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Downregulation and Subcellular Redistribution of the γ-Aminobutyric Acid$_A$ Receptor Induced by Tunicamycin in Cultured Brain Neurons

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Abstract The significance of N-linked glycosylation and oligosaccharide processing was examined for the expression of γ-aminobutyric acid$_A$ receptor (GABA$_A$R) in cultured neurons derived from chick embryo brains. Incubation of cultures with 5 μg/ml of tunicamycin for 24 h blocked the binding of ^{3}H-flunitrazepam and ^{3}H-muscimol, probes for the benzodiazepine and GABA sites on the receptor, by about 20% and 28%, respectively. The loss of ligand binding was due to a reduction in the number of binding sites with no significant changes in receptor affinity. Light microscopic immunocytochemistry also revealed that the treatment reduced approximately 13% of the intensity of GABA$_A$R immunoreactivity in the neuronal somata. Furthermore, the fraction of intracellular receptors was decreased to 24% from 34% of control in the presence of the agent, as revealed by trypsinization of cells in situ followed by ^{3}H-flunitrazepam binding. The molecular weight of the receptor subunit protein was lowered around 0.5 kDa after tunicamycin treatment, in accordance with that following N-glycosidase F digestion, indicating the blockade of N-linked glycosylation of GABA$_A$R by tunicamycin. Moreover, intense inhibitions of 91% and 44%, respectively, were detected to the general galactosylation and mannosylation in the tunicamycin-treated cells, whereas the protein synthesis was hindered by 13%, through assaying the incorporation of ^{3}H-sugars and ^{3}H-leucine. Nevertheless, treatment with castanospermine or swainsonine (10 μg/ml, 24 h), inhibitors to maturation of oligosaccharides, failed to produce significant changes in the ligand binding. In addition, in situ hybridization analysis showed that these three inhibitors did not perturb the mRNA of GABA$_A$R α_1-subunit. The data suggest that tunicamycin causes the downregulation and subcellular redistribution of GABA$_A$R by producing irregularly glycosylated receptors and modifying their localization. Both galactosylation and mannosylation during the process of N-linked glycosylation may be important for the functional expression and intracellular transport of GABA$_A$R. J. Cell. Biochem. 70:38–48, 1998.

Key words: GABA$_A$ receptor; N-glycosylation; radioligand binding; in situ trypsinization; galactosylation; mannosylation; immunoblotting; immunocytochemistry

γ-Aminobutyric acid$_A$ receptor (GABA$_A$R) is the major inhibitory neurotransmitter receptor in vertebrate brain. It is known as a multisubunit membrane glycoprotein containing an integral ligand-gated Cl^- ion channel. Through combining with the receptor, GABA increases the Cl^- ion conductance to modulate neuronal excitability. The activity of the receptor can be regulated by many therapeutically important drugs, notably the benzodiazepines, barbiturates, and steroids [Macdonald and Olsen, 1994]. A native GABA$_A$R is thought to be a 220- to 355-kDa pentamer comprising combinations from a collection of at least 18 distinct subunits, α_{1-6}, β_{1-4}, γ_{1-4}, δ, ϵ, and ρ_{1-2}, as revealed by molecular biological studies [Schofield et al., 1987; Rabow et al., 1995; Whiting et al., 1997]. Each subunit of GABA$_A$R may possess two to four numbers of potential N-linked glycosylation sites located at the extracellular N-terminal domain of the topological model [Burt and Kamatchi, 1991; Cutting et al., 1992]. Immunoblotting analysis has demonstrated the presence of N-linked oligosaccharides within the α_{1-3}, α_5, and β_{2-3} subunits of GABA$_A$R purified from brains of 5- to 10-day-old rats, by using

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specific antibodies to identify the various subunits after N-glycanase digestion [Buchstaller et al., 1991a,b; Sieghart et al., 1993].

We have previously shown, using a Na^+/H^+ ionophore monensin and ^3H-flunitrazepam (FNZ) as the probe for $GABA_AR$, that the galactosylation, i.e., the maturation of receptor glycoprotein, plays a role in maintaining regular binding capacity of the receptor of embryonic chicken brain neurons in culture [Yin, 1992]. In an earlier study, the long-term administration of tunicamycin (TM), a specific inhibitor to N-glycosylation, interrupted almost completely the Cl^- current elicited by activating $GABA_AR$ in *Xenopus* oocytes transfected by brain mRNAs [Sumikawa et al., 1988]. Moreover, a recent investigation revealed that TM hindered the insertion of $GABA_AR$ subunits to the cell surface of 293 cells expressing the receptor [Connolly et al., 1996]. With regard to the nicotinic acetylcholine receptor (nAchR), accumulated evidence has supported the idea that N-glycosylation of the receptor is linked to its stability and transport to the cell surface [Prives and Bar-Sagi, 1983; Gehle and Sumikawa, 1991]. However, mechanisms underlying the importance of N-glycosylation to the proper subcellular expression of $GABA_AR$ remain to be clarified in neurons. Castanospermine and swainsonine, blockers to maturation of N-linked oligosaccharides, have also been shown to affect the expression of a number of proteins, such as myelin basic proteins [Bhat and Zhang, 1994] and aspartate transporters [Deas et al., 1992]. Whether the two blockers alter the properties of $GABA_AR$ is unknown.

