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Influence of agmatine in adaptation of cAMP signal transduction system of opiate receptors

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KEY WORDS agmatine; opioid receptors; cyclic AMP; guanosine 5′-*O*-(3-thiotriphosphate); morphine; idazoxan; enkephalins; forskolin; radioimmunoassay; radioligand assay

ABSTRACT

AIM: To observe attenuative effects of agmatine on opiate desensitization and substance dependence. **METHODS**: Guanosine 5′-*O*-(3-[^{35}S] thiotriphosphate) ([^{35}S]GTTP) binding and cellular cyclic AMP (cAMP) level were determined by radioligand binding assay and radioimmunoassay in NG108-15 cells, respectively. **RESULTS**: Agmatine increased stimulative action of opioids on [^{35}S]GTTP binding by about 35 % and inhibitory effects of opioids on cellular cAMP concentration by about 114.3 % in NG108-15 cells pretreated with opioids. On the other hand, it also inhibited cAMP over-shooting by 214.9 % of morphine substance dependent cells precipitated by naloxone compared with that of control. These effects of agmatine were antagonized by idazoxan in a concentration-dependent manner. **CONCLUSION**: Agmatine reversed the formative process of adaptation in cAMP signal transduction cascade.

INTRODUCTION

The essence of tolerance to and substance dependence on opioids is adaptation, which might occur in prereceptor, receptor, and postreceptor[1]. Desensitization, characterized by the reduction of responsiveness of opiate receptor/inhibitory G protein (G_i) system after acute or chronic opiate stimulation, and cyclic AMP (cAMP) over-shooting after chronic opioid administrations have been considered as the molecular mechanisms underling opiate tolerance and substance dependence, respectively[2]. The chemicals which inhibit the formative processes of desensitization and cAMP over-shooting might have abilities to antagonize opiate tolerance and substance dependence.

Agmatine, an endogenous imidazoline receptor (I-R) ligand[3,4], enhanced opiate analgesia and antagonized opioid tolerance and substance dependence in mice and rats *in vivo*[5-7], and in guinea pig ileum longitudinal smooth muscle *in vitro*[8]. On the other hand, agmatine inhibited nitric-oxide synthase by substrate competitive manner and activation of I-R, which was related to its inhibition of opiate tolerance and substance dependence[9]. The aim of the paper was to observe the effect of agmatine on the desensitization of opiate receptor/G_i and substance dependence characterized by cAMP over-shooting induced by pretreatment with opioids in NG108-15 cells.

MATERIALS AND METHODS

Agents The initial stock cultures of NG108-15 cells were from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were puchased from Gibco BRL; [*D*-Pen^2, *D*-Pen^5]-enkephalin (DPDPE), forskolin, hypoxanthine, aminopterin, thymidine, Tris, edetic acid, egtazic acid and 1-methyl-3-isobutylxanthine (IBMX) were products of Sigma Co; morphine and idazoxan were purchased from Qinghai Pharmaceutical Factory and from Research Biochemicals International, respectively. Guanosine 5′-*O*-(3-[^{35}S]thiotriphos-

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phate) ([^{35}S]GTTP, 50.88 PBq·mol^{-1}) and [^{3}H]cAMP (1.37 PBq·mol^{-1}) were purchased from DuPont Co; antibody of cAMP was the product of Department of Nuclear Medicine, Shanghai Second Medical University.

Cell cultures The culture of NG108-15 cells was performed[10]. Briefly, the cells were cultured in DMEM containing HAT (hypoxanthine 0.1 mmol·L^{-1}, aminopterin 10 μmol·L^{-1}, and thymidine 17 μmol·L^{-1}) plus 10 % FBS in humidified 10 % CO_2 +90 % air.

