Volume 293, number 1,2, 145–148 FEBS 10404 November 1991

Phorbol 12-myristate-13-acetate (PMA) stimulates a differential expression of cholecystokinin (CCK) and c-*fos* mRNA in a human neuroblastoma cell line

Hans-Jürg Monstein and Ronnie Folkesson

Department of Clinical Biochemistry, KK 3014, State University Hospital (Rigshospitalet), DK-2100 Copenhagen, Denmark

(Received 21 August 1991)

Regulation of cholecystokinin (CCK) and the proto-oncogene c-*fos* mRNA expression was studied in the human neuroblastoma cell line SK-N-MC. Cells were treated either with the tumor promoting phorbolester phorbol-12-myristate-13-acetate (PMA), the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), which results in an elevated intracellular cyclic AMP (cAMP) level, or with a combination of PMA and IBMX. The level of CCK and c-*fos* mRNA was determined by Northern-blot analysis with CCK and c-*fos* specific antisense RNA probes after 4–24 h of drug treatment. Treatment with PMA and IBMX for 4–24 hours transiently raised the CCK mRNA level ~1.5–3.5 times compared to the controls, and the combination PMA and IBMX had an additive effect and elevated CCK mRNA abundance 1.5–6.5 times. Under the same experimental conditions, both PMA and IBMX elevated the c-*fos* mRNA level ~3–5.5 times. The drug combination showed a pronounced synergistic effect and raised the c-*fos* mRNA level ~3–20 times as compared to controls. Apparently, CCK and c-fos mRNA expression appears to be regulated by similar protein kinase C (PKC) and cAMP-dependent mechanisms in SK-N-MC cells.

Cholecystokinin (CCK); C-*fos*; Phorbol ester; cAMP; mRNA; Northern blot

1. INTRODUCTION

Neuropeptide gene expression in eukaryotic cells involves the induction of transcription factors which in term bind to specific *cis*-acting DNA elements surrounding the coding region of a gene [1–3]. These mechanisms use signal transduction pathways common to many types of eukaryotic cells. Signals received at the cell surface regulate the production of second messengers such as cAMP and diacylglycerol which activate protein kinase A and C, respectively. This cascade then leads to the activation of (cell)-specific transcription factors and thereby inducing expression of selected genes. [2,4–8].

Certain proto-oncogenes such as c-*fos* and *jun* are inducible by second messengers and the products of the proto-oncogenes act themselves as transcription factors [9–11]. The c-*fos* promoter contains different types of regulatory elements, which are essential for basal and stimulated c-*fos* mRNA expression. Two elements, responsible for basal c-*fos* transcription, have been identified and it was found that these elements resemble the consensus sequence of cAMP-regulated promoters (reviewed in [10,11]. An upstream enhancer region, located between −317 to −292, is required for the induction of the c-*fos* gene with e.g. serum and phorbol esters.

Correspondence address: H.-J. Monstein, Department of Clinical Biochemistry, KK 3014, State University Hospital (Rigshospitalet), DK-2100 Copenhagen, Denmark

Cholecystokinin (CCK) mRNA and bioactive CCK peptides are expressed in a cell-specific manner in the central nervous system, the intestinal tract, and in male germ cells [12–14]. Thus, several groups have investigated clonal cell lines which express CCK at the transcriptional and translational level [15–21]. In a recent study it was shown that the rat CCK gene contains a transcriptional enhancer, essential for the expression of CCK mRNA. Moreover, the rat CCK enhancer shows structural similarities with the human CCK, enkephalin A (PEA), and c-*fos* genes, containing cAMP-(CRE) and phorbolester-like response elements (TRE) [18,22].

In this study we describe experiments to investigate the effects of PMA, an activator of protein kinase C (PKC) and, IBMX, a phosphodiesterase inhibitor, and a combination of both drugs on the modulation of CCK and c-*fos* mRNA expression in a human SK-N-MC cell line.

