Corticotropin-releasing Hormone Induces Fas Ligand Production and Apoptosis in PC12 Cells via Activation of p38 Mitogen-activated Protein Kinase*

Recent experimental findings involve corticotropin-releasing hormone (CRH) in the cellular response to noxious stimuli and possibly apoptosis. The aim of the present work was to examine the effect of CRH on apoptosis and the Fas/Fas ligand system in an in vitro model, the PC12 rat pheochromocytoma cell line, which is widely used in the study of apoptosis and at the same time expresses the CRH/CRH receptor system. We have found that following, CRH induced Fas ligand production and apoptosis. These effects were mediated by the CRH type 1 receptor because its antagonist antalarmin blocked CRH-induced apoptosis and Fas ligand expression. CRH activated p38 mitogen-activated protein kinase, which was found to be essential for CRH-induced apoptosis and Fas ligand production. CRH also promoted a rapid and transient activation of ERK1/2, which, however, was not necessary for either CRH-induced apoptosis or Fas ligand production. Thus, CRH promotes PC12 apoptosis via the CRH type 1 receptor, which induces Fas ligand production via activation of p38.

Several lines of evidence suggest that corticotropin-releasing hormone (CRH) may play a role in the cellular response to noxious stimuli that promote neuron death (1). Indeed, CRH contributes to hippocampal ischemic injury, an effect prevented by the CRH antagonist α-helical CRH (αhCRH) (2). Similarly, astrocytes, a potent CRH antagonist, exerts a considerable neuroprotective effect on hippocampal cell damage following kainic acid-induced excitotoxic seizures (3). In animal models of induced status epilepticus, CRH causes neuronal loss in limbic structures, including the CA3 region of the hippocampus characterized by pyramidal cell apoptosis (4). Furthermore, administration of CRH to the brain of immature rats is associated with progressive hippocampal CA3 neuron apoptosis independent of glucocorticoids (5). These phenomena involve cell apoptosis. However, there is no information regarding the effects of CRH on the apoptotic machinery.

The Fas/Fas ligand system controls apoptosis of several types of immune cells and possibly of epithelial and neural cells including cells in hippocampus and cortex (6, 7). The aim of the present work was to examine the effect of CRH on apoptosis and Fas ligand expression in a well established in vitro model, the PC12 rat pheochromocytoma cell line, which has characteristics of epithelial and neuronal cells and is widely used as a model in the study of apoptosis. PC12 cells express the CRH/CRH receptor and the Fas/Fas ligand systems, providing a physiological model for the study of the effects of CRH on apoptosis and the intracellular signaling cascade involved (8, 9). When PC12 cells that are differentiated by nerve growth factor (NGF) are deprived of growth factors, they undergo apoptosis via expression of Fas ligand (10). Expression of Fas ligand and apoptosis in differentiated PC12 cells and primary cultures of rat sympathetic neurons depend on activation of stress-activated protein (SAP) kinase, p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) (10–12), whereas apoptosis of non-neuronally differentiated PC12 cells does not depend on JNK activation (12). Activation of p38 MAPK plays a central role in both undifferentiated and differentiated PC12 as well as in neuronal cell apoptosis because inhibition of this kinase promotes cell survival (13). Furthermore, withdrawal of survival factors from NGF-differentiated PC12 cells causes activation of p38 MAPK and down-regulation of extracellular signal regulated kinases (ERK1/2) MAPK resulting in apoptosis. Activation of ERK1/2 induced by NGF promotes PC12 cell proliferation and survival (14, 15). Indeed, sustained activation of ERK1/2 MAPK initiates proliferating and antiapoptotic signals, whereas rapid and transient activation correlates with apoptosis (16). In the first part of this work we examined the effect of CRH on apoptosis measured as the DNA fragmentation rate or fluorescent staining of apoptotic bodies. CRH was applied in the presence or absence of CRH antagonists αhCRH (9–41) and antalarmin, a pyrrolopyrimidine compound that antagonizes CRHR1-mediated effects of CRH, including pituitary ACTH release, stress behaviors, and acute inflammation (17–19). In the second part of this work we examined the mechanism through which CRH achieves its proapoptotic effect. We examined the effect of CRH in the presence or absence of its antagonists on Fas ligand production at the protein level using fluorescence-activated cell sorter analysis, Western blotting, or immunofluorescence and at the mRNA level using reverse transcription-PCR. In the third part of this work we analyzed the signaling pathways involved in CRH-induced Fas ligand production. Thus, we examined the effect of CRH on p38 MAPK and ERK1/2 activation and the effect of a MAPK-ERK kinase (MEK1) inhibitor and a p38 MAPK inhibitor on CRH-induced apoptosis and Fas ligand production.

