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Regulation of expression of cAMP response element-binding protein in the locus coeruleus *in vivo* and in a locus coeruleus-like cell line *in vitro*

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ABSTRACT **Expression of the cAMP response element (CRE)-binding protein (CREB) has been thought to be constitutive and not subject to regulation. In the course of investigating effects of chronic morphine on the cAMP pathway in the locus coeruleus, a brain region important for opiate addiction, we found that levels of CREB immunoreactivity and CRE binding were increased by chronic morphine administration. To further investigate possible mechanisms underlying this unexpected finding, we studied the regulation of CREB expression in a cell line (CATH.a) that exhibits many properties of locus coeruleus neurons. Agents that activate the cAMP pathway led to a >60% decrease in CREB mRNA in this cell line. Moreover, these alterations in CREB mRNA levels were associated with changes in levels of CREB immunoreactivity and CRE-binding activity. In contrast, the same treatments fail to alter CREB expression in PC12 pheochromocytoma cells.**

Studies of regulation of gene expression by the cAMP intracellular signaling pathway have focused on the cAMP response element (CRE)-binding protein (CREB) and related proteins. These proteins belong to a family of leucine zipper transcription factors that share certain structural motifs, bind DNA as dimers, and regulate transcription of specific genes. CREB is a 43-kDa protein that binds to specific DNA sequences termed CREs, found in the promoters of many neurally expressed genes (1, 2, 4–6). CREB can activate transcription when phosphorylated at Ser-133 by cAMP-dependent protein kinase (PKA) or Ca^{2+}/calmodulin-dependent kinases (7, 8). It is generally believed that the CREB gene is constitutively expressed and not subject to regulation (7, 9, 10).

Evidence is now accumulating for a role of CREB in the brain. For example, CREB phosphorylation has been shown to be important in pituitary proliferation and in the setting of circadian rhythms (11, 12). CREB may also have a role in mediating morphine action in the locus coeruleus (LC). The LC is the major noradrenergic nucleus in brain and is important in physical opiate dependence and withdrawal (13–15). Acute morphine exposure decreases cAMP levels in the LC and reduces CREB phosphorylation, whereas chronic morphine upregulates the constituent proteins of the cAMP pathway and increases CREB phosphorylation (15, 16). This regulation of the cAMP pathway has been shown to contribute to the electrophysiological actions of acute and chronic morphine in these neurons (3, 15). Regulation of CREB and subsequent changes in gene expression may underlie some of the long-term effects of opiates on LC neurons (17). In the course of studies aimed at further understanding morphine regulation of CREB function, we observed that chronic morphine administration increased total levels of CREB immunoreactivity, as well as CRE-binding activity, in the LC.

To examine in greater detail the mechanisms underlying this regulation of CREB expression in the LC, a continuous, "LC-like" cell line, CATH.a, was used. Suri *et al.* (18) developed this cell line from a brainstem tumor of a transgenic mouse expressing the simian virus 40 T antigen under the control of the tyrosine hydroxylase promoter. These cells exhibit a neural, noradrenergic phenotype: they express neurofilaments and synaptophysin and lack glial fibrillary acidic protein, express the enzymes tyrosine hydroxylase and dopamine β-hydroxylase, and synthesize norepinephrine. In addition, the CATH.a cells resemble LC neurons in their signal transduction profile. Adenylyl cyclase is stimulated in the cells by vasoactive intestinal peptide (VIP) and corticotropin-releasing factor but is inhibited by μ-opiate agonists, neuropeptide Y, γ-aminobutyric acid B agonists, and α_2-adrenergic agonists (19). We now show that agents that activate the cAMP pathway decrease CREB mRNA levels in the CATH.a cells. Further analysis reveals that these alterations in mRNA levels are associated with changes in CREB protein levels and CRE-binding activity.

