Identification of the convulsant opiate thebaine in mammalian brain

(radioimmunoassay/high-performance liquid chromatography/gas chromatography/mass spectrometry)

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ABSTRACT The convulsant opiate thebaine, an intermediate of morphine biosynthesis, was purified from ovine brain to homogeneity by gel filtration and high-performance liquid chromatography (HPLC) monitored by a radioimmunoassay. The immunoreactive material behaved identically to standard thebaine in two HPLC systems and was confirmed to be thebaine by combined gas chromatography/mass spectrometry. To our knowledge, the presence of thebaine in mammalian tissue has not been demonstrated previously. Codeine and morphine were also found to exist in ovine brain. The presence of thebaine in ovine brain provides strong evidence that morphine and codeine, in various mammalian tissues, are of endogenous origin and actually biosynthesized from a precursor.

Recent evidence has focused attention on the existence of endogenous opiate alkaloids in mammalian tissues (1–3). These molecules may then constitute a component of a unique regulatory pathway perhaps involving enkephalin, dynorphin, and endorphin, the more fully characterized opiate peptides. This notion is substantiated by the finding that the levels of two opiate alkaloids, morphine and codeine, are not static but rather undergo rapid alterations following physiological stimulation (3, 4), whereas bioconversion of various opiate precursors can be demonstrated in vivo (1, 5) or in vitro (5, 6). A critical issue is to establish whether any of these intermediates actually exists in brain and further to determine whether they have biological activity in their own right. We report here that one opiate, thebaine, a precursor in the biosynthesis of morphine and codeine that has convulsant properties, is present in ovine brain.

METHODS

Materials. Ovine brains (from Colorado Lamb, Denver, CO) were used for the isolation of endogenous opiate alkaloids. (−)-Codeine (Hoffmann-La Roche) was cryostabilized from methanol was used for radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), and gas chromatography/mass spectrometry (GC/MS) analysis as a standard. (−)-Codeine was from Merck. Morphine hydrochloride and 125I-labeled morphine (90–150 Ci/mmol; 1 Ci = 37 GBq) were from Hoffmann-La Roche. Other reagents were purchased from Baker or Sigma.

RIA. A rabbit antiserum that was raised against 3-carboxymethoxyphenol/morphine conjugated to bovine serum albumin (7) and 125I-labeled morphine as tracer were used in the RIA. The sensitivity of the assay to thebaine (IC50 = 0.58 ± 0.04 pmol (mean ± SEM) is about 1/10th that of morphine or codeine, both of which react equally well with the antiserum (6). The detection limit of the assay for thebaine is 0.08 pmol. With this level of sensitivity we were able to detect a thebaine-like compound during the purification steps.

