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Effects of opioid receptor agonists on cAMP second messenger system

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KEY WORDS morphine; methadone; etorphine; opioid-related disorders; cyclic AMP; delta opioid receptors; adenylate cyclase; NG108-15 cells

ABSTRACT

AIM: To study the mechanism underlying the difference in physical dependence potential of morphine (Mor), methadone (Met), buprenorphine (Bup), etorphine (Eto), and dihydroetorphine (DHE). **METHODS**: Adenylate cyclase of NG108-15 cells were used for studying the effects of different opiates on cAMP second messenger system. **RESULTS**: Bup, DHE, and Eto were distinct from Mor in naloxone-precipitated rebound response of cAMP in NG108-15 cells chronically treated with these opiates. Naloxone given to NG108-15 cells treated with Mor for 24 h produced marked rebound response of adenylate cyclase. While no such rebound response was detected when the cells were treated with Bup, DHE, and Eto for 24 h. The naloxone-induced rebound response of cAMP in chronic Met-treated NG108-15 cells was also lower than that in chronic Mor-treated NG108-15 cells. Following a prolonged exposure to Bup, DHE, and Eto for 72 h, the naloxone-induced rebound response of cAMP in these cells was still markedly lower than that in Mor-treated cells. The substitution of Mor with Bup, Met, DHE, and Eto inhibited naloxone-induced rebound response of cAMP in chronic Mor-treated NG108-15 cells. **CONCLUSION**: There were distinct differences among these opiates in regulating cAMP second messenger system, which was related to their physical dependence potential.

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INTRODUCTION

The cAMP second messenger system had been proposed to play a key role in opiate dependence. Strong evidence supported the view that the opiate-induced up-regulation of the cAMP system represented one mechanism by which opiates produced dependent changes in neurons and neuroblastoma × glioma hybrid cells[1]. The cAMP overshoot after episode of opiate exposure was first suggested as a possible mechanism underlying the opiate withdrawal syndrome[2]. This suggestion was subsequently supported by several lines of evidence[3,4]. The neuroblastoma × glioma cell line NG108-15 cell had been used widely to examine transmembrane signalling cascades, which had been a useful model system for study of opioid receptor and the effectors to which they were coupled. This cell line contains δ-type opioid receptors which had been found to play an important role in opiate dependence[5,6]. Previous studies in Mor-treated NG108-15 cells had shown a marked rebound effect upon withdrawal of the opiate or precipitation by antagonists, which was proposed to be linked with the abstinence in animals[7,8]. It was generally accepted that the adenylate cyclase of NG108-15 cells could act as a model for studying biochemical mechanism of opiate tolerance and dependence[7,9]. The physical dependence potential of different opioid agonist was distinctly different. Mor, a pure agonist, has a higher physical dependence potential than weak agonist Met and partial agonist Bup. However, DHE, a potent pure opioid agonist showed a lower physical dependence potential than Mor though the analgesic effect of DHE was several thousand times more potent than that of Mor. DHE inhibited the withdrawal symptoms of Mor-dependent animals without causing itself dependence during the treatment[10]. What were the causes underlying the difference in physical dependence

potential of these opiates? What difference was there in the effects of these opiate on the cAMP second messenger system? Because all opiates mentioned above were agonists of δ-receptors, we investigated the effects of these opiates on cAMP second messenger system in NG108-15 cells. The goal of the present study was to study the mechanism underlying the difference in physical dependence potential of these opiates.

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 was purchased form Sigma; cAMP assay kit was purchased from China Institute of Atomic Energy, Beijing.

Cell culture NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (Gbico) containing hypoxanthine 0.1 mmol·L^{-1}, aminopterin 10 μmol·L^{-1}, and thymidine 17 μmol·L^{-1}, glutamine 2 mmol·L^{-1}, supplemented with 10 % fetal bovine serum, benzylpenicillin 100 kU·L^{-1}, and streptomycin 100 mg·L^{-1}. Cells were grown in 100-mL flasks at 37 ℃ in a humid atmosphere of 10 % CO_2 + 90 % air until confluence, then transferred to 6-well culture plates, grown to confluence and maintained for 2 – 4 d.

