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Regulation of CCK mRNA in the human neuroepithelioma cell line SK-N-MCIXC in response to second messenger activators

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Regulation of cholecystokinin (CCK) expression was studied in the human neuroepithelioma cell line SK-N-MCIXC. The cells were treated with the phosphodiesterase inhibitor isobutyl-methylxanthine and the tumor promoting phorbol ester, phorbol-12-myristate 13-acetate; activators of the cyclic AMP (cAMP) and protein kinase C (PKC) second messenger pathways, respectively. Levels of CCK mRNA were determined after 6, 12 and 24 hour drug treatments, with Northern blot analysis using human CCK cDNA hybridization probes. Activation of both cAMP and PKC second messenger pathways increased CCK mRNA levels in SK-N-MCIXC cells. These results indicate that the levels of CCK mRNA in SK-N-MCIXC cells are regulated by cAMP and PKC dependent mechanisms.

Cholecystokinin; Neuronal cell line; Neuropeptide; Gene expression

1. INTRODUCTION

The biologically active brain-gut peptide CCK is synthesized in a cell specific manner in the intestinal tract, the central nervous system (CNS) [1–3], the pituitary [4], and in male germ cells [5]. CCK is present in high concentrations in several brain regions, including areas of the cerebral cortex, which are thought to mediate sensory, motor and associative processes [6,7]. A large number of studies indicate that brain CCK, predominantly present as the octapeptide CCK-8, functions as a neurotransmitter or neuromodulator [8,9]. Several biological functions associated with central CCK include effects that are directly related to the GI tract, for instance effects on gastric acid secretion, satiety and feeding behaviors [2,10–12].

The role which CCK plays is linked to the mechanisms that regulate its biosynthesis and metabolism. Since processing and metabolic enzymes are distributed similarly throughout the brain, biosynthetic rates of peptide precursors are generally determined by cellular contents of their encoding mRNA's [13]. Northern blot analysis and in situ hybridization with specific gene probes have demonstrated that proCCK mRNAs are expressed in specific brain regions (referenced in Monstein et al. [14]). Additionally, Liddle et al. [15] found that stimulation of intestinal CCK secretion is associated with an increase in CCK mRNA which results from increased CCK gene transcription.

The abundance of CCK mRNA is determined by its stability and by the rate of transcription, regulated by transcription factors, from its DNA template. In many eukaryotic cells, cell specific transcription factors are modulated through second messenger systems, primarily the cyclic adenosine 3′,5′-monophosphate (cAMP) and protein kinase C (PKC) pathways. Both cAMP and phorbol ester response elements (CRE and TRE) have been identified and characterized on the rat CCK promoter/enhancer, located upstream of the mRNA initiation (CAP) site [16]. The DNA sequence of the rat and human CCK promoter/enhancer region is highly conserved [16], therefore CREs and TREs should also be present in the human CCK gene.

In this study we investigated the effects of second messengers on CCK mRNA levels in the neuroepithelioma cell line SK-N-MCIXC, which was established after twice cloning the heterogeneous human neuroepithelioma cell line SK-N-MC. The SK-N-MC cell line produces CCK specific mRNA; however, it exhibits poor postranslational processing of CCK prohormonal material, yielding only high molecular weight CCK precursors and no immunoreactive CCK [17]. The SK-N-MCIXC cell line expresses the human CCK gene at very high levels and CCK-like peptides immunoreactive to a C-terminal CCK octapeptide antiserum are present in the cell line and its medium [18]. Additionally, SK-N-MCIXC cells cleave proCCK to completion (i.e. CCK-8) and demonstrate spontaneous and regulated release of CCK and CCK precursors [19]. This cell line should therefore provide a good in vitro model in which to study both the tissue-specific control of the induction of the CCK gene and the mechanisms responsible for the differential protein processing of the CCK precursor in neuronal systems. As a first step in examining the mech-

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anisms which influence CCK biosynthesis in these cells we have measured mRNA levels in SK-N-MCIXC cell preparations in the presence of isobutylmethylxanthine (IBMX), 8-bromo-cAMP, phorbol-12-myristate 13-acetate (PMA) and 4α-phorbol.

