Korean J Physiol Pharmacol
Vol 4: 291–299, August, 2000

Changes of the Level of G Protein α-subunit mRNA by Withdrawal from Morphine and Butorphanol

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Morphine or butorphanol was continuously infused into cerebroventricle (i.c.v.) with the rate of 26 nmol/μl/h for 3 days, and the withdrawal from opioid was rendered 7 hrs after the stopping of infusion. The expression of physical dependence produced by these opioids was evaluated by measuring the naloxone-precipitated withdrawal signs. The withdrawal signs produced in animals dependent on butorphanol (*kappa* opioid receptor agonist) were similar to those of morphine (*mu* opioid receptor agonist). Besides the behavioral modifications, opioid withdrawal affected G protein expression in the central nervous system. The G-protein α-subunit has been implicated in opioid tolerance and withdrawal. The effects of continuous infusion of morphine or butorphanol on the modulation of G protein α-subunit mRNA were investigated by using in situ hybridization study. In situ hybridization showed that the levels of Gαs and Gαi were changed during opioid withdrawal. Specifically, the level of Gαs mRNA was decreased in the cortex and cerebellar granule layer during the morphine and butorphanol withdrawal. The level of Gαi mRNA was decreased in the dentate gyrus and cerebellar granule layer during the morphine withdrawal. However, the level of Gαi mRNA was significantly elevated during the butorphanol withdrawal. These results suggest that region-specific changes of G protein α-subunit mRNA were involved in the withdrawal from morphine and butorphanol.

Key Words: Morphine, Butorphanol, G protein, In situ hybridization, Autoradiography, Withdrawal

INTRODUCTION

Butorphanol is considered to have a low abuse potential when used according to therapeutic recommendations. Nevertheless, several clinical reports document the development of dependence on butorphanol (Brown, 1985; Evans et al, 1985). The receptor action of butorphanol differs significantly from that of the classic opioid analgesic morphine. The dissimilarities between the actions of the two analgesics at opioid receptor subtypes lead the question of the relative participation of these subtypes in the development of dependence on narcotic analgesics. Specifically, it has been proposed that the relative involvement of opioid receptor subtypes in experimentally precipitated withdrawal by injection of naloxone, as an index of the degree of dependence, would differ between rats made dependent on butorphanol and those dependent on the standard agent, morphine. Butorphanol interact with the *kappa* as well as the *mu* and *delta* opioid receptors (Horan & Ho, 1991), whereas morphine binds as an agonist to primarily the *mu* and *delta* opioid receptors (Gulya et al, 1988). Although it is clear that the actions of butorphanol at the *mu* and *delta* opioid receptors are important to the development of dependence (Jaw et al, 1993c), evidence indicates that interaction with the *kappa* opioid receptor contributes significantly to the response (Jaw et al, 1993c; Feng et al, 1994). Opioid receptors modulate pain through a number of pharmacologically distinct receptor systems acting both spinally and supraspinally.

Four members of the opioid receptor family has been cloned and found to belong to the G protein-coupled seven transmembrane spanning receptor family (Reisine & Bell, 1993; Pasternak & Standifer,

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1995). The binding of agonists to G protein- coupled receptors promotes the formation of agonist-receptor-G protein complexes leading to G protein activation, exchange of bound GDP by GTP followed by dissociation of G α-GTP from G $\beta\gamma$ subunits and productive coupling to effecter systems (Hille, 1992). Opioid receptors are coupled to a variety of effecter systems, including inhibition of adenylyl cyclase, inhibition of calcium channel conductance and stimulation of potassium channel conductance, through the activation of GTP-binding proteins of the pertussis toxin-sensitive G αi/G αo family (Kurose et al, 1983; Aghajanian & Wang, 1986; Hescheler et al, 1987; North et al, 1987). The in situ hybridization technique was used to map the spatial expression of G αs, G αi, G αo mRNA transcripts throughout the rat brain and to verify any changes induced in them by the treatment. In the present study, the effects of withdrawal from morphine and butorphanol on the level of mRNA coding the G αs, G αi and G αo subunits were performed to see whether different opioid agonist induces different G protein subunit expression.

