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Differences in Simultaneous Cylic Adenosine Monophosphate Concentrations of Various Rat Tissues after Heroin Administration (39235)

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(Introduced by M. R. Nocenti)

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Acute opiate administration has been shown to have effects upon neurotransmitter metabolism and neuroendocrine functions thought to be modulated by intracellular cyclic adenosine monophosphate (cAMP) (1, 2). Changes in cAMP concentrations are often indicative of exogenously stimulated cellular functions (2-4). The ubiquity of the adenyl cyclase-cAMP system, modulating cellular responses to various membrane receptor stimuli (5), is consistent with evolutionary economy in the adaptation of cellular metabolic machinery for differentiated responses. Measurement of tissue cAMP concentrations, representative of *in vivo* conditions, seemed, therefore, a possible measure of the integrated effects of these various influences. The purposes of this study were to ascertain the interanimal reproducibility of basal cAMP concentrations, simultaneously determined in several tissues, and whether significant or tissue specific cAMP concentration differences are found after opiate administration.

Methods. Age-matched, male Sprague-Dawley rats (Simonsen Labs), weighing 250-300 g were selected for the experimental model. Total body immersion in liquid nitrogen was chosen as the method of sacrifice, in accordance with the report of Lust *et al.* (6), that freezing was found, at least in the brain, to produce the least artefactual change of the available methods suitable for approximately simultaneous fixation of all body tissues. To this end, a large bore, insulated Pyrex flask was constructed to contain sufficient liquid nitrogen to assure rapid, complete immersion of the animals. Thermal gradients and variations in internal tissue fixation times were not evaluated with this technique, but were assumed to be similar in the matched populations studied. The animals were chosen at random from a larger population for allocation to the experimental groups. Since testing of compounds requiring intraperitoneal injection was anticipated, the group of control animals was subdivided. Eight animals received 1 ml of sterile water ip. Three animals received 1 ml of normal saline, and three animals no injection. Experimental results from these controls were compared statistically to assure internal consistency in their use as a control group.

Heroin was the experimental opiate chosen. The active metabolite, 6-monoacetyl morphine, is rapidly distributed to affected tissues and crosses the blood-brain barrier into the CNS (7). The heroin (99% pure supplied by DEA) was diluted with sterile water to provide 10 mg/kg in 1-ml injections for each rat, an analgesic equivalent to the amount of morphine previously shown to significantly alter *in vivo* catecholamine metabolism (8), and effect neuroendocrine changes (1).

Duplicate frozen tissue samples were obtained from each animal by dissection in a cold box over Dry Ice, immediately were transferred to tubes containing 1 ml of 5% trichloroacetic acid, were homogenized at 0-4° with a rotary Teflon pestle, and centrifuged at 3000*g* for 20 min to separate the cAMP containing supernatent from the protein containing precipitate. The precipitate was redissolved with 10% NaOH at 90° for 10 min. Protein was assayed with the biuret reaction, using recrystallized bovine serum albumin (Sigma Chemical Co.) standards.

Cylic AMP radioimmunoassays were performed in duplicate, using extracts of the supernatant, according to the protocol of the Schwarz/Mann RIA (1973-74 Cat. No. 0750-13) based on the procedure of Steiner

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et al. (9). Unlabeled cAMP used was from Cal Biochem. The assay was sensitive to 0.025 pmol cAMP/tube (0.5 ml). Analytical results were calculated using a logit transformation (10) program on a Hewlett-Packard 9800 series calculator, and are reported as picomoles of cAMP per milligram of TCA precipitated protein.

To provide an arbitrary standard for evaluation of the experimental system, monitoring recovery and reproducibility, and for standardization between experimental series, 5% TCA homogenates of five frozen rat brains were pooled in a volume of TCA adjusted to approximate the protein concentration of the usual brain tissue samples. The material was divided in half, and cAMP added to one half to a concentration of 5 pmole/mg of protein. Aliquots of this material were frozen, or lyophilized for comparison with frozen aliquots, to evaluate their utility as long-term controls.

The analysis of variance (ANOV) technique was used for all statistical analyses. The level of significance was arbitrarily chosen to require $P < 0.005$.

Results. Table I summarizes data pertinent to establishing the reproducibility of the assay and the recovery of cAMP with this experimental technique. There are no significant differences between identical samples assayed at different times, or between frozen and lyophilized controls, at either physiologic concentrations or with cAMP added to the homogenate to provide an additional 5 pmole/mg of protein. The approximate percentage recovery of cAMP was 64.6%.

The experimental results shown in Table II are the mean simultaneous tissue cAMP concentrations from sham-injected control animals, and animals sacrificed 30 min after being injected with heroin. The values used for these calculations were the average of duplicate assays of two similar tissue samples.

After heroin treatment, the mean cAMP concentrations rose to some extent in all tissues studied. This increase was significant ($P < 0.005$) in hypothalamus (237%), cerebral cortex (247%), cerebellum (246%), liver (83%), adrenal cortex (150%), and adrenal medulla (593%). The increase was statistically less significant ($P > 0.1$) in the heart (78%), kidney cortex (17%), skeletal muscle (6%), and fat (80%).

