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Agmatine inhibited tolerance to and dependence on morphine in guinea pig ileum *in vitro*

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KEY WORDS agmatine; drug tolerance; opioid-related disorders; ileum; morphine; idazoxan; naloxone; clonidine; yohimbine; serotonin

AIM: To observe effect of agmatine (Agm) on tolerance to and substance dependence on morphine (Mor) in guinea pig ileum longitudinal muscle (GPILM). **METHODS:** The experiment was performed in electric field stimulation (EFS) test *in vitro*. **RESULTS:** Mor inhibited twitch contractions of GPILM induced by EFS [IC_{50} = 140 (107 - 182) nmol·L^{-1}]. Incubation of GPILM with Mor 270 nmol·L^{-1} for 8 h evoked a 37-fold increase in IC_{50} of Mor (tolerance) and a contractile response to naloxone (Nal, substance dependence). When the preparations were coincubated with Mor + Nal and Mor + Agm, Mor lost the ability to induce tolerance and inhibited the contractile responses of the preparations to Nal by 90 % and 75 %, respectively. These effects of Agm could be almost completely antagonized by idazoxan. **CONCLUSION:** Agm prevented the development of tolerance to and substance dependence on Mor in GPILM *in vitro* by activation of imidazoline receptors.

Tolerance and dependence induced by opioids made a lot of troubles in therapeutics and sociology for a long time. More and more attentions have been paid to the field to find their mechanism. The diversity of drug types and endogenous biological substances capable of attenuating or promoting opiate tolerance and dependence suggests that the tolerance and dependence can be modulated at multiple sites involving a variety of neurotransmitters[1-3].

Agmatine (Agm) is recently considered as an endogenous ligand of imidazoline receptors (I-R) and widely distributed in mammalian tissues[4]. Agm enhanced morphine (Mor) analgesia and inhibited tolerance to Mor[5]. On the other hand, according to our unpublished results, Agm also had weak analgesia and inhibited the development of substance dependence. The aim of the paper was to observe the inhibitory effects of Agm on tolerance to and dependence on Mor in guinea pig ileum longitudinal muscle (GPILM) *in vitro* and analyze its possible receptor mechanism using selective I-R antagonist idazoxan (Ida).

MATERIALS AND METHODS

Guinea pigs (n = 88), ♂, weighing 238 g ± s 26 g, were obtained from Animal Center of Academy of Military Medical Sciences (Certificate № 01-3041). Agm and yohimbine were purchased from Sigma Co. Ida and 5-hydroxytryptamine (5-HT) were products of Research Biochemicals International, and clonidine was product of Changzhou Pharmaceutical Factory. Choline chloride was purchased from the Third Chemical Reagent Plant of Beijing. Chemicals and glucose for preparation of Ringer's solution were purchased from Beijing Shuanghuan Chemical Reagent Plant.

Guinea pigs were fasted for at least 12 h before experiment. They were killed by a blow on the neck. A 12-cm segment of ileum (10 cm proximal to cecum) was excised and kept in Ringer's solution of the following composition in mmol·L^{-1}: NaCl 154, KCl 5.66, $CaCl_2$ 2.54, glucose 2.77, $NaHCO_3$ 5.95, and choline chloride 0.002. The longitudinal muscle with attached myenteric plexus was separated[6]. The strips were mounted in a 10-mL tissue bath containing 5 mL of Ringer's solution at 37 ℃ and bubbled with 95 % O_2 + 5 % CO_2. The preparations were allowed to equilibrate for 1 h under 0.5 g of tension. Electric field stimulation (EFS) of the preparations was performed

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with an electric stimulator (SEN-7103, Nihon Kohden Kogyo Co, Japan) through 2 parallel platinum electrodes, using repeated pulses of 100 V, 5 ms, and 0.15 Hz. The twitch contractions of the preparations were recorded with a 2-pen balance recorder through a tension transducer. For each experiment at least 5 guinea pigs were used.

