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IRAS, a candidate for I_1-imidazoline receptor, mediates inhibitory effect of agmatine on cellular morphine dependence

Ning Wu [a], Rui-Bin Su [a], Bo Xu [a], Xin-Qiang Lu [a], Yin Liu [a], Jian-Quan Zheng [a], John E Piletz [b], Jin Li [a,*], Bo-Yi Qin [a]

[a] *Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China*
[b] *Department of Biology, Jackson State University, Jackson, MS 39217, USA*

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Abstract

Agmatine, an endogenous ligand for the I_1-imidazoline receptor, has previously been shown to prevent morphine dependence in rats and mice. To investigate the role of imidazoline receptor antisera-selected protein (IRAS), a strong candidate for I_1R, in morphine dependence, two CHO cell lines were created, in which μ opioid receptor (MOR) was stably expressed alone (CHO-μ) or MOR and IRAS were stably co-expressed (CHO-μ/IRAS). After 48 h administration of morphine (10 μM), naloxone induced a cAMP overshoot in both cell lines, suggesting cellular morphine dependence had been produced. Agmatine (0.1–2.5 μM) concentration-dependently inhibited the naloxone-precipitated cAMP overshoot when co-pretreated with morphine in CHO-μ/IRAS, but not in CHO-μ. Agmatine at 5–100 μM also inhibited the cAMP overshoot in CHO/μ and CHO-μ/IRAS. Efaroxan, an I_1R-preferential antagonist, completely blocked the effect of agmatine on the cAMP overshoot at 0.1–2.5 μM in CHO-μ/IRAS, while partially reversing the effects of agmatine at 5–100 μM. L-type calcium channel blocker nifedipine entirely mimicked the effects of agmatine at high concentrations on forskolin-stimulated cAMP formation in CHO-μ and naloxone-precipitated cAMP overshoot in morphine-pretreated CHO-μ. Therefore, IRAS, in the co-transfected CHO-μ/IRAS cell line, appears necessary for low concentrations of agmatine to cause attenuation of cellular morphine dependence. An additional effect of agmatine at higher concentrations seems to relate to both transfected IRAS and some naive elements in CHO cells, and L-type voltage-gated calcium channels are not ruled out. This study suggests that IRAS mediates agmatine's high affinity effects on cellular morphine dependence and may play a role in opioid dependence.

Keywords: Opioid dependence; Imidazoline receptor; IRAS; Agmatine; cAMP overshoot

1. Introduction

Chronic administration of opioids such as morphine produces tolerance and dependence, which greatly limits their clinical use. The biological basis of tolerance and dependence induced by chronic exposure to opioids is considered as molecular, cellular, and neural network adaptations. The adaptation in intracellular signal transduction includes up-regulations of cAMP pathway, Ca^{2+} pathway, nitric oxide synthase (NOS) system, MAPK cascade signal pathway, and so on [1,2].

Recent studies show that agmatine, a decarboxylated product of L-arginine, enhances morphine analgesia, and attenuates chronic tolerance and dependence in vivo [3–7]. Many studies demonstrated that agmatine meets many criteria for a neurotransmitter and/or

Abbreviations: α_2-AR, α_2-adrenoceptors; Agm, agmatine; APH, aminoglycoside phosphotransferases; BU224, 2-(4,5-dihydroimidaz-2-yl)-isoquinolin; CHO, Chinese hamster ovary; CHO-μ, Chinese hamster ovary cells expressing μ opioid receptor; CHO-μ/IRAS, Chinese hamster ovary cells co-expressing μ opioid receptor and imidazoline receptor antisera-selected; CHO-μ/IRAS-Low, Chinese hamster ovary cells co-expressing μ opioid receptor and low level imidazoline receptor antisera-selected; Efa, efaroxan; GABA, γ-amino butyric acid; hIRAS, human imidazoline receptor antisera-selected protein; IBMX, 3-isobutyl-1-methylxanthine; I_1R, I_1-imidazoline receptor; I_2R; I_2-imidazoline receptor; IRAS, imidazoline receptor antisera-selected protein; Irs, imidazoline receptors; L-NAME, *N*ω-nitro-L-arginine; MK801, dizocilpine; Mor, morphine; MOR, μ opioid receptor; NE, norepinephrine; Nif, nifedipine; NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; rMOR, rat μ opioid receptor; VTA, ventral tegmental area

* Corresponding author. Tel.: +86 10 66932681; fax: +86 10 68211656.
E-mail address: lijin@nic.bmi.ac.cn (J. Li).

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neuromodulator in brain and is considered as a putative endogenous ligand for imidazoline receptors (IRs) [8]. IRs, also known as imidazoline binding sites or imidazoline recognizing sites, exist in at least two principal subtypes, I_1 and I_2 receptors [9]. Autoradiographic and immunohistochemical studies have demonstrated that I_1R is regionally distributed throughout the central nervous system [10]. Overlap in distributions of IRs and opioid receptors may be observed in some regions related to opioid antinociception and dependence, such as laminae I and II of spinal dorsal horn, nucleus of the solitary tract, midbrain periaqueductal gray, central gray, nucleus paraventricularis thalami, amygdala nucleus and striatum. It has also been reported that I_1R participates in the effects of clonidine on activation of dopaminergic neurons in VTA during morphine withdrawal [11]. Furthermore, after chronic morphine treatment of rats, the density of [^{3}H]idazoxan binding sites is decreased in forebrain and cerebellum [7]. These findings have suggested that I_1R and/or I_2R may be related to the modulation of morphine analgesia, tolerance or dependence.

