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Coupling of a New, Active Morphine Derivative to Sepharose for Affinity Chromatography

(6-succinylmorphine/opiate receptors/antibodies/naloxone)

ERIC J. SIMON, WILLIAM P. DOLE, AND JACOB M. HILLER

Department of Medicine, New York University Medical Center, New York, N.Y. 10016

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ABSTRACT **A new, pharmacologically active morphine derivative, 6-succinylmorphine, was synthesized. The properties of this compound and evidence for its structure are presented. Succinylmorphine was covalently coupled to ethylamino-Sepharose. Morphine–Sepharose containing up to 40 μg of morphine did not block the electrically stimulated contraction of isolated guinea pig ileum, but after alkaline hydrolysis of beads containing 2 μg of morphine the supernatant completely blocked contraction. This block was reversed by the specific morphine antagonist naloxone. Antibodies to morphine were removed from serum by morphine–Sepharose, but not by ethylamino-Sepharose, providing evidence of the efficacy of the beads for affinity chromatography.**

One of the most important unresolved problems in the study of the mode of action of the opiate narcotics is the existence and nature of the putative receptor for these drugs in the central nervous system. The highly stereospecific nature of the analgesic action of narcotics and their conversion to specific, potent antagonists by relatively minor structural changes are difficult to explain without the postulation of specific receptors.

Affinity chromatography has been applied successfully to the isolation and purification of enzymes and hormone receptors (1). To enable us to use this powerful technique in the search for opiate receptors, we synthesized the new morphine derivative, 6-succinylmorphine, and coupled this pharmacologically active compound to Sepharose beads. The only reference found to the succinylation of morphine was a report in 1875 by Beckett and Wright (2), who treated morphine with succinic acid at 180° and believed that they had obtained a disuccinylmorphine on the basis of the elementary analysis and the disappearance of the phenolic groups.

We present evidence for the structure of 6-succinylmorphine and for the covalent nature of the bond formed when 6-succinylmorphine is coupled to Sepharose. The usefulness of the morphine–Sepharose beads for affinity chromatography was demonstrated by the use of antibodies to morphine as a model of a specific opiate receptor.

MATERIALS AND METHODS

Materials. Morphine sulfate was obtained from Merck & Co., Rahway, N.J. The free base was crystallized by precipitation at pH 8, filtered, and dried. Dihydromorphine was prepared by hydrogenation of morphine sulfate in water at room temperature and atmospheric pressure with a catalyst of palladium on charcoal. Radioactive dihydromorphine was prepared by catalytic tritiation of morphine (New England Nuclear Corp., Boston, Mass.). Affinose 101 (ethylamino-Sepharose) was purchased from Bio-Rad Laboratories, Richmond, Calif., and the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, from Pierce Chemical Co., Rockford, Ill. Silicic acid-coated thin-layer plates (Silplate F-22) were purchased from Brinkmann Instrument Co., Westbury, N.Y.

Methods. Free phenolic groups were measured with the Folin phenol reagent, as described for proteins by Lowry *et al.* (3). Ultraviolet spectroscopy was done in water at pH 2 with a Zeiss spectrophotometer. Infrared spectroscopy was done for us by Dr. Gisela Witz, with a Perkin Elmer Model 421 spectrophotometer and mass spectroscopy was done by Dr. R. Servis, with a Finnigan model 1015 mass spectrometer at an ionizing potential of 70 eV. Radioactive counting of aqueous solutions and suspensions of beads was in Aquasol, a toluene-based, water-miscible scintillation fluid (New England Nuclear Corp.) in a Packard liquid scintillation counter.

