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Ghrelin-induced activation of cAMP signal transduction and its negative regulation by endocannabinoids in the hippocampus

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ABSTRACT

Increasing evidence indicates that the gut peptide ghrelin facilitates learning behavior and memory tasks. The present study demonstrates a cellular signaling mechanism of ghrelin in the hippocampus. Ghrelin stimulated CREB (cAMP response-element binding protein) through the activation of cAMP, protein kinase A (PKA), and PKA-dependent phosphorylation of NR1 subunit of the NMDA receptor. Ghrelin increased phalloidin-binding to F-actin suggesting CREB-induced gene expression might include reorganization of cytoskeletal proteins. The effect was blocked by the antagonist of the ghrelin receptor in spite of the receptor's primary coupling to Gq proteins. We also discovered inhibitory effect of endocannabinoids on ghrelin-induced NR1 phosphorylation and CREB activity. 2-arachidonoylglycerol (2-AG) exerted its inhibitory effect in the Type 1 cannabinoid receptor (CB1R)-dependent manner, while anandamide's inhibitory effect persisted in the presence of antagonists of CB1R and the vanilloid receptor, suggesting that anandamide might directly inhibit NMDA receptor/channels. Our findings may explain how ghrelin and endocannabinoids regulate hippocampal appetitive learning and plasticity.

1. Introduction

Ghrelin is a unique acylated 28 amino acid peptide that was first identified in rat stomach extracts as an endogenous ligand for the growth hormone secretagogue receptor (GHSR, or ghrelin receptor). Ghrelin initiates a release of growth hormone through the activation of Gq proteins (Kojima, 1999). In addition, ghrelin increases appetite and initiates a feeding behavior (Ferrini et al., 2009). The ghrelin receptor is localized in high concentrations in the hypothalamus (Harrold et al., 2008). However, the hypothalamus is not the only brain region that expresses the ghrelin receptor. The ghrelin receptor is also highly expressed in the hippocampus (Zigman et al., 2006). This evidence suggests an additional role of ghrelin, since the hippocampus is not considered as the primary brain area that controls appetite or the release of growth hormone. In the hippocampus, circulating ghrelin was reported to cross the blood–brain barrier and enhance long-term potentiation (LTP) (Diano et al., 2006). A well-accepted key molecule in the induction and maintenance of hippocampal LTP is CREB. Indeed, the family of CREB transcription factors has been suggested to be involved in a variety of biological processes, including the development and plasticity of the nervous system (Mayr and Montminy, 2001). Nevertheless, it is not completely understood whether ghrelin stimulates CREB and activates its signaling in the hippocampus. We investigated the expression of phosphorylated CREB (pCREB) in response to ghrelin in the cultured hippocampus, since pCREB expression is a necessary step for the occurrence of functional and structural plasticity.

Endocannabinoid (eCB) and the type 1 cannabinoid receptor (CB1R) have been implicated as key molecules in modulating a feeding behavior. eCB and CB1R stimulate hypothalamic orexigenic neurons, enhance appetite, and facilitate feeding behavior (Jo et al., 2005). Interestingly, evidence suggests that ghrelin may exert its orexigenic effect by stimulating the production of eCB in the hypothalamus (Kola et al., 2008). However, to date, there is no evidence in the hippocampus that a similar interaction might occur between the ghrelin and endocannabinoid system. In the present study, we report a novel role of eCB on ghrelin-induced cellular signaling in CREB activation.

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2. Experimental materials and methods

2.1. Slice preparation and pharmacological treatment

The hippocampal slice culture was used because: 1) chemical effects of ghrelin and anandamide could be assessed directly on the expression of pCREB by eliminating potential neuron-circuit activities produced by synapses made by extra-hippocampal neurons, which can cause secondary changes in CREB activities; and 2) a transient elevation of pCREB was reported as a possible result of decapitation and cardiac perfusion (O'Callaghan and Sriram, 2004).