[^{35}S]GTTP binding assay The cells cultured for 24 h were washed 3 times with phosphate buffer (NaCl 137 mmol·L^{-1}, KCl 2.6 mmol·L^{-1}, Na_2HPO_4 10 mmol·L^{-1}, KH_2PO_4 1.8 mmol·L^{-1}) and then harvested with 3 mL of the buffer, and pelleted at 4 ℃ at 1000 × *g* for 5 min. The pellet was resuspended with 1 mL lysis buffer (Tris 5 mmol·L^{-1}, edetic acid 5 mmol·L^{-1}, egtazic acid 5 mmol·L^{-1}, pH 7.4). After 5 min in ice bath, it was homogenized by repeated aspirations with an insulin syringe for 3 times. The cell lysate was centrifuged at 4 ℃ and at 12 000 × *g* for 30 min and the pellet was resuspended in action buffer (Tris 50 mmol·L^{-1}, edetic acid 1 mmol·L^{-1}, NaCl 100 mmol·L^{-1}, and $MgCl_2$ 5 mmol·L^{-1}, pH 7.4) on ice.

[^{35}S]GTTP binding was measured with a modification of the assay[11]. Cell membrane protein (3 – 4 μg) was mixed with reaction buffer, GDP 30 μmol·L^{-1} and [^{35}S]GTTP 7400 Bq in total volume of 100 μL. The incubation was started by addition of membrane protein to the action mixture and continued at 30 ℃ for 60 min. The reaction was terminated by rapid filtratrion through GF/C filters under vacuum and then counted in scintillation cocktail. Nonspecific binding was determined in the presence of GTTP 5 μmol·L^{-1} and substrated from total bound radioactivity. The results were expressed as bound [^{35}S]GTTP pmol·g^{-1}(protein) or % of basic binding [(Bq in drug tube – Bq in nonspecific tube) ÷ (Bq in basic tube – Bq in nonspecific tube) × 100].

Desensitization In acute pretreatment test, the cells cultured for 24 h and suspended in phosphate buffer were pretreated with normal saline, DPDPE 1 μmol·L^{-1}, and agmatine (1 or 10 μmol·L^{-1}) + DPDPE (selective agonist of δ opiate receptor) at 37 ℃ for 10 min, respectively. In chronic pretreatment test, the cells cultured for 16 h were respectively pretreated with normal saline, morphine 100 μmol·L^{-1} (nonselective opiate receptor agonist), and agmatine 10 μmol·L^{-1} + morphine for 8 h in the environment as mentioned. After being washed with phosphate buffer, the cells pretreated were homogenized, and [^{35}S]GTTP binding was determined. To observe the antagonistic effect of idazoxan on the action of agmatine, idazoxan was added into phorsphate buffer (acute pretreatment test) or culturing medium (chronic pretreatment test) 5 min prior to DPDPE or morphine + agmatine.

cAMP assay[12] NG108-15 cells were pre-challenged with control medium (basic), medium containing forskolin 10 μmol·L^{-1} + IBMX 500 μmol·L^{-1}(control) or medium containing forskolin + IBMX + DPDPE 10 μmol·L^{-1} at 37 ℃ for 10 min. The reactions were terminated with perchloric acid 1 mol·L^{-1} and neutralized with K_2CO_3 2 mol·L^{-1}. After centrifugation at 12 000 × *g* for 5 min, the supernatants were taken to measure the cAMP concentration by radioimmunoassay[11]. The results were expressed as % of basic or control.

Desensitization and substance dependence To test desensitization characterized by decrease in inhibitory effects of opioids on cAMP concentration and the preventive effect of agmatine on the desensitization, the cells cultured for 24 h were respectively pretreated with normal saline, DPDPE 10 μmol·L^{-1}, and agmatine (1 or 10 μmol·L^{-1}) + DPDPE at 37 ℃ for 10 min. After being washed with phosphate buffer, the cells were stimulated with receptor agonist and the cellular cAMP level was determined. To test substance dependence characterized by cAMP over-shooting, the new inoculated cells were respectively pretreated with normal saline, morphine 100 μmol·L^{-1} and concentrations of agmatine (1 or 10 μmol·L^{-1}) + morphine for 24 h in the invironment as mentioned. After incubation, the cells were washed with DMEM without FBS, and then were challenged with naloxone 100 μmol·L^{-1} under culture conditions for 10 min. After being washed with phosphate buffer, the cellular cAMP was determined. To observe the antagonistic effect of idazoxan on the actions of agmatine, idazoxan was added 5 min prior to addition of agmatine + DPDPE or morphine.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by *t*-test.