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‡ The abbreviations used are: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; αhCRH, α-helical CRH (9–41); CRHR1, CRH type 1 receptor; DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein-ERK kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; SAP kinase, stress-activated protein kinase.
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**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**

Rat/human recombinant CRH was purchased from Sigma. The synthetic nonpeptide CRHR1 antagonist antalarmin was provided by Dr. G. P. Chrousos (NICH, National Institutes of Health). The synthetic peptide antagonist ahCRH (9–41) was purchased from Sigma.

The pharmacological inhibitor SB203580 (p38 MAPK inhibitor-[4-(4-fluorophenyl)-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole]) and PD98059 were purchased from Sigma and Calbiochem, respectively. Mouse monoclonal antibodies against Fas or Fas ligand were obtained from Transduction Laboratories. Anti-actin monoclonal antibody was supplied by Chemicon (Temecula, CA). Anti-phospho-p38 MAPK polyclonal antibody and anti-total p38 MAPK antibody were purchased from Cell Signaling (Beverly, MA) as were anti-phospho-ERK1/2 MAPK and anti-total ERK1/2 MAPK antibodies. Secondary antibodies used were fluorescein isothiocyanate-labeled rabbit anti-mouse IgG (Chemicon), horseradish peroxidase-conjugated anti-mouse IgG (Chemicon), and horseradish peroxidase-conjugated anti-rabbit IgG (Immunotech, France).

Bovine serum albumin fraction V, dithiothreitol, NaF, and aprotinin were obtained from Sigma. Bradford Coomassie Brilliant Blue G-250 was obtained from Bio-Rad, and nitrocellulose membranes for Western blotting were purchased from Millipore (Bedford, MA). Immunoreactive bands were visualized with an enhanced chemiluminescence kit from PerkinElmer Life Sciences. All sterile tissue apparatus were obtained from Corning (Corning, NY). All other chemicals and reagents were obtained from Sigma, if not stated otherwise.

**PC12 Cell Culture**

PC12 cells were obtained from three sources: Dr. M. Greenberg (Children’s Hospital, Boston, MA), early passages from the late Dr. G. Guroff (Section on Growth factors, NICH, NIH), and the American Type Culture Collection. All cells responded similarly to CRH. Cells were grown in RPMI 1640 containing 2 mM t-glutamine, 15 mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% horse serum, and 5% calf serum (all purchased from Sigma) at 37°C.

**Apop2is Detection Assays**

*Quantitative Measurement of Apoptosis—* PC12 cells were plated in 96-well plates at an initial concentration of 30,000 cells/well. After 3 days the number of PC12 cells doubled. At that point the culture media were changed to serum-free media. As expected, PC12 cells undergo apoptosis when cultured under serum-free conditions. Apoptosis was measured by direct determination of nucleosomal DNA fragmentation with the “Cell Death Detection enzyme-linked immunosorbent assay plus” kit (Roche Molecular Biochemicals). Cells were harvested and lysed according to the manufacturer’s protocol. The mono- and oligonucleosomes contained in the cell lysates were determined using an anti-histone-biotin antibody. The data are expressed in photometric units. Each unit corresponds to 10,000 nucleosomes.