EXPERIMENTAL PROCEDURES

***In Vivo* Morphine Treatment and LC Dissection.** Male Sprague–Dawley rats (initial weight, 140–160 g) were used in this study. Chronic morphine treatment involved the subcutaneous implantation of morphine pellets (containing 75 mg of morphine base; National Institute on Drug Abuse) daily for 5 days, with rats sacrificed on day 6. This treatment results in profound states of tolerance, dependence, and withdrawal (16). Control rats received placebo pellet implants. Acute morphine treatment involved the subcutaneous injection of morphine sulfate in 0.9% saline (10 mg/kg; National Institute on Drug Abuse), with rats sacrificed 2 hr following injection, when some acute biochemical changes are maximal (20). Control rats received saline injections. LC nuclei from control and drug-treated rats were excised from 0.75-mm-thick coronal cross sections of brainstem by use of a 15-gauge syringe needle (20). LC nuclei obtained from two rats were pooled. It has been estimated that these dissections are 75% LC neurons. Frontal cortex was isolated by gross dissection.

Cell Culture. CATH.a cells were a generous gift of D. M. Chikaraishi (Tufts University) and were cultured in RPMI 1640 medium with 4% fetal bovine serum and 8% horse serum (GIBCO). For serum-free cultures, RPMI 1640 was supplemented with 1% bovine serum albumin and 20 mM Hepes (pH 7.6). PC12 cells were provided by P. DeCamilli (Yale University) and were grown in RPMI 1640 medium with 10%

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Abbreviations: CRE, cAMP response element; CREB, CRE-binding protein; LC, locus coeruleus; PKA, cAMP-dependent protein kinase; VIP, vasoactive intestinal peptide.

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horse serum and 5% fetal bovine serum. All culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml). In all experiments, 10^7 cells were passaged into 100-mm culture dishes. Media were changed 24 hr after passage and cells were treated with drug 48 hr after passage.

Preparation of Brain and Cell Extracts and Immunoblot Analysis. After drug treatment, cells were washed twice with phosphate-buffered saline (PBS), scraped into 20 mM Hepes, pH 7.9/0.4 M NaCl/20% (vol/vol) glycerol/5 mM $MgCl_2$/0.5 mM EDTA/0.1 mM EGTA, 0.1 mM *p*-aminobenzamidine/1% (vol/vol)-Nonidet P-40/1 mM phenylmethanesulfonyl fluoride/5 mM dithiothreitol/1 μM okadaic acid containing pepstatin (1 μg/ml) and leupeptin (10 μg/ml), and sonicated for 15 sec. Brain samples were Dounce homogenized in the same solution (1–2 mg of wet weight per 0.1 ml). Cell and brain homogenates were then incubated on ice for 25 min and centrifuged at 15,000 × *g* for 25 min at 4°C. The supernatant was removed, and SDS was added (1%) to those samples that were immunoblotted. In some experiments, nuclear extracts were obtained (21, 22). These yielded results equivalent to those obtained with whole cell extracts.

Aliquots of cell extracts (60 μg of protein) and brain extracts (90 μg) were subjected to SDS/PAGE (22). After electrophoretic transfer to nitrocellulose, proteins were immunolabeled with an anti-CREB antiserum (1:20,000) or an affinity-purified anti-phospho-CREB antibody (1:5000). The anti-CREB antiserum, raised against a TrpE–CREB fusion protein, and the anti-phospho-CREB antibody, raised against a phosphopeptide corresponding to CREB aa 123–136 and adsorbed with an unphosphorylated peptide, were kindly provided by David Ginty and Michael Greenberg (Harvard Medical School). The anti-CREB antiserum is specific for CREB and does not recognize other family members on immunoblots of PC12 cells (11). The conclusion that the 43-kDa protein present in CATH.a extracts is CREB is based on the finding that this protein comigrated on SDS/PAGE gels with the PC12 CREB. Moreover, in some experiments, labeling was done with the anti-CREB antiserum that had been preadsorbed with TrpE–CREB fusion protein for 6 hr at 4°C. The blots were incubated with a 1:4000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Laboratories), developed with the enhanced chemiluminescence (ECL) system of Amersham, and exposed to Hyperfilm-ECL (Amersham) for 1–2 min. Bands on the autoradiogram were analyzed with a GSXL laser densitometer. All blots were stained with amido black to confirm equal loading of protein.

