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Cyclic AMP-Dependent Regulation of Fibroblast Growth Factor-2 Messenger RNA Levels in Rat Cortical Astrocytes: Comparison with Fibroblast Growth Factor-1 and Ciliary Neurotrophic Factor

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SUMMARY

The present study was undertaken to investigate the regulatory mechanisms of fibroblast growth factor-1 and -2 (FGF-1 and FGF-2) gene expression compared with ciliary neurotrophic factor (CNTF) in rat cortical astrocytes. Glial cells represent a source of different trophic factors and cytokines that can influence the survival of multiple cell populations within the central nervous system. We found that the β-adrenergic receptor agonist (βAR) isoproterenol produced a significant induction of FGF-2 gene expression and protein in type I astrocytes. On the contrary, the gene expression for FGF-1 and CNTF is markedly reduced after exposure to isoproterenol. The changes produced by the βAR agonist is mimicked by cyclic AMP analogues (8-bromo-cAMP) or 3-isobutyl-1-methyl-xanthine, a cAMP phosphodiesterase inhibitor, which indicates that intracellular elevation of this second messenger is responsible for these effects. The regulation of neurotrophic factors by isoproterenol is not restricted to cortical astrocytes and may take place through different mechanisms. Inhibition of protein synthesis prevents the decrease in CNTF without affecting the changes in FGF-1 and FGF-2 gene expression. Coincubation of isoproterenol with actinomycin D, an inhibitor of gene transcription, prevents the modification of neurotrophic factor biosynthesis, indicating that transcriptional mechanisms are indeed involved in these regulatory pathways. However, the determination of FGF-2 mRNA half-life suggests that the effect of the βAR agonist can be in part the result of mRNA stabilization. The mechanisms that we describe can be important in the maintenance of neuronal homeostasis and may be relevant in the development of alternative strategies for the treatment of acute and chronic neurodegenerative disorders.

Many neurotrophic factors are expressed in the central nervous system. These molecules display specific patterns of distribution from development through adulthood, indicating a profound influence in the maturation of specific neuronal pathways as well as in the maintenance of cell homeostasis (1, 2).

Some of these factors are predominantly expressed in neuronal cells, whereas other molecules, such as FGF-2 and CNTF, are mainly localized in astrocytes. CNTF supports survival and differentiation of a variety of neuronal cell types, including sensory neurons, sympathetic neurons, and motoneurons (for reviews, see Refs. 3 and 4). FGF-1 and FGF-2 enhance the survival of neurons from different brain structures (5) and regulate the differentiation of selected neuronal phenotypes (6–8).

In vivo, the administration of neurotrophic molecules is capable of preventing lesion-induced degeneration of specific neuronal pathways. For example, CNTF can prevent the degeneration of facial motoneurons in the developing mouse (9), whereas FGF-2 is effective in rescuing cholinergic neurons after fimbria/fornix transection (10, 11) or thalamic degeneration after cortical infarction (12). Such neuroprotective activity is supported by *in vitro* observation that FGF-2 is effective in different models of excitotoxicity (13, 14). Therefore, the use of trophic factors for the treatment of acute and chronic neurodegenerative disorders is becoming very appealing (15). However, the direct use of these agents seems to be quite complicated in relation to their chemical properties, as they do not pass the blood-brain barrier and do not distribute properly after systemic injection. Therefore,

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ABBREVIATIONS: βAR, β-adrenergic receptor; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGF-1, fibroblast growth factor-1 (acidic); FGF-2, fibroblast growth factor-2 (basic); GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; PBS, phosphate-buffered saline; NGF, nerve growth factor.

new strategies must be developed to take advantage of their therapeutical properties; among these is the possibility of modulating their production within specific cell populations with the use of drugs with more acceptable pharmacokinetic profiles. It is therefore of primary importance to obtain detailed information regarding the mechanisms that control neurotrophic factor production within the brain. Several lines of evidence indicate that their biosynthesis can be modulated in a complex fashion by activation of specific neuronal pathways or through interaction with neurotransmitters and cytokines (16–18).

The expression of FGF-2 *in vivo* can be increased after injury or as a consequence of seizure activity (19–22). Moreover, we recently demonstrated that glucocorticoid hormones stimulate FGF-2 gene expression in cultured astrocytes and, *in vivo*, may tonically regulate the biosynthesis of this trophic molecule (23, 24).

To determine whether other systems can modulate the expression of FGF-2 and its congener FGF-1 in astrocytes, we investigated the effects of isoproterenol and cAMP elevating agents on the mRNA levels of these trophic factors compared with CNTF. Our results indicate that βAR agonist elevates FGF-2 gene expression and down-regulates mRNA levels of FGF-1 and CNTF. The effects on FGF-1 and FGF-2 depend on gene transcription but, unlike CNTF, do not require new protein synthesis. These events can contribute to the regulation of trophic factors within the brain and may play a role in adaptive mechanisms taking place after cellular activation or as a response to neuronal injury.

