Journal of Cellular Biochemistry 100:851–864 (2007)

CCK1 and 2 Receptors Are Expressed in Immortalized Rat Brain Neuroblasts: Intracellular Signals After Cholecystokinin Stimulation

Sonja Langmesser,[1] Maria I. Cerezo-Guisado,[2] Maria J. Lorenzo,[2] Luis J. Garcia-Marin,[1] and Maria J. Bragado[2]*

[1]Departamento de Fisiología, Biología Molecular y Genética, Universidad de Extremadura, Cáceres 10071, Spain
[2]Departamento de Bioquímica, Biología Molecular y Genética, Universidad de Extremadura, Cáceres 10071, Spain

Abstract Cholecystokinin (CCK) is one of the most abundant neuropeptides in the central nervous system (CNS) where it promotes important functions by activation of receptors CCK1 and CCK2. Our aim was to investigate CCK receptors expression and their downstream intracellular signaling in immortalized rat brain neuroblasts. Results show that CCK1 and CCK2 receptor mRNAs and CCK2 receptor protein are expressed in neuroblasts. CCK incubation of neuroblasts leads to stimulation in a time-dependent manner of several signaling pathways, such as tyrosine phosphorylation of adaptor proteins paxillin and $p130^{Cas}$, phosphorylation of p44/p42 ERKs as well as PKB (Ser473). Moreover, CCK-8 stimulates the DNA-binding activity of the transcription factor AP-1. The CCK2 receptor agonist gastrin stimulates ERK1/2 phosphorylation in a comparable degree as CCK does. ERK1/2 phosphorylation activated by CCK-8 was markedly inhibited by the CCK2 receptor antagonist CR2945. Incubation for 48 h with CCK-8 increases neuroblasts viability in a similar degree as EGF. In summary, our data clearly identify CCK1 and CCK2 receptor mRNAs and CCK2 receptor protein in brain neuroblasts and show that incubation with CCK promotes cell proliferation and activates the phosphorylation of survival transduction pathways. Stimulation of ERK1/2 phosphorylation by CCK is mainly mediated by the CCK2 receptor. Moreover, this work might provide a novel model of proliferating neuronal cells to further study the biochemical mechanisms by which the neuropeptide CCK exerts its actions in the CNS. J. Cell. Biochem. 100: 851–864, 2007.

Key words: cholecystokinin; receptors; neuroblasts; phosphorylation; signaling

Cholecystokinin (CCK) is a remarkable member of the brain–gut peptide family, being one of the most abundant neuropeptide in the central nervous system (CNS), particularly in the cerebral cortex, striatum, and hippocampus [Vanderhaeghen et al., 1975; Dockray, 1976]. Moreover, CCK is present in many neuronal pathways and it is co-localized with several classic neurotransmitters like dopamine [Beinfeld, 2001]. Several molecular forms of CCK of varying aminoacid length exist, being the sulphated octapeptide (CCK-8), the predominant form in the brain [Beinfeld, 2001; Tirassa et al., 2002]. CCK may act as a neurotransmitter or neuromodulator in many areas of the CNS where it is involved in a variety of behavioral functions such as satiety, anxiety, exploratory and locomotor activity, learning, and memory [Beinfeld, 2001; Tirassa et al., 2002]. Moreover, CCK also has non-behavioral effects on the CNS as a possible mediator of fever and hypothermia [Beinfeld, 2001]. It also has a neuroprotector effect against N-methyl-D-aspartate-receptor mediated glutamate cytotoxicity in cortical neurons [Akaike et al., 1991; Tamura et al.,

Grant sponsor: Ministerio de Ciencia y Tecnología; Consejeria de Sanidad y Consumo, Junta de Extremadura, Spain; Grant numbers: SAF-2001-0154, SCSS0430.

*Correspondence to: Maria J. Bragado, PhD, Departamento de Bioquímica, Biología Molecular y Genética, Facultad de Veterinaria, Universidad de Extremadura. Avda. de la Universidad, s/n, 10071 Cáceres, Spain.
E-mail: jbragado@unex.es

Received 3 November 2005; Accepted 9 October 2006
DOI 10.1002/jcb.21193

1992] and against the degeneration of cholinergic neurons in the cerebral cortex following basal forebrain lesion in rats [Tirassa et al., 2002], which is an animal model of a cholinergic deficit in the cerebral cortex, as it occurs in patients with Alzheimer's disease [Sugaya et al., 1992]. In addition, it has been postulated that CCK is involved in the development and special function of each neocortical area of the primate [Yamashita, 1992].

Two receptors mediate the effects of CCK: The CCK1 (previously CCK-A) and the CCK2 receptor [previously CCK-B) [Hill et al., 1987; Wank et al., 1992a,b]. The structures of both receptors are known and in humans show 48% homology. However, they differ in their distribution, their affinities for the natural agonists CCK and gastrin, and their affinities for a number of synthetic agonists and antagonists [Tirassa et al., 2002]. The CCK2 receptor is the predominant subtype in the CNS where it is widely distributed, however, it also occurs in abundance in the gastrointestinal tract [Hill et al., 1987; Wank et al., 1992a,b]. The CCK1 receptor has a more limited distribution with the highest densities in the hypothalamic nuclei, certain areas of the hippocampus, the septum, the dorsal motor vagal nucleus, and the interpeduncular nucleus of the brain stem [Hill et al., 1987; Wank et al., 1992a,b].

CCK receptors belong to the G-protein coupled receptor family and their activation leads to the stimulation of phospholipase C resulting in the generation of inositol phosphates and diacylglycerol, which release intracellular calcium and activate protein kinase C (for review, see Williams, 2001). The ability of CCK to activate these pathways has been extensively studied in pancreatic acinar cells, one of the most widely used target cells to study receptors and signaling mechanisms of CCK [Garcia and Jensen, 1998; Williams, 2001]. In these cells, regulation of growth, gene expression, translational control of protein synthesis, cytoskeletal organization, and energy metabolism by CCK have been shown to involve novel intracellular signal transduction mechanisms such as the mitogen-activated kinase cascades, PI3K-PKB/Akt-mTOR signaling, tyrosine phosphorylation pathways, or NF-κB signaling [Garcia and Jensen, 1998; Williams, 2001].

Despite the physiological relevance of CCK in the development and neuroprotection of the CNS, little is known about the intracellular pathways mediating CCK functions in neuronal cells from the mammalian brain. Therefore, the aims of this study were to investigate whether CCK1 and CCK2 receptors are expressed in immortalized rat brain neuroblasts and to elucidate the transduction pathways induced by CCK-8, such as ERK1/2 phosphorylation, PKB/Akt activation, and tyrosine phosphorylation of adaptor proteins. Additionally, we intended to establish a connection between CCK-8 stimulation and cell survival in rat brain neuroblasts.

MATERIALS AND METHODS

Cell Culture

Spontaneously immortalized rat brain neuroblasts, E18 cells [Muñoz et al., 1993], were kindly provided by Dr. A. Muñoz, Instituto de Investigaciones Biomédicas, CSIC Madrid, Spain. These cells were derived from 18-day-old fetal rat cerebral cortices and they were characterized as primitive neuroblasts that express the protein of neurofilament NF 68 and the primitive neuronal marker nestin but they lack the astrocyte marker, glial fibrillary acidic protein. Partial differentiation of these cells to neurons can be induced with dibutyryl-cAMP and then neuroblasts express additional neuronal markers such as neurofilament proteins NF 145, NF 220, and neuron-specific enolase (personal communication of Dr. Muñoz).

Cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and penicillin/streptomycin (100 U/100 μg/ml) at 37°C in a humidified 5% CO_2/95% air atmosphere. Cultures were passaged when confluent by trypsinization using trypsin–EDTA solution (Gibco BRL, Life Technologies, Paisley, Scotland).