**Western Blot Analysis**

After stimulation cells were harvested and lysed in a lysis buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and freshly added proteinase inhibitors phenylmethylsulfonyl fluoride (10 μg/ml) and 1 μg/ml aprotinin for the detection of Fas and Fas ligand. Lysis was performed for 30 min on ice with occasional vortexing. To detect the phosphorylated and nonphosphorylated forms of p38 MAPK and ERK1/2, cells were lysed in 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and freshly added proteinase inhibitors phenylmethylsulfonyl fluoride (10 μg/ml), 0.5 mM dithiothreitol, and 50 mM NaF. Subsequently, cells were sonicated for 5 s on ice. Solid cellular debris was removed by centrifugation at 12,000 × g for 15 min. Lysates were collected and stored at −80°C. The protein concentration of each lysate was measured by a modification of the Bradford assay. The protein concentration of each lysate was normalized versus actin. Where p38 MAPK and ERK1/2 was measured, membranes were first probed for the phosphorylated form of the protein, then stripped and probed for the total protein. The intensity of the bands was quantified using the Bio-Rad imaging system, and the quantity of the phosphorylated proteins were expressed as the ratio of the phosphorylated divided by the total protein in each case.

**Measurement of Kinase Activity**

p38 MAPK and SAP kinase/JNK activity measurements were performed with assays provided by Cell Signaling Technology. Briefly, cells were lysed in containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM freshly added phenylmethylsulfonyl fluoride. Lysis was completed by sonication on ice.

**Immunoprecipitation—** Protein lysates were incubated with immobilized anti-phospho-p38 MAPK (Thr-180/Tyr-182) monoclonal antibody in p38 MAPK assay, or c-Jun fusion protein beads in SAP kinase/JNK assay. Immune complexes were precipitated, and kinase assays were performed according to the manufacturer’s protocol. The concentration of each target protein was normalized versus actin. Where p38 MAPK and ERK1/2 was measured, membranes were first probed for the phosphorylated form of the protein, then stripped and probed for the total protein. The intensity of the bands was quantified using the Bio-Rad imaging system, and the quantity of the phosphorylated proteins were expressed as the ratio of the phosphorylated divided by the total protein in each case.

**Electrophoresis and Immunoblotting—** The phosphorylated substrates were subjected to 12% polyacrylamide gel electrophoresis, transferred to membranes, and incubated with either phospho-ATF-2 (Thr-71) for p38 kinase assay or phospho-c-Jun (Ser-63) antibodies for SAP kinase/JNK kinase.

**Immunofluorescence**

PC12 cells were grown on glass slides and treated with CRH and its antagonists for 2.5 h. Control cells were left untreated. At the end of the incubation periods, cells were fixed in 2% (w/v) formaldehyde for 10 min, permeabilized in 0.2% (w/v) Triton X-100 for 10 min, blocked in 1% fetal calf serum in PBS for 15 min, and then incubated overnight at 4°C with a mouse anti-rat Fas ligand antibody diluted 1/100 in PBS containing 1% fetal calf serum. Samples were washed with PBS and incubated for 1 h with fluorescein isothiocyanate-conjugated secondary anti-mouse antibody diluted 1/100. Cells were analyzed in a confocal laser scanning microscope (Leica TCS-NT).

**Reverse Transcription-PCR for Fas Ligand**

Primers for Fas ligand were 5′-CAG CCC TGT AAT TAG CCA TGT C-3′ (sense) and 5′-CAC TGA GAT CAA AGC ACT CC-3′ (anti-sense). Primers for β-actin were 5′-CAT CCT GTC GGC ATT GAC AGG-3′ (sense) and 5′-CTT CCG GGT CAT GAA GTC CTG-3′ (anti-sense) (10). Total cellular RNA was isolated using Trizol reagent (Invitrogen). After reverse transcription, the cDNA product was amplified by PCR, at 35 cycles, annealing to temperature of 60°C for Fasligand (55°C for β-actin). It should be noted that Fas ligand mRNA amplification at 35 cycles is still at the exponential phase. 10 μl of the amplification product was loaded on an agarose gel and resolved by electrophoresis.