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RESULTS

DPDPE and morphine respectively stimulated [^{35}S]GTTP binding with G protein and inhibited the cellular cAMP concentration in the presence of forskolin in a concentration-dependent manner (Fig 1).

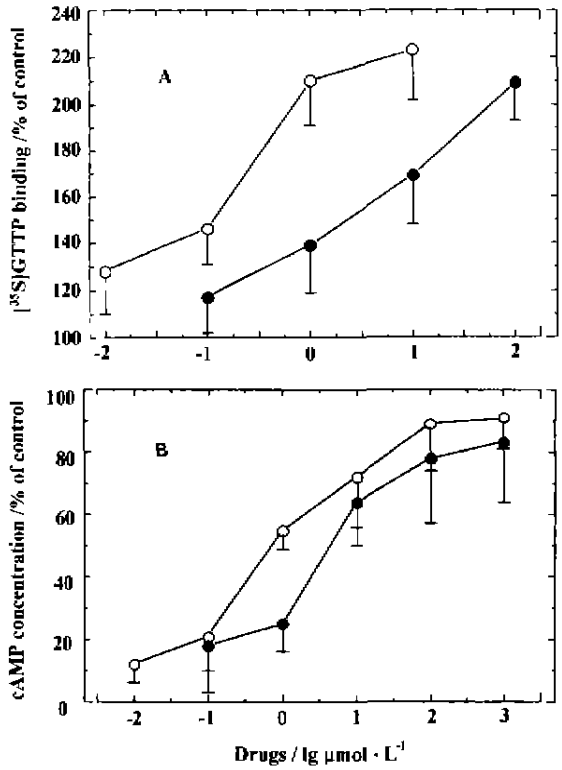

Fig 1. Stimulative effects on [^{35}S] GTTP binding (A) and inhibitory actions on cAMP concentrations (B) of DPDPE (○) and morphine (●) in naive NG108-15 cells. n = 5 tests.

Pretreatment of NG108-15 cells with DPDPE 1 μmol · L^{-1} at 37 ℃ for 10 min induced a desensitization, shown as a decrease in stimulative action of DPDPE on [^{35}S]GTTP binding and in inhibitory effect on cellular cAMP concentration. Agmatine inhibited the desensitization induced by acute or chronic pretreatment with opioids in a concentration-dependent manner. When the cells were pretreated with agmatine + DPDPE, DPDPE lost in part the ability to induce desensitization. Agmatine 10 μmol · L^{-1} increased the stimulative effect of DPDPE on [^{35}S]GTTP binding by 35 % and inhibitory effect of DPDPE on cellular cAMP concentration by 114.3 % compared with those of DPDPE group (Tab 1).

Tab 1. Effects of agmatine (Agm) on the decrease in stimulation on [^{35}S]GTTP binding and in inhibition on cAMP concentraion of DPDPE 1 μmol·L^{-1} in NG108-15 cells pretreated with DPDPE. $^{c}P < 0.01$ *vs* saline. $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* DPDPE. $^{i}P < 0.01$ *vs* Agm + DPDPE.