Thus, the present study examines effects of those inhibitors to N-glycosylation and oligosaccharide processing on the expression of $GABA_AR$ in cultured neurons. The subcellular distribution of the receptor was investigated by the treatment of cultures with trypsin followed by radioligand binding. Mechanisms for the action of the drugs on $GABA_AR$ were monitored by analyzing the general protein synthesis and glycosylation, and the expression of $GABA_AR$ α_1-subunit mRNA.

MATERIALS AND METHODS

Neuron Culture and Drug Treatment

Neurons were dissociated from brains of 7-day chick embryos, plated on collagen-coated culture dishes, and maintained in culture for 7 days, as described previously [Roca et al., 1990; Yin and Yang, 1992]. TM (MW, 800), castanospermine (189), or swainsonine (173) (Sigma) dissolved in culture medium was added to the cultures to a final concentration of 1, 5, or 10 µg/ml, and the incubation time ranged from 3 to 24 h. To assess the reversibility for the effect of the agent, after removing the TM-containing medium from a number of cultures, the cells were fed with fresh medium without TM and subjected to a radioligand binding assay. The viability of cultured neurons was examined by incubating cultures with 0.4% Trypan blue in 0.81% NaCl, 0.06% KH_2PO_4, and 0.05% methyl-p-hydroxybenzoate, pH 7.2, for 4 min. The cells were then washed with phosphate-buffered saline (PBS), coverslipped, and observed under a light microscope.

Radioligand-Binding Assay

The radioligand-binding assay was conducted according to earlier protocols [Roca et al., 1990]. In brief, cultured cell homogenates were prepared from 100-mm dishes by homogenization and centrifugation. Aliquots of the homogenates were incubated with 2–25 nM ^3H-FNZ (specific activity: 90 Ci/mmol, Amersham, Buckinghamshire, UK) or 2–300 nM ^3H-muscimol (4.6 Ci/mmol) for 60 min on ice. The incubation was terminated by filtering reaction mixtures through Whatman GF/B glass fiber filters; the radioactive remaining on the filters was counted after adding Liquiscint (National Diagnostics, Atlanta, GA). One mM flurazepam (a gift of F. Hoffman-La Roche, Basel, Switzerland) or 5 mM GABA (Sigma, St. Louis, MO) was included in half of the samples to determine nonspecific binding. The nonspecific binding value was subtracted from the total binding to yield the specific component.

In Situ Trypsinization

Exhaustive trypsin treatment of intact brain cultures was performed as depicted previously [Czajkowski and Farb, 1986]. Briefly, after washing in 25 mM HEPES buffer, cultures were incubated with or without 0.5 mg/ml bovine pancreas trypsin (Sigma) in the buffer for 90 min at 37°C. Subsequently the cultures were placed on ice and soybean trypsin inhibitor (Sigma) was added to the dishes, in order to halt trypsin activity. Cells were then collected for reversible binding assay using 5 nM ^3H-

FNZ as described above. The sensitivity of surface $GABA_AR$ to trypsin attack allows the determination of the fraction of receptors that are located intracellularly or on the cell surface.

^{3}H-Amino Acid and ^{3}H-Sugar Incorporation

Cells kept on 60-mm culture dishes were treated with or without the drugs. ^{3}H-leucine (153 Ci/mmol) at 0.4 μCi/ml, ^{3}H-mannose (13.9 Ci/ml) at 10 μCi/ml, or ^{3}H-galactose (25.5 Ci/ml) at 5 μCi/ml was applied to the cultures 2 h before terminating the drug treatment. Subsequent to washing the cultures with phosphate-buffered salt solution (PBSS) 4 times, each dish received 1 ml sodium dodecyl sulfate (SDS)/Nonidet P-40/urea (0.2%/2%/8 M) and was then placed on a shaker for 1 h. Proteins in the lysate were precipitated on ice by using 10% trichloroacetic acid (TCA) for 1 h, before the addition of 0.1 N NaOH [Yin, 1992]. TCA-precipitable proteins were collected by filtration of the samples through Whatman filters and washed with PBSS containing 5% TCA. The radioactivity associated with the filters was measured by liquid scintillation counting.

Data Analysis

All biochemical determinations were performed in triplicate, and normalized to control. Scatchard plots were analyzed by the ligand program and used to determine the maximal binding sites, B_{max}, and apparent affinity constant, K_D of the radioligand to the receptor [Munson and Rodbard, 1980]. Data are presented as means ± standard deviations. Significance in difference among data was analyzed using a two-tailed Student *t*-test.

Photolabeling, Sodium Dodecyl Sulfate Polyacrylomide Gel Electrophoresis (SDS-PAGE), and Immunoblotting

Fluorography was carried out according to previous procedures [Yin and Fan, 1990]. In brief, cultured cell homogenates were incubated with 5 nM ^{3}H-FNZ in PBSS for 60 min on ice followed by irradiation with long-wavelength ultraviolet light at a distance of 3 cm for 45 min at 4°C. Thereafter, the samples were centrifuged and washed twice. The pellets were resuspended in PBSS and subjected to SDS-PAGE using 10% acrylamide slab gels. The gels were stained, incubated with Amplify (Amersham, Buckinghamshire, UK), and dried. The dried gels were assembled with Hyperfilm-MP (Amersham) in cassettes and placed at −70°C for 3 months.

