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Phosphorylation of Cyclic AMP Response Element-Binding Protein and Induction of c-*fos* Gene Expression on Withdrawal from Chronic Treatment with Carbachol in NG108–15 Cells

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SUMMARY

Alterations in adenylyl cyclase activity in cultured cells after prolonged exposure to drugs such as morphine have been extensively studied as models for drug tolerance and withdrawal. NG108–15 cells develop increased intracellular cAMP concentrations after abrupt withdrawal from chronic treatment with the muscarinic cholinergic agonist carbachol. To determine whether this withdrawal-induced increase in cAMP modifies gene expression, we studied phosphorylation of the cAMP response element-binding protein (CREB) and expression of the c-*fos* gene, known to contain a cAMP response element, in NG108–15 cells after abrupt withdrawal from chronic treatment with carbachol. Prostaglandin E_1, which activates adenylyl cyclase, caused concentration-dependent increases in the phosphorylation of CREB and in the abundance of c-*fos* mRNA. These changes occurred with small increments in cAMP accumulation. In cells treated with carbachol for 48 hr, induction of withdrawal with the muscarinic antagonist atropine led to a small increase in intracellular cAMP concentration but an 11.6-fold increase in the phosphorylation of CREB and a 3.4-fold increase in accumulation of c-*fos* mRNA. The adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine, which attenuated the chronic carbachol-induced increase in cAMP concentration, prevented the increased phosphorylation of CREB and the enhanced accumulation of c-*fos* mRNA during atropine-induced withdrawal. These results indicate that expression of the c-*fos* gene is induced by the small increments in cAMP concentration that can occur in cells on withdrawal from chronic treatment with drugs such as muscarinic agonists.

Considerable interest has focused on molecular alterations that may contribute to pathophysiological changes that occur during abrupt withdrawal from drugs such as opiates. The opiate-regulated adenylyl cyclase system has been one focus of this research (1, 2). Sensitization of adenylyl cyclase at the cellular level was originally described in NG108–15 mouse neuroblastoma X rat glioma hybrid cells (3, 4). Prolonged treatment of these cells with drugs such as morphine (3, 4), carbachol (5), or norepinephrine (6), all of which acutely inhibit the activity of adenylyl cyclase, has been found to induce increased adenylyl cyclase activity, which is manifest when the drug is withdrawn. This increase in adenylyl cyclase activity was proposed to represent a biochemical correlate of drug withdrawal and addiction (3). Sensitization of adenylyl cyclase by chronic treatment with inhibitory drugs has been documented in several types of cells and may represent a general cellular adaptive mechanism (7, 8).

This inhibitory receptor-induced increase in adenylyl cyclase activity or cAMP accumulation in NG108–15 cells can be detected after stimulation of the enzyme activity by activation of PGE_1 receptors (3, 5, 6, 9–13). Also, relatively small increases in basal enzyme activity or in basal cAMP concentrations in intact cells have been found during drug withdrawal (5, 6, 14, 15). We wondered whether the relatively small increases in basal cAMP concentration that occur after withdrawal from chronic treatment with an inhibitory drug could have potentially important and physiologically relevant consequences for gene expression. The expression of many genes is known to be induced by cAMP (16, 17). Regulation of expression of the gene for c-*fos* has been studied extensively, and its expression is induced by a wide variety of agents, including serum factors, growth factors, phorbol esters, calcium, and cAMP (18, 19). Regulation of gene expression by cAMP is mediated by specific phosphoprotein transcription factors, CREBs, which bind to distinct enhancer elements called CREs within the 5′-flanking sequence of target genes (20). The 5′-regulatory region of the c-*fos* gene has been reported to contain two (21, 22), three (23), or four (24) CRE-like elements. c-*fos* is considered to be an immedi-

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ABBREVIATIONS: DDA, 2′,5′-dideoxyadenosine; CREB, cAMP response element-binding protein; PGE_1, prostaglandin E_1; CRE, cAMP response element; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ate-early gene in that its mRNA is expressed rapidly, transiently, and independent of *de novo* protein synthesis. The FOS protein in turn functions as a transcription factor to regulate the expression of other (late-response) genes (18, 19). Consequently, c-*fos* is likely to be involved in linking certain rapid changes within the cell to long term adaptations in cellular responsiveness.

We examined cAMP-mediated phosphorylation of CREB and induction of the c-*fos* gene in the NG108–15 cell. Phosphorylation of CREB and accumulation of mRNA for the c-*fos* gene were found to be increased in these cells by agents that increase intracellular cAMP concentration. To test the hypothesis that expression of the c-*fos* gene may be induced by an increase in cAMP on withdrawal from chronic treatment with an inhibitory drug, cells were treated chronically with the muscarinic cholinergic agonist carbachol. Carbachol was chosen for these experiments because we had found previously that the magnitude of sensitization of adenylyl cyclase induced by this drug is greater than that for other inhibitory drugs in NG108–15 cells (15). Phosphorylation of CREB and accumulation of mRNA for c-*fos* were found to be significantly increased after withdrawal from chronic treatment with carbachol. Induction of expression of the c-*fos* gene under these conditions can likely be attributed to the relatively small increase in cAMP concentration and consequent phosphorylation of CREB that occurs during drug withdrawal.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (4500 mg of D-glucose/l) and hypoxanthine/aminopterin/thymidine supplement were obtained from GIBCO-BRL (Grand Island, NY). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Carbachol, atropine, and cAMP were purchased from Sigma Chemical Co. (St. Louis, MO). Ro 20–1724 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PGE_1 was a gift from Upjohn Co. (Kalamazoo, MI). DDA was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). cAMP antiserum was purchased from Research Products International (Mount Prospect, IL). Immobilon P membranes were purchased from Millipore Corp. (Bedford, MA). Anti-CREB and anti-pCREB antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Rainbow protein molecular weight markers, anti-rabbit Ig horseradish peroxidase-linked antibody, ECL Western blotting reagents, [^{32}P]dCTP (3000 Ci/mmol), Multiprime DNA Labeling System, and Hybond-N nylon membranes were purchased from Amersham Corp. (Arlington Heights, IL). A mouse genomic DNA probe for the c-*fos* gene was obtained from American Type Culture Collection (Rockville, MD). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Cell culture. NG108–15 cells were grown in Dulbecco's modified Eagle's medium supplemented with $NaHCO_3$ (3.7 g/l), heat-inactivated fetal bovine serum (8%), hypoxanthine (0.1 mM), aminopterin (1.0 uM), and thymidine (16.0 uM) in a humidified atmosphere of 5% CO_2 at 37°.

