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EFFECTS OF NARCOTIC ANALGESIC DRUGS ON THE CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE-ADENYLATE CYCLASE SYSTEM IN RAT BRAIN

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1 The concentrations of cyclic adenosine 3',5'-monophosphate (cyclic AMP), measured in discrete brain areas removed from rats killed by microwave irradiation, rose transiently in most areas after the administration of morphine. The most pronounced changes, however, were found 2 h after doses of either 10 or 60 mg/kg morphine when cyclic AMP levels declined significantly in the hypothalamus, medulla and cerebellum. In morphine-tolerant rat brains there were no decreases in cyclic AMP levels.

2 Basal adenylate cyclase activity in crude nerve-ending fractions from discrete areas of rat brain was unaffected by the addition of active analgesic agonists, antagonists or inactive isomers to the assay medium *in vitro*, except for a nonspecific inhibition at drug concentrations of 1 mM.

3 The acute administration of morphine or levorphanol, but not dextrorphan produced transient increases in basal cyclase activity of crude nerve-ending preparations from midbrain and striatum. In morphine-tolerant rats, these changes in basal adenylate cyclase activity were no longer seen.

Introduction

The acute administration of narcotic analgesic drugs to experimental animals produces dose-related biphasic effects on the levels of biogenic amines in brain (Way & Shen, 1971) and on the rates of their biosynthesis (Clouet & Ratner, 1970; Smith, Sheldon, Bednarczyk & Villarreal, 1972). The effects of narcotic drugs on the cyclic nucleotides in brain that are involved in adrenergic transmission have been examined indirectly, for the most part; i.e. the administration of cyclic adenosine 3',5'-monophosphate (cyclic AMP) intracerebrally has been shown to reverse the depressant effect of morphine (Ho, Loh & Way, 1972). The fact that adenylate cyclase activity in the central nervous system is stimulated by catecholamines, a phenomenon which has been demonstrated both *in vitro* (Kakiuchi & Rall, 1968a and b; Daly, Huang & Shimizu, 1972; Kebabian, Petzold & Greengard, 1972) and *in vivo* (Burkard, 1972; Garelis & Neff, 1974), suggested that a study of the effects of morphine and other narcotic analgesics on the rates of biosynthesis and the concentrations of cyclic AMP in homogenates of discrete brain areas, and of the activity of adenylate cyclase in crude nerve-ending preparations from the same areas, after treatment of rats with narcotic analgesic drugs and related compounds might be rewarding.

Methods

Male Wistar rats weighing between 100 and 130 g were injected subcutaneously with 10 or 60 mg/kg morphine hydrochloride, 15 mg/kg levorphanol or dextrorphan tartrate (Hoffmann-LaRoche) or equivalent volumes of 0.9% w/v NaCl solution (saline) for acute experiments. Morphine pellets containing 75 mg of morphine base, prepared as described by Way, Loh & Shen (1969), were implanted subcutaneously to induce tolerance. Tolerance was also induced by subcutaneous injections twice daily for 10 days of morphine in increasing doses from 10 to 100 mg/kg per injection. In one experiment, four groups of rats were injected subcutaneously with saline or morphine (60 mg/kg) and two groups with naloxone (1 mg/kg) 10 min later. All rats were killed 2 h after the initial injection. (−) and (+)-Methadone and naloxone hydrochloride were obtained from Eli Lilly and Endo Laboratories, respectively.

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Assay for cyclic AMP

After appropriate treatment, rats were placed in a styrofoam box within a microwave oven (Litton Model 70/75) and irradiated for 38 s (the time found to result in highest cyclic AMP concentrations in brain), a procedure similar to that described by Schmidt, Hopkins, Schmidt & Robison (1972). The rats were decapitated, the heads cooled on ice and the brains were removed and dissected into six areas: cortex, medulla, cerebellum, hypothalamus, striatum and midbrain, as described by Glowinski & Iversen (1966). The concentrations of cyclic AMP were measured by a protein-binding assay similar to that described by Gilman (1970), except that the binding protein was isolated from rabbit skeletal muscle instead of beef muscle, and 100 mM sodium acetate buffer, pH 4.5 was used for the binding reaction (Miki, Yamamoto & Iwatsubo, 1972). Brain protein was measured after precipitation with 5% trichloroacetic acid (TCA) by the method of Lowry, Rosebrough, Farr & Randall, 1951.

