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Serotonin transporter phosphorylation modulated by tetanus toxin

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Abstract Tetanus toxin (TeTx) modifies Na^+-dependent, high-affinity 5-hydroxytryptamine (5-HT, serotonin) uptake in a synaptosomal-enriched P_2 fraction from rat brain. The effect corresponds to a rapid and non-competitive uptake inhibition, and it is preceded by induction of phospholipase C (PLC) activity and translocation and down-regulation of the classical protein kinase C (PKC-α, -β and -γ) isoforms. The effects on serotonin transport and on *c*PKC activation were similar to the effects exhibited by phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Moreover, after treatment with TeTx, an increase in Ser- and Tyr-specific phosphorylation was found. Activation of PKC by both TeTx and TPA results in a loss of transport capacity and serotonin transporter (SERT) phosphorylation, which are abolished by coapplication of the specific PKC inhibitor bisindolylmaleimide-1. Since a specific PLCγ1 phosphorylation prior to TeTx's inducing SERT phosphorylation was found, the studies suggest that part of the action of TeTx consists of modifying the signal cascade initiated in tyrosine kinase receptors on nerve tissue previous to its cellular internalization, resulting in transporter phosphorylation.

Key words: Protein kinase C; Tetanus toxin; Phospholipase Cγ1; Rat brain; Serotonin transporter; Synaptosome

1. Introduction

Tetanus toxin (TeTx) is produced by the bacilli *Clostridium tetani* and is the protein responsible for producing a deadly syndrome in humans, tetanus disease. Like other neurotoxins, the active holotoxin is comprised of two fragments, the heavy (H, 100 kDa) and light (L, 50 kDa) chains, held together by a disulfide bond [1]. The 50-kDa C-terminal domain of the H-chain would be involved in the binding and internalization of this toxin in neurons [2]; the 50-kDa N-terminal portion of the H-chain would be involved in the translocation of the L-chain in the cytosol. L-TeTx is a zinc-endoprotease, which specifically cleaves synaptobrevin via a process independent of synaptic activity [3,4].

The action of TeTx is completely neurospecific. Binding to the plasma membranes is essential for development of spastic paralysis. Polysyalogangliosides [5] and glycoproteins [6] have been proposed as candidate receptors. Nevertheless, efforts to find such protein receptors have been futile.

Not all of the clinical symptoms of tetanus intoxication can be ascribed to the inhibition of vesicular synaptic secretion. Certain symptoms such as insomnia, hyperactivity, hyperthermia, etc., seem to suggest the implication of different mechanisms, different neurons, and different areas of the CNS. Recently, it was demonstrated that TeTx can partially block neurotransmitter release by stimulating a Ca^{2+}-dependent transglutaminase activity [7]. TeTx also induces protein kinase C (PKC) translocation, even before classical tetanus symptoms are evident [8,9]. Finally, it has been demonstrated that TeTx blocks Na^+-dependent 5-hydroxytryptamine (serotonin, 5-HT) uptake in rat-brain synaptosomes [10,11]. This inhibitory effect has also been studied on other transmitters, however, only minor or no effects have been observed [12].

Serotonin transporter (SERT) is the sole molecule responsible for extracellular 5-HT transport, coupling uptake to the influx of Na^+ ion down the concentration gradient [13]. PKC has been implicated as a regulator of SERT activity. Activators of PKC, such as phorbol esters, induce the phosphorylation of SERT in Ser/Thr residues responsible for the re-uptake inhibition [14]. Our group has previously demonstrated that TeTx, or its H_C-fragment, inhibits the [^{3}H]5-HT uptake in rat-brain synaptosomes. This inhibitory action is independent of the metalloprotease activity of the neurotoxin contained in the L-chain, and its potency is two or three orders of magnitude greater than the serotonin selective re-uptake inhibitors [10,11].

In the present work, it shall be demonstrated whether or not the phospholipase C activity and PKC regulation, found after treatment with TeTx [9], belong to a transduction mechanism responsible for the inhibition of the serotonin transport via carrier phosphorylation.

2. Materials and methods

2.1. Materials

TeTx was provided by List Biological Laboratories, Inc. (Campbell, CA, USA). Tissue-culture-grade *myo*-inositol, 5-HT, carbachol, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were from Sigma Chemical (St. Louis, MO, USA). *myo*-[2-^{3}H]Inositol (55 Ci/mmol) was from NEN Research (Boston, MA, USA). 5-[1,2-^{3}H]Hydroxytryptamine (34 Ci/mmol) was from Amersham International (Buckinghamshire, UK). Nerve growth factor (NGF) was supplied by Alomone Labs.

