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Morphine-Induced Modulation of Calcitonin Gene-Related Peptide Levels

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WELCH, S. P., P. P. BASS, K. G. OLSON AND G. PUGH. *Morphine-induced modulation of calcitonin gene-related peptide levels.* PHARMACOL BIOCHEM BEHAV 43(4) 1107–1116, 1992.—Calcitonin gene-related peptide (CGRP) is a novel calcium-modulatory product of the gene that encodes for calcitonin. Acute administration of morphine decreases levels of CGRP in rat corpus striatum. Tolerance to morphine did not alter the levels of CGRP in any brain region or in the spinal cord of the rat. CGRP did not alter the tolerance to the antinociceptive effects of morphine. Chronic naltrexone increased the levels of CGRP in the hypothalamus. Concurrent chronic administration of naltrexone plus morphine raised the levels of CGRP in the medulla, midbrain, and spinal cord. CGRP enhances naloxone-precipitated withdrawal jumping in mice. In rats, during withdrawal the levels of CGRP were tripled in the corpus striatum and significantly reduced in the hippocampus and hypothalamus. In the corpus striatum, CGRP enhances forskolin-stimulated cyclic adenosine monophosphate (cAMP) accumulation when such accumulation is suppressed (as with the chronic opiate administration), but conversely depresses forskolin-stimulated cAMP accumulation under normal conditions (as with chronic vehicle administration). These data are consistent with the hypothesis that CGRP acts as a modulatory peptide in opiate-sensitive systems and tonic opioid control of CGRP levels exists in brain.

Calcitonin gene-related peptide cAMP Opiate tolerance Withdrawal jumping Radioimmunoassay

CALCITONIN (CT) gene-related peptide (CGRP) is a novel peptide product of the gene that encodes for CT (1). The binding of CGRP in the brain occurs in areas such as the brainstem and midbrain, which are important in pain perception and neuronal transmission. CGRP is one of the most abundant peptides in the spinal cord and is especially high in the dorsal horn of the spinal cord (19,21,45). CGRP produces several effects in the spinal cord that are the opposite of those produced by opiates. CGRP colocalizes and is coreleased with substance P, a major nociceptive transmitter in spinal afferents (18,23). CGRP also enhances substance P concentrations spinally, possibly by enhancing release (40) and decreasing degradation of substance P (28). Acutely administered opiates, on the other hand, decease the release of substance P (29). CGRP enhances the nociceptive effects of substance P (10). Opiates, on the other hand, decrease spinal nociception (56).

Our work has shown that both salmon calcitonin (sCT) (52) and CGRP produce biphasic modulation of calcium uptake in isolated synaptosomes from mouse brain. These biphasic effects of CGRP and sCT in vitro correlate temporally in vivo to initial antagonism and then enhancement of morphine-induced antinociception by ICV sCT and CGRP (53,54). These data, along with those of other investigators using dorsal root ganglia in culture (43), indicate that CGRP enhances calcium uptake or influx in brain and spinal afferent neurons. Work has shown that intrathecal administration of antibodies to CGRP produces antinociception in rats (24,27), while intrathecally administered CGRP itself facilitates nociception and produces slight hyperalgesic effects (10,16). These data substantiate the hypothesis that CGRP is a modulator of nociception at brain and spinal sites, possibly via modulation of calcium.

Due to the evidence for the interaction of CGRP and acutely administered opiates (3,4,53–55), as well as the evidence that CGRP acts as a neurmodulatory peptide, we hypothesized that the development of tolerance to morphine might alter the levels of CGRP in the spinal cord or brain and that withdrawal from morphine might also involve changes in levels of CGRP. Previous work has shown that the levels of CGRP are decreased by administration of μ and δ opiate receptor-selective peptides in vitro to rat dorsal spinal cord slices (41), are not altered during opiate tolerance development or withdrawal in the cat spinal cord (38), but appear to be altered in select brain regions from tolerant rats (48). Through our investigation, we wished to provide a comprehensive study of the changes in CGRP levels in brain regions and the spinal cord in the rat following acute and chronic exposure to mor-

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phine and following withdrawal from morphine. Because both morphine and CGRP have been shown to modulate the formation of cyclic adenosine monophosphate (cAMP) (17,25, 26,39,50), we examined the effects of CGRP on adenylyl cyclase activation following acute and chronic administration of opiates. We also evaluated the effects of chronic administration of CGRP on the development of tolerance to morphine and the withdrawal signs associated with naloxone-precipitated withdrawal from morphine.

METHOD

Measurement of CGRP Levels

Brain and spinal cord regions were dissected according to the method of Glowinski and Iverson (20) (brain) and Gibson et al. (19) (spinal cord). Tissue was processed using the method of Skofitsch and Jacobowitz (45). Tissue samples were added to 250 μl 0.1 N hydrochloric acid on ice and suspended in a boiling water bath for 10 min. A 20-μl aliquot was removed for protein determination by the Lowry et al. method (30). The remaining sample was centrifuged at 5,000 ×*g* for 30 min. A 200-μl aliquot of the resultant supernatant was frozen in a polystyrene tube at −70°C and dried in a vacuum centrifuge connected to a Virtis (The Virtis Company, Gardiner, NY) lyophilizer. The samples were stored at −70°C until assays were performed. The disequilibrium radioimmunoassay (RIA) was performed in accordance with the instructions included in the kit purchased from Peninsula, Inc. (Belmont, CA). The antisera to CGRP was rabbit antirat CGRP with a cross-reactivity of less than 0.02% with sCT and many other endogenous peptides (as measured by Peninsula). The samples were reconstituted in a 0.06 M phosphate buffer (200 μl), pH 7.4, containing 0.01 M EDTA and 3% bovine serum albumin. Standards containing rat CGRP were prepared in the RIA buffer (200 μl). All tubes were incubated for 48 h at 4°C with 200 μl CGRP antisera. Tubes containing buffer only (400 μl) and buffer (200 μl) plus antibody (200 μl) were used to determine background and maximal binding, respectively. After the 48 h incubation, 10,000 cpm/tube [^{125}I]Tyr rat CGRP was added in 200 μl buffer to each tube. The samples were incubated at 4°C for 1 additional day. Separation of bound from free CGRP was done by addition of 8 mg charcoal (Norit GSX, Peninsula, Belmont, CA) in 250 μl 0.06 M phosphate buffer containing 0.25% gelatin. The samples were centrifuged at 4°C for 30 min at 5,000 × *g*. The supernatant was removed immediately by vacuum aspiration and the pellet counted in a Beckman gamma counter (Beckman Instruments, Fullerton, CA). The standard curve was plotted using a linear regression program. If the correlation coefficient of the standard curve was less than 0.95, the assay was repeated. The concentration of CGRP in each tube was calculated from the standard curve. All samples were run in triplicate. Each experiment was repeated at least three times. The average concentration of CGRP for each treatment condition and brain region was determined (± SEM). The concentrations of CGRP in brain and spinal cord regions of tolerant rats or acutely morphine-treated rats were statistically compared to the corresponding concentrations in nontolerant rats or acutely vehicle-treated rats using Dunnett's *t*-test (13). In the acute studies, morphine was injected 10 mg/kg SC 20 min prior to sacrifice of rats. In the chronic studies, rats were rendered tolerant as described below. However, in one study separate groups of rats with morphine or placebo minipumps were injected every 12 h with 2 mg/kg naltrexone SC to assess for the blockade of tolerance development and subsequent alteration of CGRP levels. In withdrawal studies, rats were implanted with ALZET minipumps (Alza Corp., Palo Alto, CA) containing morphine. Rats were divided into two groups of six rats each and injected with naloxone (! mg/kg, SC) or distilled water vehicle SC. Rats were sacrificed at 10 min after naloxone injection and brain regions and spinal cord removed and CGRP levels measured. Rats were observed during the 10 min following injection of naloxone or vehicle for withdrawal signs such as wet-dog shakes, scratching, rearing, and aggression. These signs occurred in rats receiving naloxone but not in those receiving vehicle.

