Urocortin, But Not Urocortin II, Protects Cultured Hippocampal Neurons from Oxidative and Excitotoxic Cell Death via Corticotropin-Releasing Hormone Receptor Type I

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Urocortin and urocortin II are members of the corticotropin-releasing hormone (CRH) family of neuropeptides that function to regulate stress responses. Two high-affinity G-protein-coupled receptors have been identified that bind CRH and/or urocortin I and II, designated CRHR1 and CRHR2, both of which are present in hippocampal regions of mammalian brain. The hippocampus plays an important role in regulating stress responses and is a brain region in which neurons are vulnerable during disease and stress conditions, including cerebral ischemia, Alzheimer's disease, and anxiety disorders. Here we report that urocortin exerts a potent protective action in cultured rat hippocampal neurons with concentrations in the range of 0.5–5.0 pM, increasing the resistance of the cells to oxidative (amyloid β -peptide, 4-hydroxynonenal, ferrous sulfate) and excitotoxic (glutamate) insults. We observed that urocortin is 10-fold more potent than CRH in protecting hippocampal neurons

from insult, whereas urocortin II is ineffective. RT-PCR and sequencing analyses revealed the presence of both CRHR1 and CRHR2 in the hippocampal cultures, with CRHR1 being expressed at much higher levels than CRHR2. Using subtype-selective CRH receptor antagonists, we provide evidence that the neuroprotective effect of exogenously added urocortin is mediated by CRHR1. Furthermore, we provide evidence that the signaling pathway that mediates the neuroprotective effect of urocortin involves cAMP-dependent protein kinase, protein kinase C, and mitogen-activated protein kinase. This is the first demonstration of a biological activity of urocortin in hippocampal neurons, suggesting a role for the peptide in adaptive responses of hippocampal neurons to potentially lethal oxidative and excitotoxic insults.

Key words: antalarmin; cAMP; excitotoxicity; lipid peroxidation; sauvagine; stress

Corticotropin-releasing hormone (CRH) and urocortin (Urc) are two members of a family of mammalian neuropeptides that function to regulate neuroendocrine, autonomic, and immunologic responses to stress (Owens and Nemeroff, 1991; Lovejoy and Balment, 1999). CRH is expressed widely in neurons in the brain, whereas urocortin exhibits a more restricted expression pattern (Morin et al., 1999). One of the few regions of mammalian brain that contains cells expressing both CRH and Urc is the hippocampus (Morin et al., 1999). The effects of CRH and Urc are mediated by two high-affinity receptors, CRHR1 and CRHR2, which are coupled to multiple G-proteins and which activate at least two intracellular signaling pathways (Perrin and Vale, 1999; Grammatopoulos et al., 2001). Both CRH and Urc are potent activators of CRHR1, although Urc binds CRHR2 with much higher affinity than CRH, and Urc therefore is believed to be the endogenous ligand for CRHR2 (De Souza, 1995; Perrin and Vale, 1999). The expression pattern of CRHR2 in brain is distinct and more restricted than that of CRHR1 (Van Pett et al., 2000). However,

both receptors are expressed in several brain regions, such as the hippocampus (Van Pett et al., 2000). Although there is generally a poor correlation between the localization of Urc-containing projections and major sites of CRHR2 expression (Bittencourt et al., 1999), additional members of the CRH family that selectively bind CRHR2 were identified recently: UrcII (Reyes et al., 2001), UrcIII (Lewis et al., 2001), stresscopin, and stresscopin-related peptide (Hsu and Hsueh, 2001).

Studies with CRHR1 antagonists (Webster et al., 1996; Habib et al., 2000) and of mice lacking the CRHR1 gene (Smith et al., 1998; Timpl et al., 1998) suggest that this receptor is critical for activation of the hypothalamic–pituitary–adrenal (HPA) axis and stress responses. In contrast, studies of mice lacking the CRHR2 gene suggest that this receptor is responsible for the modulation of stress responses and maintenance of homeostasis after HPA axis activation (Coste et al., 2000; Kishimoto et al., 2000). The hippocampus plays a fundamental role in learning and memory processes (Martin et al., 2000) and in negative feedback regulation of the HPA axis during stress (McEwen, 1999). It is a major site of pathology in several disorders, including Alzheimer's disease (AD; Jellinger and Bancher, 1998) and ischemic stroke (Ott and Saver, 1993). Moreover, hippocampal atrophy and impairments in cognitive function occur in conditions characterized by hyperactivation of the HPA axis and excessive secretion of glucocorticoids, including AD, schizophrenia, depression, and anxiety disorders (Raber, 1998). Although CRH and Urc are critical components of the stress response, emerging evidence suggests that there are additional roles for these peptides. For

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instance, evidence has been provided that CRH can regulate learning and memory processes (Lee et al., 1993; Radulovic et al., 1999). Furthermore, CRH can protect against cell death caused by oxidative insults in tumor cell lines (Lezoualc'h et al., 2000) and in primary neuronal culture (Lezoualc'h et al., 2000; Pedersen et al., 2001), and Urc can protect primary cardiac myocytes from hypoxic/ischemic injury (Okosi et al., 1998; Brar et al., 1999, 2000). We now report that Urc exerts a potent cytoprotective activity toward cultured hippocampal neurons, suggesting a role for this peptide in adaptive responses of hippocampal neurons to insult under pathophysiological conditions.