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Abbreviations: BIM-1, bisindolylmaleimide-1; 5-HT, 5-hydroxytryptamine (serotonin); NGF, nerve growth factor; PKC, protein kinase C; PLC, phospholipase C; SERT, serotonin transporter; TeTx, tetanus toxin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TyrKRs, tyrosine kinase receptors

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(Jerusalem, Israel). All other reagents were of the highest grade possible from standard commercial sources.

2.2. Preparation of synaptosomes and subcellular fractionation

All experiments were performed with a crude synaptosomal fraction (P_2) prepared from Sprague–Dawley 4–6-week-old rat brains, according to [15], with slight modifications [10]. In the cases where a subcellular fractionation was required, the synaptosomes were collected by centrifugation after each treatment and 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 dithiothreitol, 1 mM Na_3VO_4, 50 mM NaF, 2 mM PMSF, 10 μg/ml leupeptin and 25 μg/ml aprotinin, and disrupted by sonication. The homogenate was centrifuged for 1 h at $100\,000\times g$ to separate the soluble fraction (supernatant), from the particulate fraction (precipitated). The precipitated fraction was further resuspended to the original volume using homogenization buffer supplemented with Triton X-100 (0.3%), and subsequently sonicated and incubated for 1 h at 4°C. The extract was centrifuged for 1 h at $100\,000\times g$ and the resulting supernatant was considered as the particulate fraction extract. Protein concentration was determined according to [16].

2.3. Immunoprecipitation and Western blot analysis

After treatment, synaptosomes were collected by centrifugation and the reaction medium was eliminated. Next, 1 ml of homogenization buffer supplemented with 0.3% Triton X-100 was added and the synaptosomes were disrupted by sonication. For immunoprecipitation, 1 mg of total protein was incubated by gentle rocking at 4°C overnight in the presence of 4 μg of antibody. The immunocomplex was then captured by adding 100 μl of washed Protein-A agarose bead slurry (50 μl of packed beads) previously incubated with 3% BSA/PBS to eliminate unspecific binding and gently rocked at 4°C for 2 h. The agarose beads were collected by pulsing and the supernatant was drained off. The beads were washed three times with ice-cold PBS, resuspended in 100 μl 2×non-reducing sample buffer and then boiled for 4 min. Next, 10 μl of each sample were analyzed in SDS–PAGE. The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The blotting buffer used contained 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol (pH 8.3). The membrane filters were blocked for 1 h with PBS/4% BSA in the case of the antibodies directed against phospho-amino acids, and with PBS/5% defatted powdered milk in the rest of the cases. Next, the membranes were incubated overnight with the corresponding antibody diluted in blocking buffer. The polyclonal antibodies against the PKC isoforms α, β and γ were purchased by Boehringer Mannheim GmbH (Mannheim, Germany). The specific antibody against phosphotyrosine (Clone PY99) was from Santa Cruz Biotechnology (CA, USA), and phosphoserine (polyclonal antibody) was obtained from Zymed Laboratories Inc. (San Francisco, CA, USA); the polyclonal antibody against the SERT and the SERT antibody-control peptide (AG297) were from Chemicon International (Temecula, CA, USA), and the monoclonal antibody against PLCγl was from Upstate Biotechnology (Lake Placid, NY, USA). Next, the membrane filters were incubated for 1 h with a secondary antibody conjugated with horseradish peroxidase diluted in blocking buffer. Several washes with PBS/0.05% Tween 20 were performed between the steps. The Western blots were developed with a buffer containing 0.1 M Tris–HCl pH 8.6, 0.2 mg/ml luminol, 0.01 mg/ml *p*-coumaric acid and 0.01% H_2O_2 and exposed to Amersham ECL films. The computer-assisted analysis of the bands was performed with a Bio-Rad GS700 system, and data were processed with a Bio-Rad Molecular Analyst image program using a Dell work station. Repeated scans were taken for film non-linearity corrections.

2.4. Determination of ^{3}H-inositol phosphates and ^{3}H-inositol lipids

Polyphosphoinositide hydrolysis was determined according to [17]. The crude synaptosomal fraction (P_2) was labelled in bulk with *myo*-[^{3}H]inositol for 2 h at 37°C, washed and transferred in 100 μl aliquots to tubes with 100 μl of Krebs–Ringer buffer containing 10 mM LiCl and, when appropriate, agonists. After 30 min, reactions were stopped by adding 1.2 ml of chloroform:methanol (1:2). Samples of the aqueous phases (1 ml) were neutralized with 1.5 M NH_4OH, diluted with 5 ml of water, and loaded onto columns with 0.5 ml of Dowex AG1X8 (100–200 mesh, formate form). After washing the columns with 6×4 ml of water and 2×4 ml of 60 mM sodium formate/5 mM borax to remove [^{3}H]inositol and [^{3}H]glycerophosphorylinositol, ^{3}H-inositol phosphates were eluted together with 2×4 ml of 1.0 M ammonium formate/0.1 M formic acid. After 12 ml of a toluene/Triton X-100-based scintillation cocktail were added, vials were cooled to form a clear homogeneous phase, and were then counted at a 30% efficiency rate. Accumulation of ^{3}H-inositol phosphates was calculated as the percentage of lipid labelling in the same sample, with total [^{3}H]inositol labelling of lipids monitored by counting 0.2-ml aliquots of the organic phases. When agonist effects on [^{3}H]inositol labelling of lipids were examined, synaptosomes were preincubated for 10 min with agonists and lithium prior to the addition of [^{3}H]inositol (4 μCi/ml). To decrease the background, organic phases containing ^{3}H-inositol lipids were washed with 1.5 ml of a new aqueous phase.

