. Data were normalized, taking the value of the soluble control absorbance arbitrary units as 100%. The amounts of immunoreactive PKC isoform in the soluble (○) or particulate (●) fraction (expressed as a percentage of the soluble control value) were plotted against the incubation time of H_C-TeTx. Data from three separate experiments are expressed as means ± S.D.

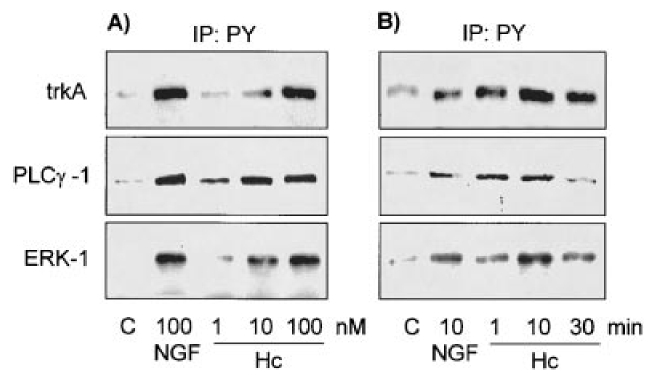

Figure 3 H_C-TeTx-induced tyrosine phosphorylation of trkA, PLCγ-1 and ERK-1

(**A**) Synaptosomes, in suspension in Krebs–Ringer buffer supplemented with 10 mM glucose, were incubated at 30 °C with NGF (100 nM), as a positive control, for 10 min or with various H_C-TeTx (Hc) concentrations (1, 10 or 100 nM) for 5 min. (**B**) Synaptosomes were incubated at 30 °C with NGF (100 nM), as a positive control, for 10 min or for various times (1, 10 or 30 min) with 100 nM H_C-TeTx (Hc). Equal amounts of lysate from each time point (1 mg protein/ml), obtained by sonication, were immunoprecipitated (IP), as described in the Material and methods section, with 4 μg of antibody directed against phosphotyrosine residues (PY). Subsequent blots were probed with anti-trkA, anti-PLCγ-1 or with anti-ERK-1. Each experiment was repeated three times and similar results were obtained.

PKC-δ showed down-regulation up to 30 min, the longest time tested (Figure 2). The PKC-α kinetics shows a rapid onset, since translocation was detectable at 1 min, followed by a modest down-regulation at 10 min and 30 min. In these experiments a loss of PKC-ϵ from the soluble fraction was also found (55 ± 13 % with respect to the control). No consistent translocation was observed in the case of PKC-ζ, even after 30 min of incubation.

Tyrosine phosphorylation of trkA, PLCγ-1 and ERK-1/2 is stimulated by H_C-TeTx

Immunoprecipitation experiments using anti-phosphotyrosine antibodies were performed in order to determine the possible effect of H_C-TeTx on cellular signal transduction pathways which involve tyrosine phosphorylation. In each experiment, whole synaptosomal homogenates were immunoprecipitated using antibodies from the PY20 clone, and the content of several proteins phosphorylated on tyrosine, i.e. trkA, PLCγ-1 and ERK-1, was determined by Western blotting. NGF, as a typical signal transduction activator, was used as a control. The amount of tyrosine phosphorylation that appeared in trkA, PLCγ-1 and ERK-1 was dependent on the H_C-TeTx fragment concentration, and reached a maximum at 100 nM (Figure 3A). The extent of response achieved with H_C-TeTx as activator of phosphorylation was similar to that of NGF, in the cases where the neurotrophin was used as a positive control. The band corresponding to the trkA receptor appeared at approx. 140 kDa molecular mass, and the band corresponding to PLCγ-1 at approx. 150 kDa. The onset of tyrosine phosphorylation was rapid and detectable within 1 min of incubation with H_C-TeTx (100 nM) in the three phosphoproteins tested (Figure 3B), although the maximum amount of signal was seen at 10 min, and had declined at 30 min. In some experiments, a band corresponding to ERK-2 appeared, indicating that parallel activation of ERK-2 also existed (results not shown). The major detection of ERK-1 in immunoprecipitates was because of the greater specificity of this antibody for ERK-1, as explained in the Materials and methods section. The PLCγ-1 phosphorylation was further supported by our finding that TeTx induced a modest, but significant, increase in polyphosphoinositide hydrolysis in rat brain synaptosomes, which is in agreement with a PLC activity enhancement [27].

