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Effect of agmatine on DAMGO-induced mu-opioid receptor down-regulation and internalization via activation of IRAS, a candidate for imidazoline I_1 receptor

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ABSTRACT

Agmatine, an endogenous ligand for imidazoline I_1 receptor, has previously been shown to prevent opioid tolerance in rats and mice, but the cellular mechanisms remain unknown. In the present study, the effects of agmatine activation on imidazoline I_1 receptor on the desensitization, down-regulation and internalization of μ opioid receptor were investigated. Two cell lines, CHO cells transfected μ opioid receptor (CHO-μ cells) and co-transfected μ opioid receptor and imidazoline I_1 receptor antisera-selected protein (IRAS) (CHO-μ/IRAS cells), were used. In both CHO-μ cells and CHO-μ/IRAS cells, agmatine (0.01–10 μM) did not affect the desensitization of μ opioid receptor induced by [D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) (10 μM) treatment for 30 min. However, agmatine (0.1–100 nM) co-pretreatment with DAMGO (1 μM) for 12 h concentration-dependently inhibited DAMGO-induced down-regulation of μ opioid receptor in CHO-μ/IRAS cells, but not in CHO-μ cells. Efaroxan, the I_1/α_2-adrenoceptors mix antagonist, completely reversed the inhibitory effect of agmatine, suggesting the participation of imidazoline I_1 receptor. In addition, agmatine (1–100 nM) inhibited DAMGO-induced internalization of μ opioid receptor in CHO-μ/IRAS cells, which was reversed by efaroxan as well. While treatment with DAMGO (1 μM) or co-treatment with agmatine (1–100 nM) for 12 h failed to affect the mRNA level of μ opioid receptor. Taken together, these results indicate that the inhibitory effect of agmatine on tolerance in vitro might be related to attenuation of the internalization and down-regulation of μ opioid receptor via activation of imidazoline I_1 receptor.

1. Introduction

Opioids are the most important analgesics used clinically for pain management. Long-term use of opioids, however, often results in tolerance. Tolerance is often defined as a decrease or loss of opioid analgesic effect following repeated treatments, so that a higher dose is required to keep equivalent analgesic effect (McQuay, 1999). Cellular tolerance following prolonged opioid receptor activation could result from alterations in receptor coupling, the number of receptors, the amount of effector protein or the capacity of an effector to be regulated by opioid receptors. These above processes are considered including the following steps: (1) acute desensitization of opioid receptors to effector coupling, (2) receptor internalization, where the receptor is sequestered from the cell surface, and (3) receptor down-regulation, which is defined as the reduction in the total number of receptors (Stafford et al., 2001; Qiu et al., 2003; Zuo, 2005; Bailey and Connor, 2005). Desensitization, internalization or down-regulation play important roles in tolerance and limit the cellular response to a continuously present stimulus.

Agmatine is an amine that is formed by decarboxylation of L-arginine and hydrolyzed by the enzyme agmatinase to putrescine. As a novel neuromodulator, agmatine binds to several target molecules in the brain, such as imidazoline receptors, *N*-methyl-D-aspartate (NMDA) receptors, α_2-adrenergic receptors, nitric oxide synthase (NOS), and is proposed as an endogenous ligand for imidazoline receptors. Our laboratory and others reported that agmatine significantly enhances morphine analgesia (Li et al., 1999a; Ruiz-Durantez et al., 2003), inhibits the development of tolerance to morphine analgesia (Kolesnikov et al., 1996; Li et al., 1998), attenuates ethanol and morphine withdrawal syndromes (Uzbay et al., 2000; Li et al., 1999b; Aricioglu-Kartal and Uzbay, 1997; Su et al., 2003), and inhibits opioid-induced self-administration (Morgan et al., 2002). These effects were reversed by blockade of imidazoline receptor, suggesting imidazoline receptors mediated the effects of agmatine.

Imidazoline receptors were first discovered by Bousquet et al. (1984) when they studied antihypertension effect of clonidine. It is now accepted that there are at least two subtypes of imidazoline receptors, imidazoline I_1 receptor and imidazoline I_2 receptor. A strong candidate for imidazoline I_1 receptor, known as imidazoline receptor antisera-selected protein (IRAS), has been cloned from human hippocampus (Piletz et al., 2000). Several evidence support the identity of native imidazoline I_1 receptor and cloned IRAS in tissue distributions, ligand binding properties, some cellular functions and downstream signal pathway (Piletz et al., 2000; Dontenwill et al., 2003a,b; Dupuy et al.,

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2004; Li et al., 2006; Piletz et al., 2003). In order to study the role of IRAS in opioid dependence and tolerance, we have established Chinese hamster ovary (CHO) cell line that stably co-expresses μ opioid receptor and IRAS (CHO-μ/IRAS) (Wu et al., 2005). In this cell model, we found that the activation of IRAS by agmatine attenuated the up-regulation of cAMP, Ca^{2+} signal pathway and downstream gene expression induced by morphine, which might be the possible mechanisms of the inhibitory effect of agmatine on morphine dependence (Wu et al., 2005, 2006).

However, the mechanisms of agmatine-inhibiting morphine tolerance remains unknown. The purpose of the study, therefore, is to investigate the role of agmatine on desensitization, internalization and down-regulation of opioid receptor which are underlying the cellular tolerance, and further elucidate the relationship between imidazoline I_1 receptor and the effect of agmatine.