Drug treatments of cells. NG108–15 cells were seeded 4 days before the final treatments in various dishes for the different analyses: at 0.06×10^6/35-mm dish to assay cAMP accumulation, at 0.18×10^6/60-mm dish to assay phosphorylation of CREB, and at 0.5×10^6/100-mm dish to prepare RNA for c-*fos* mRNA analysis. To test the effect of chronic treatment with carbachol, cells in serum-containing medium were treated with carbachol (10^{-5} M) for 48 hr. Although analysis for c-*fos* mRNA is usually performed after cells have been deprived of serum due to induction of the mRNA for c-*fos* by serum factors (23–26), a preliminary experiment revealed that chronic treatment with carbachol induced an increase in basal cAMP concentration in cells cultured in the presence of 10% serum but not in the presence of 0.1% serum (data not shown). Therefore, the usual serum-containing media were used in experiments for the analysis of cAMP, CREB, and mRNA. In another preliminary experiment, we found that extensive washing of NG108–15 cells with serum-free media induced the expression of c-*fos* mRNA. Consequently, we induced withdrawal from carbachol by adding an excess concentration of the muscarinic cholinergic antagonist atropine (10^{-5} M). In a preliminary experiment, the time of treatment with atropine was varied from 30 to 120 min. The magnitude of the increase in c-*fos* mRNA was maximal after 60 min of atropine-induced withdrawal from carbachol and declined to basal values by 120 min (data not shown). Therefore, cells were routinely treated with atropine or other drugs for 60 min before preparation of RNA. Cells were treated with atropine or other drugs for 30 min before denaturation for CREB analysis.

Assay of intracellular cAMP. Incubations were terminated by aspirating the medium from cells and adding 1.0 ml of 0.1 M HCl to the dishes. Cell counts were made from separate dishes. cAMP was measured by radioimmunoassay (27).

CREB analysis by SDS-PAGE and immunoblotting. Incubations were terminated by aspirating the medium from cells and adding a denaturing solution containing 62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. The cellular extracts were subjected to one-dimensional SDS-PAGE with a 10% acrylamide gel (28). Rainbow protein molecular weight markers were included to estimate molecular weights. Protein bands were electrophoretically transferred to Immobilon P membrane in 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11.0, containing 10% methanol, for 16 hr at 200 mA. Immobilon blots were blocked in 20 mM Tris·HCl, pH 7.4, 137 mM NaCl, 0.2% Tween 20, and 5% powdered milk for 2 hr at 37°. Membrane blots were then incubated with primary antibodies anti-CREB, diluted 1:5000, and anti-pCREB, diluted 1:1000, for 18 hr at 4°. After four washes in the buffered solution without powdered milk, blots were incubated with secondary antibody, anti-rabbit Ig peroxidase-linked antibody, diluted 1:20,000 for 1 hr at 22°. After additional washes, detection was made by the enhanced chemiluminescence method with ECL Western blotting reagents according to the manufacturer's instructions. The blots were exposed to Kodak XAR-5 film for ~5 min for CREB detection and ~30 min for pCREB detection. The developed film was scanned with an LKB Ultrascan laser densitometer.

Preparation of RNA and analysis by Northern blotting. Incubations were terminated by aspirating the medium from cells and rapidly washing the cells with cold calcium- and magnesium-free phosphate-buffered saline. Denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol was added directly to the dishes to lyse the cells. A single-step method with guanidinium thiocyanate/phenol/chloroform extraction was used to prepare total RNA from the cells (29). For analysis of RNA by Northern blotting, RNA was heated at 65° for 10 min, cooled rapidly on ice, and denatured with 6% formaldehyde. The RNA (25 μg) was fractionated by electrophoresis in a 1% agarose gel and transferred to a nylon membrane by capillary blotting. RNA bands were hybridized at 42° for 16 hr to probe for the c-*fos* or β-actin genes that were labeled with [^{32}P]dCTP by the random prime method. The nylon membrane was washed and exposed to Kodak XAR-5 film with an intensifying screen at −70° for 24–48 hr. The autoradiograms were scanned with an LKB Ultrascan laser densitometer. The quantity of mRNA for c-*fos* (the major band on the Northern blots) was normalized to that of β-actin.

Data analysis. Results (expressed as mean ± standard error) were compared by *t* tests with $p < 0.05$ taken as statistically significant.

Results

In the initial series of experiments, we examined the ability of PGE_1, an activator of adenylyl cyclase in NG108–15

cells, to enhance the phosphorylation of CREB. Immunoblots of denatured cell extracts were reacted with anti-pCREB, an affinity purified polyclonal antibody raised against a 14-amino acid CREB sequence containing a phosphorylated serine residue, that only recognizes a CREB phosphorylated at that specific residue (30). Two major bands of 37 and 43 kDa exhibited increased phosphorylation with increasing concentration of PGE_1 (Fig. 1A). Some immunoblots were also probed with the antibody directed against full-length CREB (Fig. 1B). This antibody recognizes both phosphorylated and nonphosphorylated CREB protein. The band at 37 kDa, detected with antibody to pCREB peptide, was not detected with the antibody to (nonphosphorylated) CREB and thus is likely to be a phosphorylated protein other than CREB. The 43-kDa band has the mobility of a CREB protein (31–35). Comparison of CREB phosphorylation with intracellular cAMP concentrations (Fig. 1C) indicated that CREB was phosphorylated at low concentrations of PGE_1, which resulted in only small increments in cAMP accumulation.

