VISUALIZATION OF OPIATE RECEPTORS AND OPIOID PEPTIDES IN SEQUENTIAL BRAIN SECTIONS

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Summary

Autoradiographic and immunocytochemical studies were carried out on adjacent sections from formaldehyde-perfused rat brains in order to directly correlate the distribution of opiate receptors and opioid peptides. Perfusion fixation of the brains resulted in a partial loss of specific [³H]naloxone binding with essentially no change in the pharmacological properties of the remaining sites. When the distribution of sites was compared to that of enkephalin immunoreactivity in adjacent sections, striking correlations were observed in a number of areas throughout the neuraxis. Adjacent section autoradiography-immunocytochemistry should provide a useful tool for relating the anatomical distribution of opiate receptor subtypes to different opioid peptide neuronal systems.

Following the discovery of opiate receptors in brain (1-3), the isolation of the opioid peptides, Met- and Leu-enkephalin (4), led to the suggestion that these peptides are natural ligands for opiate receptors. Subsequent studies demonstrated the existence of other opioid peptides, particularly β-endorphin (5) and dynorphin (6), which are biosynthetically and anatomically separable from each other and the enkephalins (cf. 7). Since there appear to be multiple subtypes (µ, δ and κ) of opiate receptors (8,9), for which endogenous opioid peptides exhibit different affinities (9-12), it is possible that different populations of receptor subtypes could serve as the physiological sites of action of different opioid peptide neuronal systems. Exploring this question from an anatomical viewpoint requires the visualization of β-endorphin, dynorphin and the enkephalins in very close relation to autoradiographically detected opiate receptor subtypes. In previous studies visualizing opiate receptors and opioid peptides, different brains were used, precluding a direct, comparative analysis of their distributions. The present study was carried out to demonstrate the feasibility of adjacent section analysis of opioid peptides and opiate receptors in brain. We therefore chose to study the distribution of [³H]naloxone binding sites in relation to enkephalin immunoreactivity.

Materials and Methods

Adult male Sprague-Dawley rats were given intracerebroventricular injections of colchicine (300-400 µg) to facilitate cell body immunoreactivity. After 24 or 48 hours, the animals were anesthetized and perfused through the aorta with ice-cold 0.1 M phosphate-buffered 4% formaldehyde (pH 7.4) for 30 minutes. The brains were removed, blocked, incubated in phosphate-buffered 15% sucrose at 4°C overnight, frozen in isopentane at -40°C and sectioned at 20 µm
in a cryostat. Sections were thaw-mounted onto subbed slides and stored at \(-70^\circ\text{C}\). For comparison, brains removed from unperfused rats were frozen immediately after decapitation and sectioned and mounted as above. Since cryostat-out sections from unperfused rats do not exhibit immunocytochemical staining for enkephalins, sections adjacent to those intended for receptor autoradiography were subjected to various postfixation treatments. Incubation treatments were carried out in different concentrations of 0.1 M phosphate-buffered (pH 7.4) formaldehyde (0-4%), from 10 seconds to 48 hours, at 4°C to 25°C, and in combination with other fixatives (0-2% glutaraldehyde or Perfix). These sections, as well as sections from perfused rats, were processed for immunocytochemistry using affinity-purified Leu-enkephalin antisera in the PAP procedure (13). Opiate receptor labelling was carried out by incubating sections from perfused or unperfused rats in 0.05 M Tris HCl (pH 7.4) containing 1 nM \[^{3}H\]naloxone (40 Ci/mmol, NEN) and 100 mM NaCl for 2 hours at 4°C. These incubation conditions were the same used previously to label \(\mu\)-opioid receptors (14-16), except that the incubation time was extended from 1 to 2 hours to increase specific binding, which was defined as the difference between total binding and binding in the presence of 1 \(\mu\)M levorphanol. Displacement studies were carried out using varying concentrations (0.1 nM-0.1 mM) of different unlabelled opiates (17). Following incubation, the slides were washed by agitation in 5 changes (~0 seconds each) of 200 mls of 0.1 M phosphate-buffered saline (pH 7.4) at 4°C, and dried rapidly using a hair drier in a cold room at 4°C. For quantitation of binding in the displacement studies, the sections (and underlying glass) were transferred into vials containing 10 mls fluor and shaken to extract the radioactivity, which was measured by liquid scintillation counting. Other \[^{3}H\]naloxone-labelled sections, adjacent to sections processed for Leu-enkephalin immunoreactivity, were prepared for autoradiography according to the method of Herkenham and Pert (18). Adjacent immunocytochemistry-autoradiography sections were viewed and photographed in bright field and dark field using a Leitz Orthoplan microscope.

