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Serotonin transport is modulated differently by tetanus toxin and growth factors

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Abstract

It has been previously shown that 5-HT uptake inhibition produced by tetanus toxin (TeTx) corresponds to a non-competitive inhibition, and it is preceded by phosphorylation of the tyrosine-kinase receptor trkA, phospholipase C activation and translocation of protein kinase C isoforms [FEBS Lett. 481 (2000) 177; FEBS Lett. 486 (2000) 136]. In the present work, it is shown that agonists of tyrosine-kinase receptors (NGF, EGF, basic FGF) enhance Na^+-dependent, 5-hydroxytryptamine (serotonin, 5-HT) uptake in the synaptosomal-enriched P_2 fraction from rat-brain, suggesting a divergence in the intracellular signal pathways triggered by TeTx and by agonists of TyrK receptors. Co-applications of TeTx and agonists of TyrK receptors result in a mutual and partial reversion of their effects on 5-HT transport. In spite of their differences on transport, TeTx, TPA and NGF produce an increase in serotonin transporter phosphorylation in Ser separately, which is abolished by the PKC-inhibitor bisindolylmaleimide-1. Co-application of sodium vanadate, a tyrosine-phosphatase inhibitor, partially abolishes the effect produced by TeTx, whereas genistein, a tyrosine-kinase inhibitor, does not exert any variation of TeTx inhibition. Analyses by immunoblotting of the activation of specific PKC isoforms activation, determined as translocation to the membrane compartment, reveals differences in the pattern produced by NGF and TeTx. PKCγ, δ, and ε isoforms are equally activated by both compounds, whereas the β isoform is activated in a sustained manner only by TeTx, and the α isoform is only down-regulated by NGF. The aim of the present work was to explore whether NGF have the same effect on 5-HT transport than TeTx, since both compounds share the ability of activate part of the same transduction pathways. In spite of this, growth factors and TeTx show an opposite effect on 5-HT transport, even though SERT phosphorylation is enhanced in both cases. The differential effect on α- and β-PKC isoenzymes found between NGF and TeTx action could explain this apparent discrepancy.

Keywords: Tetanus toxin; Growth factors; Serotonin (5-hydroxytryptamine); Uptake; Transporter; Synaptosomes

1. Introduction

Tetanus toxin (TeTx) produced by the *Clostridium tetani* bacilli is the protein-toxin which is responsible for the paralytic syndrome of tetanus. Together with other clostridial neurotoxins (CNTs), the active holotoxin shares a common structure composed of a heavy (H, 100 kDa) and a light (L, 50 kDa) chain, held together by a disulphide bond (Herreros et al., 1999). It has been proposed that the action of the CNTs can be dissected into four-step mechanisms (Montecucco et al., 1994; Montecucco and Schiavo, 1994): binding, internalisation, translocation and blockade of neurotransmitter release. The 50 kDa C-terminal domain of the H-chain would be necessary and sufficient for the binding and internalisation of this protein-toxin in neurons (Lalli et al., 1999); the 50 kDa N-terminal portion of the H-chain would be involved in the translocation of the amino-terminal domain of TeTx (L-chain) in the cytosol; and the L-TeTx chain, which is a zinc-endopeptidase that specifically cleaves vesicle-associated membrane protein (synaptobrevin/VAMP2) via a process independent of synaptic activity, would be responsible for the blockade of exocytosis (Schiavo et al., 1992; Link et al., 1992).

Despite the widespread distribution in cells of synaptobrevin isoforms, the action of TeTx in vivo is completely

Abbreviations: CNTs, clostridial neurotoxins; BIM-1, bisindolylmaleimide-1; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; 5-HT, 5-hydroxytryptamine (serotonin); NGF, nerve growth factor; PKC, protein kinase C; PLC, phosphoinositide–phospholipase C; SERT, serotonin transporter; SSRI, selective serotonin re-uptake inhibitor; TeTx, tetanus toxin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TyrKRs, tyrosine-kinase receptors

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neurospecific. Binding to the plasma membranes is essential for development of spastic paralysis, but the specific receptor has not been yet identified. Polysialogangliosides have been proposed as candidates (Williamson et al., 1999). However, the relative low-affinity binding to these sialoesfingolipids questioned whether gangliosides are the true membrane receptors. The existence of glycoproteins that could exercise functions of very high-affinity receptors has also been proposed (Yavin and Nathan, 1986; Pierce et al., 1986). Nevertheless, efforts to find such proteic receptors have been futile.