**Flow Cytometric Analysis of Fas Ligand Expression**

In brief, cells were washed twice with PBS, prefixed, and permeabilized as described above. Blocking was performed with 1% fetal calf serum in PBS for 15 min. Then, the primary antibody (anti-mouse Fas ligand) was added and incubated overnight at 4°C. Cells were washed twice with PBS and incubated with a fluorescein isothiocyanate-labeled rabbit anti-mouse IgG and analyzed on a flow cytometer (Epics Elite Coulter, U. K.).
**RESULTS**

**CRH Induces PC12 Cell Apoptosis**—The effect of CRH was measured on accelerated apoptosis from serum deprivation. PC12 cells were treated with CRH, and apoptosis was measured at several time points. Apoptosis became significant at 24 h and peaked at 72 h (Fig. 1A). More specifically, exposure of 60,000 cells to CRH for 24 h increased the number of apoptotic cells in 7,621 ± 223 (mean ± S.E., n = 15) compared with 4,932 ± 411 of parallel controls (Table I). At 72 h the number of apoptotic cells was 22,215 ± 888 (n = 21) (51.27 ± 1.7% of the remaining cell population) in the CRH-exposed cells compared with 14,273 ± 1,170 (30.74 ± 2.12% of the remaining cell population) of parallel controls. Apoptotic cells were counted at the same time points using DAPI staining giving similar results (data not shown). A representative example of apoptotic bodies is shown in Fig. 1D.

The effect of CRH was more profound on the 3rd day. We therefore tested whether the effect of CRH was dose-dependent on the 3rd day of exposure. Indeed, the effect of CRH was dose-dependent peaking between 10^{-9} and 10^{-8} M (Fig. 1B). More specifically, 10^{-8} M CRH increased apoptosis to 1.90 ± 0.13 photometric units (n = 21 of seven independent experiments, p < 0.001) compared with 1.14 ± 0.09 photometric units of control cells, i.e. treated with the CRH diluent only. Similarly, 10^{-7} M CRH increased apoptosis to 1.79 ± 0.23 photometric units (n = 21 of seven independent experiments, p < 0.001) compared with parallel controls.

Treatment of cells with 10^{-8} M antalarmin decreased apoptosis induced by 10^{-9} M CRH to 76 ± 17% photometric units (n = 5 of two independent experiments) compared with 167 ± 11% of parallel control cells, i.e. cells treated with CRH alone (p < 0.05) (Fig. 1C), suggesting that the proapoptotic effect of CRH was mediated by the CRHR1 receptor. In the presence of 10^{-6} M antalarmin, 10^{-9} M CRH-induced apoptosis was suppressed further, reaching 53 ± 7% of parallel controls (n = 5 of two independent experiments, p < 0.01) (Fig. 1C). It should be noted that a higher concentration of CRH (10^{-8} M) required a higher concentration of antalarmin to have its effect suppressed, suggesting that there is a stoichiometric balance between the two molecules (data not shown). In contrast, ahCRH required higher concentrations than antalarmin to reverse CRH-induced apoptosis in PC12 cells. Treatment of PC12 cells with 10^{-8} M CRH requires 10^{-6} M ahCRH to reduce apoptosis to the basal levels, e.g. 83 ± 20% of the control cells (n = 5 of two independent experiments, statistically significant compared with cells exposed to CRH alone, p < 0.05) (Fig. 1C). Because antalarmin is mainly a CRHR1 antagonist and the effect exerted by CRH was completely inhibited by antalarmin, we assume that CRH-induced apoptotic signals are mediated via CRHR1. Antalarmin alone at 10^{-6} M had a low but significant suppressive effect on serum deprivation-induced apo-