Phosphorylation of CREB by PGE_1 was also sensitive to acute regulation via activation of an inhibitory receptor coupled to adenylyl cyclase. In cells treated with PGE_1 (10^{-8} M) with or without carbachol to activate m4 muscarinic cholinergic receptors, carbachol inhibited the PGE_1 stimulation of both CREB phosphorylation and cAMP accumulation; this inhibition was blocked by atropine (Fig. 2). In four experiments, carbachol inhibited PGE_1-stimulated CREB phosphorylation by 71 ± 2% (data not shown). Consequently, the phosphorylation of CREB appeared to be very sensitive to both stimulatory and inhibitory pathways.

To determine whether expression of the c-*fos* gene could be induced in NG108–15 cells by increases in cAMP concentration, we examined the effect of treating cells with drugs that increase cAMP concentrations via different mechanisms. Cells were treated with various concentrations of PGE_1 to stimulate cAMP synthesis or with two concentrations of the phosphodiesterase inhibitor Ro 20–1724 to inhibit cAMP degradation. Cells were treated for 60 min for mRNA analy-

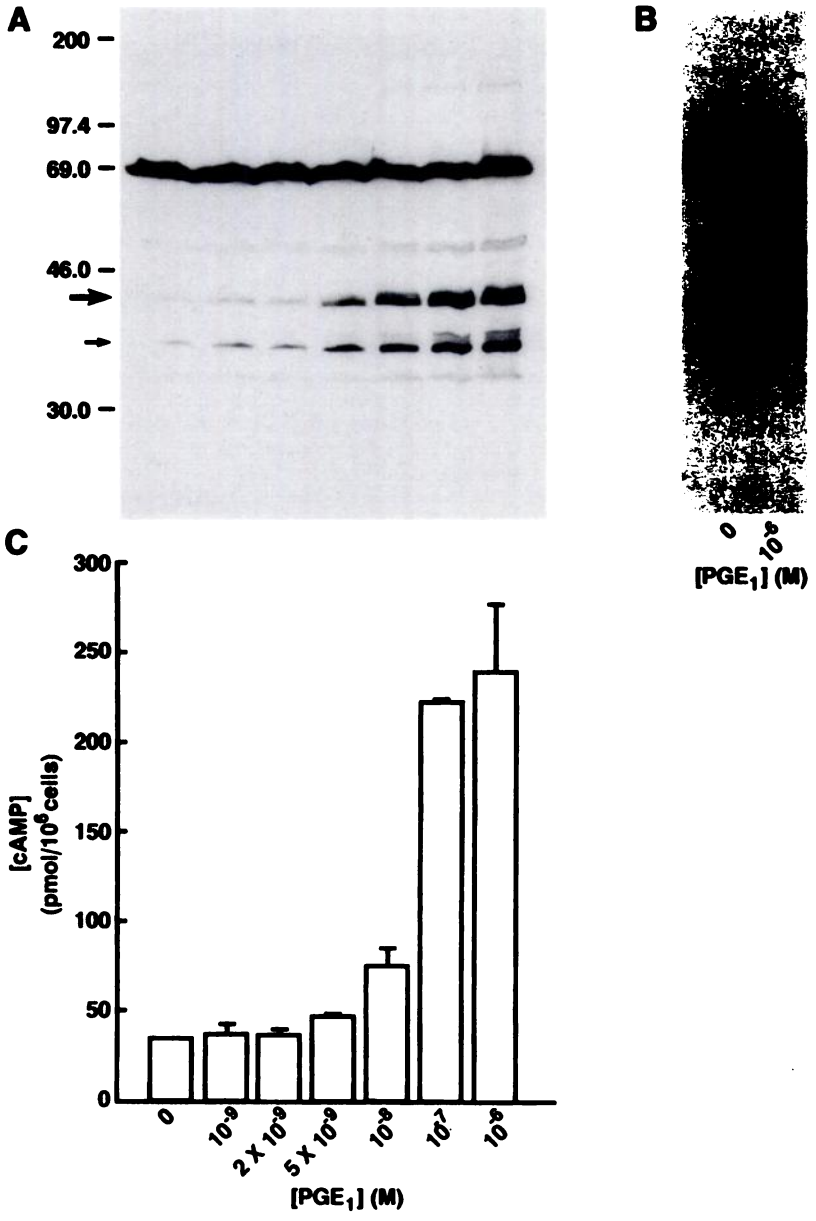

Fig. 1. CREB, CREB phosphorylation, and cAMP concentrations in NG108–15 cells. Cells were incubated with PGE_1 at various concentrations, as indicated, for 30 min. A, Denatured whole-cell extracts were subjected to SDS-PAGE and immunoblotting with antibodies to pCREB peptide. B, Denatured whole-cell extracts were subjected to SDS-PAGE and immunoblotting with antibodies to CREB. C, cAMP was assayed, as described in Experimental Procedures. Molecular weights of the Rainbow protein molecular weight markers (in kDa) are indicated next to the immunoblots. *Heavy arrow*, 43-kDa band identified as CREB; *Light arrow*, band at 37 kDa detected with antibody to pCREB peptide that was not detected with the antibody to (nonphosphorylated) CREB. cAMP data are from analogous incubations corresponding to those for pCREB analysis in this representative experiment and are the mean ± standard deviation of replicate culture dishes. The amount of immunodetected 43-kDa CREB protein did not change from basal during treatment of the cells with PGE_1. Phosphorylation of CREB in response to PGE_1 was associated with increments in intracellular cAMP concentration, and significant phosphorylation occurred at low doses of PGE_1, which resulted in only small increments in cAMP. pCREB analysis was repeated four times in this experiment; the lowest PGE_1 dose at which significant phosphorylation of CREB occurred was 5×10^{-9} M (8.8 ± 1.8-fold), and phosphorylation reached 28.8 ± 11.5-fold after treatment of the cells with 10^{-6} M PGE_1.

