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Isolation of morphine from toad skin

(nonpeptide opiate/endogenous)

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ABSTRACT **A nonpeptide opioid was found in toad skin and purified to homogeneity by using HPLC with electrochemical detection. A nonpeptide opioid also was detected in bovine brain and adrenals as well as rabbit and rat skin, by reversed-phase HPLC following Sephadex G-15 column chromatography. The material in toad skin was identical to morphine by immunological, pharmacological, and physical chemical criteria.**

Previous reports from this laboratory documented the existence of an endogenous nonpeptide morphine-like compound (MLC) in the central nervous system, urine, and cerebrospinal fluid of various species (1–3). The compound was characterized by its binding to antibodies against morphine and to the opiate receptors of the hybrid cell line NG108-15 (2). Although the morphine-like compound inhibited the guinea pig ileum contraction induced by electrical stimulation, this inhibition was not reversed by naloxone. Killian *et al.* (4) confirmed the results, although they found that the effect of the compound on the guinea pig ileum was reversed by naloxone.

Using HPLC, we found three peaks of morphine immunoreactivity in bovine brain (5). However, the concentrations of these compounds were not sufficient for purification or characterization. In the present study, several animal tissues were screened, and an immunoreactive compound was successfully purified from toad skin. We have identified the endogenous nonpeptide opioid in toad skin as morphine.

MATERIALS AND METHODS

Materials. [^{3}H]Dihydromorphine (65 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. Morphine·HCl and ^{125}I-labeled morphine (90–150 Ci/mmol) were from Hoffmann-La Roche. Toads (*Bufo marinus*) were purchased from West Jersey Biological Supply (Wenonah, NJ).

Purification. The ventral and dorsal skin was taken from 50 toads (about 800 g) and left in 4 liters of 95% methanol/5% 0.01 M HCl at 4°C for 1 week. The extract then was centrifuged at 20,000 × *g* for 10 min, and the supernatant was evaporated to dryness. The residue was dissolved in a minimum volume of 1 M Tris·HCl buffer (pH 8.6) saturated with NaCl, adjusted to pH 8.5, and then extracted into 5 volumes of 10% 1-butanol in chloroform. The organic phase then was back-extracted with 2 volumes of 0.01 M HCl (6). The recovery of morphine or ^{125}I-labeled morphine by these extraction procedures is 50–70%. The aqueous phase was evaporated to dryness, dissolved in 30 ml of 1 mM HCl, centrifuged at 25,000 × *g* for 30 min, and then applied to a Sephadex G-15 column (2.6 × 100 cm). The immunoreactive fractions were eluted with 0.1 M pyridine/acetic acid (pH 6.2), collected, evaporated, and dissolved in 2 ml of 1 mM HCl. An aliquot was chromatographed by reversed-phase HPLC, using an Alltech Model 42 system with a LiChrosorb RP-18 column (0.4 × 25 cm). The samples were eluted with a linear 0–25% (vol/vol) gradient of 1-propanol in 0.1 M pyridine/acetic acid (pH 6.2) at a flow rate of 1.5 ml/min. The immunoreactive fractions were pooled, evaporated, dissolved, reapplied to the RP-18 column, and eluted with the same linear gradient described above but in 0.1 M pyridine/acetic acid (pH 6.7). Further purification was carried out by 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; immunoreactive fractions were rechromatographed under the same conditions.

Table 1. Distribution of nonpeptide opioid

Tissue	pmol/g of tissue
Toad skin	3.01 ± 1.48
Rabbit skin*	0.29 ± 0.04
Rat skin*	0.27 ± 0.01
Bovine adrenal†	0.13 ± 0.02
Bovine brain cerebellum	0.09 ± 0.00
Bovine brain cerebral cortex	0.05 ± 0.01

Tissues were assayed by morphine RIA as described in *Materials and Methods*. Concentrations are expressed as morphine immunoequivalents.
*Rabbit and rat skins were shaved and extracted in 95% methanol/5% 0.01 M HCl.
†There was no significant difference between medulla and cortex.

Detection by HPLC. The effluent was monitored with the amperometric electrochemical detector model LC-4A (Bioanalytical Systems, West Lafayette, IN) at an oxidation potential of 0.75 V vs. Ag/AgCl electrode (7, 8).