MATERIALS AND METHODS

Primary neuronal cultures and experimental treatments. Cultures of dissociated cortical and hippocampal neurons were prepared from embryonic day 18 (E18) Sprague Dawley rats via methods described previously (Mattson et al., 1992). The cultures were maintained in Neurobasal medium with B27 supplements (Invitrogen, San Diego, CA), and all experiments were performed with 8-d-old cultures. Cell-signaling inhibitor studies and experiments with CRHR1/CRHR2 antagonists were performed in Neurobasal medium for 24 hr treatment periods to avoid toxic effects of the drugs (Pedersen et al., 2001). In all other experiments the treatments were performed in Locke's solution [containing (in mM) 154 NaCl, 5.6 KCl, 2.3 CaCl₂, 1.0 MgCl₂, 5 NaHCO₃, 5 glucose, and 5 HEPES, plus 0.010 mg/ml phenol red, pH 7.2]. Stock solutions of human/rat CRH (Calbiochem, La Jolla, CA), rat Urc (Sigma, St. Louis, MO), and mouse UrcII (custom synthesis by Research Genetics, Huntsville, AL) were prepared in 5% glacial acetic acid at 100 μ M, further diluted to 10 nM with dH₂O, and stored at –20°C. The selective CRHR1 antagonist, antalarmin (provided by Dr. K. Habib, National Institute of Mental Health, Bethesda, MD), and the selective CRHR2 antagonist, antisauvagine-30 (provided by Dr. J. Rivier, The Salk Institute, La Jolla, CA), were prepared in dimethylsulfoxide (DMSO) to a final concentration of 10 μ M and stored at –20°C. The compounds NF 449, PD 98059, bisindolylmaleimide I (BIM I), and H-89 (Calbiochem) were prepared at 200 μ M in dH₂O, at 4 mM in DMSO, at 10 μ M in DMSO, and at 100 μ M in DMSO, respectively, and stored at –20°C. A stock solution of 3-isobutyl-1-methylxanthine (IBMX; Sigma) was prepared at 50 mM in DMSO and stored at –20°C. The sources of and preparation of stock solutions of synthetic human amyloid β -peptide 25–35, 4-hydroxynonenal, ferrous sulfate, and L-glutamic acid were as described in our previous studies (Pedersen et al., 2000, 2001).

Quantification of neuronal survival. Two different methods were used to quantify cell survival. The first method involved counting the number of viable hippocampal neurons, judged by morphological criteria, in premarked microscope fields before and at indicated time points after exposure to the experimental treatments as described previously (Mattson et al., 1992). The second method involved an assessment of nuclear morphology. After treatment the cultures were fixed with 4% paraformaldehyde (in 1 $\times$ PBS) and stained with the fluorescent DNA-binding dye Hoechst 33342 (1 μ g/ml in 1 $\times$ PBS for 30 min). Cells were visualized under epifluorescence illumination (340 nm excitation; 510 nm barrier filter), using a 40 $\times$ oil immersion objective and a Nikon Eclipse TE300 inverted microscope (300–500 cells/culture were counted in four separate cultures per treatment condition). The percentage of cells with condensed and/or fragmented chromatin was determined in each culture.

Reverse transcription-PCR and sequencing. Total RNA was extracted with the use of TRIzol reagent (Invitrogen) from dissected tissues of E18 rats and from 8-d-old cultures of dissociated cortical and hippocampal neurons prepared from E18 rats. First-strand cDNAs were prepared from 2 μ g of total RNA, using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and using oligo-dT according to the manufacturer's instructions. The cDNAs were amplified directly by using PCR and Platinum PCR SuperMix (Invitrogen) with 10 μ l of first-strand reaction mix, 10 μ M forward primer, and 10 μ M reverse primer. The following sequences of the CRHR1 primers were used: forward, 5'-CAA CAC GAC AAA CAA TGG-3', and reverse, 5'-GCA AGA AGA GGA CAA AGG-3'; the following sequences of the CRHR2 primers were used: forward, 5'-TCA TCA CCA CCT TCA TCC-3', and reverse, 5'-CAG CCT TCC ACA AAC ATC-3'. Primers were designed on the basis of the published sequences for rat brain CRHR1 (Chang et al., 1993; Perrin et al., 1993) and CRHR2 (Lovenberg et al., 1995). The conditions for PCR were 94°C for 2 min, followed by 33 cycles of 94°C for

30 sec, 50°C for 30 sec, and 72°C for 45 sec, followed by 72°C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gels, extracted with the QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and subcloned into the pCR 4-TOPO vector (Invitrogen). Plasmid purification was performed with the Qiagen Plasmid Midi Kit, and plasmid DNA was sequenced by using T3 primer and an Applied Biosystems (ABI, Foster City, CA) Prism 3700 automated DNA sequencer.

