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Vasoactive Intestinal Peptide, Pituitary Adenylate Cyclase-Activating Peptide, and Noradrenaline Induce the Transcription Factors CCAAT/Enhancer Binding Protein (C/EBP)-β and C/EBPδ in Mouse Cortical Astrocytes: Involvement in cAMP-Regulated Glycogen Metabolism

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We have described previously a transcription-dependent induction of glycogen resynthesis by the vasoactive intestinal peptide (VIP) or noradrenaline (NA) in astrocytes, which is mediated by cAMP. Because it has been postulated that the cAMP-mediated regulation of energy balance in hepatocytes and adipocytes is channeled at least in part through the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, we tested the hypothesis that C/EBP isoforms could be expressed in mouse cortical astrocytes and that their level of expression could be regulated by VIP, by the VIP-related neuropeptide pituitary adenylate cyclase-activating peptide (PACAP), or by NA. We report in this study that in these cells, C/EBPβ and C/EBPδ are induced by VIP, PACAP, or NA via the cAMP second-messenger pathway. Induction of C/EBPβ and -δ mRNA by VIP occurs in the presence of a protein synthesis inhibitor. Thus, *c/ebpβ* and *c/ebpδ* behave as cAMP-inducible immediate-early genes in astrocytes. Moreover, transfection of astrocytes with expression vectors selectively producing the transcriptionally active form of C/EBPβ, termed liver-enriched transcriptional activator protein, or C/EBPδ enhance the glycogen resynthesis elicited by NA, whereas an expression vector producing the transcriptionally inactive form of C/EBPβ, termed liver-enriched transcriptional inhibitory protein, reduces this resynthesis. These results support the idea that C/EBPβ and -δ regulate gene expression of energy metabolism-related enzymes in astrocytes.

Key words: VIP; PACAP; noradrenaline; glycogen; astrocytes; energy metabolism; cAMP; transcription factors; C/EBP; immediate-early genes

There is increasing evidence that neurotransmitters exert receptor-mediated effects in astrocytes (Barres, 1991; Murphy, 1993). In particular, we have demonstrated pronounced short- and long-term effects of the vasoactive intestinal peptide (VIP) and the monoamine noradrenaline (NA) on astrocyte glycogen metabolism (Sorg and Magistretti, 1991, 1992; Magistretti et al., 1993). Thus, activation of VIP or NA receptors leads within minutes to glycogenolysis, followed within a few hours by a massive glycogen resynthesis (Sorg and Magistretti, 1991, 1992). Glycogen is the single largest energy reserve of the brain. It is localized predominantly in astrocytes, to such an extent that this cell type can be positively identified at the ultrastructural level by the presence of glycogen granules in the cytoplasm (Peters et al., 1991). Glycogen turnover in the nervous system is very rapid and is under the control of multiple regulatory mechanisms (Magistretti, 1988, 1990). Decreased synaptic activity that is achieved, for example, by anesthesia or slow-wave sleep markedly increases the glycogen content of the brain. Pathological conditions leading to neuronal loss, such as brain trauma, x-ray irradiation, or Alzheimer's disease, also cause a massive deposition of glycogen granules in the reactive astrocytes at the periphery of the lesioned areas [see Magistretti et al. (1993) and references therein]. The modulation of brain glycogen levels by VIP or NA led us to propose that the primary action of certain neurotransmitters is to regulate local-energy homeostasis within the CNS by acting on non-neuronal cells such as astrocytes (Magistretti, 1990, 1991). The massive glycogen resynthesis elicited by VIP or NA in mouse cortical astrocytes is mediated by specific receptors coupled to the cAMP signal transduction cascade, and it is suppressed by cycloheximide or actinomycin D, which implies new protein synthesis (Sorg and Magistretti, 1992).

In an attempt to characterize further this receptor-mediated translational-dependent event triggered by VIP or NA in a cAMP-dependent manner (Sorg and Magistretti, 1992), we focused our attention on a family of transcription factors called CCAAT/enhancer-binding protein (C/EBP). Like the Fos/Jun and activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) families, C/EBPs are characterized by a bipartite DNA-binding domain, composed of a basic region, contacting DNA, linked to a homo- or heterodimer-forming region called leucine zipper (for review, see Lamb and McKnight, 1991; McKnight, 1992). The first member of this family, C/EBPα, was found at high levels in adipose, hepatic, and placental tissues and at lower levels in lung and small intestine (Birkenmeier et al.,

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1989). Except for lung, each of these tissues can be considered to be part of the network of organs that facilitates the uptake, metabolism, and storage of physiological fuels. Therefore, it has been speculated that regulation of energy balance is channeled, at least in part, through C/EBPα (McKnight et al., 1989). The C/EBP family also includes C/EBPβ (this is referred to in the literature also as NF-IL6, IL-6-DBP, LAP, AGP/EBP, or CRP2), C/EBPδ (also called CRP3, CELF, or NF-IL6β), and C/EBPγ (also known as Ig/EBP-1) (for review, see McKnight, 1992; Chen-Kiang et al., 1993). Members of the C/EBP family can form homo- or heterodimers, all of which can bind to the same *cis*-regulatory elements. C/EBPα, -β, and -δ are involved in terminal differentiation of a variety of cells including adipocytes, hepatocytes, gut epithelial cells, macrophages, and myelomonocytes (Friedman et al., 1989; Cao et al., 1991; Natsuka et al., 1992; Scott et al., 1992; Chandrasekaran and Gordon, 1993; Yeh et al., 1995). Furthermore, C/EBPβ and -δ activate various genes involved in acute-phase, inflammatory, and immune responses (Akira and Kishimoto, 1992; Chen-Kiang et al., 1993) [see also Tanaka et al. (1995) and references therein]. Thus, C/EBPs appear to be pleiotropic transactivators involved in tissue-specific metabolic gene transcription, in signal transduction activated by several cytokines, and in cell differentiation. Because some C/EBP isoforms stimulate the expression of genes encoding for enzymes involved in energy-metabolism regulation and because C/EBPβ and -δ have been shown to be implicated in cAMP responsiveness (Kageyama et al., 1991; Metz and Ziff, 1991; Park et al., 1993; Vallejo et al., 1993), we tested the hypothesis that members of the C/EBP family are expressed in astrocytes and that their level of expression is regulated by VIP, by the VIP-related neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), or by NA.

In this report, we demonstrate that in mouse cortical astrocytes VIP, PACAP27 and NA increase C/EBPβ and -δ isoforms at the mRNA and protein levels via a cAMP-dependent mechanism. Induction of C/EBPβ and -δ mRNA by VIP is rapid and occurs in the presence of a protein synthesis inhibitor. *c/ebpβ* and *c/ebpδ*, therefore, can be considered to be cAMP-inducible immediate-early genes in mouse astrocytes. Moreover, overexpression of C/EBPδ or liver-enriched transcriptional activator protein (LAP), the transcriptionally active form of C/EBPβ, by transient transfection of astrocytes with expression vectors containing their cDNA sequence, showed that these transcription factors can increase NA-mediated glycogen resynthesis. This suggests that C/EBPβ and -δ are primary target genes of the signal transduction pathway triggered by VIP, PACAP27, or NA, which regulate glycogen metabolism in the brain.

MATERIALS AND METHODS

Tissue culture. Primary cultures of cerebral cortical astrocytes were prepared from 1- to 2-d-old Swiss albino mice as described previously (Sorg and Magistretti, 1992). Briefly, forebrains were removed aseptically from the skulls, the meninges were excised carefully, and the neocortex was dissected free. The cells were dissociated by passage through needles of decreasing gauges (1.2 × 40, 0.8 × 40, and 0.5 × 16 mm) with a 10 ml syringe. No trypsin was used for dissociation. The cells were seeded on poly-ornithine-coated dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and incubated at 37°C in an atmosphere containing 5% CO_2/95% air. The culture medium was renewed after 5 d of seeding and then twice per week. This procedure yields cultures in which >90% of cells are immunoreactive for glial fibrillary acidic protein (GFAP) (Sorg and Magistretti, 1992).

Exposure to neurotransmitters. During all incubations, primary cultures of astrocytes were maintained at 37°C in an atmosphere containing 5% CO_2/95% air. Twenty-four hours before treatment, the culture medium was removed and astrocytes were incubated in serum-free DMEM. Without changing the medium, the cells then were exposed to VIP, PACAP27, NA, or other pharmacological agents to attain the desired final concentrations. When required, cells were exposed for 30 min to the protein synthesis inhibitor anisomycin before VIP exposure. Similarly, the adrenergic antagonists atenolol and prazosin were added to the cells 10 min before NA treatment. At the end of the various periods of incubation, cells were washed three times with ice-cold PBS, pH 7.4, and total RNA or protein was extracted. VIP and PACAP27 were purchased from Bachem (Bubendorf, Switzerland), and all other reagents were purchased from Sigma (St. Louis, MO).