Morphine RIA. Nonpeptide opioid was determined on the basis of its competition with ^{125}I-labeled morphine for rabbit antibodies developed against 3-carboxymethylmorphine conjugated to bovine serum albumin; nonpeptide opioid was expressed as morphine equivalents (9). An aliquot of each column fraction to be tested was dried in a 1.5-ml Eppendorf centrifuge tube, using a Savant Speed-Vac concentrator. The residue was dissolved in phosphate-buffered saline (pH 7.4) and ^{125}I-labeled morphine and the morphine antibody were added to each tube. Incubation was for 1 hr at 4°C in a total volume of 250 μl. The antibody-bound ^{125}I-labeled morphine was separated by the Farr procedure (10).

Opiate Receptor Binding Assay. Whole rat brain minus cerebellum was homogenized in 30 volumes of 50 mM Tris Cl (pH 7.7) and centrifuged for 20 min at 30,000 × *g*. The pellet was suspended in Tris buffer and centrifuged, and the resultant pellet was resuspended for use in the assay. The binding assay was carried out according to the method of Pert *et al.* (11).

RESULTS

Toad skin contained the greatest concentration of morphine immunoreactivity of the tissues tested (Table 1). Morphine immunoreactivity was also found in rabbit and rat skin and bovine adrenal. The concentrations in the adrenal cortex and

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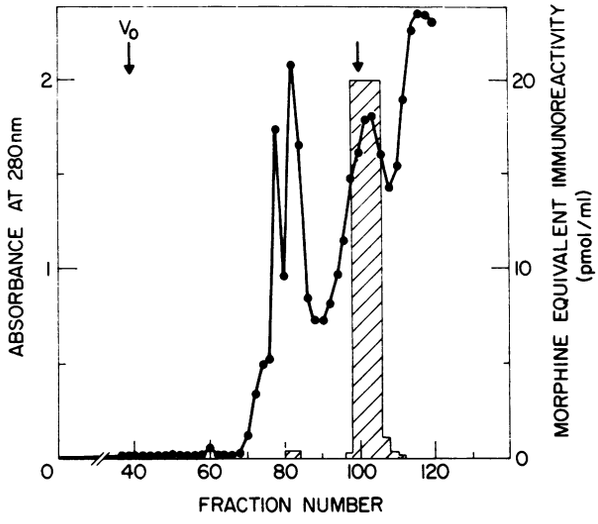

FIG. 1. Sephadex G-15 chromatogram of nonpeptide opioid. Immunoreactivity was measured as described in *Materials and Methods* and is expressed as morphine immuno-equivalents. Fraction size was 4 ml. Unlabeled arrow indicates the elution position of morphine. Closed circles represent absorbance at 280 nm, whereas the hatched bar graph represents morphine immunoreactivity.

medulla were not significantly different from one another. In contrast to bovine brain, from which three immunoreactive peaks were obtained by RP-18 chromatography (5), all other tissues examined gave only one immunoreactive peak. Of the three immunoreactive peaks from the RP-18 column, one had a mobility that was similar to that of morphine. However, we were unable to further characterize this peak because of the low concentrations found in brain tissue. Of the other two immunoreactive species, one had a greater affinity for brain μ receptors than did morphine. Incubation of the immunoreactive species from these tissues with Pronase for 1 hr at 37°C or heating them at 95°C for 10 min did not cause a loss of binding activity to antibody or receptor.

Since toad skin had the highest concentration of immunoreactivity, it was used as the source for the nonpeptide opioid. The organic solvent extraction procedure was based on the properties of morphine and its related derivatives. This procedure was effective also in eliminating substances that interfere with the RIA. Fig. 1 shows the chromatogram from Sephadex G-15, with one major immunoreactive fraction. The arrow indicates where morphine elutes from the column. The immunoreactive fraction was subjected to RP-18 reversed-phase HPLC (Fig. 2 *A* and *B*; an arrow indicates the retention time of pure morphine under each of the two pH conditions). The immunoreactive peak from toad skin was chromatographically indistinguishable from morphine. The resulting immunoreactive fraction was chromatographed on a Whatman Partisil 10 SCX ion-exchange column. As shown in Fig. 2*C*, a sharp peak was electrochemically detected that coincided with the immunoreactivity. The immunoreactive fraction was collected and rechromatographed under the same conditions; again, a single sharp peak was observed by electrochemical detection (Fig. 2*D*).

Hydrodynamic voltammograms were obtained using HPLC/electrochemical detection. The nonpeptide opioid and morphine each were chromatographed on a Whatman Partisil 10 SCX column at a flow rate of 1 ml/min, using 0.1 M pyridine/acetic acid (pH 4.7) as the mobile phase. At oxidation potential of 0.5–0.9 V, the hydrodynamic voltammogram of the nonpeptide opioid is identical to that of morphine (Fig. 3).

