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Short-Term Effects of Thyroid Hormones on Cytoskeletal Proteins Are Mediated by GABAergic Mechanisms in Slices of Cerebral Cortex from Young Rats

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SUMMARY

Thyroid hormones play important roles in brain function. However, few information is available about the effect of 3,5,3′-triiodo-L-thyronine (T_3) or thyroxine (T_4) on the *in vitro* phosphorylation of intermediate filament (IF) proteins from cerebral cortex of rats. In this study we investigated the involvement of GABAergic mechanisms mediating the effects of T_3 and T_4 on the *in vitro* incorporation of ^{32}P into IF proteins from cerebral cortex of 10-day-old male rats. Tissue slices were incubated with or without T_3, T_4, γ-aminobutiric acid (GABA), kinase inhibitors or specific GABA antagonists and ^{32}P-orthophosphate for 30 min. The IF-enriched cytoskeletal fraction was extracted in a high salt Triton-containing buffer and the *in vitro* ^{32}P incorporation into IF proteins was measured. We first observed that 1 μM T_3 and 0.1 μM T_4 significantly increased the *in vitro* incorporation of ^{32}P into the IF proteins studied through the PKA and PKCaMII activities. A similar effect on IF phosphorylation was achieved by incubating cortical slices with GABA. Furthermore, by using specific GABA antagonists, we verified that T_3 induced a stimulatory effect on IF phosphorylation through noncompetitive mechanisms involving $GABA_A$, beyond $GABA_B$ receptors. In contrast, T_4 effects were mediated mainly by $GABA_B$ mechanisms. In conclusion, our results demonstrate a rapid nongenomic action of T_3 and T_4 on the phosphorylating system associated to the IF proteins in slices of cerebral cortex of 10 day-old male rats and point to GABAergic mechanisms mediating such effects.

KEY WORDS: phosphorylation; thyroid hormones; intermediate filaments; PKA; PKCaM II; GABA.

INTRODUCTION

Intermediate filaments (IFs) are major components of the cytoskeleton and nuclear envelope in most types of eukaryotic cells. They are expressed in cell-type-specific

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patterns and play an important structural or tension-bearing role in the cell. Evidence is now emerging that IF also acts as an important framework for the modulation and control of essential cell processes, in particular, signal transduction events (Paramio and Jorcano, 2002). The neuronal cytoskeleton comprises a protein network formed mainly by microtubules (MT) and neurofilaments (NF), the IFs of neurons. Neurofilaments are composed of three different polypeptides whose approximate molecular weight are 200, 160, and 68 KDa, and are commonly referred to as heavy (NF-H), medium (NF-M), and light (NF-L) neurofilament subunits (Ackerley *et al.*, 2000). Glial fibrillary acidic protein (GFAP) is the intermediate filament of mature astrocytes (Eng *et al.*, 2000) and vimentin is the IF of cells of mesenchimal origin (Alberts *et al.*, 2002).

The amino and the carboxy-terminal tail domains of NF subunits are potential phosphorylation sites (Nixon and Sihag, 1991). The phosphorylation sites located on the amino terminal domain of the neurofilament subunits are phosphorylated by second messenger-dependent protein kinases including protein kinase C, cyclic AMP- (PKA), and Ca^{2+}/calmodulin-dependent (PKCaMII) protein kinases (Sihag and Nixon, 1990). The functional role of neurofilament phosphorylation is to date not completely clear. However, the regulation of IF polymerization by phosphorylation is well described in the literature. Vimentin filaments reconstituted *in vitro* undergo complete disassembly when phosphorylated by purified protein kinase A or protein kinase C (Inagaki *et al.*, 1987). A similar *in vitro* disassembly induced by phosphorylation has been noted for almost all major IF proteins, such as glial fibrillary acidic protein (GFAP) (Inagaki *et al.*, 1990), desmin (Inada *et al.*, 1998), keratin (Ku and Omary, 1997), α-internexin (Tanaka *et al.*, 1993), NF-L (Hashimoto *et al.*, 1998), and lamin (Peter *et al.*, 1992). On the other hand, the carboxy terminal side arm domains of NF-H and NF-M subunits are extensively phosphorylated by several protein kinases, such as glycogen synthetase kinase (GSK) 3, extracellular signal-regulated kinase (ERK), stress activated protein kinase, protein kinase K, protein kinase C, and Cdk-5 (Hisanaga *et al.*, 1993; Shetty *et al.*, 1993; Guidato *et al.*, 1996; Sun *et al.*, 1996). It has been demonstrated that *in vitro* phosphorylation of COOH-terminal domains of NF-H and NF-M straightens individual neurofilaments and promotes their alignment into bundles (Leterrier *et al.*, 1996), whereas *in vivo* phosphorylation of these proteins is associated with an increased interneurofilament spacing (Hsieh *et al.*, 1994). As a consequence, NF-H and NF-M COOH-terminal side arms extend and form crossbridges among neurofilaments and other cytoskeletal elements (Gotow *et al.*, 1994).