Though agmatine is considered as a putative endogenous ligand for IRs, whether I_1 and/or I_2 subtypes mediate agmatine's actions on regulating morphine dependence remains unclear. In fact, it has been difficult to directly demonstrate the relationship between IRs, the especially I_1 subtype, and opioid dependence. The reason is that agents binding to I_1R also bind to α_2-adrenoceptors (α_2-ARs), and selective antagonists for I_1R are still not commercially available. Though some new ligands with high affinity and selectivity for I_1R have been synthesized recently [12–14], theses compounds are still not available commercially. Furthermore, there are no suitable cell types or animal models that express I_1R and opioid receptors in the absence of α_2-AR.

An imidazoline-binding protein, named imidazoline receptor antisera-selected protein (IRAS), was cloned from human hippocampus [15]. The amino acid sequence of human IRAS (hIRAS) is distinct from known proteins, except for a mouse homologue named Nischarin, which binds integrin α5 subunit and plays a negative role in cell migration [16]. Several lines of evidence support IRAS as a strong candidate for I_1R. First, IRAS was selected with specific anti-imidazoline receptor antiserum [15]. Second, the distribution of IRAS mRNA was positively correlated with radioligand binding density (B_{max}) for I_1R in a range of rat tissues, including brain [17]. Furthermore, transient transfection of IRAS cDNA into CHO cells resulted in the appearance of high-affinity I_1 binding sites [15].

Based on these observations, we established a stably transfected CHO cell line co-expressing MOR and IRAS. In this cell line, the role of IRAS, a strong candidate for I_1R, in cellular opioid dependence was investigated.

2. Materials and methods

2.1. Materials

The origin of hIRAS-pcDNA3.1(+) has previously been described [15]. rMOR-pcDNA3 and CHO cells were the generous gifts from Dr. L.Y. Liu-Chen (Temple University School of Medicine, Philadelphia, PA, USA). pcDNA3.1/hygro(+) was purchased from Invitrogen Corporation (Invitrogen™, Carlsbad, CA, USA). [^{3}H]diprenorphine (50 Ci/mmol), [^{3}H]clonidine (55.5 Ci/mmol), [^{3}H]yohimbine (85 Ci/mmol), and [^{3}H]L-arginine (45 Ci/mmol) were purchased from PerkinElmer Life Sciences (NEN™, Boston, MA, USA). Geneticin, lipofectamine, and RPMI 1640 medium 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). Agmatine, moxonidine, IBMX, forskolin, naloxone, clonidine, yohimbine, MK801, nifedipine and Dowex 50wx8-400 resin (H^+ form) were purchased from Sigma Chemical Company (Sigma®, St. Louis, MO, USA). Efaroxan and BU224 were purchased from Research Biochemicals International (RBI, Natick, MA, USA). Morphine was purchased from Qinghai Pharmaceutic Factory (Xining, China). The kits for cAMP assays were purchased from the National Academy of Atomic Energy of China (Beijing, China).

2.2. Generation of cell lines co-expressing MOR and IRAS and cell culture

cDNA encoding rat μ opioid receptor (rMOR) was isolated by *Hin*d III digestion from rMOR-pcDNA3 plasmid, and subcloned into the mammalian expression vector pcDNA3.1/hygro(+). Transfection of CHO cells was performed with Lipofectamine according to the manufacturer's instructions. Cells transfected with rMOR cDNA were individually isolated and cloning cultured for 4–6 weeks under the selection pressure of 200 μg/ml hygromycin B. One of the positive clones (CHO-μ) was then retransfected with the hIRAS cDNA (hIRAS-pcDNA3.1(+)) and the cells were grown for another 4–6 weeks under co-selection of 1 mg/ml geneticin and 50 μg/ml hygromycin B. After cell cloning, two positive clones (CHO-μ/IRAS and CHO-μ/IRAS-Low) were selected for study. IRAS's density of CHO-μ/IRAS-Low is one-sixth of CHO-μ/IRAS.

CHO cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C with humidified atmosphere consisting of 95% air and 5% CO_2. The media for CHO-μ and CHO-μ/IRAS were the same as that for CHO cells except the former contained

50 μg/ml hygromycin B, and the latter contained 50 μg/ml hygromycin B and 200 μg/ml geneticin.

2.3. Membrane preparation and [^{3}H]diprenorphine binding assays

[^{3}H]diprenorphine binding assays were used to determine the expression level of MOR. Membrane preparation and [^{3}H]diprenorphine (NEN, Boston, MA, USA) binding assays were done according to Li et al. [18].

2.4. [^{3}H]clonidine binding assays for IRAS in membrane preparation

[^{3}H]clonidine binding assays were used to determine the expression level of IRAS. Cells were pelleted and resuspended in ice-cold lysis buffer containing 5 mM Tris–HCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM 1,10-phenanthroline, and 0.1 mM ascorbic acid at pH 7.4. After 30 min, cell suspension was passed through a 29_G3/8 syringe needle at least five times, and centrifugated (4 °C, 39,000 × *g*, 20 min). The pellets were resuspended in ice-cold 50 mM Tris–HCl buffer (pH 7.5) to pass through the syringe needle and centrifugated again. This step was repeated twice. Membrane preparation was resuspended in HME buffer contained 5 mM HEPES, 0.5 mM EDTA, 0.5 mM EGTE, 0.5 mM $MgCl_2$, and 0.1 mM ascorbic acid (pH 7.5). Brad-ford method was used to determine the protein content.