Experimental Procedures

Materials. Media and serum were obtained from Hyclone, and molecular biology reagents were obtained from Bio-Rad, Ambion, and New England Biolabs. General reagents were purchased from Sigma, whereas drugs were purchased from RBI and Sigma.

Cell cultures and treatments. Astrocytes were prepared from newborn rat cerebral cortices according to the method of McCarthy and de Vellis (25) with small modifications. Briefly, cerebral cortices were rapidly dissected in Hanks' medium, and meninges were carefully removed. The cerebral tissue was gently pressed through an 82-μm nylon filter into culturing medium. The cells were initially plated at high density (20×10^6 cells) in 75-cm^2 flasks. For the first 4 days, the medium consisted of DMEM/high glucose, 20% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and then was changed to DMEM/10% FBS.

Astrocytes were grown to confluence (12–14 days) and then shaken overnight in a thermostatic room at 37° on a rotary horizontal shaker (250 rpm). The medium was discarded, and the cellular monolayer was washed once with fresh medium, detached with trypsin/EDTA/PBS solution that did not contain Ca^{2+} or Mg^{2+}, and replated in 100-mm tissue culture dishes. These cells consisted, almost exclusively, of type 1 astrocytes as previously shown and characterized (26). Astrocytes were used at confluency (6–8 days after replating).

For treatments, culture medium was replaced with 10 ml of DMEM without FBS, and cells were used after 24 hr by adding the appropriate drug or drugs from stock solutions. After incubation, cells were harvested in guanidine thiocyanate for total RNA extraction.

RNA preparation. The cells were scraped in 4 M guanidine thiocyanate (containing 25 mM sodium citrate, pH 7.5, 0.5% sarcosyl. and 0.1% 2-mercaptoethanol) and passed through a 20-gauge needle, and total RNA was isolated by phenol/chloroform extraction according to the method of Chomczynski and Sacchi (27).

Quantification was carried out by absorption at 260 nm, and the purity of the samples was assessed by determining the ratio of A_{260nm} to A_{280nm}. RNA was then reprecipitated in ethanol for RNase protection assay. To verify that equal amounts of total RNA were used in RNase protection assay, parallel samples were loaded on an agarose/formaldehyde gel, run (35 V for 16 hr), and stained with ethidium bromide (data not shown).

RNA probe synthesis for RNase protection assay. A transcription kit (MAXI script, Ambion) was used to generate cRNA probes, and ^{32}P-CTP was used as a radiolabeled nucleotide. Plasmids (containing the appropriate cDNAs) used in our study were as follows. Plasmid RObFGF503 (Dr. Andrew Baird, The Whittier Institute, La Jolla, CA) containing a 1016-bp portion of the rat FGF-2 cDNA was linearized with *Nco*I and used to generate a ^{32}P-labeled 524-base antisense cRNA probe that included 477 bases of FGF-2 sequence and 47 bases of the pBluescript II SK$^+$ polylinker region. Plasmid HBGF-1 (Dr. S. Goodrich, Alton Jones Cell Science Center, Lake Placid, NY), containing the rat FGF-1 cDNA, was linearized with *Nco*I; *in vitro* transcription generated a 626-base antisense cRNA probe containing 568 bases of the FGF-1 sequence and 70 bases of the polylinker region. A Bluescript II SK$^+$ plasmid, containing the full coding sequence of rat CNTF (Dr. Michael Sendtner, University of Wurzburg, Germany), was linearized with *Eco*RI and used as a template for a T3 RNA polymerase to generate a 670-base antisense cRNA probe yielding a protected fragment of 600 bases.

A cRNA probe to GAPDH was used as internal standard. By *in vitro* transcription with a T3 RNA polymerase, a 376-base probe was obtained from the plasmid pTRI-GAPDH-Rat (Ambion), which, after RNase protection assay, yielded a protected fragment of 316 bases.

RNase protection assay. The RNase protection assay was performed on a 10-μg sample of total RNA as described previously (19). Briefly, after ethanol precipitation, total RNA was dissolved in 20 μl of hybridization solution (80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4, 400 mM sodium acetate, pH 6.4, 1 mM EDTA) containing 150,000 cpm of each ^{32}P-labeled cRNA probe (specific activity, $>10^8$ cpm/μg). Three or four cRNA probes were simultaneously used in the assay. After being heated at 85° for 10 min, the cRNA probes were allowed to hybridize the endogenous RNAs at 45° overnight. At the end of the hybridization, the solution was diluted with 200 μl of RNase digestion buffer (300 mM NaCl, 10 mM Tris·HCl, pH 7.4, 5 mM EDTA, pH 7.4) containing a 1:400 dilution of an RNase cocktail (1 mg/ml RNase A and 20 units/ml RNase T1) and incubated for 30 min at 30°. Proteinase K (10 μg) and sodium dodecyl sulfate (10 μl of 20% stock solution) were then added to the sample, and the mixture was incubated at 37° for an additional 15 min. At the end of the incubation, the sample was extracted with phenol/chloroform and ethanol precipitated. The pellet containing the RNA/RNA hybrids was dried, resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), boiled at 95° for 5 min, and separated on a 5% polyacrylamide gel under denaturing conditions (7 M urea). The protected fragments were visualized with autoradiography, and their sizes were determined with the use of ^{32}P-end-labeled (T4 polynucleotide kinase) *Msp*I-digested pBR322 fragments.

