C-terminal fragment of tetanus toxin heavy chain activates Akt and MEK/ERK signalling pathways in a Trk receptor-dependent manner in cultured cortical neurons

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Previous publications from our group [Gil, Chaib, Pelliccioni and Aguilera (2000) FEBS Lett. **481**, 177–182; Gil, Chaib, Blasi and Aguilera (2001) Biochem. J. **356**, 97–103] have reported the activation, in rat brain synaptosomes, of several phosphoproteins, such as neurotrophin tyrosine kinase (Trk) A receptor, phospholipase Cγ-1, protein kinase C (PKC) isoforms and extracellular-signal-regulated kinases 1 and 2 (ERK-1/2). In the present study, we examined, by means of phospho-specific antibodies, the activation of the signalling cascades involving neurotrophin Trk receptor, Akt kinase and ERK pathway, in cultured cortical neurons from foetal rat brain, by tetanus toxin (TeTx) as well as by the C-terminal part of its heavy chain (H_C-TeTx). TeTx and H_C-TeTx induce fast and transient phosphorylation of Trk receptor at Tyr^{674} and Tyr^{675}, but not at Tyr^{490}, although the potency of TeTx in this action was higher when compared with H_C-TeTx action. Moreover, H_C-TeTx and TeTx also induced phosphorylation of Akt (at Ser^{473} and Thr^{308}) and of ERK-1/2 (Thr^{202}/Tyr^{204}), in a time- and concentration-dependent manner. The detection of TeTx- and H_C-TeTx-induced phosphorylation at Ser^{9} of glycogen synthase kinase 3β confirms Akt activation. In the extended analysis of the ERK pathway, phosphorylation of the Raf, mitogen-activated protein kinase kinase (MEK)-1/2 and p90Rsk kinases and phosphorylation of the transcription factor cAMP-response-element-binding protein were detected. The use of tyrphostin AG879, an inhibitor of Trk receptors, demonstrates their necessary participation in the H_C-TeTx-induced activation of Akt and ERK pathways, as well as in the phosphorylation of phospholipase Cγ-1. Furthermore, both pathways are totally dependent on phosphatidylinositol 3-kinase action, and they are independent of PKC action, as assessed using wortmannin and Ro-31-8220 as inhibitors. The activation of PKC isoforms was determined by their translocation from the cytosolic compartment to the membranous compartment, showing a clear H_C-TeTx-induced translocation of PKC-α and -β, but not of PKC-ε.

Key words: Akt, cortical neuron, extracellular-signal-regulated kinase (ERK), neurotrophin, tetanus toxin.

INTRODUCTION

Tetanus toxin (TeTx) is among the most powerful bacterial protein toxins known. TeTx induces spastic paralysis by specifically cleaving vesicle-associated membrane protein (VAMP)/synaptobrevin, a SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) protein and a key component of the neurotransmitter exocytosis machinery. TeTx consists of a light chain (molecular mass 50 kDa) responsible for the cytotoxic activity, and a heavy chain (molecular mass 100 kDa), which can be disrupted into two fragments by the protease papain, corresponding to its C-terminal part (H_C fragment, 50 kDa, residues 864–1315 of TeTx) and its N-terminal part (H_N fragment, 50 kDa). The H_C domain is responsible for TeTx binding to the cell membrane. Once TeTx binds to nerve termini of motor neurons, it is retroaxonally transported to the central nervous system (CNS). On release and recapture by interneurons, it blocks the secretion of inhibitory neurotransmitters (i.e. γ-aminobutyric acid and glycine). The TeTx-blocking activity is due to a Zn^{2+}-dependent proteolytic cleavage of VAMP (reviewed in [1]). It is well documented that TeTx binding to neurons requires polysialogangliosides and involves the C-terminal part of the TeTx heavy chain (H_C-TeTx) [2]. However, the necessity for a protein as a co-receptor for TeTx on neurons is highly suspected [3].

Recently, growing evidence from our group suggests that TeTx can activate, through the H_C fragment, intracellular signalling pathways, involving the Trk receptor, extracellular-signal-regulated kinase (ERK) and protein kinase C (PKC) isoforms [4,5]. The neurotrophin family comprises nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 and -4/5, which exert their effects through the interaction with membrane receptors, i.e. the Trk receptors. NGF binds specifically to the TrkA receptor, whereas BDNF and neurotrophin-4/5 bind to TrkB and neurotrophin-3 binds to TrkC (reviewed in [6]). In addition, neurotrophins can bind non-specifically to a 75 kDa membrane receptor ($p75^{NTR}$) [7]. On the other hand, $p75^{NTR}$ is the high-affinity receptor for pro-neurotrophins, which are converted into mature molecules by means of proteolytic cleavage [8]. 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 [9]. Binding

Abbreviations used: BDNF, brain-derived neurotrophic factor; CNC, cortical neuron culture; CNS, central nervous system; CREB, cAMP-response-element-binding protein; DTT, dithiothreitol; E18, embryonic day 18; ERK, extracellular-signal-regulated kinase; GSK3β, glycogen synthase kinase 3β; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PI-3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; TeTx, tetanus toxin; H_C-TeTx, C-terminal part of TeTx heavy chain; Trk, high-affinity tyrosine kinase receptors from neurotrophins; VAMP, vesicle-associated membrane protein.