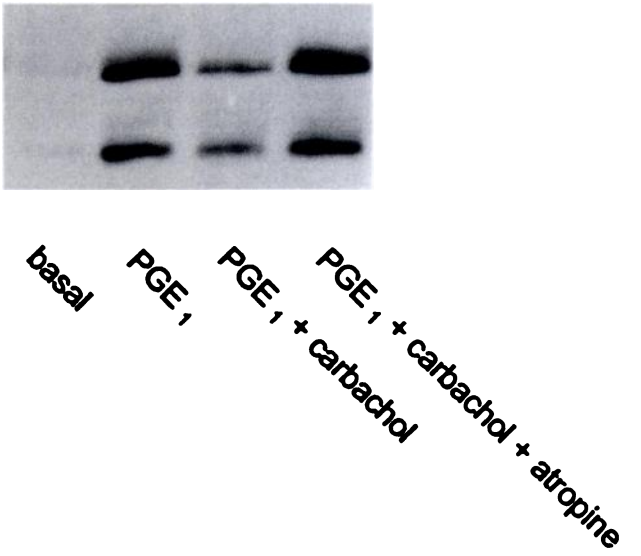

Fig. 2. CREB phosphorylation in NG108–15 cells. Cells were untreated or incubated with PGE_1 (10^{-8} M) with or without carbachol (10^{-5} M) and with or without atropine (10^{-5} M) for 30 min, as indicated. Denatured whole-cell extracts were subjected to SDS-PAGE and immunoblotting with antibody to pCREB, as described in Experimental Procedures. Only the region of the immunoblot containing CREB and the 37-kDa band is shown. cAMP concentrations (pmol/10^6 cells, mean ± standard deviation) corresponding to the treatments for pCREB analysis in this experiment were 34.4 ± 1.3 (*basal*), 75.3 ± 9.9 (*PGE1*), 45.7 ± 3.8 (*PGE1 + carbachol*), and 86.7 ± 3.3 (*PGE1 + carbachol + atropine*). Carbachol acutely inhibited PGE_1 stimulation of CREB phosphorylation and cAMP accumulation, and this inhibition was blocked by atropine. pCREB analysis was repeated four times in this experiment with similar results.

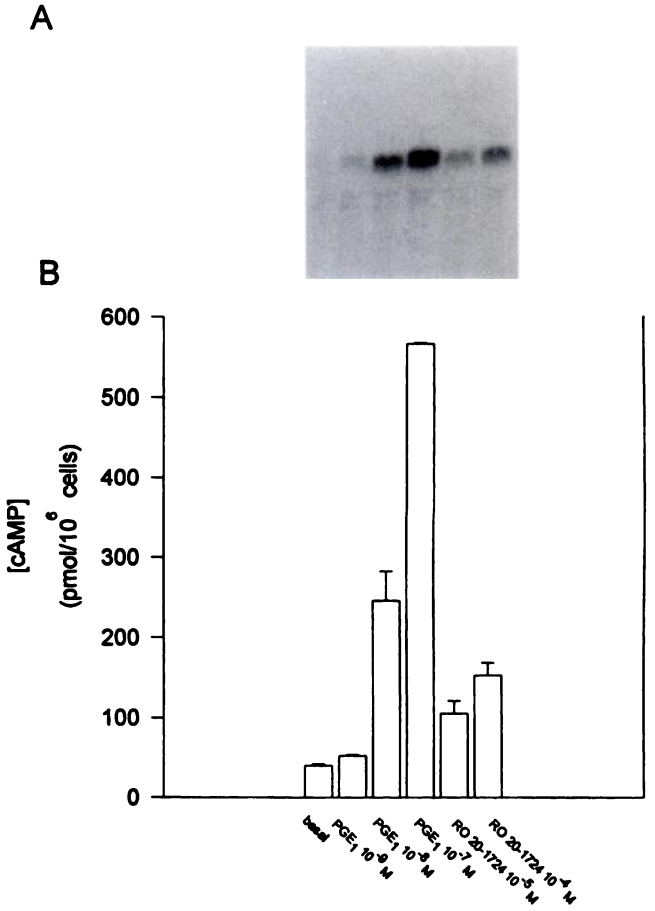

Fig. 3. c-*fos* mRNA and cAMP concentrations in NG108–15 cells. A, Cells were incubated with PGE_1 or the phosphodiesterase inhibitor Ro 20–1724 at various concentrations, as indicated, for 60 min for mRNA analysis. B, Cells were incubated with PGE_1 or the phosphodiesterase inhibitor Ro 20–1724 at various concentrations, as indicated, for 30 min for assay of intracellular cAMP concentration. See Experimental Procedures. cAMP data are from analogous incubations corresponding to those for mRNA analysis in this representative experiment and represent the mean ± standard deviation of replicate culture dishes. c-*fos* mRNA accumulation was associated with increments in intracellular cAMP concentration. Both mRNA analysis and cAMP assay were repeated once with similar results.

sis or for 30 min for assay of cAMP concentrations (Fig. 3). Cells had a very low signal for c-*fos* mRNA under basal conditions. Treatment of cells with increasing concentrations of PGE_1 or Ro 20–1724 resulted in dose-related increases in both c-*fos* mRNA accumulation and cAMP concentrations. mRNA for c-*fos* was increased at concentrations of either PGE_1 or Ro 20–1724 that were associated with increments in intracellular cAMP concentration of only 2% and 12%, respectively, of a maximal PGE_1 response (Fig. 3). These results suggest that regulation of expression of the c-*fos* gene was very sensitive to small increments in cAMP concentrations. Consequently, this gene appeared to be a good candidate for potential induction during withdrawal from chronic treatment with carbachol.