Western immunoblot analysis. For Western immunoblot analyses, total proteins from astrocytes cultured in 35 mm dishes were extracted with 50 μl of lysis buffer [50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 0.3% SDS] and heated to 100°C for 5 min. Samples then were sonicated to shear genomic DNA, and a 2 μl aliquot was used to measure the protein content following the method of Bradford (1976). Protein (50 μg) from each sample was adjusted to give a final buffer composition of 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 9% glycerol, and 0.1% bromophenol blue, and was heated to 100°C for 5 min, electrophoresed through a 12% SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. C/EBP isoforms were detected with the BM chemiluminescence detection system as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The dilution factor for each primary antibody was 1:2000 for anti-C/EBPβ and 1:3000 for anti-C/EBPδ. Anti-C/EBPβ was raised against the C-terminal 18 amino acids of C/EBPβ, whereas anti-C/EBPδ was raised against the full-length protein. These antibodies were provided by S. L. McKnight (Tularik, South San Francisco, CA). For some experiments (see figure legends for details), C/EBPδ was detected with an affinity-purified rabbit polyclonal antibody (dilution 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide corresponding to the C-terminal 22 amino acids of rat C/EBPδ. Both anti-C/EBPδ antibodies recognized a regulated band, the M_r of which corresponded to C/EBPδ. The identity of the regulated bands detected by the anti-C/EBPβ or anti-C/EBPδ antibodies was confirmed by the absence of immunoreactivity when the antibodies were preabsorbed with the peptides corresponding to the C-terminal 19 or 22 amino acids of C/EBPβ and -δ, respectively.

Northern blot analysis. Total RNA was extracted using the CsCl centrifugation procedure as described by Chirgwin et al. (1979). Twenty micrograms of each RNA sample were electrophoresed through denaturing 1.3% agarose gels containing 2 M formaldehyde, and were transferred to nylon membrane (Gene Screen, DuPont NEN, Boston, MA). Hybridization was performed for at least 18 hr at 63°C in 50% formamide, 5× SSC, 250 mM Tris-HCl, pH 7.5, 0.5% sodium pyrophosphate, 5% SDS, 1% polyvinylpyrrolidone, 1% Ficoll, 25 mM EDTA, 1% bovine serum albumin, and 150 μg/ml sheared denatured salmon sperm DNA, which contained an antisense ^{32}P riboprobe generated either from the full-length mouse C/EBPβ cDNA or from an *Eco*RI/*Xho*I fragment of the mouse C/EBPδ cDNA. Filters then were washed under high-stringency conditions (twice with 2× SSC/0.1% SDS at 65°C for 15 min and twice with 0.1× SSC/0.1% SDS at 65°C for 15 min), and autoradiographed at −70°C with an intensifying screen.

The levels of C/EBPβ or -δ mRNA were determined by scanning densitometry (BioImage system, Millipore, Milford, MA) and normalized for differences in gel loading as compared with the levels of β-actin mRNA, which were determined by rehybridization with an antisense β-actin ^{32}P riboprobe after stripping the filters.

Immunofluorescence analysis. Mouse cerebral cortical astrocytes were grown to confluence on poly-ornithine-coated 12 mm glass coverslips in 24-well microtiter dishes under the same conditions as described above. After 24 hr in serum-free DMEM, the cells were stimulated with 10^{-7} M VIP for 4 hr, washed with ice-cold PBS, fixed with acetone, and dried. They then were permeabilized with 0.01% Triton X-100 in PBS, and the nonspecific sites were blocked with normal swine serum (1:25, Dakopatts, Copenhagen, Denmark). Double labeling was performed by incubating cells overnight with a mouse monoclonal anti-GFAP antibody (1:800, Boehringer Mannheim) and affinity-purified rabbit polyclonal anti-C/EBPβ or anti-C/EBPδ antibodies (10 μg/ml, Santa Cruz Biotechnology). Anti-C/EBPβ was raised against a peptide corresponding to the C-terminal 19 amino acids of rat C/EBPβ; anti-C/EBPδ was raised against a peptide corresponding to the C-terminal 22 amino acids of rat C/EBPδ. The specificity of the anti-C/EBP antibodies was tested by neutralization with a 10-fold (by weight) excess of each peptide (100 μg/ml, Santa Cruz Biotechnology). Blocking peptides and antibodies were preincubated for 2 hr before being applied to the cells. After three washes with PBS, the

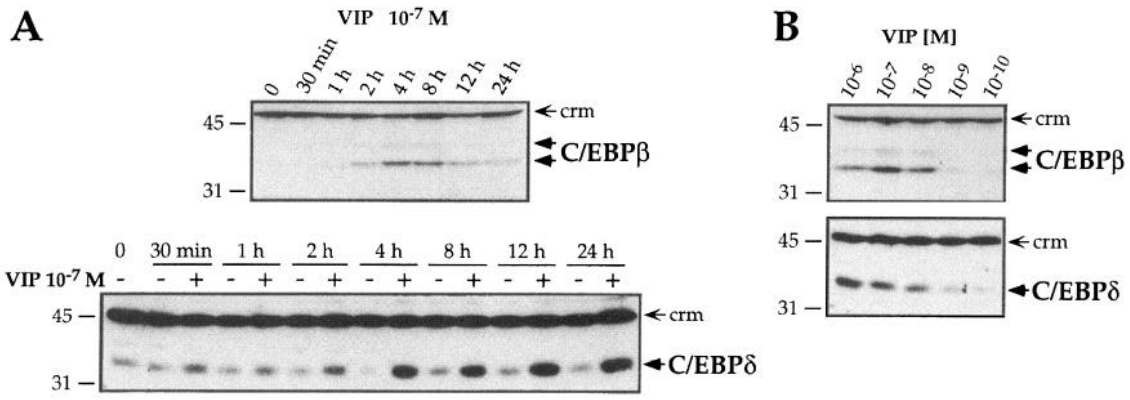

Figure 1. Induction of the transcription factors C/EBPβ and -δ by VIP in cultures of mouse cortical astrocytes. *A*, Cultures of cortical astrocytes were incubated for various periods of time (indicated on *top* of each lane) in the presence of 10^{-7} M VIP. For C/EBPδ, control cultures incubated in the absence of VIP (−) were performed at each point of the time course. Equal amounts of total protein extracts (50 μg) were analyzed by 12% SDS-PAGE, and C/EBPβ or C/EBPδ were detected by Western immunoblot analysis. Protein M_r markers are indicated (in kDa) to the *left* of the panels. Bands corresponding to C/EBPβ or -δ are indicated by *arrows* at the right. C/EBP-unrelated cross-reacting material (*crm*) (Cao et al., 1991) served as a loading control. *B*, Effect of increasing concentrations of VIP on the expression of C/EBPβ and -δ. Proteins were collected after a 4 hr stimulation in the presence of the indicated concentration of VIP and were analyzed as described in *A*. Similar results were obtained in at least three independent experiments.

cells were layered with rhodamine-conjugated sheep anti-mouse IgG (1:10, Boehringer Mannheim) and fluorescein-conjugated swine anti-rabbit IgG (1:50, Dakopatts) in PBS at room temperature for 20 min. Finally, the coverslips were washed three times with PBS and mounted, and the cells were examined by fluoromicroscopy.

Cell transfection and glycogen assay. The C/EBP protein expression vector Moloney murine sarcoma virus (MSV)-C/EBPβ and MSV-C/EBPδ (for description, see Cao et al., 1991) were provided by S. L. McKnight (Tularik). The plasmid MSV-no insert was constructed by deleting the C/EBPδ insert of MSV-C/EBPδ. MSV-LAP and MSV-liver-enriched transcriptional inhibitory protein (LIP) were constructed by PCR following the strategy and using the primers described in Yeh et al. (1995). 5′-Primers were used to introduce an optimal Kozak consensus sequence and an *Eco*RI restriction site upstream from the second or third in-frame ATG, respectively, in the C/EBPβ-coding sequence. The common downstream primer contained a *Bam*HI site for cloning into the MSV-long terminal repeat expression vector. PCR conditions for generating recombinant sequences from MSV-C/EBPβ were as follows: one cycle at 94°C for 5 min, annealing at 52°C for 2 min, extension at 72°C for 10 min, and then denaturation at 94°C for 1 min; annealing at 52°C for 2 min, extension at 72°C for 3 min, repeated for 34 cycles. Pwo DNA polymerase (Boehringer Mannheim) was used instead of *Taq* DNA polymerase to minimize the risk of errors during PCR. Reaction conditions were as recommended by Boehringer Mannheim, except that the amplification reactions also included 10% (v/v) dimethylsulfoxide. The amplified LAP and LIP cDNA fragments then were inserted to cloning sites of *Eco*RI and *Bam*HI into the MSV vector.

