Research report

The comparative analysis of proenkephalin mRNA expression induced by cholera toxin and pertussis toxin in primary cultured rat cortical astrocytes

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Abstract

In rat astrocytes, incubation with cholera toxin (CTX; 0.1 μg/ml) for 8 h increased proenkephalin (proENK) mRNA level (10-fold), which was further increased by dexamethasone (DEX; 1 μM) (2.2-fold as much as CTX alone). Although pertussis toxin (PTX; 0.1 μg/ml) did not affect the basal proENK mRNA level, DEX significantly increased proENK mRNA level in PTX-treated cells (6-fold). The inhibition of protein synthesis by cycloheximide (CHX; 15 μM) also increased proENK mRNA level in PTX-treated cells (5.2-fold), but not in CTX-stimulated cells. The treatment with CTX, but not PTX, increased c-Fos and Fra-2 protein levels as well as AP-1, CRE, or ENKCRE-2 DNA binding activity, but neither toxin affected Fra-1, c-Jun, JunB, and JunD protein levels. CHX significantly attenuated CTX-induced increase of c-Fos or Fra-2 protein level and AP-1, CRE, or ENKCRE-2 DNA binding activity, although CHX alone did not affect the basal AP-1, CRE, and ENKCRE-2 DNA binding activities. Phosphorylated CREB level was increased by both CTX and PTX, although the magnitude of phosphorylation of CREB by PTX was much less than that by CTX. In addition, CHX further or persistently increased PTX- or CTX-induced phosphorylated CREB levels in parallel with increases in proENK mRNA. However, DEX did not alter the basal or stimulated phosphorylated-CREB level. These results suggest that the elevation of phosphorylation of CREB rather than AP-1 level may be involved in CTX-induced and CHX-dependent-PTX-induced increase of proENK mRNA level. In addition, AP-1 expression or CREB phosphorylation appears not to be involved the potentiative action of DEX on proENK mRNA expression in CTX- and PTX-treated astrocytes. © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

Glia cells perform essential functions in central nervous system (CNS) development and are also believed to play important roles during gliosis in response to trauma or disease [3]. These developmental and pathological states have also been associated with CNS expression of opioid genes [26,36]. Previous studies have reported that astrocytes express proenkephalin (proENK) mRNA and its protein product [15,28,31,37,46]. However, the regulatory mechanism underlying proENK gene expression in astrocytes has not been fully understood.

In several tissues and cells, the proENK gene expression appears to be dependent upon ligand-mediated signal transduction events, such as an increase in intracellular cyclic AMP (cAMP) levels [8,33,43,44], activation of protein kinase C (PKC) [42] and the depolarization of voltage-dependent calcium channels [21,29]. G-proteins function as one of the mediators between receptors and cellular signaling effectors. Indeed, G-proteins are implicated in a broad spectrum of signaling pathways, such as an adenylyl cyclase [34,35] and a phospholipase C (PLC) [30]. The adenylyl cyclase regulates protein kinase A (PKA) via elevating cAMP, and PLC regulates intracellular
calcium level and protein kinase C via generating inositol tri-phosphate or diacylglycerol.

Cholera toxin (CTX), an exotoxin produced by *Vibrio cholerae*, is composed of a toxic A subunit covalently linked to a pentamer of B subunits. The A subunit is an enzymatic component and ADP-ribosylates the α-subunit of the G stimulatory (Gs) protein which dissociates from the βγ components, thereby activating the adenylate cyclase system. The increase of intracellular cAMP causes the increase of intracellular calcium levels and activating serum response element (SRE) or cAMP response element (CRE), suggesting that B subunit is also involved in the astrocytic cellular signal transduction pathway [12].

In addition to CTX, the exposure of primary cultured rat astrocytes to pertussis toxin (PTX; or islet-activating protein) results in ADP-ribosylation of the three α-subunits of 41-, 40-, and 39-kDa G-proteins in astrocytes [13]. However, in contrast to CTX, PTX inhibits the action of certain G-proteins, such as Gi and Go. The PTX-sensitive G-proteins are implicated in several physiological processes. For example, PTX-sensitive G-proteins have been reported to modulate the mitogenesis [23] and the formation of arachidonic acid or prostaglandins in astrocytes [14].

