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Effects of Acute and Chronic Morphine Treatment on Calmodulin Activity of Rat Brain

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SUMMARY

The cyclic AMP-phosphodiesterase assay was used to quantitate the amount of calmodulin activity in various brain areas of male rats treated acutely or chronically for 5 days with morphine. The acute treatment with morphine decreased calmodulin activity in the mitochondrial-synaptosomal P_2 fraction of the striatum, midbrain, and thalamus but had no effect on the cerebellum, which contains a low density of opiate receptors. The decrease in calmodulin activity by morphine was dose-dependent and was blocked by the opiate antagonist naloxone. In contrast, chronic treatment of rats with morphine increased calmodulin activity in the mitochondrial-synaptosomal P_2 of the striatum, midbrain, cerebral cortex, and thalamus. A highly sensitive Ca^{2+}/Mg^{2+}-ATPase assay was also used to quantitate the amount of calmodulin activity in subcellular fractions obtained from the striatum. Chronic morphine treatment caused a significant increase in calmodulin activity in the membrane containing microsomal, synaptosomal, and mitochondrial layers but only a small change in the layer that contained the soluble proteins and the synaptic vesicles. It is suggested that alteration of the content of calmodulin in specific subcellular sites may have a central role in opiate action and addiction via regulation of multiple calmodulin-sensitive biochemical pathways.

INTRODUCTION

Opiates have been widely used in medicine as potent analgesics and became especially important with the discovery of the opiate receptors and the endogenous opioid peptides, enkephalins and endorphins. Morphine constitutes an excellent drug model with which to investigate both tolerance and dependence phenomena. In early studies, Shuster (1) and Goldstein and Goldstein (2) postulated that tolerance and physical dependence are due to the overproduction of some proteins which counteract the drug effect as a compensatory response to an initial inhibition of their synthesis. This was supported by studies that showed inhibition of the development of tolerance and dependence by compounds that block protein synthesis (3, 4). Collier, instead, related addiction to an increased number of protein target receptors (5) or alteration of the metabolism of cyclic nucleotides (6). However, experiments that contradict or support either one of these models have been documented (7–9). The involvement of other neurotransmitters such as serotonin and dopamine in opiate addiction was also suggested.

Calcium is a fundamental ion in the regulation of several enzymes, including adenylate cyclase, ATPase, protein kinases, and phosphodiesterase. Interestingly, all of these enzymes are also affected by opiates. It has been found that acute treatment of rats with morphine decreases Ca^{2+} levels in various brain regions and subcellular structures, whereas chronic application of morphine increases Ca^{2+} levels in specific subcellular sites (10–12). In addition, intracisternal injection of Ca^{2+} antagonizes morphine-induced analgesia, whereas the Ca^{2+} chelator EGTA[2] and the antagonist lanthanum show the opposite effect (13).

Many Ca^{2+} effects on cellular metabolism were recently shown to be mediated by interaction of the ion with a protein of 17,000 mol wt termed calmodulin (for recent reviews see refs. 14 and 15). Upon interaction with Ca^{2+}, a Ca^{2+}-calmodulin complex is formed and this regulates the activity of phosphodiesterase (16, 17), adenylate cyclase (18, 19), Ca^{2+}/Mg^{2+}-ATPase (20, 21), protein kinases (22), and several other enzymes (14, 15). Considering the tight relationship between Ca^{2+} and calmodulin, and the close relationship between opiates and Ca^{2+}, it would seem reasonable to determine whether acute or chronic treatment of rats with morphine induces alterations in calmodulin content. In this study we used Ca^{2+}/Mg^{2+}-ATPase (23) and cyclic AMP phosphodiesterase (14–17, 24, 25) assays to quantitate the amount of biologically active calmodulin.

