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Presence and formation of codeine and morphine in the rat

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ABSTRACT **Endogenous codeine and morphine were identified in rat brain by immunological determination following HPLC. To demonstrate occurrence of a biosynthetic pathway to morphine in mammals similar to that used by the poppy plant, (+)-salutaridine, (−)-thebaine, and (−)-codeine were administered to rats intravenously. These compounds, which are intermediates in the synthesis of morphine in *Papaver somniferum*, caused a marked increase in the codeine and morphine levels in rat tissues. This provides evidence for a biosynthetic pathway to morphine in mammalians.**

The identification by Hughes *et al.* (1) of two endogenous opioid peptides, enkephalins, that interact with one of the many opioid receptors (δ) has initiated a search for the endogenous ligands for the other opioid receptors. Chavkin *et al.* (2) have proposed that another opioid peptide, dynorphin, could be the ligand for the κ receptor. Although β-endorphin has a certain affinity and selectivity for the μ-opiate receptor site, the prototypical agonist for this receptor is morphine.

Several years ago we reported the presence of a morphine-like material in mammalian tissue utilizing an antibody developed against morphine (3). This was then confirmed by Killian *et al.* (4). Recently we identified this morphine-like compound in toad skin and found it to be morphine (5). We have also been able to identify morphine in the skin of rats and rabbits (5). Goldstein *et al.* (6) reported the presence of morphine and three other closely related compounds in beef hypothalamus and adrenal. One of the issues that has to be resolved before we can definitively say that the opiate alkaloid is endogenously present and not from exogenous source is to demonstrate its synthesis in mammalian tissues. It has been shown that in the plant (+)-salutaridine, (−)-thebaine, and (−)-codeine are the direct precursors of natural (−)-morphine, with (−)-neopinone and (−)-codeinone being discrete intermediates in the conversion of (−)-thebaine to (−)-codeine (7, 8). In this study we present evidence for the conversion of these precursors to morphine in mammalian tissues.

METHODS

Male Sprague–Dawley rats weighing 200–300 g and fasting overnight were used. (+)-Salutaridine prepared from (−)-thebaine by a multistep procedure§ (12, 13) and natural (−)-thebaine crystallized from ethanol were found by GC/MS to be free from morphine and codeine. (−)-Codeine (Merck) was free of morphine as measured by HPLC and RIA. The substances were dissolved in a minimum volume of 0.1 M HCl and the pH was adjusted to 7.4 with phosphate-buffered saline (PBS). The compounds or saline were injected into the rat's tail vein; 1 hr later the animals were killed and brain, blood, small intestine, liver, and kidney were removed and placed on dry ice. The tissues were homogenized in 5 vol of acidified methanol and centrifuged at 20,000 × *g* for 10 min. The supernatant was evaporated to dryness, redissolved in PBS, and liquid/liquid extracted as described by Oka *et al.* (5).

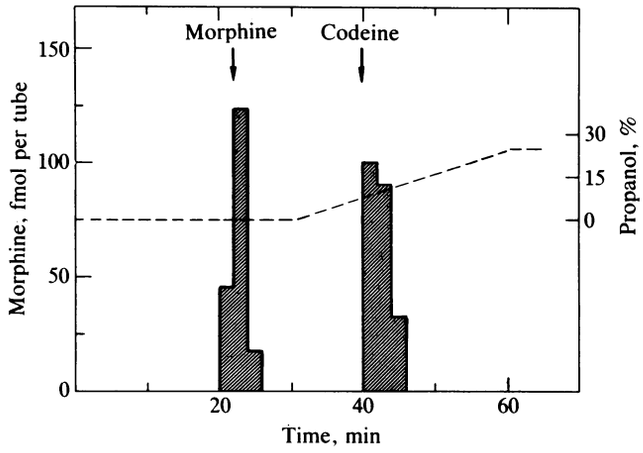

FIG. 1. Reversed-phase HPLC RP-18 of rat brain extract monitored by RIA. The brains from three rats that had been injected intravenously with (+)-salutaridine (31 nmol/g) were extracted 1 hr later.

