Previous reports [14, 15] have indicated a role for cyclic AMP in the differentiation of *H. culbertsoni* induced by biogenic amines. The present studies with imidazole suggest that the role of cyclic AMP is that of a second messenger almost comparable to its action on gluconeogenesis in liver and muscle triggered by epinephrine. Moreover, during encystment there is extensive degradation of lipids (M. K. Raizada, unpublished observations) along with glycogen [16–19].

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Central Drug Research Institute, Lucknow, India

ANIL K. VERMA
COIMBATORE R. KRISHNA MURTI

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Characterization of acute tolerance to morphine using reserpine and cycloheximide*

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It has recently been demonstrated that morphine sulfate (MS) (25 mg/kg, 30 min) produces a significant lowering of tissue calcium in regional areas of rat brain [1]. This decrease was also shown to be antagonized by naloxone. In outlining acceptable criteria to establish an action as directly due to opiate effects, it has been necessary to demonstrate selective blockade by opiate antagonists, as well as dose levels approximating a pharmacological dose range. In addition, a major criterion imposed on previous investigations has been the production of tolerance to the parameter being examined. In continuing an investigation into the role of membrane calcium in the actions of opiate analgesics, the present study was undertaken to investigate the ability of morphine to induce tolerance to the calcium depletion effect in regional brain areas of the rat.

Rats (male, Sprague–Dawley), weighing between 175 and 250 g, were used throughout all experiments. Control or drug-treated animals were sacrificed at appropriate times (see table legends) by decapitation. The skull was opened, peeled back and the brain was removed as rapidly as possible into ice-cold isotonic saline. After successive rinses in this medium to remove any adhering blood, the brains were lightly blotted dry, and regional brain dissection was performed according to the method of Glowinski and Iverson [2]. Tissues samples in the range of 10–30 mg were placed in preweighed disposable tubes (Corning disposable culture tubes, 16 × 100 mm).

Calcium levels were determined by atomic absorption spectroscopy using a Perkin Elmer model 303 unit. Concentrated nitric acid (analytical grade) (200 μl) was added and the total contents of each tube were evaporated to a dry ash over a hot plate. After cooling, the residue was resuspended in 0·5 ml of 0·1 N HCl plus 4·5 ml of 1·0% lanthanum (as the oxide) in 0·6 N HCl.

Morphine sulfate (injectable) was purchased from Lilly Laboratories, Indianapolis, Ind.; reserpine was obtained from Ciba-Geigy, Summit, N.J. (Serpasil), and cycloheximide was purchased from Sigma Co., St. Louis, Mo. Lanthanum and calcium standards were obtained from Research Chemical Corp., Sun Valley, Calif., and Fisher Scientific, Fairlawn, N.J.

The development of tolerance to morphine's ability to deplete calcium was examined in the following manner. Animals were given saline or the appropriate drug and sacrificed at the times listed in the table legends. The initial dose of morphine was repeated, and a lesser response was usually observed to the second dose. The observation of this lesser response was used as the criterion for tolerance in this study. Table 1 illustrates the effect of morphine sulfate in producing acute tolerance to the calcium depletion effect at 4 hr. Two groups of animals

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were given a single dose of morphine sulfate, 25 mg/kg, half were sacrificed at 0·5 hr and half at 4·0 hr, and the calcium content in each of eight brain regions was determined, as outlined above. A third group of animals received an initial dose of morphine sulfate at 0 hr followed by a second dose 3·5 hr later. This third group of animals was sacrificed at 4 hr. As shown in Table 1, a single dose of morphine sulfate (25 mg/kg) produces a significant decrease of regional brain calcium. Four hr after the same dose of morphine, calcium levels are returning to control values. Those animals receiving morphine at 0 time and 3·5 hr later show no significant effect from the second 30-min dose of morphine. Animals treated with saline according to the "tolerance regimen" had calcium contents unchanged and were not included in Table 1.

Tolerance to this morphine-induced calcium depletion was further characterized using a protein synthesis inhibitor, cycloheximide, and a biogenic amine-depleting agent, reserpine. Both agents have been previously demonstrated to alter the development of tolerance to morphine [3, 4]. Reserpine, 5 mg/kg, was administered 2 hr prior to the first dose of 25 mg/kg of morphine. A second dose of 25 mg/kg of MS was administered 3·5 hr after the initial MS dose. Results of this experiment are shown in Table 2. Reserpine pretreatment followed by the 4 hr tolerance regimen (see table legend) produced no significant alteration of regional brain calcium levels (see Table 1, tolerance column for comparison). In contrast, however, cycloheximide, 0·5 mg/kg, when used as pretreatment for 1 hr, followed by the 4 hr tolerance regimen (see table legend) produced a significant reduction in brain calcium levels (see Table 1, tolerance column for comparison) in eight discrete brain regions. Reserpine alone at 6 hr produced a significant depletion of calcium, while cycloheximide alone was ineffective.