Treatment with opiates and cAMP assay NG108-15 cells were exposed to different concentrations of sterile opiates in incubation medium for different periods. Culture medium was removed and each well was washed twice with serum-free DMEM 1 mL with benzylpenicillin and streptomycin, followed by the addition of serum-free DMEM 1 mL. To prevent the rebound response of adenylate cyclase prior to the addition of naloxone, the opiates were added. The rebound response of adenylate cyclase was elicited by the addition of naloxone (Nal) 10 μmol·L^{-1} in the presence of forskolin (For) 10 μmol·L^{-1}. The reaction was terminated after 10 min by placing the plates on ice-cold water, followed by rapid removal the medium, and washing twice with saline D buffer: NaCl 137, KCl 5, Na_2HPO_4 0.22, glucose 6 mmol·L^{-1}, pH 7.4 and adding ice-cold 5 % trichloroacetic acid 1 mL. The cells were scraped from the well and transferred to Eppendorff tubes and centrifuged (700 × g, 0 ℃, 10 min). The supernatants were extracted three times with water-saturated diethylether 5 mL. After evaporation of the residual ether, the cAMP concentrations were determined by competitive protein binding assay[11]. Protein concentration was determined[12], using bovine serum albumin as a standard.

RESULTS

Effects of acute opiate treatment on forskolin-stimulated cAMP accumulation When NG108-15 cells were treated with potent opioid agonists DHE, Eto, and Mor, weak agonist Met, and partial agonist Bup for 30 min, all opiate treatment groups showed a significant reduction of cAMP levels versus control (Tab 1), indicating that opiates inhibited adenylate cyclase activity during acute exposure. When precipitated with naloxone, no rebound response of cAMP was observed.

Tab 1. Acute effects of opiates on forskolin (10 μmol·L^{-1})-stimulated cAMP accumulation. NG108-15 cells were treated with different opiates for 30 min. $\bar{x} \pm s$ of triplicate determination from 5 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Opioid concentration	cAMP/nmol·min^{-1}·g^{-1}(protein) Control	Naloxone
Control	196 ± 47	169 ± 37
Morphine 10 μmol·L^{-1}	125 ± 31^b (−36.2 %)	121 ± 29
Methadone 10 μmol·L^{-1}	138 ± 35^b (−29.6 %)	129 ± 33
Buprenorphine 10 μmol·L^{-1}	141 ± 29^b (−28.1 %)	147 ± 30
Dihydroetorphine 10 nmol·L^{-1}	112 ± 24^c (−42.9 %)	116 ± 28
Etorphine 10 nmol·L^{-1}	121 ± 28^c (−38.3 %)	114 ± 21

Effects of chronic opiate pretreatment on opiate efficacy against the forskolin-stimulated cAMP accumulation When NG108-15 cells were pretreated with the opiates for 48 h, a spontaneous cAMP overshoot was observed as compared with untreated cells. At this time, if cells were exposed to

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different opiates again for 30 min, the acute inhibitory effects of Met on For-stimulated cAMP accumulation was substantially decreased. While no significant reduction of inhibition of For-stimulated cAMP accumulation was found in Mor, DHE, and Eto groups (Tab 2). Bup was also shown to decrease the acute inhibition of For-stimulated cAMP accumulation but the magnitude was markedly lower than that of Met in the same condition. This result suggested that there were different tolerances among these opiates.

Tab 2. Effects of opiate pretreatment (48 h) on opiate efficacy against forskolin-stimulated cAMP accumulation. NG108-15 cells were treated with the same opiate again for 30 min after pretreatment with this opiate for 48 h. $\bar{x} \pm s$ of triplicate determination from 5 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

	cAMP/nmol·min^{-1}·g^{-1}(protein) Control	Opiates
Control	182 ± 45	
Morphine	228 ± 39^b	151 ± 23
10 μmol·L^{-1}	(+25.3 %)	(33.6 %)
Methadone	217 ± 46^b	192 ± 41
10 μmol·L^{-1}	(+19.2 %)	(−11.5 %)
Buprenorphine	195 ± 41^b	159 ± 37^b
10 μmol·L^{-1}	(+7.1 %)	(−18.1 %)
Dihydroetorphine	203 ± 51^b	131 ± 27^b
10 nmol·L^{-1}	(+11.5 %)	(−35.5 %)
Etorphine	209 ± 34^b	152 ± 39^c
10 nmol·L^{-1}	(+14.8 %)	(−27.3 %)

Effects of pertussis toxin (PT) on forskolin-stimulated cAMP accumulation in opiate-treated NG108-15 cells As compared with that in vehicle groups, the inhibition of For-stimulated cAMP accumulation by Mor and Met was markedly decreased in PT-pretreated groups, while no significant reduction in inhibition of For-stimulated cAMP accumulation by DHE amd Eto was observed after exposure to PT (Tab 3). Bup was also shown to decrease the inhibition of For-stimulated cAMP accumulation but the magnitude was lower than that of Mor and Met. This result suggested that the mechanism underlying inhibitory effect of DHE and Eto on For-stimulated cAMP accumulation was different from those of Mor and Met.