METHODS

Chemicals

Reagents including [α-^{35}S]dATP (1,250 Ci/mmol), an oligonucleotide 3' end labeling kit and the purified oligodeoxynucleotide probes complementary to rat G protein α-subunit cDNA of G αs (5'-TCC AGA GGT CAG GAC ACG GCA GCG AAG CAG GTC CTG GTC -3'), G αi (5'-GCC CTG GGT CTT CAC ACG GGT CCG CAG CAC ATC CTG CTG-3'), and G αo (5'-GCC ATG TGT TTT GAC CCT GGT TCG GAG GAT GTC CTG-3') were obtained from DuPont-NEN (Boston, MA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals International (Natick, MA).

Animal treatment protocol

Male Sprague-Dawley rats weighing 220~240 g were acclimatized for 1 week with free access to rat chow and tap water. The temperature (24±3°C) and light (12 hr dark) of the housing environment were maintained constantly. Rats were anesthetized with Equithensin (3 ml/kg, i.p.) before standard stereotaxic surgery was performed on a Kopf stereotaxic frame. A stainless steel guide cannula (21 gauge, 10 mm long) was implanted into the right lateral cerebral ventricle (AP: −0.5 mm, LAT: +1.3 mm, and DV: −4.5 mm; Paxinos and Watson, 1986) of each rat. Rats were allowed 1 week for recovery before implantation of osmotic minipump. Morphine or butorphanol dependence was induced by continuous i.c.v infusion with morphine or butorphanol (infusion rate, 26 nmol/ μl/h) for 3 days through osmotic minipumps (Alzet 2001, Alza, Palo Alto, CA). According to previous studies (Horan & Ho, 1991; Feng et al, 1995; Tokuyama & Ho, 1996), this dose and period of opioid infusion can successfully produce physical dependence. A control group received an i.c.v. infusion of saline (1 μl/hr). Before filling into the pump, the solution was passed through 0.2 mm sterile Acrodisk filters. The minipumps were primed overnight at 35°C in sterile saline so that the normal flow rate (1 μl/hr) was attained prior to implantation. Under ether anesthesia, rats were implanted with osmotic minipumps subcutaneously between the scapulae. A Tygon tubing (0.38 mm inner diameter, Cole-Palmer, Chicago, IL) was used to connect the outlet of the minipump to a piece of 'L'-shaped stainless steel injector tubing (26 gauge, 20 mm long), which was placed into the i.c.v. guide cannula.

Measurement of withdrawal signs

Ten distinct behaviors (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penis licking, and ptosis) were scored during a 15-min period following the naloxone treatment as behavioral signs of withdrawal. Naloxone was administrated into i.c.v. (48 nmol/5 μl), 7 hr after the termination of drug infusion, performed by cutting the tubing. The development of butorphanol withdrawal is more prominent by 7 hr after discontinuation of butorphanol infusion than that of 2 or 24 hr after discontinuation (Jang et al, 1999). The reactions of each animal were evaluated by an independent observer who did not know the nature of the treatment.

Tissue preparation

Rats were sacrificed by decapitation 7-hr after stopping infusion. Brains were removed immediately and were frozen in liquid nitrogen for 20 sec. Sagittal sections, 14 μm thickness, were cut on an AMES

microtome-cryostat II (Miles Laboratories, Naperville, IL) at $-18^{\circ}C$, thaw-mounted on gelatin-coated microscope slides and stored at $-80^{\circ}C$ until used.