Transient transfection of confluent mouse cortical astrocytes was performed using the liposome-mediated gene-transfer method with the cationic lipid *N*-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethylsulfate (DOTAP) according to the manufacturer's instructions (Boehringer Mannheim). A total of 5 μg of expression vector was transfected per 35 mm dish with a DOTAP concentration of 13.4 μg/ml in serum-free DMEM. Cells were maintained for 7 hr in this medium before replacing it with DMEM containing 10% fetal calf serum and incubating further for 61 hr. Culture medium was replaced with a serum-free DMEM containing 5 mM glucose (instead of 25 mM for the culture medium). Four hours later, NA was added to the medium to a final concentration of 10^{-5} M, and cultures were maintained for 6 hr in the incubator. The reaction then was stopped by washing the cells three times with ice-cold PBS and by adding either 1.5 ml of 30 mM HCl for the glycogen assay or 50 μl of lysis buffer for Western immunoblot analysis.

For the glycogen assay, the cells were sonicated and the suspension was used to measure glycogen as described previously by Sorg and Magistretti (1992). Briefly, three 100 μl aliquots were sampled. In the first aliquot, 300 μl of acetate buffer was added. In the second aliquot, 300 μl of a solution containing 10% amyloglucosidase (10 mg/ml) in 0.1 M acetate buffer, pH 4.65, was added, and the mixture was incubated at room temperature for 30 min. After incubation with amyloglucosidase, 2 ml of 0.1 M Tris-HCl buffer, pH 8.1, containing 3.3 mM $MgCl_2$, 0.2 mM ATP, 25 μg/ml nicotinamide adenine dinucleotide phosphate (NADP), 4 μg/ml, hexokinase, and 2 μg/ml glucose-6-phosphate dehydrogenase was added, and the mixture was incubated at room temperature for 30 min. The first aliquot was treated identically. The fluorescence of the NADPH formed then was read on a fluorometer (excitation, 340 nm; emission, 450 nm). The first aliquot gave the sum of glucose and glucose-6-phosphate, and the second gave the sum of glycogen, glucose, and glucose-6-phosphate; the amount of glycogen then was determined by the difference between the first two aliquots. The third aliquot was used to measure the protein content following the method of Bradford (1976). We should note that in this report, 1 mole of glycogen corresponds to 1 mole of glycosyl units originating from glycogen. ATP, NADP, and the enzymes were purchased from Boehringer Mannheim. Statistical analyses were performed by ANOVA followed by Dunnett's test.

RESULTS

C/EBPβ and -δ are induced by VIP in mouse cortical astrocytes

To determine whether members of the C/EBP family were induced or modulated after exposure of mouse cortical astrocytes to VIP, we performed Western immunoblot analyses with antibodies against the well characterized C/EBPα, -β, and -δ isoforms. Although C/EBPα was not detected, either by Western immunoblot or by Northern blot (data not shown), we observed that the C/EBPβ and -δ isoforms were induced in a time-dependent manner by 10^{-7} M VIP (Fig. 1*A*). The antibody against C/EBPβ recognized two proteins with M_r of 39 and 36 kDa; these proteins, which have been shown to be synthesized from C/EBPβ mRNA, correspond to translation products initiated at the first and second in-frame AUGs of the C/EBPβ mRNA; the initiation at the first in-frame AUG is less efficient than at the second in-frame AUG (Descombes and Schibler, 1991). An increase in C/EBPβ protein levels occurred 2 hr after VIP exposure, reached a peak level between 4 and 8 hr, and began to decrease 12 hr after the onset of treatment (Fig. 1*A*). Analysis of the time course of the induction of C/EBPδ showed that, unlike C/EBPβ, an increase in C/EBPδ can be observed only 30 min after VIP exposure. C/EBPδ reached a maximal level after 4 hr, and unlike C/EBPβ, still remained stable at high levels of expression 24 hr after exposure to VIP. Because basal levels of C/EBPδ were higher than those of C/EBPβ and appeared to be sensitive to conditions of stress, such

as medium renewal, we performed a control manipulation (i.e., same processing of the astrocytes without adding VIP) at each point of the time course. As indicated in Figure 1*B*, VIP induced C/EBPβ and -δ expression in a concentration-dependent manner, between 10^{-8} and 10^{-6} M.

To confirm that the induction of C/EBPβ and -δ proteins actually was caused by an increase in their mRNA, we analyzed by Northern blotting total RNA extracted from astrocytes exposed to 10^{-7} M VIP for various periods of time (Fig. 2*A*). These results confirmed the induction of C/EBPβ and -δ at the mRNA level. As expected, the variations in mRNA levels preceded the variations in C/EBPβ or -δ protein levels. C/EBPβ mRNA levels increased already 30 min after VIP exposure, reached a peak between 2 and 4 hr, and decreased 8 hr after the onset of treatment. C/EBPδ mRNA levels increased gradually over time, stabilized at 4 hr, and remained maximally induced until at least 24 hr after addition of VIP (Fig. 2*A*,*B*). The increase in C/EBPδ mRNA 15 and 30 min after VIP exposure was weaker than the increase in C/EBPδ protein at 30 min. This apparent inconsistency might be explained by the fact that a single mRNA molecule can be translated several times, thus providing an amplified signal at the protein level. Taken together, these results demonstrate that in mouse cortical astrocytes, VIP induces C/EBPβ and -δ at the mRNA and protein levels in a time- and concentration-dependent manner.

C/EBPβ and -δ are induced by VIP as immediate-early genes

Because C/EBPβ and -δ are transcription factors induced relatively rapidly by VIP, we wondered whether these two C/EBP isoforms were immediate-early response genes activated directly by constitutively expressed transcription factors. An immediate-early gene is defined as a gene that can be induced without *de novo* protein synthesis and, therefore, is not blocked by inhibitors of protein synthesis (for review, see Herschman, 1991). We therefore exposed mouse cortical astrocytes to the protein synthesis inhibitor anisomycin at 20 μM for 30 min before VIP exposure, extracting total RNA 2 hr later (Fig. 3). In these conditions, protein synthesis was abolished almost completely (see legend of Fig. 3). Results indicated that C/EBPβ and -δ mRNA production still was enhanced by VIP in the presence of anisomycin, suggesting that VIP induces *c/ebpβ* and *c/ebpδ* gene transcription via a protein synthesis-independent mechanism.

Treatment of astrocytes with anisomycin alone produced an induction of the C/EBPβ and -δ mRNAs (Fig. 3). This phenomenon, called superinduction, has been described previously and is thought to reflect the loss of labile transcriptional repressors or mRNA-degrading enzymes and, in general, is believed to be a direct consequence of the inhibition of protein synthesis. Independent of its ability to block translation, anisomycin also causes side effects, by intrinsically initiating intracellular signals and immediate-early gene induction (Cano et al., 1994). Regardless of the process that caused the superinduction of C/EBPδ mRNA, we concluded from these experiments that the induction of *c/ebpβ* and *c/ebpδ* gene transcription did not require new protein synthesis and, therefore, defined *c/ebpβ* and *c/ebpδ* as immediate-early genes in mouse cortical astrocytes.