Preparation of Synaptosomes

Synaptosomes were prepared from rat brain and spinal cord using subcellular fractionation techniques described by McGovern et al. (34). Brain regions were homogenized in 0.32 M sucrose in Kreb's buffer (pH 7.4–7.5) on ice (1 g tissue/20 ml sucrose). The Kreb's buffer was composed of (mM) NaCl 120; KCl 5; NaH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 1; glucose 10; and $NaHCO_3$ 20 and were brought to pH 7.4 with 1 N HCl. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was removed and centrifuged at 18,000 × *g* for 20 min. For those experiments using the spinal cord, the P_2 pellet, which contains crude synaptosomes, was used for the assay of cAMP to obtain enough tissue. The yield of pure spinal synaptosomes following ficoll gradient separation was too low to be useful unless large numbers of rats were pooled. For those experiments involving the use of brain region tissue, the P_2 pellet was resuspended in 16% ficoll (16% wt/vol Sigma Type 400 dialyzed ficoll, Sigma Chemical Co., St. Louis, MO) that was prepared in 0.32 M sucrose. A 7.5% ficoll suspension was layered by hand over the 16% ficoll/pellet suspension to form a discontinuous gradient. The discontinuous gradient formed was centrifuged for 1 h at 40,000 × *g* using a Sorvall (Dupont, Wilmington, DE) RC2-B refrigerated ultracentrifuge with a rate zonal controller and a Sorvall SS-90 (3998383) vertical rotor head. The synaptosomes banded at the 16/7.5% ficoll interface were removed by aspiration and washed with a 1 : 1 sucrose (0.32 M)/Kreb's buffer solution. The synaptosomes were centrifuged for 5 min at 18,000 × *g* and washed again in 1 : 2 sucrose/Kreb's buffer solution and again centrifuged for 5 min at 18,000 × *g*. The synaptosomes (pellet) were resuspended in Kreb's buffer. The protein concentration was determined as described above.

Measurement of Adenylyl Cyclase Activity

Brain regions and spinal cord were removed from three rats as described above and similar regions were pooled for assay of cAMP. Binding of CGRP has been shown to be highest in the hypothalamic-pituitary area of the brian, as well as the midbrain, cerebellum, and throughout the spinal cord (21,45). The areas where this peptide have been shown to have the highest specific binding were examined in this study. The tissues were processed according to the method of Duman et al. (12) as modified for the adenylyl cyclase kit available from Amersham, Corp. (Arlington Heights, IL). The resultant synaptosomal pellet was resuspended in the buffer provided for use in the assay procedure described in the Amersham kit. Fifty micrograms of tissue were used in each tube. The formation of cAMP was initiated by addition of the tissue to buffer/regenerating system-containing tubes in at 30°C. The assay was terminated 5 min later by immersing the tubes in boiling water for 3 min.

The assay is a competitive binding assay in which [^{3}H]cAMP competes with endogenous cAMP for binding to a specific cAMP binding protein (cAMP-dependent protein kinase) provided in the kit. The more endogenous cAMP present in the sample, the less [^{3}H]cAMP that binds. Separation of the bound and free [^{3}H]cAMP was done by charcoal precipitation. Standard solutions of cAMP were prepared and a standard curve was run with each assay. All samples and the standard curve were run in triplicate. An aliquot of the supernatant was removed and placed in scintillation fluid (10 ml) for liquid scintillation counting. Forskolin (0.3-2 μM) and guanosine triphosphate (GTP) (125 μM) were added to the incubation mixture to enhance cAMP accumulation. In addition, theophylline (12.5 mM) was added to prevent the breakdown of the cAMP. A cAMP regenerating system was added to the buffer. This was composed of: adenosine triphosphate (ATP) 0.625 mM; GTP 125 μM; creatine phosphate 12.5 mM; creatine phosphokinase 12.5 IU. Basal levels of cAMP were assessed in the presence of the ATP regenerating system and theophylline but in the absence of added GTP. The total accumulated cAMP over a 5-min incubation period in the presence of drug or vehicle was quantified (pmol/mg protein/min). The drugs (CGRP or forskolin) or the respective vehicles (distilled water and 1% DMSO) were added to the incubation mixture 2 min prior to addition of the tissue to allow for temperature equilibration. The average amount formed under each experimental condition was calculated and compared to the appropriate control by use of Dunnett's t-test (13).

Morphine-Tolerant Rats and Mice

Mice were implanted with either a 75-mg morphine pellet or placebo pellet according to the method of Way et al. (51). Mice have been shown to be rendered tolerant to morphine in 72 h using this method (51). Rats were made tolerant by the chronic infusion of 60 mg/kg/day morphine for 7 days via a subcutaneously implanted ALZET osmotic minipump (ALZET Model 2ML1, Alza). This procedure has been shown to produce tolerance in 7 days (35). Alternatively, placebo control rats were implanted with minipumps containing distilled water vehicle.