Drug/μmol·L^{-1}	n	[^{35}S]GTTP /% of basic	n	cAMP /% control
Normal saline	11	220 ± 30	5	51 ± 10
DPDPE 1	11	117 ± 19^{c}	5	86 ± 7^{c}
Agm 1 + DPDPE 1	11	136 ± 13^{e}	5	50 ± 18^{f}
Agm 10 + DPDPE 1	8	158 ± 21^{f}	5	40 ± 9
Ida 0.02 + Agm 10 + DPDPE 1	5	136 ± 13	5	50 ± 7
Ida 0.1 + Agm 10 + DPDPE 1	5	106 ± 10^{i}	5	66 ± 21^{i}

Agm: agmatine. Ida: idazoxan. n = tests

Pretreatment of the cells with morphine 100 μmol · L^{-1} at 37 ℃ for 8 h also evoked a desensitization characterized by the decrease in stimulative effect of morphine or DPDPE on [^{35}S]GTTP binding. ED_{50} (95 % confident limits) of morphine [4178, (3302 – 5869) μmol · L^{-1})] in the test was increased by over 2000 times compared with that of normal saline [1.5, (1.0 – 2.2 μmol · L^{-1})] (Tab 2).

Tab 2. Inhibitory effects of Agm on decrease in stimulation of DPDPE 1 μmol·L^{-1} on [^{35}S]GTTP binding in NG108-15 cells pretreated with Mor. $^{c}P < 0.01$ *vs* saline, $^{f}P < 0.01$ *vs* Mor.

Drug/μmol·L^{-1}	Tests	[^{35}S]GTTP/ % of basic
Normal saline	6	216 ± 7
Mor 100	7	132 ± 5^{c}
Agm 10 + Mor 100	8	196 ± 33^{f}

Pretreatment of NG108-15 cells with agmatine 10 μmol · L^{-1} + morphine 100 μmol · L^{-1} for 8 h induced an increase in stimulative effect of morphine or DPDPE on [^{35}S]GTTP binding compared with those pretreated with morphine alone (Fig 2, Tab 3). ED_{50} (95 % confident limits) of morphine [12.3, (8.9 – 16.8 μmol · L^{-1})] obtained from those pretreated with agmatine + morphine was decreased tremendously compared with those pretreated with morphine alone.

The inhibitory effects of agmatine as mentioned above were antagonized by selective imidazoline receptor blocker idazoxan. When the cells were

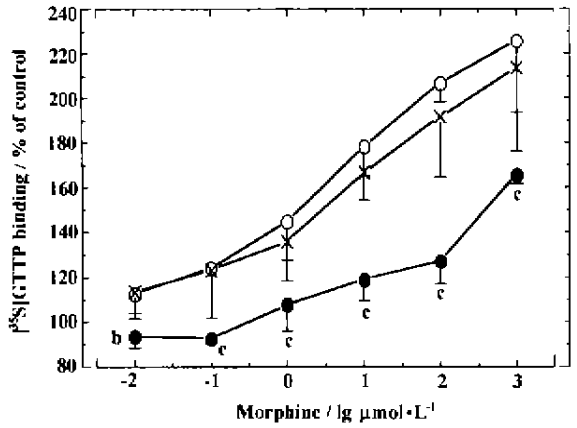

Fig 2. Stimulative effect of morphine on [^{35}S]GTTP binding in NG108-15 cells pretreated with saline (○), morphine 100 μmol·L^{-1} (●) and agmatine 10 μmol·L^{-1} + morphine (×) for 8 h. n = 5 tests. ^{b}P < 0.05, ^{c}P < 0.01 *vs* saline.

pretreated with idazoxan + agmatine + DPDPE or morphine, the inhibitory effects of agmatine on desensitization induced by pretreatment with DPDPE or morphine were disappeared, and the abilities of DPDPE or morphine to induce desensitization were restored (Fig 3).