We tested this hypothesis by comparing control cells with cells incubated with carbachol (10^{-5} M) for 48 hr. Atropine (10^{-5} M) was then added to induce withdrawal from carbachol, and the incubation was continued for 30 min (for CREB analysis and cAMP measurement) or 60 min (for RNA analysis of c-*fos*). Results of immunoblotting for CREB phosphorylation are presented in Fig. 4. Treatment of control cells with atropine did not cause a change in either CREB phosphorylation or cAMP concentration. In cells incubated with carbachol for 48 hr and then with atropine for 30 min to induce withdrawal, there was a marked increase in pCREB (Fig. 4A), which was associated with a relatively small increase in cAMP concentration (Fig. 4B). In a series of 13 experiments, the atropine-induced withdrawal from chronic carbachol treatment resulted in a 11.6 ± 2.7-fold increase in phosphorylation of CREB ($p < 0.01$ by paired t test). There was no significant increase in pCREB in cells incubated with carbachol for 48 hr but not treated with atropine.

We also tested for any possible change in the total amount of CREB protein during the chronic treatment with carbachol by immunoblotting with the anti-CREB antibody. In six experiments, the value of this CREB protein did not significantly change after chronic incubation with carbachol (data not shown). Therefore, changes observed in immunoblotting with anti-pCREB represent changes in phosphorylation only, not changes in the amount of the CREB protein.

In similar experiments, we analyzed the mRNA for the c-*fos* gene to determine whether its expression was induced during the atropine-induced withdrawal from chronic incubation with carbachol. Treatment of control cells with carbachol or atropine did not cause a change in mRNA for c-*fos* (at 60 min) or in intracellular cAMP concentration (at 30 min). In cells incubated with carbachol for 48 hr and then with atropine for 60 min to induce withdrawal, the mRNA for c-*fos* was increased, which was associated with a relatively small

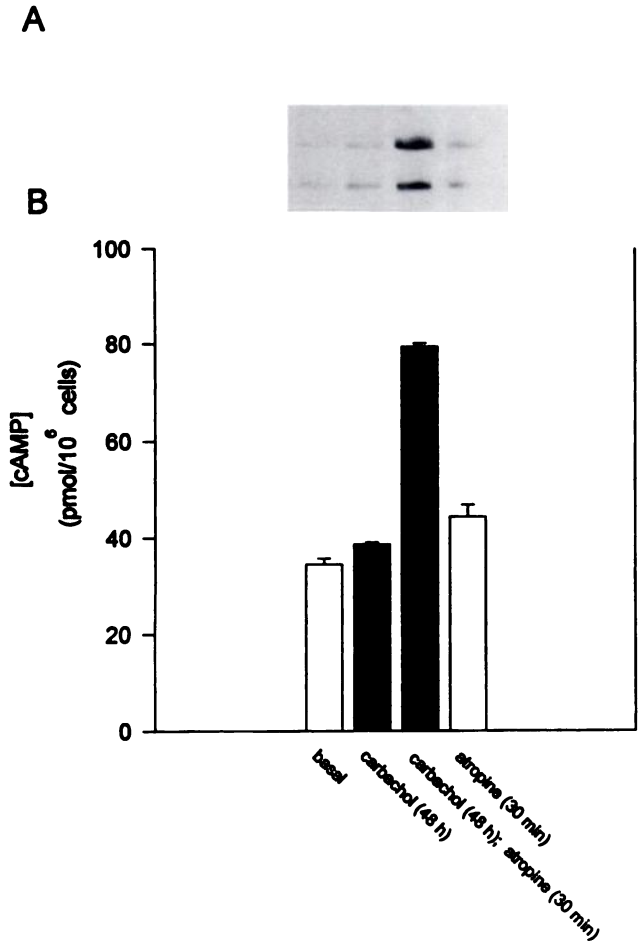

Fig. 4. CREB phosphorylation and cAMP concentrations in NG108–15 cells, incubated with or without carbachol for 48 hr. Cells were incubated or not with carbachol (10^{-5} M) for 48 hr and then left untreated or treated with atropine (10^{-5} M) for 30 min, as indicated. A, Denatured whole-cell extracts were subjected to SDS-PAGE and immunoblotting with antibody to pCREB. B, cAMP was assayed, as described in Experimental Procedures. Only the region of the immunoblot containing CREB and the 37-kDa band is shown. cAMP data are from analogous incubations corresponding to those for pCREB analysis in this representative experiment and represent the mean ± standard deviation of replicate culture dishes. Phosphorylation of CREB was associated with the relatively small increase in cAMP concentration that occurred after atropine-induced withdrawal from carbachol. pCREB analysis in this representative experiment was repeated 13 times.