Discussion. The results of these studies suggest that the effects of acute heroin administration on cAMP concentrations in these tissues can be considered in three distinct groups. The greatest percentage changes in tissue cAMP concentrations were in neural tissues or tissues derived from neural anlagen: adrenal medulla, cerebral cortex, cerebellum, and hypothalamus. This is consistent with the identification of the opiate receptor, from competitive binding studies with agonist and antagonist drugs and the localization of the specific binding to neural tissue membranes (11). De Wied has suggested, after a recent review of the literature concerning the neuroendocrine effects of opiates, that the *in vivo* effects of opiate administration are a combination of altered neurotransmitter metabolism and associated neuroendocrine changes in a complex interdependent pattern. Acute opiate administration stimulates, in rats, the release of ACTH and GH, may stimulate TSH and prolactin release, and blocks FSH/

TABLE I. ASSAYED cAMP CONCENTRATION (PMOLES PER MILLIGRAM OF PROTEIN) IN BRAIN HOMOGENATE.[a]

Series	Frozen	Lyophilized	Frozen + cAMP	Lyophilized + cAMP
I ($n = 4$) Mean ± SEM	3.98 ± 0.22	3.99 ± 0.19	7.42 ± 0.21	8.02 ± 0.56
II ($n = 6$) Mean ± SEM	4.74 ± 0.26		7.30 ± 0.36	

[a] Summary of assay reproducibility and recovery data from multiple assays of the standard brain homogenate, each done in duplicate, with and without the addition of exogenous cAMP, 5 pmoles/mg of protein. Mean, homogenates without added cAMP, 4.31; Mean, homogenates with 5 pmole cAMP/mg protein added, 7.54; Mean recovery cAMP; 64.6%.

TABLE II. COMPARISON OF MEAN TISSUE cAMP CONCENTRATIONS (PICOMOLES/MILLIGRAMS OF PROTEIN) FROM CONTROL[a] AND HEROIN-TREATED ANIMALS.[b]

Tissue	Control (n = 14) (mean ± SEM)	Heroin Treated (n = 6) (Mean ± SEM)	ANOV P
Hypothalamus	5.76 ± 0.67	19.42 ± 6.24	<0.005
Cerebral cortex	7.53 ± 1.25	26.09 ± 8.11	<0.005
Cerebellum	3.92 ± 0.50	13.55 ± 4.12	<0.005
Heart	2.71 ± 1.49	4.01 ± 0.83	NS
Liver	1.73 ± 0.22	3.22 ± 0.31	<0.005
Kidney	2.96 ± 0.40	3.46 ± 0.53	NS
Adrenal cortex	4.31 ± 0.46	10.79 ± 1.17	<0.005
Adrenal medulla	2.21 ± 0.38	15.31 ± 4.53	<0.005
Muscle	2.36 ± 0.21	2.49 ± 0.38	NS
Fat	1.49 ± 0.21	2.68 ± 0.69	NS

[a] Two series of sham-injected and uninjected control rats.
[b] Two series of control matched rats given heroin, 10 mg/kg, by ip injection.

LH secretion (1). The concept of a spectrum of direct and secondary responses is supported by the finding of significant, though smaller, cAMP concentration changes in predominantly nonneural tissues, adrenal cortex, and liver. In contrast to this, the consistent percentage of cAMP concentration increases observed in the third group of heroin-treated tissues, heart, kidney, skeletal muscle, and fat, were not statistically different from their matched controls.

It is possible the differences demonstrated by this experimental approach are tissue specific effects, the combined result of primary heroin modified neural function and secondary hormonal effects. Aminergic and hormone sensitive adenyl cyclases are present in all of the tissues studied. Studies of specific membrane receptor linked adenyl cyclase-cAMP systems modulating cellular responses are extensive (2). Hepatic adenyl cyclase modulates glycogenolysis in response to stimulation by catecholamines, glucagon, and growth hormone. Stimulation of adrenal cortical adenyl cyclase by ACTH and possibly catecholamines is associated with accelerated corticosteroid synthesis and release. Individually, components of these sequences have been found to influence neurotransmitter metabolism and activity, and various adenyl cyclase linked receptor activities (1, 2). There may, however, be other factors involved. Hypoxia is associated with tissue cAMP concentration increases (6). The role of opiate induced, presacrifice respiratory depression, and the linkage between *in vivo* hypoxia and tissue cAMP concentration changes cannot be determined from this series of experiments. In the apparently unresponsive tissues, affected cells may not comprise a percentage of the tissue sample assayed sufficient to demonstrate any of these effects.

Evidence to date has associated increased intracellular cAMP with cellular stimulation by receptor specific hormones and neurotransmitters modulating differentiated cellular functions. These experiments have revealed, therefore, an interesting inconsistency in addition to the cAMP variations associated with these experimental conditions. Cyclic AMP in the liver increased significantly while fat cAMP, usually associated with lipolysis under stimulation by the same hormones as the hepatic adenyl cyclase, remained unchanged. This inconsistency supports the suggestion that tissue or cellular cAMP concentrations may be a useful indicator of the *in vivo* activity of interdependent membrane receptors and functionally oriented subcellular metabolism. Investigations are now progressing to refine these techniques, and to combine cellular identification and quantitation of cAMP in tissue sections by immunohistochemistry.

Summary. Opiate administration has profound effects on neurotransmitter metabolism and neuroendocrine functions. Since cAMP is an intracellular metabolite common to the actions of many of the involved compounds stimulating distinct and specific membrane receptors, cAMP concentrations representative of *in vivo* conditions in heroin-treated and control animals were deter-

mined. Rats were sacrificed by immersion in liquid nitrogen 30 min after heroin injection. Tissue samples, dissected without thawing, were assayed by radioimmunoassay to determine cAMP/mg of protein. Comparisons between treated animals and sham-injected controls revealed significant cAMP concentration increases in hypothalamus, cerebral cortex, cerebellum, adrenal medulla, adrenal cortex, and liver ($P < 0.005$). Cyclic AMP concentrations in heart, skeletal muscle, kidney, and fat were not significantly different from controls. The increased tissue cAMP concentrations associated with the altered physiology of heroin administration apparently represent a pattern of integrated responses to the combination of opiate receptor binding and effects on neurotransmitter metabolism and circulating hormones.

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