Slice cultures were prepared from P6 postnatal male pups of Sprague-Dawley rats according to the method of Stoppini et al. (1991). Adequate measures were taken to minimize pain or discomfort. Experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All protocols were approved by the University of Texas at Brownsville Institutional Animal Care and Use Committee. The slices were used for the experiments after being cultured for 1 wk in media that consisted of 50% MEM, 25% HBSS, 24% horse serum, 0.5% penicillin/streptomycin solution, 0.5% 50% glucose solution, and 25 mM HEPES. Ghrelin in an octanoylated form (Phoenix pharmaceutical, Burlingame, CA) was applied to the culture media at a concentration of 200 nM for 60 min (unless specified otherwise in the text). In some experiments, the following compounds were applied to culture media:100 μM L-Dys3-GHSR-6 (Phoenix pharmaceutical, Burlingame, CA), 5 μM ifenprodil, 50 μM Rp-cAMP, 5 μM capsazepine (all from Sigma Chemical, St. Luis, MO), 100 μM APV, 5 μM AM251, 10 nM iodoresiniferatoxin (IRTX), 4 μM WIN55,212-2, 10 μM 2-AG (all from Tocris, Ellisville, MO), and 100 nM JZL184 (Cayman Chemical, Ann Arbor, MI). We applied inhibitors and antagonists to our slice culture for 2 h prior to the application of ghrelin, while agonists were applied for the identical duration of ghrelin application.

2.2. Immunohistochemistry

At the end of experiments, the slices were immersion-fixed with 4% paraformaldehyde in 1M PBS overnight, rinsed, and treated with 0.1% Triton X-100 and 10% goat (or donkey) serum. CREB phosphorylation was detected using a rabbit polyclonal antibody against pCREB (ser 133) (Cell Signaling, Danvers, MA). The ghrelin receptor was identified using a rabbit polyclonal antibody against GHSR (Phoenix Pharmaceutical, Burlingame, CA). pNR1 was detected using a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The following fluorescently-tagged secondary antibodies were used: Alexa 488 for pCREB and pNR1, and Alexa 596 for the ghrelin receptor (all from Invitrogen, Carlsbad, CA). A control for immunohistochemistry consisted of several slices from each experimental condition that were incubated with a blocking peptide before the application of the primary antibody. In some cases, the primary antibody was omitted. Dendritic spines were visualized with phallotoxin-conjugated Alexa 488 (Invitrogen, Carlsbad, CA). Results were imaged at a single cell resolution using a confocal microscope (Fluoview, Olympus, Center Valley, PA) and results were quantified using IPLab imaging software (BD Bioscience, San Jose, CA). Relative changes in the fluorescence intensity for pCREB, pNR1, and phallotoxin were normalized among slices and the results were summarized as mean ± SEM (standard error of the mean). The results were tested for statistical significance with a student *t*-test or ANOVA (analysis of variance) by comparing a given experimental group with a control. Each experiment (i.e., a pharmacological treatment) was accompanied with its own control that was taken from the slices cultured together.$P < 0.05$ was considered significant.

3. Results

3.1. Ghrelin stimulated CREB activities

The ghrelin receptor was highly expressed in our cultured hippocampus. Immunohistochemical analysis showed that the ghrelin receptor had the highest concentration in the somatic region of the pyramidal cell and to a lesser extent in the apical and basal dendritic regions in the CA1 subfield (Fig. 1A and B). This observation is in agreement with a previous report on the ghrelin binding assay, which showed biotinylated ghrelin was scattered in cell bodies of the principal layer of the hippocampal formation (Diano et al., 2006).