Although in cultured bovine adrenal chromaffin cells, both CTX and PTX were reported to enhance the proENK gene expression as well as [Met]enkephalin production [10,32], the effect of these toxins on proENK gene expression in astrocytes has not been currently characterized. Thus, in the present study, we examined the effects of CTX and PTX on the expression of proENK mRNA in relation to the proto-oncogene expression and CREB phosphorylation in cultured rat astrocytes. In addition, the effect of glucocorticoid on the CTX- or PTX-stimulated proENK expression was also investigated.

2. Materials and methods

2.1. Primary astrocyte enriched culture

Primary astrocyte-enriched cultures were prepared from the whole cortex of 1-day-old Sprague–Dawley rats. The cortex was rapidly dissected in ice-cold calcium/magnesium free Hank’s balanced salt solution (HBSS) (Life Technologies, Inc., MD) at pH 7.4. The tissue was minced and incubated in HBSS containing trypsin (2 mg/ml) for 20 min at 37°C and washed twice in plating medium; Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), 2.2 g/l sodium bicarbonate, 0.6% (w/v) d-glucose, and 20 mg/l gentamicin, and then disrupted by trituration. The cells were plated on 25 cm² culture flasks (Falcon, Franklin, NJ). The cultures were incubated at 37°C in 5% CO₂, and after 1 day the medium was completely changed to the culture medium: DMEM containing 5% heat-inactivated FBS, 2.2 g/l sodium bicarbonate, 0.6% (w/v) d-glucose, and 20 mg/l gentamicin. The cultures received half exchanges with fresh medium twice a week. After 14–15 days the cells were shaken for at least 1 day on the orbital shaker to remove the microglia, then the cells formed a dense monolayer that was composed mostly of type I astrocytes (95%), which were mostly positive for glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, MO) and rarely positive for OX42 (about 4%) (Serotech, NY), and negative for the A2B5 (Boehringer Mannheim, Mannheim, Germany) orurophil filament 68 (Sigma) antigen [37]. The cells were incubated with fresh culture medium for 24 h prior to the incubation with drugs.

2.2. Isolation of total RNA and proteins

Total cellular RNA were extracted from primary cultured astrocytes using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate [5]. Total cellular RNA in the aqueous phase was precipitated with cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted twice with an equal volume of sterile water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer (6 M guanidium chloride, 20 mM Tris–HCl [pH 8.0] and 1 mM EDTA). The protein samples were dialyzed against a renaturing buffer (20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl₂, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol) at 4°C. The concentration of protein was determined with the Coomassie Blue protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin (BSA) as a standard.

2.3. Non-isotopic Northern blot analysis

Five micrograms of total RNA was denatured and electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon hybridization membrane sheet (Amersham Pharmacia Biotech, NJ). After UV-cross
linking, the membranes were prehybridized at 68°C in prehybridization buffer (5×SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent). The digoxigenin (DIG)-labeled proENK probes were added to prehybridization buffer and incubated overnight. After the hybridization, the membranes were washed three times in 2×SSC and 0.1% SDS at room temperature, and then in 0.1×SSC and 0.1% SDS in 68°C. After equilibrating the membranes in maleic acid buffer (100 mM maleic acid and 150 mM NaCl [pH 7.5]), the membranes were blocked in maleic acid buffer containing 1% blocking reagent (Roche, IN). The chemiluminescent autoradiography detection was performed as suggested by the manufacturer (Roche) using alkaline phosphatase-conjugated anti-DIG F(ab)2 fragment (Roche) and CSPD® (Roche).