Western blot analysis. Control or isoproterenol-treated cortical astrocytes (four 100-mm dishes for each experimental point) were washed with cold PBS, scraped, and collected by centrifugation at $500 \times g$ for 6 min. Pellets were resuspended in 2 ml of cold PBS and sonicated on ice (three bursts of 15 sec/50 W, each preceded by a 30-sec pause). Samples were then clarified with the use of centrifugation at $15,000 \times g$ for 15 min at 4°. Extracts from each experimental group (~300 μg of protein) were loaded onto a 0.1-ml heparin/Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in PBS. Protein bound to the resin was then eluted by a 2-min incubation at 80° with 4× reducing (SDS-gel) sample buffer. The samples were run on a sodium dodecyl sulfate-12% polyacrylamide gel under reducing conditions, and proteins were then elec-

trophoretically transferred to a PVDF membrane (NEN Research Products, Boston, MA).

Membranes were incubated with a 1:100 dilution of rabbit affinity-purified polyclonal antibodies (kindly provided by D. B. Rifkin, New York University, New York, NY) raised against human recombinant FGF-2. This antibody is specific for FGF-2 and does not cross-react with FGF-1 in Western blot analysis, as previously reported (28). Immunocomplexes were visualized with the use of enhanced chemiluminescence with the ECL Western blotting kit (Amersham Life Science) according to manufacturer's instructions.

RNA calculation. The levels of mRNA for different neurotrophic factors were calculated by measuring the peak densitometric area of the autoradiographs analyzed with an LKB laser densitometer and normalized by the peak densitometric area of GAPDH band. To ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used.

Data are expressed as percent of control (vehicle-treated cells). The mean value of the control within a single experiment was set at 100%, and all the other values are expressed as a percent of control.

Statistical analysis. Statistical evaluation of the results was performed with use of a two-way analysis of variance. Significant changes were determined with Dunnett's *t* test (for multiple comparison).

Results

Exposure of primary culture of rat cortical astrocytes to βAR agonists produced different modifications in the expression of FGF-1, FGF-2, and CNTF, as indicated by RNase protection assay (Fig. 1). The increase in FGF-2 mRNA levels was accompanied by a marked reduction in the gene expression for CNTF and FGF-1. The mRNA levels for GAPDH did not change after treatment with isoproterenol, and its homogeneous expression among experimental groups indicates equal loading and recovery of the RNA samples throughout the RNase protection assay.

As summarized in Fig. 2A, there is a rapid elevation in FGF-2 mRNA levels within 2 hr of isoproterenol incubation, whereas at the same time point, FGF-1 and CNTF levels were not significantly altered. The maximal induction in FGF-2 was obtained at 3–6 hr of incubation, but mRNA levels had almost returned to control levels by 24 hr. Conversely, the expression for FGF-1 and CNTF was still markedly reduced after a 24-hr incubation with the βAR agonist. A continuous presence of isoproterenol was not required to produce the alterations on trophic factor mRNA levels. In fact, astrocytes exposed for 2 hr to the βAR agonist and then incubated with control medium for the following 4 hr showed the same extent of changes in neurotrophic factor expression as did cells cultured continuously with isoproterenol (see Fig. 1). The dose-response curve in Fig. 2B indicates that incubation with 10 nM isoproterenol was already effective in elevating FGF-2 and decreasing FGF-1 mRNAs, whereas maximal effects were attained at 50–100 nM.

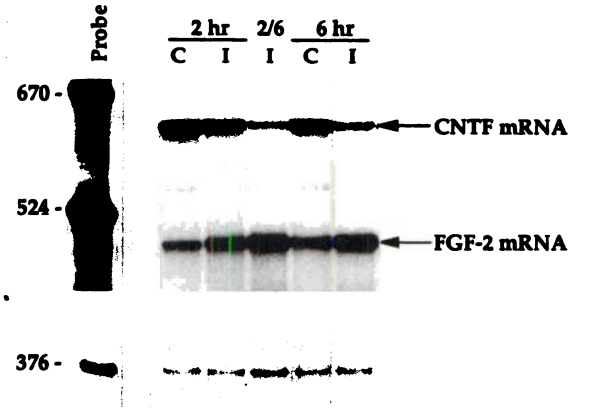

Fig. 1. Determination of FGF-2 and CNTF mRNA levels by RNase protection assay in cultured astrocytes from rat cerebral cortex. At 24 hr after serum deprivation, confluent cells were incubated with vehicle (*C*) or 1 μM isoproterenol (*I*) for 2 or 6 hr, or the βAR agonist was removed after 2 hr and astrocytes were left in control medium for an additional 4 hr (*2/6 I*). Total RNA (10 μg) was used for the determination. *Probe*, an aliquot of the hybridization solution containing the cRNA probes to FGF-2, CNTF, and GAPDH, used as internal standard. *Arrow*, protected fragments for these mRNAs. The autoradiographic film was exposed at −70° with an intensifying screen for 18 hr (FGF-2 and CNTF) or 4 hr (GAPDH).