Naloxone-induced rebound of cAMP in NG108-15 cells treated with different opiates The cAMP overshoot in Mor-treated cells fully developed after 24 h when it showed a 66 % increase over control values. The rebound response of cAMP was not apparent in other opiates-treated groups at this time except that there was slight cAMP overshoot in Met-treated group. But, following chronic opiate exposure for 72 h, the cAMP overshoot in Met and Bup groups was markedly increased. DHE and Eto groups showed an increase in the cAMP overshoot but the elevation was markedly lower than that of other groups (Tab 4).

Tab 3. Effects of pertussis toxin (PT) on forskolin-stimulated cAMP accumulation in acute opiate-treated NG108-15 cell. The cells were treated with either vehicle or PT 100 μg·L^{-1} for 3 h, and subsequently exposed to opiates for an additional 30 min. $\bar{x} \pm s$ of triplicate determination from 4 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Treatment	cAMP/nmol·min^{-1}·g^{-1}(protein) Vehicle	Inhibition/ %	PT	Inhibition/ %
Control	191 ± 57		479 ± 95	
Morphine	124 ± 28^c	35.1	427 ± 102	10.9
Methadone	139 ± 30^c	27.2	438 ± 86	8.6
Buprenorphine	132 ± 34^b	30.9	388 ± 94^b	19.0
Dihydroetorphine	116 ± 28^c	39.3	306 ± 73^b	36.1
Etorphine	121 ± 37^b	36.6	346 ± 81^b	27.8

Effects of substitution of morphine with other opiates on naloxone-induced rebound response of cAMP in NG108-15 cells When other opiates were used as substitute for Mor to treat Mor-dependent cells for additional 24 h, the cAMP overshoot was attenuated to a certain degree in Met and Bup substitute groups as compared with Mor consecutive treatment groups. Whereas a substantial reduction was observed in DHE and Eto substitute groups (Tab 5).

If the substitute treatment cells were further treated with Bup as a substitute for Met, DHE and Eto for additional 24 h, the naloxone-induced cAMP overshoot was further decreased in Met substitute groups. But no further reduction was observed in DHE and Eto groups (Tab 5).

DISCUSSION

In the present study, we demonstrated that DHE,

Tab 4. Naloxone-induced rebound response of cAMP in NG108-15 cells treated with different opiates. NG108-15 cells were treated with opiates for 24 or 72 h, then precipitated with naloxone 10 μmol·L^{-1} in the presence of forskolin 10 μmol·L^{-1}. $\bar{x} \pm s$ of triplicate determination from 6 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Opiate concentration	cAMP/nmol·min^{-1}·g^{-1}(protein) After incubation for 24 h			After incubation for 72 h		
	Control	Nal	Increase %	Control	Nal	Increase %
Control	165 ± 10	134 ± 17^b		168 ± 26	141 ± 25^b	
Morphine 10 μmol·L^{-1}	177 ± 23	294 ± 40^c	66.1	185 ± 10	307 ± 36^c	65.9
Methadone 10 μmol·L^{-1}	169 ± 39	198 ± 47^b	17.2	188 ± 23	267 ± 59^c	42
Buprenorphine 10 μmol·L^{-1}	181 ± 42	189 ± 31	4.4	181 ± 20	219 ± 66^b	21
Dihydroetorphine 10 nmol·L^{-1}	172 ± 59	179 ± 52	4.1	191 ± 64	213 ± 36^b	11.5
Etorphine 10 nmol·L^{-1}	167 ± 44	176 ± 45	5.4	185 ± 25	209 ± 26^b	13

Tab 5. Effects of substitution of morphine with other opiates on naloxone induced rebound response of cAMP in NG108-15 cells. NG108-15 cells were pretreated with Mor 10 μmol·L^{-1} for 48 h, followed by substitution of Mor with Met and Bup 10 μmol·L^{-1}; DHE, Eto 10 nmol·L^{-1} for aditional 24 or 48 h. $\bar{x} \pm s$ of triplicate determination from 7 experiments.
$^bP < 0.05$, $^cP < 0.01$ *vs* control.