Extraction and Purification Procedures. For purification of thebaine-like immunoreactive (ir) compound from ovine brain, lyophilized ovine brain (2000 g) was pulverized, defatted with two times 3 vol of methylene chloride, and dried. The defatted material was heated in 10 vol of 1 M acetic acid containing 10 mM HCl at 95–100°C for 10 min, cooled on ice, homogenized in a Polytron, and maintained at 4°C overnight. The material was centrifuged (10,000 × g, 30 min) and the supernatant was subjected to ultrafiltration (Amicon YM-10) at 4°C. The filtrate was lyophilized. Step 1 (Fig. 1A): Gel filtration at 4°C on a 5.0 × 140 cm column of Sephadex G-50 equilibrated with 1 M acetic acid. One-fourth of the extract was reconstituted with 150 ml of the eluent, centrifuged, and applied to the column; flow rate was 15 ml/min and 15-ml fractions were collected. The effluent was monitored by UV absorbance at 280 nm (model 100-40 spectrophotometer, Hitachi). Of the total volume column, every 10 fractions were combined and lyophilized. This gel filtration procedure was repeated four times. To determine which fractions from Sephadex G-50 column contain morphine-related ir substances, fractions were then applied to a Sephadex G-15 column. The following three low molecular weight fractions were found to have the ir substance: fractions 121–130, 38 g; 131–140, 44 g; 141–150, 29 g. Step 2 (Fig. 1B): Gel filtration on a 3.0 × 100 cm column of Sephadex G-15 equilibrated with 0.1 M pyridine/acetic acid, pH 4.7. One-fifth of fractions 121–130, from step 1, was reconstituted with 10 ml of buffer and applied to the column; flow rate was 1.07 ml/min and 11-ml fractions were collected. The effluent was monitored by UV detector (model 153, Altex) at 254 nm. Aliquots of each fraction were dried (Savant Speed-Vac concentrator), reconstituted in Dulbecco’s phosphate-buffered saline (pH 7.4), and assayed with a RIA for the opiate alkaloids. The ir fractions (bracket) from five successive runs were pooled and dried. Step 3 (Fig. 2): One-eighth of the pooled material from step 2 was applied to a 0.4 × 25 cm Merck LiChrosorb RP18 column (5-μm particles), using a Beckman model 322 solvent delivery system, eluted at a rate of 1.45 ml/min with 0.1 M pyridine/acetic acid, pH 4.7, containing 12% acetonitrile (vol/vol), and then flushed with 0.2 M pyridine/acetic acid, pH 4.7, containing 75% acetonitrile (vol/vol). The elution was monitored by electrochemical (EC) detector (model LC-4A, Biochemical Analytical Systems) at an oxidation potential of 500 mV vs. a Ag/AgCl electrode (6). Aliquots of fractions were assayed by RIA. The two major ir fractions (brackets) from 18 successive runs were pooled and dried. Step 4 (Fig. 3): The combined second peak (bracket) from the 18 runs in step 3 was applied to a 0.46 × 25 cm Whatman Partisil 10 SCX ion-exchange column and eluted isocratically at a rate of 1 ml/min with 0.2 M acetic acid/pyridine, pH 3.5. The effluent was monitored by EC detector at 800 mV. Aliquots of fractions were assayed by

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Abbreviations: ir, immunoreactive; EC, electrochemical.
RIA. The position of the ir peak was identical to that of standard thebaine (arrow). The fractions between 87 and 93 min were combined and evaporated to dryness. Step 5 (Fig. 4): The ir peak from step 4 was applied to the same RP18 column as in step 3 and eluted at 1.45 ml/min with 0.1 M pyridine/acetic acid, pH 4.7, containing 12% acetonitrile, giving a detectable EC response peak at 750 mV and an ir peak (total, 45 pmol). The ir peak, coeluted with standard thebaine (arrow), was evaporated to dryness and a portion (30 pmol) was used for GC/MS analysis. Portions of the HPLC solvent blank and standard thebaine that were passed through the reverse-phase HPLC system (Fig. 4) were also used for GC/MS analysis (see below). During the HPLC purification steps, standard thebaine, codeine, or morphine was assayed following each fractionation step. Following application of the standard, the injection system and the HPLC columns were washed with methanol and/or chloroform plus multiple injections of 30% acetic acid/methanol (vol/vol) or dimethyl sulfoxide to remove any standard retained in the system. After washing, immunoreactivity was not detected at the retention times of the standards.

**GC/MS Procedure.** The HPLC solvent blank at the retention time of thebaine, the putative thebaine from ovine brain (30 pmol), and standard thebaine (35 pmol), which were collected at step 5 in Fig. 4, were used for this analysis. Between each analysis, a solvent (acetonitrile) blank was also used to eliminate any possible contamination of the endogenous sample with standard sample. No trace of peaks corresponding in m/z values of major ions in thebaine spectra were observed in any of the blanks analyzed. The GC/MS system used was a VG Masslab 30–253 quadrupole mass spectrometer (electron-ir ionization mode at 25 eV and 150 μA) interfaced to a Hewlett-Packard 5890 gas chromatograph. GC column: Hewlett-Packard crosslinked methyl silicone fused silica capillary, 25 m × 0.31 mm i.d.; film thickness, 0.17 μm. Injection port and transfer line: 200°C and 220°C. GC oven conditions: 50°C held for 2 min; 10°C/min to 225°C; 225°C held for 2 min. Carrier gas: He.