Time-course experiments and subsequent analyses with antibodies specific for Thr/Tyr-phosphorylated ERK-1 (p44) and ERK-2 (p42) were performed, and the results are shown in Figure 4(A). In these experiments, the induction of p42 and p44 phosphorylation was evident, and appeared after 10 min of incubation. Equal loading of protein between samples was confirmed by detection with antibody described as specific for ERK-1, independent of phosphorylation state, although the antibody recognized both p44 and p42 when the amount of

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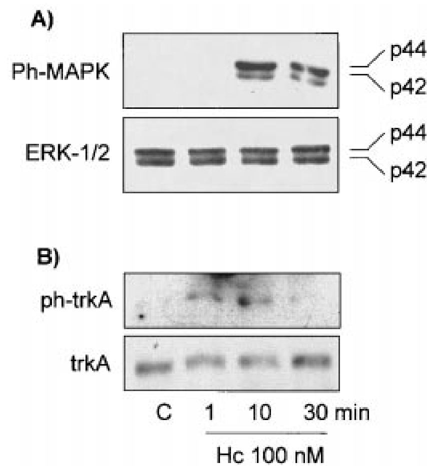

Figure 4 H_C-TeTx increases phosphorylation of ERK-1/2 and of trkA

Synaptosomes were incubated at 30 °C with the H_C-TeTx (Hc) fragment (100 nM) for various times (1, 10 or 30 min), and 20 μg/lane of total synaptosomal lysates were separated by SDS/PAGE. Protein was transferred to a PVDF membrane and immunoblotted as described in the Materials and methods section. Detection was by ECL®. (**A**) The upper blot was analysed using an antibody specific for Thr/Tyr phosphorylated ERK-1 (p44) and ERK-2 (p42) (phospho-p44/42 MAPK E10 monoclonal antibody; Ph-MAPK), and the lower blot shows the immunoreactivity obtained for the same samples with antibodies specific for ERK-1 and ERK-2 (ERK-1/2), independent of their phosphorylation state. (**B**) The upper blot was analysed using an antibody specific for phosphorylated trkA in Tyr^{490} (ph-trkA), and the lower blot shows the immunoreactivity obtained for the same samples with antibodies specific for trkA, independent of their phosphorylation state. All blots are representative of three separate experiments.

ERK-2 was great enough. Analysis of time-course experiments with an antibody which recognized the Tyr^{490}-phosphorylated trkA receptor indicated that this tyrosine kinase receptor was phosphorylated, at least on Tyr^{490}, in response to 100 nM H_C-TeTx (Figure 4B). These analyses confirm the result observed in Figure 3(A), showing that after 10 min of incubation (time of maximal Tyr^{490} phosphorylation) the signal decreased, indicating dephosphorylation of trkA. The results shown in Figure 4 point to a previous trkA activation (signal appeared after 1 min of incubation) with respect to ERK activation (signal appeared after 10 min) in response to the H_C-TeTx fragment. Equal loading of protein between samples was confirmed by detection with anti-trkA, which was independent of phosphorylation state.