Control culture homogenates were used for performing the deglycosylation experiment using N-glycosidase F following the methods by Buchstaller et al. [1991b]. The sample (60 μg protein) was dissolved in 0.5% SDS and 50 mM β-mercaptoethanol in 0.4 M Tris-HCl, pH 8.0 (170 μl), and placed in a 65°C oven for 10 min. After cooling to room temperature (RT), the sample received 1.25% Nonidet P-40, 1 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA, and its final total volume became 500 μl. Following the addition of 5 U N-glycosidase F (Boehringer Mannheim: BM, Mannheim, Germany), the sample was incubated at 37°C for 20 h. Subsequently, the proteins in the sample were precipitated with methanol/chloroform [Wessel and Flügge, 1984] and subjected to SDS-PAGE.

For immunoblotting, culture homogenates were first subjected to SDS-PAGE, followed by electrotransfer of the proteins separated on the gels onto nitrocellulose paper (Schleicher & Schuell, Dassel, Germany). The blots were incubated with 5% nonfat milk in PBS for 1 h and then a monoclonal antibody (mAb) 2C3 against chicken $GABA_AR$ (ascites fluid: 1:200 dilution) [Yin and Fan, 1990] overnight at 4°C. They were then incubated with a peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h at RT. The immunoreaction product on the blots was visualized by using 3′,3′-diaminobenzidine as the chromogen.

Immunocytochemistry

Cultures of 35-mm dishes (n = 4 for control and TM-treated, respectively) were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. They were then treated with 0.3% H_2O_2 in PBS for 10 min and 1.25% normal goat serum for 20 min and incubated with the mAb 2C3, antineurofilament 200 (Sigma), or antitubulin (Sigma) Ab over two nights at 4°C. The immunoreaction product was visualized by using a biotinylated secondary antibody and ABC-peroxidase method (Vector, Burlingame, CA). The intensity of $GABA_AR$-immunostaining in the somata of cultured neurons was quantified by employing a computer-based image-analysis system (MCID; Imagine Research, Ontario, Canada). Under a fixed level of illumination, the integrated optical density was mea-

sured for each immunopositive cell. The results are expressed as average optical densities ± standard deviations.

In Situ Hybridization

The specific 45-base antisense oligonucleotide probe, 5′-TTT CTG GCT TAA CTT CTT TGG GCT CTA TCG TTG CAC TTT TAG CAA-3′, and complementary sense probe were custom synthesized for the α_1-subunit of chicken $GABA_AR$, which corresponds to nucleotides 1356–1400 of the α_1-cDNA sequence residing within the intracellular loop [Bateson et al., 1991]. The oligonucleotides were 3′ end-labeled with digoxigenin (DIG)-11-ddUTP using a commercial labeling kit (BM).

In situ hybridization histochemistry was carried out according to earlier methods [Dickerson et al., 1994] with modifications. Briefly, 35-mm cultures were fixed in 4% paraformaldehyde, washed with 2× standard saline citrate (SSC), and dehydrated through a graded series of ethanol. The cultures were then prehybridized with the hybridization buffer (50% deionized formamide, 4× SSC, 1× Denhardt's, 500 µg/ml salmon sperm DNA, 250 µg/ml yeast tRNA, and 10% dextran sulfate) in a humid chamber at 42°C for 1 h. Following washing with 2× SSC and dehydration by ethanol, the sample was hybridized with 0.5 pmol/ml of DIG-labeled probes in the hybridization buffer at 42°C for 18 h.

Posthybridization washes included 2× SSC at RT, 15 min, 1× SSC at RT, 15 min, 0.5× SSC at 60°C, 30 min, and 0.5× SSC at RT, 15 min. After blocking with 2% normal sheep serum for 30 min at RT, the visualization of hybridization signal was achieved by incubating the samples with an alkaline phosphatase–conjugated anti-DIG antibody (BM) at 30°C for 3 h, followed by color development using the substrates (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate) for the enzyme. The nonspecific hybridization signal was determined by using a DIG-labeled sense probe.

RESULTS

Changes in Radioligand Binding

Under the treatment of 5 µg/ml of TM, the level of 5 nM ^{3}H-FNZ binding to the culture appeared in an insignificant decreasing order from 110 ± 9% to 86 ± 7% of control at 6 h to 18 h incubation of the agent. At 24 h, the level of binding was significantly decreased to 80 ± 2% (Fig. 1A). When dosage of TM was raised to 10 µg/ml, a binding level of 75 ± 6% was seen. Castanospermine or swainsonine administration at 1–10 µg/ml for 3–24 h resulted in 105 ± 9 to 92 ± 5% of control binding (Fig. 1B).

The K_D and B_{max} of ^{3}H-FNZ or ^{3}H-muscimol to $GABA_AR$ in cultures were determined in the presence or absence of 5 µg/ml of TM for 24 h. The B_{max} value of ^{3}H-FNZ to TM-treated cultures, 0.42 ± 0.01 pmol/mg protein, was about 20% lower than the control value, 0.52 ± 0.08, whereas the K_D, 2.50 ± 0.08 nM, resembled control, 2.52 ± 0.01 nM (Fig. 1C). ^{3}H-Muscimol binding revealed that the drug treatment reduced 28% of control B_{max} (3.11 ± 0.07 pmol/mg protein) to 2.25 ± 0.08; the K_D of ^{3}H-muscimol was 8.71 ± 0.51 nM and 8.75 ± 0.77 for control and TM-treated, respectively (Fig. 1D). By contrast, no changes in the B_{max} and K_D were found after 24 h incubation of cultures with 1–10 µg/ml of castanospermine or swainsonine.

