ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Jun; 20 (6): 500 - 504
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Effects of low-pH treatment on cAMP second messenger system regulated by different opioid agonists

LIU Jing-Gen[1], GONG Ze-Hui, QIN Bo-Yi
(*Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850, China*)

KEY WORDS morphine; methadone; opioid-related disorders; cultured cells; etorphine; buprenorphine; naloxone; adenylate cyclase; cyclic AMP; delta opioid receptors

ABSTRACT

AIM: To study the mechanism of opioid agonists in regulation of cAMP second messenger system. **METHODS**: Low-pH treatment was used to deplete the stimulatory G portein (G_s) function. The effects of some opiates on adenylate cyclase were compared between control and low-pH treatment membranes. **RESULTS**: In contrast to dihydroetorphine (DHE), etorphine (Eto), morphine (Mor) and methadone (Met) substantially increased the inhibitory effects on adenylate cyclase in membranes prepared from naive and chronic Mor- or Met-treated NG108-15 cells by low-pH treatment. In contrast to Mor, DHE and Eto did not result in significant decrease in the inhibitory effects on adenylate cyclase in membranes from the cells treated chronically with DHE or Eto. Marked rebound of adenylate cyclase was also not observed in membranes from chronic DHE or Eto-treated cells when precipitated with naloxone. Low-pH treatment eliminated naloxone-induced rebound of adenylate cyclase *in chronic Mor-treated cells*. **CONCLUSION**: The difference in opiate-induced functional adaptive alteration of G_s is at least one biochemical mechanism of developing opiate tolerance and dependence.

INTRODUCTION

In the previous study, we observed that DHE, and Eto were distinct from Mor in naloxone-precipitated rebound of cAMP in intact NG108-15 cells[1]. DHE and Eto have been shown to act as antagonists on excitatory opioid receptor in sensory neurons in culture and increase potency and specificity of opiate analgesics and attenuate the development of tolerance/dependence[2]. DHE might have antagonistic properties to the opioid receptor[3]. What are the causes of various opiate effects? Alteration in excitatory signal transduction pathway has been proposed to be one possible adaptive mechanism by which cells may respond to the prolonged exposure to opiate and can be contributed to the phenomenon of dependence[3]. Since adenylate cyclase in NG108-15 cells is under the control of both inhibitory and stimulatory G protein (G_i and G_s), it is possible that the increase in adenylate cyclase activity may result from increase in functional regulation of G_s. Thus, we speculated that functional adaption of G_s might be one biochemical machanism underlying opioid tolerance and dependence and the effects of different opiates on G_s function might be correlated with their potential in tolerance and dependence. To test this hypothesis, we compared the effects of different opiates on adenylate cyclase in low-pH treated membranes from naive and opiate chronic treated NG108-15 cells and investigated the effect of G_s protein on opiate tolerance and dependence.

MATERIALS AND METHODS

Chemicals Morphine was purchased from Qinghai Pharmaceutical Factory. Methadone was purchased from Tianjing Central Pharmaceutical Factory. Dihydroetorphine, etorphine, buprenorphine, and naloxone were synthesized by our institute. Forskolin, creatine phosphate, creatine phosphokinase, guanylyl-5'-imidodiphosphate [Gpp(NH)p], ATP, and GTP were purchased from Sigma; cAMP assay kit

[1] Correspondence to Dr LIU Jing-Gen. Phn 86-10-6693-1621. Fax 86-10-6021-0077. E-mail qinby@nic.bmi.ac.cn
Received 1998-03-16 Accepted 1998-10-29

was purchased from China Institute of Atomic Energy. Other chemicals were reagent grade.

Cell culture and chronic opiate treatment NG108-15 hybrid cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, hypoxanthine 100 μmol · L^{-1}, aminopterin 1 μmol · L^{-1}, thymidine 17 μmol · L^{-1}, glutamine 2 mmol · L^{-1}, benzylpeniciline 100 kU · L^{-1}, and streptomycin 100 mg · L^{-1}, in a humified atmosphere of 5 % CO_2 + 95 % air. Cells were grown in 100-mL flasks, in the presence of medium 10 mL, upon reaching subconfluent monolayers, cells were exposed to different opiates for 3 d. At the end of pretreatment, the cells were washed thrice with ice-cold medium. Flasks were kept on ice and cells were harvested, centrifuged (500 × g, 4 ℃, 10 min) and stored as pellets at − 70 ℃.