Thyroid hormones (TH) are critically involved in development and function of the central nervous system. Expression and cell distribution of cytoskeletal components have been for long time considered to be important effects of thyroid hormone during brain development, and responsible for the effects of the hormone on axonal growth, and dendritic architecture (Bernal, 2002). In this context, Paul *et al.* (1999) have reported that TH induced the expression of phosphorylated forms of vimentin in cultured astrocytes.

The classical mechanism of TH has been established as a genomic action, including binding to intracellular hormone receptors that share characteristics of nuclear transcription factors. These effects are described to occur after a given time lag

necessary to modify protein transcription (Davis *et al.*, 2002; Silva *et al.*, 2002). However, recently, a number of reports have indicated that TH exerts several effects in cells lacking classical receptors (Lin *et al.*, 1997). In addition, the effect of these hormones on the membrane transport system of chick embryo was reported to be very rapid, occurring in minutes, a time lag noncompatible with the classical scheme of a nuclear receptor action (Incerpi *et al.*, 1999a). These findings led to the identification of nonclassical TH binding elements in the plasma membrane. Through binding to these sites, TH could exert short-term effects, including those on ion fluxes at the plasma membrane (Huang *et al.*, 1999; Incerpi *et al.*, 1999b; Silva *et al.*, 2001; Volpato *et al.*, 2004), on intracellular protein trafficking (Safran *et al.*, 1992; Zhu *et al.*, 1998; Chen *et al.*, 1999), on signal-transducing cytoplasmic kinase activities (Lin *et al.*, 1996, 1999) and on the cytoskeleton (Siegrist-Kaiser *et al.*, 1990). Also, we reported a rapid nongenomic and Ca^{2+}-dependent action of T_3 on the phosphorylating system associated with vimentin in immature rat testes (Zamoner *et al.*, 2005).

In the present investigation we studied the short-term effects of thyroid hormones, T_3 and T_4, on the *in vitro* incorporation of ^{32}P into IF proteins in slices from cerebral cortex of 10-day-old rats. Additionally, the role of protein kinases on the effects exerted by T_3 and T_4 on cytoskeletal protein phosphorylation was examined. We also evaluated the influence of GABAergic system on the effects elicited by the TH, since growing evidence in the literature suggest a role for altered GABAergic function mediated by TH (Mason *et al.*, 1987; Hashimoto *et al.*, 1991; Sandrini *et al.*, 1991; Narihara *et al.*, 1994). Despite these evidences, little is known about the effects of GABA on the phosphorylating system associated to the cytoskeleton.

METHODS

Radiochemical and Compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. 3,5,3′triiodo-L-thyronine, thyroxine, gamma-aminobutyric acid (GABA), bicuculline methiodide, benzamidine, leupeptin, antipain, pepstatin, chymostatin, antibodies, acrylamide, and bis-acrylamide were obtained from Sigma (St. Louis, MO, USA). KN-93 and H-89 were obtained from Calbiochem (La Jolla, CA, USA). Phaclofen and picrotoxin were purchased from Tocris Neuramin (Bristol, UK). ECL kit was from Amersham (Oakville, Ontario).

Animals

Ten-day-old male Wistar rats were obtained from our breeding stock. Rats were maintained on a 12-h light/12-h dark cycle in a constant temperature (22°C) colony room. On the day of birth the litter size was culled to eight pups. Litters smaller than eight pups were not included in the experiments. Water and a 20% (w/w) protein commercial chow were provided *ad libitum*. The experimental protocol followed

the "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) and was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul.

Preparation and Labelling of Slices

Rats were killed by decapitation, the cerebral cortex were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper. In all experiments, slices from the same cerebral cortex were used both for control and treated groups.

Preincubation

Tissue slices were initially preincubated at 30°C for 20 min in a Krebs-Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin, and 0.7 μM chymostatin in the presence or absence of 1 mM GABA, 50 μM bicuculline, 50 μM picrotoxine, 50 μM phaclofen, 10 μM KN-93, 10 μM H-89, 1.0 μM T_3, and 0.1 μM T_4 when indicated.

Incubation

After preincubation, the medium was changed and incubation was carried out at 30°C with 100 μl of the basic medium containing 80 μCi of [^{32}P] orthophosphate with or without addition of 1 mM GABA, 50 μM bicuculline, 50 μM picrotoxine, 50 μM phaclofen, 10 μM KN-93, 10 μM H-89, 1.0 μM T_3, and 0.1 μM T_4 when indicated. The labeling reaction was normally allowed to proceed for 30 min at 30°C and stopped with 1 mL of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). Slices were then washed twice with stop buffer to remove excess radioactivity.