The Trypan blue exclusion experiment showed that approximate 5% (n = 3,452 cells counted) of neurons incorporated Trypan blue in control cultures, delineating a normal rate of cell death. A similar percentage, 6% (n = 3,564 cells counted), was seen for cultures incubated with 5 µg/ml of TM for 24 h. Thus, the treatment does not seem to induce membrane leakiness or cell death. In another experiment, cell homogenates of control cultures were subjected to a reversible binding assay using 5 nM ^{3}H-FNZ; in some incubations 5 µg/ml of TM was included. The level of binding in the presence of TM was analogous with that without it, implying a lack of interaction between TM and ^{3}H-FNZ or the receptor.

Following exhaustive trypsinization of intact control cultures, the remaining specific binding fraction resistant to trypsin was 34 ± 5%, representing the intracellular portion of receptors, as shown by using 5 nM ^{3}H-FNZ. In the presence of 1 µg/ml of TM for 3 and 24 h, the trypsin-resistant binding fractions were 32 ± 8% and 30 ± 6%, similar to control value. However, the 24-h treatment with 5 µg/ml of TM reduced significantly the fraction of trypsin-resistant binding to 24 ± 3%. Hence, the intracellular fraction of ^{3}H-FNZ binding became 19% of total specific binding of control culture (100%), whereas the ratio of cell surface binding turned 61% (Fig. 2). By contrast, treatment of the

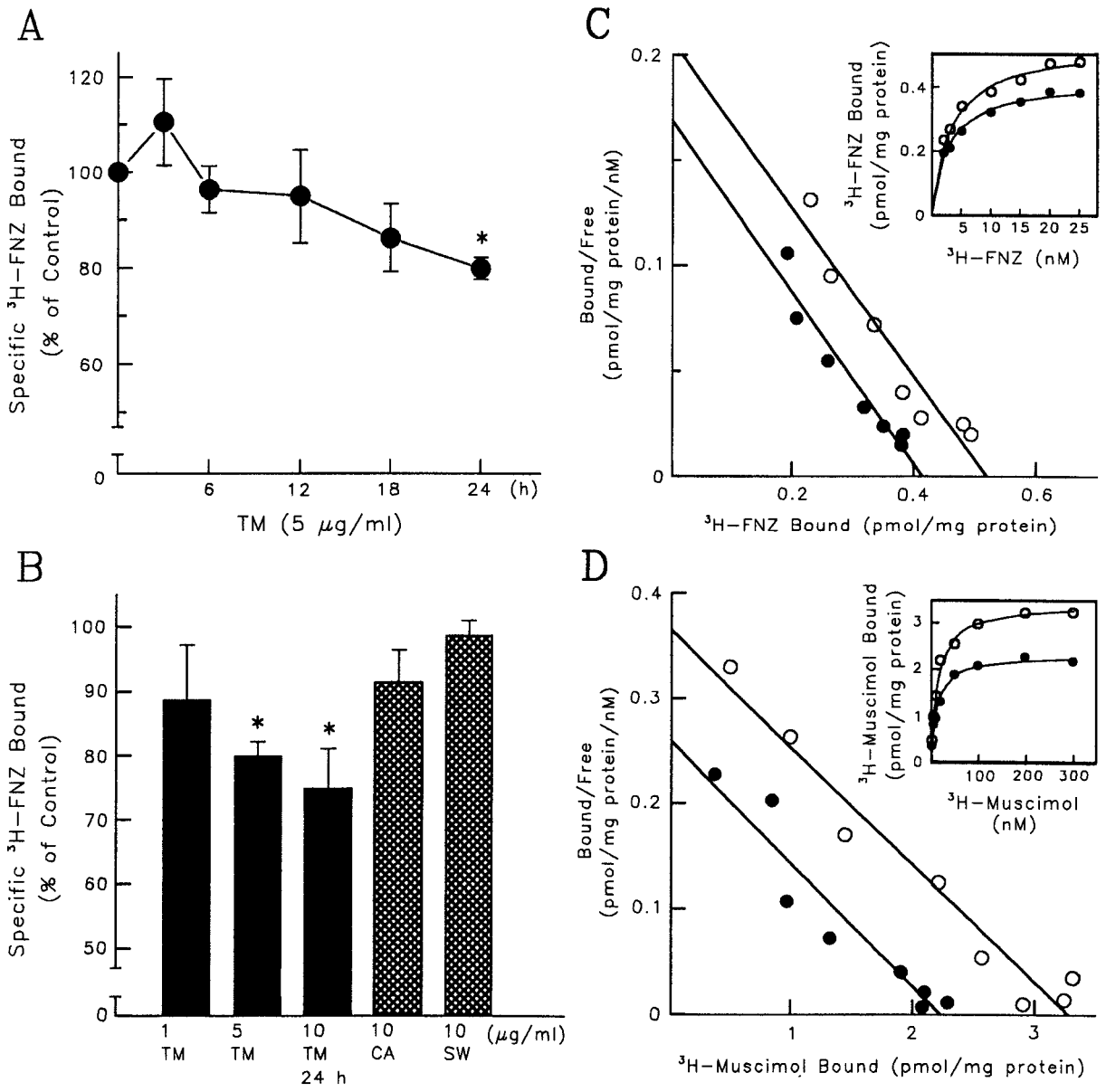

Fig. 1. TM treatment induces decreases in the number of ^{3}H-FNZ and ^{3}H-muscimol recognition sites. The cultures were incubated with 5 μg/ml of TM for various periods (**A**) or with different concentrations of TM, castanospermine (CA), or swainsonine (SW) for 24 h (**B**), followed by reversible binding of 5 nM ^{3}H-FNZ to the culture homogenates as described in Materials and Methods. Reversible binding of ^{3}H-FNZ (**C**) or ^{3}H-muscimol (**D**) to the cells was performed following a 24-h exposure to 5 μg/ml of TM. The specific binding data were analyzed and plotted according to the method of Scatchard. The **insets** show the saturation binding of ^{3}H-FNZ and ^{3}H-muscimol. The lines represent computer-analyzed 1-site fit to the data. The TM incubation reduced the Bmax of ^{3}H-FNZ and ^{3}H-muscimol by approximately 20% and 28%, respectively, from control value. Open circles: control; solid circles: TM-treated; asterisks: significantly different from control (agent concentration: 0); $P < .05$. N = 4.