Quantification of cAMP levels. The cells were pretreated for 1 hr with IBMX, an inhibitor of cAMP and cGMP phosphodiesterases, and then treated for 30 min with Urc, CRH, or UrcII in the continued presence of IBMX. After treatment the cells were washed once with 1 $\times$ PBS, incubated for 10 min in 0.1 M HCl, and scraped into microcentrifuge tubes; homogenates were sonicated briefly and stored at –80°C until use. Direct cAMP measurements were performed with the nonacetylated version of a commercial kit (Assay Designs, Ann Arbor, MI). An aliquot of each homogenate was used for determination of protein content with the Pierce (Rockford, IL) bicinchoninic acid (BCA) kit. To determine the EC₅₀ value of the Urc concentration–response, we fit a rectangular hyperbola to the data plus a constant to account for the levels of cAMP in untreated cells.

Immunoblot analysis. The cells were pretreated for 1 hr with PD 98059, BIM I, or H-89 and then treated with Urc for 15 min in the continued presence of each inhibitor. After treatment the cells were washed once with 1 $\times$ PBS and lysed in the presence of 1 $\times$ cell lysis buffer (Cell Signaling Technologies, Beverly, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride. An aliquot of each extract equivalent to 40 μ g of total protein was subjected to 15% SDS-PAGE. Proteins were transferred electrophoretically to a nitrocellulose sheet, which then was incubated overnight at 4°C in blocking solution (5% milk powder in 1 $\times$ TTBS) and incubated for 2 hr in the presence of an antibody recognizing phosphorylated ERK1/2 (mouse monoclonal IgG_{2a} at a final concentration of 200 ng/ml in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA). After washes in 1 $\times$ TTBS, the membrane was incubated for 1 hr in the presence of peroxidase-conjugated secondary antibody (goat anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) and washed further in 1 $\times$ TTBS. Immunolabeled proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Subsequently, the membrane was washed with 1 $\times$ TTBS, incubated for 30 min in Ponceau S solution (1:10 dilution with dH₂O; Sigma), washed again with 1 $\times$ TTBS, and incubated for 1 hr in blocking solution. Then the membrane was reprobed with an antibody recognizing ERK 1/2 (rabbit polyclonal IgG at a final concentration of 200 ng/ml in blocking solution; Santa Cruz Biotechnology) and processed as above with a goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch). Band intensities were quantified by densitometry with NIH Image software.

RESULTS

Potencies and effectiveness of Urc, CRH, and UrcII in protecting cultured hippocampal neurons from oxidative and excitotoxic insults

We have demonstrated previously that treatment of primary rat hippocampal cultures with CRH can protect the cells from death induced by insults potentially relevant to the neurodegenerative process in AD (Pedersen et al., 2001). Specifically, we observed that treatment of the hippocampal cultures with CRH (≥ 5 pM) protected the cells from death induced by synthetic human amyloid β -peptide (A β) 25–35, the lipid peroxidation product 4-hydroxynonenal (HNE), ferrous sulfate (FeSO₄), or L-glutamic acid (L-glut). Here, we compare the potencies and effectiveness of CRH with that of Urc and UrcII in protecting primary hippocampal neurons from death induced by these insults. Exposure of the cultures to aggregated A β 25–35 at 1 μ M resulted in neurite fragmentation and shrinkage of the cell body in many neurons during a 24 hr period, whereas the majority of neurons cotreated with Urc at 10 pM and A β 25–35 at 1 μ M had intact neurites and cell bodies with normal appearance (Fig. 1A). Cotreatment with Urc decreased the extent of A β -induced cell death in a concentration-dependent manner (Fig. 1B). Significant protection of the neurons occurred with Urc at concentrations as low as

