Cancer Metastasis–Suppressing Peptide Metastin Upregulates Excitatory Synaptic Transmission in Hippocampal Dentate Granule Cells

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Arai, Amy C., Yan-Fang Xia, Erika Suzuki, Markus Kessler, Olivier Civelli, and Hans-Peter Nothacker. Cancer metastasis–suppressing peptide metastin upregulates excitatory synaptic transmission in hippocampal dentate granule cells. J. Neurophysiol. 94: 3648–3652, 2005; doi:10.1152/jn.00590.2005. Metastin is an anti-metastatic peptide encoded by the KiSS-1 gene in cancer cells. Recent studies found that metastin is a ligand for the orphan G-protein–coupled receptor GPR54, which is highly expressed in specific brain regions such as the hypothalamus and parts of the hippocampus. This study shows that activation of GPR54 by submicromolar concentrations of metastin reversibly enhances excitatory synaptic transmission in hippocampal dentate granule cells in a mitogen-activated protein (MAP) kinase–dependent manner. Synaptic enhancement by metastin was suppressed by intracellular application of the G-protein inhibitor (MAP) kinase–dependent manner. Synaptic enhancement by metastin was suppressed by intracellular application of the G-protein inhibitor GDP-β-S and the calcium chelator BAPTA. Analysis of miniature excitatory postsynaptic currents (mEPSCs) revealed an increase in the mean amplitude but no change in event frequency. This indicates that GPR54 and the mechanism responsible for the increase in mEPSCs are postsynaptic. Metastin-induced synaptic potentiation was abolished by 50 μM PD98059 and 20 μM U0126, two inhibitors of the MAP kinases ERK1 and ERK2. The effect was also blocked by inhibitors of calcium/calmodulin-dependent kinases and tyrosine kinases. RT-PCR experiments showed that both KiSS-1 and GPR54 are expressed in the hippocampal dentate gyrus. Metastin is thus a novel endogenous factor that modulates synaptic excitability in the dentate gyrus through mechanisms involving MAP kinases, which in turn may be controlled upstream by calcium-activated kinases and tyrosine kinases.

INTRODUCTION

KiSS-1 is a metastasis-suppressor gene initially identified in melanomas (Lee et al. 1996). Recent studies discovered that a fragment of the peptide encoded by this gene is a ligand for the orphan G-protein–coupled receptor GPR54, which has also been called hOT7T175 or AXOR-12 in humans. This peptide fragment, which contains 54 amino acids and is carboxy-terminally amidated, has variously been named metastin (Ohtaki et al. 2001), kisspeptin-54 (Kotani et al. 2001), or KISS11(68–121) (Muir et al. 2001). Several proteolytically generated shorter fragments called kisspeptin-10, -13, and -14 have also been identified as endogenous ligands (Bilban et al. 2004; Kotani et al. 2001). They all contain the same amidated C-terminus and are similarly active with an affinity to GPR54 that is three- to tenfold higher than that of the parent peptide kisspeptin-54 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). GPR54 and KiSS-1 are also present in the brain. They are highly expressed in the hypothalamus in which they are regulated over the reproductive cycle through estrogen and the estrogen alpha-receptor (Lee et al. 1999; Navarro et al. 2004). Moreover, recent reports showed that central administration of C-terminal decapeptide metastin(45–54) (i.e., kisspeptin-10) regulates release of gonadal hormones and onset of puberty (Navarro et al. 2005) through GPR54 in hypothalamic neurons, and that the decapeptide is as active in this regard as the parent peptide metastin(1–54) (Gottsch et al. 2004). In accordance with this, polymorphism of GPR54 has been found to be associated with familial idiopathic hypogonadotrophic hypogonadism (de Roux et al. 2003; Seminara et al. 2003). GPR54 is also expressed at high levels in several other brain regions, including the hippocampal dentate gyrus and the amygdala, but its physiological and/or behavioral function in these regions is still unknown. Moreover, the nature of metastin’s effect on neuronal physiology has not yet been determined in any of the brain regions. Here we report that the decapeptide metastin(45–54) (hereafter called “metastin”) potentely enhances excitatory synaptic transmission in dentate gyrus granule cells through postsynaptic signaling pathways involving mitogen-activated protein (MAP) kinases, and that the peptide itself is also expressed in this region, possibly acting as a paracrine agent.