Naloxone-Precipitated Jumping in Mice

Mice were implanted with either morphine or placebo pellets and injected every 12 h for 3 days with 2 μg/mouse rat CGRP (Bachem, Inc., Torrance, CA) or distilled water vehicle. Intrathecal injection of the CGRP and vehicle was performed via the method of Hylden and Wilcox (22). On day 4 (12 h after the last injection of CGRP, a time point when CGRP does not acutely alter morphine antinociception), mice were injected with various doses of naloxone subcutaneously. Six mice were placed on individual 24-in. tall platforms. The number of mice per group of six mice that jumped from the platforms in a 10-min period, as well as the latency to jump for each mouse (in minutes), was recorded. Although it would have been optimal to perform such experiments in the rat because the RIA for CGRP levels was performed in the rat, chronic administration of CGRP to the rat would have required large quantities of the peptide, which was cost prohibitive, and the stability of the peptide over long infusions could not be guaranteed. Conversely, it would have been optimal to run the RIA for CGRP in mice. However, no mouse CGRP or antisera is commercially available. We are able to detect mouse CGRP using rat antisera and rat CGRP for the standard curve, but absolute levels of CGRP in the mouse cannot be quantified due to the lack of appropriate mouse CGRP standards.

RESULTS

Acute administration of morphine (10 mg/kg) to the rat resulted in a decrease in the levels of CGRP in the corpus striatum when rats were sacrificed 20 min after administration of the morphine (Fig. 1A). Levels of CGRP in this region were reduced from nearly 300 fmol/mg protein to less than 100 fmol/mg protein. No other significant effects of administration of morphine acutely (Figs. 1A and B) were observed although the level of CGRP in the cortex was higher and approached a significant difference following morphine administration. The concentrations of CGRP in vehicle (distilled water)-pretreated animals in this experiment are similar to those reported by other investigators (19,33).

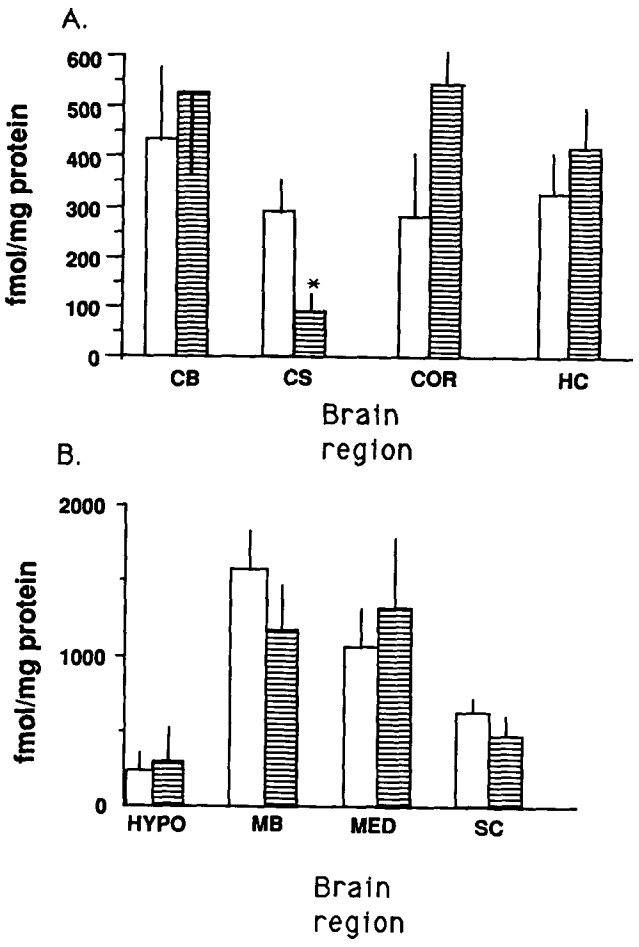

FIG. 1. Effect of acute administration of morphine on calcitonin gene-related peptide (CGRP) levels in brain regions and the spinal cord. Rats were injected SC with morphine (10 mg/kg) or distilled water vehicle. Twenty minutes later, rats were sacrificed and brain regions and spinal cord were removed and analyzed for CGRP levels as described in the Methods section. Each group represents the mean ± SEM of the levels in six separate rats. (□), vehicle (distilled water)-pretreated rats; ⊟, morphine-pretreated rats. CB, cerebellum; CS, corpus striatum; COR, cortex; HC, hippocampus; HYPO, hypothalamus; MB, midbrain; MED, medulla; SC, spinal cord. *$p < 0.05$ from vehicle-pretreted rats.

Chronic administration of morphine via ALZET osmotic minipumps to rats for 7 days induced tolerance to a challenge dose of 15 mg/kg morphine SC as tested in the tail-flick test in the rat (35). Administration of naltrexone 2 mg/kg every 12 h to rats for 7 days concurrently with the minipump totally blocked the development of tolerance to a 15-mg/kg dose of morphine as tested in the tail-flick test. The half-life of naltrexone in the rat is 11.4 h in plasma and 8 h in the brain (36). Using 2 mg/kg every 12 h, the level of naltrexone will not decrease lower than 1 mg/kg in 12 h in brain. Chronic administration of naltrexone has been shown to not alter the antagonistic effects of the drug vs. morphine in antinociceptive tests in man (32) or in the monkey (44). Chronic administration of naltrexone to those rats receiving vehicle ALZET pumps did not alter the baseline tail-flick latencies of rats. However, comparison of the levels of CGRP in naive rats treated with vehicle SC at 20 min prior to sacrifice (Fig. 1) and in rats receiving vehicle ALZET pumps for 7 days along with twice-daily injection of distilled water SC (Fig. 2) shows some differences. Levels of CGRP in the cerebellum, cortex, hippocampus, hypothalamus, medulla, and spinal cord do not differ significantly between these two groups of rats. However, in the corpus striatum and midbrain the levels of CGRP are higher in rats treated with vehicle SC (Fig. 1) than those treated chronically with vehicle via the minipump and injected SC with vehicle twice per day (Fig. 2). Thus, these two regions appear to be sensitive to either injection of the vehicle SC or chronic implantation of the minipump in the response to CGRP.

Chronic administration of morphine via minipump with injection of distilled water every 12 h did not alter the levels of CGRP in any brain region or in the spinal cord of rats from those levels observed in rats receiving vehicle ALZET minipumps. Thus, tolerance to morphine did not alter CGRP levels (Fig. 2). In addition, administration of naltrexone (2 mg/kg, SC every 12 h) to rats receiving vehicle minipumps did not significantly change CGRP levels compared to the vehicle minipump/vehicle SC group of rats except in the hypothalamus (Fig. 2A). In that region, the levels of CGRP were raised from 472 fmol/mg/protein to 1,047 fmol/mg protein by administration of the naltrexone chronically. Administration of naltrexone chronically to rats receiving morphine minipumps raised the levels of CGRP in the medulla, midbrain, and spinal cord (Fig. 2B). The levels of CGRP in the hypothalamus did not change from the elevation produced by chronic naltrexone alone (Fig. 2A). Thus, in these three regions the blockade of tolerance development by chronic naltrexone administration results in changes in CGRP levels not observed in animals receiving vehicle minipumps in conjunction with chronic naltrexone even through both treatment groups are not tolerant to morphine.