Substitutive opiates	cAMP/nmol·min^{-1}·g^{-1}(protein) For	For + Nal	Increase/%
Control (Mor)	239 ± 25	381 ± 94^b	59.4
Met (24 h)	216 ± 57	298 ± 65^b	38.0
Met, Bup	193 ± 42	222 ± 51	15.0
DHE	184 ± 39	208 ± 44^b	13.0
DHE, Bup	189 ± 36	220 ± 45^c	16.4
DHE, Met	195 ± 46	233 ± 52^b	19.5
Eto	192 ± 49	212 ± 62^c	10.4
Eto, Bup	196 ± 37	230 ± 39^b	17.3
Eto, Met	206 ± 46	247 ± 51^b	19.9
Bup	181 ± 52	219 ± 49^b	21.0

Eto, and Bup were distinct from Mor in naloxone-precipitated cAMP overshoot after chronic exposure to them. The rebound liability of cAMP induced by naloxone in these opiate-treated NG108-15 cells was approximately parallel to the physical dependence liability in these opiate-treated animals and human, indicating that the naloxone-induced rebound response of adenylate cyclase in NG108-15 cells was a useful model system for studying opiate physical dependence potential *in vitro*. This result also suggested that the different regulative effects of these opiates on cAMP second messenger system were related to their different physical dependence potential. It was reported that DHE had a high psychic dependence potential, which can produce heroin-like euphorigenic effect with a high abuse liability, though most of withdrawal symptoms produced by DHE were mild and moderate according to the criteria of opiate withdrawal scale[13]. This suggested that the mechanism underlying the physical dependence might be different from that underlying psychic dependence. Different opiates could impose different influence on opioid receptor or receptor-mediated signal transduction pathway because of their different structure and intrinsic activity. Therefore, when a combined application of opiates might result in either enforcement or alleviation of dependence development. Clinic studies showed that using DHE as substitution therapy could relieve the withdrawal symptom and to facilitate detoxification in patients with opioid addiction[14,15]. In the present study, the substitution of Mor with DHE, Eto, Met, and Bup could inhibit naloxone-induced rebound response of cAMP in Mor chronic treatment NG108-15 cells. The result was similar to that given by clinical substitution therapy studies, which strongly supported novel hypothesis[15] that pure opioid agonists could substitute each other for controlling abstinence symptoms, but the addiction of the two drugs did not add together. This could be considered as the main theoretical basis of substitution for opioid addicts.

In the present study, we also observed that DHE significantly decreased the levels of cAMP of NG108-15 cells which were pretreated with DHE for 48 h. Moreover, when NG108-15 cells were further treated

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with PT, which has been shown to inactivate irreversibly the G_i protein, DHE still markedly inhibited adenylate cyclase activity. These results suggested that DHE might inhibit adenylate cyclase via regulation G_s protein function. Since adenylate cyclase activity in NG108-15 cells was regulated by both inhibitory and stimulatory G protein. It was possible that increase in adenylate cyclase activity following chronic opiate treatment might result from an alteration in the extent of G_s and G_i regulation. Therefore we speculated that the inhibitory effects of DHE and Eto on adenylate cyclase of the chronic treatment or PT treatment of NG108-15 cells might be related to regulating G_s function but further study would be needed to confirm such speculation.

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不同的阿片受体激动剂对环腺苷一磷酸第二信使系统的作用

R971.1

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关键词 吗啡；美沙酮；埃托啡；阿片类有关的紊乱；环腺苷一磷酸；δ阿片受体；腺苷酸环化酶；NG108-15 细胞

目的：研究吗啡（Mor）、美沙酮（Met）、丁丙诺啡（Bup）、二氢埃托啡（DHE）和埃托啡（Eto）躯体依赖性差别的机制。**方法**：用 NG108-15 细胞模型，观察不同的阿片受体激动剂对 cAMP 第二信使系统的作用。**结果**：细胞分别暴露于 Mor、Met、Bup 各 10 $\mu mol \cdot L^{-1}$ 和 DHE、Eto 各 10 $nmol \cdot L^{-1}$，24 h 或 72 h 后，用纳洛酮 10 $\mu mol \cdot L^{-1}$ 催促，Mor 组 cAMP 水平明显反跳性升高，其他各组 cAMP 反跳水平虽有一定程度的升高，但幅度较 Mor 组低得多。用 Met、Bup、DHE 和 Eto 替代处理 Mor 预处理 48 h 的 NG108-15 细胞，可使 Nal 催促的 cAMP 反跳水平明显降低。**结论**：Mor、Met、Bup、DHE 和 Eto 对 cAMP 第二信使系统的作用明显不同，这与它们的躯体依赖性有关。

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