Isolation of a novel endogenous opiate analgesic from human blood
(opiate receptor/analgesia/anodynin/enkephalin/morphine)

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ABSTRACT Based upon its ability to inhibit opiate receptor binding, a low-molecular-weight substance (600) has been isolated from human plasma by extraction into butanol and ion exchange, molecular sieve, and thin-layer chromatography. When this substance, termed anodynin, is microinjected into rat periaqueductal gray matter, it causes a profound, long-lasting analgesia which is prevented by prior injection of the opiate antagonist naloxone. Anodynin (opiate receptor binding material) levels in serum from hypophysectomized rats are less than 5% of values obtained in sham-operated controls. Anodynin differs from enkephalin, a morphine-like peptide isolated from brain, in its sensitivity to enzymatic loss of opiate receptor inhibitory potency, thin-layer chromatographic mobility, and behavioral effects. Anodynin might be a hormone that acts on peripheral opiate receptors in the classical manner, but might also, due to its lipophilic nature and small size, penetrate into the brain to produce centrally mediated behavioral effects.

Opiate receptors are hypothetical stereospecific tissue constituents with which morphine and other opiate agonists complex to initiate their pharmacological effects (1, 2). Specific binding of radiolabeled opiates to nervous tissue homogenates having a number of features expected of pharmacologically relevant drug receptors has been demonstrated (3–5) and studied extensively (6, 7). Presumably, opiate receptors, which are localized on the synaptic membrane (8) of certain discrete brain areas (9–11), participate in some normal physiological function even when the organism is not exposed to opiates. The rather strict structural requirements for opiate activity have suggested to some investigators (12–14) that opiate receptors normally interact with some naturally occurring "endogenous ligand" for which morphine and its congeners provide structural analogues.

Recently Hughes and his associates (15) have isolated two pentapeptides from porcine brain on the basis of their morphine-like effects on the mouse was deferens and guinea pig ileum. They have termed these peptides "enkephalin," assigned them structures, and demonstrated that synthetic enkephalin is pharmacologically active (16). Snyder and his associates (17) and Terenius and Wahlstrom (18) have also isolated morphine-like peptides from mammalian brain, using their affinity for opiate receptors in vitro as the basis of their purification.

The endogenous ligand located in the central nervous system might function as a neuromodulator (19) or a neurotransmitter, for which the opiate receptor is a post-synaptic recognition unit which alters sodium ion conductance (6, 7). An additional function has been proposed by Goldstein and his collaborators, who have isolated a larger morphine-like peptide from bovine pituitary (20, 21) and suggested that it may be stored and released from pituitary and act at remote opiate-sensitive tissues in the manner of a typical pituitary hormone.

We now report the isolation of "anodynin," a small peptide from human blood that has a high affinity for opiate receptors in vitro. The peptide, which is not enkephalin, produces profound and long-lasting analgesia and sedation in rats, which is reversed by the opiate antagonist naloxone. Levels of anody-
Table 1. Comparison of endogenous inhibitor content and opiate receptor binding of various regions of rat brain

<table>
<thead>
<tr>
<th>Region</th>
<th>% Inhibition</th>
<th>Opiate receptor binding (pmol of [3H]naloxone bound/mg of tissue)</th>
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<tbody>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midbrain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
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<tr>
<td>Remainder of</td>
<td></td>
<td></td>
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<tr>
<td>tissue</td>
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</table>

For preparation of endogenous inhibitor, each region was homogenized in 10 volumes of glycine-HCl buffer (0.05 M, pH = 3) and the supernatant fluid was extracted into butanol after boiling and adjustment to pH 9. Inhibition by 100 λ of reconstituted extract was assessed after 60 min of incubation at 0° (NaCl = 100 mM). Values are mean ± standard error of the mean (number of extracts). Opiate receptor content was assessed as described in Materials and Methods after dissection of specified regions.

volume of 0.5 ml with [3H]naloxone (Amersham-Searle, 34 Ci/mmole) in a concentration of 1.3 nM (20,000 cpm). After rapid filtration and washing with two 7-ml portions of ice-cold standard buffer (4), membrane-laden filters were transferred and counted at 40–45% efficiency at least 6 hr after shaking with detergent scintillation fluor (Aquaosol, New England Nuclear). Control incubations containing levallophan (100 nM) or dextrallorphan (100 nM) (which did not significantly reduce binding) were included. Stereospecific binding represented 80–90% of the total bound naloxone in all experiments.