In situ hybridization

In situ hybridization was carried out by the previously described method (Oh and Ho, 1999). The oligonucelotide probe was labeled on its 3' end using terminal deoxynucleotidyl transferase and [α-^{35}S] dATP. Ten picomoles of the probe in the tailing buffer (0.1 M potassium cacodylate, 25 mM Tris base, 1.0 mM cobalt chloride, and 0.2 mM dithiothreitol, pH 7.0) and 50 pmol [α-^{35}S]dATP were incubated with 36 units of terminal deoxynucleotidyl transferase at 37°C for 30 min. The reaction was terminated by adding 400 μl cold (4°C) Nensorb reagent A (0.1 M Tris-HCl, pH 7.7, 10 mM triethylamine, 1 mM EDTA). The labeled probe was purified using Nensorb 20 column chromatography and was eluted in 50% ethanol. The probe solution was diluted with hybridization buffer [50% formamide, 10% dextran sulfate, 1% Denhardt's solution, 100 mM dithiothreitol, 0.025% tRNA from *E. Coli* and 0.05% DNA from salmon testes in 4×standard sodium citrate buffer (SSC, 1×SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0)]. Frozen slides were dried at room temperature for 10 min, and fixed in 4% paraformaldehyde in phsophate-buffered saline (PBS), pH 7.4, for 10 min. The slides were then rinsed three times in PBS for 3 min, rinsed once in 2×SSC for 3 min, rinsed briefly in deionized water and dried in a stream of room air. Each brain slice was hybridized with 30 μl hybridization buffer under a coverslip to prevent tissue from drying and incubated overnight at 40°C in a high-humidity environment. After hybridization, the coverslips were carefully removed in 1×SSC and the slides were washed in 1×SSC three times for 3 min each to remove the excess hybridization buffer. Slides were then washed four times, 15 min each, in 2×SSC + 50% formamide at 55°C, followed by washing in 1× SSC at room temperature twice for 15 min each. Finally, slides were briefly washed in deionized water and dried in a stream of room air. Competition hybridization was performed by adding an 80-fold excess of unlabeled probe, which showed negligible non-specific hybridization in the final image. Dried tissue sections were apposed to Hyperfilm-βmax (Amersham), along with ^{14}C-plastic standard slide (ARC-146, ARC, St. Louis, MO) for 2 to 3 weeks at room temperature. The film was developed in Kodak D19 at room temperature for 3 min and fixed for 5 min. Autoradiograms were analyzed by a digital scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA), operating on the image acquisition and analysis program ImageQuant 3.3 (Molecualr Dynamics). Non-specific binding was less than 5% of the total binding and was negligible for analyzing the autoradiograms.

Quantification and statistics

The density in each region was recorded by marking 4 to 10 areas of the brain according to the size and shape of the region. The mean values were determined from 5 rats and expressed as the mean±SE, in nCi/g tissue. Data were analyzed using Student's t-test and analysis of variance followed by Newman-Keul's test.

RESULTS

Withdrawal behaviors

Naloxone did not precipitate withdrawal syndrome

Table 1. Withdrawal signs elicited in morphine- or butorphanol-dependent rats

Withdrawal signs	Saline	Morphine	Butorphanol
Escape behavior	0/8	5/8*	4/8*
Wet dog shakes	2/8	8/8**	8/8**
Teeth chattering	0/8	7/8**	8/8**
Rearing	0/8	8/8**	7/8**
Locomotion	1/8	7/8*	6/8*
Stretching	0/8	6/8**	5/8*
Scratching	0/8	6/8**	7/8**
Salivation	0/8	6/8**	5/8*
Penis licking	0/8	7/8**	7/8**
Ptosis	0/8	6/8**	6/8**

Rats received i.c.v. infusion of morphine (26 nmol/μl/h) or butorphanol (26 nmol/μl/h) for 3 days and were injected with naloxone (48 nmol/5 μl) into the i.c.v. 7 h after the termination of drug infusion. Numbers denote the number of rats showing positive signs over the total number of rats tested. *$p<0.05$, **$p<0.01$, values are significantly higher than the saline values as determined by the chi-square test.