Isolation of Total RNA and RT-PCR

Total RNA was isolated as previously described by Chomczynski and Sacchi [1987], followed by RQ1 DNase (Promega, Madison, WI) treatment. RNA integrity was checked on a 0.8% agarose gel.

cDNA was synthesized from 2 μg total RNA using MMLV-RT (Promega) according to manufacturer's instructions with 1 μg Oligo$(dT)_{15}$ (Promega) as primer. Ten microliters of the reaction were used as template for a first PCR round. To ensure absence of contaminating

genomic DNA, the same PCR was perfomed on non-reverse transcribed RNA. Primers were 5′-CTGTAGGAATACATGGAC and 5′-TCTGGAACTCTACCAAGG for CCK1 and 5′-GTGCTGATGGTGGTATAG and 5′-TGACAACGCTAACCACAC for CCK2, 35 PCR cycles (30 s denaturation at 94°C, 30 s annealing at 50°C, 740 s elongation at 72°C) were run. For a second round of amplification, 1/10 of the respective reactions was used as template. Primers were 5′-GGCTGTTACTTGCAGAGG and 5′-ATACCTGGACAGCAATGC for CCK1 and 5′-AAGCTGCTGGCTAAGAAG and 5′-TAGGAGGATCCTCATCTG for CCK2, 35 PCR cycles (as above but with annealing at 55°C) were performed. Products were run on a 1.5% agarose gel, bands were excised from the gel and purified using the GENECLEAN Turbo Kit (Q-BIO Gene, Carlsbad, CA) according to manufacturer's instructions. Product identity was confirmed to be CCK1 and 2 receptors by sequencing performed by a private scientific company (Valencia, Spain).

Cell Treatment and Protein Isolation

Cells were seeded in 100-mm dishes at a density of 15,000 cells/cm^2 and grown for 3 days in medium containing 7.5% FCS. Cells were washed twice with HAM's F12 and medium was changed to HAM's F12, 2 mM glutamine, and penicillin/streptomycin (100 U/100 μg/ml). After 24 h, cells were washed once more with HAM's F12 and fresh serum-free medium was added. Cells were left to equilibrate for at least 1 h. After stimulation with CCK-8 at the concentrations and times indicated, cells were lysed in 180 μl lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 10 mM $Na_4P_2O_7$, 10% glycerol, 1% Triton-X-100, 0.5 mM Na_3VO_4, and protease inhibitor cocktail (complete EDTA-free; Roche, Mannheim, Germany)]. Lysates were centrifuged at 13,000*g* and 4°C for 15 min, and protein concentration of the supernatant was determined using the method of Bradford [1976].

Immunoprecipitation

One hundred fifty micrograms of total protein were incubated with 3 μg anti-phosphotyrosine monoclonal antibody (PY20) (Transduction Laboratories, Lexington, KY), and 30 μl recombinant protein A-agarose beads (Upstate Biotechnology, Inc., Lake Placid, NY) overnight at 4°C in a total volume of 800 μl. Beads were washed twice with phosphate-buffered saline, resuspended in Laemmli buffer (0.2 M Tris/HCl pH 6.8, 0.2 M DTT, 4% SDS, 10% glycerol, 0.01% bromophenol, 5% ß-mercaptoethanol), and subjected to Western blotting analysis.

Western Blotting

Denatured proteins from total cell extracts or immunoprecipitates were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Dassel, Germany). Membranes were blocked in Blotto (50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 5% non-fat dry milk) and incubated with primary antibodies. Incubation conditions were: anti-phospho-ERK (Cell Signaling, Beverly, MA) 1:500 for 1.5 h at room temperature, anti-ERK (Sigma, St. Louis, MO) 1:20,000 for 1 h at room temperature, anti-phospho-PKB (BD Biosciences, San Jose, CA) 1:1,000 overnight at 4°C, anti-p130^{Cas} (Transduction Laboratories) 1:750 for 2 h at room temperature, anti-paxillin (Transduction Laboratories) 1:10,000 for 1.5 h at room temperature, anti CCK1 and anti-CCK2 receptor (Santa Cruz Biotechnology) obtained against the N- and C-terminal region of human origin, respectively, 1:200 for 2 h at room temperature. Membranes were washed twice with Blotto and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (anti mouse IgG 1:6,000 or anti rabbit IgG 1:10,000) for 45 min at room temperature. After three washes (10 min each) with 50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, membranes were incubated with Super Signal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min at room temperature and exposed to Hyperfilm ECL (Amersham, Piscataway, NJ). Results were analyzed by densitometry using the MacBAS Software, version 2.2, and expressed as fold increase over control. Protein extracts from the pancreas and brain used as tissue control in CCK receptor Western blot experiments were isolated from adult rats.

Viability Assay

Cell viability was determined by the colorimetric MTT assay [Mosmann, 1983]. Briefly, cells were seeded in 24-well plates at a density of 15,000 cells/cm^2 and allowed to attach overnight. Cells were washed twice with Ham's F12 and medium was changed to serum-free

medium alone or supplemented with 1 μM or 0.1 nM CCK-8 or 10 nM EGF. After 48 h, 0.5 mg/ml MTT (Sigma) was added to the medium, and cells were incubated for 1 h. Subsequently, medium was aspirated and 500 μl of acidic isopropanol were added to solubilize the formazan precipitates. Absorbance of samples was determined at a wavelength of 570 nm with background subtraction at 630–690 nm.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as previously described by Andrews and Faller [1991]. Briefly, after stimulation with 1 μM or 0.1 nM CCK-8, cells were lysed in 10 mM HEPES/KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PSMF, and incubated on ice for 10 min. Samples were centrifuged, supernatant was discarded, and the pellet was resuspended in 20 mM HEPES/KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PSMF, and protease inhibitor cocktail (complete EDTA-free; Roche). After incubation at 4°C in continuous agitation, samples were centrifuged for 5 min at 13,000*g* and 4°C, and protein concentration of the supernatant was determined using the BioRad Protein Assay (BioRad, Munich, Germany) according to manufacturer's instructions. Extracts were stored at −80°C.

Two oligonucleotides, 5′-AGCTTGATGAGTCAGCCG-3′ and 5′-GATCCGGCTGACTCATCA-3′, were annealed to yield a double-stranded oligonucleotide probe containing the AP-1 consensus sequence. For the annealing, 10 μg of each oligonucleotide were mixed, boiled at 95°C for 5 min, and allowed to cool overnight at room temperature in a buffer containing 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. The resulting double-stranded fragment was radioactively labeled using [α^{32}P]dCTP and the Prime-a-Gene Labeling System (Promega) according to manufacturer's instructions but substituting the included buffer by Klenow dilution buffer (Gibco BRL, Carlsbad, CA). Labeled DNA was purified by phenol extraction and subsequent ethanol precipitation and resuspended at a concentration of 200,000 cpm/μl.

Ten micrograms of the nuclear extracts were incubated with 0.1 μg/μl poly(dIdC)ñpoly(dIdC) (Sigma)/250 ng/μl salmon testis DNA (Sigma) and labeled probe (200,000 cpm) in 20 mM HEPES/KOH pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 1% glycerol, and 1 mM $MgCl_2$ in a total volume of 10 μl for 20 min at room temperature. For competition studies, nuclear extracts were preincubated with a 50-fold, 100-fold, and 500-fold excess of unlabeled probe for 10 min at room temperature prior to addition of labeled probe. One microliter loading buffer (10 mM EDTA, 445 mM Tris, 445 mM borate, 50% glycerol, 0.01% bromophenol blue) was added and samples were run on 6% polyacrylamide gels. Gels were fixed, dried, and exposed to X-ray film. Results were analyzed by densitometry using the MacBAS Software, version 2.2.

Statistical Analysis

All data provided are reported as mean ± standard error of the mean (SEM). In view of the Gaussian distribution of the data gathered, each sample was compared with control using Student's *t*-test. All analyses were performed using SPSS version 11.1 for Windows software (SPSS, Inc., Chicago, IL). The level of significance was set at $P \leq 0.05$.