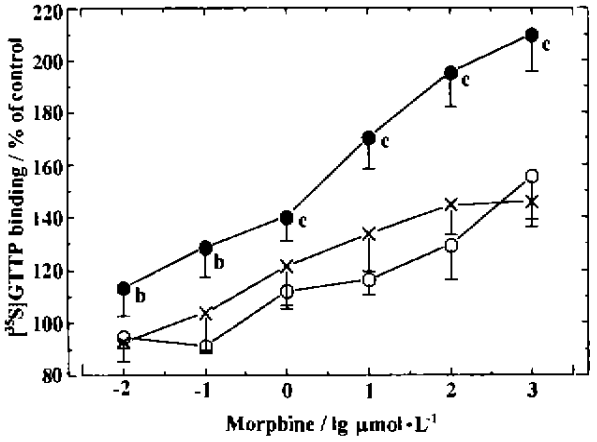

Fig 3. Influence of idazoxan in the inhibitory effect of agmatine on desensitization to stimulative action of morphine on [^{35}S]GTTP binding in NG108-15 cells pretreated with morphine 100 μmol·L^{-1} (○), agmatine 10 μmol·L^{-1} (●), and idazoxan 100 μmol·L^{-1} + agmatine + morphine (×) for 8 h. n = 5 tests. ^{b}P < 0.05, ^{c}P < 0.01 *vs* morphine.

Pretreatment of NG108-15 cells with morphine 100 μmol·L^{-1} for 24 h evoked a 2.1-fold increase in cellular cAMP concentration precipitated by naloxone (cAMP over-shooting) compared with that of control. When the cells were pretreated with agmatine 10 μmol·L^{-1} + morphine for 24 h, morphine completely lost the ability to induce the cellular cAMP over-shooting. However, the inhibitory effect of agmatine on cAMP over-shooting was antagonized by idazoxan in a concentration-dependent manner (Tab 3).

Tab 3. Inhibitory effect of Agm on cAMP over-shooting of Mor-dependent NG108-15 cells precipitated by naloxone. ^{c}P < 0.01 *vs* saline. ^{f}P < 0.01 *vs* Mor, ^{i}P < 0.05 *vs* Agm + Mor.

Drug/μmol·L^{-1}	cAMP/% of control
Normal saline	100 ± 0
Morphine 100	286 ± 126^{c}
Agm 1 + Mor 100	182 ± 23
Agm 10 + Mor 100	91 ± 6^{f}
Ida 0.1 + Agm 10 + Mor 100	338 ± 136^{i}

Agm: agmatine; Mor: morphine; Ida: idazoxan.

DISCUSSION

NG108-15 cells are neuroblastoma × glioma hybrid cells on which there are many kinds of receptors and their corresponding signal transductive systems, including δ opiate receptor and I-R. The δ opiate receptors are coupled with G_i and can be selectively activated by DPDPE[12]. According to our unpublished results and other reports, I-R distributed on NG108-15 cells is also G protein coupled receptor[13], but G protein coupled with I-R is neither G_i nor G_s, might belong to G_{12} or G_q family.

In the present test, acute or chronic pretreatment of NG108-15 cells with opioids induced a desensitization characterized by decrease in responsibility to opiate stimulative effects on [^{35}S]GTTP binding and inhibitory actions on cellular cAMP concentration. Although the accurate boundary line and relationship among the desensitization, opiate tolerance and substance dependence are not fully understood, there is no question that desensitizaton is the first step of biological adaptation and is the basis of a series of pathophysiological changes in the formative process of opiate tolerance and substance dependence[1,12]. Agmatine inhibits the desensitization,

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restores the opioid stimulative effect on [^{35}S]GTTP binding and inhibitory actions on cellular cAMP concentration. The inhibitory effects of agmatine can be antagonized by selective I-R antagonist idazoxan. These results indicate that the inhibitory effects of agmatine on desensitization are achieved by activation of I-R.

The best established molecular adaptation to chronic drug exposure is up-regulation of the cAMP pathway, a phenomenon first discovered in cultured NG108-15 cells and later demonstrated in neurons in response to repeated opiate administration[1]. In the present test, pretreatment of NG108-15 cells with morphine for 24 h induced an up-regulation of cAMP, expressing as cAMP over-shooting precipitated by naloxone. Agmatine attenuated the over-shooting by activation of I-R in a concentration-dependent manner.

