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EFFECTS OF CHRONIC EXPOSURE OF NG108-15 CELLS TO MORPHINE OR ETHANOL ON BINDING OF NUCLEAR FACTORS TO cAMP-RESPONSE ELEMENT

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SUMMARY: The gel retardation assay with a single-stranded oligo-DNA of cAMP-response element (CRE) in a somatostatin promoter region was selected to examine the possibility of transcriptional regulation of cAMP-inducible genes by chronic morphine or ethanol treatment of NG108-15 cells. When the nuclear extracts from the cells treated with morphine (50 μM) or ethanol (100 mM) for several days were assayed, the amount of DNA-protein complex was decreased about 30-40 % compared to that of the control. The decreased complex was recovered by 1-2 days after withdrawal of the drugs. Treatment of the cells with these drugs for 1 h did not change the amount of the DNA-protein complex. Thus, changes in CRE-binding proteins from the cells treated chronically with morphine or ethanol suggest that these drugs can modulate the expression of cAMP-inducible genes through which tolerance and dependence may develop.

Neuroblastoma x glioma hybrid cells (NG108-15) contain δ-opiate receptors and have provided a model system for studying the molecular mechanism of tolerance and dependence in terms of adenylate cyclase system (1). Morphine inhibits adenylate cyclase activity in the cells, and causes the increases in adenylate cyclase synthesis (2) and the down-regulation of opiate receptors (3) in the cells by several days. Chronic exposure of NG108-15 cells to ethanol also causes the decrease in mRNA of GTP-binding protein (Gs) (4). These observations suggest the possibility that the expression of many genes in the brain is modulated by morphine or ethanol during the acquisition of tolerance and dependence. Recently, it has been reported that cyclic AMP regulates a number of genes through a cAMP response element (CRE) in their promoter regions and nuclear CRE-binding proteins bind specifically to the CRE to stimulate the transcription of cAMP-responsive genes. Among these genes,

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CRE of somatostatin gene has been characterized well and binds a 43 kDa nuclear protein from rat pheochromocytoma PC12 cells (5). We therefore tried to examine whether morphine or ethanol affects on CRE-binding proteins and changes the formation of CRE-protein complex on the gel retardation assay. In this report, we describe that the complex formation of CRE with the nuclear proteins extracted from NG108-15 cells decreased markedly upon chronic morphine or ethanol treatment.

MATERIALS AND METHODS

Chemicals

T4 polynucleotide kinase and calf intestinal phosphatase were obtained from Takara Shuzo. Nitrocellulose filter and BCA protein assay reagent were purchased from Schleicher & Shuell and Pierce, respectively.

Nuclear Extract Preparation

NG108-15 cells were cultured as described previously (6). Nuclear extracts were prepared from the control or treated cells according to the method of Dignam et al (7). Morphine or ethanol was added to the cultures every day. Protein assay was carried out as described (8).

Gel Retardation Assay

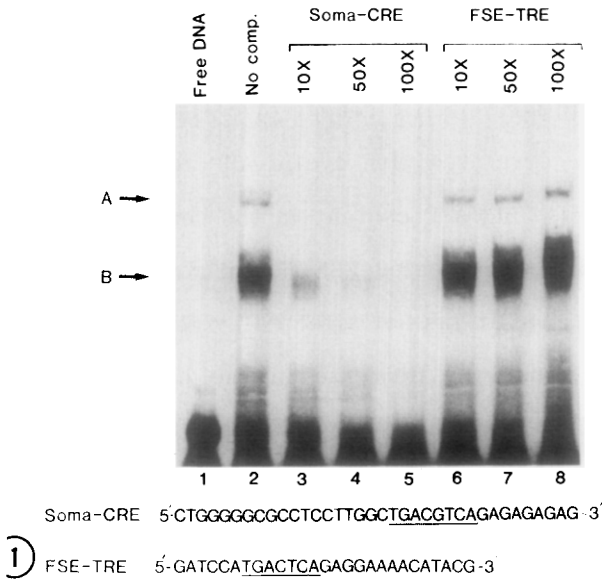
Formation of protein-DNA complex was performed with the nuclear extract (5μg protein) according to the method of Luscher et al (9). Non-radioactive competitors were also added in some experiments. DNA-protein complexes were separated on 4% polyacrylamide gel for 2 h at 150 V in a solution containing 50 mM Tris-glycine (pH 8.5) and 2 mM EDTA. The gel was autoradiographed with intensifying screens (Kodak) for 5 h at -80℃. The bands of DNA-protein complex were estimated quantitatively on autoradiogram by a densitometer (Shimazu, CS-9000) or by counting the radioactivity of the bands cut out from the gels by a scintillation counter (Aloka). Under the experimental conditions, the radioactivity or autoradiographic intensity was within a linear range. Oligo-DNAs containing a consensus single-stranded somatostatin- CRE (5'-CTGGGGGCGCCTCCTTGGCTGACGTCAGAGAGAGAG-3') (10), single-stranded complimentary somatostatin-CRE (5'-CTCTCTCTCTGACGTCAGCCAAGGAGGCGCCCCCAG-3'), and FSE-TRE (5'-GATCCATGACTCAGAGGAAAACATACG-3') in fat-specific element (FSE) (11) were synthesized on an Applied Biosystems Model 380B and end-labeled with T4 polynucleotide kinase and [γ ^{32}P]ATP (Amersham). To prepare labeled double-stranded somatostatin-CRE (Soma-ds-CRE), labeled single-stranded CRE (Soma-ss-CRE) and nonradioactive single-stranded complimentary CRE (Soma-ss-cCRE) were incubated at 88℃ for 2 min, 65℃ for 10 min, 37℃ for 10 min, and 25℃ for 5 min (12).