cultures with castanospermine or swainsonine did not change the ratios. When the TM-treated cultures were first incubated with trypsin and then exposed to Trypan blue, the number of cells that excluded the dye (94%, n = 2,866 cells counted) was similar to the cultures without trypsinization. Therefore, the incubation of trypsin does not appear to cause cell death.

General Protein Synthesis and Glycosylation

Effect of the drugs on the general protein synthesis and glycosylation was explored by

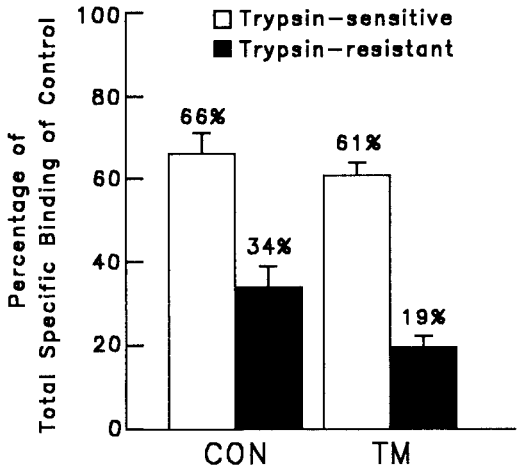

Fig. 2. Subcellular distribution of GABA$_A$R is changed by TM. The cultures were treated with (*TM*) or without (*CON*) 5 µg/ml of TM for 24 h followed by in situ trypsinization and 5 nM ^{3}H-FNZ binding. Percentages of trypsin-sensitive (cell surface) or trypsin-resistant (intracellular) binding to total specific binding of control culture (100%) were determined. N = 4.

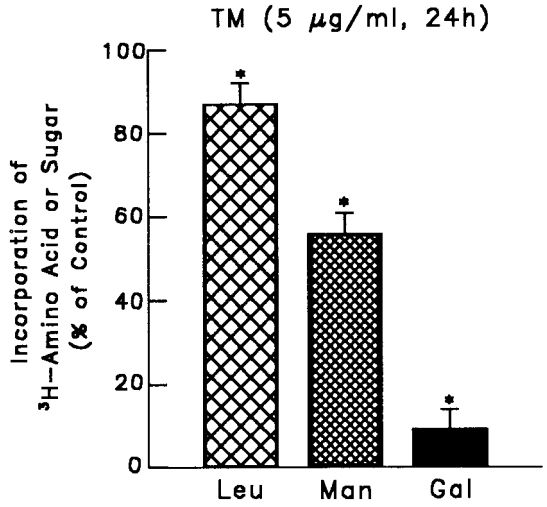

Fig. 3. TM inhibits the incorporation of ^{3}H-leucine (*Leu*), ^{3}H-mannose (*Man*), and ^{3}H-galactose (*Gal*) by the culture. Cells were incubated with 5 µg/ml of TM for 24 h; the radiolabeled amino acid or sugar was added to the medium 2 h before beginning the harvest. The incorporation percentages were determined as described in Materials and Methods. Asterisks: significantly different from control (agent concentration: 0; 100%); $P < .05$. N = 3.

incubating the cultures with ^{3}H-leucine or ^{3}H-sugars. The incorporation extent of ^{3}H-leucine into TCA-precipitable materials decreased about 13% from control (100%) in cultures incubated with 5 µg/ml of TM for 24 h. No significant changes were found following 1–10 µg/ml of castanospermine or swainsonine (24 h) treatment. The incorporation of ^{3}H-mannose was reduced by 23 ± 9%, 24 ± 4%, and 44 ± 5% in the presence of 1 µg/ml of TM for 3 and 24 h, and 5 µg/ml of TM for 24 h, respectively. Under the same conditions, the uptake of ^{3}H-galactose decreased 23 ± 10%, 65 ± 11%, and 91 ± 5% (Fig. 3). Incubation with castanospermine or swainsonine resulted in reductions of 7–25% in ^{3}H-galactose incorporation.

Molecular Weight of GABA$_A$R Subunit Proteins

Photolabeling and SDS-PAGE revealed that at least two proteins with apparent molecular masses of 50 kDa and 48 kDa were associated with ^{3}H-FNZ in the control cultures (Fig. 4A). The mAb 2C3 against the chicken GABA$_A$R recognized a major band of 50 kDa and a minor band of slightly heavier molecular weight on immunoblots (Fig. 4B,C). TM incubation (5 µg/ml, 24 h) resulted in a small reduction, around 0.5 kDa of the immunostained and ^{3}H-FNZ labeled bands (Fig. 4B). Exhaustive treatment with N-glycosidase F on the control culture homogenates, which removes all classes of N-linked carbohydrates, produced similar reduction in the molecular weight (Fig. 4C). These experiments indicate TM-induced inhibition to the N-glycosylation of GABA$_A$R proteins.