In a separate study, rats were implanted with morphine-containing ALZET pumps for 7 days. Rats were then divided into groups of six rats each and injected SC with naloxone (1 mg/kg, SC) or distilled water vehicle. Rats were sacrificed 10 min later. The levels of CGRP were raised from 400 fmol/mg protein to nearly 1,200 fmol/mg protein in the corpus striatum (Fig. 3). Levels of CGRP were significantly reduced in the hippocampus (497 vs. 98 fmol/mg protein) and hypothalamus (1,100 vs. 268 fmol/mg protein). In this study, the levels of CGRP in rats receiving morphine-containing ALZET pumps and injected with vehicle SC (Fig. 3) are not significantly different from the levels of CGRP in rats receiving morphine-containing ALZET pumps and receiving vehicle SC every 12 h (Fig. 2) with the exception of the corpus striatum, which had higher levels of CGRP in the former treatment paradigm (Fig. 3).

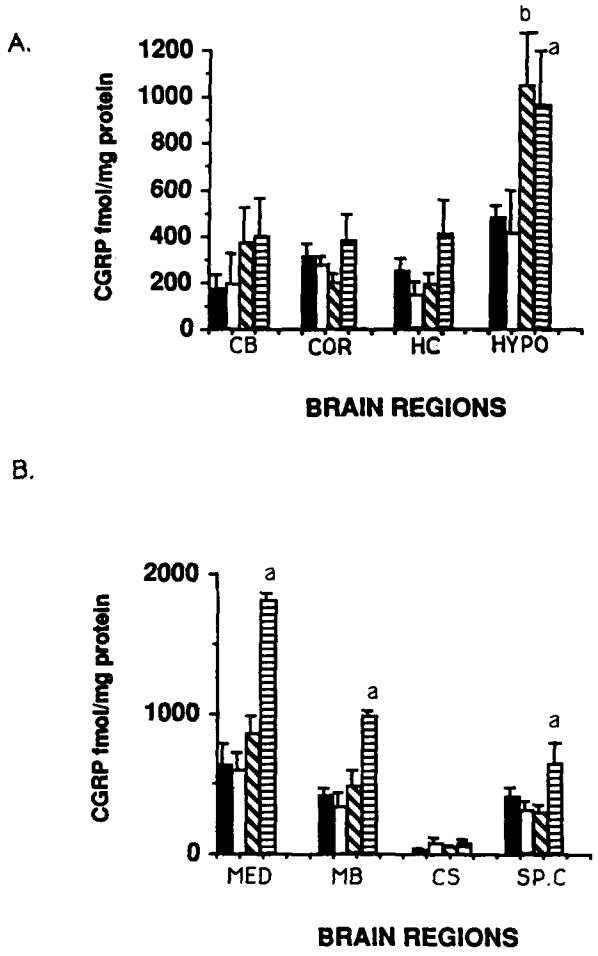

FIG. 2. Effect of chronic administration of morphine in combination with vehicle or naltrexone SC on calcitonin gene-related peptide (CGRP) levels in brain regions and the spinal cord of the rat. Rats were implanted with ALZET osmotic minipumps containing distilled water or morphine (60 mg/kg/day) for 7 days as detailed in the Methods section. Concurrently, rats were injected SC with either naltrexone (2 mg/kg) or distilled water every 12 h for the 7-day duration of the minipump. The minipump was not removed. On day 8, rats were sacrificed and brain regions and spinal cord were analyzed for CGRP levels as described in the Methods section. Each bar represents the mean (±SEM) for six separate rats. (■), vehicle minipump injected with distilled water SC, every 12 h for 7 days; (□), morphine minipump injected with distilled water SC, every 12 h for 7 days; (▨), vehicle minipump injected with naltrexone SC, every 12 h for 7 days; (▤), morphine minipump injected with naltrexone SC, every 12 h for 7 days. CB, cerebellum; CS, corpus striatum; COR, cortex; HC, hippocampus; HYPO, hypothalamus; MB, midbrain; MED, medulla; SP.C, spinal cord. [a]$p < 0.05$ from morphine minipump injected with vehicle SC for 7 days; [b]$p < 0.05$ from vehicle minipump injected with vehicle SC for 7 days.

Rats that were either tolerant to morphine or not tolerant (placebo) were evaluated for changes in CGRP alteration of adenylyl cyclase activation in the synaptosomes from the midbrain, cerebellum, corpus striatum, and spinal cord. All these areas have been shown to have high binding of CGRP (21,45). In the midbrain (Fig. 4A) and cerebellum (Fig. 4B), forskolin

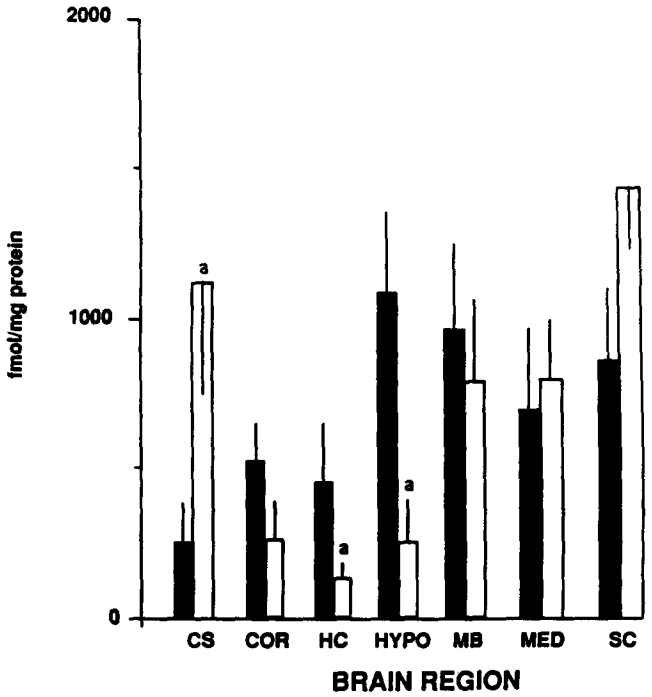

FIG. 3. Effect of naloxone-precipitated withdrawal from morphine on the levels of calcitonin gene-related peptide (CGRP) in brain regions and the spinal cord of the rat. Rats were implanted with morphine minipumps as described in the Methods section. On day 8, rats were injected with either distilled water SC or naloxone (1 mg/kg, SC). Rats were sacrificed 10 min later and brain regions and the spinal cord were analyzed for CGRP levels. Each bar represents the mean (± SEM) CGRP level present in six separate rats. (■), morphine minipump with vehicle SC at 10 min prior to sacrifice; (□), morphine minipump with naloxone SC at 10 min prior to sacrifice. CB, cerebellum; CS, corpus striatum; COR, cortex; HC, hippocampus; HYPO, hypothalamus; MB, midbrain; MED, medulla; SC, spinal cord. $^{a}p < 0.01$ from morphine minipump with vehicle SC.