Saturation binding of [^{3}H]clonidine was performed with seven concentrations from 1 to 32 nM. Binding was carried out in HME buffer, which was optimal for I_1R, containing 50 μg membrane preparation with a final volume of 250 μl in the presence of an α_2-AR ligand (100 μM norepinephrine) to mask the possibility of α_2-AR. Moxonidine (10 μM) was used to define specific binding to I_1R. After 1 h incubation at 25 °C, bound and free [^{3}H]clonidine were separated by filtration with GF/C filters under reduced pressure, and the filters were washed rapidly three times with 50 mM Tris–HCl buffer (pH 7.5). Radioactivity in filters was determined by liquid scintillation counting.

2.5. [^{3}H]yohimbine binding assays in membrane preparation

[^{3}H]yohimbine (α_2-antagonist) binding assays were used to find out whether α_2-ARs existed endogenously in the parent cell line. Membranes of CHO cells were prepared as described for [^{3}H]diprenorphine binding. Saturation binding of [^{3}H]yohimbine was carried out in 50 mM Tris–HCl buffer (pH 7.5) at 37 °C for 20 min. Radioligand concentrations ranged from 1 to 32 nM. Protein concentration was 60 μg/ml and unlabelled yohimbine (500 μM) was added for determination of non-specific binding.

2.6. Measurement of cAMP levels

Cells were plated into 6-well plates and treated with vehicle (saline) or drugs for 48 h. Following the treatment, cells were washed three times with 2 ml of RPMI 1640 medium. The levels of cAMP were measured during a 10-min incubation with IBMX (500 μM) and forskolin (10 μM). The cAMP overshoot was then elicited by the addition of 10 μM naloxone. The reaction was terminated by removing incubation medium and adding 1 ml of ice-cold 5% trichloroacetic acid. Sample preparation and cAMP level assay were performed according to kit's instructions. The results were expressed as pmol/(mg protein 10 min).

2.7. Measurement of NOS activity

NOS activity was determined as previously described [19] with some modifications. Cells were harvested and homogenized in a solution containing 50 mM Tris–HCl, 1 mM EGTA, 1 mM D,L-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin at pH 7.4. The crude homogenates were centrifuged at 12,000 × *g* for 60 min at 4 °C. The NOS activity in the supernatants was determined by the rate of conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine. Cell extracts (150 μg/ml) were incubated in the assay buffer (100 μl) at 37 °C for 30 min. The reactions were terminated by adding a buffer containing 20 mM HEPES, 2 mM EGTA, 0.2 mM EDTA, and 1 mM D,L-citrulline at pH 5.5. [^{3}H]L-citrulline in the mixture was separated from [^{3}H]L-arginine by chromatography using Dowex 50wx8-400 resin (Na^+ form). For the determination of total NOS activity, the assay buffer contained 50 mM Tris–HCl, 1 mM D,L-dithiothreitol, 100 μM β-NADPH, 2 mM $CaCl_2$, 20 μg/ml calmodulin, and 2.5 μCi/ml [^{3}H]L-arginine at pH 7.4. The assay buffer, with deletion of $CaCl_2$ and calmodulin, was used to determine the activity of inducible NOS. The activity of constitutive NOS was calculated by the total NOS activity minus the inducible NOS activity. Results were expressed as pmol citrulline/(mg protein min).

2.8. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analyses were done with one-way ANOVA followed by Dunnett *t*-test or *t*-test.

3. Results

3.1. Co-expression of MOR and IRAS in CHO cells

No specific binding was detected with 1 to 32 nM [^{3}H]yohimbine (data not shown). This indicated that there

is no α_2-AR in CHO cells, which was consistent with previous reports [15,20].

Saturation binding assays were performed with [^{3}H]diprenorphine and [^{3}H]clonidine to determine the density (B_{max}) and affinity (K_d) for MOR and I_1R, respectively, in CHO cells stably transfected with rMOR (CHO-μ) and hIRAS (CHO-μ/IRAS). Untransfected CHO cells did not exhibit any [^{3}H]diprenorphine binding or high-affinity [^{3}H]clonidine binding (data not shown). In CHO-μ/IRAS cells, the B_{max} and K_d values of MOR were 1.83 ± 0.13 pmol/mg protein and 0.24 ± 0.02 nM ($n = 5$), and the values of IRAS were 184.0 ± 12.8 fmol/mg protein and 15.75 ± 2.29 nM ($n = 3$), respectively (curve fitting by non-linear regression analysis). In CHO-μ expressing μ alone, the B_{max} and K_d values of MOR were 1.87 ± 0.20 pmol/mg protein and 0.25 ± 0.03 nM ($n = 4$), similar to those in CHO-μ/IRAS cells. In addition, morphine (1 nM–10 μM, 10 min) concentration-dependently inhibited adenylyl cyclase activity in CHO-μ/IRAS and CHO-μ (Fig. 1). The E_{max} and EC_{50} for morphine were similar; indicating that co-expression of IRAS with MOR had no direct influence on the function of MOR.

3.2. Effect of agmatine on forskolin-stimulated cAMP levels and chronic morphine-treated, naloxone-precipitated cAMP overshoot in CHO-μ and CHO-μ/IRAS cell lines

Baseline experiments were first conducted in the absence of opioid. Agmatine's concentration ranged from 10 nM to 100 μM based on the affinity for I_1R [21,22]. Pretreatment with agmatine at concentrations from 10 nM to 2.5 μM for 48 h did not affect forskolin-stimulated cAMP formation in CHO-μ or CHO-μ/IRAS (Fig. 2 A).