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[2] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Methods

Morphine treatment. Male Sprague-Dawley rats were weighed 24 hr before treatment and uniformly distributed by weight into groups. For acute treatment, morphine HCl was injected i.p. (10 or 20 mg/kg); control groups were treated with saline (4 ml/kg). Animals were made addicted to morphine in two different ways: (a) The animals received injections of morphine HCl (20, 20, 25, 25, and 30 mg/kg) on 5 consecutive days. (b) Two slow-releasing pellets (each containing 75 mg of morphine base, 75 mg of microcrystalline cellulose, 1.5 mg of calcium stearate, and 0.75 mg of $SiO_2 \cdot H_2O$) were implanted under the dorsal skin following a schedule of one pellet every 2 days; control animals were sham-operated but no pellet was implanted.

Calmodulin extraction. Animals were killed at noon by decapitation 30 min after acute treatment, 2 days after implantation of the second pellet, or 24 hr after the injection of morphine (30 mg/kg) into chronically treated rats (chronic treatment). The indicated brain areas were quickly dissected as outlined (26), and calmodulin was extracted from the whole tissue homogenate, the mitochondrial-synaptosomal P_2 pellet, or from layers obtained from further fractionation of lysed P_2 as follows. All procedures were performed at 4°. Calmodulin of the whole-tissue homogenate was prepared from various regions of the brain by homogenization with a Kinematica Polytron homogenizer (Setting 4 for 15 sec) in 2 ml of 40 mM Tris-HCl (pH 7.4 at 25°) containing 2 mM EGTA. The homogenate was centrifuged at 38,000 × *g* for 15 min, and the supernatant was separated, placed in a boiling water bath for 90 sec, and recentrifuged as before. Preliminary experiments showed that the boiling destroyed over 95% of the endogenous phosphodiesterase activity but did not change the calmodulin activity, being in line with previous studies (14). The supernatant was then dialyzed extensively against 40 mM Tris-HCl (pH 7.4 at 25°) and recentrifuged. Aliquots of the supernatant were tested for protein content (27) and the remainder was stored at −20°. Mitochondrial-synaptosomal P_2 fractions from the indicated area were prepared according to the method of Whittaker *et al.* (28), and subfractionation of osmotically lysed P_2 was performed as described previously (29). Calmodulin was extracted from these fractions with Tris-HCl containing 2 mM EGTA. The samples were placed in a boiling water bath for 90 sec and further treated as samples of the whole-tissue homogenate.

Determination of calmodulin levels by cyclic AMP-phosphodiesterase assay. Rat brain phosphodiesterase was purified, and the cyclic AMP-dependent phosphodiesterase assay in a final volume of 0.5 ml was performed according to ref. 24, with minor modifications (25), using *Crotalus atrox* 5′-nucleotidase. Pure bovine brain calmodulin (25–500 ng) was used as a standard to quantitate calmodulin levels in 0.75–1.5 μg of protein of P_2 extracts from various brain areas. The phosphodiesterase assay was continued for 15 min, and under these conditions activation of the enzyme was linear with regard to calmodulin content. All samples were tested in duplicate, the difference between duplicate samples being less than 15%.

Determination of calmodulin levels by the Ca^{2+}/Mg^{2+}-ATPase assay. The calmodulin content in the various extracts of the striatum was determined by their ability to activate Ca^{2+}/Mg^{2+}-ATPase from human red blood cells, according to the method of Muallem and Karlish (23) with minor modifications. Outdated human red blood cells (100–150 ml of packed cells) were washed three times with 10 volumes of ice-cold isotonic Tris-HCl (pH 7.4 at 25°) by centrifugation for 5 min at 2,000 × *g*, lysed in 10 volumes of distilled water by stirring for 5 min, and centrifuged for 10 min at 40,000 × *g*. The pellet was washed three to five times in 2 mM Hepes (brought to pH 7.4 at 25° with 2 mM Tris base) containing 1 mM $MgCl_2$ until it was free of hemoglobin, and finally suspended in the same buffer containing 0.1 mM $MgCl_2$. Calmodulin was extracted from these membranes by adding EGTA to 5 mM final concentration and two cycles of freezing and thawing. The membranes were then washed three times in 2 mM Hepes-Tris/0.1 mM $MgCl_2$ by a 10-min centrifugation at 40,000 × *g* to remove the EGTA; the pellet was suspended in the same buffer, divided into aliquots, and stored at −80°. The membrane-bound ATPase lost not more than 10% of activity over 3 weeks of storage, and was not used beyond this period. The protein content of the final membrane suspension in different preparations was 2.4–6.0 mg/ml.