2. MATERIALS AND METHODS

2.1. *Cell culture and drug treatment*

SK-N-MCIXC cells were plated in 172 cm^2 culture flasks in RPMI containing 10% bovine calf serum, defined, non-iron supplemented, (Hyclone, Logan, Utah), 1% penicillin/streptomycin (Sigma, St. Louis, MO) and 1% non-essential amino acids (Irvine Scientific, Santa Ana, CA). The flasks were placed in a 37°C humidified incubator, with a 5% CO_2 atmosphere. The cells were split weekly with a hypo-osmolar solution [20]. The cell line was tested with the Gen-probe mycoplasma test kit (Gen-Probe Inc., San Diego, CA) and found to be negative for mycoplasma contamination.

Approximately 2 million cells were plated in 100 × 20 mm tissue culture dishes, Falcon 3003 (Becton Dickson Labware, Lincoln Park, NJ) and grown for 4 days prior to treatment. Cells were treated with fresh media supplemented with 0.5 mM IBMX, 0.5 μM PMA and dimethyl sulfoxide (DMSO) as a vehicle control, for 6, 12 and 24 h. In addition, 0.5 mM 8-bromo-cAMP and 0.5 μM 4α-phorbol were used at the 12 h time point (all drugs purchased from Sigma, St. Louis, MO). Treatments were done in duplicate for each individual experiment. After the appropriate time the treated cells were lifted with a hypoosmolar solution, washed and resuspended in 1 ml of phosphate buffered saline. The cells were counted and 6 million cells were aliquoted into Eppendorf tubes for RNA isolation.

2.2. *Preparation of RNA and Northern blot analysis*

Total RNA was extracted from SK-N-MCIXC cells with an RNAid Plus Kit (BIO 101, La Jolla, CA). Cells were lysed with a guanidine isothiocyanate buffer and extracted with phenol–chloroform. RNA was further purified with RNAmatrix. The samples were precipitated with ethanol and resuspended in diethylpyrocarbonate (DEPC) treated H_2O. RNA in each sample was spectrophotometrically quantified at 260 nm with a Beckman model 25 spectrophotometer. Purity of the samples was checked by an additional reading taken at 280 nm. Typically a ratio of 1.6–1.8 was obtained.

Prior to electrophoresis RNA samples and RNA ladder (0.24–9.5 kb; Gibco/BRL, Grand Island, NY) were treated with 0.7% (v/v) formaldehyde, 50% (v/v) deionized formamide and 1 × MOPS (20 mM 4-morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) for 15 min at 55°C. Ethidium bromide was added to RNA ladder samples prior to loading. Electrophoretic separation of RNA samples was performed on a 1% (w/v) agarose gel containing 1 × MOPS and 6% (v/v) formaldehyde submerged in 1 × MOPS. The gel was run for 2 h at 100 mV, with the buffer circulated with a peristaltic pump. The RNA ladder was viewed on a short-wave UV box and the marker positions recorded. The samples were transferred to a Zeta-Probe GT nylon membrane (Bio-Rad, Richmond, CA) by capillary action with 6 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na_3-citrate · $2H_2O$). RNA was fixed to membranes by UV illumination in a UV Stratalinker 1800 (Stratagene, La Jolla, CA) using the Autocrosslink function.

2.3. *Hybridization and cDNA probes*

The CCK probe was a 550 bp *AluI-AluI* fragment of exon 2 of the human CCK gene [21]. The β-actin probe was a 1 kb chicken cDNA fragment. The cDNA probes were labeled using a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemica, Indianapolis, IN). [^{32}P]dCTP, 3000 Ci/mmol (NEN, Boston, MA), was used to label the probe.