Viability of Cultures During Recovery From TM

Subsequent to 24-h exposure to TM (5 µg/ml), the drug-containing medium was replaced by fresh medium in a number of cultures. At 3 h and 24 h after the replacement, the binding of ^{3}H-FNZ to the cultures was further decreased to 58 ± 3% and 41 ± 6% of control level, compared to that just before the onset of recovery (80 ± 2%). The trypan blue exclusion experiment exhibited that cell death significantly increased to 17% (n = 3,583 cells counted) and 47% (n = 3,597 cells) of cells recovered for 3 h and 24 h, respectively, from the TM exposure. A treatment of TM at a lower dose (1 µg/ml), but for a longer time (48 h), caused the detachment of a number of cells from the culture dish.

GABA$_A$R Immunoreactivity

Light microscopic observations exhibited that TM did not cause significant changes in the cell size, shape, and number in culture. Immunocytochemical localization of GABA$_A$R with mAb 2C3 demonstrated the presence of immunoreaction products in the perikarya of about 89% of neurons (n = 2,143 cells counted) in control

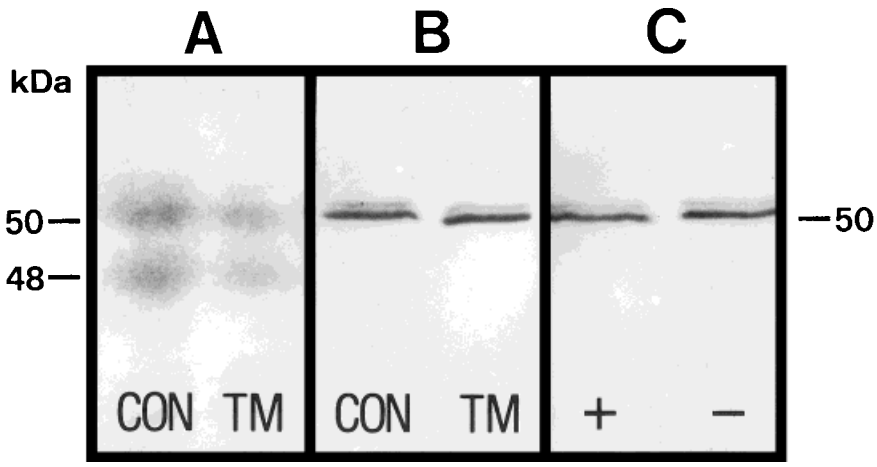

Fig. 4. TM lowers the molecular weight of $GABA_AR$ subunit proteins. **A:** Fluorogram of the receptor subunits. Photolabeling using 5 nM ^{3}H-FNZ and SDS-PAGE of the TM-treated (5 μg/ml for 24 h) (*TM*) or control (*CON*) cultures were carried out as depicted in Materials and Methods. **B:** Immunoblot of control and the drug-treated receptor subunits. SDS-PAGE and immunoblotting using mAb 2C3 against the receptor were performed. **C:** $GABA_AR$ subunit proteins after N-glycosidase F digestion. Control culture homogenates were incubated with (+) or without (−) N-glycosidase F before being subjected to SDS-PAGE and immunoblotting. A small reduction of around 0.5 kDa from control is seen for the TM-treated subunits. These experiments were performed four times with similar results.

culture (Fig. 5A). Similarly, in cultures after 24 h incubation with 5 μg/ml of TM, 90% of cells were immunostained (n = 2,080 cells), but the level of $GABA_AR$ immunoreactivity in the somata, 0.48 ± 0.05 (mean optical density, n = 2,000 cells), was significantly lower than 0.55 ± 0.07 of control (n = 2,000) (Fig. 5B). In addition, the TM administration did not seem to change neurofilament 200 kDa and tubulin immunoreactivity in cultured neurons (data not shown). This may imply the independence of the cytoskeletal elements to the actions of TM.

$GABA_AR$ α_1-Subunit mRNA

In order to explore mechanisms underlying the effects of the drugs on $GABA_AR$, the α_1-subunit mRNA was examined in the cultured neurons by in situ hybridization using DIG-labeled oligonucleotide probes. About 70% (n = 2,862 cells counted) of control neurons contained the α_1 mRNA, which appeared as dark purple reaction products in the perikarya. A similar ratio (68%, n = 2,892 cells) and intensity of labeling were observed for cultures following 24 h incubation with 5 μg/ml of TM (Fig. 6). Treatment of cultures with castanospermine or swainsonine (1–10 μg/ml, 24 h) failed to produce notable changes as well. Thus, these agents did not seem to modify the expression of the α_1 mRNA. The percentage of cells containing α_1 mRNA is lower than that immunostained by mAb 2C3. The difference probably results from that mAb 2C3 recognizes two putative α-subunits [Yin and Fan, 1990].

DISCUSSION

The present report has investigated the possible role N-glycosylation plays in the regulation of $GABA_AR$ expression in cultured brain neurons. The main conclusions relate to the adverse effect of TM and subcellular distribution of $GABA_AR$.

N-Glycosylation and Binding Capacity of $GABA_AR$

Reversible binding assay using ^{3}H-FNZ and ^{3}H-muscimol demonstrated that TM treatment (5 μg/ml, 24 h) reduced about 20% and 28% of the binding sites in neuronal cultures. Consistent with the ligand-binding data, light microscopic immunocytochemistry also exhibited the decreased intensity of $GABA_AR$-immunoreactivity in the TM-treated neurons. Similarly, reductions in the binding of ^{3}H-FNZ and ^{3}H-muscimol have been shown for the deglycosylated purified receptor from adult rat brain [Sweetnam and Tallman, 1986] and 293 cells expressing $GABA_AR$-containing N-deglycosylated α_1-subunit [Buller et al., 1994].