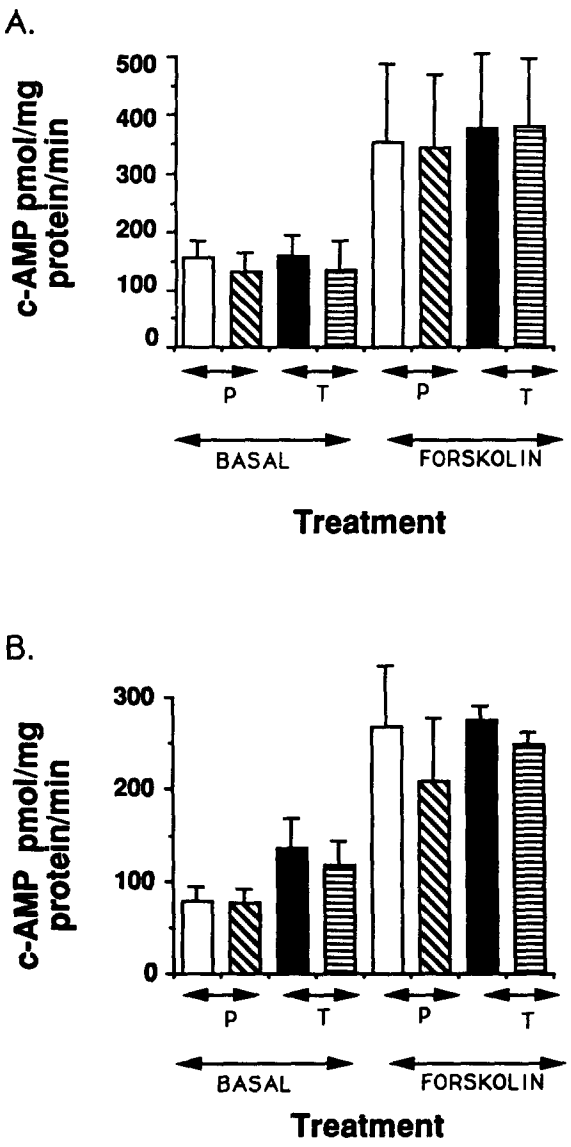

FIG. 4. Effect of calcitonin gene-related peptide (CGRP) on the accumulation of cyclic adenosine monophosphate (cAMP) in synaptosomes from the midbrain (A) and cerebellum (B) of tolerant and nontolerant rats. Rats were implanted with either morphine-containing ALZET minipumps or distilled water-containing pumps as detailed in the Methods section. On day 8 with pumps intact, rats were sacrificed and brain regions and the spinal cord were removed for the measurement of cAMP accumulation in synaptosomes (for the brain regions) or the P_2 (crude synaptosomal) pellet (spinal cord). cAMP accumulation was measured as detailed in the Methods section. Basal accumulation of cAMP was determined in the absence of added guanosine triphosphate (GTP). Forskolin stimulation of cAMP accumulation was determined in the presence of 125 μM GTP plus 1 μM forskolin. CGRP (1 μM) or distilled water vehicle was added to the sample incubation mixture at 2 min prior to addition of the tissue. After addition of the tissue, the samples were allowed to incubate for 5 min prior to termination of the assay. Each column represents the mean ± SEM of four separate preparations of synaptosomes. In each synaptosomal preparation, three rats were pooled to yield adequate tissue. (□), placebo, distilled water in vitro; (▨), placebo, CGRP in vitro; (■), tolerant, distilled water in vitro; (▤), tolerant, CGRP in vitro. P, placebo (vehicle) minipump; T, morphine minipump (rats were tolerant to morphine).

significantly enhanced the accumulation of cAMP. However, no significant differences between placebo and tolerant rats was observed in the stimulation of cAMP accumulation by forskolin. In addition, no significant differences in the accumulation of cAMP were observed in the placebo vs. the tolerant preparation upon addition of CGRP in vitro (Figs. 4A and B). A limited number of studies were performed using the medulla, hippocampus, and spinal cord, with similar results to those shown in Fig. 4.

However, in the corpus striatum (Fig. 5) significant differences in the treatment groups were observed. Basal levels of cAMP accumulation were significantly less in the synaptosomes from tolerant rats and were raised significantly by 1 μM CGRP. Forskolin (0.3 and 1 μM) produced a dose-related increase in cAMP accumulation in the placebo preparation. CGRP blocked forskolin-stimulated increases in cAMP accumulation in the placebo preparation when 0.3 μM forskolin was used, but did not block the stimulation produced by the 1-μM concentration of forskolin. Conversely, in the tolerant preparation forskolin (1 μM) produced little stimulation of cAMP accumulation. However, CGRP significantly enhanced the effects of both concentrations of forskolin in the tolerant preparation. Because tolerance to morphine altered the sensi-