Recently, it has been indicated that TeTx is a powerful neurotoxin which acts by different mechanisms inducing the alteration of relevant processes in the CNS: it was demonstrated that TeTx can partially block neurotransmitter release by stimulating Ca^{2+}-dependent transglutaminase activity (Facchiano and Luini, 1992; Facchiano et al., 1993; Ashton et al., 1995). TeTx also induces protein kinase C translocation/activation (Aguilera and Yavin, 1990; Gil et al., 1998), even before classical tetanus symptoms are evident (Aguilera et al., 1993). Finally, it has been demonstrated that TeTx, but not botulinum neurotoxin type A (BoNT/A), is able to block serotonin transport in CNS synaptosomes; this inhibitory effect has also been studied in the uptake of other transmitters, however, only minor or no effects of CNTs in the uptake of transmitters have been observed (Wellhöner, 1992; Inserte et al., 1999; Najib et al., 1999).

The serotonin transporter (SERT) belongs to a small family of integral membrane proteins which transport neurotransmitters (Nelson, 1998). SERT is the sole molecule responsible for extracellular 5-HT clearance in blood plasma or in the nervous system, coupling uptake to the influx of Na^+, K^+ and Cl^- ions (Borowsky and Hoffman, 1995; Rudnick and Clark, 1993; Worrall and Williams, 1994). Selective serotonin re-uptake inhibitors (SSRIs) have become the most successful class of marketed antidepressants, with fluoxetine (Prozac) being the most popular due to its efficacy in the treatment of various anxiety disorders and depression (DeVane, 1999). SERT is the primary binding site in the brain for antidepressant drugs, and it also interacts with cocaine and amphetamines (Ritz et al., 1990; Rudnick and Wall, 1992). Our group has previously demonstrated that TeTx, or its Hc-fragment, inhibits the Na^+-dependent, high-affinity [^{3}H]5-HT uptake in rat-brain synaptosomes. This inhibitory action is independent of zinc-endopeptidase activity, and its potency is greater than the selective inhibitors paroxetine and fenfluramine (Inserte et al., 1999; Najib et al., 1999).

Recently, Gil et al. (2000, 2001) presented convincing data in rat-brain synaptosomes demonstrating that TeTx and Hc-TeTx activate signal transduction pathways involving trkA, PLCγ1, and PKC isoforms, and finally affecting SERT phosphorylation and serotonin transport capacity. In future work, it shall be necessary to give an explanation of the different answers of some growth factors, such as NGF, with respect to TeTx and TPA.

2. Experimental procedures

2.1. Materials

Tetanus toxin was provided by List Biological Laboratories Inc. (Campbell, CA, USA). Polyclonal antibodies against PKC isoforms α, γ and ζ were purchased by Boehringer Mannheim Gmbh (Germany), as well as protein A–agarose beads. Monoclonal antibodies against PKC isoforms β, δ, and ε were from Transduction Laboratories (Lexington, KY, USA). The specific antibody against phosphoserine (polyclonal antibody) was obtained from Zymed Laboratories Inc. (San Francisco, CA, USA), and the polyclonal antibody against the serotonin transporter was from Chemicon International (Temecula, CA, USA). NGF 2.5S was supplied by Alomone Labs (Jerusalem, Israel). 5-Hydroxytryptamine, 12-*O*-tetradecanoylphorbol 13-acetate, bFGF, EGF, Tween 20 and protein A–agarose were from Sigma. 5-[1,2-^{3}H]Hydroxytryptamine ([^{3}H]5-HT) (34 Ci/mmol) was from Amersham International (Buckinghamshire, UK). Dowex AG1X8 was supplied by Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA, USA). All other reagents were of the highest grade possible from standard commercial sources.