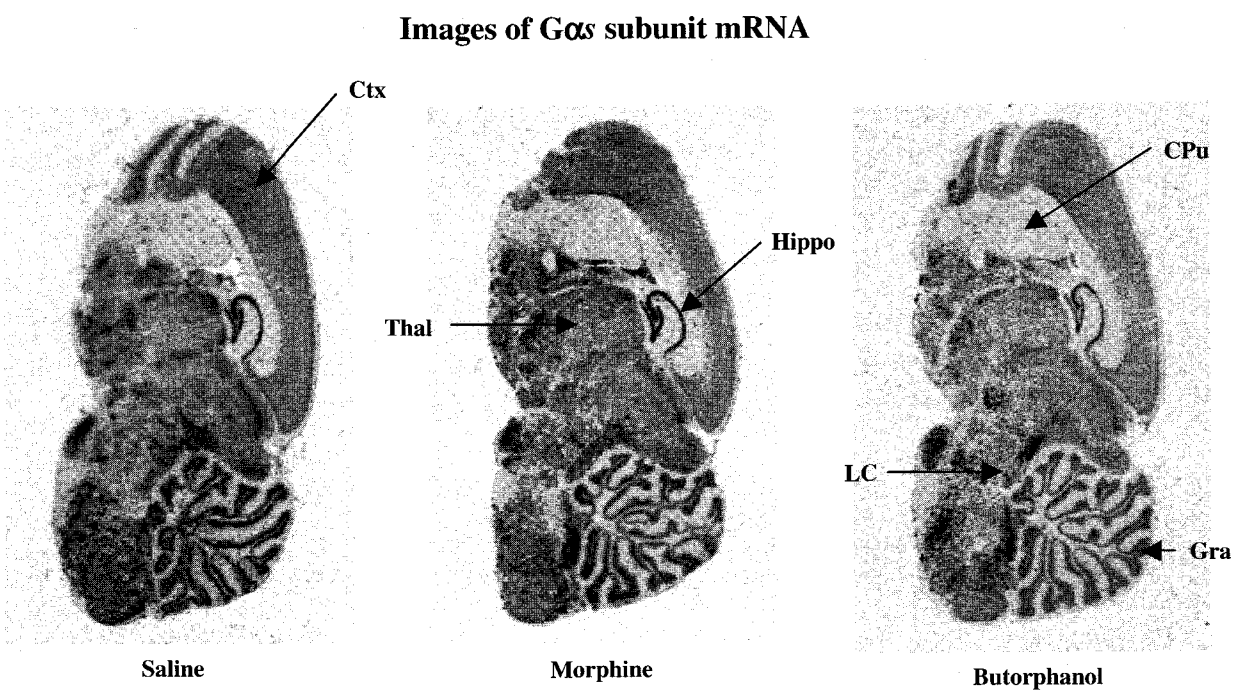

Fig. 1. Representative autoradiograms of G protein αs-subunit mRNA in sagittal rat brain sections. Exposure time was 2 weeks. Ctx, cortex; Thal, thalamus; Hippo, hippocampus; LC, locus coeruleus; Cpu, caudate putamen; Gra, cerebellar granule layer.

Table 2. Changes of the level of G protein Gs subunit mRNA in rats withdrawal from morphine and butorphanol

Regions	mRNA levels (nCi/g tissue)		
	Saline	Morphine	Butorphanol
Cortex	93.33±3.85	66.61±6.39**	76.54±5.82*
Caudate-putamen	20.53±2.91	15.78±2.42	19.13±1.93
Thalamus	65.24±8.94	50.97±2.20	64.67±4.78
Hippocampus			
CA1	121.49±10.28	102.46±12.54	126.18±10.08
CA3	135.32±12.69	139.22±11.55	142.52±13.31
Dentate gyrus	119.33±10.51	113.41±10.48	118.90±10.84
Brainstem			
Inferior colliculus	98.17±9.49	88.76±8.93	97.54±7.50
Locus coeruleus	115.98±7.85	100.67±5.08	111.47±9.78
Cerebellum			
Granule layer	147.12±10.99	127.34±8.26	113.36±9.07*

Rats were rendered dependent on morphine and butorphanol by continuous icv infusion (26 nmol/μl/hr) for 3 days. Values are expressed as mean±SE from 5~6 rats. *$p<0.05$, **$p<0.01$ for difference from respective control groups.

from saline infused animals. The continuous i.c.v. infusion of morphine (26 nmol/μl/h) or butorphanol (26 nmol/μl/h) for 3 days induced physical dependence, manifested by withdrawal syndrome (escape behavior, wet dog shakes, teeth chattering, rearing, locomotion, stretching, scratching, salivation, penis licking, ptosis) when naloxone was administrated into i.c.v. (48 nmol/5 μl) 7 hr after the termination of