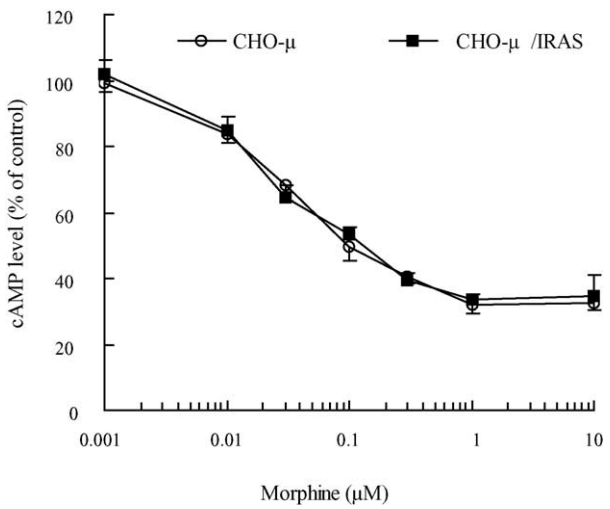

Fig. 1. Inhibition by morphine of adenylyl cyclase activity in CHO-μ and CHO-μ/IRAS. The data are mean ± S.E.M. of six separate experiments in duplicate. Control cAMP levels were 1417.6 ± 99.8 pmol/(mg protein 10 min) for CHO-μ and 1288.0 ± 76.3 pmol/(mg protein 10 min) for CHO-μ/IRAS.

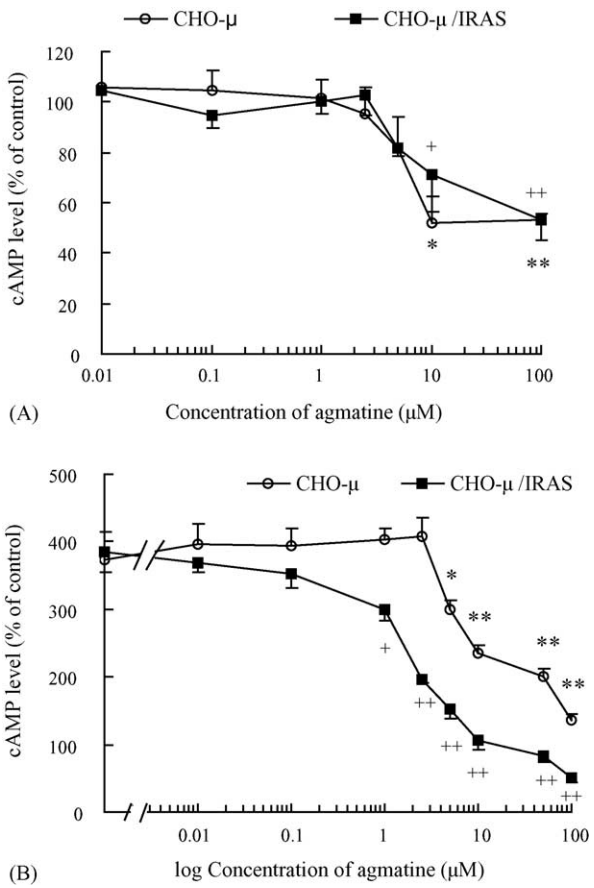

Fig. 2. Effects of agmatine on (A) forskolin-stimulated cAMP levels and (B) morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ and CHO-μ/IRAS cells. The data are mean ± S.E.M. of six separate experiments in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from vehicle-pretreated control in CHO-μ by one-way ANOVA followed by Dunnett t-test; $^{+}P < 0.05$ and $^{++}P < 0.01$, significantly different from vehicle-pretreated control in CHO-μ/IRAS by one-way ANOVA followed by Dunnett t-test. (A) Control cAMP levels were 1551.7 ± 92.1 pmol/(mg protein 10 min) for CHO-μ and 1336.8 ± 120.3 pmol/(mg protein 10 min) for CHO-μ/IRAS. (B) Control cAMP levels were 1064.4 ± 111.9 pmol/(mg protein 10 min) for CHO-μ and 1423.4 ± 128.6 pmol/(mg protein 10 min) for CHO-μ/IRAS. cAMP overshoots were $372.9 \pm 40.9\%$ for CHO-μ and $383.7 \pm 29.3\%$ for CHO-μ/IRAS.

However, above 5 μM agmatine, forskolin-stimulated cAMP formation was decreased (Fig. 2A). Except for a slight difference at 10 μM agmatine, the inhibitions were similar in both transfected cell lines, suggesting that agmatine's effect at those concentrations is independent of IRAS in the absence of opioid.

Up-regulation of adenylyl cyclase following prolonged or repeated opioid treatment is considered an important cellular mechanism underlying opioid dependence. Hence, naloxone-precipitated cAMP overshoot, also named cAMP super-sensitization, after chronic opioid treatment of cells is an in vitro model of the abstinence state. In CHO-μ and CHO-μ/IRAS, naloxone-precipitated cAMP overshoot was observed after morphine (10 μM) pretreatment for 48 h (Fig. 2B). The concentration of cAMP induced by naloxone (10 μM, 10 min) was approximately three to

four-fold higher than control cells without morphine (Fig. 2B). Co-pretreatment of CHO-μ/IRAS with 1.0 and 2.5 μM agmatine during morphine exposure for 48 h significantly decreased the naloxone-precipitated cAMP overshoot by 21.7 and 49.0%, respectively (Fig. 2B). A complete return to basal levels was obtained with 50 μM agmatine (Fig. 2B). In CHO-μ, up to 2.5 μM agmatine had no effect on chronic morphine-induced naloxone-precipitated cAMP overshoot. Only higher concentrations of agmatine in CHO-μ cells were able to attenuate cAMP overshoot in a concentration-dependent manner, but the degree of inhibition was still not 100% in CHO-μ cells even at 100 μM (Fig. 2B). The IC_{50} values of agmatine inhibiting cAMP overshoot were 3.54 ± 0.88 μM in CHO-μ/IRAS and 77.50 ± 16.86 μM in CHO-μ, respectively ($n = 6$).