Gel Shift Assays. For gel shift assays (22), the CRE probe was a double-stranded synthetic oligonucleotide (GATCGGC-TGACGTCAGAG; bold lettering denotes palindromic consensus sequence) derived from the promoter sequence of the somatostatin gene. The probe was labeled with [α-^{32}P]dGTP and [α-^{32}P]dTTP to a specific activity of 4–9 × 10^8 cpm/μg by the Klenow DNA polymerase fill-in reaction (all [^{32}P]dNTPs and -rNTPs were from NEN). For competition assays, 0, 3, 10, 30, and 100 ng of competitor DNA were added to the gel-shift reaction mixture 20 min before the labeled probe. The sequences of the mutated CRE and the human metallothionein AP-1 oligonucleotides were, respectively, GATCGGCT-GACGTCTGAG and TCGACGGGACTCAGCGCGC. For supershift experiments, 1 μl of the anti-CREB antiserum was incubated with the gel-shift reaction components for 30 min at 4°C before addition of the probe.

Preparation of RNA and Northern Analysis. After drug treatment, the cells were washed twice with PBS and lysed in 4 M guanidinium isothiocyanate. Total RNA was isolated by centrifugation on a CsCl gradient (23). RNA concentrations were measured by optical density, and 20-μg aliquots of total RNA were electrophoresed in a formaldehyde/1.2% agarose gel and transferred to nylon membranes (Biotrans, ICN) by electroblotting. Quality and quantity of RNA were confirmed by staining the blot with 0.2% methylene blue. Hybridizations were performed either with an [α-^{32}P]dATP-random-prime-labeled cyclophilin fragment at 42°C or an [α-^{32}P]rCTP-labeled CREB riboprobe (Promega kit) at 65°C in 50% formamide/750 mM NaCl/50 mM phosphate, pH 7.0/5 mM EDTA/5× Denhardt's solution/0.02% salmon sperm DNA/0.1% SDS. The plasmids containing the full-length cyclophilin (24) and CREB cDNAs were provided by S. E. Hyman (Harvard Medical School). The membranes were washed and subjected to autoradiography; bands on autoradiograms were analyzed with a GSXL laser densitometer.

RESULTS

Regulation of CREB Protein Expression and CRE Binding in the LC *in Vivo*. Immunoblot analysis revealed a low level of CREB immunoreactivity in the LC of control rats (Fig. 1*A*). Reaction with the identified 43-kDa band corresponding to CREB was selectively and completely blocked when the anti-CREB antiserum was preadsorbed with TrpE–CREB (Fig. 1*A Lower*). CREB immunoreactivity was increased by chronic morphine treatment (Fig. 1*A*). Chronic morphine also increased CRE-binding activity in the LC (Fig. 1*B*). The specificity of this CRE-binding activity was demonstrated by competition studies with unlabeled oligonucleotides (data not shown; see Fig. 4). Acute (2-hr) morphine treatment did not affect CREB immunoreactivity or CRE-binding activity (data not shown). In contrast to the LC, frontal cortex showed no change in levels of CREB immunoreactivity and CRE-binding activity after chronic morphine treatment (Fig. 1).

Regulation of CREB mRNA Expression in CATH.a Cells. The ability to regulate CREB levels *in vivo* was explored further in cultured CATH.a cells. The effect of perturbing the cAMP system on CREB expression in CATH.a cells was examined by treating the cells with forskolin, a diterpene that activates adenylyl cyclase. Northern blots of total RNA from CATH.a cells probed with the radiolabeled riboprobe for CREB revealed a single mRNA of ≈7 kb. Forskolin induced a time-dependent decrease in CREB mRNA, standardized to cyclophilin mRNA levels; after 2 hr, a 60% reduction was observed compared with control (Fig. 2). CREB mRNA levels remained reduced for up to 24 hr (data not shown).