Membranes were prehybridized in a heat sealing bag with a solution of 250 mM NaCl, 250 mM $NaHPO_4$, 50% (v/v) deionized formamide and 7% (w/v) sodium dodecyl sulfate (SDS) for 30 min at 43°C in a shaking water bath. Hybridization was carried out at 43°C for 12–16 h in fresh buffer with 50 ng of [^{32}P]dCTP labeled cDNA probe. The membranes were washed with vigorous shaking with 2 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS for 15 min each at 25°C. A final 15 min wash with 0.1 × SSC, 0.1% SDS was performed at 65°C with shaking. The moist membrane was sealed in a heat sealing bag and exposed to Kodak X-Omat AR scientific imaging film (Eastman Kodak Co., Rochester, NY) in a cassette with intensifying screens at −70°C for 2–3 days.

2.4. *Data analysis and statistics*

Autoradiograms were analyzed using a Bio-Rad model 620 densitometer (Richmond, CA). CCK levels were standardized against β-actin levels. Non-treated controls were normalized to 100. Second messenger treated values were normalized against the controls. Statistical analysis was performed using the Student's *t*-test for grouped data. Significance was set at $P < 0.05$.

3. RESULTS

To examine the affects of second messenger systems on CCK mRNA levels in SK-N-MCIXC cells, these cells were treated with activators of the cAMP or PKC regulatory systems. IBMX (0.5 mM), a phosphodiesterase inhibitor which elevates intracellular cAMP levels and 8-bromo-cAMP (0.5 mM), a non-hydrolyzable analog of cAMP, were used to investigate the effect of increasing cAMP levels on CCK mRNA in SK-N-MCIXC cells. PMA (0.5 μM) a tumor promoter and potential activator of phospholipase dependent PKC, was used to examine the effect of the PKC system on CCK mRNA levels. The cells were also treated with the inactive phorbol ester, 4α phorbol (0.5 μM), to determine PMA specificity in this system. Additionally PMA and IBMX were added to the cells in combination, to reveal possible synergistic interactions between the two second messenger pathways. Cells were also treated with the DMSO vehicle. DMSO had no effect on CCK mRNA levels at any time point (6 h, $n = 4$, df = 6, $t = 0.276$, N.S.; 12 h, $n = 4$, df = 6, $t = 0.271$, N.S.; 24 h, $n = 3$, df = 4, $t = 0.699$, N.S.) Levels of CCK mRNA treatments were determined by Northern blot analysis with a human CCK cDNA hybridization probe (Fig. 1).

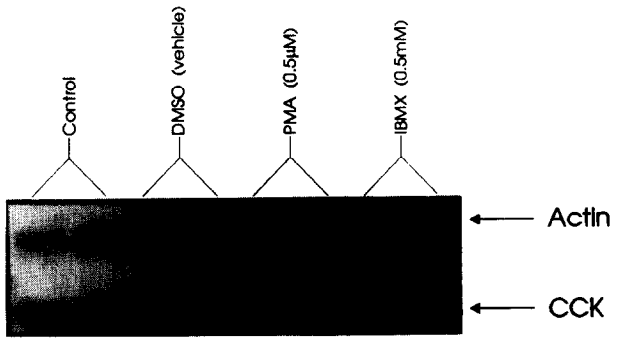

Fig. 1. Northern blot analysis with a human CCK cDNA hybridization probe demonstrating CCK mRNA bands after 12 h treatments with control media, DMSO, IBMX, PMA and PMA in combination with IBMX.

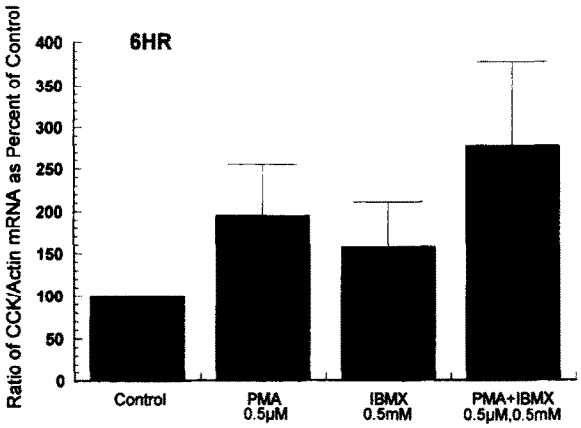

Fig. 2. Activation of both cAMP and PKC second messenger pathways increase CCK mRNA levels in SK-N-MCIXC cells. After 6 h treatments both PMA and IBMX raised the levels of CCK mRNA. PMA raised CCK levels 2.0-fold, IBMX raised the levels 1.6-fold and the drug combination increased CCK mRNA levels 2.8-fold when compared to non-drug treated controls.