The Ca^{2+}/Mg^{2+}-ATPase assay (23) was conducted as follows. A portion (10 μl) of the red blood cell membrane suspension (unless otherwise indicated) was incubated for 30 min at 37° in a final volume of 200 μl of 20 mM Tris-HCl (pH 7.4 at 25°) containing 100 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 5 mM Hepes-Tris, 0.1 mM ouabain, 0.01 mM $CaCl_2$, 100 μM ATP (containing 5 to 10 × 10^4 cpm of [γ-^{32}P]ATP), and various amounts of calmodulin-containing brain extracts from various subcellular fractions or pure bovine brain calmodulin that was added before the incubation was started. After incubation the samples were cooled to 0° for 5 min, and 100 μl of 4 N H_2SO_4 containing 4% ammonium molybate, 10% perchloric acid, and 2 mM inorganic phosphate were added. Isobutanol (800 μl) was added, and the samples were shaken and centrifuged for 30 sec in an Eppendorf microcentrifuge at about 12,000 × *g*; 0.5 ml of the isobutanol phase was then counted in toluene-Triton scintillation fluid. Ca^{2+}/Mg^{2+}-ATPase activity was calculated as picomoles of phosphate liberated from the ATP per microgram of protein per minute after subtraction of the basal activity of samples not containing brain extract or pure calmodulin. All samples were tested in duplicate, the difference between duplicate samples being less than 10%; the data were expressed as percentages of controls. Student's *t*-test was used to determine significant differences between control and treated groups.

Materials

Chlorpromazine, ouabain, ATP, cyclic AMP, *C. atrox* 5′-nucleotidase, and EGTA were obtained from Sigma Chemical Company (St. Louis, Mo.). Pure bovine brain calmodulin was obtained from Fluka, [γ-^{32}P]ATP (specific activity 3000 Ci/mmole) was obtained from Radiochemical Centre (Amersham, England), morphine HCl was obtained from Teva (Jerusalem, Israel), and nalox-

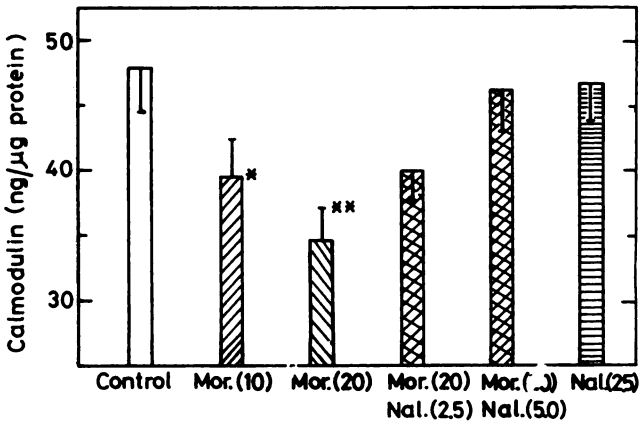

FIG. 1. *Effect of various concentrations of morphine and naloxone on the calmodulin content of P_2 fractions of rat midbrain*

Mitochondrial-synaptosomal P_2 fractions were prepared from rats treated 30 min earlier with saline (control) or morphine HCl (*Mor.*) (10 or 20 mg/kg). Naloxone (*Nal.*) at 2.5 or 5.0 mg/kg was injected 15 min prior to morphine. The calmodulin content was quantitated by the cyclic AMP-phosphodiesterase assay with pure bovine brain calmodulin as the standard. N = four to eight rats. *, **, Significantly different from control ($p < 0.01$ and $p < 0.005$, respectively).

one HCl was obtained from Endo Laboratories. All of the other chemicals used were of analytical grade.