Adenylate cyclase assay

After decapitation, the brains were removed and dissected into six areas as described above. The tissues were homogenized in 0.32 M sucrose in sufficient volumes to make tissue suspensions of 30 mg wet weight/ml, and the crude mitochondrial-synaptosomal fraction isolated by the established sucrose density gradient centrifugation procedure (Chakrin & Whittaker, 1969). For *in vitro* incubations the crude mitochondrial-synaptosomal fraction was recentrifuged in order to isolate a nerve-ending fraction. The tissue pellets were suspended in water to make final protein concentrations of approximately 2 μg/μl.

In the cyclase assay, 50 μl of tissue suspension was added to 150 μl of a mixture containing 0.5 to 1 μCi [^{32}P]-adenosine 5′-triphosphate (0.1 μmol, New England Nuclear Corp.), $MgSO_4$ (1 μmol), theophylline (2 μmol), cyclic AMP (0.2 μmol), phosphoenolpyruvate (1 μmol), pyruvate kinase (1.9 i.u. with 2.2 μmol ammonium sulphate, Sigma Chemical Co.) and tris (hydroxymethyl)-methylammonium hydrochloride (Tris) (8 μmol), pH 7.5 (and drugs, when tested *in vitro*) and incubated at 30°C for 5 minutes. In preliminary experiments, shown in Table 2, the ATP-generating system (phosphoenolpyruvate and pyruvate kinase) was omitted and caffeine (1.3 μmol) was used as a phosphodiesterase inhibitor instead of theophylline. This assay was terminated after 10 min at 30°C. In each assay, the enzyme reaction was stopped by heating the assay vessels to 100°C for 2 minutes. One ml of the Tris buffer containing 10,000 to 20,000 ct/min of [^{3}H]-cyclic AMP (New England Nuclear Corp.) was added before centrifugation in order to calculate recovery of cyclic AMP in each sample. The cyclic AMP fraction was separated from other components on a column of neutral aluminium oxide as described by White & Zenser (1971) and twice clarified by $ZnSO_4$-$Ba(OH)_2$ precipitation, as described by Krishna, Weiss & Brodie (1968). The ^{3}H and ^{32}P radioactivity in the supernatant fraction was measured in a Nuclear-Chicago scintillation spectrometer. In control experiments, (1) [^{3}H]-cyclic AMP was added to the aluminium column and carried through the procedure with 60-70% recovery; and (2) phosphodiesterase (from beef heart, Sigma Chemical Co.) was added to the assay with the elimination of radioactivity in the final supernatant fraction. Under the experimental conditions described, enzyme activity was proportional to tissue concentration and time in both assays.

Statistics

The data were examined for significance of differences by Student's *t* test.

Results

Concentrations of cyclic AMP after acute treatment with morphine

After the administration of morphine (60 mg/kg, s.c.), transient rises in cyclic AMP concentrations were found in all areas except hypothalamus and medulla (Figure 1). In the latter regions, both 10 and 60 mg/kg doses of morphine produced significant decreases in cyclic AMP concentrations within 1 or 2 hours. In the cerebellum, both doses of morphine produced biphasic changes in cyclic AMP concentrations which were maximal at 15 or 30 min and returned to control or lower levels 2 h after morphine administration.