Because forskolin has many effects other than activation of adenylyl cyclase, cells were also incubated with 1,9-

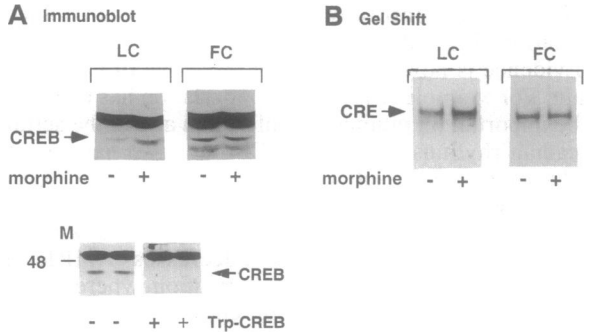

FIG. 1. Effect of chronic morphine administration on CREB (*A*) and CRE-binding activity (*B*) in rat LC. LC and frontal cortex (FC) from rats after chronic (5-day) administration of placebo or morphine pellets were analyzed by CREB immunoblotting. (*A Upper*) Representative autoradiograms illustrate morphine regulation of CREB immunoreactivity in the LC and lack of morphine regulation in the FC. This regulation in the LC was observed consistently among 11 control and morphine-treated samples (each derived from two rats). (*A Lower*) Specificity of the anti-CREB antiserum for the brain 43-kDa CREB as evidenced by the selective blockade of binding to the protein by preadsorbing the antiserum with Trp–CREB. M, molecular size standard, in kilodaltons. (*B*) Gel shift analysis of morphine regulation of CRE binding activity.

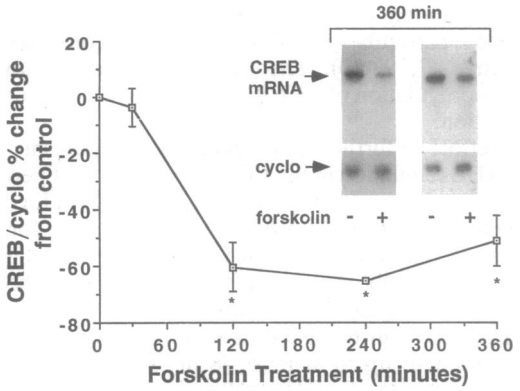

FIG. 2. Effect of forskolin on CREB mRNA in CATH.a cells. CATH.a cells were harvested 0, 0.5, 2, 4, or 6 hr after incubation with vehicle or 5 μM forskolin and analyzed for CREB and cyclophilin (cyclo) mRNA by Northern blotting. Representative autoradiograms are presented (*Inset*). The graph summarizes the time-dependent forskolin regulation of CREB mRNA in the CATH.a cells. Data are standardized to cyclophilin mRNA and expressed as percent change from control (mean ± SEM). Each experiment ($n = 6$ per time point) has been repeated two to four times with similar results. *, $P < 0.05$ by unpaired Student's t test.