Figure 1. Protective effects of Urc against A β -induced cell death in cultured rat hippocampal neurons. *A*, Cultures were exposed for 24 hr to Locke's solution alone (*Control*) or to Locke's solution containing Urc at 10 pM, A β 25–35 at 1 μ M, or the combination of Urc at 10 pM and A β 25–35 at 1 μ M; the cultures were photographed under phase-contrast optics with a 10 $\times$ objective. Many of the neurons exposed to A β 25–35 exhibited morphological signs of degeneration, i.e., cell body shrinkage and neurite fragmentation (arrows), whereas the majority of neurons treated with a combination of Urc and A β 25–35 had neurites and cell bodies with normal appearance. *B*, The neuroprotective effect of Urc is concentration dependent. Cultures were exposed for 24 hr to Locke's solution alone (*Control*) or to Locke's solution containing A β 25–35 at 1 μ M, Urc at the indicated concentrations, or the combination of A β 25–35 at 1 μ M and Urc at the indicated concentrations. The cultures were photographed under phase-contrast optics with a 10 $\times$ objective, and the percentage of remaining viable neurons was determined. Values are the mean and SD of determinations made in three cultures per treatment condition (* p = 0.0007 and # p < 0.0001 vs A β ; one-way ANOVA and Fisher's PLSD).

0.5 pM, and complete protection was afforded by Urc at concentrations in the range of 1–5 pM (Fig. 1*B*). As with CRH, we observed that Urc also protected against cell death that was induced by HNE, FeSO₄, and L-glut (Fig. 2*A*). However, a 10-fold lower concentration of Urc achieved the same extent of protection when compared with CRH (Fig. 2*B*). For instance, complete protection against HNE-induced cell death occurred with CRH at 10 pM and with Urc at 1 pM. Half-maximal effects were observed with Urc at 0.5 pM and CRH at 5 pM, and a combined treatment of the cultures with Urc and CRH at these concentrations resulted in complete protection from HNE-induced cell death (Fig. 2*B*). In contrast to Urc and CRH, treatment of the cultures with UrcII at concentrations up to 100 pM was completely ineffective at preventing cell death induced by HNE (Fig. 2*C*).

Evidence that the neuroprotective effects of Urc and CRH are mediated by CRHR1

Binding studies have demonstrated that Urc has a higher affinity for CRHR2 than does CRH (Perrin and Vale, 1999), whereas receptor expression studies have revealed that Urc potentially can activate pathways downstream of CRHR1 and CRHR2 (Grammatopoulos et al., 2000). Because Urc exhibited a greater potency than CRH in protecting neurons from insult, we performed a series of studies aimed at determining which type of CRH receptor in fact was mediating these effects of the peptides. First, we performed RT-PCR analyses to establish whether both CRHR1 and CRHR2 were expressed in the cultured hippocampal neurons and to determine the relative abundance of CRHR1 and CRHR2 mRNAs within each sample. We observed expression of both CRHR1 and CRHR2 in untreated 8-d-old hippocampal and cortical cultures, with the levels of CRHR1 mRNA being several-fold higher than the levels of CRHR2 mRNA (Fig. 3). These results are consistent with the relative abundance of CRHR1 and CRHR2 mRNAs in the hippocampus, cortex, and midbrain of E18 rats (Fig. 3). We confirmed the presence of both CRHR1 and CRHR2 in the hippocampal cultures by cloning and sequencing the PCR products (Fig. 3).

In a second set of studies we used highly selective antagonists for CRHR1 and CRHR2 to determine which of the two receptors mediated the protective effects of Urc and CRH in our cultures. In particular, we used the nonpeptide CRHR1 antagonist antalarmin (Ant; Webster et al., 1996; Chen et al., 1997) and the peptide CRHR2 antagonist antisauvagine-30 (aSVG-30; Rühmann et al., 1998; Higelin et al., 2001). As shown in Figure 4, pretreatment of the hippocampal cultures with Ant at 10 nM completely blocked the ability of Urc and CRH to protect against HNE-induced cell death. Interestingly, pretreatment of the cultures with Ant or aSVG-30 at 10 nM caused a slight potentiation in HNE-induced cell death (Fig. 5), suggesting that both CRHR1 and CRHR2 are occupied by ligand under basal conditions and serve a neuroprotective function when the cells are exposed to an insult. Significant protection by Urc against HNE-induced cell death was observed in cultures pretreated with aSVG-30, but not in cultures pretreated with Ant (Fig. 5). Collectively, the results of these studies suggest that the neuroprotective effects of exogenously added Urc and CRH are mediated exclusively by CRHR1, consistent with the much higher expression levels and therefore availability for ligand binding of CRHR1 versus CRHR2.