C/EBPβ and -δ are localized in the nucleus of GFAP-positive cells

The primary cultures of astrocytes used in this study contain >90% of cells immunoreactive for the astrocytic marker GFAP. The remaining 10% is composed of progenitor cells for oligodendrocytes,

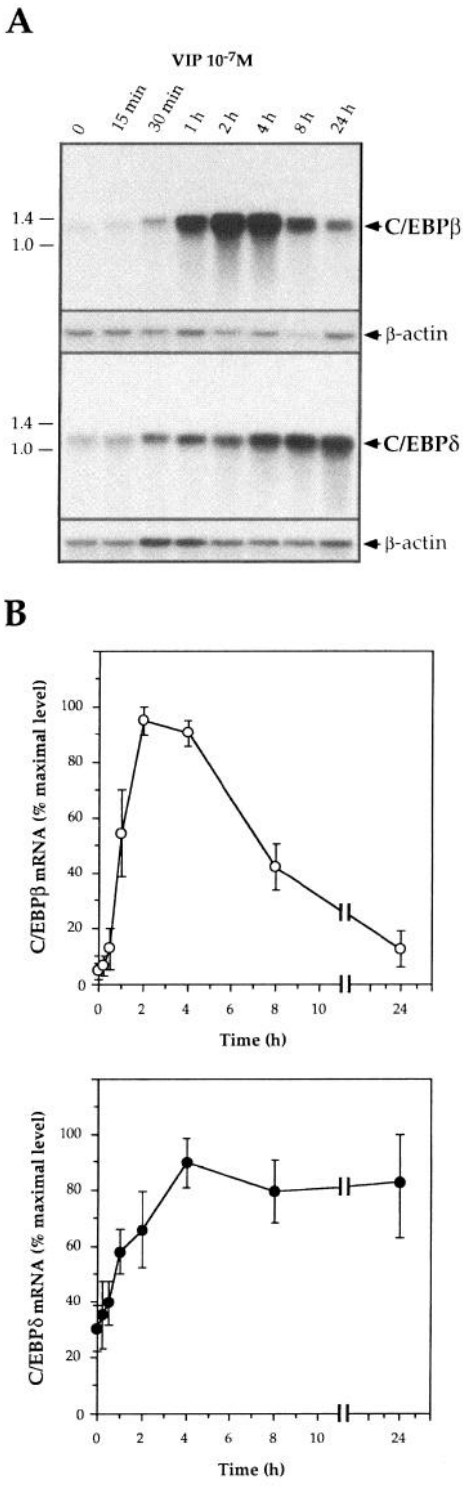

Figure 2. Induction of C/EBPβ and -δ mRNA expression by VIP in cultures of mouse cortical astrocytes. *A*, Time course of C/EBPβ and -δ mRNA accumulation after exposure of cultures of cortical astrocytes to 10^{-7} M VIP. Equal amounts of total RNA (20 μg) were electrophoresed and analyzed by Northern blotting using ^{32}P riboprobes complementary to C/EBPβ or -δ mRNAs. Similar results were obtained in three independent experiments. RNA markers are indicated (in kb) at the *left*. Blots were hybridized sequentially with C/EBP and β-actin probes; the latter was used as a loading control. *B*, Results in *A* were quantified by scanning densitometry and are expressed as mean ± SEM (n = 3) compared with the maximal level of expression for each C/EBP mRNA.

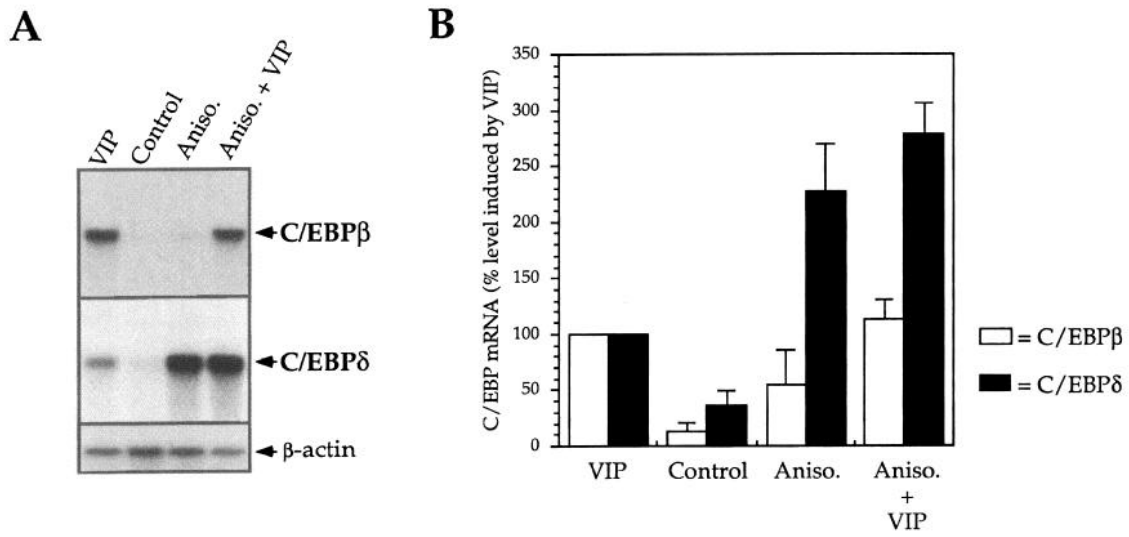

Figure 3. Induction of C/EBPβ and -δ mRNA expression by VIP is not blocked by the protein synthesis inhibitor anisomycin. *A*, Cultures of cortical astrocytes were treated with the protein synthesis inhibitor anisomycin (*Aniso.*; 2×10^{-5} M) 30 min before VIP (10^{-7} M) addition, and then they were incubated for another 2 hr. Anisomycin at this concentration caused a 97 ± 7% ($n = 3$) reduction in the incorporation of [^{35}S]methionine into total protein during the 2 hr of VIP exposure. Equal amounts of total RNA (20 μg) were electrophoresed and analyzed by Northern blotting. Similar results were obtained in three independent experiments. The blots were hybridized sequentially with ^{32}P riboprobes complementary to C/EBPβ, C/EBPδ, and β-actin mRNAs; the latter was used as a loading control. *B*, Results in *A* were quantified by scanning densitometry and are expressed as the mean ± SEM ($n = 3$) compared with the C/EBP mRNA levels evoked by VIP in the absence of anisomycin.

astrocytes, and microglia. It was necessary, therefore, to confirm that C/EBPβ and -δ indeed were expressed by GFAP-positive cells. To do this, we performed double-immunofluorescence labeling of GFAP and C/EBPβ or GFAP and C/EBPδ (Fig. 4). These results clearly showed that C/EBPβ and -δ are expressed and localized in the nucleus of mouse cortical astrocytes exposed to VIP. The use of neutralizing peptides corresponding to the C-terminal part of C/EBPβ or -δ demonstrated the specificity of the antibodies against the C/EBP isoforms. In rat pheochromocytoma (PC)-12 cells, a translocation of C/EBPβ from the cytoplasm to the nucleus after cAMP stimulation was described by Metz and Ziff (1991). Because the basal levels of C/EBPβ were barely detectable, we did not observe such a translocation in mouse cortical astrocytes after exposure to VIP. The basal levels of C/EBPδ were higher, with the protein specifically immunolocalized in the nucleus and not detected in the cytoplasm.

PACAP27 induces C/EBPβ and -δ in mouse cortical astrocytes

A major action of VIP in the cerebral cortex is the stimulation of cAMP formation (Magistretti and Schorderet, 1984); therefore, we addressed the question of whether the VIP-related neuropeptide PACAP also induced C/EBPβ or -δ, because PACAP stimulates cAMP formation more potently than VIP in rat primary astrocytes (Hashimoto et al., 1993). Two forms of PACAP, PACAP38 and PACAP27, have been identified and demonstrated to be equally potent in stimulating the adenylate cyclase (for review, see Arimura, 1992). We applied PACAP27 to mouse cortical astrocytes and found that it induced C/EBPβ and -δ expression in a concentration-dependent manner between 10^{-9} and 10^{-7} M (Fig. 5*B*). PACAP27, therefore, is 10-fold more potent than VIP in stimulating C/EBPβ and -δ expression. However, some differences in the time course of C/EBPδ induction by PACAP27 were observed compared with the time course of C/EBPδ induction by VIP. Thus, C/EBPδ protein levels increased only 4 hr after PACAP27 exposure and returned to the basal level 24 hr after PACAP27 exposure (Fig. 5*A*). Therefore, unlike VIP, PACAP27 induced C/EBPβ and -δ with a similar time course.

NA induces C/EBPβ and -δ in mouse cortical astrocytes

Given the effects of VIP or the monoamine neurotransmitter NA on the energy metabolism of mouse astrocytes, as well as the capacity of NA to stimulate adenylate cyclase, we tested whether NA could induce C/EBPβ or -δ isoforms. When NA was applied to mouse cortical astrocytes, we observed by Western immunoblot analysis the most rapid and persistent induction of C/EBPβ and -δ protein levels among the three neurotransmitters tested (Fig. 6*A*). These levels still remained high 24 hr after NA exposure, although C/EBPβ levels were lower than the maximal induction observed at 4 hr. Furthermore, NA induced C/EBPβ and -δ in a concentration-dependent manner, with an effect already apparent at 10^{-7} M (Fig. 6*B*). This induction could be mimicked by the β-adrenergic receptor agonist isoproterenol, but not by the α_1-agonist methoxamine (Fig. 6*C*). Moreover, the NA-mediated C/EBPβ and -δ induction was decreased by the β1-adrenergic receptor antagonist atenolol, but not by the α_1-antagonist prazosin. Taken together, these pharmacological analyses strongly suggest that NA induces C/EBPβ and -δ via receptors of the β-subtype that are coupled to adenylate cyclase (for review, see Summers and McMartin, 1993).