In the present study, a maximal reduction of 44% in the incorporation of ^{3}H-mannose indi-

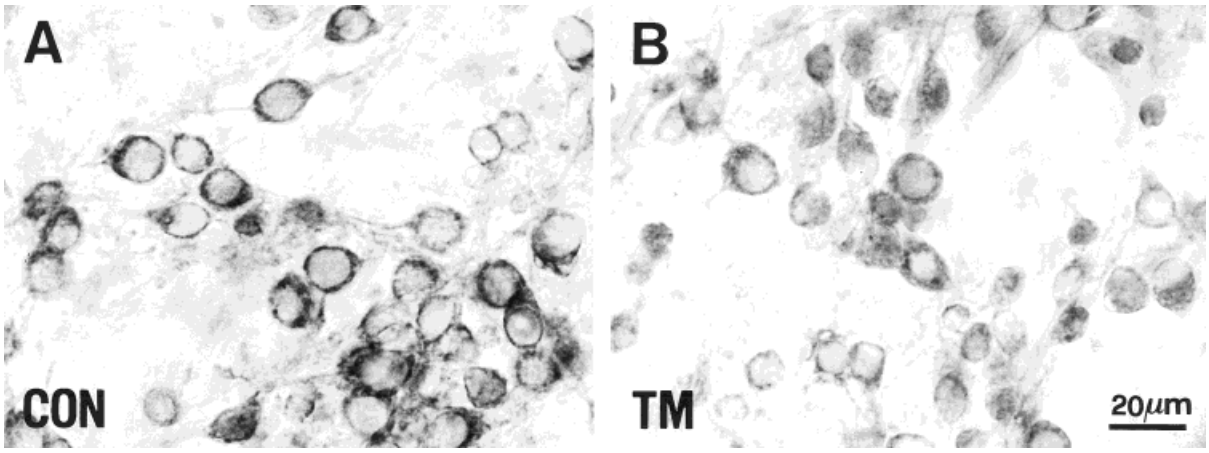

Fig. 5. TM downregulates $GABA_AR$ immunoreactivity. Light microscopic immunostaining using mAb 2C3 was performed on cultures in the presence (*TM*) or absence (*CON*) of 5 μg/ml of TM for 24 h and quantified as described in Materials and Methods. Immunoreaction products appear as dark granules heterogeneously localized in the perikarya.

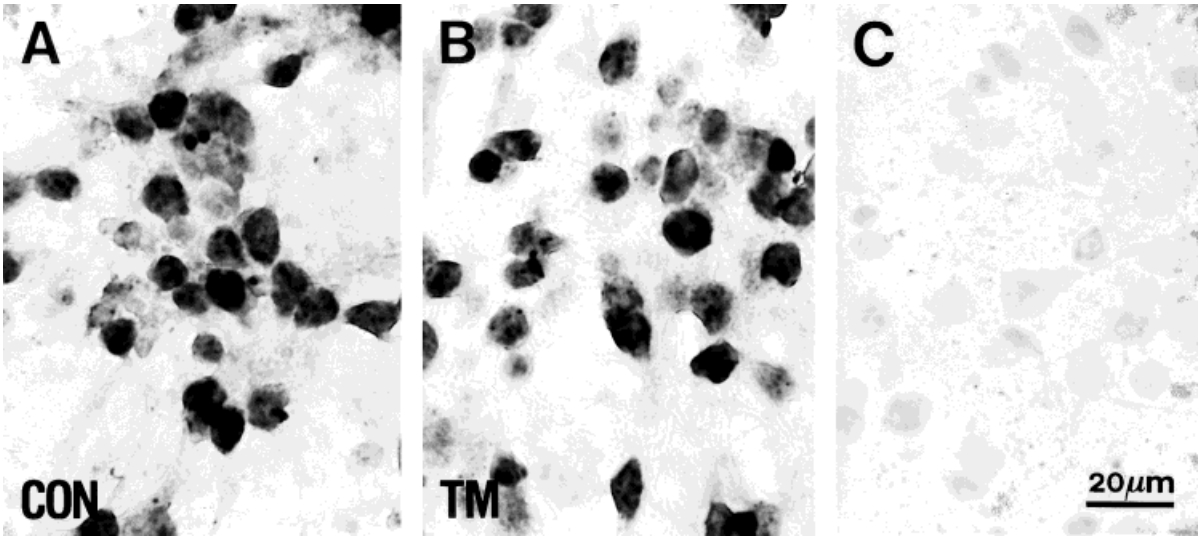

Fig. 6. TM does not change the expression of $GABA_AR$ α_1-mRNA in the cultured neurons. In situ hybridization on the cultures treated with (*TM*) or without (*CON*) 5 μg/ml of TM for 24 h was performed using either a DIG-labeled antisense oligonucleotide probe (**A** and **B**) or sense oligonucleotide probe (**C**) built to the same region of α_1-subunit cDNA as described in Materials and Methods. The experiment was repeated three times with similar results.

cates the hindrance generated by TM to the formation of N-glycosylated polypeptides. Moreover, the finding of a severe blockade, 91%, in ^{3}H-galactose uptake points to a more vigorous effect by TM on the maturation of glycoproteins. Because mannosylation occurs in rER and galactose is one of the terminal sugars to be added to the oligosaccharides in the trans compartment of Golgi apparatus [Kornfeld and Kornfeld, 1985], it is likely that TM acts at rER and Golgi apparatus to produce abnormally glycosylated receptors and thereby contribute to the decreased ligand binding. In muscle cells, ^{3}H-mannose incorporation was obstructed almost completely by TM, in agreement with the great loss of ^{125}I-α bungarotoxin binding [Prives and Olden, 1980]. This experiment suggests a different responsiveness to TM taken by other cell types.