**Table I**

Quantitation of apoptosis

|                  | Apoptotic cells | Cumulative percentage of apoptotic cells |
|------------------|-----------------|----------------------------------------|
| 24 h (n = 15)    |                 |                                        |
| Control          | 4,932 ± 411     | 8.22 ± 0.69%                           |
| CRH              | 7,621 ± 223     | 12.7 ± 0.37%                           |
| 72 h (n = 21)    |                 |                                        |
| Control          | 14,273 ± 1,170  | 30.74 ± 2.12%                          |
| CRH              | 22,215 ± 888    | 51.27 ± 1.7%                           |
| 96 h (n = 12)    |                 |                                        |
| Control          | 20,000 ± 979    | 62.19 ± 3.04%                          |
| CRH              | 21,000 ± 1,312  | 99.7 ± 0.3%                            |
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**Fig. 2. Effect of CRH on Fas ligand (FasL) production, Western blot data.** Panel A, PC12 cells were treated with vehicle (control) or 10^{-9} M CRH in serum-free medium for 2.5, 24, and 72 h. Cells were lysed, and supernatants were electrophoresed and probed by Western analysis with anti-Fas ligand monoclonal antibody followed by anti-actin antibodies. CRH increased Fas ligand peaking at 2.5 h of exposure. Panel B, the stimulatory effect of CRH was blocked by the specific CRH1 antagonist antalarmin. Fas ligand concentration was measured as described above. Data are expressed as a percentage of parallel controls exposed only to vehicles and represent the mean of four independent experiments (panel A) or three independent experiments (panel B). *p < 0.05 and **p < 0.001 denote a significant statistical difference compared with controls (panel A) or with cells exposed to CRH alone (panel B).

**Fig. 3. Effect of CRH on Fas ligand production: immunofluorescence.** Cells were treated for 2.5 h without serum (panel A), with 10^{-9} M CRH (panel B), with serum (panel C), or with 10^{-8} M antalarmin (panel D). Exposure to CRH resulted in an intense cytoplasmic staining for Fas ligand (panel B), which was prevented by antalarmin (panel D). Cells were viewed and photographed using a confocal fluorescence microscope.

CRH Induces Fas Ligand Production—Serum deprivation induced the expression of Fas ligand in a time-dependent manner (Fig. 2A, Western blot data) in agreement with previously published data (10). To test whether CRH promotes apoptosis via Fas ligand production, the Fas ligand protein levels were measured on PC12 cells exposed to CRH. CRH induced a rapid increase in the concentration of Fas ligand, peaking at 2.5 h (Fig. 2A). Thus, 10^{-9} M CRH induced-Fas ligand production at 2.5 h was 164 ± 12% (Fas ligand:actin ratio, n = 13 of four independent experiments, p < 0.001) of parallel controls, i.e. cells exposed to vehicles only. The stimulatory effect of CRH was detected for 24 h.

Staining of PC12 cells with antibody against Fas ligand showed dense cytoplasmatic staining of cells exposed to CRH 10^{-9} M for 2.5 h (Fig. 3B). Furthermore, flow cytometry analysis of PC12 cells exposed to CRH 10^{-9} M confirmed the Western blot data (Fig. 4). The analysis of the mRNA of Fas ligand by reverse transcription-PCR showed that CRH stimulated Fas ligand expression (Fig. 5). Indeed, Fas ligand mRNA started to increase at 6 h of exposure to CRH and remained elevated at 24 h.

The effect of CRH on Fas ligand production was blocked completely by antalarmin, suggesting that CRH acts via CRHR1 to activate intracellular pathways that lead to Fas ligand induction. Exposure of our cells to 10^{-8} M antalarmin suppressed CRH-induced Fas ligand production from 164 ± 0.05% of parallel controls exposed only to vehicles and represent the mean of five independent experiments, p < 0.05) (Figs. 2A, 3B, 3D, and 4E).