For stripping and rehybridization, blots were briefly washed in sterile Millipore water, then 50 mM Tris–HCl, pH 8.0, 50% dimethylformamide and 1% SDS for 0.5 h at 65°C, and rehybridized to DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase. The cRNA probes for proenkephalin [45], and cyclophilin [9] were synthesized in vitro from linearized The membranes were baked at 80°C for 20 min and then in 0.1×SSC and 0.1% SDS in 68°C. After equilibrating the membranes in maleic acid buffer (100 mM maleic acid and 150 mM NaCl [pH 7.5]), the membranes were blocked in maleic acid buffer containing 1% blocking reagent (Roche, IN). The chemiluminescent autoradiography detection was performed as suggested by the manufacturer (Roche) using alkaline phosphatase-conjugated anti-DIG F(ab)2 fragment (Roche) and CSPD® (Roche).

2.4. Western blot analysis

Fifty micrograms of total cellular protein was separated by electrophoresis in 8% polyacrylamide gels, electro-transferred to polyvinylidene difluoride (PVDF) filter and blocked with blocking buffer (3% skimmed milk, 1% BSA 10 mM trizma base [pH 8.0], 150 mM NaCl) and then incubated with antiserum against c-Fos (1:1000) (Santa Cruz Biotech., CA, USA), Fra-1 (1:1000) (Santa Cruz Biotech), Fra-2 (1:1000) (Santa Cruz Biotech), c-Jun (1:1000) (Santa Cruz Biotech), JunB (1:1000) (Santa Cruz Biotech), JunD (1:1000) (Santa Cruz Biotech), CREB (1:1000) (New England Biolabs, Beverly, MA), phospho-CREB (1:1000) (New England Biolabs) in blocking buffer for 4 h at room temperature. Filters were then washed three times with Tris-buffered saline containing 0.3% Tween-20 (TBST; 10 mM trizma base [pH 8.0], 150 mM NaCl, 0.3% Tween-20 for 5 min and then incubated with the goat anti-rabbit donkey IgG–horseradish peroxidase conjugate (1:5000) in blocking buffer at room temperature for 1 h. After washing the filters with TBST for 10 min (three times), ECL-plus solution (Amersham Life Science Co., UK) was added. Then, the membranes were exposed to Hyperfilm-MP (Amersham) for detection of light emission.

2.5. Non-isotopic electrophoretic mobility-shift assay

The AP-1 (5’-CGC-TTG-ATG-CTA-GGG-GCC-GGA-3’; Santa Cruz Biotechnology), CRE (5’-CTA-GTG-ATG-ACG-GCC-GGA-TC-3’; Santa Cruz Biotechnology), and ENKCRE-2 (5’-CTA-GTG-ATG-CAG-CCG-GAT-C-3’; Korea Biotech, Dae-Ku, Korea) were annealed by incubating an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl at 95°C for 10 min, and then the mixture was allowed to cool to room temperature. The DNA-binding assay was performed by following the instructions in the manual provided with the DIG-Gel Shift Kit (Boehringer Mannheim). Binding reactions were carried out at room temperature for 20 min and reaction mixtures contained 50 µg of total protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween20, 50 µg/ml poly (dl-dC), and approximately 0.3 pmol of specific probe labeled with DIG-ddUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Protein–DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% polyacrylamide (30:1, acrylamide:bisacrylamide) gels. Gels were run at room temperature in 89 mM Tris (pH 8.3), 89 mM boric acid, and 2 mM EDTA at a constant voltage (8 V/cm), and electroblotted onto positively charged nylon membranes. The membranes were baked at 80°C for 15 min, washed with 0.3% Tween 20 in Buffer I, and hybridized with the diluted anti-DIG–alkaline phosphatase (1:10,000 [75 mU/ml]) in Buffer II for 30 min. After two washes for 15 min with 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III for 2 min. The method for detection of chemiluminescence was identical to the method used for the non-isotopic Northern blot analysis.

2.6. Image quantification and statistical analysis

The expression of proENK mRNA induced by drugs was quantified with Bio-profil Bio-ID application (Vilber-Lourmat, France), and expressed as the percentage of the control proENK mRNA levels. One-way analysis of variance (ANOVA) was used to test for overall statistical significance. Multiple comparisons between groups were made using Fisher’s Least Significance Test.