After chronic morphine exposure, agmatine (10 nM–100 μM) co-administrated with naloxone did not affect cAMP overshoot in both cell lines (data not shown). In addition, agmatine (10 nM–100 μM, 10 min) alone did not alter forskolin-stimulated cAMP formation (data not shown).

3.3. Effect of agmatine in CHO-μ/IRAS-Low cell line

Compared to CHO-μ, aminoglycoside phosphotransferase (APH) was expressed in CHO-μ/IRAS and geneticin was added to stabilize the CHO-μ/IRAS culture. A potential artifact of the co-transfection procedure was also investigated using another CHO-μ/IRAS-Low clone. In this second cell line, it was observed that 0.1–2.5 μM agmatine did not alter cAMP overshoot even though the medium contained geneticin (Fig. 3). But, when agmatine was increased to 10 and 100 μM, the cAMP overshoot was attenuated in CHO-μ/IRAS-Low (Fig. 3). Therefore, geneticin and APH could not alone explain agmatine's inhibitory effect on the cAMP overshoot and it seems the effect of agmatine at 0.1–2.5 μM is dependent on the level of IRAS. These results suggested that the inhibitory effect of agmatine on chronic morphine-induced naloxone-precipitated cAMP overshoot is IRAS-mediated at low concentrations of agmatine.

3.4. Antagonism by efaroxan of the inhibitory effect of agmatine on naloxone-precipitated cAMP overshoot

As CHO cells lack α_2-ARs, the mixed I_1/α_2 receptors antagonist efaroxan (10 and 50 μM, according to previous reports [23,24]) was used selectively to examine the role of IRAS in mediating agmatine's effects on morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ/IRAS cells. Our preliminary studies showed that pretreatment of the cells with efaroxan alone (10 or 50 μM, 48 h) had no effect on either forskolin-stimulated cAMP formation or morphine-pretreated, naloxone-precipitated

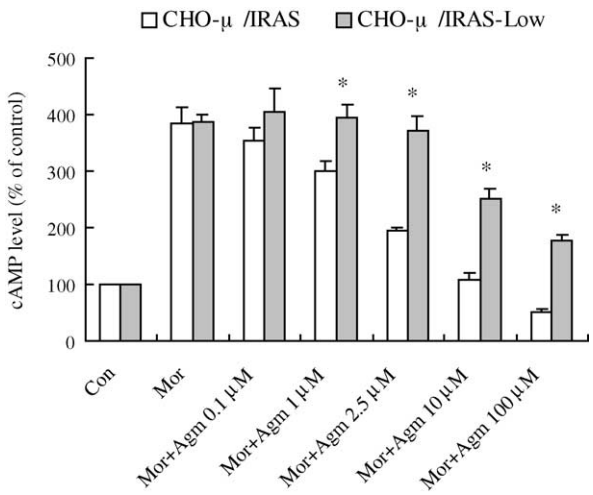

Fig. 3. Effect of agmatine on morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ/IRAS and CHO-μ/IRAS-Low cells. The data are mean ± S.E.M. of six separate experiments in duplicate. $^{*}P < 0.01$ and $^{**}P < 0.01$, significantly different from CHO-μ/IRAS by *t*-test. Control cAMP levels were 1423.4 ± 128.6 pmol/(mg protein 10 min) for CHO-μ/IRAS and 1056.4 ± 103.0 pmol/(mg protein·10 min) for CHO-μ/IRAS-Low. cAMP overshoots were 383.7 ± 29.3% for CHO-μ/IRAS and 386.1 ± 14.4%for CHO-μ/IRAS-Low.

cAMP overshoot in CHO-μ or CHO-μ/IRAS cells (data not shown). Furthermore, in CHO-μ cells, efaroxan (50 μM, 48 h) did not antagonize agmatine's (5–100 μM, 48 h) inhibition of cAMP overshoot (Fig. 4A), which indicated there is no endogenous I_1 binding site in CHO-μ cells, and agmatine's effect in this cell line might be I_1R-independent. By comparison, the inhibitory effects of 1.0 or 2.5 μM agmatine on the cAMP overshoot in CHO-μ/IRAS were significantly reduced by 10 μM efaroxan and completely blocked by 50 μM efaroxan (Fig. 4B). The effects of agmatine at 5 or 10 μM were partially reversed by 50 μM efaroxan in the CHO-μ/IRAS cell line (Fig. 4B).

3.5. Effect of BU224 on agmatine's inhibition in CHO-μ cells

In CHO-μ cells, agmatine (5 μM or higher) attenuated chronic morphine-induced naloxone-precipitated cAMP overshoot, suggesting that non-IRAS mechanisms might also be involved in the effect of agmatine. A putative I_2R antagonist, BU224, was used to determine the role of I_2R in the inhibitory effects of agmatine. Pretreatment with BU224 (100 μM) for 48 h did not alter the forskolin-stimulated cAMP level in naive CHO-μ cells or the naloxone-induced cAMP overshoot in CHO-μ cells pretreated with morphine (Fig. 5). Likewise, BU224 did not block the effects of agmatine on forskolin-stimulated cAMP level or naloxone-induced cAMP overshoot. Furthermore, it has been reported by Zhao et al. that CHO cells lack [^{3}H]idazoxan binding [25], indicating the absence of I_2R site.