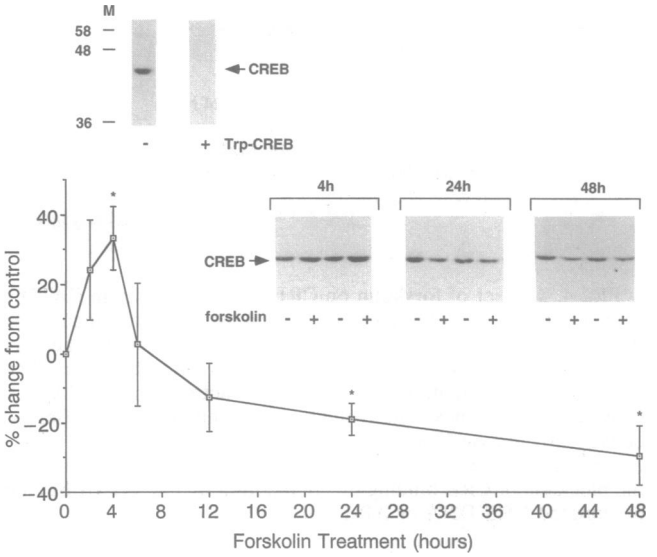

FIG. 3. Effect of forskolin on CREB in CATH.a cells. CATH.a cells were harvested 0, 2, 4, 6, 12, 24, or 48 hr after incubation with vehicle or 5 μM forskolin and analyzed by CREB immunoblotting. (*Upper*) Autoradiogram demonstrating the specificity of the anti-CREB antiserum for the 43-kDa CREB in CATH.a cells as evidenced by the blockade of binding to the protein by preadsorbing the antiserum with Trp–CREB. M, molecular size standards, in kilodaltons. (*Lower*) Representative autoradiograms of CREB immunoreactivity in the CATH.a cells are presented (*Inset*). The graph summarizes the time-dependent forskolin regulation of CREB immunoreactivity in CATH.a cells. Data are expressed as percent change from control (mean ± SEM). Each experiment ($n = 6$ per time point) has been repeated two to four times with similar results. *, $P < 0.05$ by unpaired Student's t test.

dideoxyforskolin, which does not activate adenylyl cyclase but exerts many of the other effects of forskolin (25). In contrast to forskolin, 1,9-dideoxyforskolin did not alter CREB mRNA levels in the CATH.a cells (94 ± 30% of control, $n = 6$). To further support a role of cAMP in CREB regulation, we examined CREB expression following incubation with VIP, which activates adenylyl cyclase in these cells (19), as it does in many tissues. VIP treatment for 4 hr induced a 33 ± 4% decrease in CREB mRNA (mean ± SEM, $n = 6$; $P < 0.05$ by unpaired Student's t test).

Cellular differentiation regulates levels of CREB-like proteins in F9 embryonal carcinoma cells (26). To test the possibility that CREB mRNA regulation in CATH.a cells might be a function of the modest cellular differentiation that occurs during forskolin or VIP treatment, the cells were incubated for 24 hr in serum-free medium, which induced the cells to extend small, neurite-like processes. This morphology of the cells was similar to that seen following 2–6 hr with forskolin or VIP. Serum-free medium did not affect CREB mRNA levels under basal conditions (−9 ± 4% change from serum-containing medium), nor did it influence the ability of a 2-hr forskolin treatment to decrease CREB expression (−46 ± 16% change from control; mean ± SEM; $n = 6$; $P < 0.05$ by unpaired Student's t test).

Regulation of CREB Protein Expression and CRE Binding in CATH.a Cells. Forskolin and VIP regulation of CREB mRNA expression in CATH.a cells suggested that total levels of CREB in these cells might also be regulated. Immunolabeling of total cell or nuclear extracts with an anti-CREB antiserum revealed much higher levels of CREB compared with those in brain. Forskolin treatment led to an initial increase in CREB immunoreactivity, to 35% above control values (Fig. 3 *Lower*). This increase peaked at 4 hr of forskolin treatment, and by 6 hr was largely attenuated. Longer exposure to forskolin (12–48 hr) resulted in a progressive decrease in CREB immunoreactivity, to 30% below control values at 48 hr. There was no observable change in levels of CREB immunoreactivity in control cultures over the time of these experiments. VIP treatment resulted in a similar short-term increase in CREB immunoreactivity, so that after 2 hr there was a 33 ± 9% increase in CREB from control (mean ± SEM; $n = 6$; $P < 0.05$ by t test). Both VIP and forskolin treatments were also associated with increases in CREB phosphorylation, as determined by immunoblotting with an anti-phospho-CREB antibody (data not shown). However, the alterations in signal intensity of "total" CREB shown in Fig. 3 cannot be explained by different immunoreactivity to phospho- and dephospho-CREB, because the anti-CREB antiserum used recognizes both forms equally (11). We confirmed this by phosphorylating nuclear extracts of CATH.a cells with purified PKA (16). Although such treatment resulted in a >10-fold increase in phospho-CREB immunoreactivity, there was no detectable change in total CREB immunoreactivity (data not shown).