RESULTS

Synthesis of 6-succinylmorphine

Reflux of morphine or dihydromorphine with excess succinic anhydride in benzene leads to the selective succinylation of the 6-hydroxyl group. Morphine-free base (1 g) and succinic anhydride (1 g) in benzene (20 ml) are placed in a 100-ml flask provided with a condenser and heating mantle. After 2 hr of heat at reflux temperature, additional succinic anhydride (1 g) is added and reflux is continued for 1 hr. The reaction mixture is allowed to cool to room temperature, the benzene is decanted and discarded, and residual benzene is evaporated in a stream of nitrogen. The residue is dissolved in 10 ml of water and brought to pH 2 with 2 N HCl. After filtration to remove a small amount of acid-insoluble material, the pH is raised to about 9 with 2.5 N NaOH and the solution is filtered to remove unreacted morphine. The pH is adjusted to about 5, whereupon 6-succinylmorphine crystallizes while standing in a refrigerator overnight. Crystals are harvested by filtration and dried over calcium chloride under reduced pressure. The yield of 6-succinylmorphine is 60–70% of theoretical. To prepare radioactive succinyl dihydromorphine, the reaction is performed with 1 g of [7,8-^{3}H]dihydromorphine (1–2 μCi/mg).

Chromatography

For the separation of 6-succinylmorphine from morphine by thin-layer chromatography, the best solvent is ethanol–

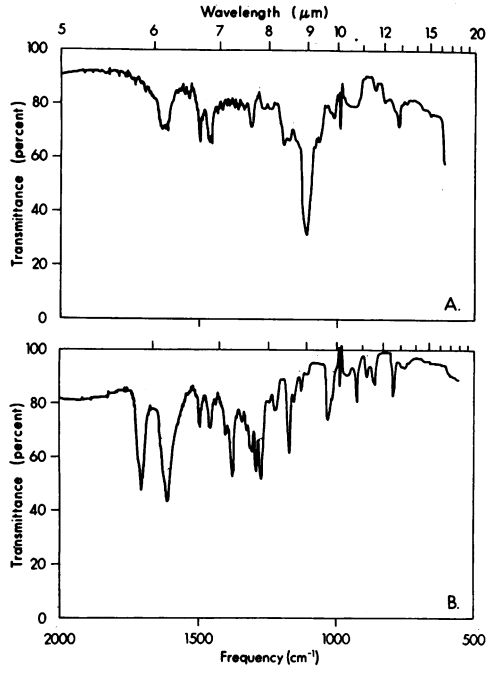

FIG. 1. Infrared spectra of morphine (*A*) and of 6-succinylmorphine (*B*).

dioxane–benzene–ammonium hydroxide 40:5:50:5, a modification of solvent S3 of Cochin and Daly (4), in which the R_F of morphine is 0.56 and that of 6-succinylmorphine is 0.14. The corresponding hydrogenated compounds have R_F values of 0.53 and 0.10. In solvent S2 of Cochin and Daly, morphine has R_F 0.52 and 6-succinylmorphine 0.60.

The product synthesized and crystallized as described above usually contains less than 2% morphine (or dihydromorphine), as determined from the distribution of radioactivity on chromatograms of 6-succinyl-[7,8-^{3}H]dihydromorphine.

Analysis. Elementary analysis* of 6-succinylmorphine, dried under reduced pressure at 60° over P_2O_5, showed: C 62.3, H 6.25, N 3.7; calculated for $C_{21}H_{23}NO_6 \cdot H_2O$: C 62.6, H 6.2, N 3.5.

Solubility

6-Succinylmorphine does not have a well-defined melting point, but decomposes above 200°. It is very soluble in water below pH 3 and above pH 8.5, but is much less soluble between these pH values (0.2 mg/ml at 2° and 0.5 mg/ml at 25°), consistent with its zwitterion structure. The compound is sparingly soluble in all organic solvents tested.