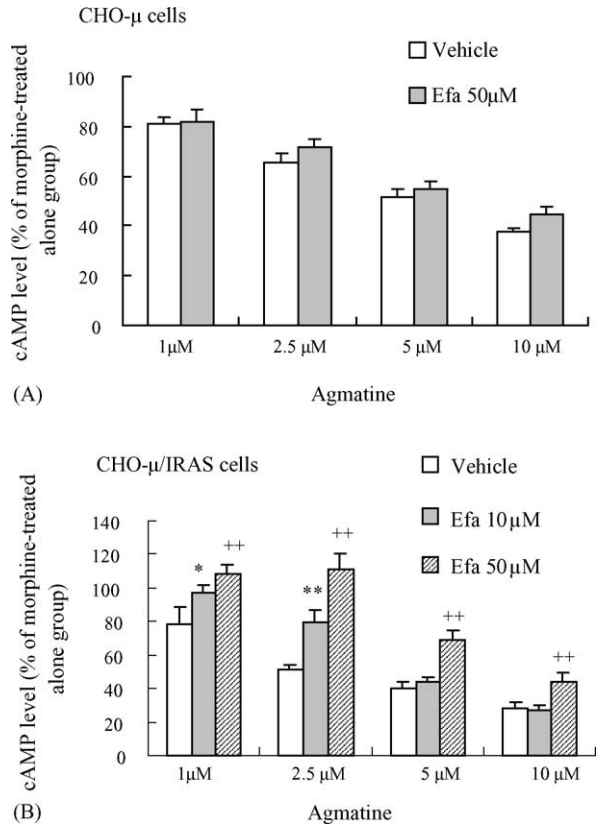

Fig. 4. Antagonism by efaroxan of the inhibitiory effect of agmatine on morphine-pretreated, naloxone-precipitated cAMP overshoot in (A) CHO-μ and (B) CHO-μ/IRAS cells. The data are mean ± S.E.M. of six separate experiments in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from vehicle group by *t*-test; $^{++}P < 0.01$, significantly different from vehicle group by *t*-test. (A) CHO-μ cells. Control cAMP levels were 1020.7 ± 138.5 pmol/(mg protein 10 min) for vehicle group and 1085.2 ± 107.4 pmol/(mg protein 10 min) for 50 μM efaroxan group, respectively. (B) CHO-μ/IRAS cells. Control cAMP levels were 1218.0 ± 66.0 pmol/(mg protein 10 min) for vehicle group, 1124.4 ± 68.4 pmol/(mg protein 10 min) for 10 μM efaroxan group, and 947.1 ± 80.7 pmol/(mg protein 10 min) for 50 μM efaroxan group, respectively.

3.6. Effect of L-NAME, MK801, or nifedipine on chronic morphine-treated, naloxone-precipitated cAMP overshoot in CHO-μ

Whether NOS inhibitor, NMDA receptors antagonist or L-type Ca^{2+} channels blocker mimic the inhibitory effect of agmatine on morphine-pretreated, naloxone-precipitated cAMP overshoot was investigated. Pretreatment with L-NAME (10 μM, 100 μM and 1 mM), a competitive inhibitor of NOS, for 48 h did not decrease the chronic morphine-induced naloxone-precipitated cAMP overshoot in CHO-μ (data not shown). Furthermore, no NOS activity was detected in naive CHO-μ cells, or in morphine pretreated CHO-μ cells, or in morphine pretreated and naloxone-precipitated CHO-μ cells (data not shown). Additionally, MK801, an antagonist of NMDA receptor, at 1 and 10 μM did not alter forskolin-stimulated cAMP level (Fig. 6). Furthermore, MK801 (1 and 10 μM) did not mimic the effect of agmatine (5-100 μM) on chronic

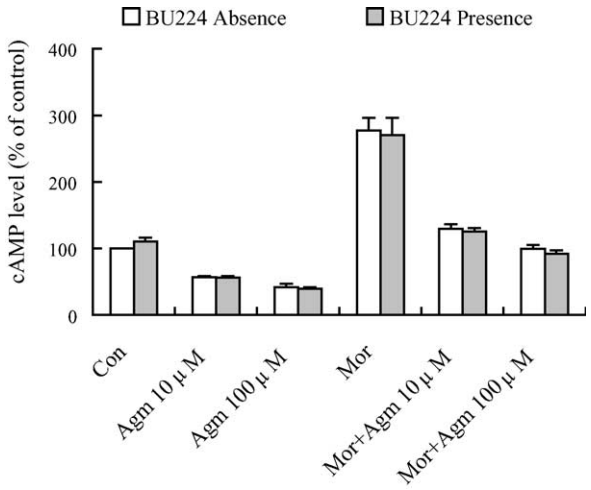

Fig. 5. Lack of effect of the putative I_2R antagonist BU224 on the inhibitory action of agmatine on forskolin-stimulated cAMP formation and morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ. The data are mean ± S.E.M. for six separate experiments in duplicate. Control cAMP level was 1035.3 ± 39.2 pmol/(mg protein 10 min). cAMP overshoots were 274.9 ± 19.2% in the group of BU224 absence and 269.8 ± 26.3% in the group of BU224 presence, respectively.

morphine-induced naloxone-precipitated cAMP overshoot in CHO-μ (Fig. 6).