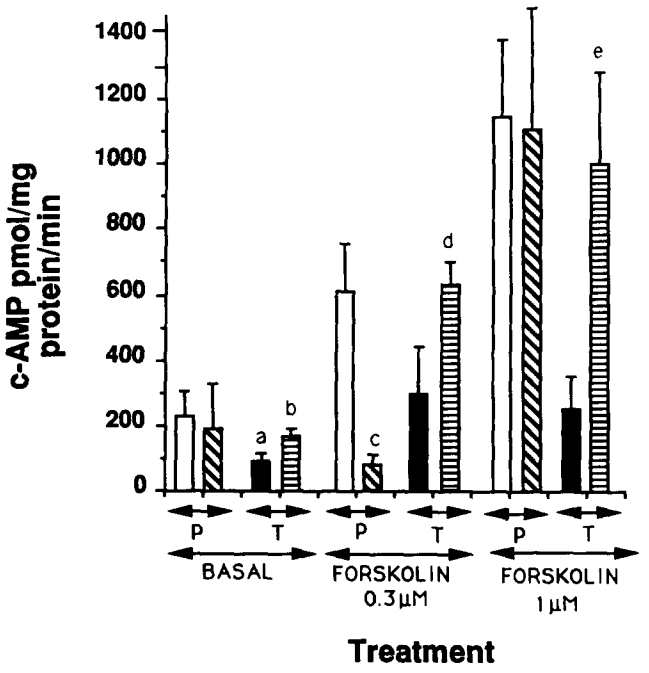

FIG. 5. Effect of calcitonin gene-related peptide (CGRP) on the accumulation of cyclic adenosine monophosphate (cAMP) in synaptosomes from tolerant and nontolerant rats. Rats were implanted with either morphine-containing ALZET minipumps or distilled water-containing pumps as detailed in the Methods section. On day 8 with pumps intact, rats were sacrificed and the corpus striatum was removed for measurement of cAMP accumulation in synaptosomes. cAMP accumulation was measured as detailed in the Methods section. Basal accumulation of cAMP was determined in the absence of added guanosine triphosphate (GTP). Forskolin stimulation of cAMP accumulation was determined in the presence of 125 μM GTP plus 0.3 or 1 μM forskolin. CGRP (1 μM) or distilled water vehicle was added to the sample incubation mixture 2 min prior to addition of the tissue. After addition of the tissue, the samples were allowed to incubate for 5 min prior to termination of the assay. Each column represents the mean ± SEM of four separate preparations of synaptosomes. In each synaptosomal preparation, three rats were pooled to yield adequate tissue. (□), placebo, distilled water in vitro; (▨), placebo, CGRP in vitro; (■), tolerant, distilled water in vitro; (▤), tolerant, CGRP in vitro. P, placebo (vehicle) minipump; T, morphine minipump (rats were tolerant to morphine). [a] $p < 0.05$ from placebo, vehicle; [b] $p < 0.05$ from tolerant, vehicle; [c] $p < 0.01$ from placebo, forskolin 0.3 μM, vehicle; [d] $p < 0.05$ from tolerant, forskolin 0.3 μM, vehicle; [e] $p < 0.01$ from tolerant, forskolin 1 μM, vehicle.

tivity to CGRP and forskolin in the corpus striatum, the effects of acute morphine on CGRP and forskolin stimulation of cAMP accumulation were evaluated (Fig. 6). Although significant differences were observed between vehicle- and morphine-pretreated rats in basal accumulation of cAMP in the presence of CGRP or accumulation of cAMP in the presence of forskolin + CGRP, these changes were not significantly different from rats receiving distilled water vehicle. Thus, administration of morphine acutely does not enhance stimulation of cAMP accumulation in this region.

Behavioral studies were performed to evaluate the effects of chronic administration of CGRP (2 μg, every 12 h, IT) on the tolerance to a challenge dose of morphine and withdrawal from morphine. Chronic administration of CGRP did not alter the response to a challenge dose of morphine. In nontolerant mice receiving distilled water IT or CGRP IT for 3 days, administration of morphine (15 mg/kg, SC) produced an antinociceptive effect [93 and 100% maximum possible effect (MPE, respectively)]. In tolerant mice, the antinociception following chronic administration of distilled water or CGRP IT did not differ significantly (15 and 20% MPE, respectively). Thus, CGRP did not alter the response to morphine in either group of mice. However, when tolerant mice were injected with naloxone SC, tolerant mice receiving chronic CGRP were much more sensitive to the effects of naloxone as measured in the withdrawal jumping test vs. morphine tolerant mice injected chronically with distilled water (Fig. 7). Mice injected with distilled water IT chronically showed a dose-related increase in the number of mice jumping as the dose of naloxone was increased. Using 0.03 mg/kg naloxone, only one of six mice jumped. However, in the group receiving chronic CGRP naloxone (0.03 mg/kg) produced jumping in five of six mice. In addition, mice that were injected with CGRP in combination with morphine had higher numbers of lethalities. Upon examination, these mice had abdominal swelling due to urine retention in the bladder. It is likely that death was due to rupture of the bladder upon injection of the drug.

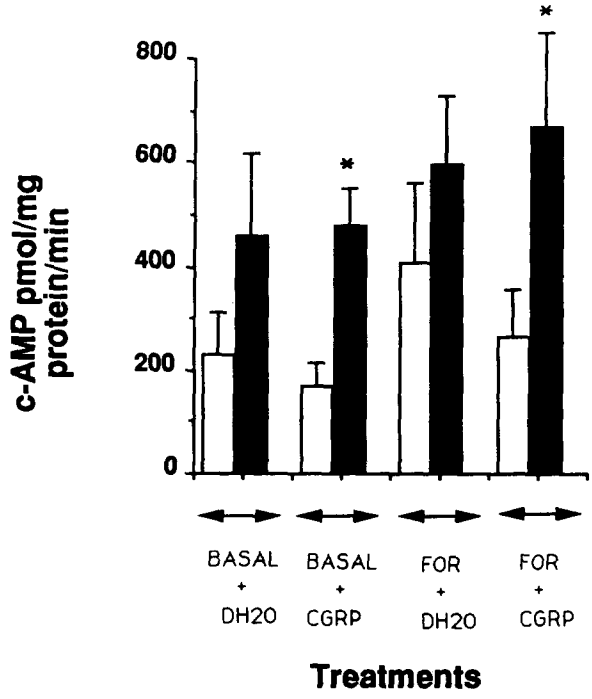

FIG. 6. Effect of acute administration of morphine or vehicle SC on the accumulation of cyclic adenosine monophosphate (cAMP) in synaptosomes from the corpus striatum of the rat. Naive rats were injected with morphine (10 mg/kg, SC) or vehicle (distilled water, SC) and sacrificed 20 min later. The corpus striatum was removed from three rats and was pooled for the measurement of cAMP accumulation as detailed in the Methods section. Basal cAMP accumulation was determined in the absence of guanosine triphosphate (GTP). Forskolin was added at a concentration of 2 μM to stimulate cAMP accumulation in the presence of 125 μM GTP. Each bar represents the mean ± SEM for the determination on three separate synaptosomal preparations. (□), rats injected with distilled water SC; (■), rats injected with morphine SC, $*p < 0.05$ from corresponding vehicle SC group.