Figure 3. Expression of CRHR1 and CRHR2 in cultured rat hippocampal and cortical neurons. *Top*, Total RNA extracted from hippocampal and cortical cultures and from the midbrain, hippocampus, and cortex of E18 rat was subjected to first-strand reverse transcription, followed by PCR that used primers specific for either CRHR1 or CRHR2. The PCR products were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and were visualized with a FUJIFilm FLA-3000 fluorescent image analyzer and Image Gauge version 3.2 software. Equal loading of PCR products onto the gel was determined by using internal primers specific for GAPDH (data not shown). Similar results were obtained in three independent experiments. *Bottom*, Identity of the PCR products corresponding to the hippocampal cultures was confirmed by cloning and sequencing. *Underlined* sequences are those recognized by the reverse primers used for PCR amplification.

The neuroprotective effect of Urc requires activation of cAMP-dependent protein kinase, protein kinase C, and mitogen-activated protein kinase

Although it is well established that increases in the levels of cAMP occur with the activation of CRHR1 and CRHR2, little is known regarding the signaling pathways that mediate responses to Urc and CRH. In addition to cAMP-dependent protein kinase (PKA), the results of recent studies suggest the involvement of

protective effects were observed with Urc at 1 μM , CRH at 10 μM , or with the combination of Urc at 0.5 μM and CRH at 5 μM (* p < 0.0001 vs HNE; * p < 0.002 vs Urc, 0.5 μM + HNE; + p = 0.0001 vs CRH, 5 μM + HNE; ** p < 0.008 vs Urc, 0.5 μM + HNE; or CRH, 5 μM + HNE; one-way ANOVA and Fisher's PLSD). C. Cultures were exposed for 24 hr to Locke's solution alone (*Control*) or to Locke's solution containing HNE at 1 μM , UrcII at 1, 10, and 100 μM , or the combinations as indicated. Values are the mean and SD of determinations made in four cultures per treatment condition. UrcII was ineffective at protecting against HNE-induced cell death at any of the concentrations that were tested.

Figure 4. The neuroprotective effects of Urc and CRH are blocked by a CRHR1 antagonist. *A*, Cultures were exposed for 1 hr to fresh Neurobasal with or without antalarmin (*Ant*) at 10 nM and subsequently were exposed for 24 hr to Neurobasal alone (*Control*) or to Neurobasal containing Urc at 10 pM, HNE at 10 μ M, *Ant* at 10 nM, or the combinations as indicated. Values are the mean and SD of determinations made in four cultures per treatment condition. The extent of cell death in cultures treated with HNE alone and in cultures treated with *Ant* followed by Urc and HNE was not statistically different. *B*, Cultures were exposed for 1 hr to fresh Neurobasal with or without *Ant* at 10 nM and subsequently were exposed for 24 hr to Neurobasal alone (*Control*) or to Neurobasal containing CRH at 10 pM, HNE at 10 μ M, *Ant* at 10 nM, or the combinations as indicated. Values are the mean and SD of determinations made in four cultures per treatment condition. The extent of cell death in cultures treated with HNE alone and in cultures treated with *Ant* followed by CRH and HNE was not statistically different.

protein kinase C (PKC; Chakravorty et al., 1999; Miyata et al., 1999) and mitogen-activated protein (MAP) kinase (Brar et al., 2000; Craighead et al., 2000; Grammatopoulos et al., 2000) in cellular responses to Urc and CRH. We have reported previously (Pedersen et al., 2001) that the protective effects of CRH in primary hippocampal cultures could be prevented by pretreatment with H-89, an inhibitor of PKA activity (O'Sullivan and Jamieson, 1992; Otmakhova et al., 2000). Thus we performed experiments to provide evidence for involvement of the cAMP–PKA pathway in the neuroprotective effect of Urc. Treatment of cultured hippocampal neurons with Urc or CRH caused an increase in cellular cAMP levels, an effect that could be blocked by

Figure 5. The neuroprotective effects of Urc are not blocked by a CRHR2 antagonist. Cultures were exposed for 1 hr to fresh Neurobasal with or without *Ant* or antisauvagine-30 (*aSVG-30*) at 10 nM and subsequently were exposed for 24 hr to Neurobasal alone (*Control*) or to Neurobasal containing Urc at 10 pM, HNE at 10 μ M, *Ant* at 10 nM, *aSVG-30* at 10 nM, or the combinations as indicated. Values are the mean and SD of determinations made in four cultures per treatment condition. Pretreatment with *Ant* or *aSVG-30* caused a slight potentiation of HNE-induced cell death (* p = 0.0001 and # p = 0.0074 vs HNE; one-way ANOVA and Fisher's PLSD). The protective effect of Urc was blocked in cultures pretreated with *Ant*, but not in cultures pretreated with *aSVG-30* (* p < 0.0001 vs HNE + *aSVG-30*; one-way ANOVA and Fisher's PLSD).

pretreatment with *Ant*, whereas UrcII treatment did not alter the cellular levels of cAMP (Fig. 6*A*). The effect of Urc on cAMP levels was concentration-dependent, with an EC_{50} value of 0.58 pM (Fig. 6*B*). Consistent with these results and our previously published observations with CRH (Pedersen et al., 2001), we observed that pretreatment of the cultures with H-89 blocked the ability of Urc to protect the neurons against HNE-induced cell death (Fig. 7*A*).