RESULTS

Identification of mRNA CCK Receptors in Neuroblasts by RT-PCR Analysis and Protein CCK Receptors by Western Blotting

mRNA expression of CCK receptors in neuroblasts was analyzed by RT-PCR using isoform-specific primers based on the published sequence of rat CCK receptors [Wank et al., 1992b; Bragado et al., 2003]. PCR yielded two products of approximately 470 and 300 bp (Fig. 1), whereas nothing could be amplified from the original RNA preparation (data not shown), indicating that amplification products do not arise from DNA contaminations. Finally, we have confirmed that the sequences of the RT-PCR products coincide with sequences of either the CCK1 or CCK2 receptor cDNAs indicating that E18 cells express the mRNAs of both receptors.

Protein expression of CCK receptors in neuroblasts was analyzed by Western blotting using specific antibodies against CCK1 and CCK2 receptors. As seen in Figure 1B, the CCK2 receptor, which is highly expressed in brain homogenates from adult rats (Fig. 1B, lanes 3 and 4), is expressed in rat neuroblasts (lanes 5 and 6) and not expressed at all in rat pancreatic acini (lanes 1 and 2), as it is well

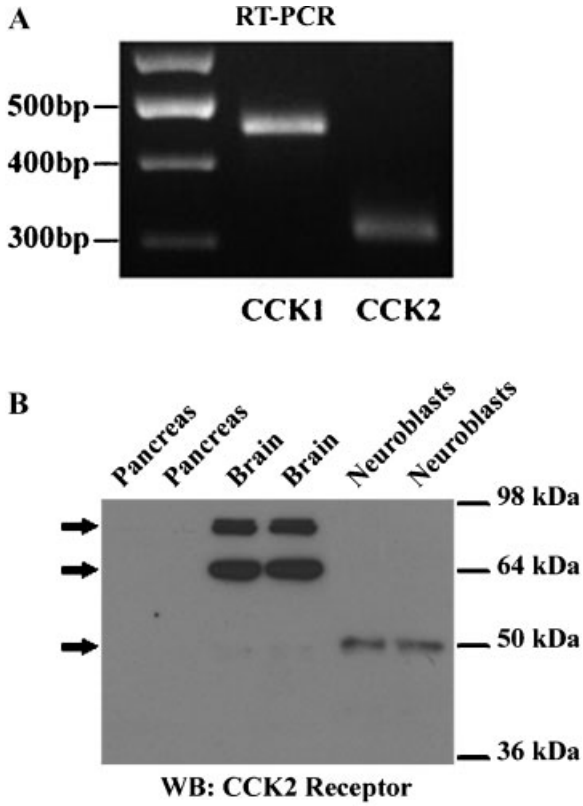

Fig. 1. Expression of mRNA (**A**) and protein (**B**) CCK1 and CCK2 receptors in rat brain neuroblasts. A: Total RNA from E18 cells was isolated and reverse transcribed to yield cDNA. By PCR using isoform-specific primers, the expected products (479 bp and 309 bp for CCK1 (**lane 2**) and CCK2 (**lane 3**) respectively, were amplified and visualized by gel electrophoresis. First lane, DNA ladder molecular weights are indicated on the left. B: Rat brain homogenates and whole cell lysates from isolated rat pancreatic acini and rat brain neuroblasts were analyzed by SDS–PAGE followed anti-CCK2 receptor Western blotting as described in Materials and Methods.

described [Zhou et al., 1995; Monstein et al., 1996; Morisset et al., 2000]. It is important to mention that the electrophoretic mobility of CCK receptor proteins is altered by the post-translational modification of glycosylation [Morisset et al., 2000]. Regarding the expression of the CCK1 receptor, in our experimental conditions, the antibody did not detect any band in rat brain neuroblasts at the correct molecular weight marker (data not shown). This antibody, however, shows a high expression of this receptor in rat pancreatic acini, as expected.

Tyrosine Phosphorylation of $p130^{Cas}$ and Paxillin in Neuroblasts in Response to CCK-8

We examined the functionality of CCK receptors by studying intracellular signaling pathways activated after incubation of neuroblasts with CCK-8. Among CCK-activated transduction pathways, those mediated by tyrosine phosphorylation have been of considerable attention [Garcia and Jensen, 1998]. We then investigated CCK-8-mediated tyrosine phosphorylation of the adaptor proteins, $p130^{Cas}$ and paxillin in neuroblasts using immunoprecipitation and Western blotting. Stimulation of neuroblasts with 0.1 nM CCK-8 rapidly induced tyrosine phosphorylation of $p130^{Cas}$ (Fig. 2) or paxillin (Fig. 3), which was found to be maximal after 5 min (2.7 ± 0.56 and 2.05 ± 0.41-fold increase over control, respectively, $P < 0.05$, $n = 4$). Interestingly, tyrosine phosphorylation levels of $p130^{Cas}$ started to decrease after 5 min (Fig. 2), whereas phosphorylation of paxillin remained markedly elevated after 60 min (Fig. 3). Treatment of neuroblasts with a higher concentration of CCK-8, 1 μM, had no effect on the tyrosine phosphorylation of any of these proteins (data not shown).

Stimulation of ERK1/2 Phosphorylation by CCK-8 in Rat Neuroblasts

CCK activates ERK1/2 by binding to CCK1 receptor in rat pancreatic acini [Duan and Williams, 1994; Williams, 2001] and by binding to CCK2 receptor in the pancreatic cell line AR42J [Seva et al., 1997]. We next determined whether CCK-8 stimulates phosphorylation of ERK1/2 and PKB in rat neuroblasts. E18 cells that had been serum-starved for 24 h were stimulated with CCK-8 at a concentration of 0.1 nM (Fig. 4A–C) or 1 μM (Fig. 4D–F) for times ranging between 1 min and 1 h. Treatment of neuroblasts with 1 μM CCK-8 caused a rapid increase in the phosphorylation of p44 (Fig. 4D,F) and p42 (Fig. 4E,F). For both proteins, stimulation with CCK-8 for 1 min was already sufficient to provoke a significant increase in phosphorylation ($P < 0.05$ in both cases). Maximal phosphorylation was reached after 15 min (p44: 4.17 ± 0.93-fold increase over control, $P < 0.05$; p42: 2.03 ± 0.11-fold increase over control, $P < 0.005$; $n = 4$ in both cases). Phosphorylation levels were no longer significantly elevated after 1 h incubation. Treatment of neuroblasts with 0.1 nM CCK-8 had a similar effect on the phosphorylation of p44 (Fig. 4A), which again was found to be maximally increased after 15 min (4.07 ± 0.46-fold increase over control, $P < 0.005$, $n = 4$). Maximal phosphorylation of p42 (Fig. 4B) occurred after 5 min

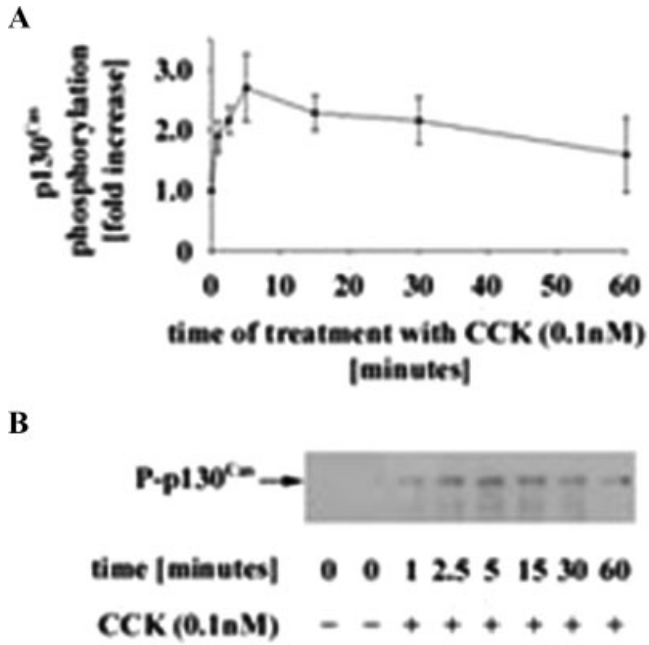

Fig. 2. CCK-8 stimulation of brain neuroblasts leads to $p130^{Cas}$ tyrosine phosphorylation in a time-dependent manner. E18 cells were treated with CCK-8 (0.1 nM) for the indicated times and then lysed. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine mAb (PY20), and then analyzed by SDS–PAGE followed anti-$p130^{Cas}$ immunoblotting as described in Materials and Methods. **Panel B**: Representative Western blot of immunoprecipitates after stimulation with CCK-8 (0.1 nM) at the indicated times. **Panel A**: Quantification of results was performed by scanning densitometry. Results shown are mean ± SEM of four independent experiments and are expressed as fold increase over control (time 0).