**RESULTS**

Thebaine-like ir compound was purified from an acetic acid-containing HCl extract of ovine brain by gel filtration, reverse-phase (RP18), and ion-exchange HPLC systems. Fractions were assayed with a RIA to detect morphine-

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**Fig. 1.** Gel filtration chromatograms and morphine-related immunoreactivity: Sephadex G-50 of ovine brain extract (A) and Sephadex G-15 of fractions 121–130 from Sephadex G-50 (B). Effluent was monitored by UV detection and RIA for morphine. Shaded bar graphs represent morphine-related immunoreactivity.

**Fig. 2.** Reverse-phase HPLC chromatogram of morphine-related ir compounds from Sephadex G-15. Effluent was monitored by EC detection and RIA. Shaded bar graphs represent immunoreactivity. The second major ir peaks (bracket) were calculated using thebaine as a standard. The arrow indicates the position of standard thebaine in the HPLC system. The first major (bracket) and the other minor ir peaks were calculated using morphine as a standard.

**Fig. 3.** Ion-exchange HPLC chromatogram of thebaine-like ir compound from reverse-phase HPLC. Effluent was monitored by EC detection and RIA. Shaded bar graphs represent thebaine immunoreactivity. The arrow indicates the position of standard thebaine in the HPLC system.

**Fig. 4.** Reverse-phase HPLC chromatogram of thebaine-like ir compound from ion-exchange HPLC. Effluent was monitored by EC detection and RIA. Shaded bar graphs represent thebaine immunoreactivity. Trace A, HPLC solvent blank; trace B, EC response peak of standard thebaine when 65 pmol of the standard was applied to the HPLC system (arrow). Portions of these samples were used for GC/MS analysis (see Fig. 5).
related ir substances, including morphine, codeine, and thebaine.

Figs. 1–4 show the purification procedures of thebaine-like ir compound from ovine brain. The ovine brain acid extract was first subjected to Sephadex G-50 filtration (Fig. 1A). The RIA was not performed at this stage because of the high concentration of acid in each fraction, which interfered with the assay. The low molecular weight material from the Sephadex G-50 filtration was applied to a Sephadex G-15 column, and a broad region of morphine-related ir compounds was found (Fig. 1B). Application of this ir fraction to a RP18 column resulted in two main ir peaks (Fig. 2). The first peak was similar to the position where standard morphine and codeine eluted. Morphine and codeine cannot be separated using this solvent system. The second peak eluted in the same position as standard thebaine. The second peak was then applied to an ion-exchange column for further purification. It was found to have an identical retention time as that of standard thebaine (Fig. 3). The thebaine-like ir peak was rechromatographed on the same RP18 column used in step 3 (Fig. 4). A single EC response peak associated with immunoreactivity occurred where standard thebaine eluted (Fig. 4). The total amount isolated was 45 pmol. Portions of this endogenous putative thebaine, and also standard thebaine and HPLC solvent blank collected at step 5 in Fig. 4, were then subjected to GC/MS analysis (Fig. 5). The electron-ionization mass spectrum of standard thebaine has a base peak corresponding to the molecular ion at m/z 311 and two major fragment ions at m/z 296 and m/z 255. These fragment ions result from loss of a methyl group and the N-methyl bridge with hydrogen transfer (C2H2N) (8), respectively. Standard thebaine and the endogenous putative thebaine eluted at the same GC oven temperature (192°C) and retention time (16.2 min), and all three of the above diagnostic ions plus numerous other characteristic lower mass fragment ions occurred in both samples with similar relative intensities (Fig. 5). From these results, we conclude that the isolated compound is thebaine. The first ir peak at step 3 (Fig. 2) was also purified by RP18 and ion-exchange columns using different solvent systems (see ref. 6), resulting in the isolation of codeine (total, 64 pmol) and a trace of morphine.