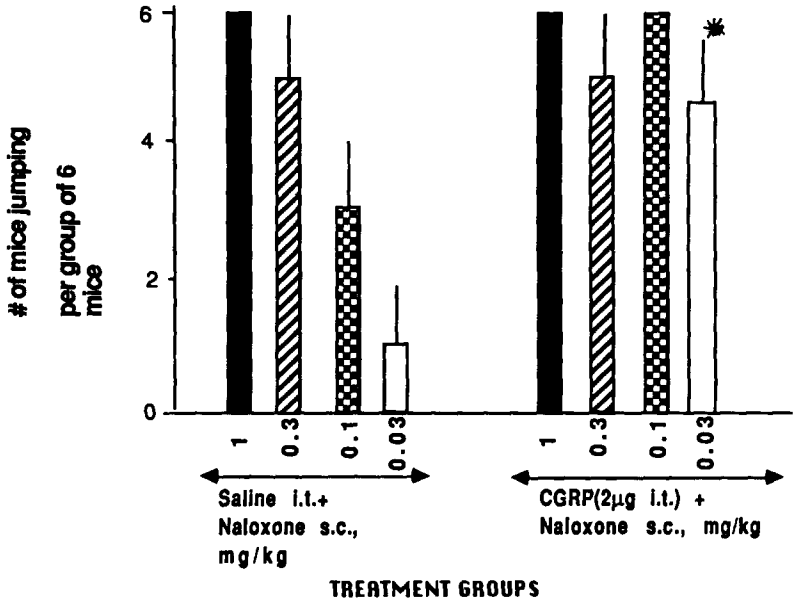

FIG. 7. Effect of chronic administration of calcitonin gene-related peptide (CGRP) or saline IT on the naloxone-precipitated withdrawal jumping response in mice. Mice were implanted with either a morphine or placebo pellet for 3 days. During those 3 days, mice were injected every 12 h with either saline (5 μl, IT) or CGRP (2 μg/mouse, IT). On day 4, 12 h after the last IT injection of either vehicle or CGRP mice were injected with naloxone (0.03–1 mg/kg, SC) and the number of mice per group of six to jump from 24-in. platforms was quantitated as a measure of naloxone-precipitated withdrawal from morphine. The average number of mice that jumped per group of six (±SEM) was determined for each dose of naloxone tested using at least three groups of six mice each. The vehicle for the naloxone, distilled water SC, was also tested in separate groups of six mice (not shown) and was devoid of activity in that it did not precipitate withdrawal jumping in any group of mice. *$p < 0.05$ from saline IT, naloxone 0.03 mg/kg SC.

DISCUSSION

Several lines of evidence have led us to hypothesize that CGRP may be involved in the production of tolerance to opiates or withdrawal signs in opiate-dependent animals. CGRP modulates the acute antinociceptive effects of morphine (3,4,53–55) and morphine-induced calcium uptake by brain synaptosomes (53). The highest binding of CGRP in the dorsal horn of the spinal cord (45) coincides with a major site of opiate binding and activity. The highest quantities of CGRP in the human brain are found in the locus coeruleus (47). Interestingly, it has been reported that the locus coeruleus is the site of upregulation of adenylyl cyclase activity upon chronic morphine administration. This effect of chronic morphine on adenylyl cyclase has been proposed to be a mechanism of tolerance production (7,9,12). Both CGRP and sCT have been shown to modulate cAMP levels (25,26). Because opiates and CGRP modulate adenylyl cyclase, CGRP could alter opiate tolerance by altering cAMP production in neurons. CGRP both enhances sympathetic outflow from the brain (15) and enhances the release of substance P spinally (40), effects that have been shown to be related to enhanced influx of calcium by CGRP. Both enhanced sympathetic tone (42) and enhanced release of substance P (2) occur during withdrawal from chronic opiates. Thus, chronic opiate-induced changes in CGRP levels in the brain could modulate opiate withdrawal or, conversely, changes in CGRP upon precipitated withdrawal from opiates could alter the withdrawal signs observed.

Our data indicate that the interaction of opiates with CGRP during the development of tolerance to and withdrawal from morphine are complex. Acute administration of morphine resulted in a decrease in the levels of CGRP in the corpus striatum. However, upon chronic administration of morphine decreased levels of CGRP are not observed in morphine-tolerant rats. This area of the brain is most associated with motor control. Because administration of morphine to rats acutely led to general hypoactivity that was not observed in opiate-tolerant rats, it is possible that the CGRP is involved as a modulator of motor responses in rats. No other evidence for such a modulatory role for CGRP exists. These data support the idea that the acute antinociceptive effects of opiates are not mediated by decreases in CGRP levels in those areas of the brain most involved in nociceptive processing. Our work differs from that of Pohl et al. (41), who show deceased release of CGRP in the dorsal spinal cord in response to μ- and δ-opioids. They suggest that the antinociceptive effects of morphine are due to presynaptic inhibition in the release of CGRP in spinal afferent fibers. The differences between our work and that of Pohl et al. (41) may be due to our use of the intact spinal cord rather than the dorsal spinal cord or the differences between administration of morphine in vivo in our system rather than in vitro. In the intact rat, the potential for

the influences of descending neuronal pathways on the release of CGRP following morphine administration might be observed. We are measuring levels, not release, of CGRP. It is possible that factors such as synthesis and degradation of the peptide altered our ability to measure changes in peptide content in the spinal cord.

Although chronic administration of morphine did not significantly alter the levels of CGRP in the brain or spinal cord (Fig. 2), we did observe a large increase in CGRP levels in rats receiving morphine via ALZET pumps along with naltrexone SC. Even though these rats were not tolerant to morphine, the levels of CGRP were significantly different in several brain regions (hypothalamus, medulla, and midbrain) and the spinal cord from those observed in vehicle-treated rats that are also not tolerant to morphine. These data indicate that CGRP may not play a major role in tolerance development. Additional evidence for the lack of CGRP involvement in tolerance development comes from our studies in mice in which chronic administration of CGRP (2 μg, IT, every 12 h for 3 days) failed to alter the antinociceptive effects of a challenge dose of morphine in either morphine-tolerant or nontolerant mice (data described in the Results section). It is possible that in these studies had we been able to administer the CGRP for a longer period of time or at a higher dose we would have observed an effect. This was impossible because of the retention of urine that occurred after a 3-day administration of CGRP IT. It has been shown that CGRP produces contraction of the urinary bladder in vitro (31). Perhaps in our system the CGRP produced constriction of the urinary bladder sphincter muscle and reduced urination. In addition, the spinal site of our injections IT has been shown to be the site for the neurons innervating the bladder (11). It is possible that chronic administration into this spinal cord region causes some destruction of the neurons in the area, resulting in urine retention. Even though chronic administration of CGRP did not alter tolerance to morphine as measured in the tail-flick test, chronic administration of the CGRP to mice resulted in an enhanced sensitivity of mice to naloxone-precipitated withdrawal jumping.