After 1 h of equilibration, the preparations were stimulated until a steady amplitude was obtained. Mor, clonidine, or 5-HT was then added to the bath in increasing concentrations to observe their effects on the twitches induced by EFS. Routinly, 5 to 6 concentrations in 3 to 5-fold steps were applied at a 20-min interval. The concentrations to inhibit or excitate one-half the maximal amplitude of the twitch contractions (IC_{50} or ED_{50}) was determined by Bliss program.

The preparations were then treated by incubation with normal saline (NS), Mor 270 nmol·L^{-1}, 5-HT 125 nmol·L^{-1}, or Mor + 5-HT. The preparations were washed with Ringer's solution containing the same concentrations of the drugs as mentioned above every 15 min over a period of 8 h. At the end of incubation, the tissues were stimulated as previously described until a steady contractile amplitude was obtained. The recoveries of the contractile responses to EFS were over 80 % or better compared with those before the incubation. The IC_{50} were then redetermined while maintaining the concentrations of Mor, 5-HT, or Mor + 5-HT in the media.

To assess the antagonistical effects of naloxone (Nal) or Agm on the development of tolerance to Mor, pairs of ileum were suspended in separated baths. After the IC_{50} of Mor had been determined, the preparations in different baths were incubated with Mor 270 nmol·L^{-1} + NS (control), Mor + Nal 100 nmol·L^{-1}, and Mor + Agm 0.1 or 1 μmol·L^{-1} for 8 h. During the period of incubation, the preparations were washed every 15 min with Ringer's solution containing the same concentrations of Mor, Nal, or Agm as mentioned above. IC_{50} of Mor was redetermined. To observe the inhibitory effect of Ida on the effect of Agm, the control preparations were incubated with Mor and Agm as mentioned above, and those in test group were done not only with Mor and Agm but also with Ida 10 or 100 nmol·L^{-1}. After 8-h incubation, IC_{50} of Mor was redetermined.

At the end of each experiment, the tolerant preparations were tested for the presence of substance dependence by adding Nal 55 μmol·L^{-1} to media to elicit a contractile response. To perform a rational comparision between the preparations, the contractile amplitudes of the preparations induced by Nal were expressed as % of those induced by KCl 30 mmol·L^{-1}.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed with t test.

RESULTS

EFS induced twitch contractions of GPILM which were inhibited by Mor or clonidine, and enhanced by 5-HT obviously. Yohimbine and Nal antagonized the inhibitory effects of clonidine and Mor. In contrast to clonidine and Mor, Agm had no effect on the twitch contractions ($n = 5$, $P > 0.05$). It did not influence the inhibitory effects of Mor and clonidine on the contractions (Fig 1).

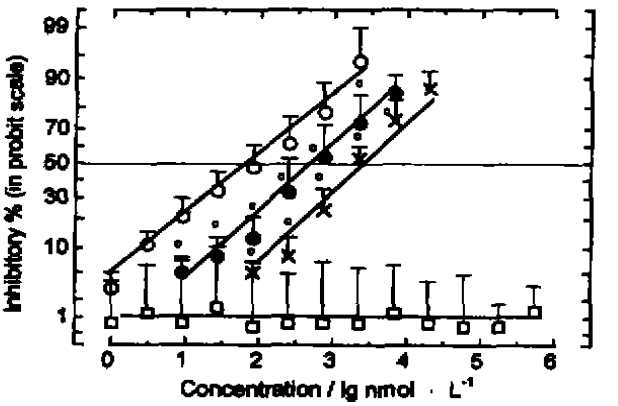

Fig 1. Inhibitory effects of clonidine (○) and Agm (□) on contractile responses of GPILM to electric field stimulation. Clonidine + yohimbine 10 (●), clonidine + yohimbine 100 nmol·L^{-1} (×). $n = 5$ guinea pigs. $\bar{x} \pm s$. $^cP < 0.01$ vs clonidine.

Incubation of GPILM with NS for 8 h had no effect on IC_{50} of Mor ($n = 3$, $P > 0.05$) compared with those before incubation (Tab 1). However, incubation of the preparations with Mor induced a decrease in the inhibitory effect of Mor and clonidine (Fig 2), and increase in IC_{50} of Mor and clonidine respectively by 37 (Tab 1) and 20 times [from 0.09 (0.07 – 0.12) to 1.79

Tab 1. Influence of coincubation with drugs on Mor IC_{50}. n = guinea pigs. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* Mor.