2.5. Assay of [^{3}H]5-HT uptake

In the standard assay, to measure the synaptosomal 5-HT uptake, 100 μl of synaptosomes, diluted with oxygenated Krebs–Ringer bicarbonate buffer to achieve a final protein concentration of 0.4–0.8 mg/ml, was mixed in a polypropylene tube with 50 μl of toxin diluted in Krebs–Ringer bicarbonate buffer containing 0.05% BSA, or with 50 μl of the same medium without toxin as a control. After a preincubation period of 30 min at 37°C in a shaking water bath, uptake was started by the addition of 50 μl [^{3}H]5-HT. For saturation experiments, the

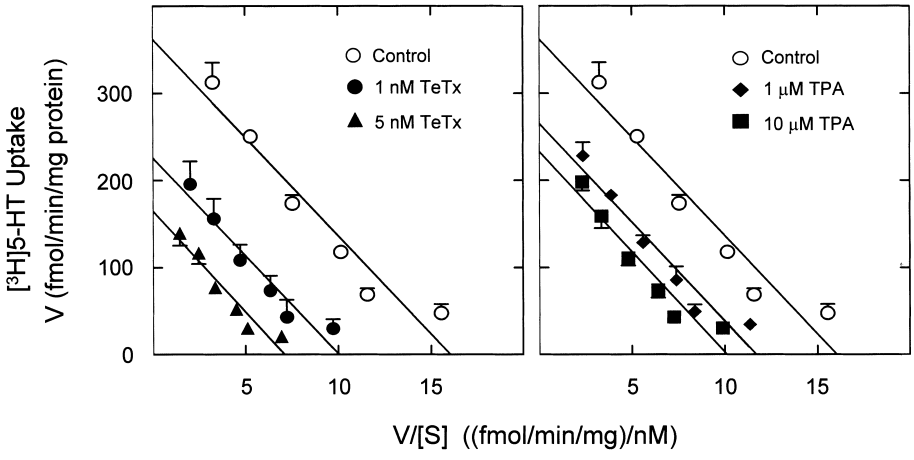

Fig. 1. Apparent kinetics parameters of serotonin transport. The effects of TeTx and TPA on [^{3}H]5-HT uptake were evaluated by saturation experiments and evaluated the kinetic parameters by bound/free versus bound analyses. Synaptosomal fractions were preincubated for 30 min with saline (○), or in the left panel with TeTx (●, ▲), and in the right panel with TPA (◆, ■). Transport activity was measured using six different concentrations of [^{3}H]5-HT (5–160 nM) for 5 min at 37°C. Data are mean ± S.D. (bars) of a triplicate determination of a representative experiment.

tritiated amine concentration ranged from 5 to 160 nM. After an incubation of 5 min at 37°C, samples were filtered in vacuum over Whatman GF/C (Whatman International Ltd., Maidstone, UK) filters in a vacuum-filtration manifold (Brandel Inc., Gaithersburg, MD, USA) and washed three times with 5 ml ice-cold Krebs–Ringer bicarbonate buffer. Filters were then dried, placed in vials with 5 ml biodegradable counting scintillante (Amersham Int. Plc, Buckinghamshire, UK) and counted in a Wallac-1409 liquid scintillation counter, with a counting efficiency of 45%. Radioactivity accumulated by synaptosomes at 0°C for 5 min was routinely subtracted, and temperature-dependent uptake was defined as the difference between uptake carried out at 37°C and at 0°C.