2. MATERIALS AND METHODS

2.1. *Cell culture and drug treatment*

SK-N-MC cells were originally obtained from the Dept. of Pathology, Uppsala University, and maintained at the Dept. of Pharmacology as described earlier [15,18]. SK-N-MC cells from passage 120 were cultured in Nunc-Petridishes (10 cm) at 37°C in 10 ml Ham's F10 and Dulbeccos modified Eagles medium (1:1), supplemented with 15% fetal calf serum, 10 ml/l non-essential amino acids (Gibco, 1000×stock solution), 190 mg/l L-glutamine, and 10 ml/l penicillin/streptomycin (10 000 U and 10 000 μg/ml) in humidified air containing 5% CO_2. Cell cultures were grown to a density of 10^7 cells/dish and harvested at appropriate times in phosphate-buffered saline (137 mM NaCl, 2.7

mM KCl and 1.5 mM Na_2HPO_4-KH_2PO_4; pH 7.4) supplemented with 0.5 mM EDTA. A cell pellet was obtained by centrifugation (1500 × *g*) at 4°C for 10 min in autoclaved Eppendorf tubes. Cells were treated with 0.5 mM IBMX, 0.5 μM PMA or the combination of both drugs as indicated in figure legends and harvested as above.

2.2. *Preparation of total RNA and Northern blot analysis*

Total RNA from 5×10^6 cells was extracted according to the method of Chomczynski and Sacchi [23], and quantitated by UV absorption at 260/280 mm. Electrophoretic separation of total RNA was on a 1.2% agarose–0.7% formaldehyde gel followed by transfer onto Hybond N membrane (Amersham, Birkerød, Denmark) in 25 mM sodium phosphate buffer (pH 6.4). Filters were fixed by UV illumination (Stratagene, UV-box). Prior to transfer, RNA preparations were routinely checked for degradation by ethidium bromide staining of the agarose gel.

2.3. *Hybridization and antisense RNA probes (cRNA)*

Hybridization was performed as described earlier [24]. Following hybridization, bands were visualized by exposure for 1–3 days at −80°C using a DuPont intensifier screen to Fuij RXO-1G or Amersham MP films. Densitometric scanning of the Northern blots was carried out on a LKB-Pharmacia XL laser densitometer. The levels of CCK and c-*fos* mRNA signals were determined relative to the mouse β-actin hybridization signal.

The construction and in vitro transcription of the rat CCK and mouse β-actin DNA templates were recently described [15]. The c-*fos* DNA template was purchased from Amersham. For each hybridization probe, ~1 μg plasmid DNA was in vitro transcribed either by RNA polymerase SF6 or T7 using Promegas Riboprobe Kit and 50 μCi [α-^{32}P]UTP (400 Ci/mmol).

3. RESULTS

Phorbol-12-myristate-13-acetate (PMA) a tumor promotor and potential activator of phospholipase dependent protein kinase C was used to study the effect on CCK and c-*fos* mRNA expression in SK-N-MC cells. To reveal possible synergistic interactions between protein kinase C and cAMP-dependent regulatory systems, treatment of SK-N-MC cells with IBMX or the combination PMA and IBMX was tested. Levels of CCK and c-*fos* mRNA in total RNA extracts were determined by Northern blot analysis using rat CCK and human c-*fos* antisense (cRNA) RNA hybridization probes. Northern blot filters initially hybridized with the CCK and c-*fos* cRNA probes, were re-probed with a mouse β-actin cRNA probe, thus allowing determination of the relative CCK and c-*fos* mRNA levels in each slot. The results of these experiments are summarized in Fig. 1. The time course revealed that the drugs used had different effects on both CCK and c-*fos* mRNA expression. PMA stimulated CCK mRNA expression ~2.5-fold with a maximum around 12 to 24 h (Fig. 1A). Similar results were obtained using the phosphodiesterase inhibitor IBMX. The CCK mRNA level rose ~2.3-fold after 12 h. The drug combination of PMA and IBMX had a small additive effect and elevated the CCK mRNA level ~6.5-fold, compared to controls (no drugs). In PMA-treated cells, it appeared that the elevated CCK mRNA level did not change significantly between 12–24 h, whereas in IBMX-, or PMA- and