The inhibition of 13% to the general protein synthesis by TM implies an insignificant reduction in the newly synthesized receptor proteins, since they make up only 4% of the total receptor as reported previously [Czajkowski and Farb,

1989]. Variable degrees of blockade to protein synthesis by TM (17–53%) were proclaimed in muscle [Prives and Olden, 1980] and endothelial cultures [Tiganis et al., 1992], whereas no changes occurred in *Xenopus* oocytes [Buller and White, 1990]. However, additional mechanisms are possible to account for the impaired receptor function, such as an increased rate of receptor degradation or internalization. It has been shown that the deglycosylated nAchR is quickly degraded and $GABA_AR$ internalization takes place when cultured neurons are exposed to receptor agonists [Blount and Merlie, 1990; Tehrani and Barnes, 1991].

Molecular Size of $GABA_AR$ Subunit Proteins After TM Treatment

In this study, both TM treatment and N-glycosidase F digestion decreased only about 0.5 kDa of the molecular size of $GABA_AR$ subunit proteins. Our previous study has found that the embryonic chicken receptor proteins are approximate 1–2 kDa lighter than perinatal and postnatal receptors, as shown by photolabeling with ^{3}H-FNZ and SDS-PAGE [Yin and Lee, 1994]. Thus, the molecular mass of N-glycans of the chicken receptor may be smaller than that of the rat receptor, which ranges from 5 to 9 kDa in neonatal brains [Buchstaller et al., 1991a,b; Sieghart et al., 1993]. Differential N-glycosylation of dopamine transporters likewise occurs during postnatal development and in different species of animals [Patel et al., 1993, 1994]. In addition, various culturing conditions caused a variable glycosylation extent of Sertoli cells [Page et al., 1990]. The common core region of N-linked oligosaccharides is approximate 1 kDa and possibly constitutes the N-glycans seen in the embryonic chicken $GABA_AR$. Further analysis of the N-glycans will aid in better understanding of their significance in the receptor function.

N-Glycosylation and Subcellular Distribution of $GABA_AR$

Here we have found that TM induced a decrease in the fraction of intracellular receptors. This may be due to altered intracellular transport of the $GABA_AR$, which appears independent of microtubules and neurofilaments. In order to sustain a proper number of cell surface receptors, the transport of abnormally glycosylated receptors might be regulated differentially, implying a plastic reaction of neurons to the drug perturbation. Similar phenomenon was found for the nAchR in TM-treated *Xenopus* oocytes; cell surface expression of the nAchR was maintained by the supply from intracellular receptors, and therefore a reduced level of intracellular receptors was seen [Buller and White, 1990]. However, mechanisms involved in receptor degradation and recycling may also partake in developing the abnormality. A different finding was reported for the TM-treated 293 cells expressing the receptor, in which the receptor ($\alpha_1\beta_2\gamma_2$) was retained internal to the cell surface of most cells [Connolly et al., 1996]. Nevertheless, the demonstration of subcellular localization of $GABA_AR$ requires electron microscopic immunocytochemistry or other further studies.

Our previous study has declared that the galactosylation of $GABA_AR$ is implicated in its functional expression but is independent of the regulation to subcellular localization [Yin, 1992]. Thus N-glycosylation is likely to participate in regulating the transport of the receptor, in addition to maintaining its normal function. Insignificant changes to the $GABA_AR$ induced by the castanospermine or swainsonine treatment could be due to their acting at later maturation stages of N-linked oligosaccharides. Nonetheless, incubation with higher dosage of the agents for longer duration is presumed to impinge considerably upon receptor properties.

Neuron Viability and TM Treatment

Following 24 h of TM incubation, Trypan blue exclusion experiment revealed an intact cell viability in the culture, but after 3 h and longer removal of the drug, the viability of cells deteriorated and the ligand binding continued to decrease, proclaiming the toxic effect brought about by TM. The embryonic neurons are probably more vulnerable than other preparations, such as cell lines and *Xenopus* oocytes, to which the effect by TM is reversible [Doss et al., 1985; Muβhoff et al., 1992]. A recent study has found that TM incubation (2 μg/ml, 24 h) has led the cultured rat sympathetic neurons to apoptosis detected under light microscopy by a staining method for nuclear condensation [Chang and Korolev, 1996]. In our study, it seems that 24-h incubation of 5 μg/ml of TM may have caused an irreversible damage indiscernible by the Trypan blue method. The confirmation of TM toxicity awaits further investigations.

In summary, the data indicate that TM impedes the N-glycosylation of $GABA_AR$ to render reductions in ligand-binding sites and redistribution of the receptor subcellularly. It is likely that TM causes changes to the intracellular transport of the receptor. Mechanisms underlying these alterations presumably involve the mannosylation and galactosylation, in addition to other relevant intracellular events. The results suggest that N-glycosylation is critical to sustaining a constant number of functional $GABA_AR$ and its regular subcellular localization in neurons.

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