Preparation of the High Salt-Triton Insoluble Cytoskeletal Fraction From Tissue Slices

After treatment, preparations of IF-enriched cytoskeletal fractions were obtained from cerebral cortex of 10-day-old rats as described by Funchal *et al.* (2003). Briefly, after the labelling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100, and the protease inhibitors described above. The homogenate was centrifuged at 15800 $\times$ *g* for 10 min at 4°C, in an Eppendorf centrifuge, the supernatant discarded, and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched

pellet, containing neurofilament subunits, vimentin, and glial fibrillary acidic protein (GFAP), was dissolved in 1% SDS and protein concentration was determined.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at -70°C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Immunoblotting Analysis

Cytoskeletal fractions (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the following monoclonal antibodies: anti NF-150 (clone NN-18) diluted 1:100, anti NF-68 (clone NR-4) diluted 1:300, anti vimentin (clone vim 13.3) diluted 1:400, and antiglial fibrillary acidic protein (GFAP) (clone G-A-5) diluted 1:400. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase-conjugated rabbit antimouse IgG diluted 1:4000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit.

Protein Determination

The protein concentration was determined by the method of Lowry *et al.* (1951) using serum bovine albumin as the standard.

Statistical Analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the *F*-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

Figure 1 shows the immunoblotting (A) and the autoradiograph (B) of the IF-enriched cytosketeton fraction from cerebral cortex of 10- day-old male rats. Lane a displays the molecular weight standards. The pattern of the Triton-insoluble IF proteins from cerebral cortex of rats is demonstrated in lane b, where the bands of NF-M, NF-L, vimentin, and GFAP can be seen. Lanes c–f show the correspondent immunoblotting where the bands of 150 and 68 kDa are immunoreactive with NF-M and NF-L antibodies, respectively (lanes c and d)., while the 54 kDa band is identified as vimentin (lane e) and the 50 kDa band as GFAP (lane f). It can be seen in the autoradiograph that NF-M, NF-L, vimentin, and GFAP are good substrates for the endogenous phosphorylation system (Fig. 1B). When tissue slices of cerebral cortex were incubated for 30 min with 1 μM T_3 or 0.1 μM T_4 we observed an increased ^{32}P incorporation pattern into the cytoskeletal proteins studied (NF-M, NF-L, vimentin, and GFAP) (Fig. 2). Then, we investigated whether PKA and PKCaMII were involved in the activating effect of TH on the phosphorylating system associated to IF proteins extracted in high-salt Triton containing buffer from cerebral cortex of 10-day-old male rats. In this series of experiments tissue slices were preincubated with the specific kinase inhibitors before treatment with the hormones. We added H-89 and KN-93, the specific protein kinase inhibitors of PKA and PKCaMII, respectively, to the incubation system in the presence of T_3 or T_4. Results showed that H-89 prevented the hyperphosphorylation induced by the TH, conversely KN-93

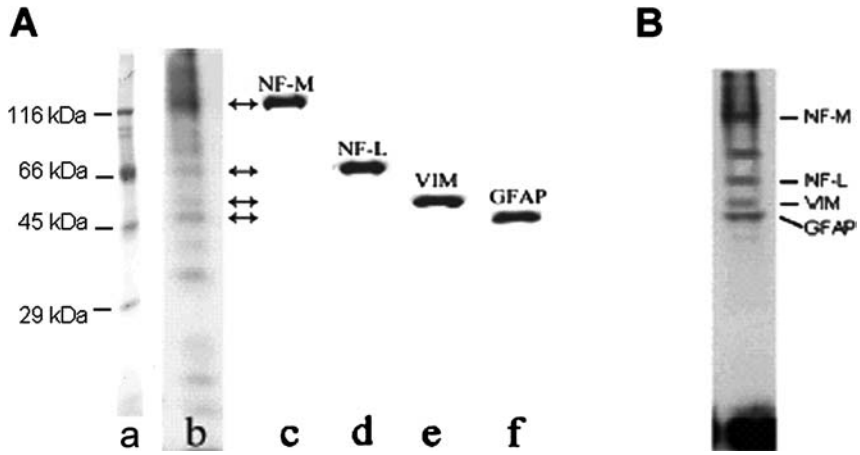

Fig. 1. Polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and autoradiograph of the cytoskeletal fraction. (A) *Lane* a, molecular weight standards (KDa), from top to bottom: β-galactosidase (116), bovine albumin (66), egg albumin (45), carbonic anidrase (29). *Lane* b, nitrocellulose membrane of the high salt Triton insoluble cytoskeletal fraction stained with Ponceau. (c–f) Immunoblotting of the IF-enriched cytoskeletal fraction with anti IF monoclonal antibodies: (c) NF-M, middle molecular weight neurofilament subunit; (d) NF-L, low molecular weight neurofilament subunit; (e) Vim, vimentin; (f) GFAP, glial fibrillary acidic protein. B) Autoradiograph of the Triton-insoluble IF-enriched cytoskeletal fraction from a 10-day-old rat.