3. Results

3.1. The effect of CTX and PTX on the expression of proENK mRNA in primary cultured astrocytes

All data shown in the present study were repeated at least three times and showed the same tendency of results. The proENK mRNA level in rat astrocytes was examined up to 8 h after CTX (0.1 µg/ml) treatment. As shown in Fig. 1A, the proENK mRNA level began to increase significantly 4 h and further increased 8 h after CTX treatment (about 10-fold). To examine the effect of glucocorticoid on CTX-induced increase in proENK mRNA level, dexamethasone (DEX, 1 µM) was co-
Fig. 1. The effects of cholera or pertussis toxin on the expression of proenkephalin mRNA in primary cultured astrocytes. Northern blot analyses were performed at specific time-points (0.5, 4, 8 h) in cells treated with (A) control medium, 0.1 μg/ml cholera toxin (CTX) alone, 1 μM dexamethasone (DEX) alone, DEX and CTX together, 15 μM cycloheximide (CHX) alone, CHX and CTX together, and (B) control medium, 0.1 μg/ml pertussis toxin (PTX) alone, DEX alone, DEX and PTX together, 15 μM CHX alone, CHX and PTX together. CHX was pre-treated for 30 min prior to the incubation with CTX. DEX was co-treated with CTX. Five micrograms of total RNA were used for determining proenkephalin (proENK) mRNA levels in Northern blot method as described under Materials and methods. The unregulated mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in the column graph indicate the S.E.M. (**P<0.01, ***P<0.001 compared to the control group; *P<0.05, **P<0.01 compared to the CTX or PTX group; n=3 independent experiments).

treated. The elevation of proENK mRNA level induced by CTX treatment was potentiated significantly by the co-treatment with DEX (about 2.2-fold as much as CTX alone at 8 h after treatment). However, DEX did not affect the basal level of proENK mRNA expression. To examine the possible involvement of on-going protein synthesis in CTX-induced increase in proENK mRNA level, cells were pretreated with cycloheximide (CHX, 15 μM), a non-
specific protein synthesis inhibitor. The pre-treatment with CHX did not change the elevation of proENK mRNA level induced by CTX treatment.

As shown in Fig. 1B, in contrast to the effect of CTX, the proENK mRNA level was not increased by PTX (0.1 μg/ml) treatment at all time-points tested. However, co-treatment with DEX and PTX markedly increased the level of proENK mRNA (about six-fold as much as control at 8 h after treatment) although DEX only did not affect the basal proENK mRNA level. In addition, the pretreatment with CHX also increased proENK mRNA level (about 5.2-fold as much as control at 8 h after treatment) in PTX treated cells without altering the basal proENK mRNA level when treated with CHX only.

3.2. The effects of RU486 and CHX on CTX-induced proENK mRNA expression

To investigate the involvement of glucocorticoid receptor in DEX-dependent increase of CTX-induced proENK mRNA transcription, RU486, a steroid receptor antagonist, was pretreated 30 min prior to DEX and CTX treatment. As shown in the Fig. 2A, the increase of CTX-induced proENK mRNA level was potentiated by co-treatment with DEX, and which was reduced by the pretreatment with RU486.

To investigate the involvement of on-going protein synthesis on DEX-dependent potentiation of CTX-induced proENK mRNA level, CHX was pretreated 30 min prior to DEX and CTX treatments. As shown in the Fig. 2B, the pretreatment with CHX did not affect DEX-dependent potentiation of CTX-induced increase in proenkephalin mRNA level.

3.3. The effect of CTX and PTX on the expression of AP-1 in primary cultured astrocytes

Western blot analyses using antibodies against c-Fos, Fra-1, Fra-2, c-Jun, JunB, and JunD proteins were carried out to examine whether there were correlations between proENK mRNA expression and proto-oncoprotein levels. c-Fos and Fra-2 protein levels were markedly increased 4 h after CTX administration (Fig. 3A). The pretreatment with CHX effectively reduced CTX-induced Fra-2 protein level. However, although CTX-induced c-Fos protein level was effectively inhibited by pretreatment with CHX 4 h after CTX treatment, it was further increased by the pre-treatment with CHX at 8 h after CTX treatment. The co-