RESULTS

Effect of acute and chronic morphine treatment on calmodulin activity. The cyclic AMP-phosphodiesterase assay was used to quantitate calmodulin activity in several brain regions. The specificity of this assay and its use for quantitation of calmodulin has been extensively studied (14–17, 24, 25). Activation of the cyclic AMP-phosphodiesterase by the brain extracts was Ca^{2+}-dependent, blocked by EGTA, and sensitive to chlorpromazine (data not shown).

The effect of chronic treatment with morphine on calmodulin activity was studied in rats made addicted by injection of morphine HCl for 5 consecutive days, as described under Experimental Procedures. The animals were addicted to morphine, as indicated by the fact that injection of the opiate antagonist naloxone (3 mg/kg) to such rats induced characteristic withdrawal symptoms, including wet-dog shakes, diarrhea, jumping, and teeth-chattering. These animals were not used for calmodulin examination. Table 1 shows that the mitochondrial membrane P_2 extracts from the striatum, midbrain, thalamus, and cerebral cortex of rats that received injections of morphine for 5 days contained 28–37% higher amounts of calmodulin than those of littermate rats that received

TABLE 1

Effect of acute and chronic morphine treatment on the calmodulin content of the P_2 extracts of several brain areas

The calmodulin content of the P_2 extracts was quantitated by the cyclic AMP-phosphodiesterase method, with pure bovine brain calmodulin as a standard. Data are means standard deviation from 8–12 rats.

Treatment and brain region	Calmodulin		(M/C) × 100
	Control (C)	Morphine (M)	
	ng/µ protein		
Chronic			
Midbrain	45.3 ± 3.2	62.1 ± 4.4[a]	137
Thalamus	83.5 ± 7.3	108.6 ± 7.9[a]	130
Cerebral cortex	123.5 ± 8.3	158.1 ± 11.5[b]	128
Striatum	116.3 ± 7.2	157.0 ± 9.2[a]	135
Acute			
Midbrain	41.7 ± 2.8	30.7 ± 3.0[a]	74
Thalamus	72.2 ± 6.1	48.4 ± 4.7[a]	67
Striatum	113.6 ± 8.1	77.2 ± 6.6[a]	68
Cerebellum	39.0 ± 3.1	38.3 ± 2.3	98

[a] Significantly different from controls ($p < 0.005$).
[b] Significantly different from controls ($p < 0.01$).