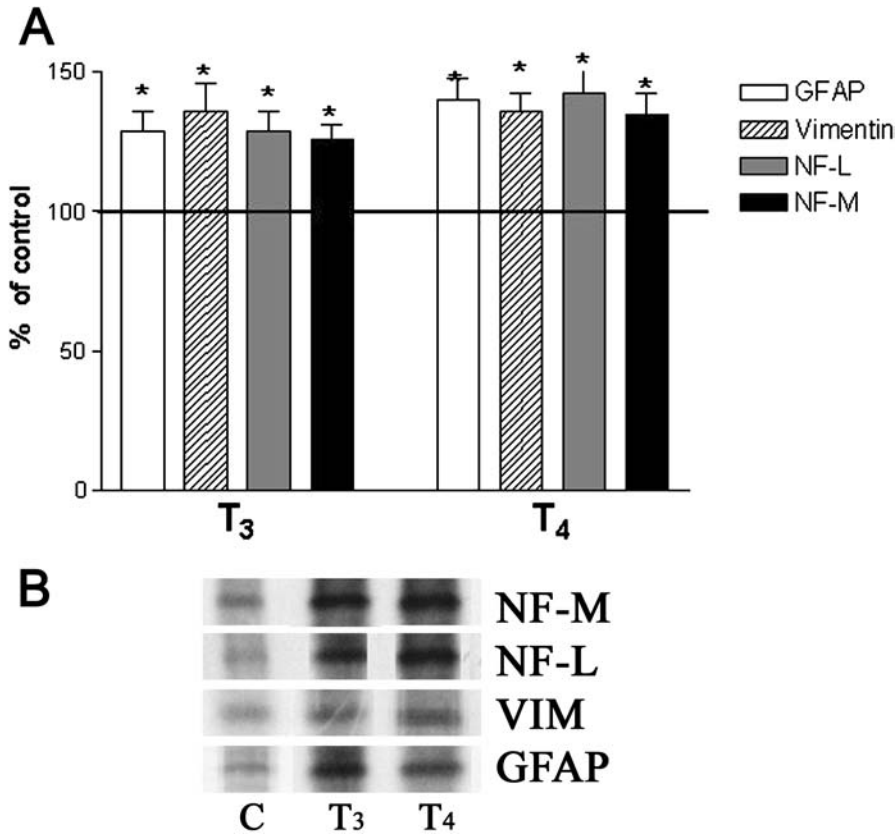

Fig. 2. Effect of thyroid hormones, T_3 and T_4, on the phosphorylation of intermediate filament subunits from cerebral cortex of rats. (A) Slices of cerebral cortex of 10-day-old male rats were incubated for 30 min with 1 μM T_3 or 0.1 μM T_4 in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into GFAP, vimentin (VIM), NF-L, and NF-M was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction, as described in Material and Methods. (B) Representative autoradiographs of the proteins studied. Data are reported as means $\pm$ SEM expressed as percentage of controls from five independent experiments using six to eight animals in each experiment. Statistically significant differences from controls, as determined by ANOVA followed by Tukey test are indicated: $^*P < 0.001$.

totally prevented the effect of T_3 but partially blocked the action of T_4 (Figs. 3(A) and (B)). These results suggest that TH activating effect on the phosphorylation of the cytoskeletal proteins from cerebral cortex of 10-day-old male rats is mediated by PKA and PKCaMII.

In order to verify whether the stimulatory effect of T_3 and T_4 on the phosphorylation of IF subunits was mediated by GABA receptors, tissue slices were incubated with ^{32}P-orthophosphate in the presence of 1.0 mM GABA. We verified that GABA was able to mimic the effect of TH. Moreover, we did not observe an additional stimulatory effect when slices were incubated with the hormones in the presence of