Table 1. Acute tolerance to morphine sulfate 4 hr after a single dose*

Brain region	Tissue calcium (μg/g wet weight, mean ± S.E.M.) Control	0·5 Hr	4 Hr	Tolerance
Hypothalamus	55·8 ± 2·0	39·8 ± 2·0	48·0 ± 2·5	50·0 ± 4·9
Hippocampus	59·0 ± 2·1	37·4 ± 1·7	49·8 ± 3·6	51·0 ± 3·7
Corpus striatum	56·6 ± 1·9	35·3 ± 2·4	50·6 ± 1·5	51·0 ± 2·1
Cortex	52·1 ± 0·7	36·8 ± 1·0	49·2 ± 0·9	47·5 ± 3·1
Cerebellum	54·8 ± 0·8	35·1 ± 2·2	44·7 ± 2·3	45·7 ± 1·2
Medulla pons	54·6 ± 1·5	36·0 ± 1·7	43·7 ± 2·3	48·0 ± 1·5
Midbrain	56·0 ± 0·9	37·4 ± 1·4	51·0 ± 4·0	51·0 ± 3·8
Thalamus	54·1 ± 1·1	33·5 ± 2·5	46·3 ± 0·8	46·6 ± 1·2

* Morphine sulfate was administered at 25 mg/kg doses for 30-min duration. Calcium determinations were made at 0·5 hr and 4 hr after a single dose of morphine. For tolerance, animals received 25 mg MS at 0 time and 3·5 hr later. Animals were sacrificed 4 hr after the initial (0 time) injection. Tolerance saline controls (0·9%) were included, using equivalent volumes of solutions and comparable injection schedules of 30 min and 4 hr, and were not different from normal saline controls. Values at 0·5 hr are significantly different from controls at $P = 0·001$. There is no significant difference between the values at 4 hr and tolerance values. All values represent the mean of eight to twelve animals.

Table 2. Effect of cycloheximide and reserpine on acute tolerance to morphine*

	Tissue calcium (μg/g wet weight, mean ± S.E.M.) HY	HI	CS	CX	CB	MP	MB	TH
Control (0·9% saline)	55·8 ± 2·0	59·0 ± 2·1	56·6 ± 1·9	52·1 ± 0·7	54·8 ± 0·8	54·6 ± 1·5	56·0 ± 0·9	54·1 ± 1·1
Reserpine† (5 mg/kg)	38·5 ± 1·4	34·4 ± 3·5	33·1 ± 2·6	34·3 ± 1·7	31·8 ± 4·4	33·7 ± 0·7	40·0 ± 1·9	39·4 ± 1·0
Cycloheximide† (0·5 mg/kg)	50·2 ± 3·6	54·9 ± 1·3	55·4 ± 2·8	57·3 ± 2·6	52·3 ± 3·3	59·3 ± 1·2	56·4 ± 1·8	56·1 ± 2·1
Reserpine‡ (5 mg/kg)	51·5 ± 4·7	53·3 ± 2·8	54·4 ± 3·7	58·4 ± 4·0	46·0 ± 2·1	53·8 ± 2·2	51·3 ± 3·0	53·0 ± 2·4
Cycloheximide‡ (0·5 mg/kg)	34·2 ± 4·1	39·6 ± 2·1	33·0 ± 1·7	36·0 ± 3·7	39·6 ± 4·4	34·7 ± 1·7	36·6 ± 0·6	36·2 ± 2·6

* Control animals received 0·9% saline in appropriate volumes. Controls for animals given reserpine and cycloheximide alone were saline treated at comparable time courses and with equivalent volumes. Reserpine was used as the injectable form supplied by Ciba-Geigy, while cycloheximide was dissolved in 0·9% saline.

† Reserpine alone at 6 hr produced a significant depletion of calcium in eight discrete brain regions. Cycloheximide alone at 5 hr had no significant effect on cerebral calcium.

‡ Reserpine (5 mg/kg) and cycloheximide (0·5 mg/kg) were used as 2 hr and 1 hr pretreatment, respectively, followed by the tolerance regimen outlined in the legend of Table 1. Reserpine pretreatment followed by the tolerance regimen produced no significant changes compared to saline controls. Cycloheximide pretreatment produced values significantly different at $P < 0·001$.

The ability of morphine to produce acute tolerance to the calcium-depleting effect is evident from the data in Table 1. Based on the criteria of tolerance that repeated doses at the same level produce less effect than the initial dose, the calcium levels if no tolerance occurred should be similar to the values at 0·5 hr. However, calcium values in eight discrete brain regions were equivalent to the values at 4 hr after a single dose of MS, and for the majority of brain regions were not significantly different from controls. Reserpine failed to alter this apparent acute tolerance to morphine-induced calcium depletion. Evidence has been presented showing that reserpine antagonized the analgesic effect of morphine [5, 6]. However, Smith *et al.* [7], in pretreating animals with reserpine, found no effect on tolerance induction by levorphanol. Studies presented in this paper would also suggest that reserpine is without effect in altering the development of tolerance.