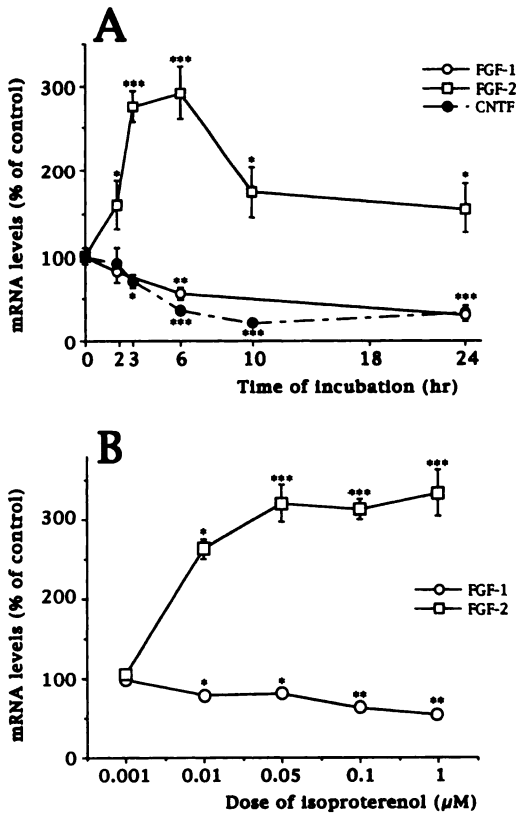

Fig. 2. Time course (A) and dose-response curve (B) of isoproterenol on FGF-1, FGF-2, and CNTF mRNA levels in primary culture of rat cortical astrocytes. Results are expressed as percent of control cells and represent the mean ± standard error of four to eight independent determinations. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ versus control cells (analysis of variance with Dunnett's *t* test).

The regulation of neurotrophic factors by isoproterenol in cortical astrocytes seems to be mediated by the formation of cAMP. Indeed, as illustrated in Table 1, exposure of astrocytes to analogues of the second messenger (8-bromo-cAMP), direct activation of adenylate cyclase by forskolin, or incubation with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) produced a marked induction in FGF-2 gene expression and a decrease in CNTF and FGF-1 mRNA levels. Similarly, in response to isoproterenol, the endogenous βAR agonist norepinephrine and the more selective β_2AR agonist clenbuterol (data not shown) were able to mod-

TABLE 1

Effect of putative cAMP elevating agents on FGF-1, FGF-2 and CNTF mRNA levels in rat cortical astrocytes

Twenty-four hours after serum deprivation, confluent cells were incubated for 6 hr with the different drugs before cell harvesting and RNA extraction. The results, expressed as percentage of control cells, represent the mean ± standard error of at least four independent determinations.

Treatment	FGF-1	FGF-2	CNTF
		% of control cells	
Control	100 ± 7	100 ± 5	100 ± 5
Isoproterenol (1 μM)	55 ± 6[b]	277 ± 31[c]	36 ± 4[c]
Norephinephrine (10 μM)	n.d.	204 ± 20[a]	30 ± 4[c]
Forskolin (10 μM)	36 ± 11[b]	260 ± 88[a]	21 ± 2[c]
8 bromo-cAMP (0.2 mM)	n.d.	219 ± 39[a]	26 ± 4[c]
8 bromo-cAMP (1 mM)	39 ± 5[b]	332 ± 52[c]	26 ± 11[c]
3-isobutyl-1-methylxanthine (1 mM)	44 ± 9[a]	251 ± 9[b]	35 ± 3[c]

[a] $p < 0.05$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).
[b] $p < 0.01$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).
[c] $p < 0.001$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).

ulate the gene expression of the above trophic molecules. The concomitant increase in FGF-2 and decrease in FGF-1 expression by isoproterenol were blocked by the βAR antagonist propranolol (Fig. 3) but not by antagonists of other adrenergic receptor subtypes, such as yohimbine (α_1) and prazosin (α_2), as indicated in Table 2.

To determine whether the changes produces by the βAR agonist on FGF-2 mRNA levels translated into protein, we performed Western blot analysis on cellular extracts from control or isoproterenol-treated astrocytes. As shown in Fig. 4, two forms of FGF-2 at 18 kDa and 21–22 kDa were recognized by our antibodies in control astrocytes, and the immunoreactivity for FGF-2 was increased after incubation with isoproterenol.

The effects produced by the βAR agonist were not restricted to cortical astrocytes. Table 3 shows that the same qualitative changes in CNTF and FGF-2 expression were observed in hippocampal or cerebellar astrocytes, although the magnitude of FGF-2 induction was higher in cerebellar glial cells.