3. Results

3.1. TeTx, like TPA, induces [^{3}H]5-HT uptake inhibition in rat-brain synaptosomes

The accumulation of [^{3}H]5-HT into synaptosomal-enriched P_2 fractions was used to determine the kinetic parameters of the SERT, and initial uptake velocity was measured at [^{3}H]5-HT concentrations that ranged from 5 to 160 nM (Fig. 1). Kinetic analysis based on non-linear regression analysis revealed a [^{3}H]5-HT uptake with an apparent V_{max} = 404.8 ± 11.7 fmol/min/mg of protein and a K_M = 48.4 ± 3.4 nM. Treatment with both TeTx and TPA significantly inhibit the [^{3}H]5-HT uptake. Significant decreases with respect to control were found in apparent V_{max} when the synaptosomes were treated for 30 min at 37°C with two concentrations of the toxin, 1 nM TeTx (252.9 ± 7.3 fmol/min/mg) and 5 nM TeTx (183.6 ± 8.7 fmol/min/mg), and with two concentrations of the PKC activator, 1 μM TPA (295.0 ± 7.6 fmol/min/mg) and 10 μM TPA (257.1 ± 7.0 fmol/min/mg). The values of P were statistically significant ($P < 0.01$, $n = 3$). No significant differences were found among the K_M of [^{3}H]5-HT uptake in all cases shown in Fig. 1. The results were evaluated by the bound/free versus bound analysis (Eadie–Hofstee plots).

3.2. TeTx induces dose-dependent translocation of PKC isoforms.

In order to examine the redistribution of PKC subspecies after incubation with TeTx, immunodetection of isoforms present in cytosol and in membranous fractions from synaptosomal preparations were assessed (Fig. 2). The three subspecies of the *c*PKC family show response. The PKC-α is slightly down-regulated (60% of the control in both fractions at the maximum TeTx concentration tested, 300 nM), while the PKC-β is clearly translocated at 5 nM TeTx, appearing down-regulation at greater concentrations. The PKC-γ shows the maximal translocation at 50 nM TeTx and is down-regulated at 300 nM TeTx.

3.3. TeTx induces both PLC activation and PLCγ1 phosphorylation at Tyr residues via tyrosine kinase receptors (TyrKRs)

Full evidence of PLC activation was obtained by measurement of sustained inositol phosphates accumulation in the tissues treated with the neurotoxin and with the endogenous agonist of the TyrKRs trkA, NGF, and were compared with the breakdown of phosphatidyl-3-inositol 4,5-diphosphates (PIP_2) mediated by muscarinic activation with carbachol. TeTx stimulated the PIP_2 hydrolysis in rat-brain synaptosomes. The stimulation observed with TeTx (1 nM) was similar to that obtained with the NGF (100 nM)-treated synaptosomes, 129 ± 30% and 139 ± 13%, respectively. Carbachol (1 mM) was more effective in stimulating PIP_2 hydrolysis, 230 ± 21% (Fig. 3A), probably due to the different PLC isoenzymes induced during each activation. Since it known that NGF produces its action via activation/phosphorylation of a PLCγ isoenzyme, this prompted our group to identify whether TeTx produces specific phosphorylation of PLCγ1 via some TyrKRs. To determine the identity of this phosphorylated