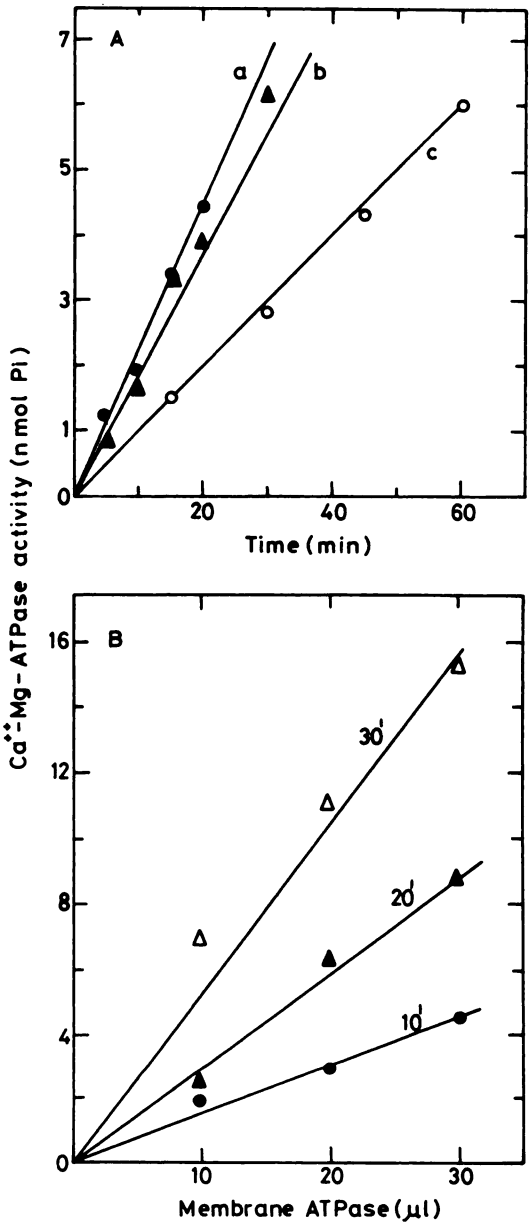

FIG. 2. *Basal Ca^{2+}/Mg^{2+}-ATPase activity of red blood cell membranes*

A. The basal activity of three red blood cell membrane preparations (*a*, *b*, and *c*) (2.4–6.0 mg of protein per milliliter) was determined at the indicated times. No calmodulin was added.

B. The basal activity of different amounts of red blood cell membranes from the same preparations was tested at 10, 20, or 30 min.

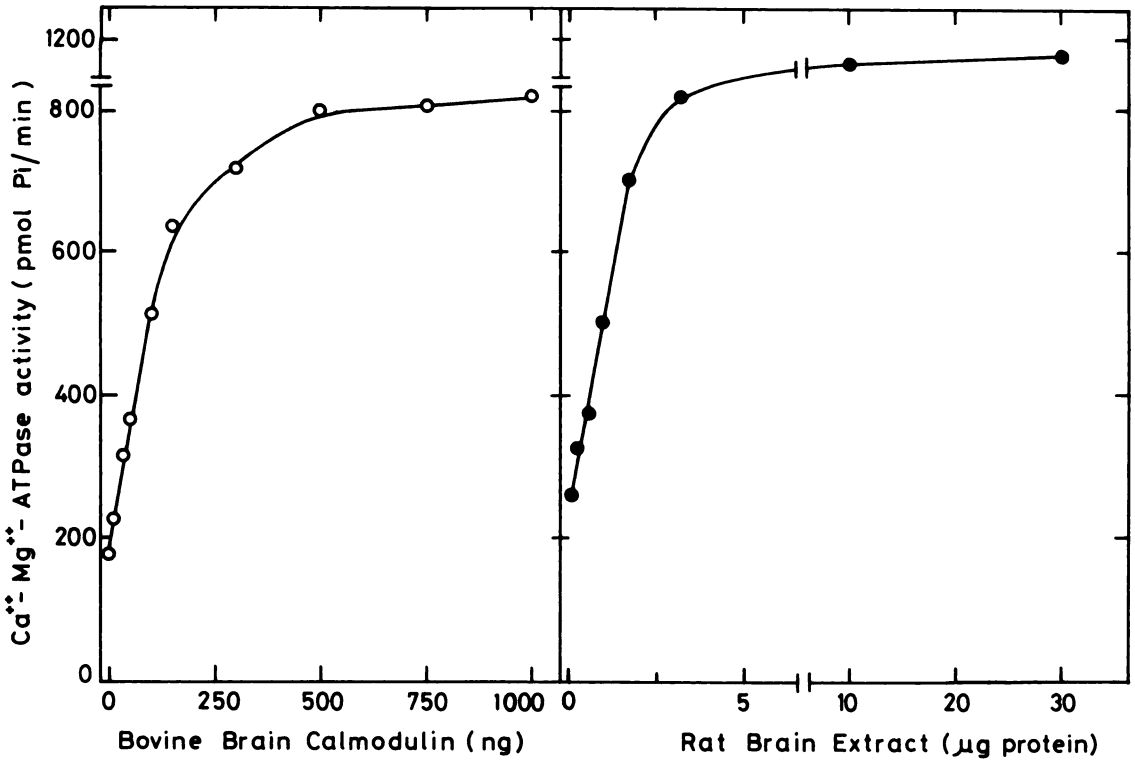

FIG. 3. *Stimulation of Ca^{2+}/Mg^{2+}-ATPase by bovine brain calmodulin or rat striatal extract*