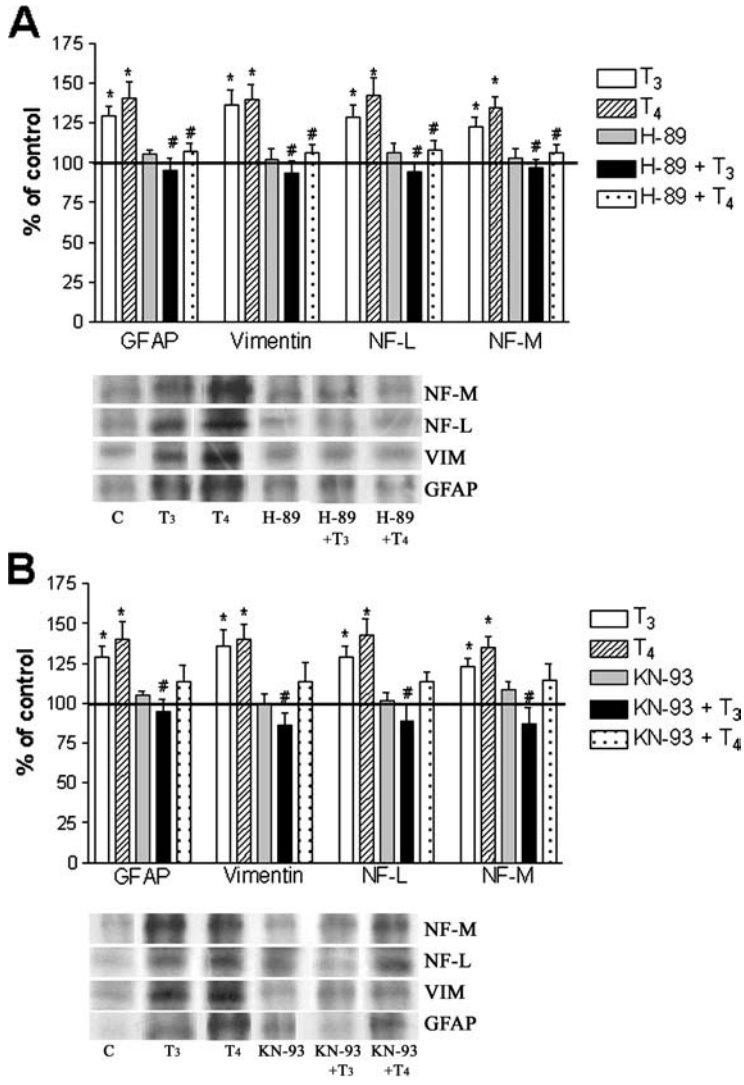

Fig. 3. Effect of treatment of tissue slices with T_3 or T_4 on cAMP- or Ca^{2+}/calmodulin-dependent *in vitro* incorporation of ^{32}P into IFs in the cytoskeletal fraction of cerebral cortex of 10 day-old male rats. Slices of cerebral cortex were preincubated and incubated with 1 μM T_3 or 0.1 μM T_4 and ^{32}P-orthophosphate, in the presence or absence of 10 μM H- 89 (A) or 10 μM KN-93 (B), as described in Material and Methods. The cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M) was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction. Representative autoradiographs of the proteins studied are shown in the insets. Data are reported as means ± SEM expressed as percent of controls from five independent experiments using six to eight animals for each experiment. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. $^{*}P < 0.001$ hormones compared with control group; $^{\#}P < 0.01$ "drug + hormone" compared with respective hormone group.

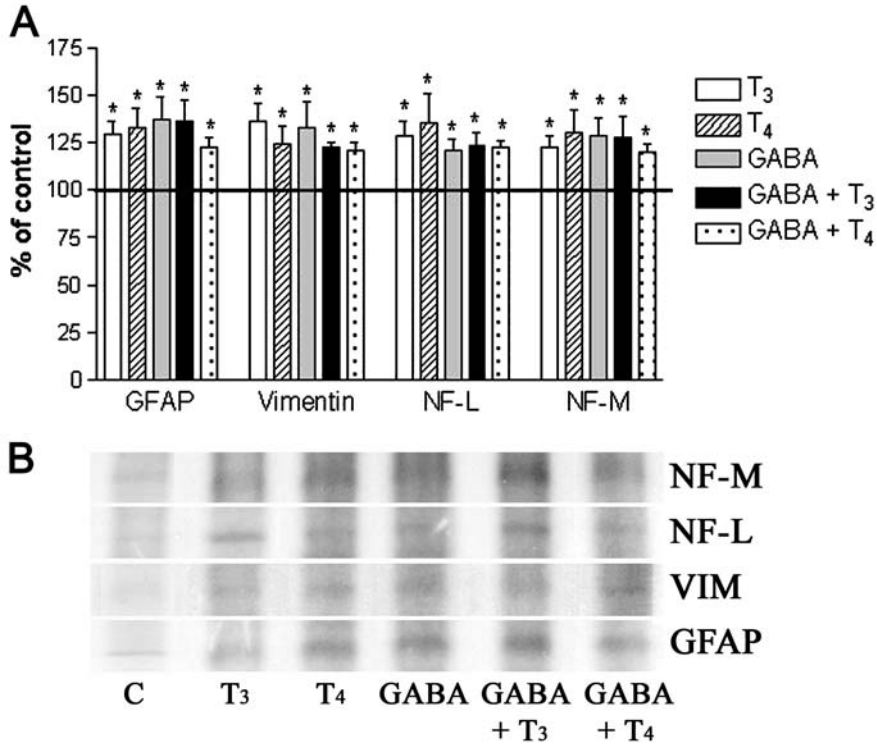

Fig. 4. Effect of GABA on T_3 and T_4-induced phosphorylation of intermediate filament subunits of cerebral cortex of rats. (A) Slices of cerebral cortex of 10-day-old male rats were incubated with 1 μM T_3 or 0.1 μM T_4 and/or 1 mM GABA in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M), was measured from the autoradiographs of the SDS-PAGE of the phosphorylated-enriched cytoskeletal fraction. (B) Representative autoradiographs of the proteins studied. Data are reported as means ± SEM expressed as percentage of controls from five independent experiments using six to eight animals for each experiment. Statistically significant differences from controls, as determined by ANOVA followed by Tukey–Kramer multiple comparison tests are indicated: $^*P < 0.001$.