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of neurotrophin to its Trk receptor activates the protein kinase intrinsic to the receptor, leading to autophosphorylation at tyrosine residues, which allows recognition of the phosphorylated tyrosine residues of the receptor by several intracellular signalling proteins that contain Src homology 2 domains, such as phospholipase Cγ-1 (PLCγ-1), Shc proteins and the p85 phosphatidylinositol 3-kinase (PI-3K) subunit (reviewed in [10]). Such interactions lead to the activation, by means of a kinase cascade, of the Raf/mitogen-activated protein kinase (MAP kinase) kinase (MEK)/ERK pathway. ERK is a family of protein serine/threonine kinases of which the best-characterized members are ERK-1 (p44) and ERK-2 (p42). These become activated after phosphorylation at threonine and tyrosine residues [11]. Once activated, ERK phosphorylates and activates other protein kinases. Among the substrates of ERK is the family of p90 ribosomal S6 kinases (p90Rsk), which is activated by virtually all extracellular signalling molecules that stimulate the ERK pathway. On the other hand, ERK induces phosphorylation of the transcription factor cAMP-response-element-binding protein (CREB), an action mediated by the protein kinases mitogen- and stress-activated kinases 1 and 2 [12]. ERK activity can also be enhanced by the direct action of PKC on Raf kinase [13]. PKC is an extended family of protein serine/threonine kinases, especially abundant in the nervous system, composed of homologous isoforms, which can be divided into three subfamilies depending on their activation requirement: 'classical' PKCs (α, βI, βII and γ isoforms), 'novel' PKCs (δ, ε, θ and η isoforms) and 'atypical' PKCs (ζ and ι isoforms) [14]. Some activators can cause the translocation of PKC from the cytosolic fraction to the membrane, an event directly related to their activation [15].

As has been pointed out previously, activation of Trk receptors can also trigger a pathway initiated by PI-3K, which leads to the activation of Akt, a 60 kDa serine/threonine kinase. It is well documented that Akt is a general mediator of survival signals and that Akt is both necessary and sufficient for survival of eukaryotic cells [16]. In the current model for Akt activation, Akt is first recruited from the cytoplasm to cellular membranes by interaction of its pleckstrin homology domain with phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, which are lipid products of PI-3K [17]. Akt activity is not stimulated by translocation itself but is dependent on phosphorylation at two residues (Ser^{473} and Thr^{308}). Thr^{308} is phosphorylated by the phosphoinositide-dependent kinase 1, which also contains a pleckstrin homology domain [18]. The mechanism of phosphorylation of Ser^{473} remains elusive; although phosphoinositide-dependent kinase 1 is a possible candidate, the integrin-linked kinase and very recently autophosphorylation have also been implicated [19]. Mutational analysis has shown that although Thr^{308} is sufficient to activate Akt, both residues are required for maximal activation [20].

In the present study, new actions of TeTx exerted through the interaction of this toxin with the cellular membrane are described. Since both TeTx and its H_C fragment are internalized through an endocytic mechanism and subsequently retroaxonally transported, the possibility of an interaction between TeTx and the Trk receptors arises. This possible mechanism could be used by *Clostridium* to transport the TeTx to the CNS, without excluding possible advantages for the pathogen in affecting host signalling.

MATERIALS AND METHODS

Materials

Biologically active TeTx was supplied by List Biological Laboratories (Campbell, CA, U.S.A.). Polyclonal anti-TrkB and anti-α-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti-PLCγ-1 was supplied by Upstate Biotechnology (Lake Placid, NY, U.S.A.). A phosphotyrosine-specific antibody (clone PY20) was obtained from Zymed Laboratories (San Francisco, CA, U.S.A.). All of the antibodies raised against phosphorylated residues, i.e. E10 monoclonal antibodies against phospho-p44/42 MAP kinase, polyclonal antibodies against phospho-Akt (Ser^{473} and Thr^{308}), phospho-glycogen synthase kinase 3β (GSK3β) (Ser^{9}), phospho-C-Raf (Ser^{259}), phospho-MEK-1/2 (Ser^{217}/Ser^{221}), phospho-p90Rsk (Ser^{380}), phospho-CREB (Ser^{133}), phospho-specific Trk receptor (Tyr^{490} and Tyr^{674}/Tyr^{675}) and anti-Akt antibody were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Monoclonal antibody raised against ERK-1 and anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase, as well as monoclonal antibodies against PKC isoforms β and ε, were from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal antibody against PKC isoform α and Protein A–agarose beads were purchased from Boehringer Mannheim (Mannheim, Germany). Despite the anti-ERK-1 antibody being described as specific for this kinase, it also detected ERK-2, though with less potency. Human recombinant BDNF and wortmannin were supplied by Sigma (St. Louis, MO, U.S.A.), whereas tyrphostin AG879, PD98059 and Ro-31-8220 were from Calbiochem (Darmstadt, Germany). All other reagents were of the highest grade possible from standard commercial sources.

Primary cortical neuron cultures (CNCs)

Timed-pregnant Sprague–Dawley rats were purchased from the Servei d'estabulari (Cerdanyola, CAT, Spain). After the mother was killed by decapitation under diethyl ether anaesthesia, the foetuses were removed on embryonic day 18 (E18), their brains were removed and placed in ice-cold PBS and the cortices were dissected. Then, single cells were dissociated using a glass pipette and cultured at a density of 1.5×10^6 cells/ml in basal Eagle's medium (PAN Biotech GmbH, Aidenbach, Germany), supplemented with 5 % (v/v) heat-inactivated bovine calf serum, 5 % (v/v) heat-inactivated foetal horse serum, 0.5 % glucose, 2 mM L-glutamine and antibiotics (penicillin and streptomycin). Cultures were maintained at 37 °C in a humidified 5 % CO_2 atmosphere. After culture for 7 days *in vitro*, on day 7 the medium was changed to basal Eagle's medium, supplemented with 10 % (v/v) heat-inactivated bovine calf serum and cytosine arabinoside (10 μM) to remove non-neural cells. Only mature cultures (10–12 days *in vitro*) were used for experiments.