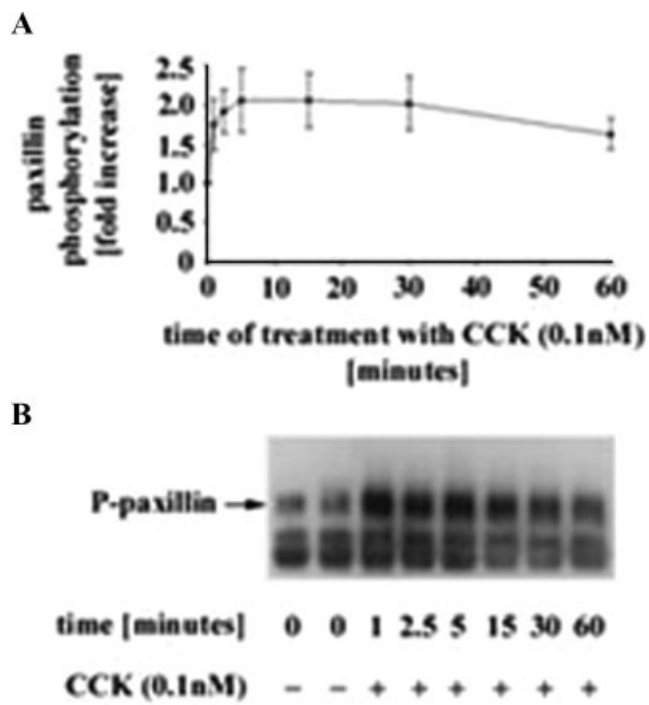

Fig. 3. CCK-8 stimulation of brain neuroblasts leads to paxillin tyrosine phosphorylation in a time-dependent manner. E18 cells were treated with CCK-8 (0.1 nM) for the indicated times and then lysed. Whole cell lysates were immunoprecipitated with anti-phosphotyrosine mAb (PY20), and then analyzed by SDS–PAGE followed by anti-paxillin immunoblotting as described in Materials and Methods. **Panel B**: Representative Western blot of immunoprecipitates after stimulation with CCK-8 (0.1 nM) at the indicated times. **Panel A**: Quantification of results was performed by scanning densitometry. Results shown are mean ± SEM of four independent experiments and are expressed as fold increase over control (time 0).

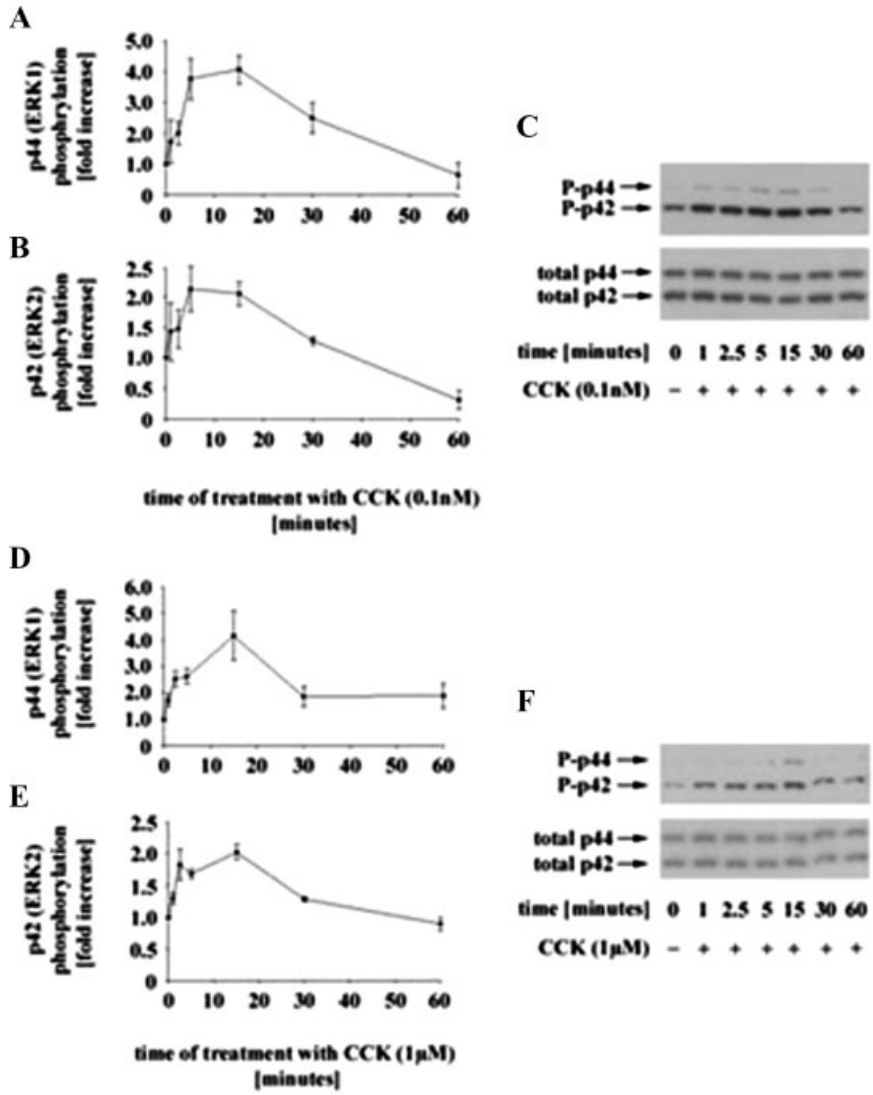

Fig. 4. CCK-8 stimulation of brain neuroblasts leads to ERK1/2 activation in a time-dependent manner. Serum-starved E18 cells were treated for the indicated times with 0.1 nM (**A**–**C**) or 1 μM CCK-8 (**D**–**F**) and then lysed. Cell lysates were analyzed by SDS–PAGE and Western blotting using anti-phospho-ERK1/2 (P-p44 and P-p42) or anti-ERK1/2 (total p44 and p42) specific antibodies. Panel C shows the time course of ERK1/2 activation after stimulation with 0.1 nM of CCK-8; Quantification of p44 (A) and p42 (B) bands was performed by scanning densitometry. Panel F shows the time course of ERK1/2 activation after stimulation with 1 μM of CCK-8. Quantification of p44 (D) and p42 (E) bands was performed by scanning densitometry. Values shown are mean ± SEM of four independent experiments and are expressed as fold increase over control (time 0).