GABA, suggesting that GABA and the hormones act through the same mechanism (Fig. 4). Next we tested the effect of 1.0 μM T_3 or 0.1 μM T_4 in the presence of GABAergic receptor antagonists in preincubation and incubation medium. Incubation of tissue slices with T_3 or T_4 in the presence of the competitive $GABA_A$ antagonist bicuculline showed that this antagonist was not able to prevent the effect of the hormones on the phosphorylating system associated to the cytoskeletal proteins (Fig. 5(A)). However, picrotoxin, a noncompetitive $GABA_A$ inhibitor, was able to prevent hyperphosphorylation induced by T_3 treatment but it was ineffective in preventing T_4 effects (Fig. 5(B)). Finally, when tissue slices were incubated with T_3 following preincubation with the specific $GABA_B$ antagonist phaclofen, the stimulatory effect of the hormone on the *in vitro* phosphorylation of the various

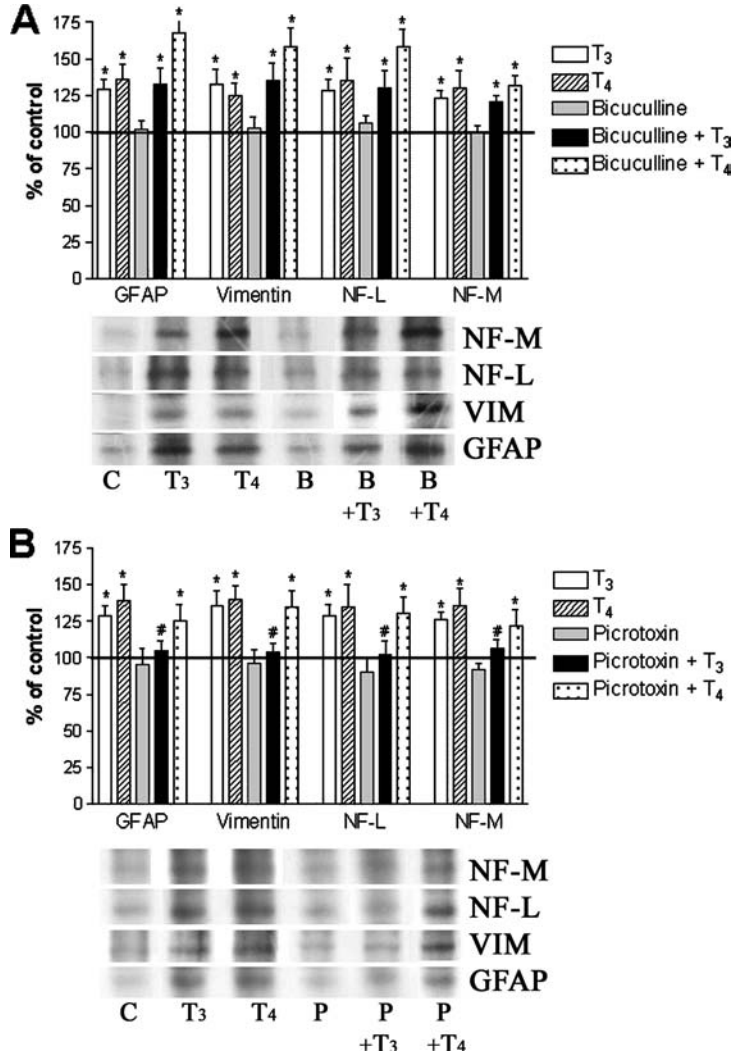

Fig. 5. Effect of the $GABA_A$ and $GABA_B$ antagonists on T_3 and T_4-induced phosphorylation of intermediate filament subunits of cerebral cortex of rats. Slices of cerebral cortex of 10-day-old male rats were incubated with 1 μM T_3 or 0.1 μM T_4 and/or 50 μM bicuculline (A), 50 μM picrotoxin (B), 50 μM Phaclofen (C) in the presence of ^{32}P-orthophosphate. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into glial fibrillary acidic protein (GFAP), vimentin (VIM), low molecular weight neurofilament subunit (NF-L), and middle molecular weight neurofilament subunit (NF-M), was measured from the autoradiographs of the SDS-PAGE of the phosphorylated enriched cytoskeletal fraction. Representative autoradiographs of the proteins studied are shown in the insets. Data are reported as means ± SEM expressed as percentage of controls from five independent experiments using six to eight animals for each experiment. Statistical analysis: one-way ANOVA followed by Tukey–Kramer multiple comparison test. $^*P < 0.001$ hormones compared with control group; $^\#P < 0.01$ "drug + hormone" compared with respective hormone group.