Expression and purification of H_C-TeTx

Escherichia coli M15 cells were induced to express a pQE3-derived expression vector (Qiagen, Chatsworth, CA, U.S.A.) containing the cDNA for the H_C-TeTx fragment–His_6 fusion protein by the addition of 0.4 mM isopropyl β-D-thiogalactoside. Cells were pelleted after 4 h, resuspended in 50 mM NaH_2PO_4/300 mM NaCl (pH 8) and sonicated on ice for two 60 s periods with a probe sonicator. The suspension was centrifuged at 15 000 ***g*** for 20 min, and the clear supernatant was applied to an Ni^{2+}-nitrilotriacetate–agarose pre-cast column (Qiagen). Protein purification was performed by following 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 and analysed by SDS/PAGE followed by staining with Coomassie Blue. Those fractions containing purified H_C-TeTx were concentrated by centrifugation using ultrafree-MC filtration tubes (Millipore; molecular mass cut-off value was 10 kDa) and stored at − 80 °C in aliquots.

Subcellular fractionation

In the cases where subcellular fractionation was required, after each treatment, cells were resuspended in 0.3 ml of homogenization buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 50 mM NaF, 2 mM PMSF, 10 μg/ml leupeptin and 25 μg/ml aprotinin and disrupted by sonication in a Dynatech Sonic Dismembrator. The homogenate was centrifuged for 1 h at 100 000 ***g*** to separate the soluble fraction, corresponding to the cytosolic compartment, from the particulate fraction, corresponding to the membranous compartment. The precipitated fraction was further resuspended to the original volume using homogenization buffer, supplemented with Triton X-100 (0.1 % final concentration) and subsequently sonicated and incubated for 1 h at 4 °C. The extract was centrifuged for 1 h at 100 000 ***g*** and the resulting supernatant was considered as the particulate fraction. A fraction (25 μl) of each sample was analysed by SDS/PAGE.

Western-blot analysis

At the end of the incubation, the medium was removed and cells were rinsed with cold PBS. The cells were lysed by scraping them in ice-cold lysis buffer [62.5 mM Tris (pH 6.8)/2 % SDS/10 % (v/v) glycerol/50 mM DTT/0.1 % Bromophenol Blue], and subjected to SDS/PAGE. The separated proteins were transferred to Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), using a Mini TransBlot Cell 3 (Bio-Rad, Hercules, CA, U.S.A.) at 100 V for 1 h. The blotting buffer used contained 25 mM Tris, 200 mM glycine and 10 % (v/v) methanol. The membrane filters were blocked for 1 h with Tris-buffered saline, supplemented with 0.1 % Tween 20 and 5 % (w/v) defatted powdered milk. Then the membranes were incubated overnight with the corresponding antibody diluted in blocking buffer. Next, the membrane filters were incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase diluted in blocking buffer. Several washes with Tris-buffered saline/0.1 % Tween 20 were performed between all of the steps. The Western blots were developed using ECL® detection reagents from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.) and exposed to Amersham ECL® hyperfilms. The computer-assisted analysis of the bands was performed with Scion Image.

Immunoprecipitation

After treatment, cells were resuspended in 0.5 ml of homogenization buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 1 mM Na_3VO_4, 50 mM NaF, 2 mM PMSF, 10 μg/ml leupeptin and 25 μg/ml aprotinin and disrupted by sonication in a Dynatech Sonic Dismembrator. A fraction (500 μg) of the total protein was incubated by gently rocking at 4 °C overnight in the presence of 4 μg of PY20 antibody. The immunocomplex was then captured by adding 50 μl of washed Protein A–agarose bead slurry (25 μl of packed beads) and was gently rocked at room temperature (20 °C) for 2 h. The agarose beads were collected by pulsing in a microcentrifuge and the supernatant was drained off. The beads were washed three times with ice-cold PBS and resuspended in 100 μl of 2× reducing sample buffer and boiled for 2 min. After this, the agarose beads were separated by pulsing, and 25 μl of each sample was analysed by SDS/PAGE and Western blotting.

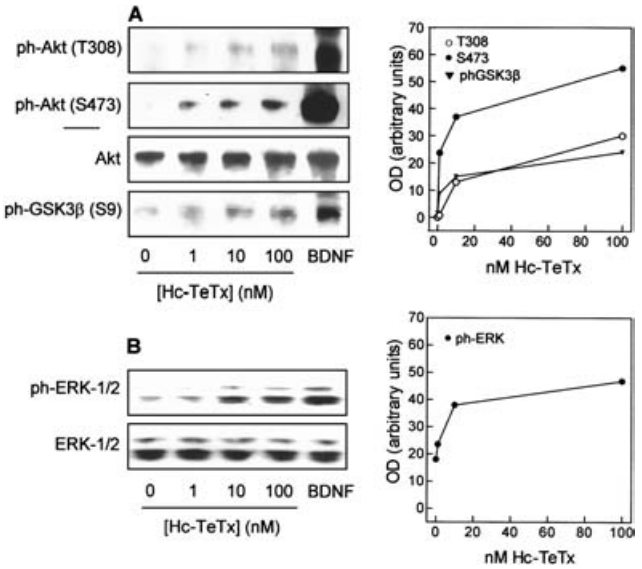

Figure 1 H_C-TeTx fragment induces phosphorylation of ERKs, Akt and GSK3β, an Akt substrate

Primary cortical cultures were treated with increasing concentrations of H_C-TeTx (1, 10 and 100 nM H_C-TeTx) or with 60 ng/ml BDNF as control, for 15 min. Total lysates were separated by SDS/PAGE and subjected to immunoblotting with the indicated antibodies. (**A**) Analysis of Akt activation. The graphs on the right represent the densitometry of the blots shown corresponding to phospho-Akt (ph-Akt) signals (○, phospho-Thr^{308}; ●, phospho-Ser^{473}) and to phospho-GSK3β (ph-GSK3β) (▼). (**B**) Analysis of the phospho-ERK-1/2 (ph-ERK-1/2) signal. The data are representative of two or three independent experiments. OD, absorbance.