The rat striatal extract was prepared from the whole-tissue homogenate by boiling in an EGTA-containing buffer as described under Experimental Procedures. The incubation period was 30 min for both the striatal extract and the pure bovine brain calmodulin.

injections of saline. In contrast, rats injected for the first time with 20 mg/kg of morphine HCl and killed 30 min later (acute treatment) had lower calmodulin levels in the thalamus, striatum, and midbrain as compared with controls (Table 1). No significant decrease was observed in the P_2 fractions prepared from the cerebellum, which contains a low density of opiate receptors.

Figure 1 shows that the effect of morphine on calmodulin levels was dose-dependent, 10 mg/kg being less active than 20 mg/kg. In addition, the effect of morphine was blocked by 40 ± 8% and 85 ± 16%, respectively, when 2.5 or 5.0 mg/kg of the opiate antagonist naloxone was injected 15 min prior to morphine. Naloxone by itself had no effect (Fig. 2).

TABLE 2

Calmodulin activity of striatal P_2 subfractions obtained from a sucrose gradient

The striatal mitochondrial-synaptosomal P_2 prepared from 16 control or morphine-addicted (by pellets) rats were lysed, fractionated on a sucrose gradient, and assayed for calmodulin activity by the Ca^{2+}/Mg^{2+}-ATPase assay. Values for calmodulin activity of Subfractions 1, 2, 3, and 4 of the controls were 3670, 945, 1123, and 1666 pmoles of P_i/min/μg of protein, respectively.

P_2 subfraction	Stimulated Ca^{2+}/Mg^{2+}-ATPase activity of morpine-addicted rats
	% of control
1. 0–0.4 M: Soluble proteins + synaptic vesicles	117 ± 6
2. 0.4–0.6 M: Microsomes + membranes	153 ± 13[a]
3. 0.6–1.0 M: Synaptosomal membranes	150 ± 25[a]
4. 1.2 M: Mitochondrial pellet	176 ± 19[a]

[a] Significantly different from controls ($p < 0.01$).

Calmodulin activity in subfractions of the striatal P_2 assayed by the Ca^{2+}/Mg^{2+}-ATPase. The sensitive Ca^{2+}/Mg^{2+}-ATPase assay was used to localize further the changes in calmodulin activity upon morphine treatment. It was first important to characterize the Ca^{2+}/Mg^{2+}-ATPase that was used. The release of phosphate increased linearly with time (Fig. 2A) and was proportional to the amount of membranes added (Fig. 2B). However, basal activities in different preparations were not identical, as indicated in Fig. 2A; therefore, the samples from control and morphine-treated rats of a given experiment were always tested with the same ATPase preparation. Thus, under the experimental conditions used the substrate concentration does not constitute a limiting factor for the enzyme for up to 30 min. This was also true with regard to the stimulated Ca^{2+}/Mg^{2+}-ATPase, which was linear during this period of incubation. EGTA that was used during the preparation of the red blood cell membranes removed the endogenous calmodulin, so that added pure bovine brain calmodulin or rat brain extracts increased the Ca^{2+}/Mg^{2+}-ATPase activity 5- to 7-fold in a dose-dependent manner, being linear with 50–1000 ng of brain extract protein (Fig. 3). The saturation levels observed with high concentrations of bovine brain calmodulin or the rat brain extracts occurred because the enzyme was maximally activated and not because of shortage of ATP, since addition of the substrate had no effect. These results suggest that the tested brain extracts contained calmodulin-like activity and that the Ca^{2+}/Mg^{2+}-ATPase assay could be used for quantitation of these calmodulin preparations.