Evidence for the structure of 6-succinylmorphine

Evidence that the morphine molecule was esterified with succinate, and that esterification occurred exclusively in position 6 is as follows: (*i*) Reaction of 6-succinylmorphine with Folin phenol reagent demonstrated quantitative recovery of free phenolic groups. (*ii*) When we tried to succinylate levorphanol, which lacks the 6-hydroxyl group, only unreacted levorphanol was recovered. (*iii*) Titration of 6-succinylmorphine with NaOH showed the presence of one titratable group, with a pK of 4.2, typical of a carboxyl group. (*iv*) Acid hydrolysis of 6-succinylmorphine yields morphine, as identified by thin-layer chromatography. (*v*) The UV spectrum of 6-succinylmorphine is indistinguishable from that of morphine, in which the chromophore responsible for the peak at 285 nm is the phenolic ring. This is further evidence for the absence of reaction at this site. (*vi*) Portions of the infrared spectra of 6-succinylmorphine and morphine are shown in Fig. 1. The spectrum of 6-succinylmorphine has lost the peak at 1100 cm^{-1} characteristic of a free secondary alcohol group. The succinyl group is indicated by a new C=O stretching peak at 1700 cm^{-1} and by peaks characteristic of a COO^- group at 1615 and 1380 cm^{-1}. (*vii*) Mass spectrometry of 6-succinylmorphine gave primary fragments at m/e 41 (100%), 44 (50%), 266 (17%), 384 (M-1) (5%), and 385 (M+) (2%). The peak at 385 agrees with the calculated molecular weight for 6-succinylmorphine. The other peaks are consistent with a fragmentation scheme expected for 6-succinylmorphine.

Pharmacology of 6-succinylmorphine

In preliminary experiments, 6-succinylmorphine was about as active in mice as morphine with respect to production of the Straub reaction and hyperactivity. It also inhibited electrically-stimulated contraction of guinea pig ileum. Detailed pharmacological studies will be published elsewhere.

Coupling of 6-succinylmorphine to Sepharose

Affinose 101 is a commercially available preparation in which ethylenediamine side arms have been introduced into Sepharose 4B beads (ethylamino-Sepharose). Coupling of affinity groups to the beads by means of side arms of various lengths was found by Cuatrecasas (5) to be essential to prevent steric inhibition of binding of enzymes and receptors during affinity chromatography. We have usually done coupling with the radioactive dihydro derivative of 6-succinylmorphine to facilitate determination of the number of bound morphine residues. Reaction was by a procedure similar to that described by Cuatrecasas (5) for coupling succinylestradiol to Sepharose. 6-Succinyl-[7,8-^{3}H]dihydromorphine (50 mg; 1 μCi/mg) was dissolved in 20 ml of H_2O. The solution was added to 12 ml of settled Affinose 101 beads. The beads had been washed with 20 volumes of 0.1 N NaCl–0.01 N Tris·HCl, pH 7 to remove sodium azide (present to prevent bacterial growth). The pH

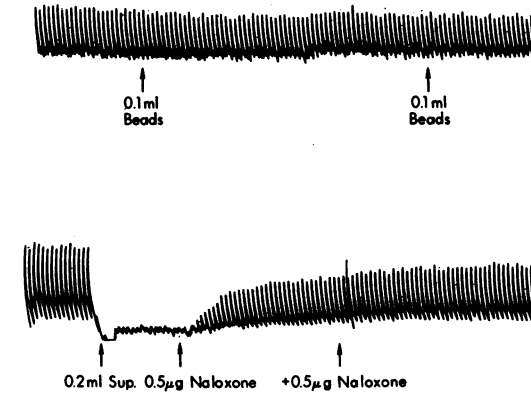

FIG. 2. Effect of morphine–Sepharose on contractions of isolated guinea pig ileum upon electrical stimulation. The ileum was stimulated at 0.2 Hz, 40 V, and 0.4 msec current duration. *Sup.*, supernatant fraction from hydrolyzed beads.

* Performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. 11377.

of the reaction mixture was adjusted to 5 and 250 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added slowly over a 5-min period. The pH was monitored frequently and readjusted to 5 with 0.1 N HCl as required, especially during the first 30–60 min. The reaction was allowed to proceed at room temperature for 20 hr. The mixture was poured into a chromatography column, and the beads were washed successively with 1 liter of 0.1 N NaCl–0.01 N Tris·HCl pH 7, with 100 ml of 1 N NaCl, and finally with 1 liter more of 0.1 N NaCl–0.01 N Tris·HCl. No radioactivity was detected in 100 ml of the last wash after lyophilizing and redissolving the residue in 1 ml of buffer. The yield of radioactive dihydromorphine bound was 0.3–0.5 μmol/ml of settled beads. The beads were stored in a refrigerator and sodium azide (0.025%) was added to prevent bacterial contamination. Immediately before use the beads were washed with at least 20 bed volumes of buffer to remove the sodium azide and any radioactivity that might have been released.