RESULTS

H_C-TeTx fragment induces Akt and ERK-1/2 phosphorylation in cultured cortical neurons

To characterize further the activation in rat brain synaptosomes of signalling pathways induced by H_C-TeTx, observed in previous reports of our group, we tested the activation of ERK and Akt kinase in cultured cortical neurons from E18 foetal rats. The incubation of cultures with increasing concentrations of H_C-TeTx (0, 1, 10 and 100 nM) for 15 min induces the phosphorylation of Akt at Ser^{473} and at Thr^{308}, and of ERK-1/2 at Thr^{202} and at Tyr^{204}, as assessed by Western blotting using phospho-specific antibodies (Figures 1A and 1B). The activation of Akt activity was confirmed by the detection of phosphorylation at Ser^{9} of a well-known Akt substrate, GSK3β. BDNF (60 ng/ml) was used as a classical signalling activator. Equal loading of proteins between samples was confirmed by detection with antibodies against total Akt and against total ERK-1/2. The maximal amount of phosphorylation was reached at 100 nM H_C-TeTx, but without an important difference from the signal which appeared at 10 nM H_C-TeTx, as is shown by densitometric quantification.

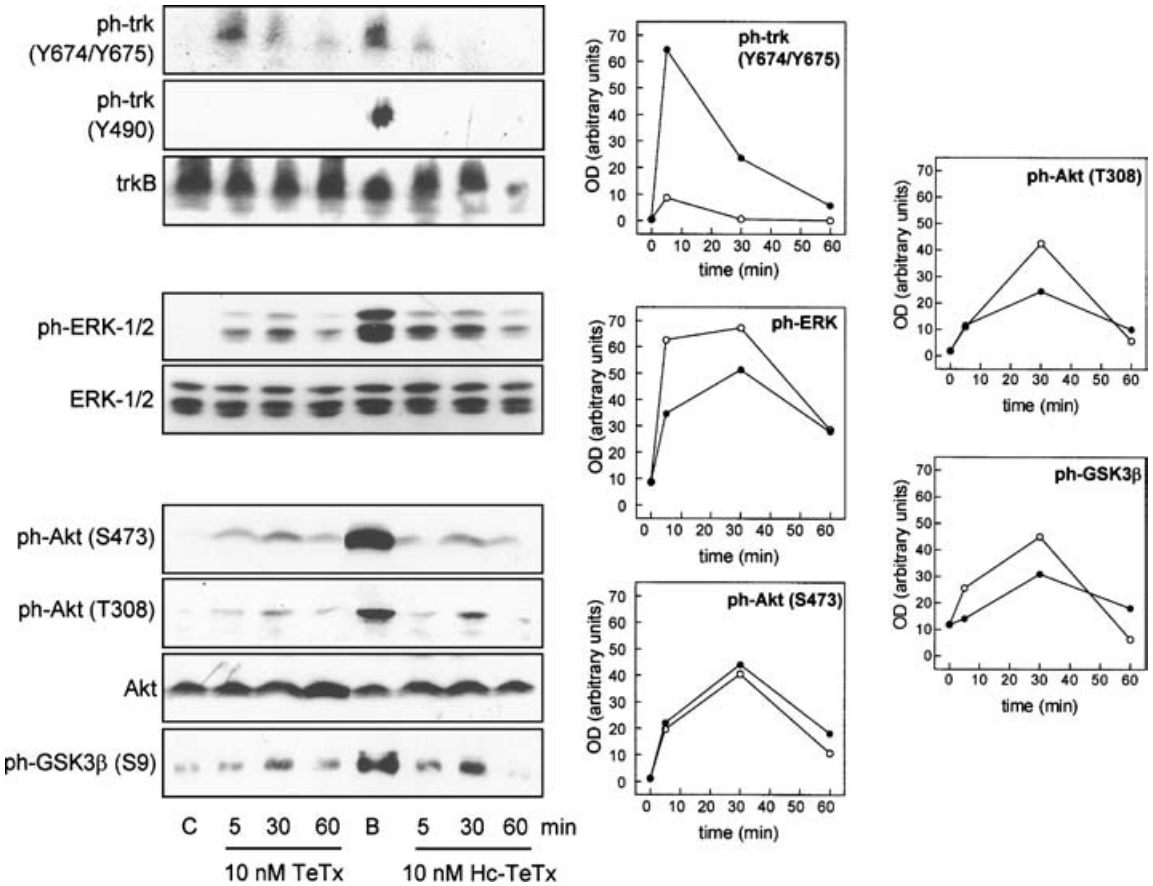

Figure 2 Time course of Trk receptor, ERK and Akt phosphorylation

Primary cortical cultures were treated with 10 nM whole TeTx or with 10 nM H_C-TeTx fragment for 0 (C, control), 5, 30 or 60 min. As positive control, 60 ng/ml BDNF (lanes indicated by 'B') was added for 15 min. Total lysates were separated by SDS/PAGE and subjected to immunoblotting with the indicated antibodies. The graphs represent the densitometry of the blots shown corresponding to the Trk receptor(s) (ph-trk) phosphorylated at Tyr^{674}/Tyr^{675} or at Tyr^{490}, phospho-ERK-1/2, phospho-Akt and phospho-GSK3β. ●, Phosphorylation due to TeTx; ○, phosphorylation due to the H_C-TeTx fragment. The data are representative of two or three independent experiments. OD, absorbance.