2.2. Preparation of synaptosomes from rat-brain

All experiments were performed with a crude synaptosomal fraction (P_2) prepared from Sprague–Dawley 6-week-old rat-brains. The whole brain was homogenised in 40 volumes (w/v) of phosphate buffer at pH 7.4 supplemented with 0.32 M sucrose. Homogenisation was performed with 12 strokes (900 rpm) using a Potter homogeniser with a Teflon pestle (0.1–0.15 mm clearance). The homogenate was centrifuged at 4 °C for 5 min at 1000 × *g*, and the resultant supernatant was centrifuged at 12,000 × *g* for 20 min. The crude synaptosomal pellet obtained from one brain was gently re-suspended 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, and gassed before use with a mixture of 95% O_2 and 5% CO_2 for 20 min and then adjusted to pH 7.4.

2.3. Subcellular fractionation

When subcellular fractionation was required, synaptosomes were collected by centrifugation after each treatment and re-suspended in 0.5 ml of homogenisation 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 phenylmethyl sulphonyl fluoride (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 re-suspended to the original volume using homogenisation buffer supplemented with Triton X-100 (0.3% 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.

2.4. Immunoprecipitation and Western blot analysis

Synaptosome suspension was diluted with Krebs–Ringer buffer to a final protein concentration of 1 mg/ml, and split into the necessary aliquots. After treatment, synaptosomes were collected by centrifugation and the reaction medium was eliminated. Next, 1 ml of homogenisation buffer supplemented with 0.3% Triton X-100 was added and synaptosomes were disrupted by sonication (three times, 10 s). For immunoprecipitation, 1 mg of total protein was incubated by gentle rocking at 4 °C overnight in the presence of 4 μg of anti-SERT 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 PBS–3% BSA to eliminate unspecific binding and gently rocked at 4 °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 re-suspended in 100 μl 2× of non-reducing sample buffer and boiled for 4 min. Next, the agarose beads were separated by pulsing, and 10 μl of each sample were analysed in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). All of the electrophoresis reagents were of analytical grade (Pharmacia Biotech, Upsala, Sweden). The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane, using a mini TransBlot Cell II (Bio-Rad, Hercules, CA, USA) at 100 V for 1 h. 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. Following that, the membranes were incubated overnight with the corresponding antibody diluted in blocking buffer. Then, 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 each step. 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. Computer-assisted analysis of the bands was performed with a Bio-Rad GS700 system (Bio-Rad, Hercules, CA, USA), and data were processed with a Bio-Rad Molecular Analyst image programme using a DELL workstation. Repeated scans were taken for film non-linearity corrections.

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

In the standard assay, to measure synaptosomal 5-HT uptake, 100 μl of a synaptosomal-enriched P_2 fraction 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 neurotoxin diluted in Krebs–Ringer bicarbonate buffer containing 0.05% BSA, or with 50 μl of the same medium without neurotoxin 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 tritiated amine concentration range was from 5 to 160 nM, and for the rest of the experiments a concentration of 60 nM was used. 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., Gaitherburg, 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 Scintillant (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.

2.6. Data and statistical analysis

Uptake was calculated as femtomoles of [^{3}H]5-HT per minute per milligram of synaptosomal protein. The kinetic constants or the apparent kinetic constants, K_m and V_{max}, of synaptosomal amine uptake have been analysed by means of the Eadie–Hofstee plot where the data points were calculated by least-square regression analysis. Part of the data are presented as means with standard deviation (mean ± S.D.), and differences among groups were compared using the Student's *t*-test. Differences between groups were compared using the one-way ANOVA test and one-way Dunnett's test for multiple comparisons. Differences were considered to be significant when $P < 0.01$.