With the exception of the hypothalamus, the changes in CGRP levels in morphine-tolerant mice (Fig. 3) are not attributable alone to naltrexone but are observed when morphine is administered in combination with naltrexone. In the hypothalamus, these data indicate that the level of CGRP may be under tonic inhibitory opioid control because naltrexone administration results in increases in CGRP. Alternatively, if CGRP release were under excitatory opioid control the chronic presence of the opiate may have resulted in decreased CGRP release, which was reversed by naltrexone. However, in other studies we find no effect of acute naltrexone (1 mg/kg, SC) or naltrexone (2 mg/kg, SC) on CGRP levels in the hypothalamus (data not shown). Thus, the rise in CGRP levels in the presence of chronic naltrexone most likely represents a homeostatic compensatory response to the chronic presence of the drug, possibly via an upregulation of the opiate receptor in the presence of the chronic antagonist. Such receptor upregulation has been shown following chronic naltrexone administration (57). These data indicate that opioid-containing neurons may synapse presynaptically on CGRP-containing neurons in the hypothalamus and thus impact on CGRP release. Both morphine and CGRP have binding sites on the hypothalamus and have been shown to alter the release of hypothalamic and pituitary peptides (14,37,46). However, no specific studies have addressed possible interaction of the opiates and CGRP on hypothalamic or pituitary function.

The mechanism by which the combination of naltrexone and morphine results in a rise in CGRP levels in the medulla, midbrain, and spinal cord (Fig. 3) not due to the effects of naltrexone alone is difficult to explain. One possible explanation is that the CGRP levels in these regions are under tonic opioid control either directly or via the release of other neuromodulators. Because naltrexone was not infused, the levels of naltrexone would not remain constant. Although the levels of naltrexone are great enough to block the development of tolerance, they may not remain high enough to continuously block the tonic opioid control of the system. These effects may reflect compensation of the system for the variable naltrexone levels. It is unlikely, however, that rats are undergoing withdrawal due to decreases in naltrexone levels because a different profile of the effects of withdrawal on CGRP levels was observed (Fig. 4).

During withdrawal precipitated rapidly by naltrexone, the levels of CGRP nearly triple in the corpus striatum. This rise in CGRP levels could indicate an increase in synthesis, release, or storage or a decrease in degradation. The exact nature of the rise in CGRP cannot be determined from these studies. However, because the corpus striatum is involved in motor control and rats exhibited classic withdrawal signs such as excessive shaking, tremors, and hyperactivity upon injection of the naloxone it is possible that the rise in CGRP levels contributes to these withdrawal signs. Additional evidence for this hypothesis is the increased sensitivity of morphine-tolerant mice to naloxone-precipitated jumping following chronic administration of CGRP (Fig. 7) and studies that indicate that CGRP enhances sympathetic discharge from the brain (15). Opiate withdrawal is accompanied by increased sympathetic outflow (42). It is also possible that the increased CGRP contributes to the rise in substance P release observed during withdrawal from morphine. It has been shown that the levels of substance P rise significantly and exclusively in the corpus striatum during opiate withdrawal (2). Our data indicate that the levels of CGRP fall significantly in the hypothalamus and hippocampus. Although the result of such decreases in CGRP on opiate withdrawal is not known, these data may provide further evidence that CGRP acts to maintain homeostasis in brain regions. Evidence exists that CGRP acts as a neuromodulator that "fine-tunes" neurochemical processes, possibly by the modulation of intracellular calcium or cAMP (25,26,53).

Although evidence for the modulation of cAMP production by CGRP exists in the brain (25,26), it has been shown that CGRP does not alter cAMP in the spinal cord. We found no alteration in basal or forskolin-stimulated cAMP accumulation in synaptosomes from morphine-tolerant or nontolerant rats by CGRP in the midbrain or cerebellum (Fig. 4) or the medulla, hippocampus, or spinal cord (data not shown). We did find significant differences in the corpus striatum (Fig. 5). In this region, using synaptosomes from nontolerant rats, CGRP had no effect on basal cAMP accumulation and significantly decreased forskolin (0.3 μM)-induced stimulation. CGRP was not able to overcome the stimulation produced by 1 μM forskolin. These data indicate that in this region the binding of CGRP is coupled to modulation of cAMP. Tolerance to morphine results in a synaptosomal preparation that has less basal accumulation of cAMP and is less sensitive to forskolin stimulation of cAMP accumulation. It has been previously reported that the levels of cAMP do not differ in striatal synaptosomes from nontolerant and tolerant rats even though the supersensitivity to the effects of GTP and dopamine are observed in the preparation from tolerant rats (5,6). Our data differ somewhat from the previous studies in that we

observe small, but statistically significant, decreases in basal cAMP accumulation in the preparation from tolerant rats. The accumulation of cAMP was not enhanced by 1 μM forskolin in tolerant synaptosomes, while levels of cAMP were increased fivefold by the same concentration of forskolin in the nontolerant preparation. These effects are not observed in rats pretreated acutely with morphine (Fig. 6). These data indicate that opiate tolerance either alters the functionality of the Gs subunit of adenylyl cyclase or enhances Gi activation. The effects of chronic morphine on Gi coupling have been shown (9,49). The data indicate that CGRP restores the functionality of the Gs subunit in the synaptosomes from tolerant rats. CGRP (1 μM) in vitro increased forskolin stimulation to those levels observed in the nontolerant preparation. The CGRP may directly increase Gs stimulation. Alternatively, it may decrease Gi activation. The coupling of CGRP binding to G proteins has been shown. Even though CGRP levels in the corpus striatum were not altered by chronic administration of morphine alone (Fig. 2), the existing levels of CGRP may be sufficient to counter morphine-induced decreases in the activation of adenylyl cyclase because the sensitivity of the Gs/Gi protein to CGRP activation/inactivation is enhanced. The data suggest that CGRP enhances forskolin-stimulated cAMP accumulation when such accumulation is suppressed (as with chronic opiate administration), but conversely depresses forskolin-stimulated cAMP accumulation under normal conditions (as with chronic vehicle administration). These data are consistent with the hypothesis that CGRP acts as a modulatory peptide.

In summary, the effects of acute and chronic morphine administration and opiate withdrawal on the levels of CGRP in the brain and spinal cord are complex. The data suggest that the corpus striatum is a site that is sensitive to opiate-induced and withdrawal-induced alterations in CGRP. In this region, CGRP may serve a neuromodulatory role via interaction with adenylyl cyclase. However, the data suggest that CGRP does not directly alter the development of opiate tolerance but may instead be involved in the autonomic and motor effects associated with the withdrawal syndrome. In addition, the data suggest that tonic opioid control of CGRP levels exists in other brain areas, especially the hypothalamus. These data suggest that CGRP release may modulate the neuroendocrine effects of the opiates, an effect that could alter indirectly the tolerance process.

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