Evidence for the covalent binding of morphine to Sepharose

In the neutral pH range morphine–Sepharose is quite stable. Salt concentrations as high as 1 M do not release bound more phine. This observation provides evidence against binding by ionic linkage. When the coupling reaction is performed without addition of carbodiimide, no radioactivity remains with the washed beads. Carbodiimides are known to promote the formation of covalent (e.g., ester, peptide) bonds. Treatment of morphine–Sepharose with dilute alkali or neutral hydroxylamine results in the quantitative release of radioactivity into the supernatant.

Effect of morphine–Sepharose on guinea pig ileum

It has long been known that morphine and related narcotics block the electrically stimulated contraction of isolated guinea pig ileum at concentrations as low as 0.1 μM (6, 7). This effect of narcotic analgesics is well correlated with their pharmacological potency in animals, and is reversed by narcotic antagonists (8).

In collaboration with Dr. S. Ehrenpreis, we studied the effect of Sepharose-bound morphine on guinea pig ileum. Fig. 2 shows that morphine bound to the beads had no effect on contraction of guinea pig ileum, nor was there inhibition with amounts of beads containing as much as 40 μg of morphine. After treatment of beads containing 2 μg of bound morphine with 0.1 N NaOH for 15 min the supernatant completely blocked contraction. This block was readily reversed by the specific morphine antagonist, naloxone (Fig. 2).

Specific binding of morphine antibodies to morphine–Sepharose beads

Morphine–Sepharose and aminoethyl-Sepharose beads (not coupled to morphine) were incubated overnight with antiserum to morphine obtained from Dr. S. Spector. Aliquots of the supernatants were tested for residual antibody by their ability to bind tritiated dihydromorphine, as described by Spector and Parker (9). Morphine–Sepharose removed from solution over 90% of the antibody, while no decrease in antibodies was seen in the supernatant from aminoethyl-Sepharose.

DISCUSSION

The specific succinylation of the 6-hydroxyl group of morphine results in a derivative useful for the coupling of morphine to solid matrices for affinity chromatography and to proteins for the preparation of antibodies. An important advantage of 6-succinylmorphine is the availability of all functional groups essential for pharmacological activity. Coupling is done via the 6-hydroxyl group, which is unnecessary for pharmacological activity. Levorphanol, which lacks the alcohol group in position 6, is, in fact, a more potent analgesic than morphine (10). Eddy and Howes (11) showed that when the 6-hydroxyl group in morphine is covered with methyl or acetyl groups increased pharmacological activity also results.

The reason for specific esterification on the 6-hydroxyl group is not completely understood, but may result from the rapid selective hydrolysis of a 3,6-disuccinyl ester. Thus, treatment of 3,6-diacetylmorphine (heroin) with boiling water or with hydroxylamine (11, 12) yields 6-acetylmorphine. The presence of a free carboxyl group in a molecule labilizes nearby ester linkages, especially when a 5- or 6-membered ring intermediate is possible. This effect may speed hydrolytic cleavage of the 3-succinyl ester, so that it occurs rapidly in water at room temperature. While the 6-succinyl ester is probably also more labile than the corresponding acetyl ester, it evidently survives in water at normal temperature and pH.

The specific binding of morphine antibodies by the morphine–Sepharose beads provides evidence for the usefulness of these beads for affinity chromatography.

In collaboration with Dr. S. Spector, we are currently preparing antibodies to morphine linked to bovine serum albumin via the 6-succinyl group. We are using the morphine–Sepharose beads for the purification of morphine antibodies and for the attempted isolation of morphine receptors presumed to be present in animal brain and in other tissues reactive to morphine, such as guinea pig ileum.

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