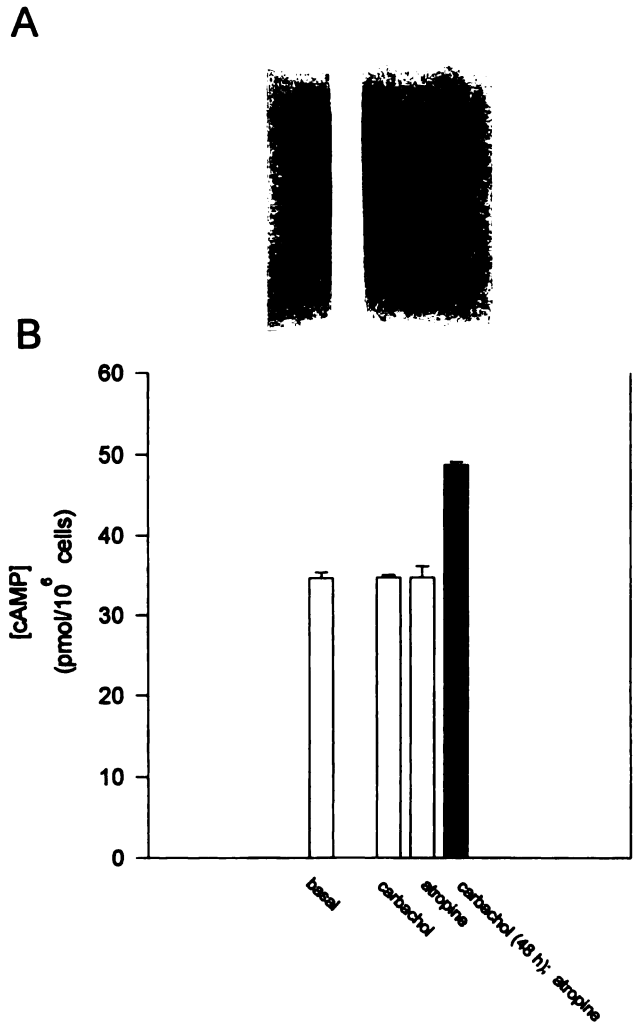

Fig. 5. c-*fos* mRNA and cAMP concentrations in NG108–15 cells incubated with or without carbachol (10^{-5} M) for 48 hr. Control cells were then left untreated (basal) or treated with carbachol (10^{-5} M) or atropine (10^{-5} M), as indicated. Cells incubated with carbachol for 48 hr were treated with atropine (10^{-5} M) as for control cells. A, Cells were treated for 60 min for mRNA analysis. B, Cells were treated for 30 min for assay of intracellular cAMP concentration. See Experimental Procedures. The Northern blot was reprobed for β-actin mRNA, the quantity of which was similar for all samples (data not shown). cAMP data are from analogous incubations corresponding to those for mRNA analysis and represent the mean ± standard deviation of replicate culture dishes. Atropine and carbachol had no acute effects on control cells. c-*fos* mRNA accumulation was associated with the relatively small increase in cAMP concentration that occurred after atropine-induced withdrawal from carbachol. This experiment was repeated twice with similar results.

increase in cAMP concentration (Fig. 5), as in the experiments for analysis of pCREB. Also similar to the results for pCREB, cells incubated with carbachol for 48 hr but not treated with atropine did not exhibit an increase in mRNA for c-*fos* (data not shown), presumably because the cAMP concentration was not sufficiently elevated without atropine-induced withdrawal (Fig. 6). In a series of eight experiments comparing control cells with cells treated with carbachol for 48 hr (each group was subsequently treated with atropine to induce withdrawal in the carbachol-treated cells), mRNA for c-*fos* was increased 3.4 ± 0.6-fold ($p < 0.01$ by paired t test) and cAMP concentration was increased 43 ± 7% ($p < 0.001$ by paired t test) in the cells treated chronically with carbachol.

In an effort to determine whether the phosphorylation of CREB and induction of c-*fos* expression were due to the relatively modest increases in intracellular cAMP concentration that occur during withdrawal from chronic treatment with carbachol, we used DDA, an inhibitor of adenylyl cyclase, to attenuate the increase in cAMP concentration that occurs during withdrawal. DDA inhibits adenylyl cyclase independent of cell surface receptors, possibly through a "P site" on the catalytic unit of adenylyl cyclase (36, 37). In these experiments, cells were treated with or without carbachol for 48 hr; each group was subsequently treated with atropine alone or with atropine in the presence of DDA. A detailed analysis of the cAMP concentrations during the 60-min withdrawal period in these four groups of cells is presented in Fig. 6. DDA caused small decrements in cAMP concentrations in

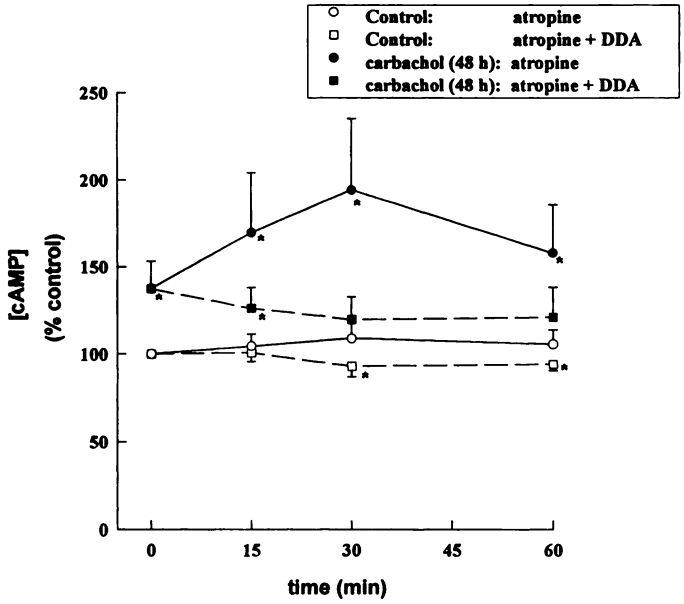

Fig. 6. cAMP concentrations in NG108–15 cells incubated for 48 hr with or without carbachol and then treated acutely with atropine, with or without DDA. Cells were treated or not with carbachol (10^{-5} M) for 48 hr, and then atropine (10^{-5} M) was added to some of the cells, with or without DDA (10^{-4} M), for various times up to 60 min. Data represent mean ± standard error of four experiments. *, $p < 0.05$ by paired t test, values significantly different from control (with atropine alone). Note that the concentration of cAMP was significantly increased before atropine-induced withdrawal but was elevated further after treatment with atropine. DDA significantly attenuated the increase in cAMP concentration that occurred during atropine-induced withdrawal from carbachol.

control cells not treated with carbachol. In contrast, the drug caused significant decrements in cells treated chronically with carbachol, and the increase in cAMP that occurred during atropine-induced withdrawal was prevented by DDA after 30 or 60 min of treatment. Thus, DDA prevented the increase in cAMP concentration that occurred during the withdrawal from chronic treatment with carbachol.