(2.12 ± 0.37-fold increase over control, $P < 0.05$, $n = 4$) and was sustained after 15 min of stimulation with CCK-8. Equal loading was confirmed by reprobing the same membranes with a total ERK1/2 antibody (Fig. 4C,F, lower films)

Stimulation of DNA-Binding Activity of AP-1 by CCK-8 in Rat Neuroblasts

Activation of ERK1/2 has been shown to stimulate the transcription factor AP-1 [Wang and Prywes, 2000]. In order to find out whether this occurs in E18 cells stimulated with CCK-8, we performed an EMSA using a radioactively labeled oligonucleotide probe containing the AP-1 consensus sequence (Fig. 5). Treatment of neuroblasts with 0.1 nM and 1 μM CCK-8 stimulated the DNA-binding activity of AP-1 by 1.37 ± 0.07 and 1.27 ± 0.05-fold increase over control, respectively (Fig. 5, $P < 0.005$; $n = 5$) Preincubation of nuclear extracts with an excess of unlabeled probe as cold competitor

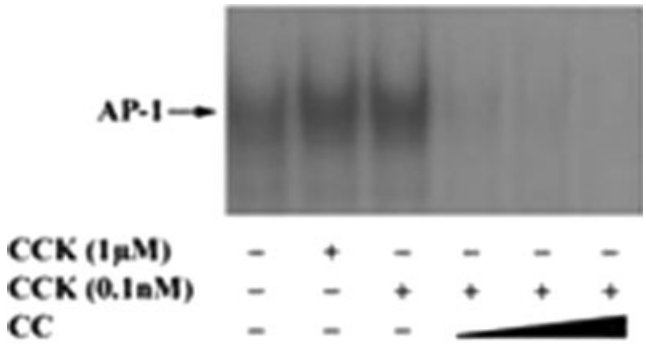

Fig. 5. Activation of AP-1 as a response to stimulation of brain neuroblasts with CCK-8. E18 cells were serum-starved. Nuclear extracts of non-stimulated cells and cells stimulated for 30 min with 0.1 nM or 1 μM CCK-8 were incubated with a radiolabeled oligonucleotide probe containing the AP-1 consensus sequence. Reaction products were analyzed in a 6% polyacrylamide gel. **Lane 1**, untreated cells, **lane 2**, cells treated with 1 μM CCK-8 for 30 min, **lane 3**, cells treated with 0.1 nM CCK-8 for 30 min, **lanes 4–6**, nuclear extract of cells treated with 0.1 nM CCK-8 for 30 min preincubated with increasing concentrations of unlabelled probe. One film representative of five independent experiments with similar results is shown. CC, cold competitor.

abolished the complex formation, thus proving the interaction to be specific (Fig. 5, lanes 4–6). Activity levels of AP-1 were maintained even after 1 h of stimulation (data not shown).

Stimulation of PKB/Akt Phosphorylation by CCK-8 in Rat Neuroblasts

Recent results show that activation of CCK2 receptor in the pancreatic cell line AR42J promotes cell survival through the induction of PI3-K/PKB/Akt signaling pathways [Todisco et al., 2001]. Therefore, we determined phosphorylation levels of PKB/Akt in rat brain neuroblasts after CCK-8 stimulation. PKB/Akt phosphorylation followed a similar pattern as that of ERK1/2 (Fig. 6). Stimulation with CCK-8, both 0.1 nM (Fig. 6A) and 1 μM (Fig. 6B), caused a significant increase in the phosphorylation of PKB already after 1 min ($P < 0.05$). Maximal phosphorylation levels were observed after 15 min of stimulation with 0.1 nM CCK-8 (4.5 ± 1.33-fold increase, $P < 0.05$, $n = 5$) or with 1 μM CCK-8 (3.49 ± 0.23-fold increase, $P < 0.005$, $n = 4$), followed by a decrease towards basal levels. We have ensured equal loading using ERK1/2 antibody since phosphorylation of ERK1/2 (Fig. 4) and PKB/Akt were analyzed on the same membranes.

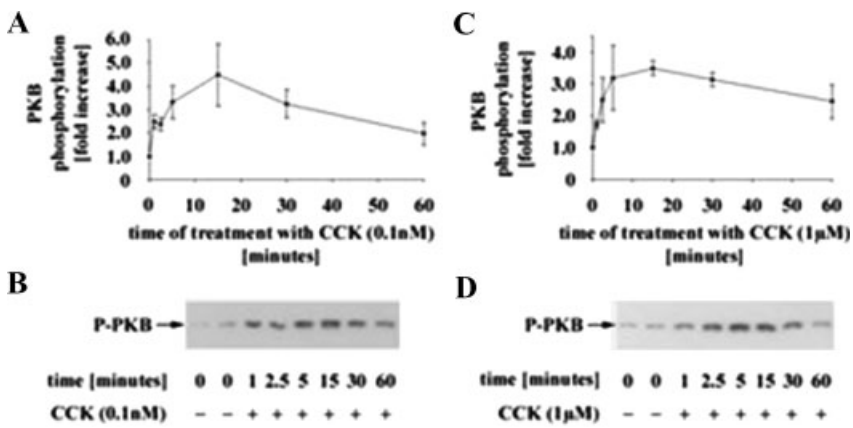

Fig. 6. CCK-8 stimulation of brain neuroblasts leads to phosphorylation of PKB/Akt at Ser473 in a time-dependent manner. Serum-starved E18 cells were stimulated with CCK 0.1 nM (**A**, **B**) or 1 μM (**C**, **D**) for the times indicated and then lysed. Cell lysates were analyzed by SDS–PAGE and Western blotting using a phospho-specific (Ser473) anti-PKB/Akt antibody. Panel B shows the stimulation of Ser473 phosphorylation of PKB/Akt with CCK-8 (0.1 nM), panel A shows quantification of bands performed by scanning densitometry and expressed as fold increase over control (time 0). Panel D shows the stimulation with 1 μM CCK-8 of Ser473 phosphorylation of PKB/Akt. Panel C shows the quantification of bands performed by scanning densitometry and data are expressed as fold increase over control (time 0). Values shown in both graphs are mean ± SEM of four independent experiments.

Inhibition of ERK1/2 Phosphorylation Using the CCK2 Receptor Antagonist CR2945 After Stimulation of Rat Brain Neuroblasts With CCK-8 and Gastrin

In order to elucidate which CCK receptor is responsible for the intracellular signals elicited after CCK-8 stimulation in neuroblasts, we have studied the effect on intracellular signaling of gastrin, hormone that acts as a CCK2 agonist and does not bind to CCK1 receptor. In addition, we have used the well-described potent and selective non-peptide CCK2 receptor antagonist [Makovec and D'Amato, 1997] that inhibits CCK2 receptor signaling in vivo [Makovec et al., 1999]. In particular, we have carried out experiments to study the ERK1/2 phosphorylation stimulated by CCK-8 or gastrin when CCK2 receptor was pharmacologically blocked with CR2945 in rat neuroblasts. As seen in Figure 7, gastrin that acts only through the CCK2 receptor, (lanes 4, 5) is able to stimulate p42 (3.4 ± 0.8-fold increase for 1 μM) and p44 (2.3 ± 0.4-fold increase for 1 μM) phosphorylation in a comparable degree as CCK at the same μM range concentrations (3.1 ± 0.7 and $2.8 \pm$ 0.5-fold increase for p42 and p44, respectively). As expected, the increase on p42 and p44 phosphorylation stimulated by the CCK2-receptor agonist gastrin is totally inhibited by CR2945 (lanes 8, 9). Treatment of rat neuroblasts with 1 μM CR2945 inhibited by 52 and 73% the increase on p42 phosphorylation observed after 1 or 0.1 μM CCK-8 incubation, respectively. Similar percentages of inhibition (62 and 73%) were observed in p44 phosphorylation stimulated by 1 or 0.1 μM CCK-8 incubation, respectively.