METHODS

Slice preparation and whole cell recording

Hippocampal slices (400 μm) were prepared from Sprague–Dawley rats of postnatal days 15–18. The animals were anesthetized with halothane before decapitation according to an institutionally approved protocol and the guidelines of the National Institutes of Health. Slices were prepared as described previously (Arai et al. 2004). In brief, a brain block was cut horizontally with a Leica VT1000S vibratome. After 1 h of recovery, a slice was transferred to the recording chamber constantly infused at 0.5 ml/min with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, NaH2PO4 1.25, CaCl2 2, MgCl2 1, NaHCO3 5, glucose 10, and HEPES 10 (pH 7.4). The N-methyl-D-aspartate (NMDA) receptor antagonists D-2-amino-5-phosphonopentanoic acid (d-AP5, 50 μM) and MK-801 (10 μM) and the γ-aminobutyric acid type A (GABAa) receptor antagonist picrotoxin (50 μM) were included in all the experiments. Whole cell recording was made from granule cells in the dentate gyrus and pyramidal neurons in the field CA1 under visualization of neurons with an infrared microscope (BX50WI, Olympus) with differential interference contrast configuration. The borosilicate glass pipette (5–10 MΩ) was filled with the internal solution containing (in mM):

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Cs gluconate 130, MgCl₂, 2, HEPES 10, and ATP Na₂, 2 (pH 7.35 adjusted with CsOH, 280 mOsMol). In some experiments, 1 mM GDP-β-S or 10 mM BAPTA was included in the recording electrode. Synaptic responses were evoked by a bipolar nichrome stimulation electrode positioned in the inner molecular layer of the dentate gyrus and in the stratum radiatum in CA1. Stimulation intensity was adjusted to obtain 30–50% of the maximum amplitude and constant current stimulation was delivered every 15 s. A brief voltage jump of −10 mV for 60 ms was applied to monitor access resistance. Experiments with changes in access resistance of >30% were excluded from analysis. Excitatory postsynaptic currents (EPSCs) were recorded with AxoPatch 200B. Signals were filtered at 5 kHz and digitized at 10 kHz with Digidata1200B/pClamp 9. The holding potential was −70 mV. Experiments were carried out at 22–24°C. Miniature EPSCs (mEPSCs) were recorded in the presence of 1 μM tetrodotoxin. The decay phase of the response was fitted with a monoexponential function and the quality of the fitting was assessed from correlation statistics.

Local pressure application of peptide through a multibarrel pipette

The drug application pipette was positioned 25–50 μm from the recording site, aiming at the proximal dendritic region where the stimulation electrode was positioned. Solution in the drug application pipette was ejected with compressed nitrogen (8–10 psi) with 200- to 400-ms pulses given every 2 s with Picospritzer III (General Valve). The drug application pipette was pulled from three-barrel glass capillaries (WPI). The opening of each tip was approximately 25 μm. The final peptide concentration at the target site was estimated to be about five times lower than the pipette concentration based on separate experiments using the a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). In these tests, an intrapipette concentration of 10 μM CNQX resulted in 50% inhibition of EPSCs; the same degree of inhibition was produced by 2 μM of bath-applied CNQX when tested on synaptic transmission in hippocampal slices (Andreassen et al. 1989).

Trypsin treatment of metastin

Metastin (20 nmol in 100 μl) was treated for 6 h at 37°C with 200 milliunits of activated trypsin-agarose and then collected by removing the beads by centrifugation.

KiSS-1 and GPR54 gene expression

Hippocampal slices were prepared as for physiological recordings. Messenger ribonucleic acid (mRNA) was extracted from these slices and subjected to RT-PCR. For KiSS-1, position 4–159 (156 bp) was amplified using the 5′-primer 5′-ATC TCG CTG GCT TCT TGG CAG-3′ and the 3′-primer 5′-ATA CCG CGG GCC CTG TTG CC-C3′. This primer pair brackets an intron region of 2.2 kb. That the PCR product represents KiSS-1 expression was confirmed by digestion with the endonuclease Mse I, which should produce two fragments of 128 and 28 bp. For GPR54, a 110-bp sequence was amplified using the 5′-primer 5′-CAG TTC TTG CGT GTG CTT CAA-3′ and the 3′-primer 5′-CGC AGA ATT GCT GTA GGA CATG-3′. Digestion with Bgl II produces two fragments of 76 and 34 bp.

Peptide and drugs

Metastin(45–54) (YNWNSFGLYamide) was synthesized by Phoenix Pharmaceuticals. Aliquots of a 1 mM stock solution in distilled water were stored at −20°C and diluted before every experiment. The drugs were purchased from Tocris (CNQX, staurosporine, PP2, wortmannin, PD98059, U0126, SB 203580), Sigma (d-AP5, tetrodotoxin, picrotoxin, GDP-β-S, genistein, trypsin-agarose), Calbiochem (BAPTA, KN-92), and Alexis (KN-93). Slices were generally incubated with inhibitors while in the holding and recording chamber.