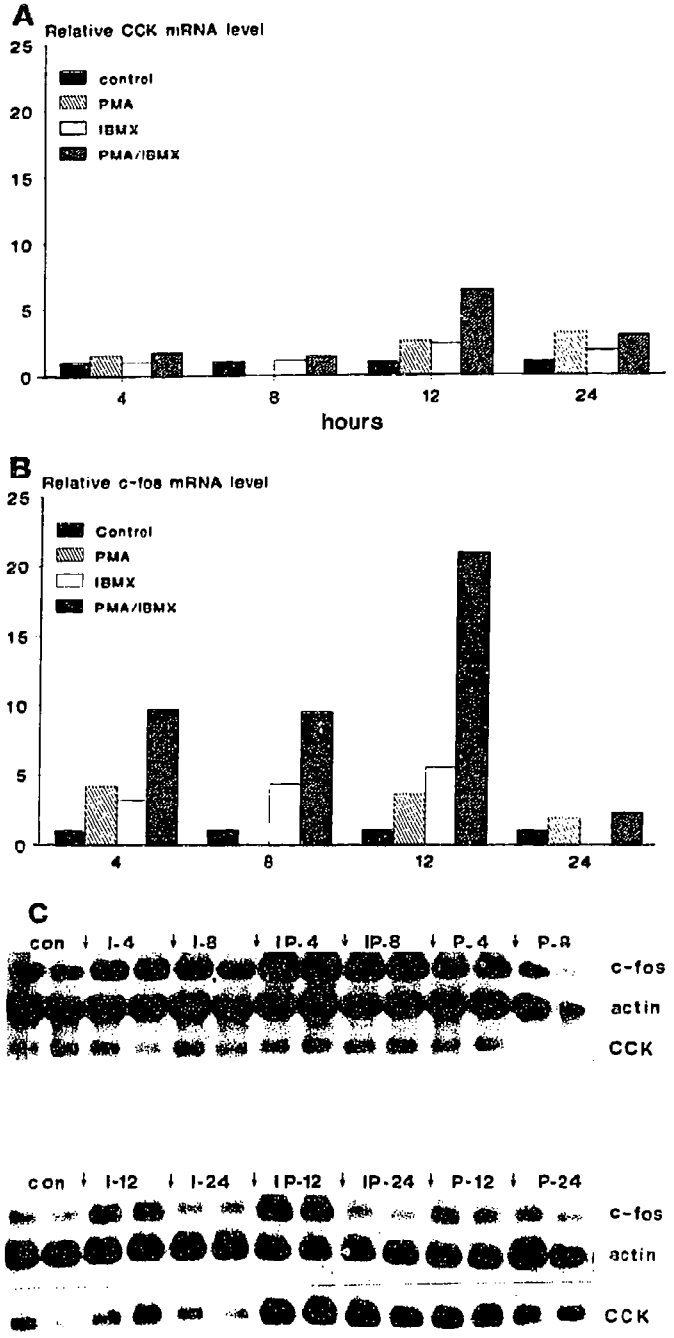

Fig. 1. Time course of the effects of PMA and IBMX, alone or in combination on the CCK (A) and c-*fos* mRNA (B) abundance in SK-N-MC cells. Cultures of SK-N-MC cells were treated with 0.5 μM PMA and/or 0.5 mM IBMX for the times indicated. The mRNA abundances are expressed as ratios to the control level at zero times (no drug). Each lane received 15 μg total RNA and autoradiograms were derived from successive hybridization of the same Northern blot (C). Histograms represent the mean densitometric values obtained from two experiments. (Con, control, no drugs; I, IBMX; P, PMA).

IBMX-treated cells, a slight decrease of the CCK mRNA levels was observed (Fig. 1A).

Under the experimental conditions described, the c-

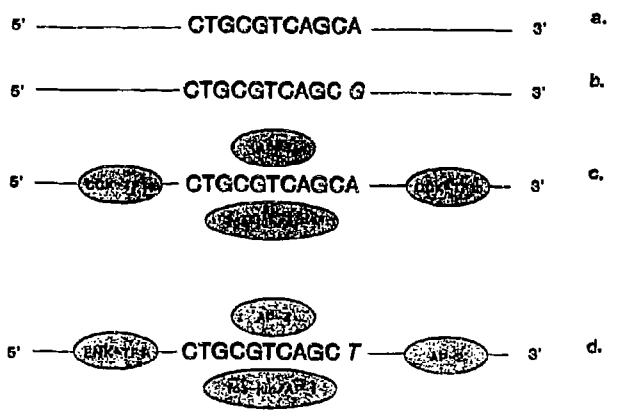

Fig. 2. Schematic illustration of the conserved c-*fos* −292 element (a), present in the human CCK (b), rat CCK (c), and human PEA (d) enhancers. Italics in (b) and (d) indicate base differences. *trans*-Acting factors, identified by DNA binding assays [1,22] are cross-hatched.