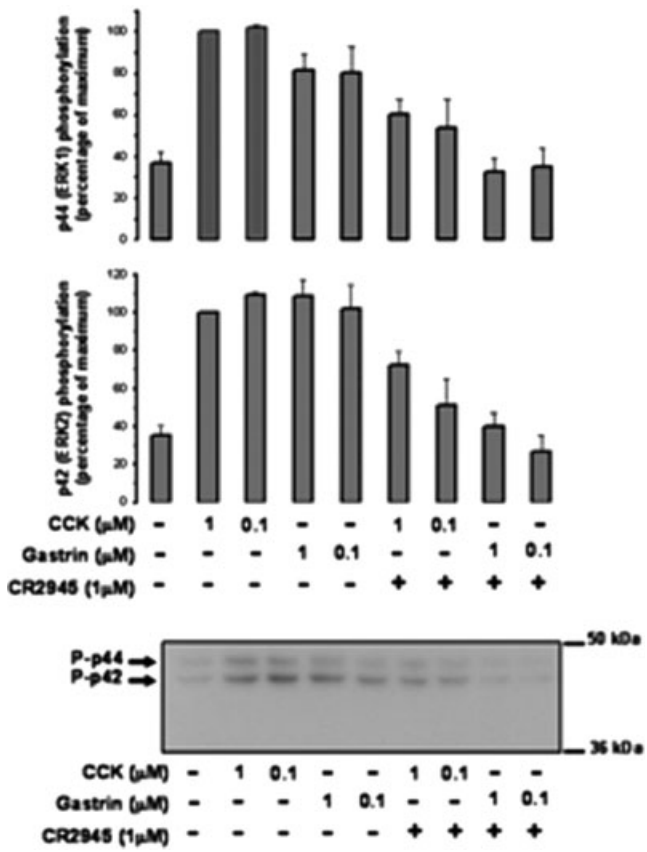

Fig. 7. Effect of the CCK2 receptor antagonist CR2945 in the CCK and gastrin stimulation of ERK1/2 phosphorylation in rat brain neuroblasts. Serum-starved E18 cells were treated for the indicated times with 1 μM of the CCK2 receptor antagonist CR2945, and indicated concentrations of CCK-8 or gastrin and then lysed. Cell lysates were analyzed by SDS–PAGE and Western blotting using anti-phospho-ERK1/2 (P-p44 and P-p42) or anti-ERK1/2 (total p44 and p42) specific antibodies. Quantification of p44 and p42 bands was performed by scanning densitometry. Values shown are mean ± SEM of four independent experiments and are expressed as percentage of maximal stimulation obtained with 1 μM CCK. One film representative of three independent experiments with similar results is shown at the **bottom panel**.

Improvement of the Viability in Serum-Deprived Neuroblasts by CCK-8. Since activation of CCK receptors in many systems showed a mitogenic effect (see review Williams, 2001), we decided to test whether activation of CCK receptors increase the number of viable cells in serum-starved cultures of E18 cells. As a control, we used growth factors such as EGF, which has been described to promote proliferation of neuroblasts in the absence of other growth factors [Ray and Gage, 1994; Lu et al., 1996]. Viability of cells in serum-free medium with or without addition of 0.1 nM or 1 μM CCK-8 or 10 nM EGF, respectively, was assessed after 48 h using MTT (Fig. 8). The number of viable neuroblasts in presence of 1 μM CCK-8 was significantly increased until $132.6 \pm 9.7\%$ as compared to control E18 cells ($n = 9$). A similar significant increase could be observed in neuroblasts cultured in presence of 10 nM EGF ($130.8 \pm 11.3\%$ as compared to control, $n = 8$). However, lower concentration of CCK-8 such as 0.1 nM did not cause significant changes in the number of viable cells (Fig. 8).

DISCUSSION

CCK is a classical brain–gut peptide that exerts a variety of physiological actions in the gastrointestinal tract and CNS being the most abundant neuropeptide in the brain (for review, Harro et al., 1993; Beinfeld, 2001; Tirassa et al., 2002). The existence of two types of CCK receptors, CCK1 and CCK2, has been demonstrated [Hill et al., 1987; Wank et al., 1992a,b] and both have been found in the CNS [Hill et al., 1987; Wank et al., 1992b; Bragado et al., 2003], but the existence of CCK receptors in rat brain neuroblasts has not been documented to date. Our results in this study clearly show that both CCK1 and CCK2 receptor mRNAs are expressed in these neuronal E18 cells. However at the protein level, only CCK2 receptor could be detected in our experimental conditions. It is important to mention that we have observed bands with different molecular mass, likely due to the fact that the electrophoretic mobility of CCK receptor proteins could be altered by the post-translational modification of glycosylation, as also suggested by Morisset et al. [2000]. The explanation for the diference in the CCK2 receptor bands observed in rat brain and in E18 neuroblasts (Fig. 1B) could also be due to the fact that the smaller form of CCK2 receptor (unglycosylated) might be highly expressed in early stages of the rat brain development (E18 neuroblasts) and its level of expression might diminish in the adult period (brain homogenates from adult rats), where the glycosylated form is more abundant. Our results suggest that

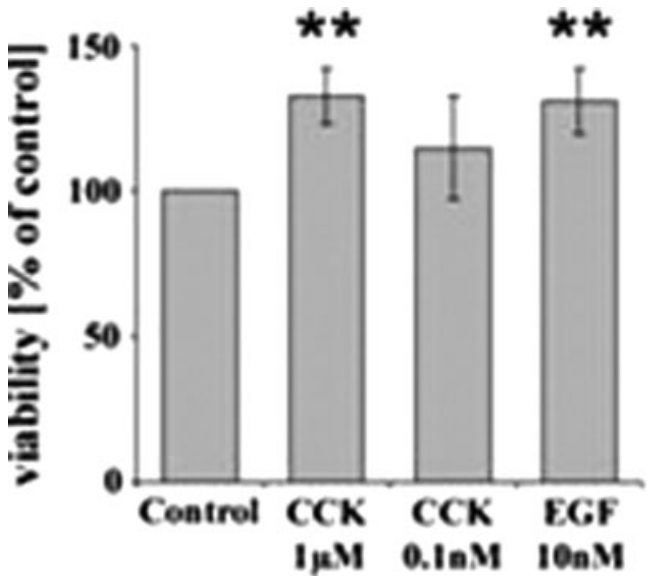

Fig. 8. Effects of stimulation of rat brain neuroblasts with CCK-8 and EGF on cell viability in serum-free medium. E18 cells were cultured for 48 h in serum-free medium containing 1 μM or 0.1 nM CCK-8 or 10 nM EGF, control cells were kept in serum-free medium without supplements. After this time, cell viability was assessed by MTT method as described in Materials and Methods. Results are expressed as mean ± SEM of six independent experiments. $^{**}P < 0.05$ compared to untreated cells.

these E18 cells could be in the future used as a good model to study the intracellular signaling pathways mediating CCK functions in the CNS.

The neuropeptide CCK binds to cell membrane CCK1 or 2 receptors and activates intracellular transduction pathways involving serine/threonine and tyrosine phosphorylation [Garcia et al., 1997a,b; Garcia and Jensen, 1998; Ferris et al., 1999; Tapia et al., 1999; Williams, 2001]. In the present study, we report that stimulation of neuroblasts with CCK activates several intracellular pathways, which suggest that CCK receptors expressed in rat neuroblasts should be functional. Thus, treatment of neuroblasts with CCK-8 rapidly induces tyrosine phosphorylation of both paxillin and $p130^{Cas}$, two key components of focal adhesion complexes [Sakai et al., 1994; Harte et al., 1996; Schlaepfer et al., 1999], to a comparable degree as is observed in non-neuronal [Garcia et al., 1997a; Garcia et al., 1997b; Ferris et al., 1999; Tapia et al., 1999] and neuronal cell types [Bragado et al., 2003] after CCK stimulation. The relevance of tyrosine phosphorylation in the CNS is demonstrated by the fact that in rat retina the natural stimulus, light, stimulates tyrosine phosphorylation of proteins in rat rod outer segments [Ghalayini et al., 1998]. Tyrosine phosphorylation has recently been implicated in the action of a number of neuropeptides, including CCK [Garcia and Jensen, 1998], that act as potent cellular growth factors in mediating both their growth effects as well as cytoskeletal changes [Shores and Maness, 1989; Gurd, 1997]. The CCK-induced tyrosine phosphorylation in neuroblasts is time-dependent, being in agreement with previous CCK results involving a rapid induction of paxillin and $p130^{Cas}$ tyrosine phosphorylation in an epithelial cell type [Garcia et al., 1997a,b; Ferris et al., 1999; Tapia et al., 1999] and $p130^{Cas}$ tyrosine phosphorylation in rat neuronal cells [Bragado et al., 2003]. It is well established that tyrosine phosphorylation of $p130^{Cas}$ and paxillin results in the formation of phospho–protein complexes which in turn can activate intracellular cascades involved in the formation of focal adhesion complexes, actin cytoskeleton regulation, and cellular growth [Sakai et al., 1994; Harte et al., 1996; Klemke et al., 1998; Schaller, 2001]. Thus, $p130^{Cas}$ and paxillin represent a convergence point in the intracellular response to different stimuli [Klemke et al., 1998]; therefore, it has been proposed that tyrosine phosphorylation of $p130^{Cas}$ might likely function as an important regulator of different intracellular signals activated by CCK-8 in rat neuronal cells [Bragado et al., 2003].