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Fig. 2. The effect of RU486 or cycloheximide on cholera toxin-induced proenkephalin mRNA expression in primary cultured astrocytes. Northern blot analyses were performed at a specific time-point (8 h) in cells treated with (A) control medium, 0.1 μg/ml cholera toxin (CTX) alone, 1 μM dexamethasone (DEX) alone, DEX and CTX together, 10 μM RU486 alone and RU486 and CTX together, and (B) control medium, CTX and DEX, 15 μM cycloheximide (CHX) alone, and CTX, DEX, and CHX together. RU486 and CHX were pre-treated for 30 min prior to the co-incubation with CTX and DEX. Five micrograms of total RNA were used for determining proenkephalin (proENK) mRNA levels in Northern blot method as described under Materials and methods. The unregulated mRNA encoding cyclophilin (CPN) was used as an internal loading control. The vertical bars in the column graph indicate the S.E.M. (***P<0.001, **P<0.01, *P<0.05 compared to the control group; **P<0.01 compared to the CTX or PTX group; *P<0.05, compared to the CTX plus DEX group; n=3 independent experiments).
Fig. 3. The effect of cholera or pertussis toxin on the expression of AP-1 proteins in primary cultured astrocytes. Western blot analyses were performed at specific time points (0.5, 4, 8 h) in cells treated with (A) control medium, 0.1 μg/ml cholera toxin (CTX) alone, 1 μM dexamethasone (DEX) alone, DEX and CTX together, 15 μM cycloheximide (CHX) alone, CHX and CTX together, and (B) control medium, 0.1 μg/ml pertussis toxin (PTX) alone, DEX alone, DEX and PTX together, 15 μM CHX alone, CHX and PTX together. CHX was pre-treated for 30 min prior to the incubation with CTX. DEX was co-treated with CTX. The phorbol-13-myristate-12-acetate (PMA; 2.5 μM) treated group was used as expression control of each Jun protein. Fifty μg of cellular proteins were used for determining of c-Fos, Fra-1, Fra-2, c-Jun, JunB, and JunD protein levels. Polyclonal antibodies against these proteins were used at a 1:1000 dilution.

3.4. The effect of CTX and PTX on total and phosphorylated CREB in primary cultured rat astrocytes

To determine if there was a correlation between the expression of proENK mRNA level and phosphorylation of CREB protein, Western immunoblot analyses for CREB and phosphorylated CREB (a phosphorylated at Ser133) expression were performed (Fig. 4A). CTX, CHX, and CTX plus CHX did not affect the basal level of total CREB at all time points. However, phosphorylated CREB level was markedly increased at 0.5 h (about 6.3-fold) and returned to the basal level 8 h after CTX treatment.
Fig. 4. The effect of cholera or pertussis toxin on the phosphorylation of CREB in primary cultured astrocytes. Western immunoblot analyses were performed at specific time-points (0.5, 4, 8 h) in cells treated with (A) control medium, 0.1 μg/ml cholera toxin (CTX) alone, 1 μM dexamethasone (DEX) alone, DEX and CTX together, 15 μM cycloheximide (CHX) alone, CHX and CTX together, and (B) control medium, 0.1 μg/ml pertussis toxin (PTX) alone, DEX alone, DEX and PTX together, 15 μM CHX alone, CHX and PTX together. CHX was pre-treated for 30 min prior to the incubation with CTX. DEX was co-treated with CTX. Fifty micrograms of cellular proteins were used for determining total CREB and phosphorylated (Ser133) CREB protein levels. Polyclonal antibodies against CREB and phospho-specific CREB were used at a 1:1000 dilution.

addition, although the pretreatment with CHX increased the CTX-induced phosphorylated CREB level with small magnitude (about 1.1-fold as much as CTX alone), it persistently maintained CTX-induced increase of phosphorylated CREB level up to 8 h after the toxin treatment.