Akt and ERK-1/2 phosphorylation, as well as Trk receptor activation, is caused by whole TeTx

Time-course experiments using Western-blot analysis and phospho-Trk-specific antibodies show that TeTx induces a rapid induction of Trk receptor phosphorylation at Tyr^{674} and Tyr^{675}, which lie within the catalytic domain. Phosphorylation at this site reflects Trk kinase activity. This antibody can detect the corresponding residues in TrkA, TrkB and TrkC. When cells are treated with 10 nM TeTx, the maximal signal observed is at 5 min, and the level of signal produced is similar to that produced by 60 ng/ml BDNF. Trk phosphorylation subsequently decreases, still being detectable at the longest time tested (60 min; Figure 2). The same analysis using 10 nM H_C-TeTx as the activator shows a similar pattern, although at a much lesser level, the phospho-Trk signal being detectable only after 5 min of incubation. When the blot is reprobed using specific antibodies for Trk phosphorylated at Tyr^{490}, no detectable signal due to either 10 nM TeTx or H_C-TeTx appears, whereas 60 ng/ml BDNF induces a clear Trk phosphorylation at Tyr^{490}. Phosphorylation at Tyr^{490} is required for Shc association and activation of the Ras/ERK cascade. This antibody can also detect the corresponding residues in TrkA, TrkB and TrkC.

A time-dependent phosphorylation of ERK-1/2 and of Akt is also observed, using specific antibody against ERK-1 and -2 dually phosphorylated at Thr^{202} and Tyr^{204}, and antibodies against Akt phosphorylated at Ser^{473} or at Thr^{308}. For ERK-1/2, a series of experiments at 5, 30 and 60 min shows a comparable effect of TeTx and of H_C-TeTx (10 nM), both producing the highest signal after 30 min of incubation, with a subsequent decrease. Nevertheless, in no case does a signal due to any toxin form reach the strong phosphorylation exerted by 60 ng/ml BDNF at 15 min. Exactly the same pattern is observed in the analysis of Akt phosphorylation, and the strongest signal is detectable at 30 min of incubation, followed by a subsequent decrease (Figure 2). Phosphorylation state of GSK3β was also detected to confirm Akt activation. GSK3β shows the maximal phosphorylation after 30 min of TeTx or H_C-TeTx incubation, this signal being transient as in the other cases analysed.

Both H_C-TeTx and TeTx enhance phosphorylation of several members of the Raf/MEK/ERK pathway

To expand the knowledge about signalling pathways triggered by TeTx, Western-blot analysis of time-course experiments was performed, using specific antibodies against phosphorylated Raf (Ser^{259}), MEK-1/2 (Ser^{217}/Ser^{221}), p90Rsk (Ser^{380}) and CREB (Ser^{133}). In all cases, both TeTx and H_C-TeTx enhanced the phosphorylation of the proteins tested, and with a similar time-dependent pattern. The maximal signal observed in the four cases was at 5 min, which subsequently decreased (Figure 3). Equal

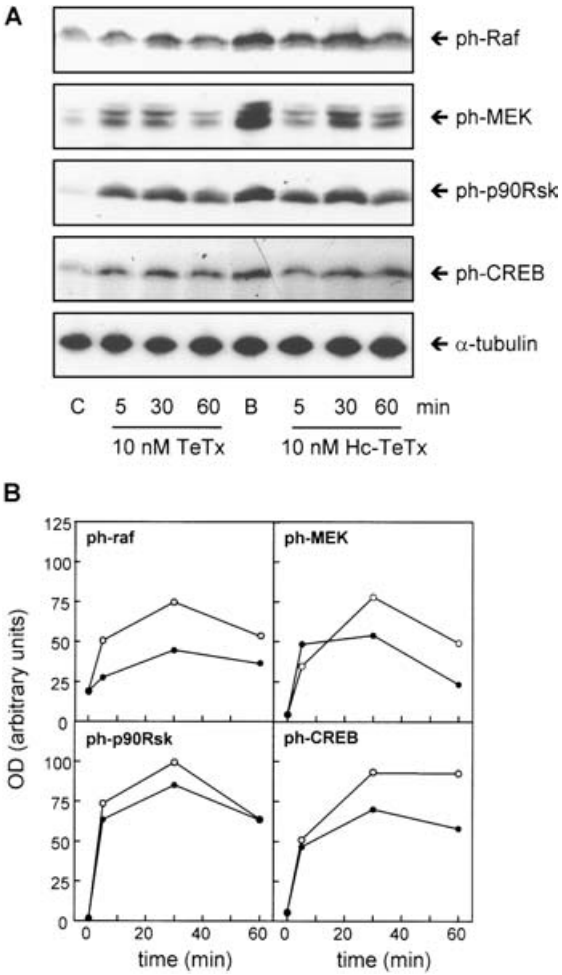

Figure 3 Time course of phosphorylation of several members of the ERK pathway

Primary cortical cultures were treated with 10 nM whole TeTx or with the H_C-TeTx fragment (10 nM) for 0 (C, control), 5, 30 or 60 min. As positive control, 60 ng/ml BDNF (lanes indicated by 'B') was added for 15 min. Total lysates were separated by SDS/PAGE and subjected to immunoblotting with antibodies against phospho-Raf (ph-Raf), phospho-MEK-1/2 (ph-MEK), phospho-p90Rsk (ph-p90Rsk) or phospho-CREB (ph-CREB). An antibody raised against α-tubulin was used as loading control. The graphs represent the densitometry of the blots shown. ●, Phosphorylation due to TeTx; ○, phosphorylation due to the H_C-TeTx fragment. The data are representative of three independent experiments. OD, absorbance.

loading of proteins among samples was confirmed by detection with antibody against α-tubulin. The blot corresponding to phospho-MEK-1/2 showed a doublet, which could correspond to different MEK phosphorylation statuses, since the antibody used reacts with MEK-1/2 dually phosphorylated at Ser^{217} and Ser^{221}, and also with MEK-1/2 singly phosphorylated at Ser^{217}, but not with MEK-1/2 singly phosphorylated at Ser^{221}.