8-(4-Bromo)-cAMP and forskolin induce C/EBPβ and -δ in mouse cortical astrocytes

The foregoing results provided a strong indication that VIP, PACAP27, and NA likely induce C/EBPβ and -δ by increasing intracellular cAMP levels in mouse cortical astrocytes. This was confirmed further by the observation that the cAMP analog 8-(4-bromo)-cAMP [8-(4Br)-cAMP] and the direct adenylate cyclase activator forskolin also induced C/EBPβ and -δ (Fig. 7). The kinetics of the induction of C/EBPβ and -δ by 8-(4Br)-cAMP or forskolin was similar to that observed with VIP, PACAP27, or

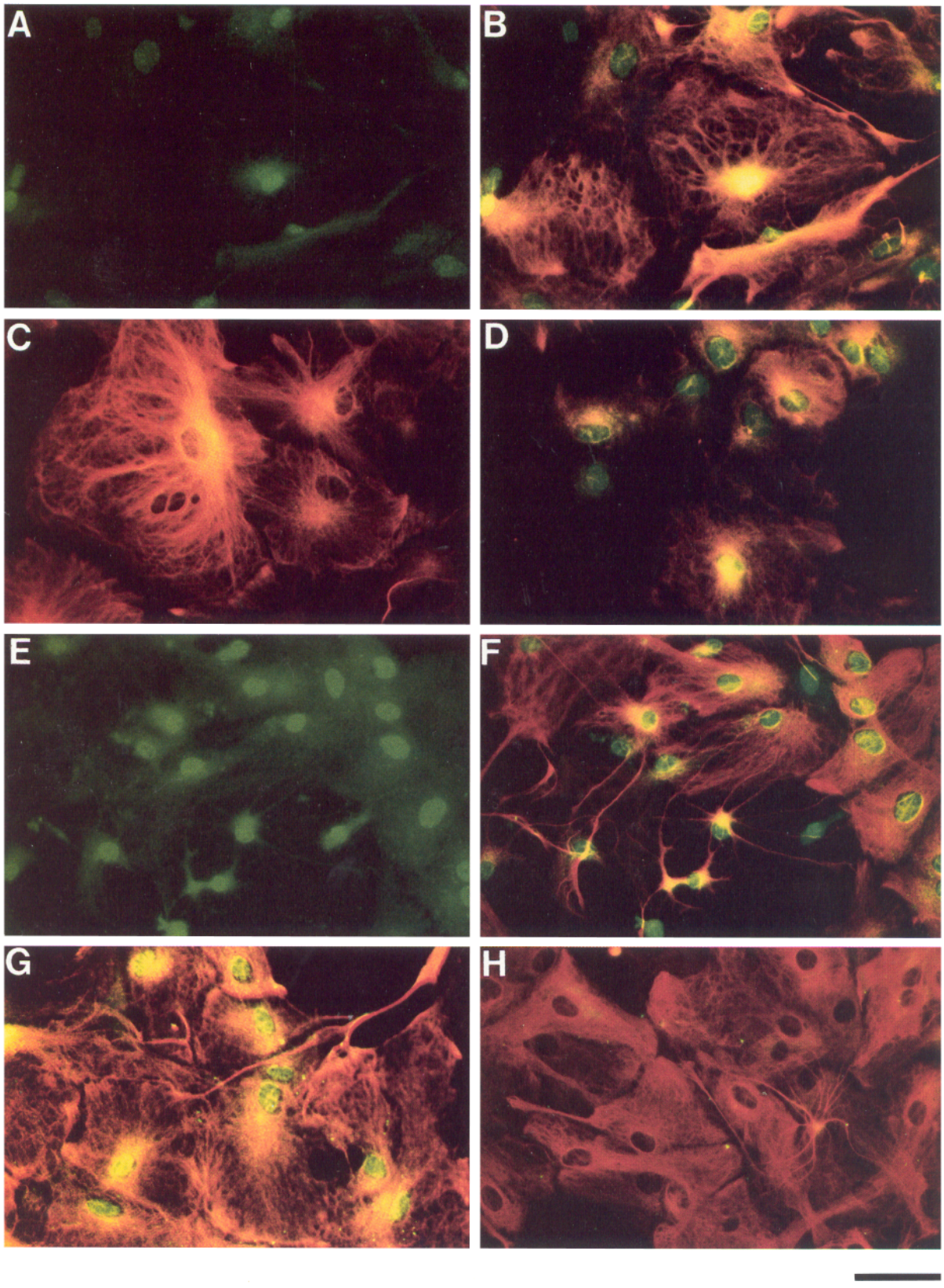

Figure 4. Immunohistochemical localization of C/EBPβ and -δ in the nucleus of GFAP-positive cells. Cultures of mouse cortical astrocytes exposed to VIP (10^{-7} M) for 4 hr were fixed with acetone, and double-immunofluorescence microscopy was performed using antibodies against C/EBPβ and GFAP (*A–D*) or C/EBPδ and GFAP (*E–H*). *A*, Fluorescein staining of C/EBPβ. *B*, Rhodamine staining of the astrocytic marker GFAP and fluorescein staining of C/EBPβ within the field shown in *A*. C/EBPβ is localized in the nucleus of the GFAP-positive cells. *C*, Control for nonspecific fluorescein labeling with the antibody against C/EBPβ. Neutralization of anti-C/EBPβ antibody with the peptide against which it has been raised caused a total loss of nuclear staining. *D*, Control of the anti-C/EBPβ antibody specificity. Preincubation of the antibody against C/EBPβ with the peptide corresponding to C/EBPδ

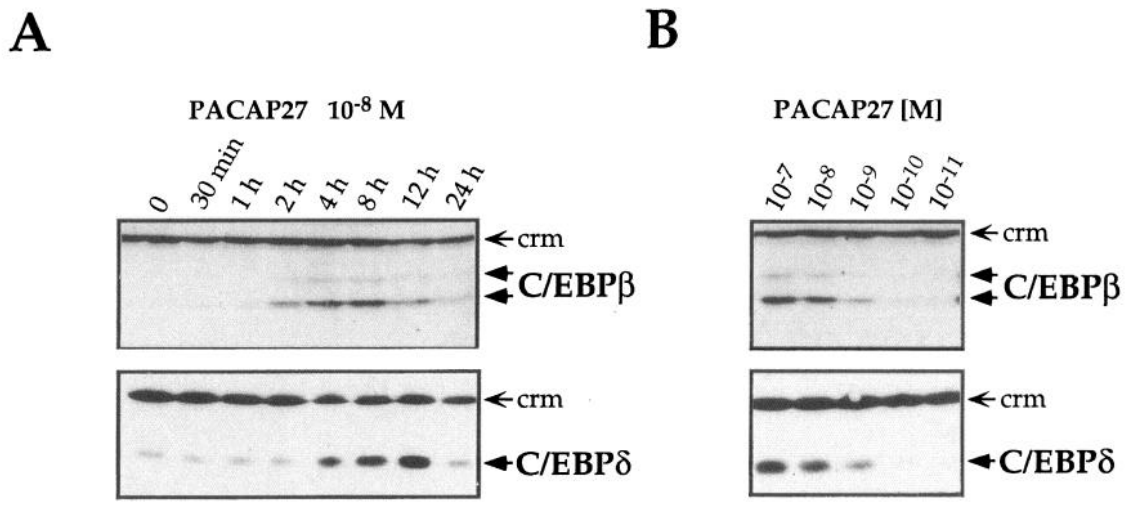

Figure 5. Induction of C/EBPβ and -δ by PACAP27 in cultures of mouse cortical astrocytes. *A*, Cultures of cortical astrocytes were incubated for various periods of time (indicated at the *top* of each lane) in the presence of 10^{-8} M PACAP27. Equal amounts of total protein extracts (50 μg) were analyzed by 12% SDS-PAGE, and C/EBPβ or -δ were detected by Western immunoblot analysis. Bands corresponding to C/EBPβ or -δ are indicated by *arrows* at the *right*. C/EBP-unrelated cross-reacting material (*crm*) (Cao et al., 1991) served as a loading control. *B*, Effect of PACAP27 at increasing concentrations on the expression of C/EBPβ and -δ. Proteins were collected after a 4 hr stimulation in the presence of the indicated concentration of PACAP27 and were analyzed as described in *A*. Similar results were obtained in three independent experiments.