Our results demonstrate that activation of both cAMP and PKC second messenger pathways increases CCK mRNA levels in SK-N-MCIXC cells. After 6 h treatments (Fig. 2) both PMA and IBMX raised CCK mRNA levels. PMA raised the levels 2.0-fold ($n = 4$, df = 6, $t = 1.605$, N.S.) in comparison to controls, IBMX raised CCK levels 1.6-fold ($n = 4$, df = 6, $t = 1.072$, N.S.). The two drugs in combination increased CCK mRNA levels 2.8-fold ($n = 4$, df = 6, $t = 1.784$, N.S.).

After 12 h treatments (Fig. 3) our results were similar to those seen at 6 h. PMA increased CCK mRNA levels in SK-N-MCIXC cells approximately 2.5-fold ($n = 4$, df = 6, $t = 5.949$, $P < 0.01$), IBMX raised the levels 1.8-fold ($n = 3$, df = 4, $t = 2.758$, $P < 0.05$), and the drug combination increased CCK mRNA levels 2.7-fold ($n = 3$, df = 4, $t = 4.615$, $P < 0.01$) (Fig. 3). At the 12 h time point we did two additional treatments. To the test the affect of a second activator of the cAMP pathway we treated the cells with 8-bromo-cAMP, this produced a 1.4-fold increase in CCK mRNA levels ($n = 3$, df = 4, $t = 3.053$, $P < 0.05$) (Fig. 3). We also treated the cells with 4α phorbol, as expected this drug had no effect on CCK mRNA levels ($n = 3$, df = 4, $t = 0.562$, N.S.) (Fig. 3).

At 24 h we continue to see an increase in response to both PMA and IBMX. However, the value for PMA, a 1.6-fold increase compared to the control, starts to decrease ($n = 3$, df = 4, $t = 1.185$, N.S.) in relation to CCK mRNA levels in response to PMA after 6 and 12 h treatments. Conversely, IBMX increased CCK mRNA levels in SK-N-MCIXC cells approximately 2.5-fold ($n = 4$, df = 6, $t = 5.949$, $P < 0.01$) somewhat higher than at previous time points. PMA combined with IBMX raised CCK mRNA levels 3.3-fold ($n = 3$, df = 4, $t = 5.206$, $P < 0.01$) compared to controls.

4. DISCUSSION

Recently, Haun and Dixon [16] found that the rat CCK promoter/enhancer region contains CREs and TREs. Sequence homology between the promoter/enhancer region of rat and human CCK suggests that these elements would also be present in the human CCK gene. The results in this paper demonstrate that second messengers affect the abundance of CCK mRNA in the homogenous human neuroepithelioma cell line SK-N-MCIXC. The increase in CCK mRNA levels is most likely due to increased CCK gene transcription and suggests that SK-N-MCIXC cells contain both CREs and TREs. Our values for CCK mRNA levels in response to PMA and IBMX, are comparable to values seen by

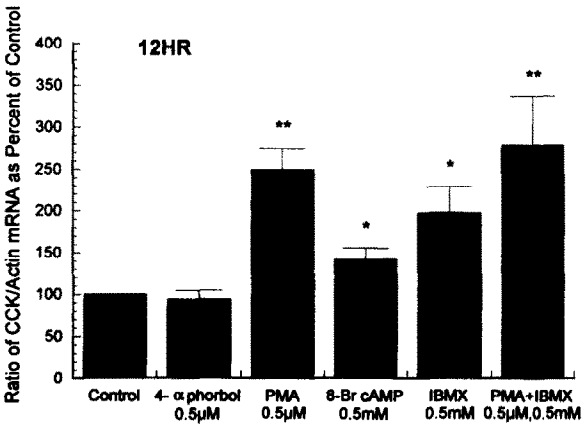

Fig. 3. Twelve hour histogram demonstrating increased CCK mRNA levels in response to PMA (2.5-fold), IBMX (1.8-fold), 8-bromo-cAMP (1.4-fold), and the PMA/IBMX combination (2.7-fold). Four α-phorbol had no effect on CCK mRNA levels. $*P < 0.05$, $**P < 0.01$.