Preparation of membranes for adenylate cyclase assays[5] All procedures were carried out at 0 ℃ − 4 ℃. Cells were thawed. The pellet was resuspended in 50 volumes of Tris · HCl 50 mmol · L^{-1}, pH 7.4 with egtazic acid (EGTA) 1 mmol · L^{-1}, and was homogenized with polytron. The homogenate was centrifuged at 500 × g for 10 min, and the supernatant was placed on ice. The pellet was resuspended in Tris-EGTA and centrifuged at 500 × g for 10 min again. The two supernatants were combined and centrifuged at 22 500 × g at 4 ℃ for 30 min. The pellet was resuspended in the original volumes of the buffer and centrifuged at 22 500 × g for 30 min again. The pellets were finally resuspended in Tris-EGTA with a final concentration of 1 g protein · L^{-1}, and liquots were stored at − 70 ℃. Membrane protein levels were determined by the method of Bradford[6].

Inactivation of G_s by low-pH treatment[7] Membranes were recovered by centrifugation at 22 500 × g at 4 ℃ for 10 min and were pretreated at low pH by resuspension in pH 4.5 buffer [1 L · g^{-1} (protein)]: sodium acetate 50, dithiothreitol (DTT) 1, $MgCl_2$ 5 mmol · L^{-1}, and incubated on ice for 30 min. Control membranes were pretreated in pH 7.4 buffer: Tris-acetate 50, NaCl 50, DTT 1, $MgCl_2$ 5 mmol · L^{-1}, as described above. The incubation was terminated by addition of 6 − 8 mL Tris buffer. Membranes were isolated by centrifugation at 22 500 × g at 4 ℃ for 10 min and resuspended in Tris buffer and immediately used.

Adenylate cyclase assay NG108-15 cell membranes (10 μg/assay) was incubated with or without various drugs at 32 ℃ − 35 ℃ for 10 min[5]. The assay system contained Tris · HCl 50, egtazic acid 0.2, DTT 0.2, NaCl 100, $MgCl_2$ 10, ATP 0.5, phosphocreatine 5 mmol · L^{-1}, GTP 10 μmol · L^{-1}, creatine phosphokinase 10 units, pH 7.4, in a final volume of 100 μL. The reaction was terminated by boiling for 2 min. The amount of cAMP generated was measured by competitive protein binding assay[8].

RESULTS

Effect of low-pH treatment on adenylate cyclase in NG108-15 cell membranes Low-pH treatment reduced the stimulation of adenylate cyclase by NaF (from 374 % of basal activity in control membranes to 113 % in treated membranes) and Gpp (NH) p (from 233 % of basal activity in control membranes to 119 % in treated membranes) and forskolin (from 460 % of basal activity in control membranes to 228 % in treated membranes). Low-pH treatment did not alter basal adenylate cyclase activity (Tab 1).

Tab 1. Effect of low-pH treatment on adenylate cyclase in naive NG108-15 cell membranes. $\bar{x} \pm s$ of triplicate determination from 4 experiments.
$^bP < 0.05$, $^cP < 0.01$ *vs* pH 7.4. Cyclic AMP levels are given as nmol · min^{-1} · g^{-1} (protein).

	pH 7.4 nmol · min^{-1} · g^{-1}	%	pH 4.5 nmol · min^{-1} · g^{-1}	%
Control	7.2 ± 1.6	100	6.9 ± 1.1	96
NaF				
10 mmol · L^{-1}	26.9 ± 6.1	374	8.1 ± 1.5^b	113
GPP (NH) P				
10 μmol · L^{-1}	16.8 ± 3.2	233	8.6 ± 1.9^b	119
Forskolin				
10 μmol · L^{-1}	33.1 ± 4.8	460	16.4 ± 3.7^c	228

Effect of low-pH treatment on opiate-inhibited adenylate cyclase in naive NG108-15 cell membranes When normal or low pH-treated membranes prepared from naive NG108-15 cells were treated with opiates with or without naloxone 10 μmol ·

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Jun; 20 (6)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

L^{-1} in the presence of forskolin 10 μmol·L^{-1} at 32 ℃ for 10 min, low-pH treatment increased the inhibition of adenylate cyclase by Mor (from 30.2 % in control membranes to 53.6 % in treated membranes) and Met (from 26.1 % in control membranes to 48.4 % in treated membranes). Low-pH treatment also statistically increased the inhibition of adenylate cyclase by DHE, Eto, and Bup, but the magnitude was substantially lower than that of Mor and Met (Tab 2).