Southwestern Analysis

Nuclear extracts were separated on 10% SDS-polyacrylamide gel, transferred to a nitrocellulose and hybridized with 5'-end-labeled double-stranded oligo-DNA as described (13).

RESULTS AND DISCUSSION

Cyclic AMP response element (Soma-CRE) in a somatostatin gene promoter contains a consensus sequence of TGACGTCA. Gel retardation assay was used to



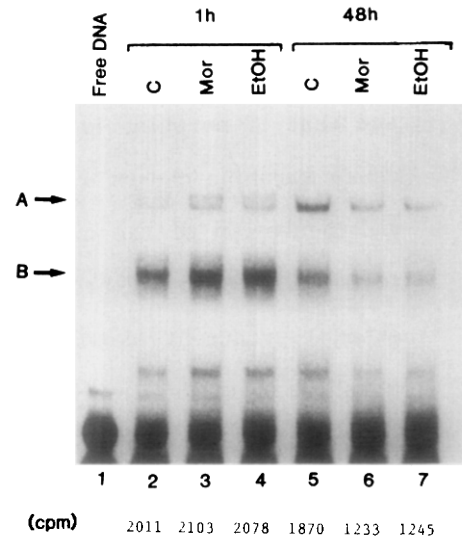


Figure 1. Gel retardation assay with competitors. Nuclear extracts (5μg protein in each lane) were prepared from NG108-15 cells and utilized for the gel retardation assay. The probe and competitors used in this experiment are diagramed below the panel. CRE and TRE sequences are underlined. The bands A and B derived from Soma-CRE-protein complex are indicated by arrows. Lane 1, no nuclear extract added; lane 2, no competitor; lane 3-5, excess unlabeled Soma-CRE as indicated; lane 6-8, excess unlabeled FSE-TRE.

Figure 2. Effects of morphine or ethanol on CRE-protein complex formation. NG108-15 cells were incubated for 1 h (lane 2-4) or 48 h (lane 5-7) with no addition (C), 50μM morphine (Mor), and 100 mM ethanol (EtOH). Nuclear extracts from the cells were utilized for the gel retardation assay. The numbers below each lane show the radioactivity of band B cut out from the gel.

detect specific CRE-protein complexes in NG108-15 cells. Soma-CRE oligo-DNA labeled at 5'-end with [^{32}P]ATP and nuclear proteins extracted from the NG108-15 cells treated with morphine or ethanol were mixed and incubated for 30 min at 25°C. When the mixture was subjected to electrophoresis on 4% polyacrylamide gel, two retarded bands were detected (Fig. 1, lane 2). Competition experiments were performed to validate the specificity of the two bands identified as a complex of CRE and its binding proteins. The bands A and B were decreased with increasing amounts of the same unlabeled oligo-DNA (Fig. 1, lane 3-5). In contrast, fat specific element (FSE) which contains a TGACTCA sequence (TPA response element), but lacks one G residue from CRE sequence did not compete out two bands observed with CRE probe (Fig. 1, lane 6-8). Thus, the bands A and B are thought to be specific to Soma-CRE.