To investigate the functional significance of alterations in CREB immunoreactivity, CRE-binding analysis was performed. Treatment with forskolin for 30 min, which did not alter CREB levels, also did not alter CRE-binding activity (Fig. 4 *Upper*). However, at time points at which forskolin led to a change in total CREB immunoreactivity, there was a corresponding change in CRE-binding activity: 4 hr of forskolin treatment significantly increased CRE-binding activity, whereas 24 hr of forskolin significantly decreased CRE-binding activity. The demonstrated CRE-binding activity was specific: it was blocked by unlabeled CRE DNA competitor but not by mutated CRE DNA or AP-1 DNA (Fig. 4 *Lower*). In addition, this band was specifically "supershifted" by the anti-CREB antiserum (data not shown).

Lack of Regulation of CREB Expression in PC12 Cells. Forskolin regulation of CREB mRNA and protein levels was also studied in PC12 cells, a neural crest-derived cell line used in many studies of CREB function. Previous studies have reported that CREB protein expression is not altered in PC12 cells up to 4 hr after forskolin treatment (9). CREB mRNA expression following perturbation of the cAMP system had not been studied directly in PC12 cells. Forskolin did not regulate CREB mRNA or protein levels in PC12 cells (Fig. 5), in contrast to CATH.a cells. Forskolin did stimulate CREB

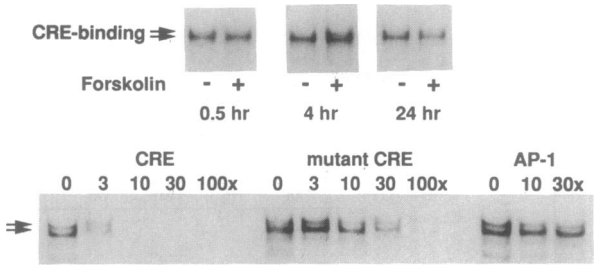

FIG. 4. Effect of forskolin on CRE-binding activity in CATH.a cells. CATH.a cells were harvested 0.5, 4, or 24 hr after incubation with vehicle or 5 μM forskolin and analyzed for CRE-binding activity by gel shift analysis. (*Upper*) Representative autoradiograms illustrate forskolin regulation of CRE-binding activity in the CATH.a cells at selected times. The regulation at 4 and 24 hr was observed consistently among 12 control and forskolin-treated samples. (*Lower*) Specificity of the CATH.a CRE-binding activity (as evidenced by the blockade of CRE binding by competition by CRE DNA but not by mutated CRE DNA or AP-1 DNA).

phosphorylation in PC12 cells (data not shown), consistent with previous reports. This finding provides further evidence that alterations in phospho-CREB *per se* do not alter levels of total CREB immunoreactivity under the experimental conditions used.

DISCUSSION

There is a growing body of evidence that chronic morphine administration upregulates the cAMP second-messenger and protein phosphorylation pathways in the LC, but not in several other brain regions, including frontal cortex (15, 17). Chronic morphine increases expression of certain G-protein α subunits, adenylyl cyclase, PKA, and a number of specific substrates for PKA in the LC. Electrophysiological and behavioral experiments have provided direct evidence for a role of the upregulated cAMP pathway in mediating aspects of opiate tolerance, dependence, and withdrawal in LC neurons (15, 17). In an effort to understand the molecular mechanisms by which morphine upregulates the cAMP pathway in the LC, we have studied morphine regulation of several transcription factors, including CREB. In addition to regulating CREB phosphorylation (16), as would be expected given morphine regulation of the cAMP pathway, chronic (but not acute) morphine administration was found in the present study to increase total levels of CREB immunoreactivity, an effect seen in the LC but not in the frontal cortex. The increase in CREB immunoreactivity in the LC was associated with an equivalent increase in CRE binding.