**Results and Discussion**

Postfixation of slide-mounted sections from unperfused rat brain failed to result in any detectable immunocytochemical staining for Leu-enkephalin, precluding the use of unperfused brain for adjacent section immunocytochemistry-autoradiography. Brain sections from rats perfused with formaldehyde exhibited good binding of \[^{3}H\]naloxone (over 1000 dpm bound/slice), 80-90% of which could be displaced by 1 \(\mu\)M levorphanol. Stereospecificity of binding was confirmed by the lack of displacement in the presence of 1 \(\mu\)M dextrorphan. Despite the loss of some \[^{3}H\]naloxone binding sites as a result of the perfusion, displacement studies indicated that the pharmacological properties of the remaining binding sites were essentially unchanged (17). Furthermore, the anatomical distribution of sites was very similar to that observed previously in brain sections from unperfused rats (14,16,18). In the striatum, for example, the characteristic patches and subcallosal streak of binding sites were clearly evident (Fig. 1A), and the specificity of this pattern was confirmed in the adjacent section by blockade with 1 \(\mu\)M levorphanol (Fig. 1B). When the distributions of \[^{3}H\]naloxone binding sites and Leu-enkephalin immunoreactivity were compared in adjacent sections, a number of correlations were observed. For example, in the habenula, a dense zone of \[^{3}H\]naloxone binding sites (Fig. 1C) corresponded to the location of a field of intensely stained enkephalin immunoreactive fibers surrounded by a zone of more sparsely distributed immunoreactive fibers (Fig. 1D). A further example of correspondence is shown in the parabrachial nucleus, where the distribution of \[^{3}H\]naloxone binding sites (Fig. 1E) closely matched that of immunoreactive enkephalin perikarya and fibers (Fig. 1F). We have noted, and described elsewhere (17), many other areas of correspondence, as well as non-correspondence, between presumed opiate receptors and enkephalin...
Fig. 1
Adjacent section autoradiography-immunocytochemistry. Caudate-putamen: \([^{3}H]naloxone\) binding in the absence (A) and presence (B) of 1 \(\mu\)M levorphanol. Habenula: \([^{3}H]naloxone\) binding (C) and Leu-enkephalin immunoreactivity (D). Parabrachial nucleus: \([^{3}H]naloxone\) binding (E) and Leu-enkephalin immunoreactivity (F). v: ventricle. bc: brachium conjunctivum. Bar = 100 \(\mu\)m.
neuronal systems in rat brain. Despite the preferential labelling of µ receptors by low concentrations of [3H]naloxone, and the preferential binding of enkephalin to δ receptors, some correlation between µ receptor and enkephalin distributions was expected on the basis of earlier autoradiographic and immunocytochemical studies carried out on different brains (e.g., 19). Furthermore, the anatomical loci of µ receptors may be relevant to some sites of enkephalin action, a suggestion supported by the finding that [3H]D-Ala², D-Leu⁵-enkephalin, a prototypical δ ligand, can exhibit a "µ-like" anatomical binding pattern with "δ-like" pharmacological properties (20). We are presently carrying out autoradiographic-immunocytochemical studies using different radiolabelled opiate receptor ligands under various incubation conditions to explore the relationships between opiate receptor subtypes and enkephalin, dynorphin and ß-endorphin neuronal systems in brain.

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References