In contrast to the apparent lack of effects using reserpine, cycloheximide has a very significant effect on the tolerance development induced by morphine. Cycloheximide, a protein synthesis inhibitor, has been reported to prevent the development of tolerance and physical dependence and to block the increase in brain serotonin accompanything this tolerance and physical dependence [8]. Further, Cox and Osman [9] have demonstrated that cycloheximide prevented the development of tolerance after continuous morphine infusion. Data presented here demonstrate that cycloheximide, but not reserpine, prevents the development of tolerance within a 4-hr period. This effect at a lower dose level than previously reported for cycloheximide studies (0·5 mg/kg), together with the lack of effect from a biogenic amine-depleting agent, suggests that development of acute tolerance to morphine may depend on very rapid changes in the protein synthesis which may collectively be considered acute cellular adaptation. Possible sites of action for this effect must include protein synthesis at the neuronal cell membrane. Morgan and Austin [10] have demonstrated that, while cycloheximide inhibits protein synthesis in the synaptic membrane or synaptosomal cytoplasm, chloramphenicol causes inhibition in the mitochondrial subfraction. In the

studies of Cox and Osman [9], chloramphenicol had no effect on the development of tolerance to morphine.

The studies here indicate that morphine can cause a very rapid development of tolerance to calcium depletion. The blockade of this effect by a protein synthesis inhibitor, cycloheximide, indicates that morphine may be inducing a rapid alteration of membrane protein synthesis in producing tolerance to calcium depletion.

DAVID H. ROSS
SHERWOOD C. LYNN*

Departments of Pharmacology and Psychiatry, University of Texas Health Science Center, San Antonio, Texas 78284, U.S.A.

* Recipient of an NIMH Psychiatry Training Grant Medical Student Fellowship.

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Formation of angiotensin II from tetradecapeptide renin substrate by angiotensin-converting enzyme*

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Tetradecapeptide renin substrate (TDP, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) was first isolated from a partial tryptic digest of renin substrate (angiotensinogen) [1]. Although TDP can serve as a substrate for both renin [2] and pseudorenin [3], it has not been shown to occur naturally. In the anesthetized, pentolinium-treated rat in our laboratory, TDP elicits a pressor response which is different from that of angiotensin I both in intensity (1:50†) and duration (2×). The ratio of pressor activity of TDP relative to angiotensin II has been reported to be 1:37 [4], while the ratio of contractile activity in the isolated rat colon has been reported as 1:9 [5]. A ratio of 1:15 has been reported for the stimulation of release of catecholamines from the isolated cat adrenal gland [6]. Montague *et al.* [7] have reported a TDP:angiotensin II ratio of 1:12 for both pressor activity in the rat and contractile activity of isolated guinea pig ileum. It is not known whether these biological activities are due to the complete 14-residue sequence of TDP or are a function of a smaller peptide (e.g. angiotensin II) produced by hydrolysis of TDP.

Angiotensin-converting enzyme has been shown to act on a wide variety of peptide substrates *in vitro* by removing dipeptide units from the C-terminus of the peptide chain [8]. Our present study reports the finding that angiotensin-converting enzyme can form angiotensin II from TDP by the successive removal of three dipeptides: Tyr-Ser, Leu-Val and His-Leu. Since the enzyme cannot hydrolyze a peptide bond involving the imino group of proline [9], further hydrolysis is prevented, and angiotensin II is the limit peptide.

TDP and the nonapeptide (His^6-Ser^{14})‡ were synthesized by the solid phase method as described previously [3], and angiotensin-converting enzyme was purified from hog lung [10]. His-Leu was synthesized in this laboratory [11], Leu-Val was purchased from Fox Chemical Co. and Tyr-Ser was made by reducing Cbz-Tyr-Ser (Cyclo Chemical Co.) with H_2/Pd. The concentrations of solutions of the peptides were determined by amino acid analysis after acid hydrolysis. The synthetic peptide < Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro was a gift from Dr. J. W. Ryan, Papanicolaou Cancer Research Institute. Biological assays were performed in the anesthetized, pentolinium-treated rat, using angiotensin I as a standard as previously described [12].

Initial reaction rates were determined with a Technicon Auto Analyzer using the ninhydrin-reaction procedure described previously [13]. This procedure measures continuously the formation of new free amino groups as peptide bonds are hydrolyzed. TDP ($9{\cdot}2 \times 10^{-6}$ M) was incubated at 37° with converting enzyme in 0·05 M sodium-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7·5, with NaCl at the indicated concentration in a total volume of 10 ml. The incubation mixtures were sampled into the AutoAnalyzer continuously for 18 min, and enzyme velocities were calculated from the slopes of the recordings. Rates are expressed as μmoles dipeptide formed/min/mg of enzyme in terms of leucine color equivalents. Color values of the dipeptides relative to leucine (100) are: Tyr-Ser, 87; Leu-Val, 90; and His-Leu, 52. Hydrolysis rates for the nonapeptide (His^6-Ser^{14}) were determined in a similar manner.

The initial velocity of TDP hydrolysis by converting enzyme is dependent on chloride, as shown in Table 1.

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† Ratios of biological activity of TDP:angiotensin have been calculated on a molar basis.

‡ Peptide fragments of TDP are numbered according to their position in the TDP sequence.