Concentrations of cyclic AMP in brains of tolerant animals

Rats made morphine-tolerant by 10 days of morphine injections were killed 2 h after the last injection. In brain regions of these rats, the concentrations of cyclic AMP were unaltered from control values in all areas except cortex and striatum (Table 1), in which there were significant increases. Compared to cyclic AMP concentrations attained 2 h after the initial injection of 60 mg/kg, the levels of the nucleotide were higher in all areas

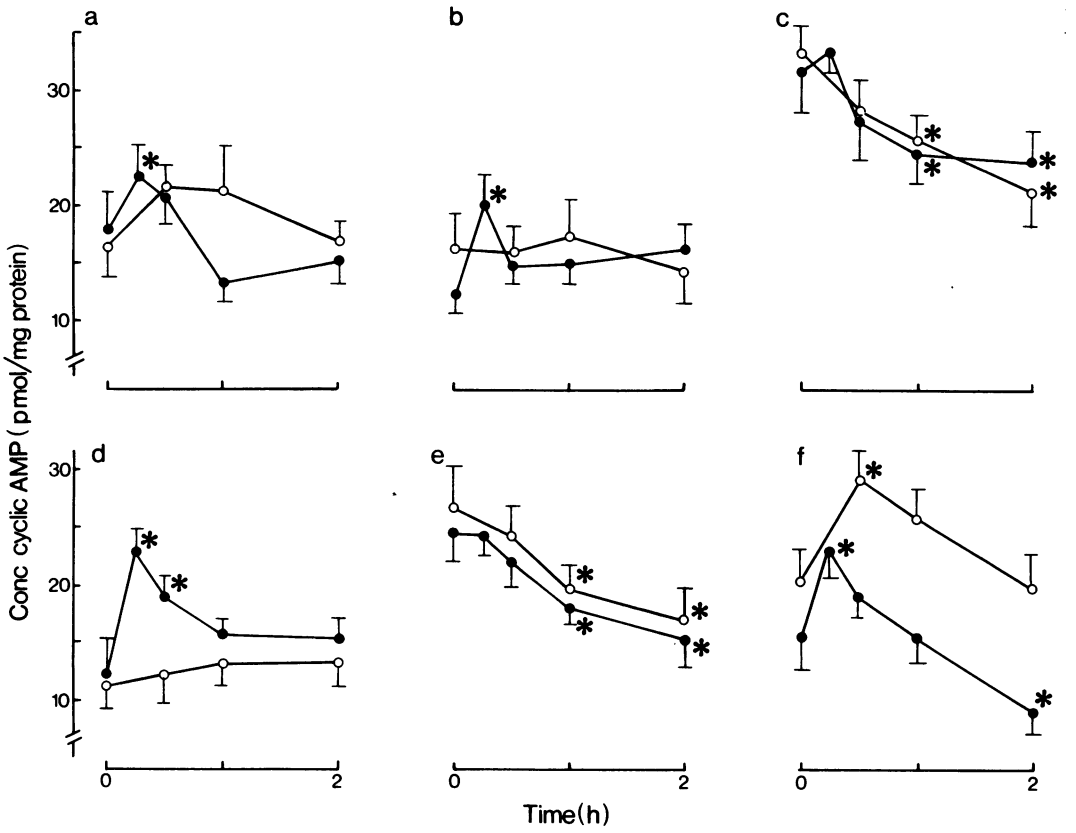

Figure 1 Concentration of cyclic AMP in areas of rat brain after the acute administration of morphine: (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. (o) Morphine 10 mg/kg s.c.; (•) 60 mg/kg s.c. Morphine was administered to rats that were subsequently killed at intervals up to 2 hours. The zero-time values are from untreated rats. Each value represents the mean from four rats. Vertical lines show s.e. mean. *Values differ significantly from appropriate zero-time control values ($P < 0.05$).

Table 1 Concentrations of cyclic AMP in brain areas from morphine-tolerant rats

Brain area	*Control* (pmol cyclic AMP/mg protein)	*Morphine-tolerant†*
Midbrain	26.5 ± 0.7	27.1 ± 0.6
Striatum	12.8 ± 0.3	*21.4 ± 0.3
Medulla	26.9 ± 0.3	27.1 ± 0.3
Cortex	9.2 ± 0.1	*16.6 ± 0.4
Hypothalamus	21.6 ± 0.7	24.6 ± 0.8
Cerebellum	26.0 ± 0.3	24.2 ± 0.1

† Rats were made tolerant by injections twice daily with morphine in increasing doses from 10 to 100 mg/kg for 10 days, and were killed 2 h after the last injection. Control animals received saline in equivalent amounts. Each value represents the mean ± s.e. mean of four animals.
* Significantly different from control values at $P < 0.001$.

in the tolerant animals. Thus, the depleting effect of morphine administered acutely was not found in tolerant animals.