3. Results

The accumulation of [^{3}H]5-HT into synaptosomal-enriched P_2 fractions was used to determine the kinetic parameters of serotonin transport, and initial uptake velocity was measured at serotonin concentrations that ranged from 5 to 160 nM. Previous to specific treatment, kinetic analysis based on non-linear regression analysis revealed a [^{3}H]5-HT uptake with an apparent $V_{max} = 363.1 \pm 21.9$ fmol/(min mg

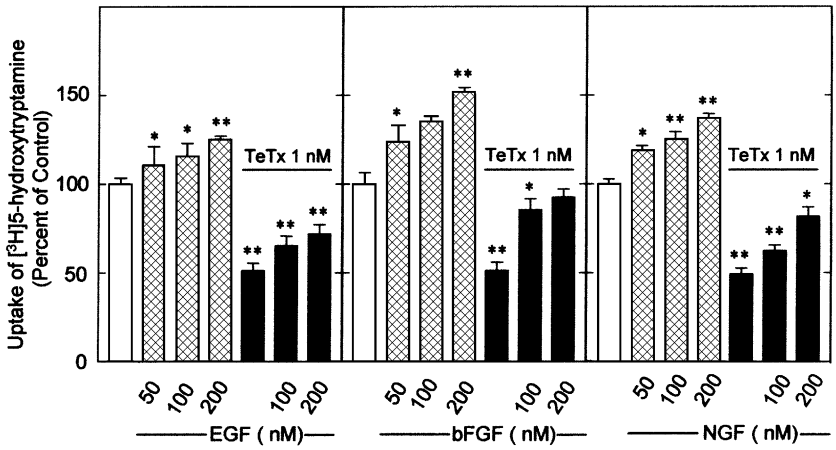

Fig. 1. Growth factors and tetanus toxin modulate [^{3}H]5-hydroxytriptamine uptake. Synaptosomal preparations were incubated in the presence (black bars) or absence (clear bars in Control and squared bars in TyrKR agonists treatments) of TeTx. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or nerve growth factor (NGF) treatments, at different concentrations, correspond to squared bars. [^{3}H]5-HT uptake was performed with serotonin 60 nM. Vertical bars represent mean ± S.E.M. values, with respect to Control of three separate experiments, each one in triplicate. Differences between groups were compared using the one-way ANOVA test and one-way Dunnett's test for multiple comparisons. Asterisks represent values significantly different ($^*P < 0.05$ and $^{**}P < 0.01$) from Control group.

of protein) and an apparent $K_m = 46.2 \pm 6.6$ nM at 37 °C. The non-specific uptake measured at 0 °C represented between 30 and 40% of the total [^{3}H]5-HT uptake in synaptosomal fractions. As was described in previous works, significant decreases with respect to Control were found in apparent V_{max}, without changes in K_m, in treatments with both TeTx and phorbol ester TPA (Najib et al., 1999, 2000). As can be seen in Fig. 1, treatment with a moderate TeTx concentration of 1.0 nM was able to block the specific [^{3}H]5-HT uptake in P_2 fractions. On the other hand, the growth factors used, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), which are endogenous agonists of some tyrosine-kinase receptors, were unable to produce the same effect at the indicated concentrations in Fig. 1. On the contrary, the growth factors tested produced significant increases in [^{3}H]5-HT uptake (127.2% of Control with 200 nM EGF, 151.2% with 200 nM bFGF, and 138.5% with 200 nM NGF). Co-application of the neurotoxin (1 nM) with each one of the indicated growth factors was able to produce a progressive, dose-dependent reversion of the TeTx effect.

In preliminary trials not shown here, [^{3}H]5-HT uptake was found to be rapidly blocked by genistein, with an IC_{50} of 172.2 ± 5.4 μM, in our synaptosomal preparations. The 5-HT uptake inhibition was not raised significantly by simultaneous application of 200 μM of genistein with the neurotoxin at 1 nM concentration (Table 1). On the other hand, sodium vanadate (200 μM), a non-specific tyrosine-phosphatase inhibitor, produced a significant activation of [^{3}H]5-HT uptake, with respect to the Control group (124.5%), and was able to produce a partial blockade (74.5%) of the TeTx effect when toxin and sodium vanadate were applied simultaneously.