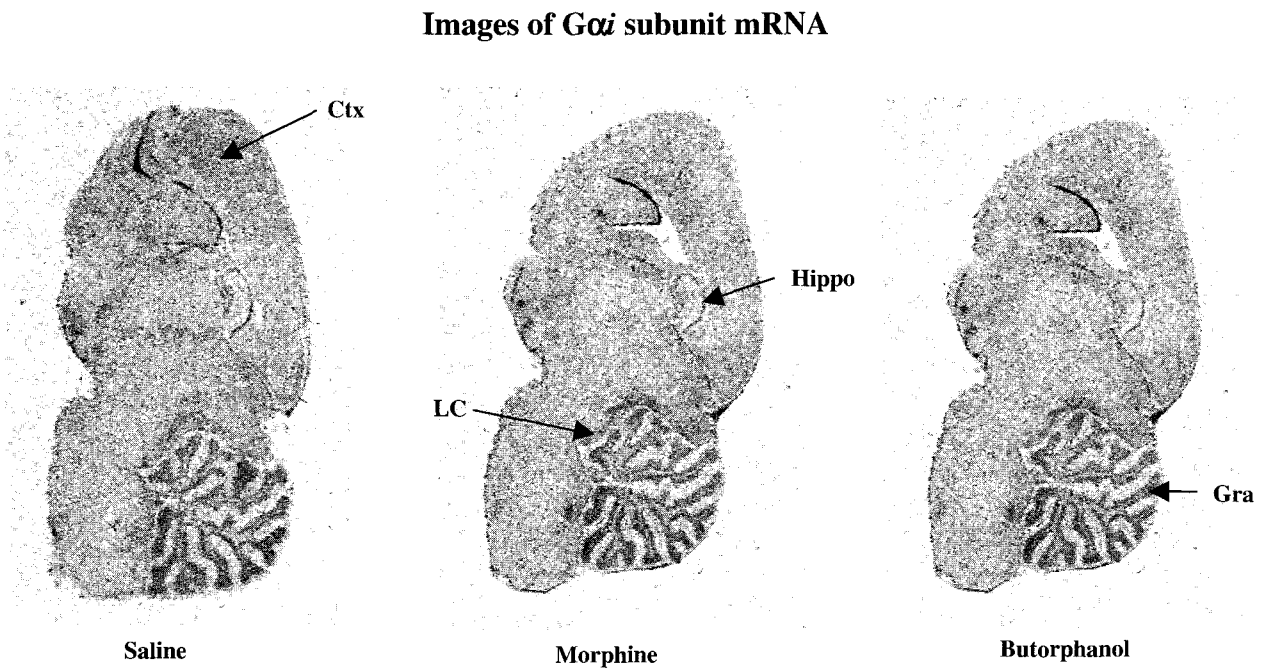

Fig. 2. Representative autoradiograms of G protein αi-subunit mRNA in sagittal rat brain sections. Exposure time was 4 weeks. Ctx, cortex; LC, locus coeruleus; Hippo, hippocampus; Gra, cerebellar granule layer.

Table 3. Changes of the level of G protein Gi subunit mRNA in rats withdrawal from morphine and butorphanol

Regions	mRNA levels (nCi/g tissue)		
	Saline	Morphine	Butorphanol
Cortex	23.30±1.32	19.59±1.34	25.30±1.55
Hippocampus			
CA1	35.18±2.37	27.87±2.06	38.94±2.17
CA3	30.45±2.19	27.96±2.31	35.01±1.99
Dentate gyrus	33.45±2.47	25.48±2.21*	35.11±1.83
Locus coeruleus	26.95±1.03	28.29±1.30	33.23±2.17*
Cerebellum			
Granule layer	38.76±1.78	32.75±2.13*	36.35±1.46

Rats were rendered dependent on morphine and butorphanol by continuous icv infusion (26 nmol/μl/hr) for 3 days. Values are expressed as mean±SE from 5~6 rats. *$p<0.05$ for difference from respective control groups.

drug infusion (Table 1). Without the administration of naloxone, no obvious withdrawal syndrome was observed in morphine- or butorphanol-dependent rats (data not shown).