Drug/μmol·L^{-1}	n	IC_{50}/nmol·L^{-1} (95 % confidence limits) Before pretreatment	After pretreatment	Ratio
NS	3	421 (312 – 568)	360 (273 – 475)^c	0.85
Mor 0.27	6	156 (125 – 196)	5864 (4814 – 7141)	37.59
Nal 0.1 + Mor 0.27	6	128 (100 – 163)	143 (113 – 181)^c	1.12
Agm 0.1 + Mor 0.27	6	129 (101 – 147)	244 (194 – 306)^c	1.89
Agm 1.0 + Mor 0.27	6	130 (102 – 166)	142 (111 – 183)^c	1.09
Ida 0.01 + Agm 0.1 + Mor 0.27	5	102 (81 – 127)	2201 (1791 – 2703)^b	21.68
Ida 0.1 + Agm 0.1 + Mor 0.27	5	140 (107 – 182)	5433 (4469 – 6606)^a	38.81
Mor 0.27 + 5-HT 0.125	10	227 (176 – 291)	1081 (841 – 1391)^c	4.76

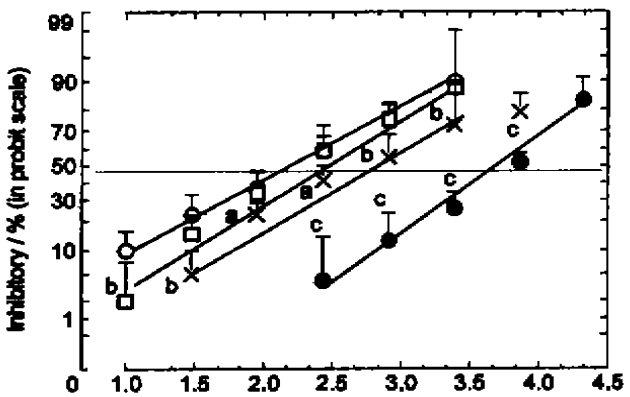

Fig 2. Influence of incubation with NS (○), Mor 270 nmol·L^{-1}(●), and Agm 0.1 (×) or 1 (□) μmol·L^{-1} + Mor for 8 h in Mor inhibitory concentration-response curves of GPILM to electric field stimulation. n = 6 guinea pigs. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* NS.

(1.39 – 2.32) μmol·L^{-1}, $n = 6$] compared with those before incubation. On the other hand, the treatment enhanced response of GPILM to 5-HT and ED_{50} value of 5-HT was decreased by >76 % [from 116.8 (98.4 – 137.5) to 28.3 (20.1 – 39.6) nmol·L^{-1}, $n = 6$].

Coincubation with Mor + Nal or Mor + Agm prevented the development of tolerance to Mor and IC_{50} of Mor had no significant changes ($P > 0.05$, $n = 6$) compared with that of incubation with NS (Tab 1, Fig 2). The inhibitory effect of Agm on the tolerant development induced by incubation with Mor was antagonized by Ida in a concentration-dependent manner (Fig 3).

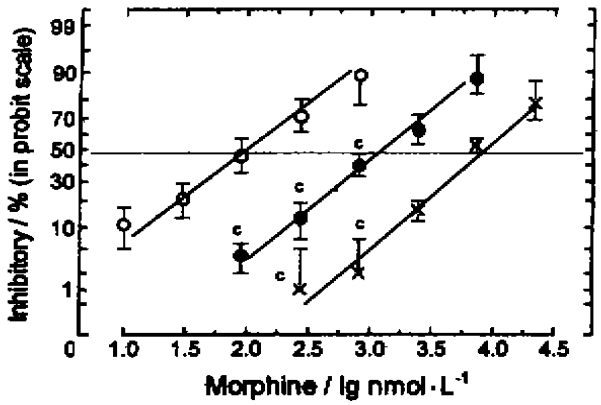

Fig 3. Influence of coincubation of preparations with NS (○), Ida 10 (●), or 100 (×) nmol·L^{-1} + Agm 1 μmol·L^{-1} + Mor 270 nmol·L^{-1} for 8 h on Mor inhibit of contractile responses of GPILM to electric field stimulation. n = 5 guinea pigs. $\bar{x} \pm s$. $^cP < 0.01$ *vs* NS.