Table 1
Effect of *o*-vanadate and genistein on tetanus toxin-induced inhibition of serotonin transporter activity

	Control	TeTx (1 nM)
[^{3}H]5-Hydoxytryptamine uptake (percentage with respect to Control)		
Saline	100.0 ± 5.1	47.60 ± 7.4*
Genistein (200 μM)	79.7 ± 4.4$^\#$	40.87 ± 3.5*
o-Vanadate (200 μM)	124.5 ± 9.4$^\#$	74.50 ± 5.1$^{*,\#}$

Synaptosomes were incubated with or without 1 nM TeTx for 30 min at 37 °C and treated simultaneously with or without genistein or sodium vanadate. Points represent the mean ± S.E.M. of seven separate experiments, each one in triplicate. The symbol (∗) denotes $P < 0.01$ and significantly different with respect to its corresponding Control. The symbol (#) denotes $P < 0.01$ and significantly different with respect to its saline treatment.

Transport capacity of SERT has been extensively described as modulated by phosphorylation. Induction of SERT phosphorylation in serine residues by tetanus toxin (1 nM), 12-*O*-tetradecanoyl-phorbol 13-acetate (100 nM) or nerve growth factor (50 nM) was compared with the basal phosphorylation level present in the vehicle synaptosomes, as can be seen in Fig. 2. By means of immunoprecipitation with anti-SERT antibody and probing with anti-phosphoserine antibody, an increase in the density of the bands corresponding to the molecular weight of serotonin transporter can be detected. The synaptosomes were incubated for 30 min at 37 °C with the vehicle or with the corresponding drug, and the following increases in signal were detected: 163% of SERT phosphorylation, with respect to Control (1 nM TeTx), 232% (0.1 μM TPA), and 126% (50 nM NGF). The specific phosphorylation exhibited by all three substances, as well as the basal signal, were diminished by co-incubation with 5 μM of the protein kinase C inhibitor bisindolylmaleimide-1 (BIM-1). In the cases of

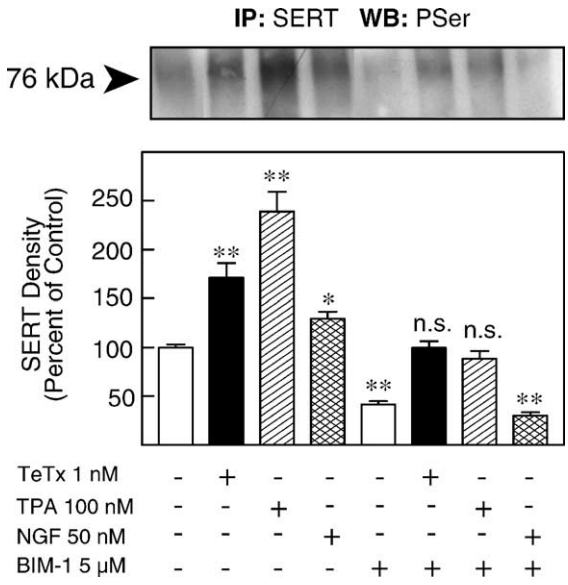

Fig. 2. Inhibition of TPA-, NGF- and TeTx-induced SERT phosphorylation by the PKC-inhibitor BIM-1. Synaptosomes were incubated with 5 μM BIM-1 or with vehicle for 30 min at 30 °C. Next, 100 nM TPA, 1 nM TeTx, 45 nM NGF or vehicle as Control were added and incubated for 30 additional minutes at 30 °C. Subsequently, the immunoprecipitation was performed as described in Section 2. Quantification by densitometry of results obtained by immunodetection are represented in the histogram. Results are expressed as mean ± S.D. of percentage of three separate experiments. Differences among groups were compared using Student's *t*-test. Asterisks represent values significantly different ($^{*}P < 0.05$ and $^{**}P < 0.01$) from Control group.

TeTx and TPA, phosphorylation returned to the basal level in the presence of BIM-1, whereas it disappeared completely in the cases of NGF and Control. The specificity of the band was previously demonstrated by incubation with the blocking peptide from SERT (Najib et al., 2000).