Nifedipine (1 and 10 μM), a blocker of L-type calcium channels, concentration-dependently decreased forskolin-stimulated cAMP levels in CHO-μ (Fig. 7 A) and also attenuated naloxone-induced cAMP overshoot compared with cells treated with morphine alone (Fig. 7B). In the presence of 1 and 10 μM nifedipine, cAMP overshoot was decreased by 17.8 and 39.6%, respectively. Thus, only nifedipine mimicked the actions of agmatine (5–100 μM) in CHO-μ cells. This suggests that some of the inhibitory effects of agmatine (5–100 μM) on cAMP overshoot may involve blockade of L-type calcium channels.

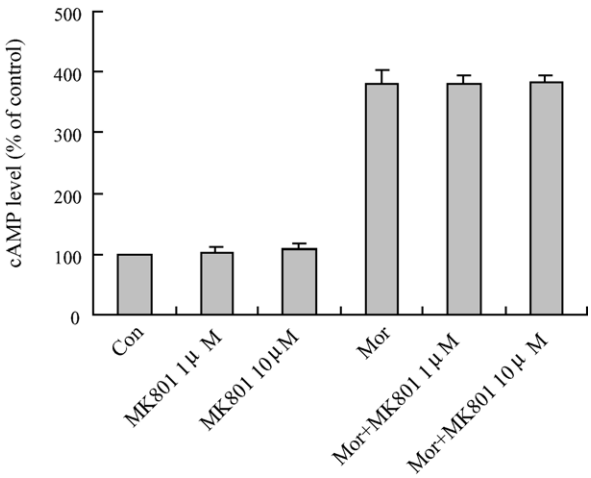

Fig. 6. NMDA receptor antagonist MK801 does not mimic agmatine on forskolin-stimulated cAMP formation and morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ. The data are mean ± S.E.M. for six separate experiments in duplicate. Control cAMP level was 945.0 ± 60.1 pmol/(mg protein 10 min) and the cAMP overshoot was 379.4 ± 25.1%.

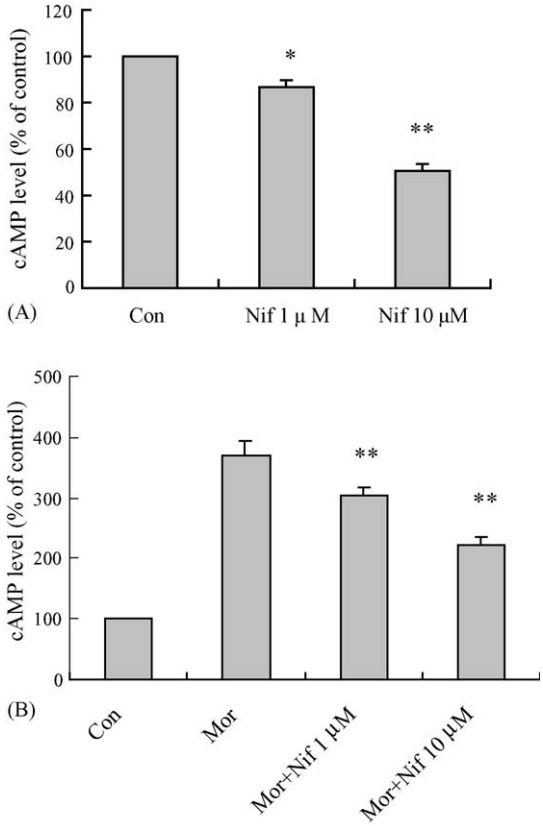

Fig. 7. Effects of the L-type Ca^{2+} channel blocker nifedipine on (A) forskolin-stimulated cAMP formation and (B) morphine-pretreated, naloxone-precipitated cAMP overshoot in CHO-μ. The data are mean ± S.E.M. for six separate experiments in duplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from vehicle-pretreated control by one-way ANOVA followed by Dunnett *t*-test. (A) Control cAMP level was 991.7 ± 126.5 pmol/(mg protein 10 min). (B) Control cAMP level was 991.7 ± 126.5 pmol/(mg protein 10 min) and the cAMP overshoot was 369.1 ± 25.5%.

4. Discussion

It has previously been shown that the CHO cell is a suitable cell type to express MOR, with the expressed receptor showing ligand-binding selectivity [26,27]. This transfected cell line therefore provides an excellent model to study signal transduction mechanisms and cellular adaptations induced by chronic exposure to opioids [28,29]. Herein, we have established the first stable co-expression system of rMOR and hIRAS in CHO cells. It is believed that this is a useful system to study possible interactions between MOR and IRAS, a candidate for I_1R, because the background (parental CHO cells) lacks native MOR, I_1R, I_2R, NMDA receptors and α_2-AR [15,20,30]. Thus, the possible reaction between MOR and IRAS as well as the effects of drugs can be studied in a defined system. Our research provides the first evidence for participation of IRAS in the inhibitory effects of agmatine on opioid substance dependence.