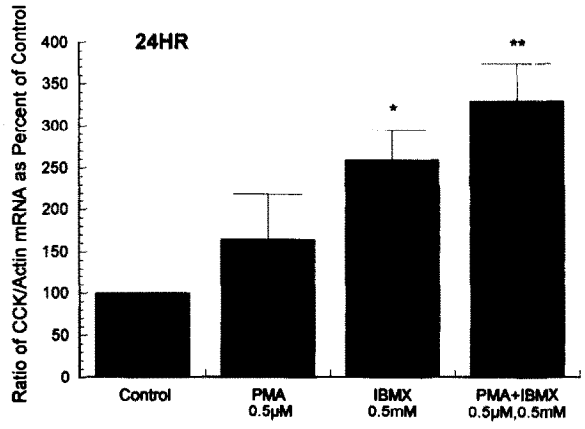

Fig. 4. At 24 h CCK mRNA levels in response to PMA (1.6-fold), IBMX (2.6-fold) and the drug combination (3.3-fold) are still increased relative to the control. At this time however, the value for PMA has begun to decrease when compared to PMA values after 6 and 24 h. $*P < 0.05$; $**P < 0.01$.

Monstein and Folkesson [22] in the heterogeneous cell line SK-N-MC. However, when these activators were added in combination to SK-N-MCIXC cells they did not produce an additive response, whereas in SK-N-MC cells there was an approximate 2.5-fold increase over either individual drug, after 12 h treatments [22]. These results indicate that SK-N-MCIXC cells do not respond additively to these drugs at the concentrations tested, or CCK mRNA levels in SK-N-MCIXC cells reach a maximum response, of approximately a 3-fold increase over controls, under these experimental conditions.

Presently information on the control of CCK gene induction or the mechanisms responsible for protein processing of the CCK precursor at different anatomical loci of CCK production is limited at the molecular biological level. It is important to identify the mechanisms that control the induction of the CCK gene and additionally to further clarify the molecular and physiological control mechanisms responsible for protein processing of CCK precursors, both peripherally and in the CNS. Although ultimately these questions should be approached in vivo, the SK-N-MCIXC cell line; which unlike the parental SK-N-MC cell line, not only expresses CCK prohormonal material, but also carries out posttranslational peptide processing [17,19]; is a unique model system in which we can begin to elucidate some of the mechanisms involved in neuronal CCK expression and processing.

SK-N-MCIXC cells, in addition to expressing CCK mRNA, demonstrate regulated and constitutive release of proCCK and its cleaved products, including CCK-58, CCK-22 and CCK-8 [19]. These cells also contain mRNA for the intracellular processing enzymes prohormone convertase 1 and 2 (PC1, PC2) and furin [19] which preferentially cleave prohormones at the carboxyl side of pairs of basic amino acid residues [23–25]. The presence of PC1, PC2 and furin mRNA in SK-N-MCIXC cells suggests that they may cleave at the dibasic amino acid pair Arg-Arg of the C-terminal part of proCCK, as is predicted from the most frequent proprotein processing site motifs [26]; or they may also cleave at an Arg-X-X-Arg arrangement, a special class of paired basic amino acid residue-processing motif, which is present at the N-terminal of CCK-58 in the proCCK sequence. Distribution of mouse PC2 (mPC2) transcripts in the cortical and subcortical areas of the brain also suggests an involvement of mPC2 in the processing of proCCK [27].

Additionally, SK-N-MCIXC cells also express the extracellularly localized, membrane-associated CCK-metabolizing enzyme neutral endopeptidase 24.11, which is also present in rat cerebral cortex rich in CCK and other brain regions high in CCK [19]. In light of the above information and the results from this study, we believe that SK-N-MCIXC cell line is a good tool to study drug, neurotransmitter and steroid effects on neuronal CCK biogenesis and processing.

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