To elucidate further the neuroprotective signaling mechanisms evoked by Urc treatment of cultured hippocampal neurons, we determined the effects of compounds that inhibit the activation of G-proteins, PKC, and MAP kinase on the ability of Urc to protect against HNE-induced cell death. Pretreatment of the cultures with NF449, a selective antagonist of $G_{s\alpha}$ (Hohenegger et al., 1998), the selective PKC inhibitor bisindolylmaleimide I (BIM I; GF109203X; Heikkilä et al., 1993; Huang et al., 1999), or the MAP kinase inhibitor PD 98059 (Dudley et al., 1995) prevented or attenuated the neuroprotective effect of Urc, although none of the compounds affected the basal level of neuron survival (Fig. 7*A*). Two prominent MAP kinases that are known to be inhibited by PD 98059 are ERK1 (44 kDa) and ERK2 (42 kDa). MAP kinases are activated by phosphorylation on serine residues and have been shown to be substrates of PKA (Park et al., 2000) and PKC (Schonwasser et al., 1998). We therefore determined whether Urc does, in fact, activate MAP kinase in cultured hippocampal neurons and whether this requires PKA and/or PKC activities. As shown in Figure 7, *B* and *C*, Urc treatment of the cultures caused an increase in the levels of phosphorylated ERK1 and ERK2. This effect was prevented by pretreatment with PD 98059 or BIM I and was attenuated by pretreatment with H-89, suggesting that the ability of Urc to activate MAP kinases in hippocampal cultures is dependent on

Figure 6. Elevation in cAMP concentrations by Urc and CRH in cultured rat hippocampal neurons. *A*, Cultures were exposed for 1 hr to Locke's solution with 50 μ M IBMX and then were exposed for 30 min to Locke's solution alone (as the control) or to Locke's solution containing Urc at 1 pM, CRH at 10 pM, or UrcII at 10 pM in the continued presence of IBMX. Additional cultures were pretreated for 1 hr with IBMX at 50 μ M and Ant at 10 nM and subsequently were treated for 30 min with Urc at 1 pM or CRH at 10 pM in the continued presence of both IBMX and Ant. Values are the mean and SD of determinations made in four cultures per treatment condition, expressed as a percentage of control cAMP levels (58.8 ± 8.7 pmol/mg protein). There was a statistically significant increase in the levels of cAMP in Urc- and CRH-treated groups ($p < 0.0001$ vs control; one-way ANOVA and Fisher's PLSD), but the levels of cAMP in control, UrcII, Urc + Ant, and CRH + Ant groups were not statistically different. *B*, Cultures were exposed for 1 hr to Locke's solution with 50 μ M IBMX and then were exposed for 30 min to Locke's solution containing Urc at 0, 0.1, 0.5, 1.0, 5.0, or 10.0 pM in the continued presence of IBMX. Values are the mean and SD of determinations made in four cultures per treatment condition. There was a statistically significant increase in the levels of cAMP in cultures treated with Urc at concentrations ≥ 0.5 pM ($p < 0.005$ vs Urc at 0 or Urc at 0.1 pM; one-way ANOVA and Fisher's PLSD).

PKC activity and, to a lesser extent, on PKA activity. Our results suggest that the neuroprotective effect of Urc is dependent on G-protein-coupled receptor activation of signaling pathways involving PKA, PKC, and MAP kinase.

DISCUSSION

We have demonstrated the ability of Urc to protect cultured hippocampal neurons against cell death induced by amyloid β -peptide, oxidative stress, a product of membrane lipid peroxidation, and the excitatory neurotransmitter glutamate. We have demonstrated previously that CRH can protect primary hippocampal and cortical neurons from cell death induced by each of these insults, although a 10-fold higher concentration of CRH was required to achieve the same extent of neuroprotection than with Urc (Pedersen et al., 2001; this study). Although the present findings are the first to document a cytoprotective action of Urc in neurons, it has been reported that Urc protects cardiac myocytes cultured from neonatal rat against lethal ischemic injury and reduces the infarct size of an intact rat heart when applied before and after an ischemic insult (Okosi et al., 1998; Brar et al., 1999, 2000). In these studies Urc was applied at concentrations in the nanomolar range, whereas in cultured hippocampal neurons we observed that Urc can protect against cell death at concentrations as low as 0.5 pM. There is a discrepancy between the reported binding affinities of Urc and CRH toward CRHR1/CRHR2 and the concentrations of the peptides used in our studies (De Souza, 1995; Perrin and Vale, 1999). For instance, the binding affinity of CRH is in the range of 200–400 pM for CRH receptors in adult brain homogenates across a variety of species, including rat (De Souza, 1995). In addition to our studies, it was shown that CRH at a concentration of 10 pM protects IMR-32 cells from an A β -induced decrease in MTT reduction, although nanomolar concentrations were required to achieve full protection (Lezoualc'h et al., 2000). Overall, these results demonstrate that Urc and CRH can promote survival in various cell types. The potency of Urc and CRH toward embryonic neurons may be attributable to higher CRH receptor densities in these cells, based on the fact that CRH receptor densities peak in the CNS during early development (De Souza, 1995).