DISCUSSION

In this paper we report the presence of the alkaloid thebaine in mammalian tissue. In addition to serving as an intermediate in the synthesis of codeine and morphine (1, 6), thebaine may also exert some distinct physiological effect of its own.

Higher concentrations of codeine and morphine can be detected in tissues after boiling and acid hydrolysis with 10% HCl, since these alkaloids exist in a conjugated form or bound to protein (3). As thebaine is unstable under acid hydrolysis, this procedure was omitted. The codeine and morphine isolated in this study were probably free in tissue. Thebaine can bind to plasma protein and albumin (9); therefore, it is possible that thebaine might exist in tissue bound to proteins.

In the poppy plant, 

Papaver somniferum, the morphine-type alkaloid thebaine is biosynthesized from two molecules of L-tyrosine (10) by way of several intermediates, such as norcoctaaine, coctaine (11, 12), reticuline (13), and salutaridine (14). It has recently been shown that norcoctaaine and coctaine are true precursors of reticuline and thebaine (11, 12), though tetrahydropapaveroline (THP, norlaudanosolane) had long been assumed to be the precursor (15, 16). The possible role of THP as a precursor in the biosynthesis of morphine in mammals is unclear. THP, which is formed by the condensation between dopamine and an aldehyde derived from a second dopamine molecule (17), has been identified in the brain of rats receiving L-3,4-dihydroxyphenylalanine (L-dopa) (18) as well as in the urine of parkinsonian patients who have been given L-dopa (19). However, it has not been detected in normal mammalian tissue nor has it been shown to be a precursor for reticuline in mammalian tissue. In plants (20) morphine is formed from thebaine by way of two pathways: (i) from codeine or (ii) from oripavine. In mammalian tissue it has been shown that salutaridine can be formed from reticuline (5) and that the concentration of morphine and codeine increased following administration of thebaine or salutaridine (1). The possibility that the thebaine found in the ovine brain is of a dietary origin cannot be excluded since the brain was obtained from a slaughterhouse and we were unable to test for the presence of thebaine in the food. However, we have recently shown that microsomes from various mammalian tissues in the presence of cofactors can convert thebaine to morphine; the biosynthetic pathway was similar to that that exists in plants (6).

Therefore, the presence of thebaine as well as codeine and morphine in ovine brain provides strong evidence that morphine and codeine, which had been identified in mammals, are of endogenous origin and biosynthesized from the precursor in mammalian tissue.

Thebaine exerts profound physiological and pharmacological effects. It has slight analgesic and depressant effects and does not elicit physical dependence (21, 22). However, thebaine can stimulate the central nervous system and cause convulsions (21–25). Morphine and codeine also produce convulsion but only at high doses (21, 23, 26), whereas at low doses, morphine can exert an anticonvulsant action (26). Thebaine can reverse some of the effects of morphine (27, 28). Electrophysiological studies indicate that the spasmodic effect of thebaine was different from that of morphine and codeine but was similar to that of strychnine (29). Thebaine has been shown to inhibit binding to the glycine receptor and the y-aminobutyric acid receptor (30). Both receptors seem to be involved in the convulsive properties of the opiates (31, 32). The finding of endogenous thebaine in mammalian brain

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**Fig. 5.** Electron-ionization mass spectra recorded for the GC/MS analysis: putative thebaine isolated from ovine brain (A) and standard thebaine (B). The HPLC solvent blank at the retention time of thebaine, the putative thebaine from ovine brain (30 pmol), and standard thebaine (35 pmol), which were collected as shown in Fig. 4, were used for this analysis.
tissue opens further areas of investigation as to the role of thebaine and its relationship to codeine and morphine.

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