2. Materials and methods

2.1. Materials

The establishment of CHO-μ and CHO-μ/IRAS cell lines have been described previously (Wu et al., 2005). RPMI DMEM/F12 medium and geneticin were purchased from Invitrogen Corporation (Gibco™, Grand Island, NY, USA). Hygromycin B was purchased from Roche Diagnostics GmbH (Roche, Mannheim, Germany). Fetal bovine serum was purchased from HyClone-Pierce (HyClone®, South Logan, UT, USA). [^{3}H]diprenorphine (50 Ci/mmol) and [^{35}S]guanosine 5′-[γ-thio] triphosphate ([^{35}S]GTPγS) were purchased from PerkinElmer Life Sciences (NEN, Boston, MA, USA). Agmatine, naloxone and [D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Cell culture

CHO-μ cells were cultured in RPMI DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μg/ml hygromycin B at 37 °C with humidified atmosphere consisting of 95% air and 5% CO_2. Medium for CHO-μ/IRAS cells was the same as that for CHO-μ cells except for 200 μg/ml geneticin contained.

2.3. Membrane preparation

Membrane proteins were isolated following the method of Zhu et al. (1997). In brief, cells were washed and lysed in ice-cold lysis buffer for 30 min (5 mM Tris–HCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin, pH 7.4), before being passed through a 29G3/8 syringe needle at least five times, and centrifuged at 34,000 ×*g* for 20 min. The pellet was resuspended in 50 mM Tris–HCl (pH 7.4), then passed through the syringe needle and centrifugated again in the same way. The aliquots were frozen at −80 °C. Bradford method was used to determine the protein content. These membranes were used for [^{3}H]diprenorphine and [^{35}S]GTPγS binding assays.

2.4. Measurement of desensitization of μ opioid receptor by [^{35}S]GTPγS binding assay

Cells were cultured for 24 h to reach 80–90% confluence and incubated with DAMGO (10 μM) at 37 °C for 0–120 min. After incubation, cells were rinsed three times with ice-cold phosphate-buffered saline to terminate the reaction and then harvested to prepare membrane for [^{35}S]GTPγS binding assay. To observe the effect of agmatine on desensitization of μ opioid receptor, vehicle or agmatine (10 nM–100 μM) was added for 5 min prior to DAMGO incubation.

[^{35}S]GTPγS binding to G proteins was conducted as a modification of previous method described by Zhu et al. (Zhu et al., 1997). Aliquots of frozen cell membranes were diluted in assay buffer (50 mM HEPES, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, pH 7.4). Cell membrane protein (30 μg) was then added to a tube with a total volume of 500 μl containing 15 μM GDP, 0.2 nM [^{35}S]GTPγS. Nonspecific binding was defined by GTPγS (40 μM). Agonist stimulation of basal [^{35}S]GTPγS binding was estimated by the addition of 10 μM DAMGO. Basal binding was defined as [^{35}S]GTPγS binding in the absence of agonist. After 1 h of incubation at 25 °C, binding was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris–HCl, 5 mM $MgCl_2{\cdot}H_2O$, 100 mM NaCl, pH 7.4), and then the mixture was filtered through GF/C filters and counted in scintillation fluid. Results (from triplicate determinations) were presented as the percentage of stimulation binding relative to basal binding.

2.5. Measurement of down-regulation of μ opioid receptor by [^{3}H]diprenorphine binding assay in membranes

Cells were treated with DAMGO (1 μM) for 12 h at 37 °C. After incubation, cells were rinsed three times with phosphate-buffered saline to remove DAMGO and then harvested to prepare membrane for measuring [^{3}H]diprenorphine binding at 1 nM. Cell membranes (20 μg protein per test tube) were incubated at 37 °C for 30 min in Tris–HCl buffer (50 mM, pH7.4), in a total volume of 500 μl. Nonspecific binding was determined in the presence of 10 μM naloxone. Incubation was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris–HCl, pH 7.4) followed by filtering through GF/C filters and counted in scintillation fluid. Down-regulation of μ opioid receptor was the decreased [^{3}H]diprenorphine binding with μ opioid receptor relative to basal binding (without DAMGO preincubation). In the assay of determining the effect of agmatine and efaroxan, cells were treated with agmatine, DAMGO (0.1–100 nM) and/or efaroxan (10 μM), then harvested for [^{3}H]diprenorphine binding.

2.6. Measurement of internalization of μ opioid receptor by [^{3}H]diprenorphine binding assay in intact cells

Cells were cultured for 24 h to reach 80–90% confluence and harvested. After being washed three times, the cell pellet was resuspendend in Kreb's buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, pH 7.4) with 5×10^5/0.5 ml and incubated with DAMGO (1 μM) at 37 °C for 30 min. The reaction was terminated by adding ice-cold Kreb's buffer and cells were centrifuged at 1500 ×*g*, 4 °C for 5 min. After being washed twice, the cell pellet was resuspendend in Kreb's buffer. To observe the effect of agmatine on internalization of μ opioid receptor, vehicle or agmatine (1 nM–1 μM) was added for 5 min prior to DAMGO incubation.

μ opioid receptor binding in intact cells was performed using [^{3}H] diprenorphine as previously described (Zhang et al., 2002). Total receptors were assessed by binding with 1.5 nM [^{3}H]-diprenorphine at 4 °C for 2 h, and with 10 μM naloxone as the non-specefic binding. Surface receptors were measured by binding with 1.5 nM [^{3}H]-diprenorphine, and 1 μM DAMGO as the non-specific binding. Naloxone, a highly lipophilic antagonist, can bind to both cell surface and intracellular receptors, whereas DAMGO, a hydrophilic agonist, binds only to the cell surface receptors. Thus, the difference between total receptor binding and cell surface receptor binding represents binding to the intracellular receptor pool. An increase in intracellular [^{3}H]-diprenorphine binding over the basal provides a quantitative measure of internalized receptors.

2.7. RT-PCR analysis

Cells were treated with 1 μM DAMGO plus vehicle or agmatine for 12 h at 37 °C. After treatment, cells were rinsed three times with phosphate-buffered saline to remove drugs and then harvested for RT-