In addition to the effects of CTX, although PTX, CHX, and CTX plus CHX did not affect the basal level of total CREB at all time-points, phosphorylated CREB level was increased 0.5 h (about 2.4-fold) and returned to the basal level 4 h after CTX treatment (Fig. 4B). In addition, the pretreatment with CHX further enhanced the increase of CTX-induced phosphorylated CREB level at all time points (about 2.6–3.2-fold as much as PTX alone), although the treatment with CHX also increased the basal phospo-CREB level 4 h after drug treatment.

3.5. The effect of CTX and PTX on AP-1, CRE, or ENKCRE-2 DNA binding activity

As revealed by the electrophoretic mobility shift assay, the basal AP-1 and ENKCRE-2 DNA binding activities were extremely low, but they were markedly increased 0.5 h after CTX treatment (Fig. 5). In addition, the increases of AP-1 and ENKCRE-2 DNA binding activities induced by CTX were inhibited by the pre-treatment with CHX at all time-points. The co-treatment with DEX also reduced the CTX-induced increase in AP-1 and ENKCRE-2 DNA binding activities at 8 h after the toxin treatment. Although the basal CRE DNA binding activity was higher than AP-1 or ENKCRE-2 DNA binding activity, it was also enhanced by CTX, and which was inhibited by either CHX or DEX (Fig. 5B).

The diminution of AP-1, CRE, and ENKCRE-2 DNA binding activities by self-competition with 50× excess of unlabeled (cold) probes indicates that these binding activities were specific (Fig. 5C). The cross-competitions between AP-1, CRE, and ENKCRE-2 showed that the factors that recognize AP-1 DNA oligomer can bind CRE and ENKCRE-2 DNA oligomers, and the factors that recognize ENKCRE-2 DNA oligomer can bind AP-1 and CRE DNA oligomers. However, the factors that recognize CRE DNA oligomer did not bind AP-1 and ENKCRE-2 DNA oligomers.

In contrast to the effects of CTX on AP-1, CRE, and ENKCRE-2 DNA binding activities PTX did not affect the basal level of total ENKCRE DNA binding activity at all time-points tested (Fig. 5B).

4. Discussion

This study has demonstrated that CTX increases the proENK mRNA expression in astrocytes. This observation is in agreement with a previous study that the exposure of primary cultured bovine adrenal chromaffin cells to CTX results in an increase in enkephalin peptide levels [10]. However, our data are not consistent with a study in bovine adrenal chromaffin cells, in which the treatment
Fig. 5. The effect of cholera or pertussis toxin on the DNA/protein binding activities of AP-1, CRE, and ENKCRE-2 in primary cultured astrocytes. Electrophoretic mobility gel shift analyses of AP-1, CRE, and ENKCRE-2 were performed at specific time points (0.5, 4, 8 h) in cells treated with (A) control medium, 0.1 μg/ml cholera toxin (CTX) alone, 1 μM dexamethasone (DEX) alone, DEX and CTX together, 15 μM cycloheximide (CHX) alone, CHX and CTX together, and (B) control medium, 0.1 μg/ml pertussis toxin (PTX) alone, DEX alone, DEX and PTX together, 15 μM CHX alone, CHX and PTX together. CHX was pre-treated for 30 min prior to the incubation with CTX. DEX was co-treated with CTX. Fifty micrograms of total proteins were used for determining AP-1 DNA binding activity. For self- or cross-competition study (C), the electrophoretic mobility shift assays were performed in the presence or absence of 50-fold molar excess of unlabeled double stranded oligonucleotide (AP-1, CRE, or ENKCRE-2).

with an equal concentration of PTX caused a pronounced increase in met-enkephalin secretion as well as proENK mRNA level [32], suggesting that the mechanism underlying Gs stimulation- or Gi inhibition-evoked proENK mRNA level in astrocytes and chromaffin cells may be different.

No alteration of CTX-induced proENK mRNA level by CHX suggests that CTX exerts its action on the induction of proENK mRNA expression via using pre-existing proteins rather than newly synthesized protein factors. However, in the presence of PTX, the pretreatment of astrocytes with CHX elevated the proENK mRNA level, suggesting that certain pre-existing proteins inhibit the action of PTX leading to up-regulate proENK mRNA expression.