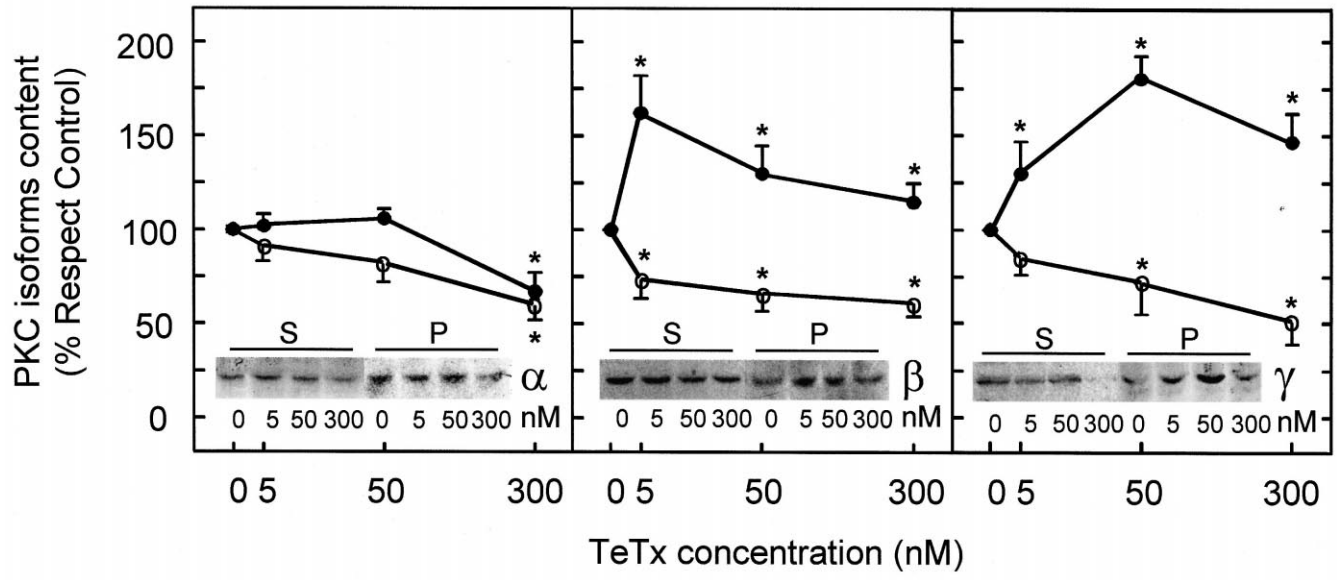

Fig. 2. Effect of TeTx on the subcellular *c*PKC isoforms distribution. Aliquots of 1 ml from a synaptosomal fraction suspension were treated with vehicle as control, and TeTx for 30 min at 30°C. After treatment, the synaptosomes were collected by centrifugation and the cytosolic '○' and particulate '●' fractions separated, as indicated in Section 2. Thereafter, the samples from each fraction were electrophoresed in 10% acrylamide resolving gels, transferred to a PVDF membrane and probed separately with antibodies against PKC-α, -β, and -γ isoforms. The intensity of the bands of each fraction was determined by scanning and expressed as a percentage, with respect to its control. The results are expressed as means ± S.D. of the percentage of three separate experiments and asterisks represent values significantly different from the control group ($P < 0.05$). Insets: Representative gels of the subcellular redistribution of PKC isoforms.

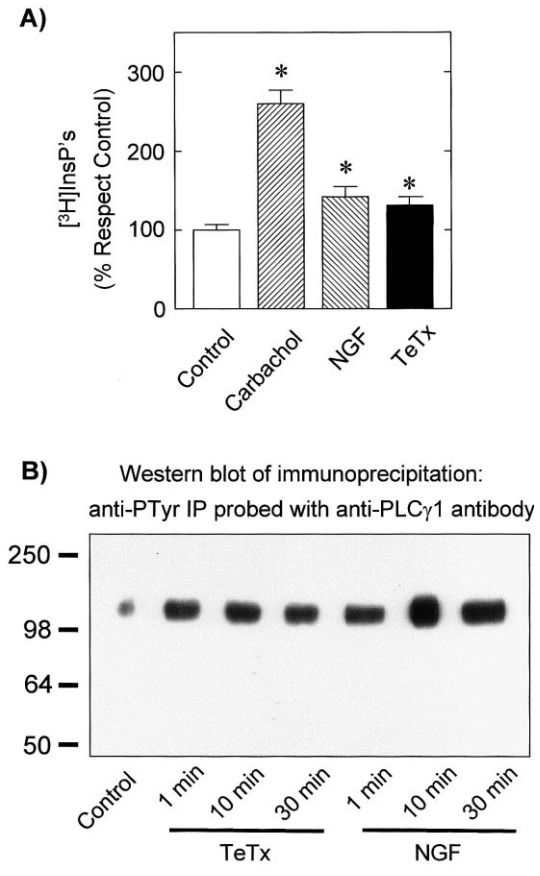

Fig. 3. A: Stimulation of PIP_2 hydrolysis by carbachol, NGF and TeTx. Synaptosomes relabelled with *myo*-[^{3}H]inositol were incubated in Krebs–Ringer buffer containing 10 mM LiCl with no further additions (control), 1 mM carbachol, 100 nM NGF or with 1 nM TeTx. The radioactivity detected in the control corresponded to 7.3 ± 1.2% of the total radioactivity. Data are expressed as means ± S.D. Asterisks represent values significantly different form the control group $P < 0.01$ ($n = 4$–7). B: Time course of TeTx and NGF-induced PLCγ1 phosphorylation. Synaptosomes were incubated at 30°C with TeTx (1 nM) or NGF (50 nM). Equal amounts of lysate from each timepoint, were immunoprecipitated with 4 μg of antibody (clone PY99) directed against PTyr residues, and blots were probed with anti-PLCγ1 antibody. ←

protein (Fig. 3B), the synaptosome lysate was immunoprecipitated using polyclonal antibodies directed against phosphotyrosine residues (Clone PY99) and blotted with anti-PLCγ1 antibody. The synaptosomes were treated with TeTx (1 nM) or with NGF (50 nM) for 1, 10 or 30 min. Fig. 3B shows that the elevation of protein phosphorylation of PLCγ1 was evident in both treatments at 1 min and they displayed maximal phosphorylation by 10 min, which remained above the basal level even after 30 min, as was observed by the increase of the bands of 150 kDa.