H_C-TeTx fragment induces translocation of PKC isoforms to membrane

The activation of PKC isoforms induces their translocation from cytosol to membrane compartments. Therefore the amount of PKC present in membrane is directly correlated to its degree of activation. In experiments aimed at studying the effect of H_C-TeTx on PKC isoforms in cortical neurons, separation of cytosol (soluble fraction) and membrane (particulate fraction) was

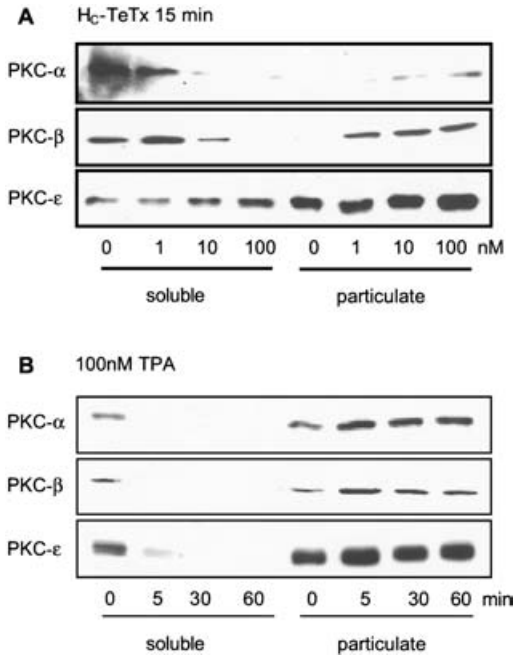

Figure 4 H_C-TeTx-induced translocation of PKC isoforms

Primary cortical cultures were treated with (**A**) increasing concentrations of H_C-TeTx (1, 10 and 100 nM H_C-TeTx) for 15 min or (**B**) 100 nM PMA ('TPA') for 0, 5, 30 or 60 min. Subsequently, soluble (cytosol) and particulate (membrane) fractions were separated, as indicated in the Materials and methods section. Western-blot analysis was performed using antibodies against PKC isoforms α, β and ε.

performed, with subsequent Western-blot analysis against PKC isoforms. This set of experiments shows that incubation of CNC at increasing concentrations (0, 1, 10 and 100 nM) of H_C-TeTx for 15 min induces changes in PKC isoform distribution (Figure 4A). Sustained translocation is observed of the classical isoforms PKC-α and -β, whereas no significant change is observed in the novel isoform PKC-ε. A parallel set of time-course experiments, for 0, 5, 30 and 60 min, using 100 nM PMA (a direct and non-physiological activator of diacylglycerol-dependent PKC isoforms) was performed to test the reliability of the system. The translocation was clear for the three isoforms tested (α, β and ε), even at only 5 min of PMA incubation (Figure 4B), proving that the compartment fractionation was effective in the system used.

H_C-TeTx fragment-induced activation of Akt and ERK pathways is abolished by tyrphostin AG879 and by wortmannin, but not by Ro-31-8220

The effect of different kinase inhibitors on H_C-TeTx-dependent activation of Akt and ERK pathways was examined. Pretreatment of cells with 10 μM tyrphostin AG879, an inhibitor of Trk, or with 100 nM wortmannin for 30 min abolished the H_C-TeTx-dependent activation of Akt, MEK-1/2, ERK-1/2 and p90Rsk, as assessed by Western-blot analysis (Figure 5A). This result points to the necessary implication of Trk receptors in H_C-TeTx action on Akt and on ERK-1/2 pathways, and to the necessary implication of the PI-3K/Akt pathway in the subsequent activation of MEK/ERK by H_C-TeTx. On the contrary, H_C-TeTx-dependent activation of Akt, MEK-1/2, ERK-1/2 and p90Rsk was not inhibited after pretreatment of cells with 1 μM Ro-31-8220 for 30 min. This result indicates that activation of

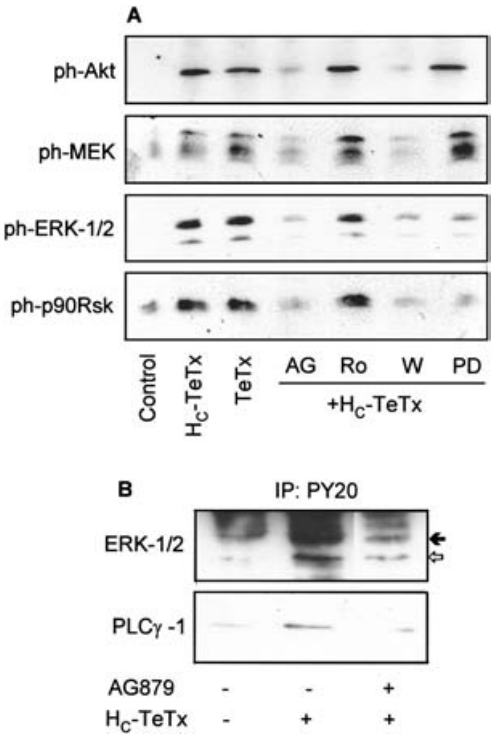

Figure 5 Effect of various kinase inhibitors on induction by the H_C-TeTx fragment of Akt and ERK pathways

(**A**) Primary cortical cultures were pretreated for 30 min, where indicated, with 10 μM AG879 (AG), 1 μM Ro-31-8220 (Ro), 100 nM wortmannin (W) or 50 μM PD98059 (PD), and then stimulated with 10 nM H_C-TeTx for 30 min. No pretreatment was performed in the controls with 10 nM H_C-TeTx or 10 nM TeTx, both for 30 min. Treatment with vehicle was performed in the control. Total lysates were separated by SDS/PAGE and subjected to immunoblotting with the indicated antibodies. Blots shown are representative of three independent experiments. (**B**) Cultures were pretreated with or without 10 μM AG879 for 30 min where indicated, and H_C-TeTx was then added (10 nM for 30 min) where indicated. In the control lane, only vehicle was added. Then immunoprecipitation (IP) was performed as indicated in the Materials and methods section, using PY20 antibody against phosphotyrosine residues and, subsequently, Western-blot analysis using antibodies against ERK-1/2 and PLCγ-1 was performed. Arrows indicate bands corresponding to ERK-1 (black) or ERK-2 (white). Blots shown are representative of two independent experiments.