Effects of opioids on adenylate cyclase activity in vitro

The effects on basal adenylate cyclase activity of the addition of morphine, levorphanol, and its less active isomer, dextrorphan, (−)- and (+)-methadone and the antagonist, naloxone, are shown in Table 2. At 1 mM concentration, levorphanol and dextrorphan reduced cyclase activity by about 50%, and the other opioids had lesser inhibitory activity. At lower drug concentrations, there was either no effect or slight inhibition of adenylate cyclase activity in the ruptured nerve-ending fractions.

Effects of acute treatment with opioids on adenylate cyclase activity

After the 60 mg/kg dose of morphine adenylate cyclase activity was increased in the crude nerve-ending preparations from midbrain, striatum and medulla, and decreased in cerebellum (Figure 2). After levorphanol 1.5 mg/kg, adenylate cyclase activity was increased in midbrain and striatum and decreased in cerebellum, while the same dose of dextrorphan produced no significant changes in cyclase activity. A dose of naloxone (1 mg/kg) that blocked the analgesic and hypothermic responses to morphine (60 mg/kg) did not block the increases in adenylate cyclase activity in midbrain and striatum 2 h after the morphine injection (Table 3). There was a trend toward increased adenylate cyclase activity in the medulla and a trend toward decreased adenylate cyclase activity in the cerebellum, although the differences were not statistically significant in this experiment.

Effects of tolerance on adenylate cyclase activity

Rats were made morphine-tolerant by the implantation of one morphine pellet subcutaneously on day 1, two pellets on day 5, followed by twice daily injections of morphine (100 mg/kg) on days 9 and 10. Tolerant rats in one group (B) were killed 18 h after the last injection, while those in another group (C) received another injection 2 h before they were killed. The untreated rats (group A) were sham-operated for pellet implantation on days 1 and 5. There were no changes in the basal activity of adenylate cyclase in any region in group B (Figure 3). However, in group C, there were significant increases above control values in several areas of brain, namely midbrain, cerebellum and cortex (Figure 3).

Discussion

The transient changes in the levels of cyclic AMP

Table 2 Effect of narcotic analgesics and related compounds on adenylate cyclase activity in nerve-endings *in vitro*

Adenylate cyclase activity (pmol cyclic AMP min^{-1} mg $protein^{-1}$)	*Midbrain* 218 ± 87	*Striatum* 430 ± 49	*Medulla* 193 ± 22	*Cortex* 392 ± 47	*Hypothalamus* 380 ± 36	*Cerebellum* 617 ± 75
Drugs (mM)			*(% of control activity)†*			
Morphine						
0.1	99	96	102	91	105	115
1.0	98	90	99	70*	89*	108
Levorphanol						
0.1	81*	101	91	90	72*	104
1.0	37*	49*	47*	56*	42*	59*
Dextrorphan						
0.1	84*	106	87*	98	82*	100
1.0	37*	57*	39*	48*	49*	50*
(−)-Methadone						
0.1	103	93	103	93	96	88*
1.0	78*	68*	95	57*	89*	74*
(+)-Methadone						
0.1	92	84*	95	71*	100	93
1.0	85*	73*	85*	71*	96	73*
Naloxone						
0.1	87*	110	91	113	104	108
1.0	81*	105	97	94	81*	108

† The drugs were added to purified ruptured nerve-ending preparations at the final concentrations specified, and adenylate cyclase activity was measured (by a different assay from that used otherwise throughout the paper, see Methods sections for details). Adenylate cyclase activity in the presence of drugs is expressed as % of the control values shown in the top line and is the average of three observations.