Distribution of G αs, G αi, and G αo Subunit mRNAs

The G αs mRNA in the present study was localized widely throughout the rat brain with high levels in the cerebellar granule layer, hippocampus, locus coeruleus, and high in the cortex, thalamus, brain stem, and low in the caudate-putamen (Fig. 1, Table 2). The level of G αi mRNA was high in the cerebellar granule layer and moderate in cortex, hippocampus, and locus coeruleus whereas low in the caudate-putamen (Fig. 2, Table 3). The level of G αo mRNA

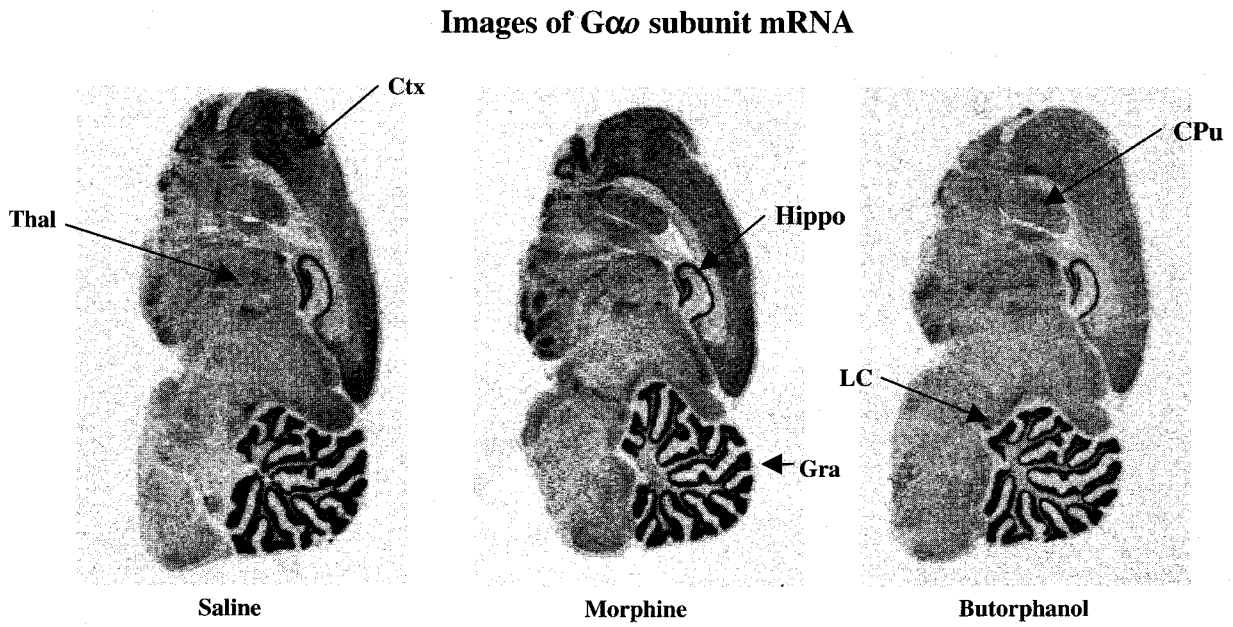

Fig. 3. Representative autoradiograms of G protein αo-subunit mRNA in sagittal rat brain sections. Exposure time was 2 weeks. Ctx, cortex; Thal, thalamus; Hippo, hippocampus; Gra, cerebellar granule layer; LC, locus coeruleus; Cpu, caudate putamen.

