Identification of Distinct Binding Site Subunits of μ and δ Opioid Receptors†

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ABSTRACT: Iodinated human β-endorphin was affinity-cross-linked to opioid receptors present in membrane preparations from bovine frontal cortex, bovine striatum, guinea pig whole brain, and rat thalamus. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography revealed covalently labeled peptides of 65, 53, 41, and 38 kilodaltons (kDa). The 65- and 38-kDa peptides were present in all four tissues. The 41-kDa peptide was seen only in bovine caudate and guinea pig whole brain while the 53-kDa peptide was absent in rat thalamus. All four labeled peptides were constituents of opioid receptors since their labeling was fully suppressed by the presence of excess opiates, such as bremazocine, during binding. The distribution and levels of the labeled species in the brain tissues examined and, in earlier work, in the neuroblastoma × glioma NG 108-15 cell line suggested that the 65-kDa peptide is a binding component of μ receptors while the 53-kDa peptide is a binding subunit of δ receptors. This result was strongly supported by the finding that the labeling of the 65-kDa peptide is selectively reduced by the presence of the highly μ-selective ligand Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol (DAMGE) during binding, while the labeling of the 53-kDa peptide is selectively reduced or eliminated by the highly μ-selective ligand [D-Pen^2,D-Pen^5]enkephalin (DPDPE). The labeling of the 41- and 38-kDa bands was reduced by either DAMGE or DPDPE. The relationship of these lower molecular weight opioid-binding peptides to μ and δ receptors is not understood. Several possible explanations are presented.

Considerable evidence has accumulated in recent years that supports the concept that the opioid receptor system is composed of multiple types (Martin et al., 1976; Kosterlitz et al., 1983; Simon & Hiller, 1984). Pharmacological, electrophysiological, and radioreceptor studies have demonstrated the existence of three major types, termed μ, δ, and κ. However, little is known about the molecular basis of opioid receptor heterogeneity. Some progress has been made in addressing this question through the preliminary identification of the ligand binding subunits of μ and δ receptors. Using [^{3}H]-fentanyl isothiocyanate as an affinity probe, Klee et al. (1982) described the specific, covalent labeling of a peptide of 58 kilodaltons (kDa) from the δ sites exclusively present in neuroblastoma × glioma (NG 108-15) cells. Newman & Barnard (1984) have reported the affinity labeling of a subunit of μ binding sites of 58 kDa from rat brain with the opioid alkylating agent [D-[^{3}H]Ala^2,Leu^5]enkephalin chloromethyl ketone, suggesting that the ligand binding subunits of μ and δ receptors possess similar molecular weights. In a recent report (Howard et al., 1985) our laboratory has described the specific, covalent labeling of a major peptide of 65 kDa derived from μ binding sites and a major peptide of 53 kDa from δ sites.

The identification of distinct μ and δ receptor types has been facilitated by the recent synthesis of highly type-selective ligands. Ligands that approach specificity for a given site include DAMGE[1] (~150-fold more selective for μ than for δ sites) and DPDPE (~200-fold more selective for δ than for μ sites) (Kosterlitz et al., 1981; Mosberg et al., 1983). In contrast to the recent report of Newman & Barnard (1984), the present work, using these highly selective ligands, provides strong evidence that the major ligand binding peptides of μ and δ receptors are indeed distinct entities of different molecular size.

Materials and Methods

Materials. ^{125}I-β-end$_H$ (~2000 Ci/mmol) was purchased from Amersham. DPDPE was a gift from Drs. H. Mosberg and T. Burks of the University of Arizona. DAMGE was obtained from Reckitt and Coleman, Hull, England. BSCOES was purchased from Pierce. Electrophoresis chemicals and premixed standards were from Bio-Rad. X-ray film (XAR-5) was from Kodak, and intensifying screens (Cronex Lightning Plus) were from Du Pont. Other supplies were from Sigma or from sources previously reported (Itzhak et al., 1984).

Membrane Preparations. Crude membrane fractions were prepared from rat, guinea pig, and bovine brain tissue as described (Ruegg et al., 1981) and stored at a concentration of 9–11 mg/mL protein in 0.32 M sucrose at −70 °C. Protein was determined by the method of Bradford (1976).