Coincubation of the preparations with Mor + Agm + Ida in the same concentrations induced 39-fold increase in IC_{50} of Mor *vs* those incubated with Mor and Agm alone (Tab 1).

Incubation of GPILM with Mor increased their responses to excitatory effect of 5-HT. However, coincubation of the tissues with Mor and 5-HT resulted in decreased respose of GPILM to 5-HT. ED_{50} of 5-HT was increased to over 100 times (Fig 4).

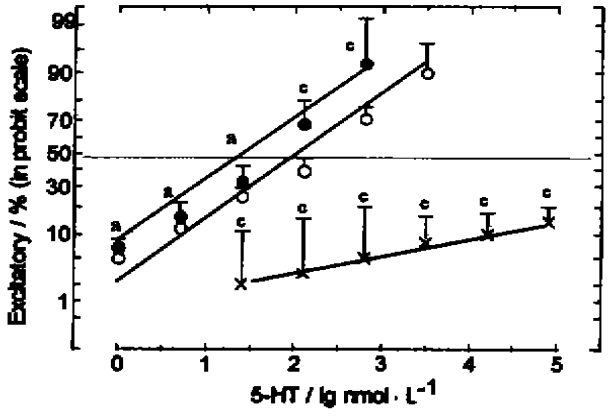

Fig 4. Influence of coincubation of preparations with NS (○), Mor 270 nmol·L^{-1}(●), and Mor + 5-HT 125 nmol·L^{-1} (×) for 8 h on 5-HT excitation of contractile responses of GPILM to electric field stimulation. n = 6 guinea pigs. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* NS.

Under the same condition, the coincubation

could also induced a tolerance to Mor, but the degree was much lighter than that incubated with Mor alone (Fig 5).

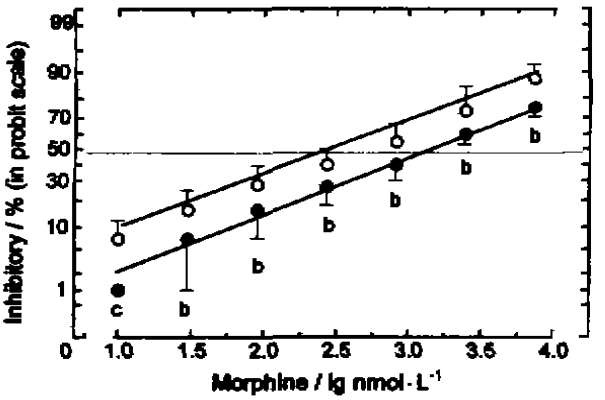

Fig 5. Influence of coincubation of preparations with NS (○), or with Mor 270 + 5-HT 125 nmol·L^{-1}(●) for 8 h on Mor inhibition of contractile responses of GPILM to electric field stimulation. $n = 6$ guinea pigs. $\bar{x} \pm s$. $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* NS.

Incubation with Mor under the same condition induced substance dependence of GPILM characterized by their contractile response to Nal (Tab 2). These results were consistent with those reported earlier[7]. Coincubation of GPILM with Mor + Nal or Mor + Agm prevented the development of the substance dependence characterized by the decrease in amplitude of the contraction induced by Nal. The inhibitory effect of Agm was antagonized by Ida. On the other hand, coincubation of GPILM with Mor + 5-HT induced a mixed tolerance to Mor and 5-HT and at the same time, it evocked a significant decrease in contractive amplitude of the preparations induced by Nal ($P < 0.01$, $n = 5$).

Tab 2. Effects of drugs on contractile reponse of GPILM to naloxone 55 μmol·L^{-1}. $n = 6$ guinea pigs. $\bar{x} \pm s$. $^{c}P < 0.01$ *vs* Mor.