To gain insight in the molecular mechanisms of isoproterenol action, we investigated these effects in the presence of

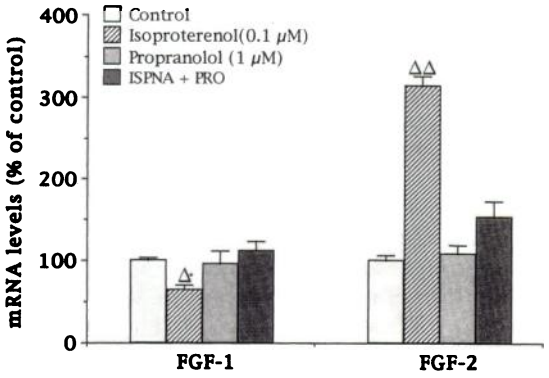

Fig. 3. Effect of the βAR antagonist propranolol on isoproterenol-induced changes in FGF-1 and FGF-2 mRNA levels. Rat cortical astrocytes were exposed to 0.1 μM isoproterenol (*ISPNA*) for 6 hr in the presence or absence of 1 μM propranolol (*PRO*). RNA was then extracted and used for FGF-1 and FGF-2 mRNA determination by RNase protection assay. The data (expressed as percent of control cells) represent the mean ± standard error of at least three independent determinations. Δ, $p < 0.05$; and ΔΔ, $p < 0.001$ versus control cells (analysis of variance with Dunnett's t-test).

TABLE 2

Isoproterenol dependent regulation of FGF-1, FGF-2 and CNTF mRNA levels in rat cortical astrocytes: modulation by α-adrenergic antagonists

Twenty-four hours after serum deprivation, confluent cells were incubated with isoproterenol (1 μM) either alone or in the presence of prazosin (10 μM) or yohimbine (10 μM). After 6 hr incubation, cells were harvested and RNA extracted. The results, expressed as percentage of control cells, represent the mean ± standard error of three to five independent determinations.

Treatment	FGF-1	FGF-2	CNTF
		% of control cells	
Control	100 ± 4	100 ± 8	100 ± 16
Isoproterenol	64 ± 3[a]	223 ± 23[a]	34 ± 3[b]
Prazosin (α_1)	116 ± 10	113 ± 7	91 ± 3
Isoproterenol + Prazosin	55 ± 9[a]	232 ± 55[a]	29 ± 5[b]
Yohimbine (α_2)	103 ± 17	107 ± 12	89 ± 12
Isoproterenol + Yohimbine	52 ± 6[a]	285 ± 25[b]	31 ± 4[b]

[a] $p < 0.01$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).
[b] $p < 0.001$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).

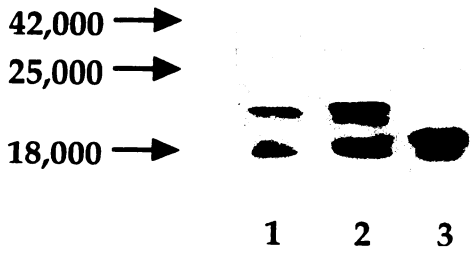

Fig. 4. Specific detection of FGF-2 by Western blot analysis. Astrocytes extracts were purified by heparin Sepharose and processed as described in Experimental Procedures. Two or three immunoreactive bands (18 kDa and 21–22 kDa) were evident in control (*lane 1*) or isoproterenol-treated (*lane 2*) astrocytes. *Lane 3*, 100 ng of human recombinant FGF-2. *Numbers at left*, molecular mass.

TABLE 3

Effect of isoproterenol on CNTF and FGF-2 gene expression in hippocampal and cerebellar astrocytes in culture

Twenty-four hours after serum deprivation, confluent cells were incubated for 6 hr with isoproterenol before cell harvesting and RNA extraction. The results, expressed as percentage of control cells, represent the mean ± standard error of three independent determinations.

Brain region	FGF-2	CNTF
	% of control cells	
Hippocampus	207 ± 42[a]	25 ± 7[b]
Cerebellum	400 ± 41[b]	21 ± 4[b]

[a] $p < 0.05$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).
[b] $p < 0.01$ vs. vehicle-treated cells (analysis of variance with Dunnett t test).

an inhibitor of either protein synthesis (cycloheximide) or gene transcription (actinomycin D). Fig. 5 shows an RNase protection assay for FGF-1 indicating that coincubation with the protein synthesis inhibitor cycloheximide does not prevent the reduction of its gene expression after incubation with isoproterenol. Cycloheximide produced a significant increase in FGF-2 and NGF (data not shown) mRNA levels, an effect that is probably taking place through mRNA stabilization, as previously described (29). The data, summarized in Fig. 6, indicate that coincubation of protein synthesis inhibitor with the βAR agonist does not influence the changes in FGF-1 and FGF-2 mRNA levels but rather prevents the decrease in the gene expression of CNTF. The presence of actinomycin D from the beginning of the incubation prevented the effects of isoproterenol on the expression for all trophic molecules. In another set of experiments, the gene