Affinity Cross-Linking of ^{125}I-β-end$_H$. Crude membranes were diluted in binding buffer (50 mM K_2HPO_4, pH 7.4, 50 μg/mL bacitracin) to a final concentration of 1.6 mg/mL protein and bound with 2 nM ^{125}I-β-end$_H$ (1200 Ci/mmol) in the presence and absence of competing ligand(s) for 1 h at 25 °C. An aliquot of this suspension was checked for specific binding by rapid vacuum filtration on glass fiber filters presoaked in 0.4% BSA and 0.01% polylysine. Specific binding (~85–90% of total) is defined as the difference in binding observed in the absence and presence of 1 μM bremazocine

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[1] Abbreviations: β-end$_H$, β-endorphin (human); ^{125}I-β-end$_H$, [27-(3-[^{125}I]iodotyrosyl)]-β-endorphin (human); DAMGE, Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol; Pen, penicillamine; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSTLE, Tyr-D-Ser-Gly-Phe-Leu-Thr; BSCOES, bis[2-[[(succinimidooxy)carbonyl]oxy]ethyl] sulfone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)-aminomethane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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or naloxone. The remaining membranes were diluted 2-fold in ice-cold binding buffer and centrifuged at 20000*g* for 15 min at 4 °C. The resulting membrane pellet was resuspended in the original volume of binding buffer, and cross-linking was initiated by the addition of BSCOES (freshly prepared in dimethyl sulfoxide) to a final concentration of 1 mM. After 15 min at 0 °C, cross-linking was terminated by the addition of a 10-fold excess of 50 mM Tris-HCl and 1 mM EDTA buffer (pH 7.4). The duration of the cross-linking reaction was found to be optimal at 15 min. As reported previously (Howard et al., 1985), BSCOES was the most useful reagent tested for the adequate visualization of radiolabeled proteins. The membranes were then centrifuged at 20000*g* for 15 min at 4 °C, and the cross-linked pellet was solubilized in SDS–PAGE sample buffer (3% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromophenol blue, 100 mM dithiothreitol).

SDS–PAGE. Prior to electrophoresis, insoluble material was removed by centrifugation. The resulting supernatant was boiled for 2 min and applied to a 5–15% linear gradient gel according to the procedures of Laemmli (1970). The gel was fixed (30% methanol, 10% acetic acid), stained with Coomassie blue R-250 to locate molecular weight standards (phosphorylase *b*, M_r 92 000; BSA, M_r 66 200; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 31 000; soybean trypsin inhibitor, M_r 21 000; lysozyme, M_r 14 400), dried, and subjected to autoradiography (XAR-5 film with intensifying screens for 3–5 days at −70 °C). A plot of log M_r vs. R_f of the standards was used for the determination of the M_r of the radiolabeled proteins. The molecular weights of the radiolabeled proteins as reported here include the molecular weight of β-end$_H$ (~3000).

RESULTS

In a previous report from this laboratory, ^{125}I-β-end$_H$ was shown to bind to μ and δ sites with similar high affinity (K_D ~2–4 nM) while exhibiting negligible affinity at κ sites (Howard et al., 1985), similar to results observed for [^{3}H]-β-end$_H$ (Ferrara et al., 1979) and in competition experiments for unlabeled β-end$_H$ against [^{3}H]DAMGE and [^{3}H]DADLE (Kosterlitz et al., 1983). As described below, cross-linking of ^{125}I-β-end$_H$ to μ and δ receptors was performed in a number of tissues that differ significantly in their μ:δ receptor ratio. The μ:δ ratios in bovine caudate (~3.5:1), bovine frontal cortex (~1:2), rat thalamus (~4.5:1), and guinea pig whole brain (~1:1) have been reported (Ninkovic et al., 1981; Chang et al., 1979; Kosterlitz & Paterson, 1980) and were confirmed in our laboratory by performing radioreceptor assays using the most site-selective ligands currently available. To this end, saturation curves were generated for the binding of [^{3}H]-DAMGE (μ sites) and [^{3}H]DSTLE in the presence of 20 nM unlabeled DAMGE (δ sites). Receptor ratios were also obtained from competition studies with unlabeled DAMGE and DPDPE (δ sites) against ^{125}I-β-end$_H$, and our results are in full agreement with the ratios reported in the literature (data not shown). To label μ and δ sites covalently, membrane preparations were incubated with 2 nM ^{125}I-β-end$_H$ in the absence and presence of one or more of the following competing ligands: DAMGE, DPDPE, and bremazocine. Samples were washed to remove unbound or loosely bound ligand and cross-linked with 1 mM BSCOES. Equal amounts of soluble protein from these preparations were subjected to electrophoresis on a 5–15% linear gradient SDS–polyacrylamide gel, followed by autoradiography. Control labeling patterns (absence of competing ligand) are similar for all tissues examined, but there are some distinct differences. All tissues demonstrate