The results of immunoblotting for CREB phosphorylation during withdrawal in the presence of DDA are presented in Fig. 7, including a representative immunoblot (Fig. 7A) and a summary of the quantitative densitometric data from eight experiments (Fig. 7B). Chronic treatment of the cells with carbachol resulted in an 8.5 ± 2.1-fold increase in phosphorylation of CREB during atropine-induced withdrawal ($p < 0.01$ by paired t test); however, when DDA was present during the withdrawal period, there was no significant increase in CREB phosphorylation.

The results of analysis for c-*fos* mRNA during withdrawal in the presence of DDA are presented in Fig. 8, including a representative Northern blot (Fig. 8A) and a summary of the quantitative densitometric data from six experiments (Fig. 8B). Chronic treatment of the cells with carbachol resulted in a 3.0 ± 0.5-fold increase in mRNA for c-*fos* during atropine-induced withdrawal ($p < 0.02$ by paired t test); a significant increase did not occur when DDA was also present during the withdrawal period, similar to its capacity to prevent significant phosphorylation of CREB.

Discussion

In the present study, we addressed the possibility that the increase in cAMP concentration occurring in NG108–15 cells after withdrawal from chronic treatment with carbachol induces changes in gene expression via phosphorylation of CREB. Expression of the proto-oncogene c-*fos* was studied because it is regulated by cAMP through several CREs (21, 23, 24) and the product of this immediate-early gene, the FOS protein, is a transcriptional factor that regulates the expression of other genes and thus functions in long term cellular responsiveness (18, 19). We have found that drugs such as PGE_1 or Ro 20–1724, which increase intracellular cAMP concentration via different mechanisms, enhance both

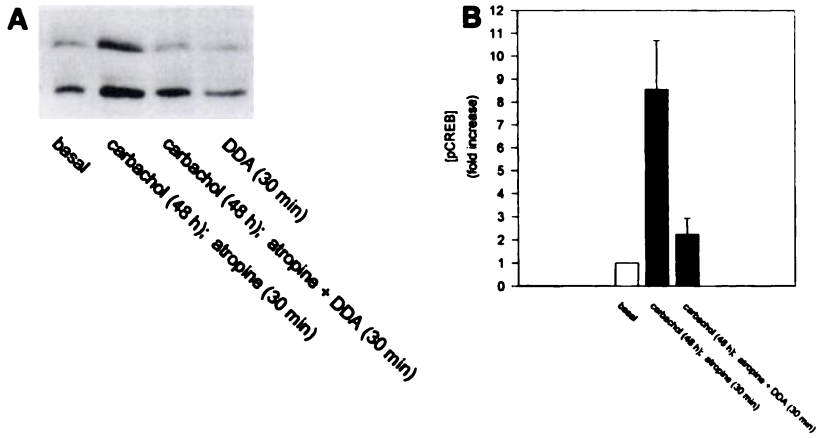

Fig. 7. CREB phosphorylation in NG108–15 cells incubated with or without carbachol for 48 hr and then left untreated or treated with atropine with or without DDA. Cells were incubated or not with carbachol (10^{-5} M) for 48 hr. Cells not incubated with carbachol were left untreated (basal) or treated with DDA (10^{-4} M) for 30 min, as indicated. Cells incubated with carbachol were treated with atropine (10^{-5} M) or atropine plus DDA for 30 min, as indicated. Denatured whole-cell extracts were subjected to SDS-PAGE and immunoblotting with antibody to pCREB, as described in Experimental Procedures. A, Representative immunoblot. Only the region of the immunoblot containing CREB and the 37-kDa band is shown. B, Summary data. pCREB was quantified by densitometry, and the values were normalized to those of untreated controls in each experiment. Values represent mean ± standard error of eight experiments. DDA inhibited the increase in phosphorylation of CREB that occurred after atropine-induced withdrawal from chronic carbachol treatment.

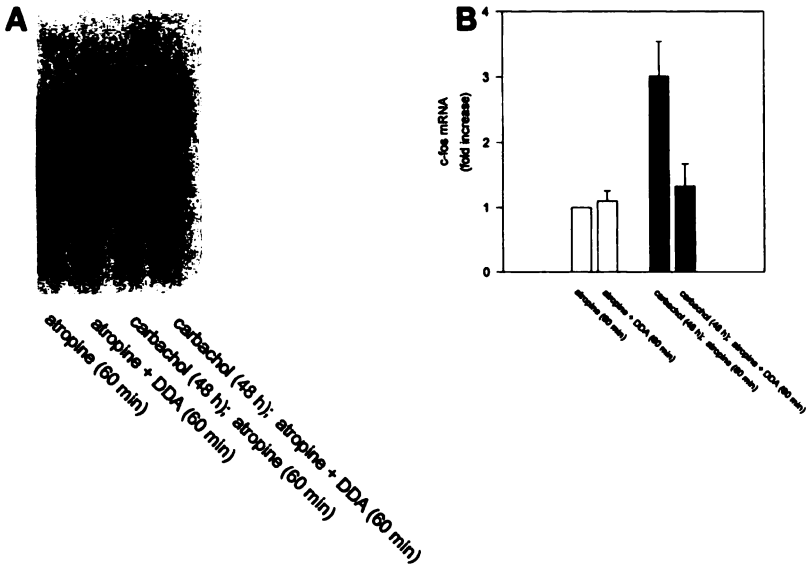

Fig. 8. Effect of DDA on accumulation of c-*fos* mRNA in NG108–15 cells during atropine-induced withdrawal from carbachol. Cells were treated or not with carbachol (10^{-5} M) for 48 hr and then treated with atropine (10^{-5} M) with or without DDA (10^{-4} M) for 60 min for mRNA analysis, as described in Experimental Procedures. A, Representative Northern blot. B, Summary data. mRNA for c-*fos* was quantified by densitometry and normalized to mRNA for β-actin, and the values expressed relative "atropine" in each experiment. Values represent the mean ± standard error of six experiments. DDA inhibited the increase in c-*fos* mRNA accumulation that occurred after atropine-induced withdrawal from carbachol.