Assay of Rat Blood Samples. Rats were killed by decapitation and blood was collected. One milliliter of serum from each rat was obtained by centrifugation at 40,000 X g for 10 min. Serum was adjusted to pH 3 with HCl, heated briefly in a boiling-water bath, and extracted into 2 ml of water-saturated butanol after pH adjustment to 9.0 with Tris buffer. After evaporation (Evapo-Mix, Buchler Instruments), samples were reconstituted in 1 ml of standard Tris buffer and aliquots were assayed for opiate receptor inhibitory activity.

Hypophysectomized Rats. Hypophysectomized male rats weighing 150 g and sham-operated controls were purchased from Hormone Assay, Chicago. Pituitaries were aspirated after parapharyngeal entry into trachectomized rats under light ether anesthesia. Sham-operated rats had identical manipulation with the exception of aspiration. One week after surgery, hypophysectomized and control rats were alternately killed by decapitation and their serum was collected, extracted, and assayed for opiate receptor inhibitory activity.

Assessment of Analgesia. Eight rats were stereotaxically implanted with chronic indwelling cannulae guides constructed from 23 gauge TW stainless steel tubing. The cannulae tips were aimed for an area 2 mm dorsal to the periaqueductal gray matter (AP + 0.6, Lat + 0.5, DV + 3.0, in the coordinates of ref. 22), an area which has been shown to be critical for mediating the analgetic actions of opiates (23, 24). Testing was initiated approximately 1 week following surgery. The antinociceptive actions of morphine and anodynin were assessed using a modification of the D’Amour and Smith paradigm in which the latency for a rat to remove its tail from under a radiant heat source is measured in seconds. Baseline “tail-flick” latencies were always determined approximately 30 min prior to a drug or control injection by four trials separated by 10 sec. A trial was automatically terminated if an animal failed to remove its tail within 12 sec.

All animals received the following drugs and drug combi-
nations in a counterbalanced order separated by 5 days: 4 μl of anodynin, 4 μl of anodynin + 20 mg/kg of naloxone 15 min prior, 4 μl of 1.25 μg/μl of morphine sulfate + 20 mg/kg of naloxone 15 min prior, and 4 μl of sterile water (vehicle for morphine). The solutions were injected into the central gray matter with 30 gauge injectors which extended 2 mm past the tip of the guide cannula. The injection rate was 4 μl/min. Testing for antinociception was initiated immediately after an injection. Animals were tested at 1-min intervals for 5 min and then at 10, 15, 30, and 60 min. At the end of the study, all animals were sacrificed and their brains were prepared following standard histological procedures to localize cannulae placements.

Enkephalin. Synthetic standards were the generous gift of Beckman Instruments, Bioproducts Division, Palo Alto, Calif.

RESULTS

Purification

The major purification step is the efficient extraction into butanol, which removes most protein and yields a lipid-rich fraction. This step was suggested by the very hydrophobic nature of the preliminary amino acid content of enkephalin proposed by Hughes et al. (25). Table 1 demonstrates that the butanol extraction procedure, when applied to rat brain, yields an endogenous inhibitor of opiate receptor binding which parallels the previously reported (3, 11) distribution of opiate receptor content in rat brain. This suggests that nonspecific interference in the opiate receptor assay has been minimized in the chosen conditions, and the majority of inhibitory activity is associated with a specific opiate receptor ligand.

No opiate receptor inhibitory activity could be detected in any fraction of the neutral washes of the Dowex column to which butanol extracts of midbrain-striatum, cerebellum, or plasma had been applied. The acidic eluent of midbrain striatum and plasma, but not cerebellum, demonstrated significant inhibition of [3H]naloxone binding after adjustment to neutrality. Molecular weight determination of the blood inhibitory substance (Fig. 1) indicates a molecular weight (600) which is not significantly different from that of synthetic enkephalin.