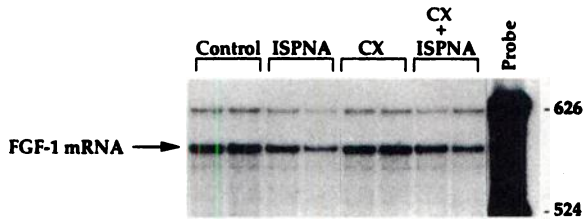

Fig. 5. Determination of FGF-1 mRNA levels by RNase protection assay in cultured astrocytes from rat cerebral cortex. At 24 hr after serum deprivation, confluent cells were incubated for 6 hr with vehicle (*Control*) or 1 μM isoproterenol (*ISPNA*) in the presence or absence of 5 μg/ml cycloheximide (*CX*). Total RNA (10 μg) was used for the determination. *Probe*, an aliquot of the hybridization solution containing the cRNA probe to FGF-1. *Arrow*, protected fragments for FGF-1. The autoradiographic film was exposed at −70° with an intensifying screen for 3 days.

transcription inhibitor was added after 3 hr of incubation with either vehicle or isoproterenol to evaluate the effects of the drug treatment on FGF-2 mRNA stability. Although the slopes of the lines in Fig. 7 were not statistically different from each other, the extrapolated value for FGF-2 mRNA half-life indicates a slight increase in isoproterenol-treated astrocytes (control $t_{1/2}$ = 9.2 hr; isoproterenol $t_{1/2}$ = 13.3 hr). This effect was observed despite the continuous presence of the βAR agonist, as its removal before the addition of actinomycin D produced the same elevation in mRNA half-life (data not shown).

Discussion

In the central nervous system, several neurotrophic factors are produced, although at different levels, in resting astrocytes. These cells can undergo functional changes as a consequence of cell injury (30) and may therefore constitute a reservoir of trophic molecules able to participate in the adaptive processes taking place after cell damage.

The purpose of the current study was to examine in cultured astrocytes the regulatory mechanisms of neurotrophic factor production, as they may contribute to the maintenance of cellular homeostasis. Our results indicate that isoproterenol, a βAR agonist, is able to up-regulate the mRNA levels for FGF-2 while decreasing the expression of FGF-1, another member of the FGF family, and CNTF, a trophic molecule highly expressed in cultured astrocytes (3, 4). The changes in CNTF mRNA levels are in agreement with the results of Rudge *et al.* (31) who demonstrated a similar effect in hippocampal astrocytes. The changes produced by the βAR agonist are mediated by the accumulation of cAMP as confirmed by the results with 8-bromo-cAMP, an analogue of the intracellular messenger, or forskolin, a direct activator of adenylate cyclase. The modifications of FGF-1 and CNTF mRNAs seem to be long lasting, whereas FGF-2 mRNA had almost returned to basal levels within 24 hr of the start of isoproterenol incubation. Our data, together with the results from other laboratories, indicate that the accumulation of cAMP in astrocytes can produce a wide array of effects on the regulation of trophic molecules and their receptors. Previous reports have shown that the expression of neurotrophic molecules other than CNTF can be altered by cAMP elevating agents. Brain-derived neurotrophic factor and neurotrophin-3 mRNA levels are induced by norepinephrine or by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine in