As can be seen in Fig. 3, phorbol ester TPA (100 nM) induces clear translocation in α, β, γ, and δ PKC isoforms, even with notable down-regulation in the case of the α isoform. On the other hand, ε isoform shows no apparent translocation, but significant down-regulation appears in the three treatments tested (TPA, NGF and TeTx). Finally, ζ isoform shows no consistent changes in any case. Some of the PKC isoforms tested (γ, δ, and ε) present similar behaviour in response to NGF (50 nM) as well as to TeTx (1 and 10 nM), whereas the pattern of α isoform is slightly different. In this case no translocation is observed between treatments, but a clear down-regulation appears when synaptosomes are treated with 50 nM NGF (with only 54% of the total initial isoform remaining), whilst no changes are found at TeTx 1 nM, concentration where SERT phosphorylation is induced. A notable down-regulation of the β isoform (55% of total initial isoform) is observed in response to NGF, but, on the contrary, clear translocation is enhanced by 1 nM TeTx. Increasing of the TeTx concentration to 10 nM induces slight down-regulation of the β isoform (12%), also detectable in the α isoform.

4. Discussion

Serotonin is a neurotransmitter in the central and peripheral nervous systems which modulates a wide spectrum of behaviours (Fozard, 1989; Jacobs and Azmitia, 1992). Serotonin is also present in other tissues, including platelets, where it represents a major secretion product of activated cells (Qian et al., 1995). The action of 5-HT, and other neurotransmitters, in these tissues is terminated by active transport (Reith, 1997), and a single gene product encoding the serotonin transporter appears to be the sole gene responsible for extracellular clearance of synaptic and plasma 5-HT (Lesch et al., 1993; Ramamoorthy et al., 1993). Acute and long-term regulations of serotonin transport have been extensively studied (Blakely et al., 1997), 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 (Myers et al., 1989; Anderson and Horne, 1992). In Fig. 1, the [^{3}H]5-HT uptake inhibition induced by TeTx and the [^{3}H]5-HT uptake activation induced by growth factors EGF, bFGF or NGF are represented. The inhibitory effect by TeTx can be observed at low concentrations, 1 nM for 30 min, and produces a decrease in transport capacity (between 40 and 50%) with no change for the apparent affinity to serotonin (K_m) in rat-brain synaptosomes. These results are similar to those presented by Anderson et al. (1995) with phorbol ester TPA and by our group with both TPA and TeTx in synaptosomal-enriched P_2 fraction (Najib et al., 1999; Inserte et al., 1999) and also with primary neuronal cultures (Pelliccioni et al., 2001). Nevertheless, TeTx was three orders of magnitude more potent than TPA in producing a decrease in transport capacity (Inserte et al., 1999; Najib et al., 1999) and these results are in agreement with the hypothesis that a transduction mechanism is modulated by the neurotoxin. However, no change was found in [^{3}H]noradrenaline uptake or in [^{3}H]dopamine uptake when the synaptosomes were treated with TeTx in the same conditions (Inserte et al., 1999), and these data are not in agreement with the finding that the activation of protein kinase C with phorbol ester also produces the inhibition of other biogenic amine neurotransmitter uptake (Huff et al., 1997; Apparsundaram et al., 1998b).

As is demonstrated in the present work, co-application of TeTx with the growth factors was able to produce a progressive reversion of the neurotoxin's effect (Fig. 1). Both stimuli, TeTx and growth factors, share the ability to activate signal transduction cascades involving activation/phosphorylation of tyrosine-kinase receptors, phospholipase Cγ-1, and extracellular regulated kinases (ERKs) 1 and 2 (Gil et al., 2000, 2001), events in which tyrosine phosphorylation is produced. Therefore, the possibility that genistein reverts TeTx-induced inhibition of transport was contemplated. As seen in Table 1, genistein does not block the effect of the toxin. Taking into account that genistein