Thin-layer chromatographic analysis in chloroform–methanol–20% ammonia (60:30:5, vol/vol/vol) of the opiate receptor inhibitory activity pooled from fractions 86–110 of the Bio-Gel column revealed the presence of four spots (RF values = 0.17, 0.58, 0.67, and 0.91), none of which corresponded to either met- or leu-enkephalin (RF = 0.47 and 0.48), which were chromatographed on the same plate (Fig. 2). After elution of the spots, significant (>15%) opiate receptor inhibitory activity was found to reside only in the slow-moving spot with RF equal to 0.17. No significant opiate receptor inhibitory activity was detectable in the eluent of the area next to synthetic met- or leu-enkephalin. The isolated inhibitory blood factor was found to run as a single spot when eluted and rechromatographed in the same solvent system (RF = 0.19) and two other solvent systems [ethanol and water, 7:3, (vol/vol) and chloroform–methanol–20% ammonia 60:45:5 (vol/vol/vol)] where RF values of 0.28 and 0.24, respectively, were obtained. The mobility of met- and leu-enkephalin also differed from that of anodynin in the two other solvent systems, where RF values for met- and leu-enkephalin of 0.75 and 0.90, respectively (ethanol/water), and 0.75 and 0.74 (chloroform/methanolic/ammonia) were obtained. Moreover, a mixture of purified anodynin and the enkephalin standard run in all solvent systems resulted in distinctly different spots with RF values closely similar to those obtained when enkephalin and anodynin were run in separate lanes.
Properties

The ability of the blood inhibitory factor eluted from the thin-layer plate to be degraded by enzymes present in brain was examined by incubating the blood substance with membranes or supernatant fluid from rat brain, terminating the enzyme action by boiling, and subsequently adding the product to the opiate receptor assay (Table 2). The blood substance appeared to be relatively resistant to loss of inhibitory activity. By contrast, enkephalin inhibition of opiate receptor binding was completely and rapidly reversed by brain enzymatic activity.

The blood inhibitory factor inhibits opiate receptor binding of [3H]naloxone with a slope that is not significantly different from that of morphine (Fig. 3) and other opiates (7).

Behavioral effects

Anodymin produced an immediate, profound, long-lasting analgesia as assessed by the rat "tail-flick" test (Fig. 4). Four microliters, containing sufficient anodymin activity to cause approximately 20% inhibition in the opiate receptor assay, caused an increase in tail-flick latency equivalent to that produced by a total of 5 μg of morphine sulfate in 4 μl of water. The antinociceptive actions of morphine as well as anodymin were blocked by pretreatment with 20 mg/kg of the opiate antagonist naloxone injected intraperitoneally. A repeated measured analysis of variance revealed a significant treatments effect (P

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Met-enkephalin</th>
<th>Anodymin</th>
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<tbody>
<tr>
<td>Buffer—1 hr, 37°</td>
<td>65</td>
<td>29</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr, 0°</td>
<td>65</td>
<td>29</td>
</tr>
<tr>
<td>0.25 hr, 37°</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td>1 hr, 37°</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr, 37°</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>

Rat brains were homogenized in 100 volumes (wt/vol) of standard buffer and the particulate fraction and supernatant fluid were obtained by centrifugation for 10 min at 40,000 x g. After incubation under the specified conditions in a total volume of 1 ml with met-enkephalin or anodymin, the samples were boiled for 2 min and aliquots (300 μl) of the supernatant fluid were assessed for inhibition in the standard opiate receptor assay. The values are means from triplicate determinations of two separate experiments which varied less than 30%.
hypophysectomized rats opiate receptor

Sham-operated rats 5

5

67

0.49

0.03

0.01

FIG. 3. Inhibition of stereospecific [³H]naloxone binding by morphine and anodynin. Anodynin was eluted from a thin-layer chromatogram, as described in Fig. 2 legend. Control stereospecific binding was 2109 ± 126 cpm. The ordinate is a probit scale.

< 0.05). Orthogonal comparisons between the effects of anodynin and anodynin plus naloxone and morphine and morphine plus naloxone also proved to be significant (P < 0.05). Both morphine and anodynin caused a stuporous immobility whose reversibility by naloxone was not assessed.