The expression levels of CD95/Fas did not change during serum deprivation with or without CRH stimulation (data not shown). The preceding observations suggest that CRH initiates signals through CRHR1 which lead to Fas ligand induction and apoptosis.

p38 MAPK Mediates the Proapoptotic Effect of CRH—In this group of experiments we examined the effect of CRH on p38 MAPK and the role of the latter in CRH-induced apoptosis because p38 MAPK plays a central role in regulating apoptosis in PC12 cells (22), also affecting Fas ligand production (10). For this purpose we used SB203580, a specific inhibitor of p38 MAPK. Treatment of PC12 cells with different doses of SB203580 inhibited CRH-induced apoptosis (Fig. 6A). The presence of the 20 μM SB203580 resulted in inhibition of the effect initiated by 10^{-9} M CRH of apoptosis from 167 ± 11% to 112 ± 9% (n = 6 of two independent experiments, statistically significant compared with cells exposed to CRH alone, p < 0.05).

In a second set of experiments, we examined the role of p38 MAPK in CRH-induced Fas ligand production. Thus, whereas treatment with 10^{-9} M CRH increased the concentration of Fas ligand to 164 ± 12% of parallel controls (Fig. 6B), the presence of SB203580 diminished the effect of CRH to 118 ± 3% of parallel controls (n = 4 of two independent experiments, statistically significant compared with cells exposed to CRH alone, p < 0.05).

To confirm that CRH activates p38 MAPK, PC12 cells were treated with CRH, and p38 MAPK activity (Fig. 7A) and phosphorylation (Fig. 7B) were measured at different time points. CRH exerted a rapid but transient activation of p38 MAPK which peaked 10 min after treatment and came back to baseline levels after 1 h (Fig. 7D). To test whether the phosphorylation of p38 MAPK is provoked by CRH, cells were incubated with CRH and
supplemented with SB203580 for 10 min, 1 h, and 2.5 h. Treatment with SB203580 blocks the CRH-mediated enhancement, bringing the levels of p38 MAPK back to serum deprivation levels (Fig. 7C). Cumulative data are shown on Fig. 7D.

CRH Induces ERK1/2 Phosphorylation, Which Is Not Associated with Fas Ligand Expression or Apoptosis—Activation of ERK1/2 is known to participate in apoptosis. Indeed, transient activation leads to proapoptotic signals, whereas prolonged activation of ERK1/2 is involved in mediating mitogenic and antiapoptotic signals that could be blocked by PD98059. To determine a possible involvement of ERK1/2 in mediating CRH-initiated signals to induce apoptosis, we treated PC12 cells with PD98059 (MEK1 inhibitor) and measured apoptosis as described previously. Treatment of PC12 cells with PD98059 did not affect CRH-induced apoptosis. PC12 cells were treated with PD98059 and stimulated with CRH for a period of 1–3 days, and apoptosis was evaluated. Our data revealed that inhibition of the ERK1/2 MAPK kinases with PD98059, a MEK1 inhibitor, had no significant effect on CRH-induced apoptosis (Fig. 8A). Similarly, treatment with the same inhibitor did not alter Fas ligand production (Fig. 8B). CRH receptors were shown previously to activate ERK1/2 in different cell types through both CRHR1 and CRHR2 after stimulation by the CRH homolog urocortin (23, 24). Thus, we tested whether CRH activates ERK1/2 phosphorylation in PC12 cells. Indeed, CRH directly activated the ERK1/2 MAPK pathway in PC12 cells. Cells were treated with CRH, and the levels of ERK1/2 MAPK phosphorylation at different time points were measured by Western blotting using a specific anti-phospho-ERK1/2 antibody. Phosphorylation of ERK1/2 MAPK was induced quickly after treatment with 10−9 M CRH, showing a 2.5-fold induction within 10 min which is reduced to base-line levels within 20 min (Fig. 8C), indicating that CRH exerts a rapid and transient activation of ERK1/2. The transient activation induced by CRH may initiate proapoptotic signals that are not inhibited by PD98059, which blocks the endogenous sustained ERK1/2 activation by blocking MEK1. Alternatively, CRH-induced ERK1/2 phosphorylation may lead to events that are not essential for Fas ligand expression and apoptosis. As a result there is no change in Fas ligand protein expression levels and, therefore, no difference in the apoptosis levels with or without inhibition of ERK1/2 (Fig. 8, A and B).