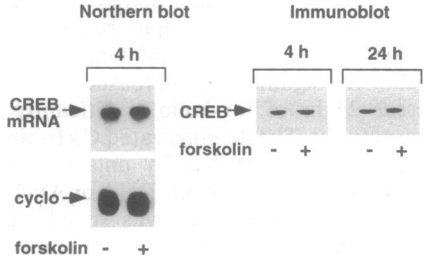

FIG. 5. Lack of effect of forskolin on CREB mRNA and protein in PC12 cells. PC12 cells were harvested 4 or 24 hr after incubation with vehicle or 5 μM forskolin and were analyzed for CREB and cyclophilin mRNA by Northern blotting (*Left*) and for CREB immunoreactivity by immunoblotting (*Right*). Forskolin had no effect on CREB mRNA as standardized to cyclophilin (cyclo) mRNA (−6 ± 2% change from control) or on CREB immunoreactivity at 4 and 24 hr (−8 ± 7% and 10 ± 5% change from control, respectively). $n = 6$; each experiment was repeated two to four times with similar results.

CREB regulation in the LC is surprising because CREB expression, particularly in brain, is generally considered to be constitutive (7, 9, 10). The functional role of morphine-induced upregulation of CREB in the LC now warrants direct investigation.

In an effort to further understand the mechanisms underlying CREB regulation in the LC *in vivo*, effects of perturbing the cAMP pathway on CREB in the LC-like CATH.a cell line were studied. CREB mRNA was decreased by forskolin, whereas the forskolin effect on CREB levels was biphasic. CREB was initially increased by forskolin but showed a progressive decrease thereafter. The decrease in CREB mRNA and protein persisted as long as forskolin exposure was maintained, up to at least 48 hr. Induction of modest differentiation in the CATH.a cells with serum-free medium, similar to that seen with forskolin, had no effect on basal or forskolin-regulated CREB expression. The findings that VIP (which activates adenylyl cyclase) mimics forskolin regulation of CREB expression, whereas 1,9-dideoxyforskolin (which does not activate adenylyl cyclase) does not, support the conclusion that cAMP is a likely mediator of these effects. CRE-binding experiments suggest that the observed alterations in CREB protein levels may be associated with altered function of this transcription factor. Thus, binding to the CRE probe (derived from the somatostatin promoter) was increased after 4 hr of forskolin treatment and decreased after 24–48 hr of forskolin treatment. Since binding to the somatostatin CRE site (a symmetric, or "high-affinity," site) has been shown not to be regulated by the phosphorylation state of CREB (27), these changes in CRE binding demonstrate that relatively small changes in CREB protein levels can have measurable functional consequences. Other evidence that relatively small changes in CREB protein levels could have important functional consequences has been reported recently. Injection of antisense oligonucleotides to CREB into a specific brain region leads to an ≈30% decrease in total CREB protein yet nearly eliminates amphetamine-induced c-*fos* induction (which is downstream of CREB activation) in this region (28).

Regulation of CREB expression by the cAMP pathway shows some cellular specificity, since forskolin failed to alter levels of CREB mRNA or protein in PC12 cells. These results suggest that the mechanisms governing both the transient increase in CREB protein and the sustained reduction in CREB expression observed in CATH.a cells involve factors not present in PC12 cells. Further evidence that CREB protein regulation is tissue-specific is provided by the lack of a morphine effect on CREB levels in the rat frontal cortex. Identification of cell-type specific factor(s) involved in this stimulus and region-specific CREB regulation should provide important information concerning the mechanisms that control CREB expression and function.

The long-term decrease in CREB could be explained by the decrease in CREB mRNA. The short-term increase in CREB, given the absence of any detectable increase in CREB mRNA, is paradoxical and may reflect increased translatability of CREB mRNA or reduced turnover of CREB. Such a change in turnover could result from increased CREB phosphorylation, a possibility that warrants direct investigation. Such a phosphorylation-induced decrease in CREB turnover could explain why the degree of change in protein does not reach the degree of change in mRNA over the time of the experiment (compare Figs. 2 and 3).