Drug/μmol·L^{-1}	% Of contraction induced by KCl 30 mmol·L^{-1}
Mor 0.27	75.3 ± 5.6
Nal 0.1 + Mor 0.27	6.3 ± 4.6^{c}
Agm 1 + Mor 0.27	13.5 ± 5.9^{c}
Ida 0.1 + Agm 1 + Mor 0.27	89.4 ± 19.8
Mor 0.27 + 5-HT 0.125	17.4 ± 1.6^{c}

DISCUSSION

Many researchers use guinea pig ileum *in vitro* as a model to study the mechanism of opiate tolerance and substance dependence, and the mechanism of effects of drugs on the tolerance and substance dependence. On the other hand, it has been reported that Agm, an endogenous ligand of I-R, exists in gastrointestinal tract with the hightest concentration compared with other tissues[8] and I-R agonist regulated many functions of gastrointestinal tract[9,10]. So GPILM is chosen as a model to observe the inhibitory effect of Agm on tolerance to and substance dependence on Mor.

In current study, both Mor and clonidine inhibit the twitch contractions of naive GPILM induced by EFS, which could be antagonized by Nal and yohimbine, respectively. In contrast to clonidine and Mor, Agm has no significant effect on the twitch contractions and dose not influence the inhibitory effects of Mor and clonidine on the contractions. Our unpublished results show that Agm (0.01 – 1000 μmol·L^{-1}) can not competitively inhibit the binding of ^{3}H-Nal with opiate receptors in cell membrane preparations of rat forebrain. These results indicate that Agm might not directly interact with opiate receptors and α_2-adrenoceptors on the preparations.

Incubation of GPILM with Mor induces an obvious tolerance not only to Mor but also to clonidine, and substance dependence on Mor. Although Agm has no action on opiate receptors as mentioned above, it prevents the development of tolerance to and substance dependence on Mor. The preventive effect of Agm can be antagonized by selective I-R antagonist Ida. These results indicate that the mechanism of inhibitory effects of Agm on the development of tolerance to and substance dependence on Mor in GPILM might be related to its influence on the opiate post-receptor mechanism by activation of I-R. The data strongly supports the compensatory hypersensitive theory[1] to be used to explain the mechanism of development of tolerance to and substance dependence on opioids.

Mor inhibits the release of acetylcholine and the twitch contractions induced by EFS by activation of opiate receptors on presynaptic membrane of myenteric plexus of GPILM.

However, 5-HT stimulates the release of acetylcholine and enhances the amplitude of the twitch contractions by activation of 5-HT receptors on presynaptic membrane of myenteric plexus[11]. After incubation of GPILM with Mor, the excitatory effect of 5-HT on contractile response of GPILM to EFS is enhanced. However, after coincubation with Mor and 5-HT, the degree of tolerance to and substance dependence on Mor is inhibited significantly, although both of them still exist. These results suggest that the formative processes of tolerance to and substance dependence on Mor result from functional changes of not only opiate system but also 5-HT system in the current experiment.

In conclusion, Agm prevents the development of tolerance to and substance dependence on Mor in the test by activation of I-R.

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胍丁胺体外抑制豚鼠回肠的吗啡耐受和依赖

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关键词 胍丁胺; 药物耐受性; 阿片类有关的紊乱; 回肠; 吗啡; 咪唑克生; 纳洛酮; 可乐定; 育亨宾; 血清素

目的: 观察胍丁胺对吗啡耐受和物质依赖豚鼠回肠纵肌(GPILM)的作用. **方法**: 本实验在离体电场刺激实验中进行. **结果**: 吗啡抑制 EFS 引起的 GPILM 收缩[IC_{50} = 140 (107 – 182) nmol·L^{-1}]. 用吗啡 270 nmol·L^{-1}与 GPILM 温浴使吗啡 IC_{50}增大 37 倍(耐受), 对纳络酮发生收缩反应(物质依赖). 分别用吗啡加纳络酮和吗啡加胍丁胺与 GPILM 温浴使吗啡失去此致耐受作用, 使标本对纳络酮收缩反应幅度分别减少了 90 %和 75 %. 胍丁胺的这些作用几乎可被咪唑克生完全阻断. **结论**: 胍丁胺通过激活咪唑啉受体抑制离体 GPILM 对吗啡耐受和物质依赖的形成过程.