Table 4. Changes of the level of G protein Go subunit mRNA in rats withdrawal from morphine and butorphanol

Regions	mRNA levels (nCi/g tissue)		
	Saline	Morphine	Butorphanol
Cortex	110.84±5.31	109.22±7.41	94.93±5.47
Caudate-putamen	112.86±7.28	95.79±8.27	95.57±8.67
Thalamus	88.67±7.58	73.83±2.78	68.18±5.22*
Hippocampus			
CA1	189.95±15.37	171.06±9.55	166.35±9.30
CA3	215.40±19.28	200.98±14.42	184.94±13.58
Dentate gyrus	160.47±10.13	152.16±11.47	140.07±8.61
Brainstem			
Inferior colliculus	89.86±7.27	77.61±5.65	79.97±5.87
Locus coeruleus	68.80±5.62	67.88±4.46	65.23±4.44
Cerebellum			
Granule layer	229.93±10.73	213.74±12.93	225.03±7.08

Rats were rendered dependent on morphine and butorphanol by continuous icv infusion (26 nmol/μl/hr) for 3 days. Values are expressed as mean±SE from 5~6 rats. *$p<0.05$ for difference from respective control groups.

was high in the cerebellar granule layer and hippocampus, moderate in the cortex, caudate-putamen and, thalamus, brain stem and locus coeruleus (Fig. 3, Table 4).

Effects of morphine and butorphanol Withdrawal on G protein α-subunit mRNAs

Morphine or butorphanol was continuously infused

into intracerebroventricle with the rate of 26 nmol/ μl/ h for 3 days. Withdrawal was produced 7 hrs after stopping opioid infusion. The level of G-protein α-subunit mRNA was changed by opiate withdrawal in a region-specific manner. The level of Gs mRNA was significantly decreased in the cerebral cortex (29%) by morphine withdrawal and decreased in the cerebral cortex (18%) and cerebellar granule layer (23%) by butorphanol withdrawal rats compared to saline treated group (Table 2). In morphine withdrawal rats, the level of G αi mRNA was decreased in the dentate gyrus (24%) and cerebellar granule layer (15%). In butorphanol withdrawal rats, the level of G αi mRNA was elevated in the locus coeruleus (23%) (Table 3). The level of G αo mRNA was not changed in the morphine withdrawal, although, the level was significantly decreased in the thalamus (23%) in the butorphanol withdrawal rats (Table 4).

DISCUSSION

Morphine and butorphanol are equipotent in the induction of dependence, as demonstrated by the behavioral signs elicited by challenge with the non-selective opioid antagonist naloxone in this experiment. It should also be noted that the general pattern for the behavioral response to precipitated withdrawal of morphine and butorphanol is similar. Butorphanol is a mixed agonist/antagonist that acts on the μ-, δ- and κ-opioid receptors with an affinity ratio of 1 : 4 : 25 (Chang et al, 1983; Lahti et al, 1985). Butorphanol produces its physical dependence primarily through κ- and/or δ-opioid receptors because β-funaltrexamine (a μ-opioid receptor selective antagonist) failed to precipitate withdrawal in butorphanol dependent rats (Jaw et al, 1993a), while both naltrindole (a δ-opioid receptor selective antagonist) and nor-binaltorphimine (a κ-opioid receptor selective antagonist) have been shown to precipitate withdrawal signs similar to those precipitated by the non-selective opioid receptor antagonist, naloxone (Jaw et al, 1993b; Jaw et al, 1993c). In contrast to the μ-selective agonist, κ-opioid receptor agonist induces an increase of thymidine incorporation in brain cell aggregates. Also unlike the μ-selective agonist, the action of κ-opioid receptor agonist is sensitive to pertussis toxin (Barg et al, 1993). The κ-agonist U-50,488H produces a concentration dependent increase in the accumulation of phosphoinositide, whereas the μ-selective agonist and the δ-selective agonist are ineffective (Periyasamy & Hoss, 1990). These results suggest that the κ-opioid utilize a different mechanism of signal transduction than μ agonists.