NA. Thus, *c/ebpβ* and *c/ebpδ* can be considered to be cAMP-responsive immediate-early genes in mouse cortical astrocytes.

PACAP27 and NA induce C/EBPβ and -δ mRNA in mouse cortical astrocytes

To confirm that the induction of C/EBPβ and -δ by PACAP27 or NA was caused by an increase in their mRNA, we analyzed by Northern blotting total RNA extracted from astrocytes exposed for 2 hr to these two neurotransmitters. As shown in Figure 8, 10^{-8} M PACAP27 or 10^{-5} M NA induces C/EBPβ and -δ mRNAs to a similar extent as 10^{-7} M VIP.

Involvement of C/EBPδ and LAP in glycogen resynthesis

Because we demonstrated that the β and δ isoforms of the C/EBP family of transcription factors were induced rapidly by VIP, PACAP27, or NA in mouse cultured cortical astrocytes, we considered the possibility that these factors mediate the cAMP- and protein synthesis-dependent resynthesis of glycogen elicited by VIP or NA in these cells (Sorg and Magistretti, 1992). To test this hypothesis, we first attempted to overexpress C/EBPβ or -δ by transfecting astrocytes with expression vectors containing their cDNA. We monitored the expression of the C/EBP isoforms by Western immunoblotting. Although C/EBPδ was overexpressed efficiently, C/EBPβ was expressed only poorly (data not shown). The failure to overexpress C/EBPβ likely was attributable to an inefficient initiation of translation, because the C/EBPβ mRNA can be translated from three in-frame AUGs to yield three proteins called LAP*, LAP, and LIP (Descombes and Schibler, 1991). Therefore, we decided to construct the MSV-LAP and MSV-LIP expression vectors as described by Yeh et al. (1995). The introduction of an optimal Kozak consensus sequence upstream from the second or third in-frame AUG allowed the selective overexpression of the transcriptionally active form of C/EBPβ, termed LAP, or the transcriptionally inactive form of C/EBPβ, termed LIP, respectively. Although LAP and C/EBPδ were expressed at high levels after the transfection of astrocytes, glycogen levels were not enhanced significantly (data not shown). Of interest, however, overexpression of LIP reduced glycogen levels significantly, which were 18.2 ± 0.9 nmol of glycogen/mg of protein for the astrocytes transfected with MSV-no insert and 11.2 ± 2.8 nmol of glycogen/mg of protein for the astrocytes transfected with MSV-LIP, which suggests that glycogen synthesis was inhibited by the LIP-mediated inhibition of C/EBP isoforms. One possible explanation for the inefficiency of LAP and C/EBPδ to increase glycogen levels is that a physiological response implying new protein synthesis, such as the resynthesis of glycogen in astrocytes, rarely is controlled by a single transcription factor. Tissue-specific gene transcription depends on a combination of *cis*-acting elements, which are recognized by several transcription factors that, by binding simultaneously to the gene promoter, favor the formation of the transcription initiation complex and the subsequent RNA synthesis by the RNA polymerase II. Therefore, if resynthesis of glycogen elicited by VIP or NA requires the induction of several transcription factors, then the overexpression of only one of these transcription factors would not be sufficient to increase glycogen levels. Because of this, we decided to transfect astrocytes with the expression vectors and then, after a period sufficient to overexpress LAP, LIP, or C/EBPδ, expose the transfected astrocytes to NA. The rationale for this protocol was that because glycogen resynthesis is a transcriptionally regulated cAMP-dependent phenomenon (Sorg and Magistretti, 1992), increases in cAMP levels triggered by NA could induce or activate other

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did not cause a loss of immunoreactivity in the nucleus of GFAP-positive cells. *E*, Fluorescein staining of C/EBPδ. *F*, Rhodamine staining of the astrocytic marker GFAP and fluorescein staining of C/EBPδ within the field shown in *E*. C/EBPδ is localized in the nucleus of the GFAP-positive cells. *G*, Control of the anti-C/EBPδ antibody specificity. Preincubation of the antibody against C/EBPδ with the peptide corresponding to C/EBPβ did not cause a loss of immunoreactivity in the nucleus of GFAP-positive cells. *H*, Control for nonspecific fluorescein labeling with the antibody against C/EBPδ. Neutralization of anti-C/EBPδ antibody with the peptide against which it has been raised caused a total loss of nuclear staining. Scale bar, 50 μm.

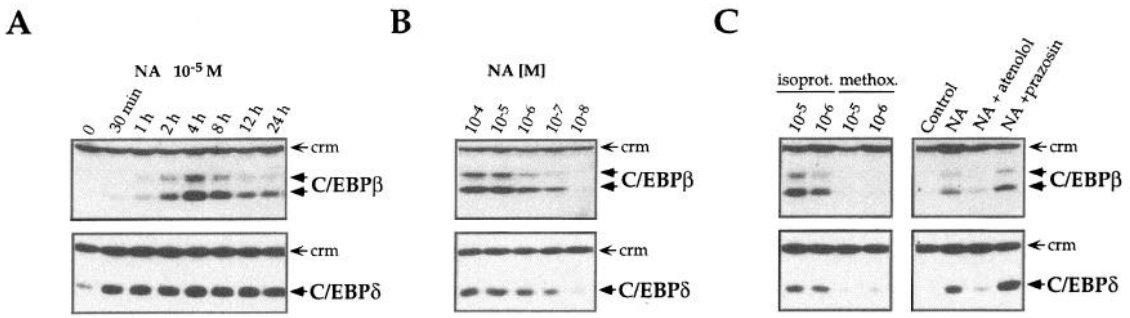

Figure 6. Induction of C/EBPβ and -δ by NA in cultures of mouse cortical astrocytes is mediated by receptors of the β-subtype. *A*, Cultures of cortical astrocytes were incubated for various periods of time (indicated at the *top* of each lane) in the presence of 10^{-5} M NA. Equal amounts of total protein extracts (50 μg) were analyzed by 12% SDS-PAGE, and C/EBPβ or -δ were detected by Western immunoblot analysis. Bands corresponding to C/EBPβ or -δ are indicated by *arrows* at the *right*. C/EBP-unrelated cross-reacting material (*crm*) (Cao et al., 1991) served as a loading control. *B*, Effect of NA concentration on the expression of C/EBPβ and -δ. Proteins were collected after a 4 hr stimulation in the presence of the indicated concentration of NA and were analyzed as described in *A*. *C*, Pharmacological characterization of the induction of C/EBPβ and -δ by NA. Proteins were collected after a 4 hr stimulation in the presence of the β-adrenergic agonist isoproterenol (*isoprot.*) or the α_1-adrenergic agonist methoxamine (*methox.*) at concentrations indicated at the *top* of each lane (panels on the *left*). The adrenergic receptor subtype implicated in the C/EBPβ and -δ induction by NA was confirmed further by exposing the cells for 10 min to the β_1-adrenergic antagonist atenolol (10^{-5} M) or to the α_1-adrenergic antagonist prazosin (10^{-5} M) before the addition of NA (10^{-6} M; panels on the *right*). Western immunoblotting was performed as described in *A*. Similar results were obtained in at least three independent experiments.

transcription factors that could synergize with the overexpressed LAP or C/EBPδ to stimulate glycogen resynthesis maximally. Moreover, we decided to expose transfected astrocytes to NA for 6 hr to measure glycogen levels when the rate of resynthesis is high (Sorg and Magistretti, 1992). A set of cultures was used to determine glycogen levels (Fig. 9*A*), and another set was used to monitor the overexpression of LAP, LIP, or C/EBPδ by Western immunoblot analysis (Fig. 9*B*). Although the observed increase over basal glycogen levels was not proportional to the LAP or C/EBPδ level, the overexpression of LAP or C/EBPδ significantly and reproducibly increased the NA-induced glycogen resynthesis of the transfected astrocytes compared with astrocytes transfected with the control vector. Moreover, the overexpression of LIP reduced the NA-induced glycogen resynthesis. These results suggest that C/EBPδ and the transcriptionally active form of C/EBPβ play a role in the glycogen resynthesis triggered by NA.