DISCUSSION

We have found that CRH promotes PC12 cell apoptosis via its CRHR1 receptor. Its apoptosis involves activation of p38 MAPK and the Fas/Fas ligand system. Activation of p38 MAPK appears to be crucial for the proapoptotic effect of CRH because inhibition of the action of p38 MAPK blocked the stimulatory effect of CRH on both Fas ligand production and apoptosis. The effect of CRH appears to be mediated by the CRHR1 because it was inhibited by the CRHR1 antagonist antalarmin. Based on our data we propose the following sequence of events: CRH activates the CRHR1 transmitting signals, which results in phosphorylation/activation of p38 MAPK leading to Fas ligand production and apoptosis. The association is supported by previously published reports showing that p38 MAPK affects Fas ligand expression and apoptosis in PC12 cells (10, 22).
In NGF-differentiated PC12 cells, withdrawal of serum and NGF results in up-regulation of p38 MAPK and JNK, leading to Fas ligand expression and apoptosis. Furthermore, inhibition of p38 MAPK promotes survival in primary neuronal cell cultures and in NGF-differentiated PC12 cells, emphasizing the significance of p38 MAPK in apoptosis (13).

CRH receptors are not death receptors per se, i.e. they cannot directly induce activation of apoptotic mechanisms. CRH receptors are G protein-coupled receptors (25) that initiate signals through several intracellular pathways such as that of cAMP, protein kinase C, and MAPKs (23, 26), leading to activation of transcription factors such as the cAMP response element-binding protein (27). The presence of a particular subtype of CRH receptor and the cell type can determine activation of a specific intracellular signaling pathway by CRH. For example, forced expression of CRH receptors in HEK293 and Chinese hamster ovary cells, which do not normally express these receptors, leads to activation of ERK1/2 MAPK and protein kinase C (23).

In this model, urocortin activates ERK phosphorylation through both CRH receptors, but CRH fails to do so. Similarly, in primary cardiac myocytes in culture which do not express CRHR1, urocortin promotes cell survival and induces a rapid phosphorylation of ERK1/2 (26). Finally, in human cytotrophoblast cells, a model in which CRH and CRH receptors play a physiological role, CRHR1 stimulation leads to the formation of inositol triphosphate and protein kinase C activation but fails to induce cAMP formation (28).

In the present work we employed the model of undifferentiated, chromaffin-like PC12 cells that undergo apoptosis under serum starvation conditions. In this system p38 MAPK is an essential component for Fas ligand induction after CRH exposure. Even though JNK plays an important role in Fas ligand production in NGF-differentiated PC12 cells, it does not appear to be involved in chromaffin-like PC12 cells because treatment with the inhibitor CEP-1347, which indirectly blocks JNK, does not prevent serum starvation-induced apoptosis (12). Indeed CRH did not provoke changes in JNK phosphorylation or JNK kinase activity in PC12 cells (data not shown). After treatment of Chinese hamster ovary cells with CRH, its receptors failed to
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FIG. 8. CRH activates ERK1/2, which is not required for the induction of Fas ligand or apoptosis. Cells were exposed to 10^{-8} M CRH with and without 50 μM PD98059 in serum-deprived RPMI 1640 medium for 3 days and lysed as described under “Experimental Procedures.” Panel A, measurement of apoptosis. Panel B, Fas ligand production expressed as the Fas ligand/actin ratio. Panel C, cells were exposed to 10^{-8} M CRH for 10 min, 20 min, 1 h, and 2.5 h. Western blot analysis was performed using antibodies specific for phosphorylated and total ERK1/2. CRH provoked a transient activation of ERK1/2. Data are expressed as percentage of parallel controls exposed only to vehicles and represent the mean of two independent experiments. **p < 0.01 denotes a statistical significance compared with parallel controls, i.e., cells exposed only to vehicles.

activate JNK, indicating that JNK is not a target of the signals initiated by the CRHR1 receptor (23). These observations suggest a signaling difference between neuronal-like differentiated and chromaffin-like undifferentiated PC12 cells according to which different pathways may be employed in each case for the induction of apoptosis.