The mechanism by which CREB expression is regulated *in vivo* and *in vitro* is unclear, but several interesting possibilities exist. Alternative splicing of the CREB gene, identified by Northern blotting, has been shown to be regulated by cAMP in Sertoli cells, where it controls a cyclical positive autoregulation of CREB expression (29, 30). However, in the CATH.a cells, Northern blotting has given no evidence of

such CREB splice variants under basal conditions or after forskolin treatment. Analysis of the CREB promoter has revealed putative response elements for CREB and other transcription factors (29–31). Studies by Meyer *et al.* (31) indicate that the human CREB promoter exhibits cAMP- and phorbol ester-stimulated transcriptional activity in transient-transfection assays. They demonstrated further that the CREs present within the CREB promoter conferred cAMP responsiveness and that CREB or CREB-like proteins bind to the CREB-gene CREs (31).

Our results differ from these transfection-expression studies in that in a nontransfected, intact cell system (i.e., CATH.a cells), negative regulation of CREB expression occurs. Although the decrease in CREB mRNA could be due to alterations in mRNA stability, especially given the speed of decay of the message, the presence of CRE sites within the CREB promoter suggests that CREB downregulation could be a result of transcriptional repression of the gene by CREB or a CREB-like protein—e.g., the transcriptional repressor CREM (32). This observed regulation of CREB could represent a form of cellular autoregulation in which forskolin initially leads to a positive regulation of CREB function via stimulation of the cAMP pathway, activation of CREB by phosphorylation, and increases in CREB protein (possibly through a phosphorylation-mediated stabilization of the protein). Compensating for the activation of CREB, agents which activate the cAMP pathway might then lead at 24–48 hr to a more persistent negative regulation of CREB mRNA and protein expression. Conversely, morphine, which acutely inhibits the cAMP pathway in the LC, might be expected to produce an opposite effect on CREB expression—namely, upregulation of CREB, as reported in this study. A remaining major question is the relationship between the observed regulation of CREB expression and more long-term regulation of the cAMP pathway (17). Thus, morphine leads to upregulation of the cAMP pathway in the LC after chronic administration, and, conversely, forskolin may lead to a downregulation in the pathway (K.L.W. and E.J.N., unpublished observations) in CATH.a cells after long-term administration. An interesting possibility, which must now be studied directly, is whether up- or downregulation of CREB expression and the reciprocal regulation of the cAMP pathway are causally related.

The cAMP-mediated downregulation of CREB could also have important consequences *in vivo* and *in vitro* in terms of changes in basal CREB levels. There is evidence that dephospho-CREB plays an important role in the cell and may be required for basal expression of some genes (33). Basal CREB also appears to function synergistically with other transcription factors (e.g., Isl-1, glucocorticoid receptor, and CCAAT enhancer-binding protein β), in some cases in a cAMP-independent fashion (34–36). In addition, CREB can interact with DNA topoisomerase II to modify its function (37). That basal levels of dephospho-CREB may have important functional consequences raises the possibility that the chronic changes in CREB levels observed in the LC and CATH.a cells may play a role in more than negative autoregulation of a cAMP-stimulated cellular pathway.

Here we present direct evidence that CREB expression can be regulated in the LC *in vivo* and in CATH.a cells *in vitro*. Alterations in CREB levels may be important both in terms of autoregulation of cAMP-induced gene transcription and in terms of control over CREB functions which are cAMP-independent. This study also demonstrates that CREB expression is subject to regulation in a cell type-specific fashion both *in vivo* and *in vitro*. The findings thereby provide additional regulatory mechanisms by which genomic effects of CREB and the cAMP pathway can be achieved. This information will guide subsequent investigations into the role of CREB phosphorylation and expression in the functional regulation of specific neuronal cell types in the brain in response to long-term perturbation.

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