DISCUSSION

Results reported in the present study indicate that in mouse primary cortical astrocytes the level of expression of the transcription factors C/EBPβ and -δ is regulated by the neurotransmitters VIP, PACAP27, or NA, which stimulate the accumulation of cAMP in these cells. C/EBPβ and -δ are induced by a cAMP-dependent mechanism, because the cAMP analog 8-(4Br)-cAMP and the adenylate cyclase activator forskolin also increase these C/EBP isoforms. *c/ebpβ* and *c/ebpδ* can be considered to be cAMP-inducible immediate-early genes in mouse astrocytes, because their induction by VIP still occurs in the presence of the

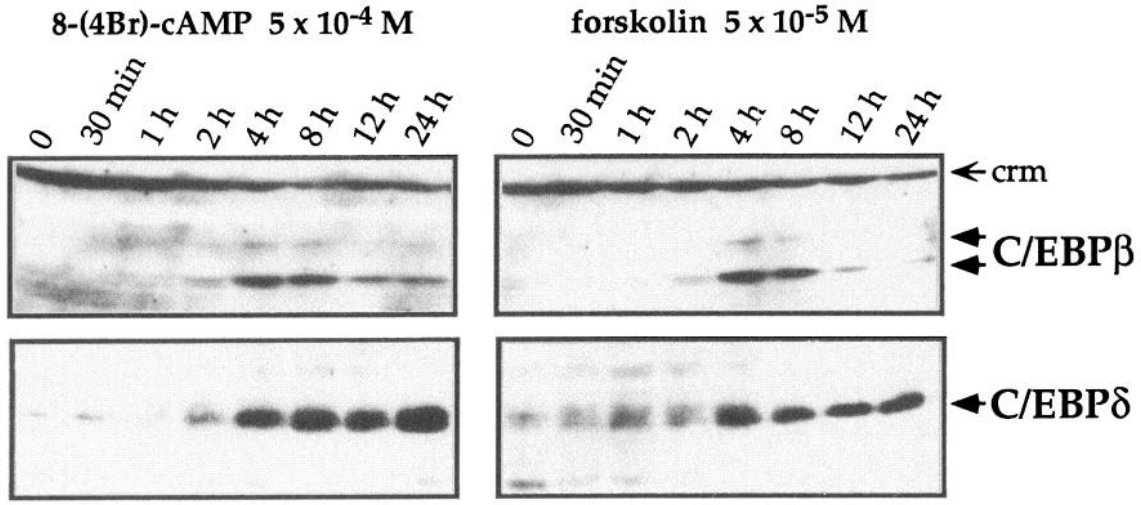

Figure 7. Induction of C/EBPβ or -δ expression via the cAMP second-messenger pathway. Proteins were collected after a 4 hr stimulation in the presence of the cAMP analog 8-(4Br)-cAMP at 5×10^{-4} M or the adenylate cyclase activator forskolin at 5×10^{-5} M. Equal amounts of total protein extracts (50 μg) were analyzed by 12% SDS-PAGE, and C/EBPβ or -δ were detected by Western immunoblot analysis. In this experiment, C/EBPδ was detected with an affinity-purified rabbit polyclonal antibody (1:500, Santa Cruz Biotechnology). Bands corresponding to C/EBPβ or -δ are indicated by *arrows* at the *right*. C/EBPβ-unrelated cross-reacting material (*crm*) (Cao et al., 1991) served as a loading control. Similar results were obtained in at least three independent experiments.

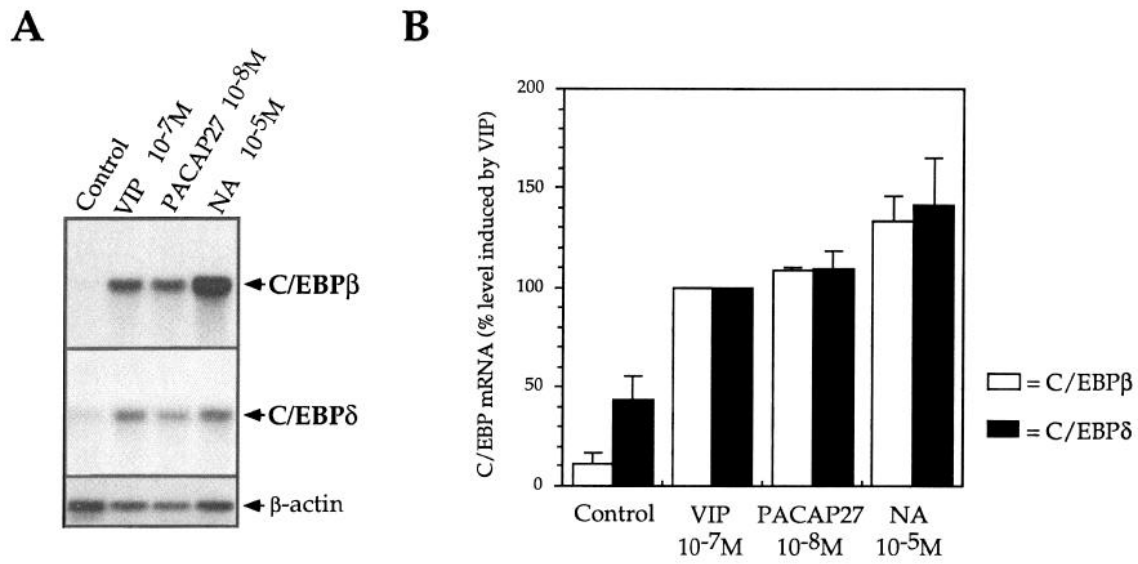

Figure 8. Comparison of the induction of C/EBPβ and -δ mRNA 2 hr after exposure to VIP, PACAP27, or NA. *A*, Cultures of cortical astrocytes were exposed for 2 hr to VIP (10^{-7} M), PACAP27 (10^{-8} M), or NA (10^{-5} M). Equal amounts of total RNA (20 μg) were electrophoresed and analyzed by Northern blotting. Similar results were obtained in three independent experiments. The blots were hybridized sequentially with ^{32}P riboprobes complementary to C/EBPβ, C/EBPδ, and β-actin mRNAs; the latter was used as a loading control. *B*, Results in *A* were quantified by scanning densitometry and are expressed as mean ± SEM (n = 3) compared with C/EBP mRNA levels evoked by VIP.

protein synthesis inhibitor anisomycin. Moreover, this study provides evidence for a role of C/EBPβ and -δ in the glycogen resynthesis elicited by VIP or NA. This suggests that *c/ebpβ* and *c/ebpδ* are the primary target genes of VIP or NA and that these C/EBP isoforms then activate late genes, encoding for enzymes implicated in glycogen synthesis. If this hypothesis is true, the induction of C/EBP isoforms by VIP or NA would represent an intermediate step between the cAMP-dependent short-term (Sorg