Results from the present work show that stimulation of E18 cells with CCK-8 rapidly leads to phosphorylation, and in turn activation, of p44 and p42 ERK1/2 in a time and efficacy comparable to the CCK response in other cell types. In our study, we show that ERK1/2 phosphorylation is promoted also after stimulation with gastrin (Fig. 7), hormone that acts as a well known CCK2 receptor agonist, with same efficacy which suggests that the effect of CCK on the phosphorylation of this survival pathway in rat neuroblasts could be mediated at least by the CCK2 receptor activation. This suggestion could be further confirmed by the fact that a well-described CCK2 antagonist, CR2945, is able to markedly inhibit (up to 73%) the increase on ERK1/2 phosphorylation stimulated by CCK in rat brain neuroblasts. Therefore, we can conclude that CCK2 receptors are very important in mediating some of the CCK intracellular signals in rat brain neuroblasts, but we cannot rule out the effect of CCK1 receptors as the intracellular action is also partially CCK2 receptor antagonist independent. In agreement with the involvement of both types of CCK receptors, it has been shown in other cell types that the CCK effect on ERK1/2 phosphorylation is mediated by binding to both CCK1 and 2 receptor [Williams, 2001]. We also report that not only is ERK1/2 phosphorylation stimulated, but also its complete transduction pathway is functional, as treatment of E18 cells with CCK-8, even for short times as in the present work, activates an end point of the ERK1/2 pathway, the DNA-binding activity of the transcription factor AP-1. ERK1/2 are serine–threonine kinases known to be involved in the control of cell growth, differentiation, survival, apoptosis, and cytokine production [Roux and Blenis, 2004].

In this study, we report that CCK activates intracellular pathways leading to PKB/Akt phosphorylation at the Ser 473 in rat neuroblasts, which indirectly means that the Akt has achieved its full kinase activity [Scheid and Woodgett, 2003]. The activation of PKB/Akt has been described after CCK2 receptor stimulation in AR42J cells [Todisco et al., 2001] and esophageal cell lines [Harris et al., 2004]. In

neuroblasts, we have found that PKB phosphorylation is dependent of the kinase activity of PI3-K [Cerezo-Guisado et al., 2005]. Activation of PKB/Akt is dependent on the PI3-K lipid product, which recruits Akt to the plasma membrane, where it is activated after sequential phosphorylation at two specific sites, Thr 308 and Ser 473. It is well known that the PI3-K/PKB transduction pathway regulates a variety of cell functions including growth, protein translation, metabolism, mitogenesis, and cell survival [Scheid and Woodgett, 2003].

Our data demonstrate that CCK-8 treatment causes a modest but reproducible increase on neuroblasts viability to the same degree as the growth factor EGF, which had been previously implicated in neuroblasts growth and proliferation [Ray and Gage, 1994; Lu et al., 1996]. Based on the fact that CCK has growth effects both in normal tissues [Reeve et al., 1994] and neoplastic tissues [Smith et al., 1991; Reeve et al., 1994] and that the intracellular pathways activated by CCK in neuroblasts have been widely shown to be involved in the regulation of cell growth, it can be suggested that the CCK-mediated increase in neuroblasts viability could be due to the stimulation of cell growth. An additional plausible explanation is that CCK might exert a neuroprotective role in rat brain neuroblasts by reducing neuroblasts death induced by serum deprivation as the CCK-activated pathways in neuroblasts are also involved in cell survival such as ERK1/2 and PKB. This hypothesis is supported by previous studies showing that CCK acts as a neuroprotector in cortical neurons in vitro [Akaike et al., 1991; Tamura et al., 1992] where it protects against the glutamate-induced neurotoxicity. It has also been documented that CCK protects the degenerative process of cholinergic neurons after basal forebrain lesion in rats [Sugaya et al., 1992; Tirassa et al., 2002] and recently, it has reported that CCK-8 protects the aging process of experimental neuronal aging based on serum-free culture conditions [Sun et al., 2005]. CCK neuroprotective effects have also been demonstrated in vivo as systemic administration of CCK resulted in the recovery of memory impairment and reduced neuronal cell death in the hippocampus [Eigyo et al., 1992].

In conclusion, in the present study, we show that immortalized rat brain neuroblasts express both types of CCK receptors at the mRNA level and the CCK2 receptor at the protein level. We report that neuroblast stimulation with CCK induces intracellular signaling cascades leading to tyrosine phosphorylation of the focal adhesion proteins $p130^{Cas}$ and paxillin in a time-dependent manner. Furthermore, CCK incubation of neuroblasts stimulates both the ERK1/2 and the PKB/Akt intracellular pathways involved in cell survival and proliferation in a time and concentration-dependent manner. Some of the signals such as ERK1/2 phosphorylation seem to be mainly mediated by the CCK2 receptors, although with our data we cannot exclude the involvement of CCK1 receptors. Finally, CCK increases the number of viable neuroblasts, which suggests that it might have a mitogenic or neuroprotector effect in these neuronal cells. This work provides further biochemical information for a better understanding of the physiological role of CCK in the CNS. Moreover, it reports a putative model of proliferating neuronal cells to further study intracellular mechanisms by which this neuropeptide exerts its biological actions in neurons.

ACKNOWLEDGMENTS

The authors thank J. Ricardo Argent for his expert technical assistance and Dr. Alberto Quesada for his kind help with the RT-PCR. S.L. was supported by a fellowship from the Studienstiftung des deutschen Volkes, Germany, and M.I. Cerezo-Guisado was supported by a PhD fellowship from Junta de Extremadura, Spain.

REFERENCES

Akaike A, Tamura Y, Sato Y, Ozaki K, Matsuoka R, Miura S, Yoshinaga T. 1991. Cholecystokinin-induced protection of cultured cortical neurons against glutamate neurotoxicity. Brain Res 557:303–307.

Andrews NC, Faller DV. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499.

Beinfeld MC. 2001. An introduction to neuronal cholecystokinin. Peptides 22:1197–1200.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Bragado MJ, Perez-Marquez J, Garcia-Marin LJ. 2003. The cholecystokinin system in the rat retina: Receptor expression and in vivo activation of tyrosine phosphorylation pathways. Neuropeptides 37:374–380.