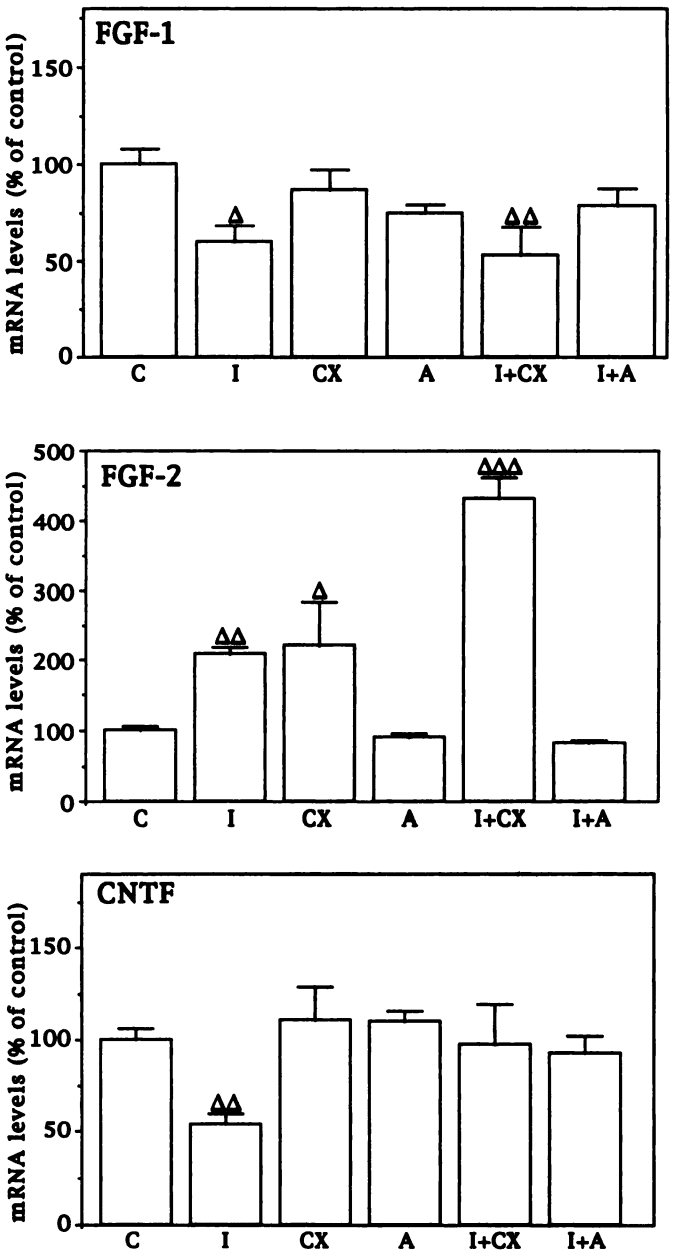

Fig. 6. Effect of protein synthesis (cycloheximide) or gene transcription (actinomycin D) inhibition on FGF-1, FGF-2, and CNTF mRNA levels after isoproterenol treatment. Rat cortical astrocytes were exposed to 1 μM isoproterenol (*I*) for 6 hr in the presence or absence of 5 μg/ml cycloheximide (*CX*) or 10 μg/ml actinomycin D (*A*). RNA was then extracted and used for trophic factor mRNA determination by RNase protection assay. The data [expressed as percent of control cells (*C*)] represent the mean ± standard error of three to six independent determinations. FGF-1: Δ, $p < 0.01$ versus control cells; ΔΔ, $p < 0.05$ versus control cells and cycloheximide-treated cells (analysis of variance with Dunnett's *t*-test). FGF-2: Δ, $p < 0.05$; and ΔΔ, $p < 0.01$ versus control cells; ΔΔΔ, $p < 0.001$ versus control cells and $p < 0.01$ versus cycloheximide-treated cells (analysis of variance with Dunnett *t*-test). CNTF: ΔΔ, $p < 0.01$ versus control cells (analysis of variance with Dunnett's *t*-test).

rat cortical astrocytes, and this effect is coupled to an increased expression of their high affinity receptor *trk*s (32, 33).

The activation of the cAMP pathway in glial cells, enriched in type I astrocytes, can therefore generate opposite effects in the expression pattern of neurotrophic molecules. Such ef-

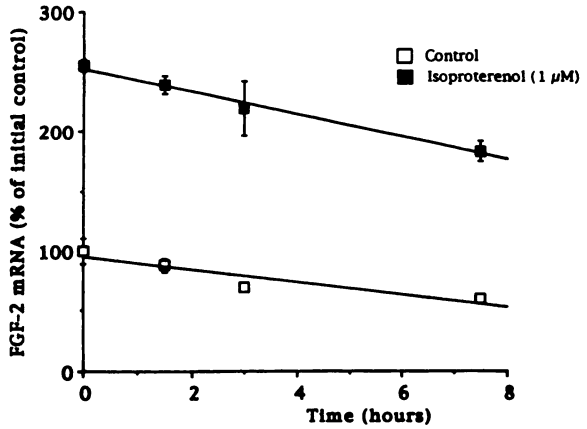

Fig. 7. Effect of isoproterenol (1 μM) on FGF-2 mRNA stability in primary culture of rat cortical astrocytes. Actinomycin D (10 μg/ml) was added to control cells or to astrocytes that have been pretreated for 3 hr with isoproterenol. RNA was extracted at different times after exposure to the gene transcription inhibitor. Results are expressed as percent of control cells before the addition of actinomycin D. Data represent the mean ± standard error of three to five independent determinations. The calculated half-life for FGF-2 mRNA was 9.2 hr for control and 13.3 hr for isoproterenol-pretreated cells.

fects are probably occurring through different mechanisms, as indicated by the results obtained with inhibitors of gene transcription and protein synthesis. Coincubation of cycloheximide prevented the down-regulation of CNTF expression by isoproterenol, suggesting that the synthesis of intermediate proteins may be necessary to produce the changes in CNTF mRNA levels. Confirming our previous results (23), cycloheximide was able to increase FGF-2 and NGF gene expression, probably as a result of mRNA stabilization (29). However, the inhibition of protein synthesis did not prevent the induction of FGF-2 as well as the decrease in FGF-1 mRNA after isoproterenol incubation. Therefore, the effect of the βAR agonist on gene expression of FGF seems to be more direct, probably through phosphorylation of transcription factors by cAMP-dependent protein kinases (34). Stachowiak *et al.* (35) recently reported that the adenylate cyclase activator forskolin is able to induce FGF-2 mRNA and protein in cultured adrenal medullary cells (35). However, unlike our observations in cortical astrocytes, the elevation of FGF-2 expression in adrenal medullary cells was prevented by coincubation with cycloheximide. In our study, in different cell populations, activation of the cAMP pathway can up-regulate the gene expression of FGF-2 through distinct mechanisms, which may or may not involve the synthesis of new proteins. Actinomycin D prevented the changes in all neurotrophic factor biosynthesis, suggesting that transcription mechanisms are indeed involved in the regulation of these molecules. However, post-transcriptional mechanisms may also participate in the regulation of FGF-2 mRNA. In fact, kinetic experiments with actinomycin D indicated that after isoproterenol treatment, the half-life of FGF-2 mRNA was slightly increased.