In vitro transfection assays have suggested that AP-1 is a critical factor for the proENK transcription in various cell types such as PC12 cells [24] and bovine chromaffin cells [1,38]. Therefore, in the present study, to investigate the possible involvement of AP-1 in the proENK mRNA
expression, the effect of CTX or PTX on Fos and Jun expression was examined. CTX increases the protein levels of c-Fos and Fra-2 but not Fra-1, c-Jun, JunB, and JunD. However, PTX failed to increase the protein levels of c-Fos and Fra-2 as well as Fra-1, c-Jun, JunB, and JunD. CTX-induced selective increases of c-Fos and Fra-2 is similar to an observation in forskolin-stimulated astrocytes, in which forskolin also increased c-Fos and Fra-2 protein levels, while it did not affect Fra-1, c-Jun and JunD protein levels [39].

Although three Jun proteins were not induced by CTX, the correlation of phosphorylated CREB level and proENK mRNA expression suggests the involvement of phosphorylated CREB, rather than AP-1 expression in the increases of AP-1 protein levels as well as AP-1, ENKCRE-2 or CRE DNA binding activity. In the present study, we found that CTX was able to increase the DNA binding activity of AP-1 as well as CRE and ENKCRE-2. Because the Fos leucine zipper has been reported to form a relatively unstable homodimer, the instability of homodimers provides a thermodynamic driving force for a preferential heterodimer formation with Jun [25]. Therefore, it is still obscure how CTX increases an AP-1 DNA binding activity without altering the levels of Jun proteins. Although the explanations for these findings are not clearly revealed, some unknown Jun-like protein that can form heterodimers with Fos or the basally expressed Jun family protein might be involved in CTX-induced increase of AP-1 DNA binding activity.

In contrast to proENK mRNA, CTX-induced c-Fos or Fra-2 protein level and AP-1, CRE, or ENKCRE-2 DNA binding activities were effectively reduced by the pretreatment with CHX, suggesting that CTX-induced c-Fos and Fra-2 increases may not be involved in the increase of proENK mRNA expression. This hypothesis is further supported by the result from the co-treatment with DEX, where although DEX increased CTX-induced proENK mRNA level, it reduced CTX-induced c-Fos or Fra-2 protein level as well as AP-1, ENKCRE-2 or CRE DNA binding activity. In addition, in PTX-treated cells, although DEX and CHX increased proENK mRNA level, there were no changes in the expression of AP-1 and DNA binding activity of ENKCRE-2. Therefore, these results suggest that the increases of AP-1 proteins and AP-1, CRE, or ENKCRE-2 DNA binding activity may not be involved in CTX-induced increase of proENK mRNA expression and its enhancement by DEX. Furthermore, in PTX-treated cells, DEX- or CHX-dependent increase of proENK mRNA expression may also be involved in the increases of AP-1 and ENKCRE-2 DNA binding activity.

In the mobility shift assay of CRE, we found the basal CRE DNA binding activity is much higher than the basal AP-1 or ENKCRE-2 DNA binding activity. In addition, although CHX inhibited CTX-induced increase of CRE DNA binding activity, its basal activity was not affected by the pretreatment with CHX, suggesting that pre-existing factors as well as newly synthesized protein factors may be involved in the formation of CRE DNA binding activity in the basal or CTX-stimulated astrocytes. Among the CRE binding proteins, CREB has been known to possess a transactivity via phosphorylating specific serine residues rather than increasing of its protein level [4]. In the present study, total CREB level was not altered by CTX, DEX, CHX, or any combination of these drugs. However, the phosphorylated form of CREB protein is increased by CTX and to a lesser extent by PTX. In addition, phosphorylated CREB level was further increased by the pretreatment of CHX in PTX-treated cells similar to an action of CHX on proENK mRNA. Although there was no remarkable enhancement of CTX-induced CRE phosphorylation by CHX at 1 h after treatment, it caused the prolongation of early-phosphorylated CREB level when astrocytes were stimulated by CTX up to 8 h.