Tab 2. Effects of opiates on adenylate cyclase activity (nmol · min^{-1} · g^{-1}) in control and low pH-treated membrane prepared from naive and chronic opiate-treated NG108-15 cells. $\bar{x} \pm s$ of triplicate determination from 3 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* opiate groups. The opiate inhibition of the forskolin-stimulated activity is in parenthesis.

	pH 7.4		pH 4.5	
	Opiate	Opiate + Nal	Opiate	Opiate + Nal
Naive NG108-15 cells				
Mor				
10 μmol·L^{-1}	23 ± 9 (−30 %)	33 ± 6^b	16 ± 4 (−54 %)	34 ± 6^b
Met				
10 μmol·L^{-1}	25 ± 5 (−26 %)	34 ± 8^b	18 ± 5 (−48 %)	35 ± 6^b
Bup				
10 μmol·L^{-1}	31 ± 7 (−22 %)	40 ± 9^b	25 ± 3 (−34 %)	38 ± 5^b
DHE				
10 nmol·L^{-1}	19 ± 5 (−38 %)	30 ± 6^b	16 ± 4 (−45 %)	30 ± 6^b
Eto				
10 nmol·L^{-1}	18 ± 4 (−39 %)	29 ± 10^b	15 ± 3 (−47 %)	28 ± 5^c
Opiate-treated NG108-15 cells				
Mor				
10 μmol·L^{-1}	45 ± 10 (−29 %)	64 ± 10	19 ± 5 (−36 %)	30 ± 7^b
Met				
10 μmol·L^{-1}	39 ± 7 (−10 %)	44 ± 9	23 ± 5 (−33 %)	35 ± 8^b
Bup				
10 μmol·L^{-1}	34 ± 6 (−14 %)	39 ± 8	30 ± 6 (−24 %)	39 ± 7^b
DHE				
10 nmol·L^{-1}	27 ± 4 (−32 %)	40 ± 7^b	16 ± 3 (−43 %)	29 ± 8^b
Eto				
10 nmol·L^{-1}	29 ± 4 (−30 %)	41 ± 8^b	15 ± 3 (−40 %)	25 ± 7^b

This result suggested that there was a difference in regulating G_s-stimulated function among these opiates.

Effect of low-pH treatment on opiate-inhibited adenylate cyclase in chronic opiate-treated NG108-15 cell membranes When NG108-15 cells were chronically treated with opiates for 3 d, an up-regulation in adenylate cyclase was observed in membranes prepared from chronic Mor- and Met-treated NG108-15 cells, whereas the inhibitory effects of Bup and Met on adenylate cyclase were decreased as compared with the membranes drived from naive NG108-15 cells (Tab 2).

Up-regulation in adenylate cyclase and reduction in inhibitory effect of DHE, Eto, and Bup on adenylate cyclase were also found in membranes prepared from chronic DHE, Eto, and Bup-treated NG108-15 cells, but the magnitude was also obviously lower than that of Mor and Met. When low pH-treated membranes prepared from chronic opiate-treated NG108-15 cells were treated with the opiate for chronic treatment with or without naloxone 10 μmol·L^{-1} in the presence of forskolin 10 μmol·L^{-1} at 35 ℃ for 10 min, low-pH treatment substantially increased the inhibition of adenylate cyclase by Mor and Met in membranes prepared from NG108-15 cells chronically treated with Mor and Met while no such effect was observed in membranes from chronic DHE-, Eto-, and Bup-treated NG108-15 cells (Tab 2).