To assist further in the identification of the morphine-like immunoreactivity, we developed mouse monoclonal antibodies to two immunogens, 3-carboxymethylmorphine and *N*-carboxymethylnormorphine. These monoclonal antibodies recognize different epitopes of morphine (unpublished results). Three different hybridoma clones were selected to study the cross-reactivity of the purified nonpeptide opioid. The IC_{50} values of the nonpeptide opioid to each monoclonal antibody were identical to those of morphine (Table 2).

Table 2 is a summary of the properties of the nonpeptide opioid as compared to morphine. There was no significant difference between the nonpeptide opioid and morphine in binding to either the polyclonal or monoclonal antibodies. This was also true when the comparison was made using the brain receptor assay. We also investigated the effect of sodi-

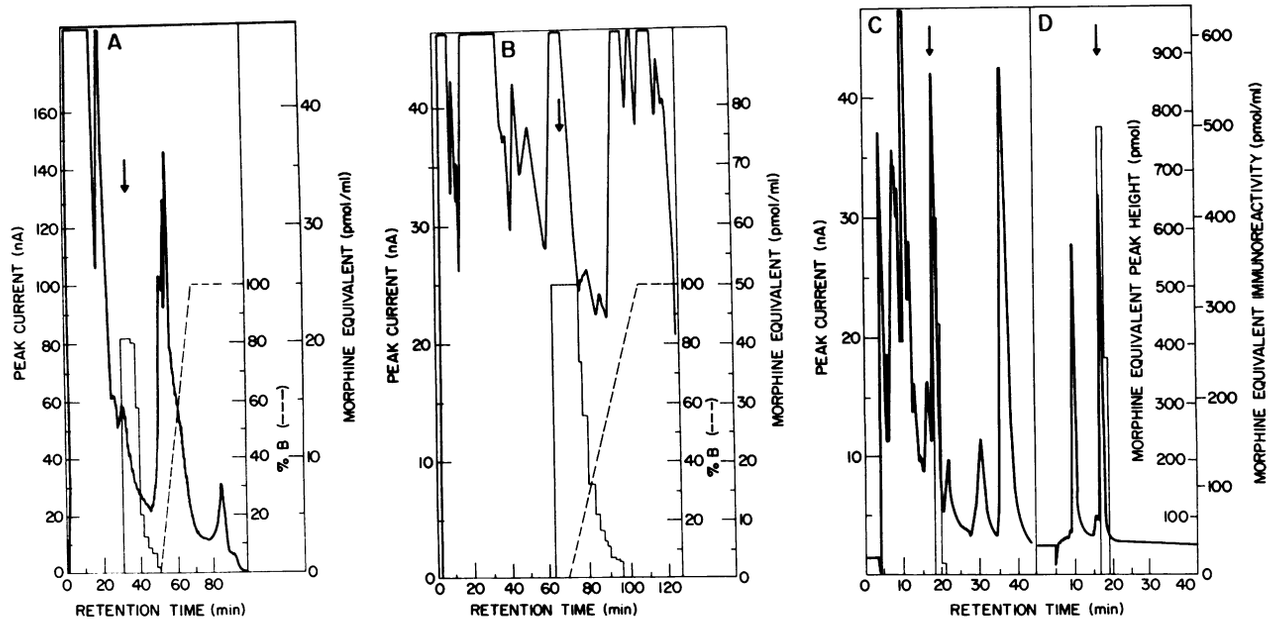

FIG. 2. HPLC chromatogram of nonpeptide opioid. Active fraction from Sephadex G-15 was pooled, concentrated, and applied to LiChrosorb RP-18 (*A* and *B*) and then to Whatman Partisil 10 SCX (*C* and *D*). The conditions are described in *Materials and Methods*. Fraction size was 2 min (*A* and *B*) or 1 min (*C* and *D*). The arrows indicate the retention times of morphine. Peak current is the darker curve, and morphine immunoreactivity is the lighter curve. % B represents the linear gradient used for elution.