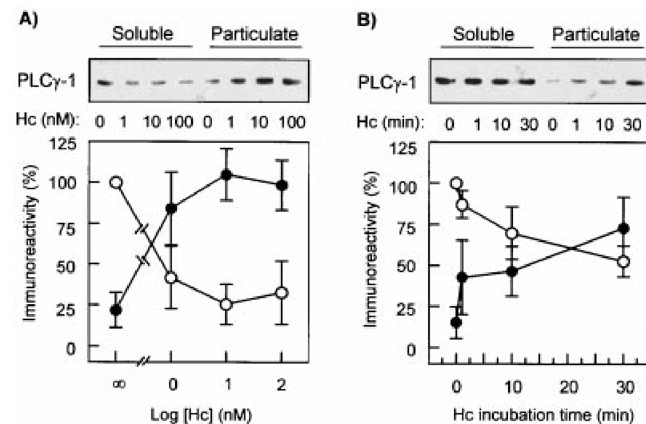

Figure 5 PLCγ-1 translocation induced by H_C-TeTx

(**A**) Dependence of H_C-TeTx concentration of PLCγ-1 translocation. Aliquots of 1 ml from a suspension of synaptosomes (2 mg protein/ml) were exposed to 1, 10 or 100 nM H_C-TeTx (Hc) for 30 min, and soluble and particulate fractions were prepared as described in the Materials and methods section. (**B**) Time course of PLCγ-1 redistribution. Synaptosomes (2 mg protein/ml) were treated with vehicle, as the control, for 30 min, or with 100 nM H_C-TeTx (Hc) for 1, 10 or 30 min, at 30 °C. For SDS/PAGE, in both cases, protein loading was 20 μg/lane for each fractions. Data were normalized, taking the soluble control arbitrary absorbance unit value as 100%. The amount of immunoreactive bands in the soluble or particulate fraction (expressed as a percentage of the soluble control value) were plotted against log H_C-TeTx concentration and incubation time. Data from three separate experiments were expressed as means $\pm$ S.D.

H_C-TeTx induces PLCγ-1 translocation to particulate fraction

A similar series of experiments to those described in Figures 1 and 2 were carried out in order to assess the effect of H_C-TeTx on PLCγ-1 translocation. Treatment of synaptosomes with 1, 10 or 100 nM H_C-TeTx for 30 min indicated that translocation of PLCγ-1 from the soluble fraction to the particulate fraction was concentration dependent (Figure 5A). The translocation of PLCγ-1 at a fixed H_C-TeTx concentration of 100 nM was detectable after 1 min of treatment, and increased further after 10 and 30 min of H_C-TeTx treatment (Figure 5B).

DISCUSSION

The first work on the influence of TeTx on members of the signal transduction pathways described a decrease in total PKC activity in TeTx-treated macrophages and in the spinal cord of mice with generalized tetanus [8]. Later, our group described the TeTx-induced activation of PKC in the perinatal rat brain, which paralleled an elevation in 5-hydroxytryptamine levels [12]. The findings that intraventricular administration of TeTx into adult rats induced translocation and down-regulation of PKC [9], and that treatment of primary neuron cultures from fetal rat brain enhanced translocation of PKC activity and increased poly-phosphoinositide hydrolysis [11] pointed in the same direction. Since PKC activity is the sum of various molecular PKC entities, a detailed investigation into which PKC isoforms are affected by the H_C-TeTx fragment was performed and is described in the present paper. In previous work by our group, it was determined that the H_C-TeTx fragment inhibits 5-hydroxytryptamine uptake in rat brain synaptosomes, an action similar to that of TeTx in its whole di-chain form, as well as in the single-chain form [27,11]. This inhibition of 5-hydroxytryptamine transport is directly related to the TeTx-induced phosphorylation of serine residues in the 5-hydroxytryptamine transporter by PKC [28]. Thus the H_C-TeTx fragment could exert similar effects on some cellular events as entire TeTx. In addition, we have shown now that H_C-TeTx acts in a similar way to the whole toxin in the activation of intracellular signalling pathways, an effect described recently [25]. The triggering of this effect could be related to the interaction of TeTx through its H_C fragment with a still undefined membrane component.

The PKC translocation observed in this work is directly related to the activation of PLCγ-1, assessed through the increase in its phosphotyrosine content, since the products of phospholipases are activators of PKC activity and translocation. Moreover, this PLCγ-1 phosphorylation is in accord with enhancement of polyphosphoinositide hydrolysis by TeTx observed in primary neuron cultures from fetal rat brain and in slices from adult rat cerebral cortex [11]. The increase in PLC activity, which is modest but significant, is very similar to that exerted by NGF in rat brain synaptosomes [29]. Stimulation of inositol phospholipid hydrolysis in response to TeTx has also been detected in rat brain synaptosomes, and at a similar level to that exerted by NGF [27]. NGF is a signalling molecule, which acts through specific PLCγ