Effect of low-pH treatment on naloxone-induced rebound of adenylate cyclase activity in membranes derived from NG108-15 cells chronically exposed to different opiates When the membranes prepared from chronic Mor- and Met-treated NG108-15 cells were exposed to naloxone 10 μmol·L^{-1} in the presence of forskolin 10 μmol·L^{-1}, a significant rebound of adenylate cyclase was observed while only a rather small increase in cAMP levels was found in membranes prepared from chronic DHE-, Eto-, and Bup-treated NG108-15 cells, which was consistent with the result previously obtained from intact cells. However, when the membranes prepared from chronic Mor- and Met-treated NG108-15 cells were exposed to low pH for eliminating G_s function, no significant rebound of adenylate cyclase was observed when precipitated with naloxone in the presence of forskolin (Tab 3). This result suggested that G_s protein might play an important role in opiate tolerance and dependence.

Tab 3. Effect of low-pH treatment on naloxone-induced rebound of adenylate cyclase activity (nmol·min^{-1}·g^{-1}) in membranes prepared from NG108-15 cells chronically treated with different opiates. Data are $\bar{x} \pm s$ of triplicate determination from 5 experiments. $^bP<0.05$, $^cP<0.01$ *vs* control. The naloxone-precipitated rebound of activity is in parenthesis.

	pH 7.4		pH 4.5	
	Control	Naloxone	Control	Naloxone
Mor				
10 μmol·L^{-1}	52 ± 11	75 ± 14^b (+45 %)	38 ± 9	40 ± 8 (+4 %)
Met				
10 μmol·L^{-1}	58 ± 14	78 ± 14^c (+35 %)	45 ± 12	49 ± 8 (+8 %)
Bup				
10 μmol·L^{-1}	47 ± 12	54 ± 13 (+16 %)	36 ± 5	37 ± 5 (+4 %)
DHE				
10 nmol·L^{-1}	43 ± 11	50 ± 12 (+16.9 %)	36 ± 9	34 ± 6 (−7 %)
Eto				
10 nmol·L^{-1}	40 ± 9	46 ± 9 (+15 %)	30 ± 7	28 ± 7 (−6 %)

DISCUSSION

We recently observed that naloxone-precipitated rebound of cAMP liability of those opiates in NG108-15 cells was parallel to their physical dependence liability in animals and human. This suggested that naloxone-precipitated rebound of cAMP in chronic opiate-treated NG108-15 cells was a useful *in vitro* model system for study of opiate physical dependence potential. Since opiate dependence cells are generally manifested as elevation cAMP production upon withdrawal of the drug, it is reasonable to speculate that G_s protein is involved in development of opiate dependence. For this reason, to study adaptive alteration in G_s function after opiate exposure may help us to understand the difference of those opiates in development of dependence and gain an insight into the mechanism of opiate dependence.

Low-pH treatment is a valid tool for studying G_s function. Exposure of striatal membranes to low pH has been reported to eliminate G_s function, whereas intrinsic adenylate cyclase activity remains unaffected[7]. Previous studies also showed that low-pH treatment selectively decreased G_s protein-mediated adenylate cyclase activity without affecting G_i protein-mediated opioid inhibition of adenylate cyclase[4,9], and increased the efficacy of opioid inhibition of adenylate cyclase[10] in NG108-15 cell membranes. Our results are consistent with those findings. The stimulation produced by the agents known to activate adenylate cyclase through G_s-sodium fluoride, the nonhydrolyzable GTP analog Gpp (NH) p, and forskolin, was significantly decreased by low-pH treatment.

We have observed that low-pH treatment can eliminate naloxone-induced rebound of adenylate cyclase in membranes prepared from Mor and Met chronic treated NG108-15 cells, indicating that G_s protein might play an important role in opiate tolerance and dependence. In the present and previous study, we have also demonstrated that Mor and Met are distinct from DHE and Eto in the following: 1) Low-pH treatment markedly increased inhibition of adenylate cyclase by Mor and Met but slightly increased inhibition of that by DHE and Eto in membranes prepared from naive NG108-15 cells; 2) Low-pH treatment sinificantly increased inhibition of adenylate cyclase by Mor and Met in membranes prepared from Mor and Met chronic treated NG108-15 cells while no marked increase in inhibition of adenylate cyclase by DHE and Eto was observed in membranes prepared from DHE and Eto chronic treated NG108-15 cells; 3) inactivation of G_i with pertussis toxin significantly decreased inhibition of adenylate cyclase by Mor and Met while no such reduction of inhibition of that by DHE and Eto was detected in intact naive NG108-15 cells. These results led us speculate that DHE and Eto might have inhibitory effects on G_s-mediated excitatory signal transduction pathway, by which DHE and Eto still substantially inhibited adenylate cyclase in NG108-15 cells after inactivation of G_i with pertussis toxin, and only slightly increased inhibition of adenylate cyclase in membranes prepared from naive and chronic DHE and Eto-treated NG108-15 cells following elimination of G_s with low-pH treatment. But further experiments are needed to confirm this speculation. Our results strongly suggest that there is difference of those opiates in regulating of G_s function, which may underlie the difference of those opiates in regulation cAMP second