Effect of hypophysectomy

Blood serum of rats whose pituitaries had been surgically removed 1 week previously was examined for opiate receptor inhibitory activity (Table 3). While sham-operated control rats had opiate receptor inhibitory activity that was equivalent to 0.67 nmol of morphine per ml of plasma, levels in serum of hypophysectomized rats had fallen by 96–98%.

DISCUSSION

Human blood apparently contains a small (600 daltons) peptide with morphine-like behavioral and opiate receptor binding activity. This substance, which we have termed anodynin, has been highly purified, as evidenced by the appearance of a single spot in three solvent systems for thin-layer chromatographic analysis. Anodynin is derived from the English word anodyne (Greek, anodyneos), which means a drug that calms and allays pain. The ability to inhibit opiate receptor binding in vitro, as previously demonstrated by Snyder and his associates (17), is a useful method for assessing purification progress. While anodynin may be a close structural analog to enkephalin, it differs from it in its sensitivity to brain enzymatic degradation, its duration of analgesia, and its thin-layer mobility in three solvent systems. It has been shown previously (26) that enkephalin, which is rapidly destroyed in brain, requires doses at least 40 times higher than morphine to elicit analgesia, which disappears completely 1–2 min after microinjection. By contrast, anodynin appears to be relatively resistant to inactivation by brain enzymes in vitro and elicits naloxone-reversible analgesia which is, at low doses, only slightly diminished 1 hr after microinjection. Since this analgesia is blocked by naloxone, it is due to a specific interaction with brain opiate receptors and cannot be attributed to a nonspecific incapacitation of the animals.

The fact that several peptides exist, the enkephalins in porcine brain and anodynin in human blood, could have functional significance or could be attributed to species differences. In any case, it is highly unlikely that anodynin is a degradation product of enkephalin or vice versa, since neither substance gives evidence of the generation of the other upon repeated thin-layer chromatographic analysis and storage.

Since hypophysectomy almost completely abolishes blood anodynin, it may be concluded that anodynin is stored in and released from the pituitary in the manner of classical hormones,

Table 3. Levels of anodynin in rat serum

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>% Inhibition of stereospecific binding</th>
<th>Morphine equivalence (nmol/ml of plasma)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>Sham-operated rats</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Hypophysectomized rats</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Butanol extracts of blood serum were prepared as in the Materials and Methods, and aliquots from each sample (150 µl) were assayed for opiate receptor inhibition, as described. Morphine inhibitory equivalence was calculated from Fig. 3. The decrease in anodynin levels in hypophysectomized rats is significant (P < 0.02).
or that a pituitary factor is required for its maintenance. Goldstein and coworkers (20, 21) originally suggested the possibility of a morphine-like hormone, based upon their purification from bovine pituitary of a higher molecular weight peptide with morphine-like properties (POP-1). The relationship between POP-1, which may be a hormone precursor, and anodyin is unclear. In addition, Hughes et al. (16) have drawn attention to the fact that the enkephalin peptide sequence is contained within the structure of the high-molecular-weight β-lipotropin (26). Anodyin, with its low molecular weight and high butanol solubility, is clearly not β-lipotropin; β-lipotropin might, however, be a prohormone for anodyin or POP-1.

Since "raw" plasma contains many substances that interfere in the opiate receptor binding assay, including adrenocorticotropic hormones (28), and cannot be directly assayed, estimation of recovery of the purification procedure is not possible. It should be noted that the possibility of other morphine-like substances' existing in blood cannot be rigorously excluded. However, if enkephalin were present in rat or human blood, its physiocochemical properties make it likely that it would have been purified with anodyin in our procedure.

The physiological function of circulating blood anodyin hopefully will be elucidated by comparison of levels in various normal and pathological states. The unperturbed levels obtained in plasma of normal rats are sufficient to maintain peripheral opiate receptors in a state of slightly more than half saturation, presuming they are in equilibrium with plasma. In addition, because of its hydrophobic nature, anodyin may pass into the brain after release from the pituitary via the blood circulation and thus produce centrally mediated behavioral effects such as analgesia, sleepiness, or euphoria.

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