A transient expression system of hIRAS was previously established by transfection into CHO cells [15]. In that study, it was shown that expression of hIRAS requires host-cell specificity. Despite Western blot evidence for the expression of the hIRAS protein in other transiently transfected cells (Sf9 and COS-7), only the CHO cell line was able to produce I_1R binding sites [15]. In our experiment, the parental CHO cell line was shown to lack α_2-AR binding sites for [^{3}H]yohimbine, which was consistent with another previous report [20]. Additionally, no specific [^{3}H]idazoxan I_2 binding was detected in untransfected CHO cells [25]. Though a low level of atypical non-adrenergic binding of [^{3}H]clonidine and *p*-[^{125}I]iodoclonidine was observed in CHO cells [15,21], these atypical bindings lack the pharmacological characteristics of I_1 or I_2 binding sites. By comparison, when hIRAS-pcDNA3.1(+) and rMOR-pcDNA3.1/hygro(+) were stably co-transfected into CHO cells, the transfectant CHO-μ/IRAS cells exhibited high affinity binding sites for [^{3}H]diprenorphine to MOR and [^{3}H]clonidine to I_1R compared with untransfected cells. The specific [^{3}H]clonidine binding was observed in membrane preparation of transfected cells. Our results thus confirm that the IRAS protein expressed in CHO cells has I_1R binding characteristics.

Even though IRAS's candidacy as I_1R still be debated, our present study provides evidence for the participation of IRAS in morphine dependence. In IRAS-negative CHO-μ cells exposed to morphine (48 h), pretreatment with low concentrations of agmatine (0.01–2.5 μM) did not alter the naloxone-precipitated cAMP overshoot. By comparison, in IRAS-positive CHO-μ/IRAS cells, low-concentrations of agmatine significantly attenuated the naloxone-induced cAMP overshoot. Furthermore, the mixed I_1/α_2 receptor antagonist efaroxan (50 μM) completely reversed agmatine's effect (1.0 and 2.5 μM). For lack of α_2-AR in CHO cells, IRAS seems mediates the inhibitory effect of agmatine at low concentrations on chronic morphine-pretreated, naloxone-induced cAMP overshoot.

Compared to CHO-μ cells, co-transfected CHO-μ/IRAS cells additionally have to express APH as a genetic selectivity mark. Correspondingly, geneticin is added during CHO-μ/IRAS cell culture. Therefore, the question arose whether geneticin or APH themselves influenced the cAMP overshoot and agmatine's inhibitory effect. Firstly, as shown in Fig. 2B, the degree of cAMP overshoot in CHO-μ/IRAS was similar to that of CHO-μ cells, suggesting that geneticin and APH did not overtly affect the cAMP overshoot. Secondly, we examined another co-transfected cell line that expressed one-sixth the level of IRAS (CHO-μ/IRAS-Low). This second co-transfected cell line was correspondingly found to be much less responsive to agmatine (Fig. 3), even though selected for APH with geneticin. Thus, agmatine's effect at low concentrations seemed related to the level of IRAS, but not to geneticin and APH. These results tend to rule out secondary mechanisms and imply strongly that agmatine attenuates morphine dependence in proportion to the level

of IRAS. If the connection with IRAS and I_1R is settled, our present study supports that the participation of I_1R in opioid dependence.

Higher concentrations of agmatine (5–100 μM) reduced morphine-pretreated, naloxone-precipitated cAMP overshoot both in the presence and absence of IRAS. This result infers that non-IRAS mechanisms may also be involved in the effects of agmatine. Besides I_1R and I_2R, agmatine also binds to α_2-ARs with moderate affinity [21,22], block ligand-gated ion channels including NMDA receptor [31], and inhibits all isoforms NOS activity [32]. The participation of α_2-ARs, NMDA receptors and NOS in opioid dependence is well known [33–35]. Other studies have shown that I_2R might also be related to opioid dependence [36,37]. However, neither α_2-ARs, I_2 binding sites nor NOS activity was detected in our cell lines. Uchino et al have further shown the absence of L-Glu plus Gly-induced Ca^{2+} mobilization coupled to the NMDA receptor in CHO cells [30]. Thus, the effect of agmatine in the CHO cell model may not relate to these mechanisms.

L-type voltage-gated calcium channels with sensitivity to dihydropyridine have been detected in CHO cells [38]. It has also been reported that agmatine can allosterically inhibit [^{3}H]nitrendipine binding to L-type Ca^{2+} channels [39]. A previous study showed agmatine could attenuate calcium currents in isolated neurohypophysial terminals and in cultured rat hippocampal neurons [40,41]. Another study demonstrated that prolonged opioid dependence leads to an increase in density of [^{3}H]dihydropyridine binding sites [42] and levels of intracellular calcium [43] in rat brain and neuroblastoma cells, respectively. Such regulatory effects have been suggested to underlie tolerance to and dependence on opioids. Our present study also shows the L-type Ca^{2+} channel blocker nifedipine mimics the inhibitory effects of higher concentrations of agmatine (5–100 μM) on the forskolin-stimulated cAMP formation and chronic morphine-induced naloxone-precipitated cAMP overshoot. Thus, the inhibitory effects of agmatine at high concentrations on chronic morphine-induced naloxone-precipitated cAMP overshoot may include blockade of L-type Ca^{2+} channels. Whether the blockage is direct or indirect remains unclear. At lower concentrations of agmatine, we also cannot rule out the possibility that the co-expression of IRAS with MOR leads to a higher affinity of agmatine for the L-type Ca^{2+} channel, but this seems unlikely given the reversal we observed of the effect of agmatine by the I_1R antagonist, efaroxan.

In conclusion, our present study demonstrates a role of IRAS in the mechanism by which agmatine inhibits morphine dependence. This inhibitory effect of agmatine was clear at low concentrations on chronic morphine-pretreated naloxone-precipitated cAMP overshoot, and was dependent on IRAS co-transfection. The effect of agmatine at higher concentrations may also relate to the L-type voltage-gated calcium channels in CHO cells.

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