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Jun; 20 (6)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

messenger system and in produing dependence. These results also support that alteration in G_s-mediated stimulatory pathway of adenylate cyclase may be at least one biochemical mechanism underlying the development of opiate dependence.

REFERENCES

1 Liu JG, Gong ZH, Qin BY. Effects of opioid receptor agonists on cAMP second messenger system. Acta Pharmacol Sin 1999; 20: 452 – 6.
2 Shen KF, Crain SM. Antagonists at excitatory opioid receptors on sensory neurons in culture increase potency and specificity of opiate analgesics and attenuate development of tolerance/dependence. Brain Res 1994; 636: 286 – 97.
3 Niwa M, al-Essa LY, Ohta S, Kohno K, Nozaki M, Tsurumi K, *et al*. Opioid receptor interaction and adenylyl cyclase inhibition of dihydroetorphine: direct comparison with etorphine. Life Sci 1995; 56: PL395 – 400.
4 Ammer H, Schulz R. Coupling of prostaglandin E_1 receptors to the stimulatory GTP-binding protein G_s is enhanced in neuroblastoma × glioma (NG108-15) hybrid cells chronically exposed to an opioid. Mol Pharmacol 1993; 43: 556 – 63.
5 Vachon L, Costa T, Herz A. GTPase and adenylate cyclase desensitize at different rates in NG108-15 cells. Mol Pharmacol 1987; 31: 159 – 68.
6 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 – 54.
7 Childers SR. Opiate-inhibited adenylate cyclase in rat brain membranes depleted of G_s-stimulated adenylate cyclase. J Neurochem 1988; 50: 543 – 53.
8 Xu SY, Bian RL, Chen X, editors. Experimental methodology of pharmacology. 2nd ed. Beijing: The People's Medical Publishing House; 1991. p 415 – 20.
9 McKenzie FR, Milligan G. δ-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi_2. Biochem J 1990; 267: 391 – 8.
10 Selley DE, Breivogel CS, Childers SR. Modification of G protein-coupled functions by low-pH pretreatment of membranes from NG108-15 cells: increase in opioid agonist efficacy by decreased inactivation of G proteins. Mol Pharmacol 1993; 44: 731 – 41.

低 pH 处理对阿片受体激动剂调节 cAMP 第二信使系统的作用

刘景根[1]，宫泽辉，秦伯益 (军事医学科学院毒物药物研究所，北京 100850，中国)

关键词 吗啡；美沙酮；阿片类有关的紊乱；培养的细胞；埃托啡；丁丙诺啡；纳洛酮；腺苷酸环化酶；环腺苷—磷酸；δ阿片受体

目的：研究不同的阿片类物质对 cAMP 第二信使系统不同作用的机制. **方法**：用低 pH 方法失活 G_s 蛋白，用蛋白竞争法测 NG108-15 细胞膜腺苷酸环化酶(AC)活性. **结果**：与二氢埃托啡(DHE)和埃托啡(Eto)不同，低 pH 处理明显增加吗啡(Mor)和美沙酮(Met)对正常和自身慢性处理细胞的膜 AC 活性的抑制作用. 与 Mor 等不同，DHE 和 Eto 对自身慢性处理细胞的膜 AC 活性抑制作用降低不明显，纳洛酮催促. DHE 和 Eto 慢性处理细胞的膜 AC 活性也未见显著反跳性升高. 低 pH 处理使纳洛酮催促的 Mor 慢性处理细胞的膜 AC 活性反跳性升高作用消失. **结论**：不同的阿片类 cAMP 信使系统的不同作用与它们对 G_s 功能调节差异有关，G_s 与阿片类物质的耐受和依赖密切相关.

(责任编辑 李 颖)