The ability of a CRHR1 antagonist to block neuroprotection by Urc and CRH demonstrates that the effect is, in fact, receptor-mediated. The compound Ant is a highly selective and potent antagonist of CRHR1, displacing 125 I-ovine-CRH binding from rat frontal cortex homogenates with a K_i of 1.4 nM (Webster et al., 1996). In rhesus monkeys Ant has been shown to attenuate neuroendocrine, autonomic, and behavioral stress responses (Habib et al., 2000). We determined that both CRHR1 and CRHR2 are expressed in our neuronal cultures. However, given that the neuroprotective effect of Urc and CRH was blocked completely by Ant and unaltered by the selective CRHR2 antagonist aSVG-30, our results indicate that only CRHR1 is required for these peptides to protect neurons from cell death. Furthermore, we observed that UrcII, which selectively binds CRHR2 (Reyes et al., 2001), does not protect cultured hippocampal neurons from cell death. Although not tested in our study, we would expect a similar lack of neuroprotective function for UrcIII and stresscopin on the basis of their highly selective binding for CRHR2 (Hsu and Hsueh, 2001; Lewis et al., 2001). Although Urc is believed to be the cognate ligand for CRHR2, both Urc and CRH can bind and potentially activate CRHR1 (Perrin and Vale, 1999). Notably, mice lacking the CRH gene exhibit normal behavioral responses to stressors, which can be attenuated by a

Figure 7. The neuroprotective effect of Urc requires G-protein-coupled receptor activation of PKA, PKC, and MAP kinase. *A*, Cultures were exposed for 1 hr to fresh Neurobasal with or without NF 449 at 200 nM, PD 98059 at 4 μ M, BIM I at 10 nM, or H-89 at 100 nM; subsequently, the cultures were exposed for 24 hr to Neurobasal alone (*Control*) or to Neurobasal containing Urc at 10 pM, HNE at 10 μ M, NF 449 at 200 nM, PD 98059 at 4 μ M, BIM I at 10 nM, H-89 at 100 nM, or the combinations as indicated. Values are the mean and SD of determinations made in four cultures per treatment condition. Pretreatment with each of the inhibitors followed by treatment with Urc and HNE caused a statistically significant increase in the extent of cell death ($p < 0.0001$ vs Urc + HNE; one-way

selective CRHR1 antagonist related to Ant (Weninger et al., 1999). In these mice the behavioral responses to stressors may be attributable to actions of Urc. It is possible, therefore, that some of the effects of Urc *in vivo* are mediated by CRHR1, and the involvement of CRHR1 versus CRHR2 in mediating the various effects of Urc may be determined by the relative abundance of the receptors at sites of action. The lack of involvement of CRHR2 in the protective effects of Urc in cultured hippocampal neurons is likely attributable to a much greater availability of CRHR1 for binding compared with CRHR2.

All of the known effects of Urc and CRH involve receptor-coupled activation of adenylate cyclase and an increase in cellular levels of cAMP, and our data are consistent with this signaling pathway in the neuroprotective effect of these peptides. Furthermore, it is well documented *in vitro* that activation of the cAMP signaling pathway protects against neuronal death caused by a variety of insults, including FeSO₄ and HNE in rat hippocampal cultures (Keller et al., 1998) and glutamate in rat cortical cultures (Morio et al., 1996). In addition to G_s and the cAMP signaling pathway, CRH receptors are coupled to several other G-proteins, including G_{q/11} linked to phospholipase C activation, IP₃ production, and PKC activation (Grammatopoulos et al., 2000, 2001). Downstream targets of PKC include the MAP kinases ERK1 and ERK2. Indeed, Urc and CRH were reported to activate ERK1/2 in primary rat cardiac myocytes (Brar et al., 2000) and cortical neurons (Craighead et al., 2000), respectively. Our observations that neuroprotection by Urc could be prevented or attenuated by inhibitors of G_s, PKA, PKC, or MAP kinase indicate that the effect is dependent on both G_s- and G_{q/11}-activated signaling pathways. Note that Urc is 10-fold more potent in activating G_{q/11} in cells expressing only CRHR1 (Grammatopoulos et al., 2000), which may explain the differences in potencies of Urc and CRH observed in our study. Similar to our findings, it was shown that CRH-induced cell shape changes in mollusk immunocytes require PKA and PKC, as well as PKB/Akt (Malagoli et al., 2000). Substrates of PKA (Park et al., 2000; Cavanaugh et al., 2001) and PKC (Schonwasser et al., 1998) include ERK1/2, and our results with BIM I and H-89 suggest that both PKA and PKC are responsible for Urc-induced phosphorylation of ERK1/2 in primary rat hippocampal neurons. Thus, emerging evidence supports a scenario in which at least two interdependent signaling pathways mediate the effects of Urc and CRH.