**Statistics**

Data are expressed as means ± SE. Student’s t-test was used for statistical analysis.

**Results**

Metastin increases EPSCs in dentate granule cells but not in pyramidal cells

AMPA receptor–mediated EPSCs were evoked by stimulation in the inner molecular layer of the dentate gyrus. Topically applied metastin significantly increased the amplitude of EPSCs at 1 μM in the application pipette, which produced an estimated final concentration of about 200 nM in the tissue (see METHODS), and a maximum increase over baseline of 60–80% was obtained at 3 μM (Fig. 1B). The increase in amplitude reached a maximum within minutes and was readily reversed on washing out the peptide. No effects were observed on the decay time constant of the response (Fig. 1B). Treating metastin with trypsin yields a peptide that lacks the amidated last amino acid and that has more than 1,000-fold lower affinity for GPR54 (Kotani et al. 2001; Ohtaki et al. 2001). As expected, this trypsin-treated metastin failed to enhance the EPSCs (8.1 ± 4.2%, P < 0.0005, six pairs, Fig. 1, C and D). Also, metastin at concentrations of 3 μM (Fig. 1, E and F) or higher (25 μM, not shown) had no effect on synaptic responses in CA1 pyramidal cells that express GPR54 at best at very low levels (Lee et al. 1999). The latter observations corroborate that the effects observed in the dentate gyrus are specifically produced by stimulation of the GPR54 receptor.

Metastin enhances EPSCs through postsynaptic mechanisms activated by Gq

Stimulation of recombinant GPR54 was shown in various cell lines to activate phospholipase C by the G-protein Gq and to increase cytosolic calcium (Kotani et al. 2001). In accordance with these findings, the increase in the dentate gyrus EPSCs was abolished when recordings were made with an intracellular solution containing 1 mM GDP-β-S, an inhibitor of G-protein activation (Fig. 2A; 7.2 ± 4.6% over baseline, n = 13), and when intracellular calcium was chelated with BAPTA (−3.9 ± 3.0%, n = 6, Fig. 2, C–E). Moreover, analysis of mEPSCs showed that metastin significantly increased the mean amplitude by 26.1 ± 4.3% (P < 0.001; 23.5 ± 2.3 vs. 18.7 ± 1.8 pA for control, eight experiments) but had little effect on event frequency (2.6 ± 0.7 vs. 2.5 ± 0.8 Hz; Fig. 1, G and H). Taken together these results indicate that activation of Gq and mobilization of intracellular calcium are essential for metastin’s actions, that the receptor for metastin is located in the dentate gyrus granule cells, and that the mechanisms responsible for enhancing EPSC amplitudes are postsynaptic.

Metastin increases EPSCs through activation of the ERK1/2 MAP kinase pathway

Studies with cancer cell lines provided evidence that the antimetastatic effects of metastin involve MAP kinases and
focal adhesion kinase (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). To examine whether similar processes may be operative in neurons we tested two inhibitors of MAP kinase activation. As shown in Fig. 2, G and H, the inhibitors PD98059 (50 μM) and U0126 (20 μM) completely eliminated the effects of metastin on EPSCs. These effects were specific for this particular subtype of MAP kinases because the p38 inhibitor SB203580 did not block the increase in the EPSCs (Fig. 2, I and J). Upstream activation of the Ras/Raf/ERK2 MAP kinase pathway by GPR54 may involve several Ras-GEFs, which can be activated for instance by calcium/calmodulin-dependent kinases (CaMK) and tyrosine kinases. Inhibitors of these kinases indeed blocked metastin’s action. Thus metastin failed to enhance EPSCs in the presence of 50 μM KN-93, an inhibitor of calcium/calmodulin kinases (0.9 ± 5.0%, n = 9, Fig. 2F) but was fully effective in the presence of the control compound KN-92. Metastin’s effect was also substantially reduced by 30–60 μM genistein, a broad-spectrum inhibitor of tyrosine kinases (14.5 ± 5.0%, n = 12). However, PP2 (2 μM), a specific inhibitor of the Src family tyrosine kinases, was ineffective (61.1 ± 9.9%, n = 10, Fig. 2F). Gq-coupled receptors by mobilizing calcium often activate one of the protein kinase C (PKC) isoforms. However, the broad-spectrum PKC inhibitor staurosporine (1 μM) did not block the effects of metastin (Fig. 2F). The PI3 kinase inhibitor wortmannin (1 μM) had no effect. These results suggest that enhancement of synaptic transmission by metastin specifically involves the Ras/Raf/ERK2 MAP kinase pathway, a calcium/calmodulin kinase, and a tyrosine kinase.