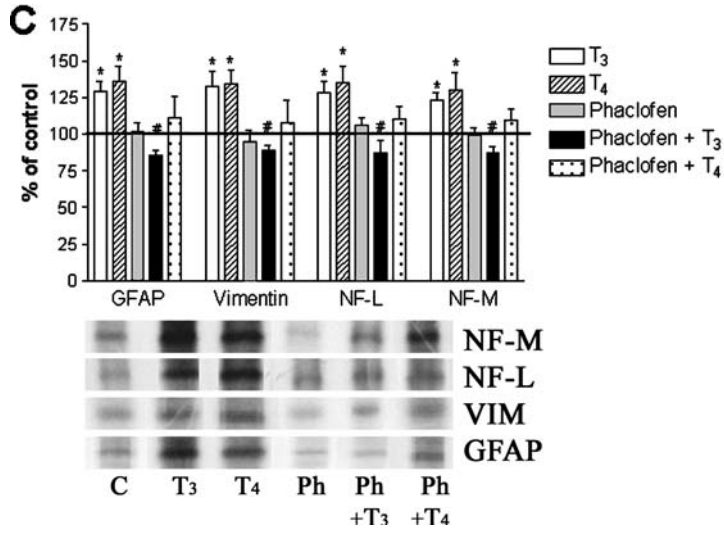

Fig. 5. Continued.

cytoskeletal proteins was prevented. Nonetheless, we observed that phaclofen only partially prevented the effect of T_4 (Fig. 5C). Taken together, these results suggest that T_3 increased the *in vitro* phosphorylation of IF subunits through mechanisms mediated by $GABA_B$ and noncompetitive $GABA_A$ receptors, while T_4 effects are mediated only partially by $GABA_B$ receptors (Fig. 5(C)).

DISCUSSION

The classical genomic concept of TH acting through nuclear receptors is well recognized (Lazar 1993; Davis *et al.*, 2000). However, nongenomic actions of TH are now widely described and characterized by rapid responses, since they occur through plasma membrane transport systems (Incerpi *et al.*, 1999b; Davis *et al.*, 2000; Silva *et al.*, 2001; Davis *et al.*, 2002; Silva *et al.*, 2002; Volpato *et al.*, 2004). Although short-term effects of T_4 on actin polymerization have been described in cultured astrocytes (Siegrist-Kaiser *et al.*, 1990) little is known about short-term effects of TH on the activity of the phosphorylating system associated to the IF proteins in the central nervous system of young rats. In the present report we investigated some mechanisms underlying the short-term effect of thyroid hormones T_3 and T_4, on the *in vitro* phosphorylation of IF proteins from cerebral cortex slices of 10-day-old male rats. We have chosen 1 μM T_3 and 0.1 μM T_4 to undertake our experimental approach since these concentrations have been reported to elicit nongenomic effects. One μM T_3 increased the *in vitro* ^{32}P incorporation into vimentin in 15-day-old rat testis (Zamoner *et al.*, 2005), and elicited membrane hyperpolarization in Sertoli cells (Silva *et al.*, 2001). Otherwise 1 μM T_3 and 0.1 μM T_4 increased amino acid transport and induced changes in the membrane potential in Sertoli cells (Segal 1989; Silva *et al.*, 2001; Volpato *et al.*, 2004).

We observed that T_3 and T_4 were able to increase the *in vitro* ^{32}P incorporation into neurofilament subunits as well as into vimentin and GFAP after 30 min incubation of tissue slices and these effects were mediated by the protein kinases PKA and PKCaMII. Our conclusions are based on the experiments using the protein kinase inhibitors H-89, a cell-permeable, selective, and potent inhibitor of PKA (Chijiwa *et al.*, 1990) and KN-93, a specific PKCaMII inhibitor (Tokumitsu *et al.*, 1990), in the presence of T_3 or T_4. Using this approach, we have previously identified that these kinases are associated to the cytoskeletal fraction from cerebral cortex of young rats (de Freitas *et al.*, 1995). However, considering T_4 action, the partial prevention of hyperphosphorylation observed in tissue slices coincubated with T_4 and KN-93 suggests the involvement of other protein kinases in such effect.