The insults used in the present study are relevant to the pathogenesis of several different neurodegenerative disorders. Mechanisms involving oxidative stress, lipid peroxidation, and/or excitotoxicity have been implicated in the degeneration of hippocampal and cortical neurons in AD, substantia nigra dopaminergic neurons in Parkinson's disease, spinal cord motor neurons in amyotrophic lateral sclerosis, and of the various neuronal populations affected in ischemic stroke (Olney et al., 1997; Matt-

ANOVA and Fisher's PLSD). *B*, Immunoblot analysis demonstrating Urc activation of MAP kinases ERK1 and ERK2. Cultures were exposed for 1 hr to PD 98059 at 4 μ M, BIM I at 10 nM, or H-89 at 100 nM and subsequently were exposed for 15 min to Urc with or without each of the inhibitors. The increased phosphorylation of ERK1 (p44) and ERK2 (p42) caused by Urc treatment is abolished in cultures pretreated with PD 98059 or BIM I and is attenuated in cultures pretreated with H-89. The levels of ERK1 and ERK2 in these samples are shown for comparison. *C*, Densitometric analysis of the immunoblots shown in *B*. The levels of phosphorylated ERK1 and ERK2 were normalized to the total levels of ERK1 and ERK2.

son, 1998; Dirnagl et al., 1999; Ludolph et al., 2000; Sayre et al., 2001). We have provided evidence that $\alpha\beta$ causes apoptosis in neurons and increases their vulnerability to excitotoxicity by a mechanism involving membrane lipid peroxidation and the production of HNE (Mattson, 1997). Although speculative at present, Urc and/or CRH may serve a neuroprotective role analogous to neurotrophic factors that are upregulated in response to CNS injury (Mattson and Lindvall, 1997). Furthermore, upregulation in the levels of CRH and Urc during stress (Givalois et al., 2000; Weninger et al., 2000) may protect hippocampal neurons from the potentially damaging effects of glucocorticoids, released from the adrenal gland with the activation of the HPA axis (Raber, 1998; McEwen, 1999). This would provide a self-regulatory neuroprotective mechanism for the stress response axis. Thus, the functions of Urc and CRH in classical stress responses also may be extended to include the regulation of neuroprotective mechanisms.

The levels of CRH mRNA are increased markedly in the cerebral cortex and amygdala of rat brain in response to focal cerebral ischemia (Wong et al., 1995), and CRH immunoreactivity is increased in certain brain regions after kainic acid administration to rats (Piekut and Phipps, 1998). However, it has been shown that neuronal damage in these models can be attenuated by CRH receptor antagonists (Strijbos et al., 1994; Maecker et al., 1997). These results suggest that Urc and/or CRH promote neuronal death, yet evidence has been provided both *in vitro* and *in vivo* that CRH is not directly toxic to neurons (Craighead et al., 2000). One explanation for these observations is that Urc and/or CRH activate glial cells, which express CRH receptors (Bishop et al., 2000), causing them to release substances that are toxic to neurons. It also should be considered that the neuroprotective effects of Urc and CRH may be limited to only certain types of injury and modes of cell death. For instance, it has been reported that CRH is unable to protect primary rat cortical neurons from cell death induced by NMDA (Craighead et al., 2000). In this study, survival was assessed by measuring the release of lactate dehydrogenase, which is an indicator of necrotic cell death. The conditions under which the insults were used in our study would result in primarily an apoptotic form of cell death (Kruman et al., 1997; Guo et al., 1998). Interestingly, neuronal cell death by necrosis was shown to occur by a $G_{\alpha s}$ - and cAMP-mediated pathway in *Caenorhabditis elegans* (Berger et al., 1998). Thus, Urc and CRH may be able to protect neurons under conditions of acute injury, resulting in apoptosis, but actually promote neuronal death under conditions of chronic injury that result in necrosis. Although the exact roles of Urc and CRH in modulating neuronal injury *in vivo* remain to be determined, agonists of CRHR1 potentially may be used for the treatment of neurodegenerative conditions involving an apoptotic form of cell death.

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