fos mRNA level increased ~4 to 6-fold but with an apparently different time course. In cells treated with PMA or IBMX, the c-*fos* mRNA content elevated ~4 times after 4 h and remained at this level. The combination of PMA and IBMX showed a synergistic effect on c-*fos* mRNA expression. After 4 h of treatment with PMA and IBMX the c-*fos* mRNA level increased ~10 times. A further increase of c-*fos* mRNA was observed after 12 h, where it reached its maximum expression (~20 times above controls), which then gradually fell back to the control level after 24 h (Fig. 1B). Obviously, the combination PMA and IBMX had its maximal synergistic effect on c-*fos* mRNA expression at 8–12 h treatment. These findings were confirmed by a different set of experiments. In three different experiments, SK-N-MC cells were treated for 10 h with PMA (0.5 μM), IBMX (0.5 mM) or PMA and IBMX in combination. PMA increased CCK mRNA 3.18 ± 0.70 and c-*fos* mRNA 3.31 ± 0.63 (SEM; n = 3)-fold that of controls. Similar results were obtained with IBMX, where CCK mRNA increased 2.65 ± 0.38 and c-*fos* mRNA 3.13 ± 0.37 (SEM; n=3)-fold as compared to the controls. The synergistic effect of the combination PMA and IBMX on c-*fos* mRNA or the additive effect of the drug combination on CCK mRNA modulation as observed during the time course was confirmed. The CCK mRNA content increased 5.38 ± 0.35-fold and the c-*fos* mRNA 13.09 ± 1.68 (SEM; n=3)-fold. In previous experiments we were able to show that noradrenaline and dbcAMP increased the level of CCK mRNA to a maximum of ~1.5–2.5 times that of controls between 9 and 18 h [18], which is in agreement with the results of the present study using IBMX.

4. DISCUSSION

Recent studies of the human neuroblastoma cell line SK-N-MC have revealed that expression of the CCK gene is modulated by a cAMP dependent mechanism at the mRNA level [18,21]. Using deletion mutation analysis and DNA footprinting it was recently shown that the rat CCK promoter/enhancer, located distally to the mRNA initiation (CAP) site, contains phorbol ester (TRE) and cAMP response elements (CRE) [22]. Phorbol esters exert its effects by activating protein kinase C, which may directly effect gene activity, whereas IBMX is capable of inhibiting cAMP phosphodiesterase activities [5,25] and thereby elevating intracellular cAMP levels.

Surprisingly, it was found that the DNA sequence of the rat and human CCK promoter region between −119 and −81 are not only highly conserved but also contain an identical 5′-CTGCGTCAGC-3′ element, present within the c-*fos* −296 element [22], known to be important for the transcriptional regulation of the c-*fos* gene [10,11]. Moreover, the responsive element 5′-CTGCGTCAGC-3′, found in the rat and human CCK gene at −85, is also identical to the human proenkephalin A (ENKCRE-2) element (Fig. 2), which has been shown to bind a variety of transcription factors [3,6] such as AP-1, AP-4, and CREB, which are inducible by PMA, cAMP, or both. This study shows that in SK-N-MC cells not only c-*fos* mRNA but also the CCK mRNA abundance is modulated by PMA, IBMX, or the drug combination. However, their mRNA levels appear to be differently regulated by the drugs used in this study (Fig. 1). This suggests that in human SK-N-MC cells, phorbolester and cAMP inducible transcription factors may bind to similar putative TRE and CRE DNA sequences present in both the human CCK and c-*fos* genes. We therefore conclude that the elevated CCK and c-*fos* mRNA levels may be due to increased transcription of the CCK and c-*fos* genes. However, it can not be excluded that changes in mRNA stability also contribute to the change in CCK and c-*fos* mRNA levels.

From the data it also appears that the c-*fos* mRNA increase precedes the elevation of CCK mRNA (Fig. 1). Previous studies revealed that c-*fos* mRNA expression in the spinal cord after peripheral inflammation and in the hippocampus after seizure [26–28] precedes the increase of proenkephalin A (PEA) and prodynorfin (DYN) mRNA, respectively. Findings, which led to the conclusion that the PEA and the DYN genes may be a target for the *fos-jun*/AP-1 complex, which binds to an AP-1 DNA motif, thereby modulating basal and enhanced PEA and DYN mRNA expression [9,26,28]. Based on these observations and the fact that the CCK and PEA genes share structurally related regulatory elements [1,18,22] and Fig. 2) it cannot be excluded that the c-*fos* gene product may act in concert with *jun*/AP-1 [29,30] on the putative AP-1 response element, and thereby modulating CCK mRNA expression. Alternatively, c-*fos* may activate CCK mRNA expression in an indirect way by inducing transcription of cell specific

transcription factor(s), necessary for the elevated CCK mRNA expression described in this study.