In the present report we also showed that the action of the hormones was mimicked by GABA. In order to verify and better understand the involvement of the GABA receptors in such effect, we used the $GABA_A$ antagonists bicuculline and picrotoxin and the $GABA_B$ antagonist phaclofen in the next set of experiments. The convulsant bicuculline is the classical $GABA_A$-receptor antagonist, which, by competing with GABA for binding to one or both sites on the $GABA_A$ receptor (Olsen and DeLorey, 1999) reduces ionic current by decreasing the opening frequency and mean open time of the channel (MacDonald and Olsen, 1994; Johnston, 1996). On the other hand, the convulsant compound picrotoxin is an ionic channel blocker, which causes a decrease in mean channel open time. Picrotoxin works by preferentially shifting opening channels to the briefest open state (1 ms) (Olsen and DeLorey, 1999). We showed that bicuculline was not able to prevent hyperphosphorylation elicited either by T_3 or T_4, while picrotoxin was effective in preventing only T_3-induced hyperphosphorylation of the IF proteins studied, strongly suggesting the participation of noncompetitive $GABA_A$ receptors in T_3 action. We also observed that the potent selective $GABA_B$ receptor antagonist phaclofen totally prevented the stimulatory effect of T_3 on the *in vitro* phosphorylation of IF proteins. Nonetheless, the effect of T_4 was only partially prevented by this antagonist. Taken together, it can be concluded that T_3 induced a short-term stimulation of the *in vitro* phosphorylation of the proteins studied through noncompetitive $GABA_A$, in addition to $GABA_B$ receptors, while T_4 acted through $GABA_B$ receptors in slices from cerebral cortex of 10-day-old male rats. Moreover, on the basis on our data showing a partial involvement of $GABA_B$ receptors in the T_4 action, we could suppose a more complex signaling mechanism involved in the short-term effect of this hormone on the cytoskeletal proteins. At this point, it is important to emphasize that our findings further support the action of T_4 at the cell membrane as an enhancer of signal transduction. Moreover, the differential mechanisms of action of T_3 and T_4 support that besides the important actions via nuclear receptors, TH can nongenomically alter distinct signal-transducting pathways targeting the cytoskeleton.

Our results showing short-term effects of TH mediated by GABAergic mechanisms are supported by previous reports. In this context, Martin *et al.* (1996) have demonstrated that TH could modulate $GABA_A$ receptors. Moreover, the short-term effects of the TH mediated by the activity of GABA receptors that we are evidencing in the present report are in line with Chapell *et al.* (1998), who described that micromolar concentrations of T_3 can directly activate $GABA_A$ receptor chloride channels, and these responses were inhibited by picrotoxin but not

bicuculline. Our results indicated that T_3 activated the $GABA_A$ receptor in a noncompetitive manner consistent with a channel-opening mechanism of action. The inability of the competitive $GABA_A$ receptor antagonist bicuculline to block T_3-induced hyperphosphorylation suggests that T_3 does not act at the GABA-binding site on the $GABA_A$ receptor, but acts at an unidentified site on the receptor complex.

Otherwise, $GABA_B$ receptors are known to be present in both presynaptic terminals and postsynaptic cells (Harrison, 1990) as well as in astrocytes (Gaiarsa *et al.*, 2002). Komatsu (1996) has described that postsynaptic $GABA_B$ receptors are involved in the induction of LTP of inhibitory synaptic transmission and that this effect was mediated at least in part by facilitation of the monoamine-induced IP_3 formation, which then causes Ca^{2+} release from the internal stores in postsynaptic cells. The increased intracellular Ca^{2+} levels could be on the basis of PKA and PKCaMII activation induced by T_3 and T_4 that we have observed in this report

Moreover, our results showing that the short-term TH treatment increased the astrocytic IF protein (GFAP) phosphorylation, via GABAergic mechanisms is supported by previous reports demonstrating that astrocytes also express a large variety of receptors for neurotransmitters and neuropeptides (Porter and McCarthy, 1997). Furthermore, Runquist and Alonso (2003) suggested that GFAP expression and the morphology of adult astrocytes are affected by GABAergic signaling, reinforcing the phisiological role of GABA pathway modulating the cytoskeleton. In addition, our present results showing that the stimulatory effects of TH on GFAP phosphorylation were prevented by GABA antagonists are in line with these findings indicating that astrocytes express high levels of GABA receptors (Rosewater and Sontheimer, 1994).

However, the exact mechanisms underlying the involvement of GABA receptors mediating the altered phosphorylation of cytoskeletal proteins in short-term effects of TH are still unknown. Growing evidence in the literature demonstrate an interaction between $GABA_A$ receptors and the cytoskeleton, which is presumably needed for receptor trafficking, anchoring, and/or synaptic clustering (Wang and Olsen, 2000). On the other hand, it has been described that microtubules participate in the maintenance of normal subcellular distribution of $GABA_A$ receptors in neurons and that the organization of microfilaments may play a role in modulating the gene expression of $GABA_A$ receptor subunits (Ho *et al.*, 2001).

In summary, our results provide the first evidence of a rapid response mechanism on the phosphorylation level of IF proteins mediated by TH both in neuronal and glial cells through GABAergic pathways. Thus, the complexity of the processes underlying the differential mechanism of action to T_3 and T_4 suggest the existence of multiple sites of regulation to the TH. However, further experiments will be necessary to clarify the physiological implications of the GABAergic mechanisms involved in the rapid action of TH on the phosphorylating system associated with the cytoskeletal proteins of rat brain.

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