Fractionation of the lysed P_2 pellet from the striatum

showed that Subfraction 1, which included the soluble components and the synaptic vesicles, contained 57% of the total calmodulin activity, with the rest being distributed unevenly in the microsomal, synaptosomal, and mitochondrial fractions. Chronic treatment of the rats with morphine (by pellets) increased calmodulin activity in the membrane-containing fractions by 50–76% as compared with equivalent fractions from control rats (Table 2). Subfraction 1 of the morphine-treated rats had only 17 ± 6% higher calmodulin activity than the controls, a small increase that may reflect alteration in the synaptic vesicles that were also included in this layer.

DISCUSSION

The important finding of these experiments was that acute and chronic treatment of rats with morphine induced opposite effects on the content of calmodulin in the nerve ending-enriched fractions of several brain regions. The specificity of the morphine effect was shown by the facts that acute administration of morphine had no significant effect on the calmodulin content of the cerebellum and naloxone blocked the acute effect of morphine. Acute treatment reduced the calmodulin content in the mitochondrial-synaptosomal P_2 fraction but did not change the total content of calmodulin in the brain (data not shown), whereas chronic application of morphine increased calmodulin P_2 levels. It seems that the major change in calmodulin content occurred in membrane-containing nerve ending structures, whereas the fraction that contained soluble proteins and synaptic vesicles indicated only a small change. According to these findings, an intracellular translocation of calmodulin upon acute treatment with morphine would be a conceivable conclusion. A previous study showed that incubation of striatal slices for 30 min with morphine increased the cytosolic content of calmodulin (30), supporting the idea that morphine can induce intracellular translocation of calmodulin. Such translocation and release of calmodulin to the cytosol may be the result of the decrease in Ca^{2+} levels that was observed under acute morphine treatment (10–12), since calmodulin binding to the cell membranes is Ca^{2+}-dependent. It is tempting to suggest that the decrease in the calmodulin content of the mitochondrial-synaptosomal fraction alters the normal equilibrium between active and nonactive calmodulin as presented in the equation

$$Ca^{2+} + \text{calmodulin}_{(\text{nonactive})} \rightleftarrows (Ca^{2+} \cdot \text{calmodulin})_{(\text{active})}$$

and subsequently the whole repertoire of cellular events that are controlled by this key protein. As a working hypothesis we suggest that the continuous presence of morphine in chronically treated rats initiates a compensatory system(s) that increases the levels of calmodulin in the nerve ending. The details of the mechanism by which the increase may occur are still obscure. The early studies showing that morphine addiction is dependent on protein synthesis (3, 4) might support the possibility that the compensatory system involves increased synthesis of calmodulin.

The abnormal behavior of morphine-addicted subjects is especially noticeable after cessation of morphine application or injection of an antagonist, both of which induce a set of characteristic withdrawal symptoms. It is conceivable that some of these signs may result from enhanced activity of calmodulin-dependent systems such as release of neurotransmitters or cyclic AMP metabolism (18, 19). Therefore, the continuous presence of morphine in morphine-dependent subjects might be necessary to prevent enhanced activity of such calmodulin-dependent biochemical pathways. The interrelationship between opiate receptors and membrane-bound calmodulin was also indicated in cultures of neuroblastoma-glioma hybrid cells that possess a high density of opiate receptors. We have also selected mutant clones of neuroblastoma-glioma cells with altered calmodulin and opiate receptor density (31). Whether other addictive compounds alter the intracellular distribution of calmodulin is as yet unknown, but it is of interest that alcohol has been reported to induce changes in Ca^{2+} brain levels and that chronic treatment with antipsychotic drugs increases striatal calmodulin levels.

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