In conclusion, it appears that maximal activation of both the PKC and PKA pathways in SK-N-MC cells may subsequently lead to the induction of basal and cell specific CCK and c-*fos* transcription factors, which bind to putative TRE and CRE elements, thereby modulating a co-ordinate but differential expressin of CCK and c-*fos* mRNA in the human neuroblastoma cell line SK-N-MC. Experiments to establish the nature of the transcription factor(s) and the DNA binding sites, involved in the modulation of CCK mRNA expression, are in progress in our laboratory.

Acknowledgements: The technical assistance of S. Hummelgaard and Ch. Vienberg is greatly appreciated. We thank T. Geijer (Uppsala) for supporting us with SK-N-MC cells and initial help in setting up the experiments; K. Pedersen and W.W. van Solinge for help in designing the computergraphs. We also thank Dr J.F. Rehfeld and K. Pedersen for critical reading and comments on the manuscript. This work was supported by the Danish MRC, The Danish Cancer Society, and the Lundbeck Foundation (to R.F.).

REFERENCES

[1] Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. and Goodman, H.M. (1988) EMBO J. 7, 3793–3805.
[2] Goodman, R.H. (1990) Annu. Rev. Neurosci. 13, 111–127.
[3] Hyman, S.E., Comb, M., Lin Y.-S., Pearlberg, J., Green, M.R. and Goodman, H. (1988) Mol. Cell. Biol. 8, 4225–4233.
[4] Farago, A. and Nishizuka, Y. (1990) FEBS Lett. 268, 350–354.
[5] Houslay, M.D. (1991) Eur. J. Biochem. 195, 9–27.
[6] Hyman, S.E., Comb, M., Pearlberg, J. and Goodman, H.M. (1989) Mol. Cell. Biol. 9, 321–324.
[7] Montminy, M.R., Gonzalex, G.A. and Yamamoto, K.K. (1990) Trends Neurosci. 13, 184–188.
[8] Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J. Biol. Chem. 263, 9063–9066.
[9] Morgen, J.I. and Curran, T. (1989) Trends Neurosci. 12, 459–462.
[10] Ransone, L.J. and Verma, I.M. (1990) Annu. Rev. Cell Biol. 6, 39–57.
[11] Verma, I.M. and Sassone-Corsi, P. (1987) Cell 51, 513–514.
[12] Persson, H., Rehfeld, J.F., Ericsson, A., Schalling, M., Pelto-Huikko, M. and Hökfelt, T. (1989) Proc. Natl. Acad. Sci. USA 86, 6166–6170.
[13] Rehfeld, J.F. (1989) in: S.G. Schultz, G.M. Makhlouf and B.B. Rauner (eds.) Handbook of Physiology: The Gastrointestinal System, Oxford University Press, New York, pp. 337–358.
[14] Rehfeld, J.F. (1988) J. Mol. Endocr. 1, 87–94.
[15] Geijer, T., Folkesson, R., Rehfeld, J.F. and Monstein, H.-J. (1990) FEBS Lett. 270, 30–32.
[16] Haun, R.S., Beinfeld, M.C., Ross, B.A. and Dixon, J.E. (1989) Endocrinology 125, 850–856.
[17] Lapps, W., Eng, J., Stern, A.S. and Gubler, U. (1988) J. Biol. Chem. 263, 13456–13462.
[18] Monstein, H.-J., Folkesson, R. and Geijer, T. (1990) J. Mol. Endocrin. 4, 37–41.
[19] Schneider, B.S., Helson, L., Monahan, J.W. and Friedman, J.M. (1989) J. Clin. Endocrin. Metab. 69, 411–419.
[20] Verbeeck, M.A.E. and Burbach, J.P.H. (1990) FEBS Lett. 268, 88–90.
[21] Verbeeck, M.A.E., Draaijer, M. and Burbach, J.P.H. (1990) J. Biol. Chem. 265, 18087–18090.
[22] Haun, R.S. and Dixon, J.E. (1990) J. Biol. Chem. 265, 15455–15463.
[23] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[24] Monstein, H.-J. and Geijer, T. (1989) Biosci. Reports 8, 255–261.
[25] Bressler, J.P. and Tinsely, P. (1990) J. Neurosci. Res. 25, 81–86.
[26] Draisci, G. and Iadarola, M.J. (1989) Mol. Brain Res. 6, 31–37.
[27] Fitzgerald, M. (1990) Trends Neurosci. 13, 439–440.
[28] White, J.D. and Gall, C.M. (1987) Mol. Brain Res. 3, 21–29.
[29] Caceres, J., Glikin, G., Bravo, R. and Weinmann, R. (1990) Oncogene 5, 59–67.
[30] Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988) Cell 54, 541–552.