the phosphorylation of CREB, as detected by a pCREB-specific antibody, and the expression of the c-*fos* gene, as assessed by probing for its mRNA. Significant increases in phosphorylation of CREB and accumulation of mRNA for c-*fos* were observed even when the increase in cAMP concentration was far less than maximal. In addition, pCREB and mRNA for c-*fos* were significantly increased in cells treated chronically with carbachol when atropine was used to promote rapid withdrawal from carbachol. The approximately 12-fold increase in pCREB and 3-fold increase in mRNA for c-*fos* in these cells during the withdrawal period were associated with only a ~40% increase in cAMP concentration above basal values. The role of cAMP in mediating the phosphorylation of CREB and expression of c-*fos* was tested by including an inhibitor of adenylyl cyclase, DDA, during the withdrawal period. DDA substantially attenuated the increase in cAMP concentration in carbachol-treated cells during withdrawal and attenuated or prevented the increases in pCREB and mRNA for c-*fos* in similarly treated cells. Consequently, phosphorylation of CREB and expression of the c-*fos* gene appeared to be induced by modest increments in cAMP concentration that occur during withdrawal from chronic treatment with carbachol.

We assessed the phosphorylation state of CREB with a commercially available polyclonal antibody raised against a 14-amino acid CREB peptide containing a phosphorylated serine residue. This residue, Ser-133, has been identified as the major site of phosphorylation by both cAMP-dependent protein kinase (32) and Ca^{2+}/calmodulin-dependent protein kinase (38). The anti-pCREB antibody recognizes full-length CREB protein phosphorylated *in vitro* on Ser-133 but does not recognize CREB that is not phosphorylated at this serine residue (30). On our immunoblots of cell extracts from NG108–15 cells, this pCREB antibody reacted with three major protein bands of molecular weights 37, 43, and 70 kDa. The 37- and 43-kDa bands reacting with anti-pCREB increased in intensity in concert with increasing PGE_1-stimulated cAMP accumulation, but the 70-kDa band did not change. The anti-pCREB antibody reacts with 30-, 37-, and 70–80-kDa proteins apparently unrelated to CREB;[1] we have seen similar bands in our experiments. On our immunoblots probed with the anti-CREB antibody, which recognizes both the phosphorylated and nonphosphorylated CREB protein, only the 43-kDa band, not the 37- or 70-kDa bands, was observed. Therefore, the 43-kDa band represents the CREB protein, whereas the 37-kDa band is likely a protein other than CREB, the phosphorylation of which changes in concert with cAMP-induced phosphorylation of CREB.

cAMP-induced phosphorylation of CREB has usually been observed after treatment of cells with a high concentration of exogenous cAMP analogue (33) or with drugs such as forskolin that result in large increases in intracellular cAMP concentration (31, 32, 34, 35). Similarly, cAMP-mediated expression of the gene for c-*fos* has usually been studied by treatment of cells with a cAMP analogue at a high concentration (24, 25) or with drugs such as forskolin (21, 23). The relationship of the concentration of cAMP with abundance of c-*fos* mRNA has not been extensively studied; typically, large increases in cAMP concentrations have been found with c-*fos* induction after stimulation of cells with isoproterenol (e.g., Ref. 22). The results of the present study demonstrate that phosphorylation of CREB and expression of the c-*fos* gene can be induced by small increments in the concentration of intracellular cAMP, such as occurs in the NG108–15 cell during withdrawal from chronic treatment with carbachol. Thus, phosphorylation of CREB and expression of the c-*fos* gene may be very sensitive to changes in the concentration of cAMP.

Studies in animals of the effect of prolonged administration of an opiate drug have also addressed the phosphorylation state of CREB and the regulation of c-*fos* during withdrawal from an inhibitory drug. Chronic treatment of rats with morphine and subsequent withdrawal from the drug have been reported to result in an increase in the phosphorylation of CREB in the locus ceruleus (39) and an increase in mRNA for c-*fos* in the locus ceruleus, nucleus accumbens, and amygdala (2, 40). A small increase in adenylyl cyclase

[1] Product bulletin, Upstate Biotechnology Inc.

activity in membrane preparations from these brain regions from morphine-treated rats has also been described in studies from these investigators (2). Results of our study on NG108–15 cells suggest that this increase in c-*fos* mRNA in the rat brain may be mediated by increases in cAMP concentration and phosphorylation of CREB during drug withdrawal.

A specific alteration in gene expression, resulting in an increase in mRNA for the c-*fos* gene, occurs in rat brain and NG108–15 cells after prolonged treatment and withdrawal from drugs that acutely inhibit and chronically sensitize adenylyl cyclase. Enhanced expression of the FOS protein may have subsequent effects on the expression of other, late-response genes. Such alterations may be responsible for cellular and organismal adaptations to prolonged exposure to drugs, including those with addictive liability. Drugs that directly inhibit adenylyl cyclase may have potential novel therapeutic application to combating the manifestations of opiate withdrawal. NG108–15 cells represent a model system with which to evaluate these possibilities.

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