3.4. *TeTx, like TPA, induces protein phosphorylations in Ser residues and specific SERT phosphorylation*

Induction of the Ser-phosphorylation by TeTx (1 nM) or by TPA (100 nM) was compared with the basal phosphorylation level present in the vehicle synaptosomes. As can be observed, in Fig. 4A, the increase of general Ser-phosphorylation was observed with respect to the control. Both treatments also produce a specific density increase of a band with a molecular weight close to the SERT molecular weight (~76 kDa). SERT is expressed equally in treated and untreated synaptosomes, demonstrated by Western blot using anti-SERT antibody (data not shown). However, by prior immunoprecipitation with anti-PSer antibody and probed with anti-SERT or vice versa immunoprecipitation with anti-SERT antibody and probed with anti-PSer antibody, an increase of density of the bands corresponding to the molecular weight of SERT can be seen (Fig. 4B). The synaptosomes were incubated for 30 min at 37°C with vehicle, TeTx (1 nM) or TPA (100 nM). The incubation of membranes in Western blot experiments with anti-SERT blocked with the antigenic peptide dramatically abolished the band of 76 kDa corresponding to SERT (Fig. 5). On the other hand, the specificity of the anti-PSer antibody

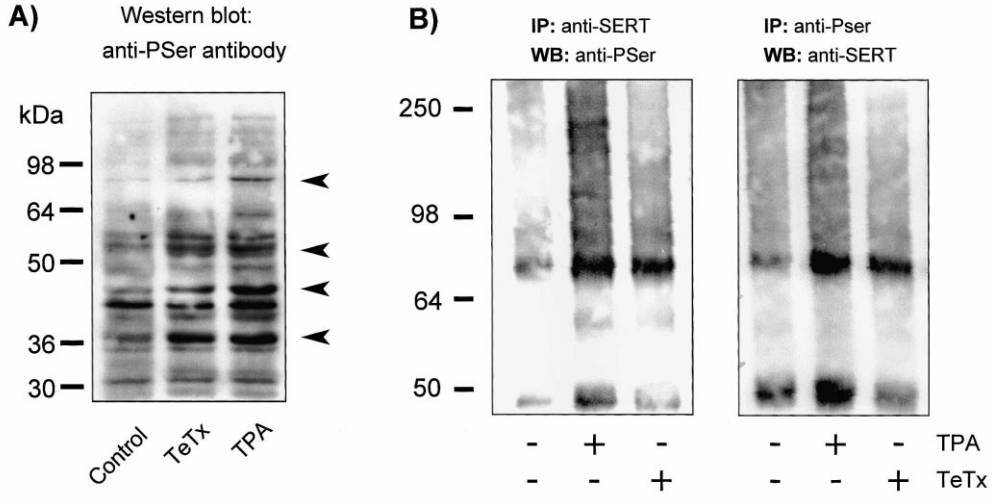

Fig. 4. Effect of TeTx (1 nM) and TPA (100 nM) on SERT phosphorylation. A: Induction of Ser-phosphorylation, the arrowheads show the Ser-phosphorylated proteins as consequence of the incubation with TeTx and TPA. B: The blot on the left corresponds to the immunoprecipitations (IP) of the Ser-phosphorylated proteins with anti-PSer antibody, in response to TeTx or to TPA, and probed with anti-SERT antibody. Meanwhile, the blot on the right corresponds to the IP of SERT with anti-SERT antibody and probed with anti-PSer antibody, in the same conditions.

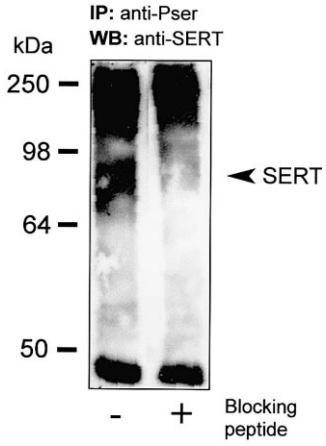

Fig. 5. Blocking of the specific immunoreactive band observed with anti-SERT with the antigenic peptide. Synaptosomes were incubated with 100 nM TPA for 30 min at 30°C. Subsequently, the immunoprecipitation was performed as described in Section 2, using 4 μg of antibody directed against PSer, and blots were probed with anti-SERT antibody with or without being previously blocked with the SERT control peptide, corresponding to a 15-amino acid sequence between transmembrane domains 7 and 8 of the rat SERT.

was assessed by blocking the PSer prior to membrane incubation treatment that induced diminution of the 76-kDa band corresponding to SERT (results not shown).