The extract was applied to a Sephadex G-15 column (0.9 × 60 cm) and eluted with 0.1 M pyridine/acetic acid, pH 6.2. The morphine-immunoreactive fractions of gel chromatography were further separated by reversed-phase HPLC (LiChrosorb RP-18 column, 0.4 × 25 cm, Merck). The samples were eluted with 0.1 M pyridine/acetic acid, pH 6.2, followed by a gradient of 1-propanol/0.1 M pyridine/acetic acid at a flow rate of 1.5 ml/min. Two-minute fractions were collected and assayed for morphine and codeine by a sensitive RIA. The antiserum was raised against 3-carboxymethylmorphine conjugated to bovine serum albumin (9) and used in a final dilution of 10^{-6} with ^{125}I-labeled morphine (Hoffmann–La Roche) as tracer. Codeine crossreacts with the antibody equally as well as morphine. The crossreactivity with thebaine is 9% and with salutaridine 0.004%, but these substances have an elution pattern on RP-18 HPLC that is distinct from codeine and morphine and therefore can be readily separated from codeine and morphine. The detection limit of the assay was 15 fmol of morphine or codeine per tube. The immunoreactive fractions obtained from reversed-phase HPLC at the position of the standards (Fig. 1, morphine and codeine) were subjected to ion-exchange HPLC using a Whatman Partisil 10 SCX column (0.4 × 25 cm) with 0.1 M acetic acid/pyridine, pH 3.5, as the mobile phase at a flow rate of 1 ml/min. Recovery of morphine and codeine through the procedure was 50–60%. Mouse monoclonal antibodies were developed against the immunogens 3-

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§Details on the preparation of pure salutaridine {EtOAc, mp 199–201°C; $[\alpha]20_D$ +87° (*c* 0.49, $CHCl_3$)} and pure thebaine {EtOH, mp 193–195°C; $[\alpha]20_D$ −227° (*c* 10, $CHCl_3$)} and additional measurements of chemical purity will be published elsewhere.

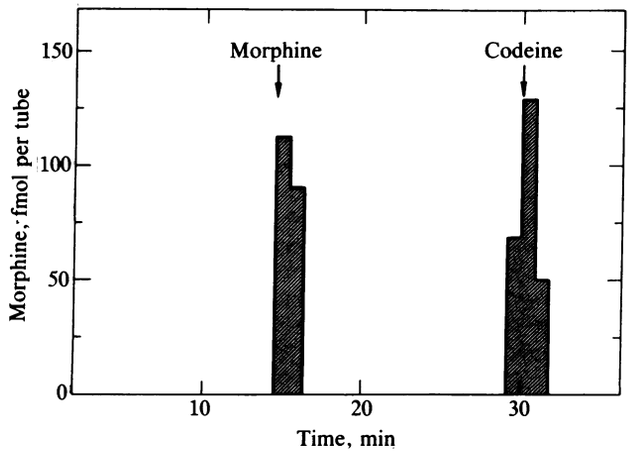

FIG. 2. Ion-exchange HPLC of rat brain extract monitored by RIA. The brains from three rats given 31 nmol of (+)-salutaridine per g intravenously 1 hr earlier were extracted.

carboxymethylmorphine and *N*-carboxymethylmorphine. The monoclonal antibodies were used to confirm the immunoreactivity of codeine and morphine from the Sephadex G-15 and HPLC columns detected by the polyclonal antibodies. The polyclonal antibodies were then used in these studies as they had a higher affinity for the alkaloids (5).

RESULTS AND DISCUSSION

For quantitative determination of morphine and codeine, the tissue extracts were assayed by a RIA after reversed-phase HPLC separation (Fig. 1). Since codeine crossreacts equally as well as morphine with the antibody, codeine concentrations were assayed in that peak where the codeine standard eluted. The endogenous substances and standards for morphine and codeine also showed identical retention time on ion-exchange HPLC (Fig. 2). Thus, the endogenous substances had identical chromatographic behavior to the standards on two different HPLC systems, and in the RIA the substances produced a parallel displacement of labeled morphine from an antibody when compared with the standard curves.