There is increasing evidence that astrocytes may be the target of catecholamines in the mature brain (36, 37). The great majority of astrocytes isolated from the adult rat brain express βAR binding whose density, as well as βAR-mediated cAMP responses, is increased in reactive cells (38). The regulation of FGF-2 and CNTF expression by isoproterenol in astrocytes is not strictly area specific, as glial cultures obtained from other brain regions (hippocampus and cerebellum) respond in the same way after exposure to the βAR agonist. We did observe quantitative differences in the induction profile of FGF-2 mRNA expression in astrocytes prepared from these brain regions. Such effects may be the results of changes in experimental conditions (cell density) or may depend on the pattern of βAR expression, as reported previously (38).

Although our experiments were performed on astrocytes prepared from newborn rats, it is likely that these regulatory mechanisms also occur in the adult animals and can be operative in different brain regions under the control of noradrenergic fibers. Follesa and Mocchetti (39) showed that systemic injection of clenbuterol, a lipophilic β_2AR agonist, increases FGF-2 mRNA levels in the rat central nervous system, specifically in hippocampus, cerebral cortex, and cerebellum. Moreover, Hayes *et al.* (40) recently demonstrated, by *in situ* hybridization, that FGF-2 induction after clenbuterol injection takes place mainly at glial levels. According to our results, astrocytes prepared from all of these brain regions respond to isoproterenol with an elevation in FGF-2 mRNA levels, suggesting that *in vivo* the direct stimulation of astroglial βARs can also elevate the gene expression of this trophic molecule.

It remains to be established whether the reduction in FGF-1 and CNTF gene expression after activation of βARs in astrocytes is indeed occurring *in vivo*. Although such effects may be difficult to detect under control conditions, alterations in cAMP levels might modulate FGF-1 and CNTF mRNA levels in circumstances, such as after neuronal injury, that lead to activation of trophic factor gene expression (41).

An important issue that requires further investigation is the extent to which these mechanisms can take place after alterations in the extracellular levels of norepinephrine. Several scenarios, in which there is an elevation of norepinephrine levels, can be encountered, including seizures, ischemia, stress, and chronic treatment with drugs known to affect norepinephrine metabolism and reuptake (42, 43). The concentration of this neurotransmitter can therefore reach levels able to modulate the gene expression of neurotrophic factor in astrocytes and, possibly, in neurons. If changes in norepinephrine availability lead to alterations in the gene expression for neurotrophic factors, these effects may contribute to the adaptive changes taking place after activation of the noradrenergic system. Data obtained after both pharmacological manipulation and selective lesions support the idea that noradrenergic fibers originating from the locus ceruleus have a protective role in ischemia and in status epilepticus (44, 45). These observations match the reported neuroprotective activity of several neurotrophic molecules. FGF-2 in able to prevent degeneration of septal neurons after fimbria/fornix lesion (10, 11) or thalamic degeneration after cortical infarction (12). Furthermore, *in vitro* models of excitotoxicity indicate that FGF-2, as well as other neurotrophic factors, can antagonize the neurotoxic effects produced by an elevated concentration of glutamate or by hypoglycemia (13, 14).

Neurons and astrocytes can therefore be programmed to synthesize trophic factors to enhance survival after various types of insult. FGF-2 gene expression can be induced in astrocytes by the excitatory amino acid glutamate, which is also very potent in increasing neurotrophin gene expression in cultured neuronal cells (17, 32, 46). Several systems can

cooperate in determining the expression of trophic molecules. Seizure activity, produced in different models of epilepsy, elevates neurotrophic factor gene expression according to specific spatiotemporal patterns (19, 20, 47, 48). Moreover, we have shown that glucocorticoid hormones increase FGF-2 mRNA levels in glial cells, whereas the reduction in circulating glucocorticoids after adrenalectomy produces a marked decrease in the expression for this trophic factor in several brain regions (23, 24).

Trans-synaptic and hormonal stimuli can therefore regulate the expression of neurotrophic molecules, which in turn may serve as a mediator of the genomic response of the cell. Such events can either render the cell more resistant to external stimuli or contribute to the adaptive mechanisms that take place after prolonged alterations in synaptic levels of neurotransmitters.

The understanding of the molecular mechanisms regulating neurotrophic factor production in neurons as well as in glial cells may be important to modulate specifically the levels of neurotrophic molecules in the brain. The possibility of influencing endogenous protective systems can be of great importance in several pathological conditions that are characterized by increased vulnerability of specific neuronal cells.

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