KiSS-1 and GPR54 are expressed in the dentate gyrus

RT-PCR experiments confirmed previous findings that GPR54 is expressed in the hippocampus (Lee et al. 1999; Fig. 2K). More important, these tests also showed that mRNA for KiSS-1 can be detected in whole hippocampal tissue and in dentate gyrus sections prepared from postnatal day 17 animals. This suggests that GPR54 may be activated by peptide released from cells within the dentate gyrus.

Discussion

This study has shown that submicromolar concentrations of metastin reversibly potentiate excitatory synaptic transmission in hippocampal dentate granule cells. This effect is most likely mediated by the G-protein–coupled receptor GPR54, which was identified in several studies as the selective target for full-length metastin and several naturally occurring shorter fragments, including the decapetide used here. This is further supported by our observation that the effect on transmission was abolished after removal of the amidated terminal amino acid by trypsin and by the lack of effect of metastin on synaptic transmission in CA1, which expresses very low levels of this receptor. Moreover, a BLAST search based on various sequences of four amino acids present in the decapetide found no homology in other peptides, which makes cross-reactivity with other neuropeptides acting on G-protein–coupled receptors unlikely.

The enhancement of synaptic transmission by metastin was reliably blocked by two compounds that inhibit ERK1 and ERK2 activation and thus appears to depend on activation of the Ras/Raf/ERK2 MAP kinase pathway. This finding may relate to a growing body of evidence according to which the MAP kinase pathway is linked with excitatory synaptic transmission in a reciprocal fashion. Thus intense synaptic stimulation and subsequent calcium influx through NMDA receptors have been shown to activate MAP kinases, and changes in synaptic strength during long-term potentiation (LTP) have been pro-
they were blocked by intracellular application of GDP-β-S, but the effects of metastin were clearly postsynaptic because phosphorylation of synapsin I (Jovanovic et al. 2000). How to enhance transmitter release after BDNF application by synapses under the control of ERK1/2 (English and Sweatt 1995), by CaMKII acting on Raf-1 (Illario et al. 2003), or by a cascade involving CaM KK and CaMKI (Schmitt et al. 2004). This would accord with our observations that metastin’s effects were blocked by KN-93, a general inhibitor of calcium/calmodulin-activated kinases (Schmitt et al. 2004). The mechanisms underlying the potentiation of AMPA-receptor–mediated currents by metastin remain to be determined. CaMKII can phosphorylate AMPA receptors and increase channel open time (Derkach et al. 1999), but this mechanism cannot readily account for the role of MAP kinases and tyrosine kinases. In view of the suggested role of MAP kinases in LTP (Zhu et al. 2002), a more plausible possibility may be that GPR54 activates ERK1/2, which in turn orchestrates AMPA receptors to move from extrasynaptic to synaptic pools, perhaps aided by an action of GPR54 on actin filaments. However, one notable difference from LTP would be that the processes regulated by GPR54 must be such that they can be rapidly reversed after washout of metastin.

Important questions to be addressed in the future concern the source of metastin in the hippocampus, the factors that regulate its expression, and the physiological role of the metastin/GPR54 system in this brain region. Because the KiSS-1 gene was found to be expressed within the dentate gyrus, metastin may act locally in an autocrine or paracrine fashion. The exact site of expression within this structure remains to be determined, but one plausible scenario would be that metastin originates from hilar neurons, which send projections to the inner molecular layer in which our recordings were made. An alternative possibility is that the peptide is released from axon terminals originating in other brain regions. The most likely source in this case would be the dorsomedial hypothalamic nucleus, which contains metastin-positive cells (Brailoiu...
et al. 2005) and projects to the hippocampus (Thompson et al. 1996). To address these questions it will also be important to determine whether metatin’s effects are limited to a specific input to the dentate granule cells. Finally, a recent study showed that expression of KiSS-1 and GPR54 in the hypothalampus is regulated over the reproductive cycle (Navarro et al. 2004). It is thus possible that metatin levels in the hippocampus exhibit similar cyclic changes and that this serves to synchronize behavior with the reproductive cycle. The high expression of GPR54 in the cortical nucleus of the amygdala and in the habenula, two regions that play a prominent role in reproductive behaviors, lends further support to this interpretation.

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