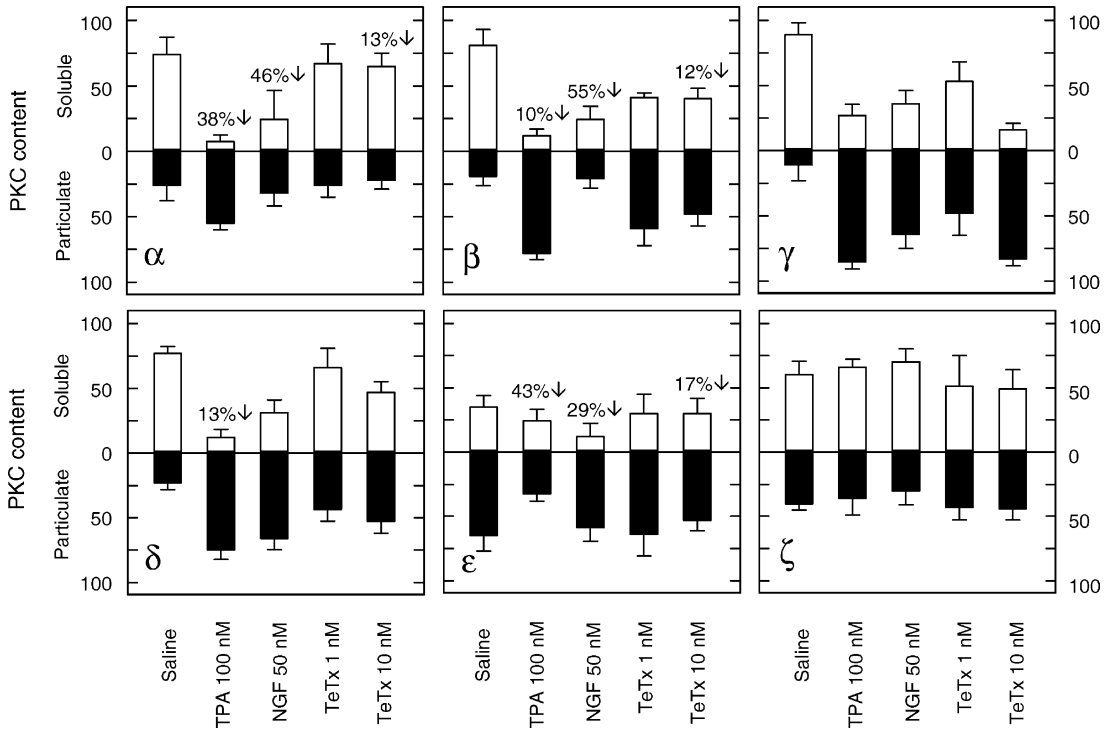

Fig. 3. Effect of treatment with TPA, NGF and TeTx on the subcellular distribution of protein kinase C isoforms in rat-brain synaptosomes. As indicated in Section 2, PKC isoforms α, β, γ, δ, ε and ζ were immunodetected by means of Western blotting. The intensity of the bands of each fraction was determined by scanning and expressed as a percentage, with respect to the respective total Control (particulate plus soluble). The results are expressed as mean ± S.D. of the percentage of three to five separate experiments, and the percentages over the bars represent a significant decrease (down-regulation) of the total immunoreactivity (if one exists).

alone induces a partial blockade of serotonin transport in our system, the lack of reversion in the TeTx effect becomes an expected result. This apparent discrepancy could be explained by the wide spectrum of action of genistein, inhibiting the general tyrosine-kinase activity, whereas TeTx should act over a reduced number of events, which could obviously be affected by genistein. The regulation of serotonin uptake by tyrosine-kinase inhibitors is also described in human platelets (Helmeste and Tang, 1995), an event that could be involved in treatment of affective disorders. It is unlikely that inhibition of serotonin uptake by tyrosine-kinase inhibitors is due to the release of endogenous serotonin stores, since genistein is actually known to inhibit rather than enhance serotonin release (Murphy et al., 1993). On the other hand, *o*-vanadate, a selective inhibitor of tyrosine-phosphatase activity, increases transport capacity, a result which confirms the necessity of general tyrosine-kinase activity to maintain serotonin uptake. Co-applications of TeTx and *o*-vanadate result in a partial reversion of the blockade exerted by the toxin, in agreement with the action of *o*-vanadate alone.