* Values are different from control values at $P < 0.05$.

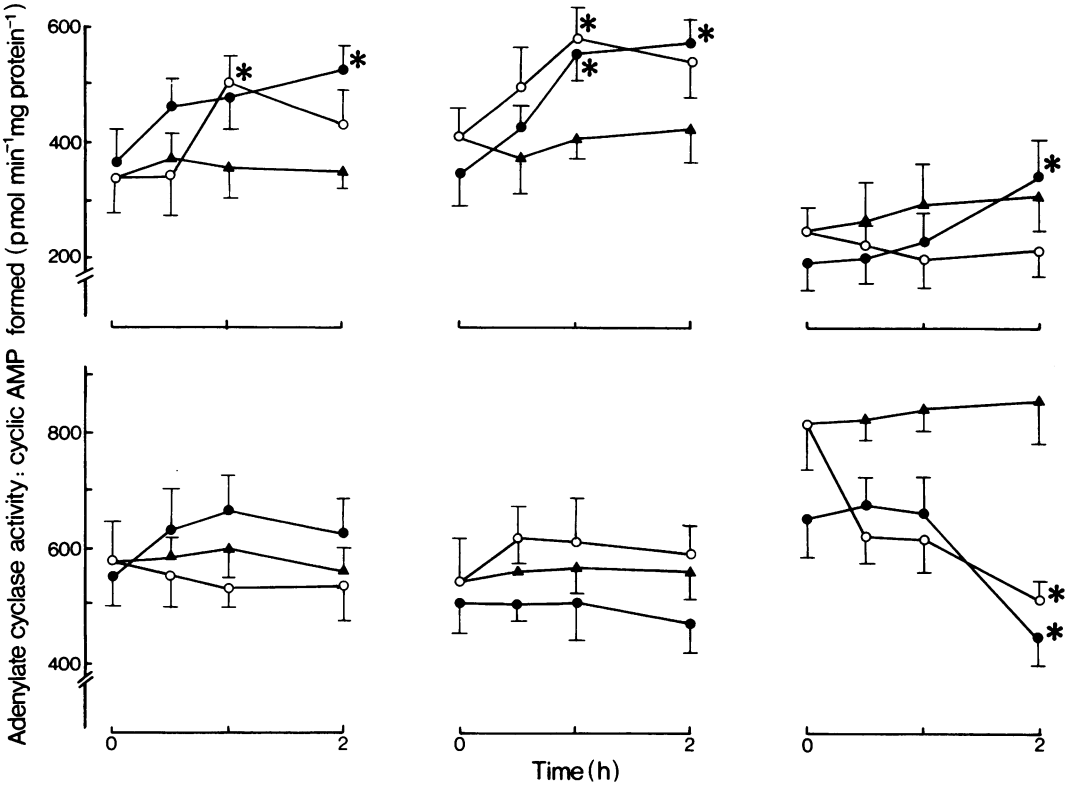

Figure 2 Adenylate cyclase activity (expressed as cyclic AMP formed) in crude nerve-ending preparations from rat brain areas after the acute administration of morphine, levorphanol or dextrorphan: (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. (●) Morphine 60 mg/kg; (○) levorphanol 15 mg/kg; (▲) dextrorphan 15 mg/kg. Each value represents the mean from three rats. Vertical lines show s.e. mean. *Values are significantly different from respective zero-time control values ($P < 0.05$).

Table 3 Effect of naloxone and/or morphine administration on adenylate cyclase activity in nerve-endings

Treatment	*Midbrain*	*Striatum*	*Medulla*	*Cortex*	*Hypothalamus*	*Cerebellum*
			(% of control activity)†			
Saline	100 ± 7	100 ± 10	100 ± 13	100 ± 9	100 ± 13	100 ± 11
Morphine	*149 ± 8	*148 ± 9	129 ± 12	100 ± 10	100 ± 11	79 ± 8
Saline + naloxone	106 ± 7	98 ± 2	103 ± 17	98 ± 8	99 ± 9	98 ± 7
Morphine + naloxone	**183 ± 8	**167 ± 12	*143 ± 9	103 ± 10	102 ± 12	84 ± 11

† Morphine (60 mg/kg, s.c.) or saline was injected into four groups of rats each. Naloxone (1 mg/kg, s.c.) was injected 10 min later into the last two groups, and all rats were killed 2 h after the initial injection. Each value represents the mean ± s.e. mean % of adenylate cyclase activity in nerve-ending preparations from the same brain regions in saline-treated control rats.