PKC is not required for activation of Akt and ERK pathways. Finally, pretreatment of cells with 50 μM PD98059 for 30 min abolished the H_C-TeTx-dependent activation of ERK-1/2 and of p90Rsk, but not the activation of Akt and MEK-1/2 (Figure 5A). Trk-dependent activation of signalling pathways was confirmed and was further characterized by immunoprecipitation with an anti-phosphotyrosine monoclonal antibody (clone PY20), followed by Western blotting with anti-ERK-1/2 or anti-PLCγ-1. Pretreatment of cells with 10 μM AG879 for 30 min abolished phosphorylation at tyrosine residues of ERK-1/2 and PLCγ-1 enhanced by 10 nM H_C-TeTx for 30 min (Figure 5B). PLCγ-1 is a PLC isoform, which by binding to tyrosine-phosphorylated membrane receptors, such as Trk receptors, becomes activated and then hydrolyses phospholipids to inositol trisphosphate and diacylglycerol, leading to PKC activation. PLCγ-1 is also tyrosine-phosphorylated by receptors when activated by them. These results reinforce the requirement for Trk receptor activation by H_C-TeTx to induce ERK-1/2 phosphorylation, and show that H_C-TeTx also enhances PLCγ-1 phosphorylation in a Trk-dependent manner. This last result is directly related to the observed activation of PKC isoforms, since PLC products are known to activate PKC.

DISCUSSION

It is shown, in the present study, that incubation of cortical neurons with TeTx can activate two parallel biochemical cascades: a MAP kinase pathway (Raf/MEK/ERK) and an Akt pathway. This effect is dependent on the interaction of TeTx with the cellular membrane and not on the catalytic activity of the toxin, since H_C-TeTx alone can activate the same signals as TeTx. Activation of both the H_C-TeTx-triggered pathways studied is totally dependent on PI-3K. Typically, dual activation of the PI-3K/Akt pathway and the ERK-1/2 cascade in neurons has been shown to occur through the activation of neurotrophin Trk receptors [21]. As was determined in previous reports of our group, both TeTx and H_C-TeTx activate TrkA phosphorylation in rat brain synaptosomes [4,5], showing phosphorylation at Tyr^{490}. This result pointed to a role of Shc adaptor proteins in TeTx-induced signalling, since phosphorylated Tyr^{490} is the target for Src-homology-2-domain-containing Shc proteins [11]. In the CNC used in the present study, the TrkA receptor cannot be studied without transfection techniques, since these cells lack TrkA and express only TrkB and TrkC [22]. Therefore TrkB activation was studied, using BDNF as the control of activation and antibodies that recognize all three phosphorylated Trk forms, as indicated by the manufacturer. Surprisingly, we failed to detect phosphorylation of Trk, due to H_C-TeTx or TeTx, at Tyr^{490}, indicating that Shc proteins are unlikely to participate in subsequent signalling by TeTx in CNCs. The existence of a phenylarsine oxide-sensitive phosphatase in cortical neurons that rapidly dephosphorylates the tyrosine residue in Shc-binding site of TrkB, i.e. Tyr^{490}, has been described previously [23]. Nevertheless, the phosphorylation of Tyr^{490} by BDNF action would be insensitive under the conditions tested to this phosphatase, as shown in Figure 2. A strong BDNF-triggered interaction between Trk and PLCγ-1 has also been described previously [23]. Tyrosine phosphorylation of PLCγ-1 has been suggested to be necessary for its activation in intact cells, but the detailed mechanisms involved are still not clear. However, it appears that binding to the activated receptor is insufficient for PLCγ-1 activation, and that tyrosine phosphorylation is required for PLCγ-1 activation in intact cells [24]. Our findings that H_C-TeTx induces tyrosine phosphorylation of PLCγ-1 in a Trk-dependent manner (Figure 5B) are in agreement with these results and are consistent with the activation by TeTx of polyphosphoinositide hydrolysis described in CNCs by our group [25]. On the other hand, TeTx- and H_C-TeTx-induced phosphorylation of Trk receptor(s) at Tyr^{674} and Tyr^{675} has been observed. These tyrosine residues become autophosphorylated when the agonist binds to the Trk receptor and are required for efficient signal transduction and promotion of biological responses [26]. The extent of phosphorylation induced at Tyr^{674}/Tyr^{675} is clearly higher for TeTx when compared with H_C-TeTx, a fact that could be due to the need for some other region for optimum binding to the membrane. In agreement with this, TeTx appears to be clearly superior to H_C-TeTx in the capacity for neuronal binding and internalization, in studies performed in cortical neurons [27]. Nevertheless, the small amount of H_C-TeTx-induced Trk phosphorylation seems to be enough to trigger the signalling described in the present study, and with a similar potency as TeTx. Another intriguing point, in our opinion, is that BDNF, the physiological activator of the TrkB receptor, and TeTx induce Trk phosphorylation at Tyr^{674}/Tyr^{675} to a similar extent, but the subsequent activation of ERK and Akt kinase is remarkably higher for BDNF. This result reveals that divergences must exist between BDNF and TeTx downstream signalling, one of them being the action of Shc proteins mentioned above. Recently, it has

been shown that activation of Trk receptor tyrosine kinases can also occur via a G-protein-coupled receptor mechanism, without the involvement of neurotrophins [28]. The possibility of Trk transactivation due to the effect of another membrane receptor in our results is unlikely, since transactivation events typically show a slow time course (2–3 h), whereas TeTx-induced Trk phosphorylation is fast (5 min).