Phospho-amino acid analyses revealed labelling at Ser residues for PKA and both Ser and Thr residues for PKC (Blakely et al., 1997). Mutation of Ser 13 in the NH2 terminus and Ser 599 in the COOH terminus significantly reduced phosphorylation by PKA. No single mutation could eliminate phosphorylation by PKC, but, rather, evidence for increased phosphorylation at other sites was found, suggesting that phosphorylation by PKC is not linked to a single site and may be hierarchical (Blakely et al., 1998). Moreover, the action of another unidentified Ser/Thr kinase which participates in SERT phosphorylation is also described, adding complexity to SERT regulation. The existence of this kinase is in agreement with the signal remains due to SERT phosphorylation in Ser after BIM-1 treatment, as seen in Fig. 2. The reason why TeTx and NGF exerts opposite effects in transport capacity, whereas both induce SERT phosphorylation in Ser, is difficult to assess, and could be related to the specific phosphorylation site or to the presence or absence of additional phosphorylations. Differential PKC isoenzyme activation is most probably related to this point.

Activation of PKC by TPA results in a loss of transport capacity as a consequence of phosphorylation of multiple members of the Na^+/Cl^--coupled neurotransmitter transporters (Qian et al., 1997; Huff et al., 1997; Apparsundaram et al., 1998a,b; Blakely et al., 1997). Although a coincidence in signals triggered by NGF and TPA, including PKC activation, has been described (Gil et al., 2000), the effects on SERT transport capacity are the opposite, and thus a divergence must exist at some point. As an approach to the specific role played by different PKC isoforms in SERT transport modulation, a correlation between activation of

some PKC isoforms and enhancement (in the case of NGF) or decrease (in the cases of TPA and of TeTx) of SERT capacity was searched for, without forgetting the possible participation of other kinases. The results from Fig. 3 show that γ, δ, and ε isoforms present a very similar pattern when synaptosomes are treated with TPA, NGF and TeTx, with clear translocation and no or slight down-regulation in the γ and δ cases, and with no translocation, but with moderate or high down-regulation in the ε case. Therefore, these isoenzymes cannot be considered as being responsible for the differences between NGF and TeTx in the SERT transport capacity. The specific sensitivity to down-regulation of the different PKC isoenzymes has been described in several reports (Matsushima and Nakamura, 1994; Olivier and Parker, 1994), being highly dependent on the system used, a fact which reflects a notable complexity in the pathways that lead to PKC degradation. The behaviour of the α isoform differs partially between NGF treatment (no translocation, but high degree of down-regulation) and TeTx treatment (no translocation and no, or slight, down-regulation). Therefore, the amount of PKCα activated, i.e. in the particulate fraction, does not change in either case. A different pattern is shown by PKCβ, where only down-regulation is detected in response to NGF, but a clear increase in the particulate fraction appears when TeTx is present. So, differential activation of PKC superfamily members by NGF and TeTx could be related to their opposite effects exerted over SERT transport capacity. SERT phosphorylation has been linked to the loss from the plasma membrane of the transporter by means of a putative endocytotic pathway (Apparsundaram et al., 1998a,b). We have no evidence of sequestration of SERT after phosphorylation as has been demonstrated by other authors (Ramamoorthy and Blakely, 1999), and this is an important aim of our present work.

In summary, the present work shows the enhancement of serotonin uptake by several growth factors in synaptosomal preparations, as well as the ability of these growth factors to partially revert serotonin uptake inhibition exerted by TeTx. Since both NGF and TeTx activate the same kind of intracellular signalling, as has been described, the existence of a divergence in the respective activated cascades must exist. The differential activation of PKC isoforms by TPA, NGF and TeTx is used in this work as an approach to understand the opposite effect exerted by both molecules on the transport capacity of SERT observed in this work, and to determine the differences in the intracellular signals triggered by NGF and TeTx.

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