* $P < 0.05$; ** $P < 0.02$, (significance of differences between morphine-treated and appropriate control groups). There were no differences between morphine and morphine plus naloxone values.

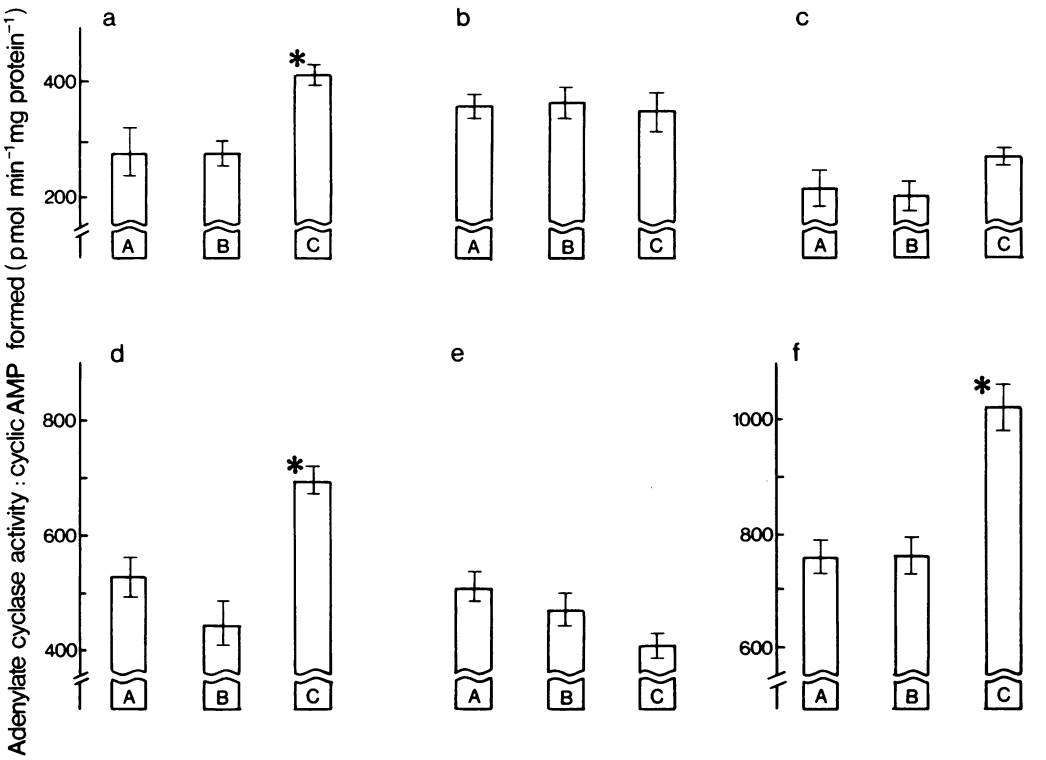

Figure 3 Adenylate cyclase activity in crude nerve-ending preparations from brain areas from morphine-tolerant rats: (a) midbrain, (b) striatum, (c) medulla, (d) cortex, (e) hypothalamus, (f) cerebellum. Columns A: Sham-operated control rats. B: Rats treated chronically with morphine as described in the text and killed 18 h after the last injection. C: Rats treated chronically with morphine as in group B, but with an additional injection of morphine 100 mg/kg 2 h before they were killed. Each column represents the mean result from four rats. Vertical lines show s.e. mean. *Values differ significanty from sham-operated controls ($P < 0.05$).