The use of specific inhibitors shows that H_C-TeTx action on Akt, ERK and PLCγ-1 pathways is totally dependent on Trk, since low concentrations of AG879 abolish phosphorylation in all three proteins. The effect of H_C-TeTx on TrkA was described by our group [5], and the results of the present study showing activation of TrkB expand the possibilities of action of this toxin. This action could be direct by means of a ligand–receptor-type interaction, a fact that would have implications in the transport of TeTx from the peripheral nervous system to the CNS, since Trk receptors, together with their ligands, are retroaxonally transported [9]. The coincidence between neurotrophin receptor and TeTx retroaxonal transporters has recently been communicated [29], supporting the role of neurotrophin receptor machinery in the entry of TeTx into the CNS. Another possibility is an indirect interaction with Trk through an adaptor protein or ganglioside. There is a general agreement that TeTx shows the highest affinity for the polysialogangliosides GT1b (a membrane glycosphingolipid containing tri-sialic acid) and GD1b (containing di-sialic acid) [30], and these gangliosides are known to activate Trk receptors and ERK phosphorylation in rat brain slices, although not nearly at as high a level as monosialoganglioside G_{M1} (ganglioside containing mono-sialic acid) [31]. Taking these results together, the possibility of an active ternary complex made up of TeTx, ganglioside and Trk arises.

As can be seen in Figure 5, the use of wortmannin reveals that PI-3K action is necessary for Akt phosphorylation as well as for the activation of the ERK pathway, as demonstrated by the inhibition of MEK, ERK-1/2 and p90Rsk phosphorylation by wortmannin. The dependence of MEK and downstream targets on PI-3K has been described in several works. For example, in PC12 and in dorsal root ganglia cells, PI-3K is required for TrkA internalization and participates in NGF signalling to ERKs via distinct actions on the small G-proteins Ras and Rap1 [32]. On the other hand, PI-3K-dependent activation of ERK by kainate [33] has been demonstrated in striatal neurons. The ERK pathway can also be dependent on the action of some PKC isoforms, this action probably being through a direct interaction between PKC isotypes and Raf [34]. In the CNC used in the present study, PKC can activate the ERK pathway since low concentrations of PMA can transiently activate ERK-1/2 phosphorylation (results not shown). Nevertheless, this does not seem to be the case with the signal activated by TeTx. Although TeTx activates PKC-α and -β isoforms in CNCs (as their translocation to membrane indicates), they act in an independent manner with respect to the ERK pathway since Ro-31-8220 cannot eliminate phosphorylation of MEK, ERK-1/2 or p90Rsk (Figure 5). Enhancement of the association of PKC activity with cell membrane in CNC due to TeTx has been described by our group [25]. Regarding Raf, this kinase is phosphorylated in the system used in the present study at Ser^{259} in response to BDNF, TeTx or H_C-TeTx (Figure 3). This is an intriguing result, since Akt [35] and cAMP-dependent kinase [36] have been described as being responsible for phosphorylation at this residue, and causing Raf inhibition, by promoting complex formation with 14-3-3 proteins [37]. However, mutant Raf proteins in which phosphorylatable residues responsible for activation have been changed can still be activated after membrane association, indicating that an additional mechanism(s) of Raf activation exists [38]. On the other hand, other authors have established that activation of Raf is independent of phosphorylation at Ser^{259} [39], whereas a Raf-independent activator has been described in PC12 cells [40]. These results point to a possible MEK activation even when Raf is inactivated.

The interference of bacterial proteins in signal transduction pathways of the host is a topic well described in the literature [41]. Pathogens such as *Listeria monocytogenes* [42] or *Bacillus anthracis* [43], among others, use the ERK pathway in their pathological mechanisms. On the other hand, several bacterial pathogens require or purportedly activate PI-3K-mediated signalling pathways to gain entry into non-phagocytic cells. For example, invasin-mediated entry of *Yersinia* into epithelial cells requires PI-3K activity [44] and InlB, a surface protein from *L. monocytogenes*, triggers activation of PI-3K and subsequent bacterial entry [45]. Moreover, type 1 pilus-mediated invasion of bladder epithelial cells by uropathogenic *E. coli* has been shown to require PI-3K activity associated with local cytoskeleton arrangements [46]. The first bacterial protein, and until now the only one, to be identified as an Akt activator was SigD from *Salmonella typhimurium* [47]. SigD, once injected into the host cell, dephosphorylates a variety of soluble inositol polyphosphates as well as inositol phospholipids, an action that could induce a conformational change in recruited Akt, favouring its phosphorylation at Thr^{308} and Ser^{473} and leading to full activation. Therefore *Clostridium tetani* could use PI-3K and Akt activation to enhance TeTx endocytosis by host cell, in the same way that PI-3K participates in NGF-induced TrkA endocytosis [32].

In summary, the activation of ERK/Akt pathways and Trk receptors by TeTx opens new possibilities in understanding how *C. tetani* interacts with the plasma membrane, how it uses cellular mechanisms for its own possible benefit and, thus, exerts its high capacity of toxicity. Whether the effects produced by TeTx on transcriptional control have physiological significance in the short or long term remains to be seen.

This work was supported by grant no. SAF2001-2045 from Ministerio de Educación y Cultura, Dirección General de Enseñanza Superior e Investigación Científica. We acknowledge the gift of *E. coli* strain containing H_C-TeTx recombinant plasmid from Dr J. Blasi (Departament de Biologia Cellular i Anatomia Patològica, Campus de Bellvitge, Universitat de Barcelona).

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Received 27 February 2003/4 April 2003; accepted 23 April 2003
Published as BJ Immediate Publication 23 April 2003, DOI 10.1042/BJ20030333