The level of CREB activity was assessed with immunohistochemical identification of phosphorylated CREB (pCREB). Quantification of pCREB-immunoreactive neurons was conducted using an auto-segmentation tool (midpoint analysis) provided by IPLab imaging software (Fig. 1C and D). Ghrelin stimulated the expression of pCREB. Low concentrations of ghrelin in 50 and 100 nM did not have any effect on pCREB expression. However, when 200 nM of ghrelin was used, the expression of pCREB increased 4-fold compared to control ($p < 0.01$)(Fig. 1I). We examined higher concentrations of ghrelin in 500 nM and 1 μM; however, the magnitude of pCREB expression did not increase any further in response to these concentrations. Magnitude of pCREB expression was not different among 200 nM and higher doses of ghrelin. Ghrelin binds to its receptor when octanoylated. The major circulating form of ghrelin is the des-acyl ghrelin, the biologically inactive form of ghrelin (at least on the GHS-R), and that very little is known about des-acyl ghrelin catabolism and ghrelin anabolism. Ghrelin can be degraded by both desoctanoylation and N-terminal proteolysis at several cleavage sites (De Vriese et al., 2004). Thus, a steep change in response to differing concentrations of ghrelin in our study may be explained by the unique process of ghrelin desoctanoylation. Finally, the effect of ghrelin was mediated by the ghrelin receptor, since the antagonist of the ghrelin receptor L-Dys3-GHSR-6 (100 μM) reduced the expression of pCREB ($p < 0.02$)(Fig. 1J).

3.2. Effect of Rp-cAMP on ghrelin-induced pCREB expression

A primary molecular constituent for the ghrelin receptor is a Gq protein. Although Gq is not linked to the cAMP/protein kinase A (PKA) signaling cascade that is necessary for CREB activation, the ghrelin receptor can cause a robust activation of CRE-mediated gene transcription (Holst et al., 2003). Therefore, we examined whether PKA was involved in the ghrelin-induced phosphorylation of CREB. Slices were incubated in the inhibitor of PKA (Rp-cAMP, 50 μM) before the application of ghrelin. Rp-cAMP blocked the ghrelin's stimulatory effect on the expression of pCREB (Fig. 2A–D). This result suggested that cAMP-dependent activation of PKA was required in ghrelin-induced activation of CREB.

Gq activation can mobilize cytoplasmic calcium ($[Ca^{2+}]_i$) by translocating IP_3 to the endoplasmic reticulum and initiate a release of Ca^{2+} from stores. Increase in cytosolic Ca^{2+} can stimulate cAMP production via the activation of Ca^{2+}-dependent adenylate cyclases. Thus, we tested the possibility for whether the IP_3 receptor was involved in the ghrelin-induced increase of pCREB in the present study. Pre-incubation of the hippocampal slices with 5 μM of Xestspongin-C, a specific antagonist of the IP_3 receptor, for 1 h before the application of ghrelin was not selective for inhibiting the ghrelin-induced up-regulation of pCREB immunoreactivity.

PKA has many well characterized cAMP-dependent roles in cell physiology, which includes the phosphorylation of the NMDA receptor (Leonard and Hell, 1997). Phosphorylation potentiates NMDA receptor function and increases the receptor-mediated currents (Skeberdis et al., 2006). The increased current permits an enhanced Ca^{2+}-permeation through the NMDA receptor and facilitates the induction of synaptic plasticity by promoting CREB signaling. Thus, we examined whether ghrelin-induced CREB expression depended on the activation of the NMDA receptor and whether NMDA receptor functions were amplified by ghrelin-induced amplification of PKA in a cAMP-dependent manner.

3.3. Effects of NMDA receptor antagonists on ghrelin-induced pCREB expression

The competitive antagonist of the NMDA receptor APV (100 μM) blocked ghrelin-induced expression of pCREB ($p < 0.001$). Surprisingly, APV lowered the level of pCREB expression below the level of pCREB detected in our control slices (Fig. 2E–I). This finding suggests that CREB may have some constitutive activities in the hippocampal slice culture. In order to test this possibility, we applied APV alone without ghrelin. APV decreased the basal level of pCREB expression in our cultured slices ($p < 0.05$, Fig. 2I),