3.5. *Bisindolylmaleimide-1 (BIM-1) blocks the TeTx and TPA induction of SERT phosphorylation*

Western blot of immunoprecipitations demonstrated that coapplication of TeTx (1 nM), or TPA (100 nM), in the presence of PKC-specific inhibitor BIM-1, 5 μM) substantially blunted the PKC-dependent SERT phosphorylation in rat-brain synaptosomes (Fig. 6A). The quantification by densitometry of the results obtained with a set of identical experiments to the upper blot ($n = 3$–5) demonstrated the statistical significance of the increase of SERT phosphorylation in the tissue treated with TeTx or with TPA. The bands revealed a two-fold stimulation of SERT phosphorylation after TeTx or TPA application. This phosphorylation was abolished by co-incubation with BIM-1. In Fig. 6, a clear and significant reduction of the basal PKC-dependent SERT phosphorylation in control synaptosomes treated with BIM-1 can also be observed. Like activation/phosphorylation of PLCγ1, SERT phosphorylation occurs over a brief period of time and it reaches at the maximal band density after 10 min of treatment, which remained above the basal level even after 30 min, as was observed by the increase of the density bands of 76 kDa, presented in Fig. 7.

4. Discussion

Serotonin is a neurotransmitter in the CNS and PNS which modulates a wide spectrum of behaviors. Serotonin is also present in other tissues, including platelets, where it represents a major secretory product of activated cells [18]. The action of 5-HT, and other neurotransmitters, in these tissues is terminated by active transport [19], and a single gene product encoding the SERT appears to be the sole gene responsible for extracellular 5-HT clearance [20,21]. Acute and long-term regulations of serotonin transport have been extensively studied

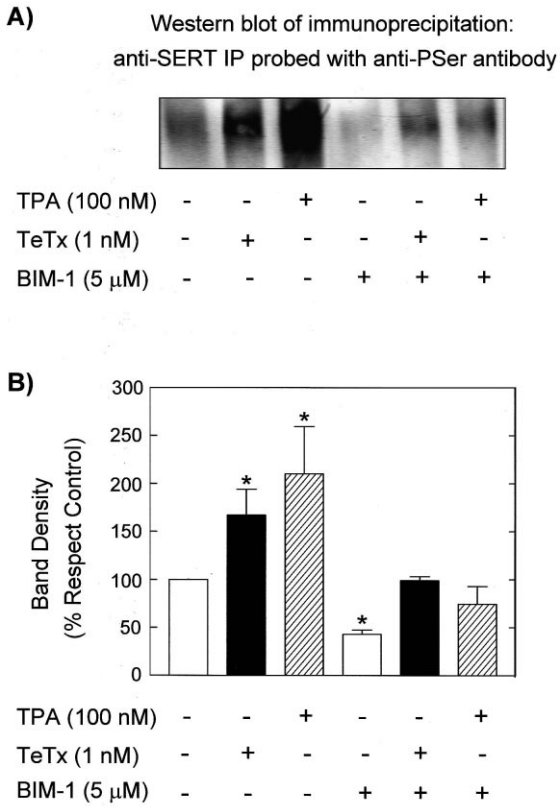

Fig. 6. A: Inhibition of TPA and TeTx-induced SERT phosphorylation by the PKC inhibitor BIM-1. Synaptosomes were incubated with BIM-1 or with vehicle for 30 min at 30°C. Next, TPA, TeTx or vehicle were added and incubated for 30 additional min at 30°C. Subsequently, the immunoprecipitation was performed as described in Section 2, using 4 μg of antibody directed against SERT, and blots were probed with anti-PSer antibody. B: Quantification by densitometry of the results obtained in A. The results are expressed as means ± S.D. of the percentage of 3–5 separate experiments, and asterisks represent values significantly different ($P < 0.05$) from the control group.

[22], and SERT gene expression is, in general, positively regulated by hormones and growth factors. Some other transduction mechanisms have been implicated in acute regulation of expressed SERT, and PKC has been implicated in the inhibition and down-regulation of this type of transporters [23,24].

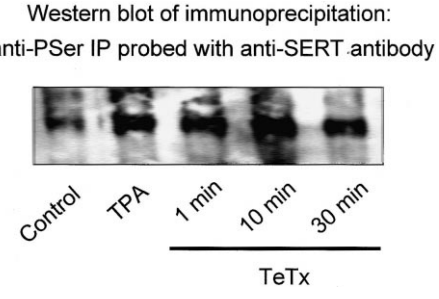

Fig. 7. Time-course of TeTx-induced SERT phosphorylation. Synaptosomes were incubated at 30°C with TeTx (1 nM) for 1, 10 or with TPA (100 nM) for 30 min. Equal amounts of lysate from each timepoint, obtained by sonication and with a protein concentration of 1 mg/ml, were immunoprecipitated with 4 μg of antibody directed against phosphoserine residues, and blots were probed with anti-SERT antibody.