The correlation of phosphorylated CREB level and proENK mRNA expression suggests the involvement of phosphorylated CREB, rather than AP-1 expression in the regulation of CTX-induced or CHX-dependent PTX-induced proENK mRNA expression. This result is similar to the result observed in forskolin-stimulated astrocytes, in which forskolin-induced proENK mRNA and AP-1 protein levels were well attenuated by CHX, while phosphorylated CREB protein level was further increased by CHX. Indeed, the involvement of phosphorylated CREB on proENK has been demonstrated in rat striatum, where phosphorylated CREB rather than Fos actively participates in the transactivation of proENK gene expression in the rats treated with haloperidol [22]. However, in PMA-stimulated astrocytes, even further enhancement of CREB phosphorylation by CHX, the pretreatment of CHX effectively inhibited PMA-induced proENK mRNA as well as AP-1 protein levels [39,40]. Therefore, even the increase of phosphorylated CREB level in astrocytes, its participation in the up-regulation of proENK mRNA expression may be determined according to the types of stimulus.

The several previous studies have suggested that glucocorticoid enhances the cAMP-dependent increase in proENK mRNA expression in C6 rat glioma cells [18]. However, prednisolone and DEX inhibit the normal proENK gene expression in B cells [2]. Therefore, the role of glucocorticoid in the proENK gene expression appears to have a cell or tissue specificity. In the present study, we observed that DEX synergistically increases the proENK transcription in CTX- or PTX-treated astrocytes. Since, CHX failed to alter the proENK mRNA level induced by DEX plus CTX, this enhancing effect induced by DEX appears not to be mediated through the newly synthesized certain transcription factors. In addition, c-Fos and Fra-2 protein levels or AP-1 and ENKCRE-2 DNA binding activities were reduced by the DEX. Furthermore, the co-treatment with DEX did not change the CTX- or PTX-induced phosphorylation of CREB. This lack of correlation observed between proENK mRNA expression and the increase of AP-1 factors or phosphorylated CREB suggests that the synergistic effect of glucocorticoid on proENK...
gene expression in CTX- or PTX-treated astrocytes may not be mediated through ENKCRE-2, AP-1, or CRE. This result is in line with the result observed in C6 rat glioma cells, in which c-fos transcription is also negatively controlled by glucocorticoids [41]. Although the mechanism underlying glucocorticoid-dependent permissive synergism in proENK mRNA expression has not been clearly understood, one of the possible mechanisms might be the presence of a glucocorticoid response element in the proENK promoter or enhancer region. Indeed, the transient transfection assay has suggested that GRE+ can mimic the response of the endogenous proENK gene to DEX and forskolin, and intensify the possibility of the presence of a positive GRE site in a proENK promoter/enhancer region [17].

Although several previous studies have suggested AP-1 is a major factor in the regulation of proENK gene expression, in the present study, these AP-1 factors appear not to be involved in CTX-induced proENK mRNA expression. In addition, PTX alone did not affect the proENK mRNA level. However, the blockade of protein synthesis caused the enhancement of proENK mRNA level in the presence of PTX, suggesting that a certain pre-existing protein (this may also inhibit the basal AP-1 factors expression) may inhibit the PTX-mediated signals to activate the proENK gene expression. Since phosphorylated CREB level was further increased by the blockade of protein synthesis, the phosphorylation of CREB has a good correlation with the proENK transcription. Previous studies have suggested that AP-2, AP-4, NFkB, and NF-1 also bind to the proENK promoter regions and regulate the expression of the proENK gene [6–8,16,27]. These studies have implied that the regulation of proENK gene expression is not as simple as the recognition of ENKCRE-2 enhancer domain by CREB or AP-1. Furthermore, it was shown that only a fraction of cultured astrocytes can express proENK mRNA [20]. Therefore, the CTX-induced changes of proENK mRNA level could either be due to changes in cells already expressing this mRNA or the induction of expression in previously non-expressing cells. Indeed, there is a possibility that the regulatory mechanism underlying proENK mRNA expression by CTX or PTX could be different according to populations. The exact nature of population specific induction of proENK mRNA expression by CTX or PTX needs to be more fully examined.

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