Prior exposure of NG108-15 cells to 50μM morphine or 100mM ethanol for 48 h reduced the formation of CRE-protein complex by about 34% or 33%, respectively

(Fig. 2). In contrast, one hour incubation of the cells with morphine or ethanol exhibited no reduction of the CRE-protein complexes, suggesting that the effect of ethanol is not due to the non-specific damage of cell membranes and that of morphine is not directly correlated with cyclic AMP levels, because morphine causes a marked decrease in cAMP content in the cells at 1 h (2). Incubation of the nuclear proteins with labeled FSE-TRE oligo-DNA also formed a specific TRE-protein complex , but it was not affected by morphine or ethanol treatment (data not shown). Since the decrease in the band B by morphine or ethanol was more consistent than band A, we measured the intensity of the band B by a densitometer or counting the radioactivity. When the time course of changes in the complex formation were examined with 50μM morphine or 100 mM ethanol, a gradual decrease of CRE-protein complex was observed until 1-2 days and thereafter reaching a steady level (Fig. 3). A rebound increase of cAMP level is demonstrated in NG108-15 cells after the withdrawal of morphine (2). When the cells were treated with morphine for 4 days and then morphine was withdrawn, the levels of DNA-protein complex were gradually returned to the control level by 30 h, but no rebound increase in DNA-protein complex formation was observed. We next examined the effects of

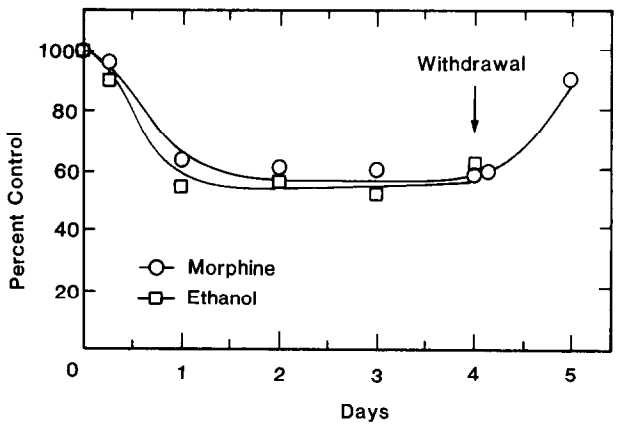


Figure 3. Time course for the inhibition of Soma-CRE complex formation by morphine or ethanol treatment. NG108-15 cells were incubated with 50μM morphine or 100 mM ethanol for indicated time. Nuclear extracts from the cells were utilized for the gel retardation assay. After 4 days, morphine was withdrawn as indicated by an arrow and then cells were cultured in the absence of morphine. Each point represents the mean percentage of the amount of Soma-CRE-protein complex (band B) from morphine- or ethanol-treated cells compared to the control cells from 3 independent experiments.

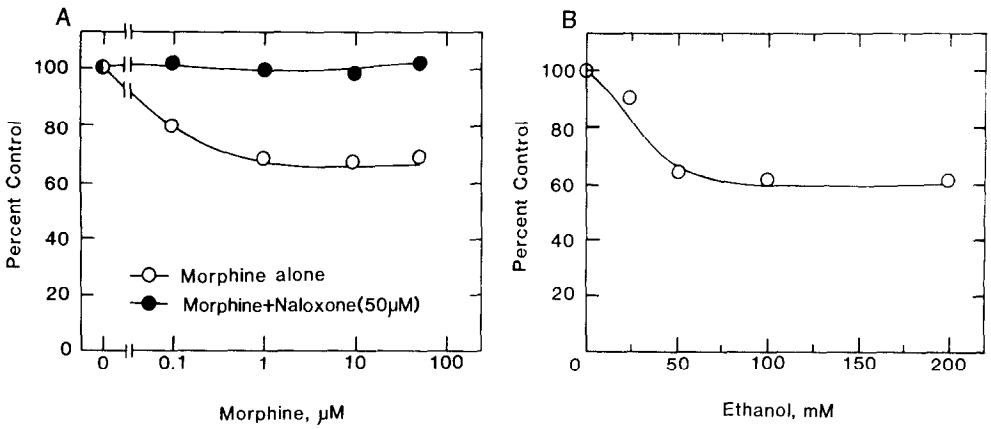


Figure 4. Dose-response curve for morphine- or ethanol-induced changes in Soma-CRE-protein complex formation. A; NG108-15 cells were incubated with indicated concentrations of morphine in the absence (- ○ -) or presence (-●-) of 50μM naloxone for 2 days. B; NG108-15 cells were incubated with indicated concentrations of ethanol for 2 days. Nuclear extracts from the cells were utilized for the gel retardation assay. Each point represents the mean percentage of the amount of Soma-CRE-protein complex (band B) from morphine- and ethanol-treated cells compared to the control cells from 3 independent experiments.

various concentrations of morphine or ethanol on the complex formation (Fig. 4). Incubation of the cells with various concentrations of morphine for 2 days caused a dose-dependent decrease in the formation of CRE-protein complex (Fig. 4A). The maximal inhibition of CRE-protein complex formation was observed at 10 μM, within a range of the pharmacological concentrations. The effect of morphine was blocked in the presence of a morphine antagonist, naloxone (50μM). Ethanol also decreased the formation of CRE-protein complex at the concentration of as low as 25 mM and a maximal inhibition was obtained at more than 50 mM (Fig. 4B), which is within range of blood alcohol levels in intoxicated humans (14). A concentration of ethanol (100 mM) required for the maximal reduction of CRE-protein complex is comparable to that for the decrease in mRNA and amount of G protein (4, 15).