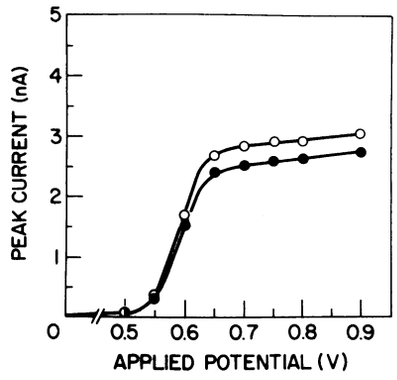

FIG. 3. Hydrodynamic voltammograms of nonpeptide opioid (18 nmol) and morphine (20 nmol). Peak heights were plotted against the oxidation potential. ○, Morphine; ●, nonpeptide opioid. Each value is an average of duplicate experiments.

um on binding affinities, using naloxone as a labeled ligand. Again, no significant difference between the nonpeptide opioid and morphine IC_{50} values were observed in the presence or absence of 0.1 M NaCl. The ratio of IC_{50} values in the presence and absence of NaCl suggest that the nonpeptide opioid is an agonist to rat brain opiate receptors (11).

Analysis by gas chromatography/mass spectrometry showed the same molecular ion for morphine and the nonpeptide opioid (Table 2).

The nonpeptide opioid also inhibited the guinea pig ileum contraction induced by electrical stimulation, at a concentration similar to that required for inhibition by morphine. At 8 nM, morphine inhibited the electrically stimulated contraction of the guinea pig ileum 30%; the extracted material likewise caused a 30% inhibition at 8 nM morphine immunoequivalents. This inhibition was reversed by naloxone.

Table 2. Properties of nonpeptide opioid from toad skin

	Nonpeptide opioid	Morphine
Retention time on HPLC		
Reversed-phase		
pH 6.2	33 min	33 min
pH 6.7	68 min	68 min
Ion-exchange	18 min	18 min
IC_{50} for rabbit anti-morphine antibody	0.42 nM	0.39 nM
IC_{50} for mouse monoclonal antibodies: 9B1	5.6 nM	5.8 nM
7C2	7.7 nM	8.3 nM
2B4	9.1 nM	9.3 nM
IC_{50} for rat brain opiate receptor*:		
Dihydromorphine	7.8 nM	7.1 nM
Naloxone	9.6 nM	9.3 nM
Naloxone plus 0.1 M NaCl	65 nM	87 nM
Molecular ion†	285.1402	285.2365

IC_{50} values for nonpeptide opioid, based on peak height ratios from HPLC with electrochemical detection, are expressed as morphine equivalent peak height. The rabbit antibody and monoclonal antibodies 9B1 and 7C2 were developed against 3-carboxymethylmorphine conjugated to bovine serum albumin. Monoclonal antibody 2B4 was developed against *N*-carboxymethylmorphine conjugated to bovine serum albumin.

*Receptor-binding study was performed as described in *Materials and Methods* using dihydromorphine (1.25 nM) or naloxone (1.25 nM) as ^{3}H-labeled ligands.

†Determined with an Associated Electronics Industries model MS-30 mass spectrometer.

DISCUSSION

In previous reports, a nonpeptide opioid referred to as MLC (morphine-like compound) was identified (1–3). In the present study, we have isolated the compound from toad skin and have identified it as morphine. Although the compound was detected in other tissues, the concentrations were too low for characterization; however, the nonpeptide opioid in these tissues competes with morphine for binding to a polyclonal morphine antibody and to an opiate receptor. Toad skin had the greatest concentration of the nonpeptide opioid of all the tissues from various species analyzed and, therefore, was used for its isolation.

The original estimations of concentration of the morphine-like compound reported by Gintzler *et al.* (1) and later by Killian *et al.* (4) were much higher than that reported here. This discrepancy could be attributed to an over-estimation in the earlier studies because of some nonspecific binding in the RIA. Alternatively, since the isolation procedure has been modified, the morphine isolated could be different from the morphine-like compound previously reported. Immunoreactivity was also noted at the solvent front from the reversed-phase HPLC column. Since this fraction was not purified in these studies, it might account for the reduced estimation of morphine-like immunoreactivity.

One of the issues raised by Hazum *et al.* (12) was that morphine can be derived from dietary sources. We did not determine whether morphine in the toad might be present due to ingestion. However, we could not detect any morphine in toad tissues other than skin; if the morphine were from an exogenous source, one would not expect it to be so limited in its localization. Since toad skin had relatively high concentrations of the nonpeptide morphine-like compound as compared to other tissues, we investigated rat and rabbit skin and found that concentrations of the morphine-like compound were higher than in other tissues. We tested rat and rabbit chow for the presence of morphine but were unable to detect any. Thus, we feel that the morphine we find in these tissues is not due to dietary sources. Two questions then have to be addressed. First, is the biosynthesis of morphine similar to that found in the poppy plant, *Papaver somniferum*? Second, what is the function of morphine in skin? Morphine causes dilatation of cutaneous blood vessels and, therefore, may be involved in regulating body temperature.

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