In Fig. 1, the [^{3}H]5-HT uptake inhibition induced by both TeTx and TPA is represented. The inhibitory effect by TPA can be observed a low concentrations, 1–10 μM for 30 min, and produces a decrease in transport capacity (30–40%) with no change for the apparent affinity to serotonin (K_M) in rat-brain synaptosomes; these results are similar to those presented by Anderson and coworkers [25] and by our group (data not shown) in primary neuronal cultures. But the effects of TeTx, and its similitude, modifying the kinetic parameters of 5-HT inhibition with respect to TPA have been introduced here. Nevertheless, TeTx was three orders of magnitude more potent than TPA in producing a decrease in transport capacity (V_{max}); in our tissue concentrations of 1–5 nM of TeTx for 30 min, it produces a decrease in V_{max} of around 40–50%. The data corroborate prior characterization of TeTx as an inhibitor of the Na^+-dependent serotonin uptake [10,11] and open the possibility of inferring the transduction mechanism modulated by the toxin.

PKC is specifically activated and down-regulated by the phorbol ester TPA [26] and by TeTx [27]. We must consider that the enzymatic assay reflects the sum of all Ca^{2+}- and phospholipid-dependent protein kinases; therefore, to obtain more information, work with specific PKC isoforms must be done. In the present work, the Western blots showed a loss of immunoreactivity of the soluble fraction, due to TeTx, in the *c*PKC (α, β and γ) isoforms. The particulate fractions showed a loss of immunoreactivity of the PKC-α and an increase of PKC-β and -γ with a tendency to total (soluble and particulate) down-regulation common to the three isoforms (Fig. 2). These results are very close to those previously presented by Gil and coworkers [28,29] with NGF (1–100 nM) and TeTx (1–100 nM), with respect to the *c*PKC subspecies, and differ from those obtained under the same conditions with TPA (5–1000 nM). TPA was more effective to down-regulate both soluble and particulate fractions. The differences would be due to the interaction of TeTx and NGF with some receptors which promote the activation of PLC, in comparison to the vis-à-vis interaction between TPA and PKC.

In Fig. 3A, stimulation of PIP_2 hydrolysis by carbachol, NGF and TeTx was performed to investigate the last proposal. Although the accumulation of [^{3}H]inositol phosphates due to the action of TeTx and NGF is relatively modest compared to the synaptosomes treated with carbachol, the responses are significant and probably the activation of only one type of PLC, i.e., PLCγ is involved. Western blot for immunoprecipitation with anti-PTyr and probed with anti-PLCγ1 antibodies make the presence of a connection between PLCγ1 and PKC manifest. TeTx (1 nM) as well as NGF (50 nM) mediate a fast and specific Tyr-phosphorylation of the PLCγ1.

The receptor-dependent activation/phosphorylation of PLCγ1 is responsible for the specific activation (translocation) of some PKC isoforms, and the activated PKC could be responsible for the increase of phosphoproteins found after treatment with TeTx or with TPA, (data obtained by Western blot with anti-PSer antibody (Fig. 4A)). Activation of PKC results in a loss of transport capacity as a consequence of phosphorylation of multiple members of the Na^+/Cl^--coupled neurotransmitter transporters [14,22,30,31]. We present the coincidence among activation/phosphorylation of PLCγ1, activation (translocation) of *c*PKC isoforms, and non-competitive serotonin transport inhibition (a decrease in transport capacity, V_{max}, rather than changes in substrate affinity, K_M, was observed). In Figs. 4B, 6 and 7, it has been demonstrated that one phosphoprotein obtained as a consequence of TeTx treatment is SERT; it could be significantly phosphorylated, with respect to the basal phosphorylation, after treatment with low doses of TeTx or with moderate doses of TPA. There is no evidence of sequestration of SERT after phosphorylation, as has been demonstrated by other authors [32]. Phosphorylation of the SERT is demonstrated by a set of elegant experiments which play with the specificity of two antibodies with affinity to the SERT. This set of antibodies could substitute the use of radioactivity experiments with [^{32}P]orthophosphate with similar efficiency. Finally, that SERT phosphorylation produced by both, TeTx and TPA, could be efficiently prevented with the specific PKC inhibitor BIM-1 is demonstrated. PLC activation leads to PKC activation and then leads to SERT phosphorylation; however, it is not possible to conclude from our results that phosphorylation and reduction of serotonin uptake are directly produced by PKC, since other targets of PKC could contribute to the final effect.

Recently Gil and coworkers [29] presented convincing data demonstrating that TeTx activates signal transduction pathways involving trkA, PLCγ1, and PKC isoforms. The final conclusions of these studies are that part of the action of TeTx consists of modifying the signal cascade initiated in some TyrKRs on nerve tissue previous to their cellular internalization, modifying the activity of a PLC isoform, *c*PKC distribution (activation) and, finally, affecting SERT phosphorylation and serotonin transport capacity.

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