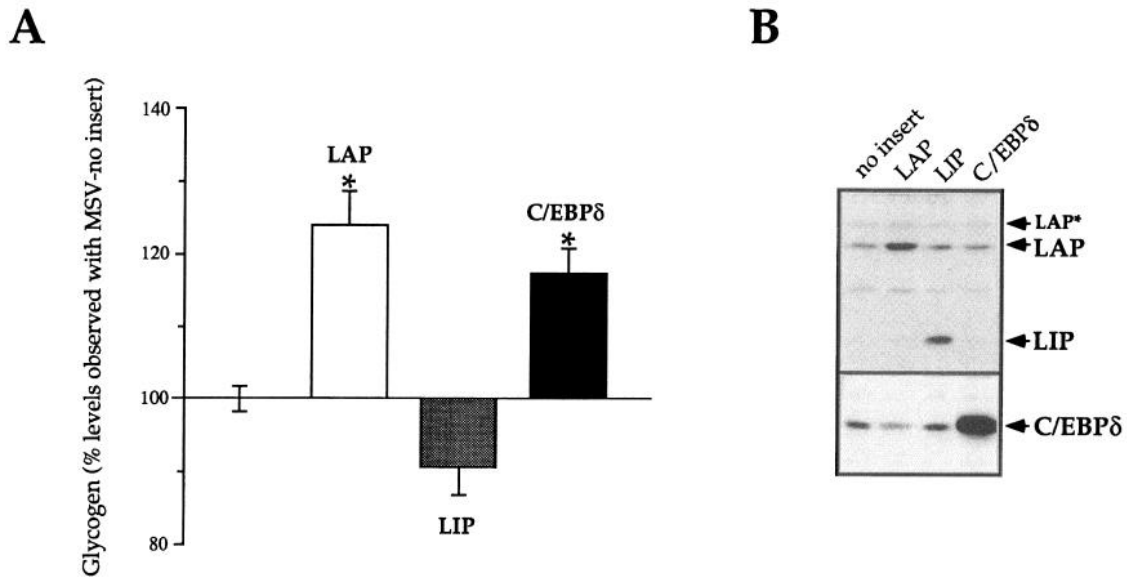

Figure 9. Effect of the transfection of mouse cortical astrocytes with LAP, LIP, or C/EBPδ expression vectors on NA-mediated glycogen resynthesis. Cultures of cortical astrocytes were transfected transiently using the cationic lipid DOTAP with the MSV-no insert control plasmid (*no insert*) or with MSV-LAP, MSV-LIP, or MSV-C/EBPδ expression vectors. Three days after transfection, astrocytes were incubated in the presence of 10^{-5} M NA, and glycogen levels were assayed 6 hr later. *A*, Results are expressed as the percent glycogen level in astrocytes transfected with the MSV-no insert control plasmid, which was 347.6 ± 9.7 nmol of glycogen/mg of protein. Results are expressed as mean ± SEM of six separate determinations from two experiments, repeated twice with similar results. *Asterisks* indicate significant difference from MSV-no insert by ANOVA followed by Dunnett's test ($p < 0.01$). The *error bar* at the *left* represents SEM of MSV-no insert. *B*, Western blot monitoring the expression of LAP, LIP, or C/EBPδ in astrocytes transiently transfected with MSV-no insert, MSV-LAP, MSV-LIP, or MSV-C/EBPδ. Parallel astrocyte cultures treated as in *A* were analyzed by Western immunoblot to detect C/EBPβ (LAP or LIP) and C/EBPδ isoforms. Equal amounts of total protein extracts (50 μg) were separated by 12% SDS-PAGE. The bands identified as *LAP** and *LAP* correspond to the proteins of 39 and 36 kDa, respectively, designated C/EBPβ in the other figures. C/EBPδ was detected with an affinity-purified rabbit polyclonal antibody (1:500, Santa Cruz Biotechnology). Note that the chemiluminescent blots were exposed for a brief period to avoid saturation of the signal for the overexpressed LAP, LIP, and C/EBPδ. Consequently, the signal of C/EBPβ and -δ was reduced considerably in the other lanes.

and Magistretti, 1991) and long-term (Sorg and Magistretti, 1992) effects of both neurotransmitters on glycogen metabolism.

This study demonstrates a regulation of C/EBP transcription factors by neurotransmitters in cells of the vertebrate CNS. The presence of C/EBP isoforms in the mammalian brain has not been established clearly. Northern blot analysis of total RNA from mouse brain failed to reveal the presence of C/EBPα, -β, or -δ mRNA (Cao et al., 1991), whereas *in situ* hybridization experiments localized C/EBPα mRNA in adult mouse brain, where it was concentrated primarily in the hippocampus, cerebellar Purkinje cells, and layers II and III of the cortex (Kuo et al., 1990). Such analysis has not been performed for C/EBPβ or -δ mRNAs. However, the human homolog of C/EBPβ has been identified in a glioblastoma cell line (Akira et al., 1990). Directly relevant to the present study is the report by Kageyama et al. (1991) who, while attempting to clone rat brain cDNAs on the basis of their ability to bind the cAMP-responsive element (CRE) of the substance P and K precursor gene promoter, cloned in fact the rat homolog of C/EBPδ, which they termed C/EBP-like factor (or CELF). In this latter study, C/EBPδ mRNA could be detected in rat whole brain by Northern blot analysis performed with polyA$^+$ RNA. C/EBPβ is regulated by the cAMP second-messenger pathway also in the rat PC12 cell line (Metz and Ziff, 1991). The activity, as well as the expression, of C/EBP family members is also regulated by cAMP. Thus, the cAMP-stimulated phosphorylation of C/EBPβ is associated with its translocation to the nucleus, in which it can induce transcription of the cellular proto-oncogene c-*fos* via interactions with the c-*fos* serum response element. A *c/ebp* gene was shown recently to be activated by stimulation of serotonin receptors, which are coupled to the cAMP pathway in sensory neurons of the marine snail *Aplysia* (Alberini et al., 1994) (for review, see Stevens and Verma, 1994). Serotonin, a neurotransmitter released from neurons involved in a simple form of learning called sensitization, rapidly induced *Aplysia* C/EBP (ApC/EBP), even in the presence of protein synthesis inhibitors. Moreover, ApC/EBP plays a crucial role in the consolidation of stable, long-term synaptic plasticity in *Aplysia*. The induction of C/EBPβ and -δ by VIP, PACAP27, or NA in mouse cortical astrocytes reported in this study shares a number of similarities with the induction of ApC/EBP by serotonin in *Aplysia* sensory neurons. Thus, ApC/EBP mRNA is induced by 8-(4Br)-cAMP or forskolin, and its induction by serotonin is not blocked by anisomycin (Alberini et al., 1994). It is of interest that regulatory mechanisms of C/EBP family members have been conserved during evolution from neurons in a simple organism like *Aplysia* to glial cells in a mammalian brain.

In this study, we also show that the overexpression of C/EBPδ and LAP significantly increases the NA-induced glycogen accumulation of transfected astrocytes. However, the increase in glycogen is not proportional to the increase in C/EBPδ or LAP. This is not surprising, because it is well known that transient transfection of expression vectors causes a small percentage of productively transfected cells. For instance, the transfection of hepatoma (HepG2) cells with the expression vector MSV-C/EBPα leads to the appearance of C/EBPα overexpression in only 1% of the cells (Friedman et al., 1989). This observation also applies to the mouse primary cultures of astrocytes transfected with MSV-C/EBPδ or MSV-LAP, because we observed by immunocytochemistry only a small percentage (3–5%) of cells expressing high levels of C/EBPδ or LAP (data not shown). Therefore, the increase in glycogen levels in productively transfected astrocytes would be reflected only marginally in total glycogen levels. Moreover, C/EBPδ and LAP likely are not the only limiting transcription factors of glycogen synthesis, and their overproduction cannot increase glycogen levels proportionally if the other limiting factors are not expressed to the same extent as C/EBPδ or LAP. One such transcription factor could be activator protein-1, because the c-*fos* gene is induced by VIP or NA in cultured astrocytes (Arenander et al., 1989; Martin et al., 1992). In view of these considerations, the significant increase in glycogen levels after transfection of astrocytes with the C/EBPδ or LAP expression vector strongly supports an involvement of C/EBPβ and -δ in the transcriptionally regulated cAMP-dependent resynthesis of glycogen evoked by VIP and NA.

Which could be the target genes of C/EBP isoforms, the induction of which would lead to an increase in glycogen levels? A number of enzymes are potential candidates for such regulation. For example, transcription of the glycogen synthase gene might be enhanced by VIP or NA via the activation of *c/ebpβ* or *c/ebpδ* genes. The glycogen synthase cDNA from mouse astrocytes has been cloned recently in our laboratory, and the level of expression of its mRNA indeed is increased by VIP or NA (Pellegri et al., in press). The promoter of the glycogen synthase gene has not been studied and, therefore, it is not known whether it contains C/EBP-binding sites. However, very recently an impaired-energy homeostasis in C/EBPα knock-out mice has been reported (Wang et al., 1995). Of interest, mice homozygous for the targeted deletion of the *c/ebpα* gene did not store hepatic glycogen and died from hypoglycemia within 8 hr after birth. In these mutant mice, the amounts of glycogen synthase mRNA were 30–50% of normal, which strongly suggests that this gene is regulated by C/EBP isoforms. Glycogen synthesis could be controlled also by the availability of glucose-6-phosphate, which is regulated by the sequential activity of the glucose transporter and hexokinase. There are several glucose transporters and several isoforms of hexokinase. As for glycogen synthase, the promoter of the brain isotypes of these proteins has not been studied. However, C/EBP might play a role in the transcriptional regulation of these genes, because C/EBP-binding sites were observed in the promoter of the insulin-responsive glucose transporter gene (Kaestner et al., 1990), whereas in the liver the dynamics of the pattern of expression of C/EBPα mRNA were virtually identical to that of the hexokinase IV (glucokinase) mRNA (Moorman et al., 1991). It is conceivable, therefore, that VIP or NA, by inducing C/EBPβ and -δ in mouse cortical astrocytes, increases the transcription of the glucose transporter and/or the hexokinase genes, leading to a stimulation of glycogen synthesis.

In addition to the involvement of C/EBP isoforms in the glycogen resynthesis elicited by VIP or NA, this study provides evidence that neurotransmitters can influence gene transcription in astrocytes, therefore demonstrating yet another level of regulation in neuron–glia interactions. Moreover, as noted in the introductory remarks, the C/EBP transcription factor family plays several well documented roles in peripheral tissue, which provide useful clues to define the functions of these transcription factors in the CNS.

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