As can be seen from Tables 1 and 2, rat tissues contain morphine and codeine; the concentration of codeine in brain tissue is greater than that of morphine. The presence of the precursor for morphine in mammalian tissues provides evidence for a biosynthetic pathway of morphine with codeine being the immediate precursor.

Tables 1 and 2 show the increase of codeine and morphine content in various tissues following the intravenous injection of the precursors. The greatest conversion was from codeine to morphine. Conversion of codeine to morphine involves demethylation of the methoxy group at C-3. In a previous report, we demonstrated a good conversion rate of codeine to morphine (10). To ascertain whether the conversion of codeine to morphine was reversible, 7 nmol of morphine per g was injected intravenously into rats. If there were any

Table 1. Codeine concentration (fmol/g) in rat tissues

Tissue	Saline	Salutaridine	Thebaine
Brain	79 ± 16	161 ± 45*	392 ± 103*
Intestine	24 ± 6	322 ± 89*	346 ± 68*
Liver	12 ± 2	189 ± 55*	348 ± 60*
Kidney	21 ± 5	161 ± 42*	820 ± 201*
Blood	8 ± 2	64 ± 22*	101 ± 19*

The codeine was measured 1 hr after the intravenous injection of salutaridine (31 nmol/g), thebaine (10 nmol/g), or saline (control). Values are given as mean ± SEM (n = 4–6).
*$P < 0.05$.

Table 2. Morphine concentration (fmol/g) in rat tissues

Tissue	Saline	Salutaridine	Thebaine	Codeine
Brain	26 ± 6	59 ± 22	96 ± 22*	16,045 ± 2,911*
Intestine	17 ± 6	99 ± 36*	112 ± 19*	182,412 ± 17,503*
Liver	11 ± 2	136 ± 37*	150 ± 27*	114,685 ± 24,074*
Kidney	16 ± 5	139 ± 37*	398 ± 94*	491,460 ± 63,900*
Blood	2 ± 1	17 ± 6*	13 ± 3*	9,063 ± 2,915*

The morphine content was measured 1 hr after the intravenous injection of salutaridine (31 nmol/g), thebaine (10 nmol/g), codeine (17 nmol/g), or saline (control). Values are given as mean ± SEM (n = 4–6).
*$P < 0.05$.

formation of codeine from morphine, it would have been below the level of detection by our assay. Salutaridine and thebaine were converted in brain, intestine, liver, kidney, and blood to codeine and morphine. These were the only tissues we analyzed, but this conversion may occur in other tissues as well. The extent to which salutaridine or thebaine forms codeine seems to be greater than their further conversion into morphine. It is possible that codeine has two roles: (*i*) it is a precursor for the synthesis of morphine and (*ii*) it has a role and localization independent of morphine.

There are a number of variables to be kept in mind in this *in vivo* study that could explain the apparently low conversion rate of the precursors salutaridine and thebaine to morphine. It is likely that these intermediates undergo rapid metabolism and excretion. Newly synthesized codeine and morphine might be localized in distinct structures of tissues; we assayed their concentration in whole organs.

It is interesting to note that the kidneys, which are the primary avenue of excretion (11), have the highest concentration of the alkaloids. We also have made the assumption that in mammalian tissue exactly the same biosynthetic pathway exists as in the poppy plant, although the K_m values for some of the enzymes and intermediates may not be identical. A critical point is that the concentrations of morphine required for an effective interaction with μ-opiate receptors are in the order of 1 nM. Thus, it would require amounts greater than we obtained with the precursors salutaridine and thebaine; however, the conversion of codeine to morphine is sufficiently high for interaction with the μ receptor. Rate of formation of codeine and morphine may be accelerated under different physiological and pathological conditions.

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