The ERK1/2 system does not appear to be involved in CRH-induced apoptosis. It should be noted that both CRHR1 and CRHR2 activate ERK1/2 when force expressed in Chinese hamster ovary cells. ERK1/2 is important in the protection of PC12 cells from apoptosis, thus acting as an opposing pathway to the proapoptotic signal of p38 MAPK (22, 29). Characteristic of this system is that sustained activation of ERK1/2 correlates with cell survival in several cell types, whereas transient activation is associated with induction of apoptosis. Inhibition of sustained ERK1/2 activation abolishes the antiapoptotic effect and promotes apoptosis. Here we show that CRH exerts a transient activation of ERK1/2, which, however does not appear to be associated with apoptosis because treatment with the MEK1 inhibitor PD98059 had no effect on CRH-induced apoptosis or CRH-induced Fas ligand production. The effect of CRH on ERK1/2 may be important for other effects of CRH on chromaffin cells including regulation of catecholamine production and secretion.

Our data showing that CRH induced apoptosis of PC12 cells which derive from neural crest are in agreement with recently published reports suggesting that CRH may play an important role in neuronal cell survival. Indeed, it has been shown that CRH provokes hippocampal CA3 neuron loss independently of glucocorticoids (5). This deleterious effect of CRH in hippocampus appears to be direct and paracrine, i.e., from locally synthesized CRH by CRHergic neurons localized in several areas of the developing rat hippocampus including the CA3 pyramidale and oriens strata, the lacunsum-molecular of Ammon’s horn, and the granule cell layer and hilus of dentate gyrus (30).

However, the effect of CRH on cell survival appears to be more complicated than a mere inducer of apoptosis because in primary neuronal cell cultures, CRH exerts a protective effect against cell death caused by amyloid-β peptides, lipid peroxidation, or glutamate (31). The protective effect of CRH is blocked by CRH receptor antagonists.

We consider the proapoptotic effect of CRH to be physiologically relevant for at least two reasons. First, the range of CRH concentrations found to be effective on apoptosis was similar to the range believed to be present within the adrenal medulla (32–36). Second, normal chromaffin cells, like the PC12 cells, have specific CRH binding sites (37, 38). The major sources of adrenal CRH are the medullary chromaffin cells (9), adrenal resident immune cells (39), and preganglionic nerve terminals. This characteristic of normal adrenal chromaffin cells appears to be retained by at least some pheochromocytomas because several human pheochromocytomas and the human (Kat45) and rat (PC12) pheochromocytoma cell lines produce CRH (9, 36, 39–44). The physiological role of adrenal CRH appears to be confined within the gland being paracrine or autocrine. It is now believed that a CRH-containing system exists within the adrenal gland regulating adrenal pre- and postnatal growth, daily steroidogenic activity, catecholaminergic tone, and response to stress (9, 45–47). Furthermore, adrenal CRH appear to be crucial in the adrenal cortical cell response to pituitary-derived systemic ACTH because antalarmin attenuates adrenal responsiveness to ACTH (19). Thus, induction of apoptosis by CRH may be an additional regulatory mechanism within the adrenal gland.

In conclusion, our data indicate that CRH is an inducer of cell apoptosis in the PC12 rat pheochromocytoma cells. The proapoptotic effect of CRH is mediated by CRHR1 and involves activation-phosphorylation of the p38 MAPK, which induces Fas ligand production causing acceleration of apoptosis.

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