Opioid withdrawal is characterized by neuronal hyperactivity that is particularly pronounced in the pontine locus ceruleus (Foote et al, 1983; Rasmussen & Aghajanian, 1989), and by behavioral excitation (Foote et al, 1983; Maldonaldo et al, 1992). The locus ceruleus neuronal hyperactivity associated with opioid withdrawal is accompanied by elevations in extracellular levels of the excitatory amino acids (Feng et al, 1995; Aghajanian et al, 1994; Zhang et al, 1994). Experimental evidence supports the hypothesis that opioids modulate glutamate release. Neurochemical evidence obtained from in vivo microdialysis studies has shown that an elevation in extracellular fluid levels of glutamate and aspartate within locus coeruleus during naloxone-precipitated withdrawal in both morphine and butorphanol dependent rats (Aghajanian et al, 1994; Zhang et al, 1994; Feng et al, 1995). Acutely, morphine, a μ-opioid receptor agonist, is shown to prevent the evoked release of endogenous glutamate in the sensorimotor cortex of conscious rats (Coutinho-Netto et al, 1980) and in both brain slices and synaptosomes from cerebral cortex (Crowder et al, 1986). Kappa-opioid receptor agonists have been reported to inhibit glutamate release from guinea pig hippocampal mossy fiber terminals (Gannon & Terrian, 1991) and from rat cerebrocortical slices (Nicol et al, 1996). These results suggest that the μ-opioid receptor agonist and κ-opioid receptor agonist have similar effect on the glutamatergic nerve system. Therefore, morphine and butorphanol show equipotent naloxone-induced withdrawal syndrome in this experiment although these opioids induce different expression of G protein α-mRNA.

There are several interesting reports suggesting that G protein α-subunit has a different role in opioid-induced analgesics and dependence. Morphine treatment alters the levels of G αs regulatory proteins in rat locus coeruleus (Nestler et al, 1989) and in the guinea-pig myenteric plexus (Lang & Schulz, 1989). After chronic exposure of cultures of striatal neurons to morphine, the level of G αs is increased (Van Vliet et al, 1993). Antibodies against α-subunit of G αi and G αo but not of G αs injected 24 hr before the naloxone challenge reduces the number of mice presenting the jumping behavior as well as the average

number of jumps (Sanchez-Blazquez & Garzon, 1994). However, the antibody blocking Gs subunit given before beginning the chronic morphine treatment, produces a significant reduction of the jumping behavior (Sanchez-Blazquez & Garzon, 1994). Therefore, it is highly conceivable that Gs-subunit participates in the cellular mechanisms opposing to the morphine effect and in a lesser extent in those mediating the expression of the withdrawal syndrome.

The in situ hybridization technique has enabled us to map the spatial expression of each α-subunit throughout the brain, providing a detailed description of G protein alteration during the withdrawal syndrome. The main finding of my study is that both morphine and butorphanol induce similar pattern of withdrawal syndrome but change the level of G αi mRNA in different manner. In the present experiments, both morphine and butorphanol decrease the level of G αs mRNA in the cortex and cerebellar granule layer and decrease the level of G αo mRNA in the thalamus. Morphine lowers the level of G αi mRNA in the dentate gyrus and cerebellar granule layer, while butorphanol elevates the level of G αi mRNA in the locus coeruleus. It has been reported that the chronic administration of morphine to rats increased the G protein in the locus coeruleus and decreased the G αi in the nucleus accumbens (Nestler et al, 1989; Terwilliger et al, 1991). However, the level of G αo mRNA or G αi mRNA is not decreased in locus coeruleus in morphine withdrawal rats but the level of G αi mRNA is elevated in butorphanol withdrawal rats in this experimental results. These observations suggest that the extremely complex homeostatic changes in the CNS that underlie the development of opioid tolerance and dependence. Although the reason for the discrepancy with regard to the brain regional distribution of α-subunit is not clear, the different dose and the route of administration of opioid might be responsible. Chronic activation of the opioid receptors by morphine or butorphanol may induce homeostatic changes that involve multiple signal transduction pathways.

In conclusion, present results demonstrate that the expression of G protein a subunit mRNAs in rat brain is significantly altered, with remarkable regional selectivity by chronic morphine and butorphanol withdrawal although these opioids were equipotent in the induction of physical dependence. These findings emphasize the need to map the precise intracellular pathways activated by chronic opioids during the development of dependence.

ACKNOWLEDGEMENTS

This study was supported by a research grant from Korea Food and Drug Administration (2000).

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