Since Soma-CRE oligo-DNA has a palindrome structure and complimentary Soma-CRE oligo-DNA also contains a TGACGTCA sequence, we examined the complimentary somatostatin-CRE oligo-DNA for a similar ability to interact with specific proteins. The complimentary Soma-CRE oligo-DNA (Soma-ss-cCRE) did not form any specific DNA-protein complex (Fig. 5, lane 5-8). A 28-mer oligo-DNA lacking a CRE palindrome sequence did not reduce Soma-CRE-protein complexes (data not

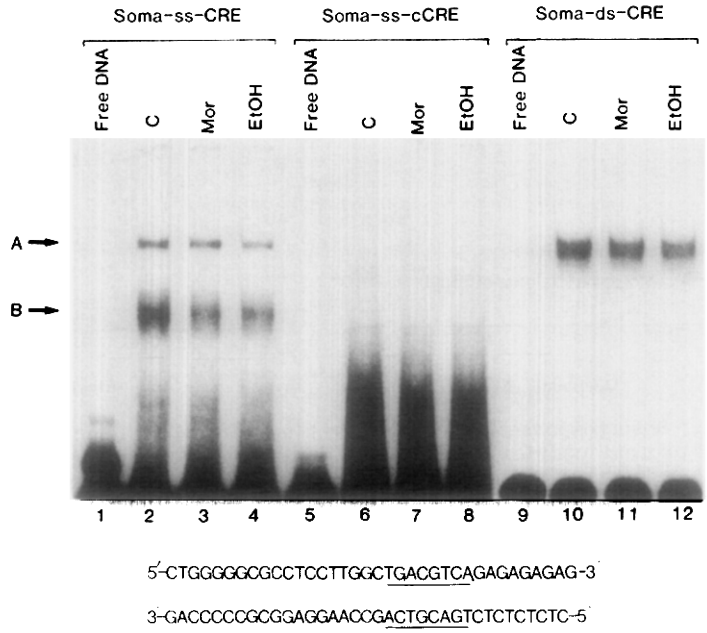


Figure 5. Gel retardation assays with single- or double-stranded Soma-CRE oligo-DNA. NG108-15 cells were incubated for 48 h with no addition (C), 50μM morphine (Mor), and 100 mM ethanol (EtOH). The probes used in this experiment are diagramed below the panel. Lane 1-4, single-stranded somatostatin CRE oligo-DNA (Soma-ss-CRE) as probe; lane 5-8, single-stranded complimentary somatostatin CRE oligo-DNA (Soma-ss-cCRE) as probe; lane 9-12, double-stranded somatostatin CRE oligo-DNA (Soma-ds-CRE) as probe.

shown). These data suggest that CRE and its adjacent sequences are necessary for the formation of CRE-protein complex. When double-stranded Soma-CRE oligo-DNA (Soma-ds-CRE) was used, one intense band of DNA-protein complex was observed, but the effects of morphine or ethanol were much less significant (Fig. 5, lane 9-12).

We next performed Southwestern blot to know how many proteins bind with CRE oligo-DNA. At least five proteins with about 110, 66, 43, 35 and 30 kDa were detected and these bands disappeared in the presence of a 100-fold excess of unlabeled Soma-CRE oligo-DNA (data not shown).

It has been demonstrated that the phosphorylation of CRE-binding protein by cAMP-dependent protein kinase does not affect the binding ability of the protein to CRE sequence (5). Pretreatment of the nuclear extracts with calf intestinal phosphatase did not change the DNA-protein complex formation (data not shown). These data suggest that inhibitory effect of morphine on the

complex formation does not result from phosphorylation or dephosphorylation of the CRE-binding proteins.

The data from Southwestern blot demonstrated that several proteins could bind to Soma-CRE and we are now examining which protein(s) is responsible for the effect of morphine or ethanol. It is also necessary to clarify which genes are regulated by CRE-binding proteins in NG108-15 cells for elucidating the mechanism by which tolerance and dependence develop.

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