Cerezo-Guisado MI, Garcia-Marin LJ, Lorenzo MJ, Bragado MJ. 2005. Lovastatin inhibits the growth and survival pathway of phosphoinositide 3-kinase/protein

kinase B in immortalized rat brain neuroblasts. J Neurochem 94:1277–1287.
Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.
Dockray GJ. 1976. Immunochemical evidence of cholecystokinin-like peptides in brain. Nature 264:568–570.
Duan R-D, Williams JA. 1994. Cholecystokinin rapidly activates mitogen-activated protein kinase in rat pancreatic acini. Am J Physiol 267:G401–G408.
Eigyo M, Katsuura G, Shintaku H, Shinohara S, Katoh A, Shiomi T, Matsushita A. 1992. Systemic administration of a cholecystokinin analogue, ceruletide, protects against ischemia-induced neurodegeneration in gerbils. Eur J Pharmacol 214:149–158.
Ferris HA, Tapia JA, García LJ, Jensen RT. 1999. CCKA receptor activation stimulates p130Cas tyrosine phosphorylation, translocation and association with Crk in rat pancreatic acinar cells. Biochemistry 38:1497–1508.
Garcia LJ, Rosado JA, Gonzalez A, Jensen RT. 1997a. Cholecystokinin-stimulated tyrosine phosphorylation of $p125^{FAK}$ and paxillin is mediated by phospholipase C-dependent and -independent mechanisms and requieres the integrity of the actin cytoskeleton and participation of p21rho. Biochem J 327:461–472.
Garcia LJ, Rosado JA, Tsuda T, Jensen RT. 1997b. CCK causes rapid tyrosine phosphorylation of $p125^{FAK}$ focal adhesion kinase and paxillin in rat pancreatic acini. Biochim Biophys Acta 1358:189–199.
Ghalayini AJ, Guo XX, Koutz CA, Anderson RE. 1998. Light stimulates tyrosine phosphorylation of rat rod outer segments in vivo. Exp Eye Res 66:817–821.
Gurd JW. 1997. Protein tyrosine phosphorylation: Implications for synaptic function. Neurochem Int 31:635–649.
Harris JC, Clarke PA, Awan A, Jankowski J, Watson SA. 2004. An antiapoptotic role for gastrin and the gastrin/CCK-2 receptor in Barrett's esophagus. Cancer Res 64: 1915–1919.
Harro J, Vasar E, Bradwejn J. 1993. CCK in animal and human research on anxiety. Trends Pharmacol Sci 14: 244–249.
Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT. 1996. p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesion and binds to focal adhesion kinase. J Biol Chem 271:13649–13655.
Hill DR, Campbell NJ, Shaw TM, Woodruff GN. 1987. Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. J Neurosci 7:2967–2976.
Garcia LJ, Jensen RT. 1998. Tyrosine phosphorylation of $p125^{FAK}$ and paxillin by cholecystokinin and other neuropeptides. In: LeRoith D, editor. Advances in Molecular and Cellular Endocrinology. Stamford (USA): JAI Press, pp 117–151.
Klemke RL, Leng J, Molander R, Brooks PC, Vuori K, Cheresh DA. 1998. CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol 140:961–972.
Lu N, Black IB, DiCicco-Bloom E. 1996. A paradigm for distinguishing the roles of mitogenesis and trophism in neuronal precursor proliferation. Brain Res Dev Brain Res 94:31–36.
Makovec F, D'Amato M. 1997. CCK_B/gastrin receptor antagonists as potential drugs for peptic ulcer therapy. DDT 2:283–293.
Makovec F, Revel L, Letari O, Mennuni L, Impicciatore M. 1999. Characterization of antisecretory and antiulcer activity of CR 2945, a new potent and selective gastrin/CCK_B receptor antagonist. Eur J Pharmacol 369:81–90.
Monstein HJ, Nylander AG, Salehi A, Chen D, Lundquist I, Hakanson R. 1996. Cholecystokinin-A and cholecystokinin-B/gastrin receptor mRNA expression in the gastrointestinal tract and pancreas of the rat and man. A polymerase chain reaction study. Scand J Gastroenterol 31:383–390.
Morisset J, Wong H, Walsh JH, Laine J, Bourassa J. 2000. Pancreatic CCK(B) receptors: Their potential roles in somatostatin release and delta-cell proliferation. Am J Phys Gastr Liver Physiol 279:G148–G156.
Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.
Muñoz A, Wrighton C, Seliger B, Bernal J, Beug H. 1993. Thyroid hormone receptor/c-erbA: Control of commitment and differentiation in the neuronal/chromaffin progenitor line PC12. J Cell Biol 121:423–438.
Ray J, Gage FH. 1994. Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor. J Neurosci 14:3548–3564.
Reeve JR, Jr., Eysselein VE, Ho FJ, Chew P, Vigna SR, Liddle RA, Evans C. 1994. Natural and synthetic CCK-58. Novel reagents for studying cholecystokinin physiology. Ann NY Acad Sci 713:11–21.
Roux PP, Blenis J. 2004. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68:320–344.
Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y, Hirai H. 1994. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J 13:3748–3756.
Schaller MD. 2001. Paxillin: A focal adhesion-associated adaptor protein. Oncogene 20:6459–6472.
Scheid MP, Woodgett JR. 2003. Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Lett 546:108–112.
Schlaepfer DD, Hauck CR, Sieg DJ. 1999. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71:435–478.
Seva C, Kowalski-Chauvel A, Daulhac L, Barthez C, Vaysse N, Pradayrol L. 1997. Wortmannin-sensitive activation of p70S6-kinase and MAP-kinase by the G protein-coupled receptor, G/CCKB. Biochem Biophys Res Commun 238:202–206.
Shores CG, Maness PF. 1989. Tyrosine phosphorylated proteins accumulate in junctional regions of the developing chick neural retina. J Neurosci Res 24:59–66.
Smith JP, Kramer ST, Cheung JY. 1991. Effects of cholecystokinin on cytosolic calcium in human pancreatic cancer cells. Regul Peptides 36:299–310.
Sugaya K, Takahashi M, Kubota K. 1992. Cholecystokinin protects cholinergic neurons against basal forebrain lesion. Jpn J Pharmacol 59:125–128.
Sun XJ, Lu QC, Cai Y. 2005. Effect of cholecystokinin on experimental neuronal aging. World J Gastroenterol 11: 551–556.

Tamura Y, Sato Y, Akaike A, Shiomi H. 1992. Mechanisms of cholecystokinin-induced protection of cultured cortical neurons against N-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. Brain Res 592:317–325.

Tapia JA, Ferris HA, Jensen RT, Garcia LJ. 1999. Cholecystokinin activates PYK2/CAKbeta by a phospholipase C-dependent mechanism and its association with the mitogen-activated protein kinase signaling pathway in pancreatic acinar cells. J Biol Chem 274:1261–1271.

Tirassa P, Manni L, Aloe L, Lundeberg T. 2002. Cholecystokinin-8 and nerve growth factor: Two endogenous molecules working for the upkeep and repair of the nervous system. Curr Drug Targets CNS Neurol Disord 1:495–510.

Todisco A, Ramamoorthy S, Witham T, Pausawasdi N, Srinivasan S, Dickinson CJ, Askari FK, Krametter D. 2001. Molecular mechanisms for the antiapoptotic action of gastrin. Am J Physiol Gastrointest Liver Physiol 280:G298–G307.

Vanderhaeghen JJ, Signeau JC, Gepts W. 1975. New peptide in the vertebrate CNS reacting with antigastrin antibodies. Nature 257:604–605.

Wang Y, Prywes R. 2000. Activation of the c-fos enhancer by the erk MAP kinase pathway through two sequence elements: The c-fos AP-1 and p62TCF sites. Oncogene 19:1379–1385.

Wank SA, Harkins R, Jensen RT, Shapira H, de Weerth A, Slattery T. 1992a. Purification, molecular cloning, and functional expression of the cholecystokinin receptor from rat pancreas. Proc Natl Acad Sci USA 89:3125–3129.

Wank SA, Pisegna JR, de Weerth A. 1992b. Brain and gastrointestinal cholecystokinin receptor family: Structure and functional expression. Proc Natl Acad Sci USA 89:8691–8695.

Williams JA. 2001. Intracellular signaling mechanisms activated by cholecystokinin-regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. Annu Rev Physiol 63:77–97.

Yamashita A. 1992. Ontogeny of cholecystokinin-immunoreactive structures in the primate cerebral neocortex. Int J Neurosci 64:139–151.

Zhou W, Povoski SP, Bell RH, Jr. 1995. Characterization of cholecystokinin receptors and messenger RNA expression in rat pancreas: Evidence for expression of cholecystokinin-A receptors but not cholecystokinin-B (gastrin) receptors. J Surg Res 58:281–289.