In conclusion, the preventive and theraputic effects of agmatine on opiate tolerance and substance dependence *in vivo* might be related to its antagonistic action on formative process of adaptation in cAMP signal transduction cascade.

REFERENCES

1 Nestler EJ, Aghajanian GK. Molecular and cellular basis of addiction. Science 1997; 278 (5335): 58 - 63.
2 Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Hanoune J, *et al*. Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. Science 1996; 273 (5275): 657 - 9.
3 Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous 'clonodine-displacing' substance in the brain. Science 1994; 263: 966 - 9.
4 Herman ZS. Agmatine — a novel endogenous ligand of imidaziline receptors. Pol J Pharmacol 1997; 49 (2 - 3): 85 - 8.
5 Kolesnikov Y, Jian S, Pasternak GW. Modulation of opioid analgesia by agmatine. Eur J Pharmacol 1996; 296: 17 - 22.
6 Aricioglu-Kartal F, Uzbay IT. Inhibitory effect of agmatine on naloxone-precipitated abstinence syndrome in morphine dependent rats. Life Sci 1997; 61 (18): 1775 - 81.
7 Li J, Li X, Pei G, Qin BY. Effects of agmatine on tolerance to and substance dependence on morphine in mice. Acta Pharmacol Sin 1999; 20: 232 - 8.
8 Li J, Li X, Pei G, Qin BY. Inhibitory effects of agmatine on tolerance to and substance dependence on morphine in guinea pig ileum *in vitro*. Acta Pharmacol Sin 1998; 19: 564 - 9.
9 Li J, Li X, Pei G, Qin BY. Correlation between inhibitions of morphine withdrawal and its nitric-oxide synthase by agmatine. Acta Pharmacol Sin 1999; 20: 375 - 80.
10 Law PY, Hom DS, Loh HH. Opiate receptor down-regulation and desensitization in neuroblastoma × glioma NG108-15 hybrid cells are two separate cellular adaptation processes. Mol Pharmacol 1983; 24: 413 - 24.
11 Tian WN, Duzic E, Lanier SM, Deth RC. Determinants of α_2-adrenergic receptor activation of G proteins: evidence for a precoupled receptor/G protein state. Mol Pharmacol 1993; 45: 524 - 31.
12 Cai YC, Ma L, Fan GH, Zhao J, Jiang LZ, Pei G. Activation of *N*-methyl-*D*-aspartate receptor attenuates acute responsiveness of δ-opioid receptors. Mol Pharmacol 1997; 51: 583 - 7.
13 Molderings G, Moura D, Fink K, Bomsch H, Gothert M. Binding of ^{3}H-clonidine to I_1-imidazoline sites in bovine adrenal medullary membranes. Naunyn Schmiedebergs Arch Pharmacol 1993; 348: 70 - 6.

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胍丁胺对阿片受体环腺苷一磷酸信号转导系统代偿适应的影响

R971.2

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关键词 胍丁胺; 阿片受体; 环腺苷一磷酸; 鸟苷5′-*O*-(3-硫三磷酸); 吗啡; 咪唑克生; 脑啡肽类; 弗司扣林; 放射免疫测定; 放射配位体测定

目的: 观察胍丁胺对阿片类所致脱敏和物质依赖的作用. **方法**: 分别用放射配体结合实验和放免法测定 γ-[^{35}S]-三磷鸟苷([^{35}S]GTTP)结合量和环腺苷一磷酸(cAMP)浓度. **结果**: 在经阿片类药物预处理的 NG108-15 细胞实验中, 胍丁胺使阿片类药物刺激 [^{35}S]GTTP 结合作用增强 35 %; 使阿片类药物对 cAMP 抑制作用增强 114.3 %; 使纳洛酮引起的吗啡物质依赖细胞 cAMP 超射幅度和对照组相比减小 214.9 %. 胍丁胺的上述作用均可被咪唑克生浓度依赖性阻断. **结论**: 胍丁胺通过激活咪唑啉受体阻止 cAMP 信号转导通路代偿适应过程的形成.

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