in some brain areas after the administration of morphine are not incompatible with a 'second messenger' role for cyclic AMP in the responses to morphine. However, the changes were not related temporally to basal adenylate cyclase activity in nerve-ending preparations from the same brain areas, with the possible exception of the cerebellum in the second hour after acute morphine. The time course of alterations of cyclic AMP concentrations was also not related temporally to transient changes in the concentrations of catecholamines in the same brain areas after acute morphine treatment, which we described earlier (Johnson, Ratner, Gold & Clouet, 1974). However, a close correlation between the rate of catecholamine release and cyclic AMP concentrations in a brain area should not be anticipated because the cyclic AMP synthesized by neurotransmitter-sensitive adenylate in membranes may be a small part of the total pool of cyclic AMP measured in tissue homogenates.

The addition of narcotic agonists, the antagonist naloxone, or relatively inactive stereoisomers of agonists, at drug concentrations below 1 mM to purified nerve-ending membrane preparations *in vitro* caused little change in adenylate cyclase activity. At 1 mM concentration, most of the narcotic drugs and related compounds inhibited the enzyme activity to some extent, with highest inhibition by the isomers, levorphanol and dextrorphan, showing that there was no stereospecificity in the inhibition of cyclase activity.

Adenylate cyclase activity in the crude mitochondrial-nerve ending fraction was higher in some areas (midbrain, medulla and striatum) and lower in cerebellum after acute morphine administration at a dose of 60 mg/kg. A lower

dose (25 mg/kg) of morphine did not cause any change in adenylate cyclase activity of crude mitochondrial fractions of mouse cerebral cortex (Naito & Kuriyama, 1973). Similarly, a dose of 15 mg/kg morphine did not alter either the activities of adenylate cyclase or concentrations of cyclic AMP in homogenate of rat cortex, cerebellum and thalamus or hypothalamus 1 h after the injection (Singhal, Kacew & Lafreniere, 1973). Thus, it seems that only a high dose of morphine, which caused profound sedation and catalepsy, could produce significant changes in basal adenylate cyclase activity.

When the effect of morphine administration on adenylate cyclase activity in the isolated crude mitochondrial fraction was compared with that of levorphanol at an equianalgesic dose to morphine and of dextrorphan at the same dose, only levorphanol produced some effects similar to those of morphine on adenylate cyclase activity while dextrorphan had no effect on cyclase activity. Thus, the effect of narcotic drugs *in vivo* on the adenylate cyclase activity seemed to show stereospecificity. Pharmacological specificity, however, was not demonstrated, since a dose of naloxone that blocked the pharmacological responses to morphine did not prevent the increases in cyclase activity induced by morphine. It may be noted that, although there were no statistically significant differences in samples from morphine or morphine plus naloxone treated rats, the trend was toward reversing the decrease in cerebellar activity and enhancing the increase in cyclase activity in other areas. Should this trend be confirmed in further studies, one possible interpretation would be that naloxone, when administered after morphine as in our experimental conditions, was acting as an antagonist in the cerebellum and as an agonist in the medulla and striatum.

In tolerant animals, basal adenylate cyclase activities were the same as in naïve animals in all brain areas, unless an additional injection of morphine (100 mg/kg) was administered 2 h before the rats were killed. The concentrations of cyclic AMP in tolerant rats killed 2 h after the last injection of morphine were higher than those in animals treated acutely, an indication either of tolerance in the response of the cyclic AMP-adenylate cyclase system, or of tolerance in interacting systems in the brain. That the largest changes in the concentration of cyclic AMP and in adenylate cyclase activity produced by the acute administration of morphine are found in the cerebellum, a tissue in which stereospecific opiate binding in *in vitro* systems is low (Kuhar, Pert & Snyder, 1973; Hiller, Pearson & Simon, 1973), suggests that the responses to opiates in the adenylate cyclase system are not a direct consequence of opiate-receptor binding *in situ*, but rather secondary responses (possibly transmitted by the noradrenergic pathway from the locus coeruleus).

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