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activation by means of its association with trkA, autophosphorylated on tyrosine residues [30]. Thus the modest increase in polyphosphoinositide hydrolysis in synaptosomes by NGF and TeTx described by our group, when compared with the muscarinic agonist carbachol [11,29], can be related to isoform-specific PLC activation. On the other hand, PLCγ-1 translocation has been described in several studies, and this event has been related to its phosphotyrosine content and to the activation of tyrosine kinase receptors [31]. In addition to the probable role of PLCγ-1 in TeTx-triggered pathways, the H_C-TeTx-induced phosphorylation of trkA in Tyr^{490} points to a parallel interaction between Shc proteins and trkA, an event described as important in neuronal differentiation, due to tyrosine kinase receptors. Since Shc adaptor proteins are the first link in a pathway that results in ERK-1/2 activation [18], this putative association with trkA is additionally supported by the ERK-1/2 phosphorylation observed in the present work. In addition to this, changes in the c-*fos* and Fos-like immunoreactivity have been observed in the cortex of rats with TeTx-induced epilepsy [32], and these could be a direct consequence of the activation of the MAPK pathway, since growth-factor-induced c-*fos* expression is activated through transcription factors, such as Elk-1 or SAP-1, which are targets of Ras/MAPK or PKC-mediated phosphorylation [33].

The atoxic H_C fragment of TeTx has the same ability to bind nerve tissue and to be retrogradely transported through synapses and reach the central nervous system as the native whole toxin, but without causing clinical symptoms [34]. On the other hand, studies on dissociated fetal cortical neurons indicate that TeTx is clearly superior to the ganglioside binding H_C-TeTx fragment in its capacity for neuronal binding and internalization [35]. Moreover, the H_C-TeTx fragment also exerts the same effect as the holoprotein in inhibiting 5-hydroxytryptamine uptake in rat brain synaptosomes [27]. This effect is not due to the action on the 5-hydroxytryptamine high-affinity site on the 5-hydroxytryptamine transporter, since binding of [^{3}H]imipramine and 5-[^{3}H]hydroxytryptamine did not change after TeTx treatment [11]. Probably, the inhibition of 5-hydroxytryptamine transport and the activation of transducer enzymes are a consequence of the interaction between TeTx and a membrane component through the H_C-TeTx domain. Although gangliosides containing '1b' structures have been described as high affinity membrane receptors for TeTx [36], a two-receptor model has been postulated, since TeTx binding to cells and neuronal membranes is sensitive to proteases [37]. In this model, the initial toxin binding to gangliosides is followed by lateral movement in the membrane until the toxin reaches the proteic receptor, and is then internalized through a vesicle endocytosis mechanism [38]. The identity of this neurospecific proteic receptor for clostridial neurotoxins is still unknown. Recently, the binding of the H_C fragment from BoNT/A to three proteins of 150, 120 and 75 kDa in rat synaptosomes has been reported [39]. The fact that BoNT/A is the only retroaxonally transported botulinum neurotoxin known is remarkable, although it is transported at a slower rate than TeTx [40]. In the case of TeTx, the C-terminal half of its H_C fragment has been identified as being responsible for neuronal binding and interaction with a putative protein receptor with an apparent molecular mass of 15 kDa [41].

The differential translocation of synaptosomal PKC isoforms in response to TeTx, as well as the induction of phosphorylation of trkA, PLCγ-1 and ERK-1/2 and activation of PKC isoforms, has recently been demonstrated by our group [25]. As a consequence of the results presented in the present work, we can attribute this signal activation to the H_C-TeTx fragment. This observation is consistent with the extensive descriptions in the literature of H_C-TeTx being responsible for toxin binding to the cell membrane, and thus being the trigger of signal transduction pathways by TeTx. The putative interaction between TeTx and trkA would explain the finding of the toxin in endocytic vesicles, as well as representing the way by which TeTx is carried from the injury site to its target tissues, i.e. the peripheral and central nervous systems, since the trk receptors have been described as retroaxonally transported. On the other hand, the activation of signal transducers could represent a collateral effect as a consequence of the binding of the toxin to the trkA receptor. In any case, further studies are required to establish the specific role of trkA in TeTx action, membrane binding and mobility, as well as the implications of the activation of signal transduction pathways by the neurotoxin.

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