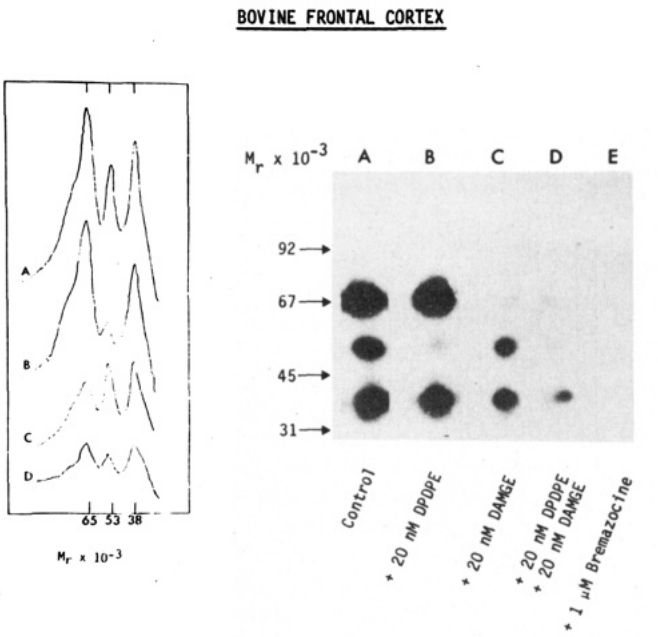

FIGURE 1: Affinity cross-linking of bovine frontal cortex membranes. Membranes (300 μg) that had been bound with 2 nM ^{125}I-β-end$_H$ in the absence and presence of competing ligand(s) were cross-linked with 1 mM BSCOES (15 min at 0 °C). Cross-linking was terminated by the addition of an excess of 50 mM Tris–EDTA buffer, and samples were solubilized in SDS–PAGE sample buffer. Equal amounts of protein (containing ~2000–10 000 cpm) were applied to a 5–15% linear gradient gel with a 4.5% stacking gel. Electrophoresis was conducted for 3–4 h at 25-mA constant current. The fixed, dried gel was then subjected to autoradiography: (lane A) control, no competing ligand added; (lane B) +20 nM DPDPE; (lane C) +20 nM DAMGE; (lane D) +20 nM DPDPE and 20 nM DAMGE; (lane E) +1 μM bremazocine. The positions of the molecular weight standards are shown. Also presented is a densitometric scan of lanes A–D.

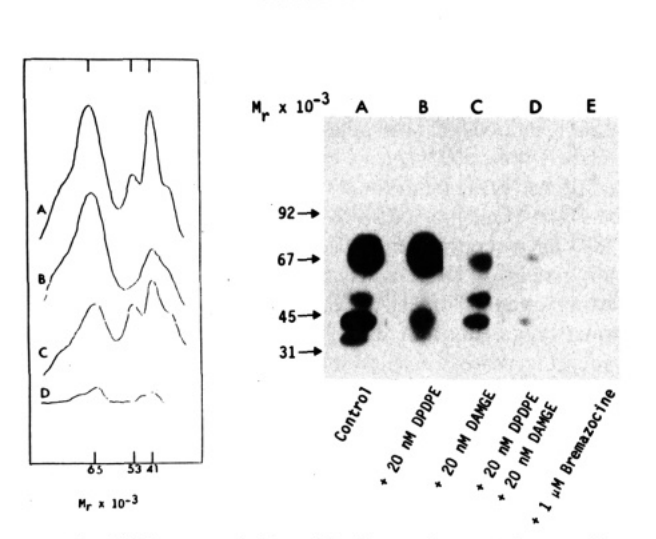

FIGURE 2: Affinity cross-linking of bovine caudate membranes: (lane A) control, no competing ligand added; (lane B) +20 nM DPDPE; (lane C) +20 nM DAMGE; (lane D) +20 nM DPDPE and 20 nM DAMGE; (lane E) +1 μM bremazocine. The positions of the molecular weight standards are shown. Also presented is a densitometric scan of lanes A–D.

significant labeling of a peptide of 65 kDa as well as variable labeling of a peptide of 38 kDa (Figures 1–4, lanes A). In addition, labeling of a peptide of 53 kDa was observed in all tissues except for rat thalamus, and the labeling of a 41-kDa species was seen only in bovine caudate and guinea pig brain. Densitometric scanning of autoradiograms indicated that the ratio of incorporated radioactivity into the 65- and 53-kDa

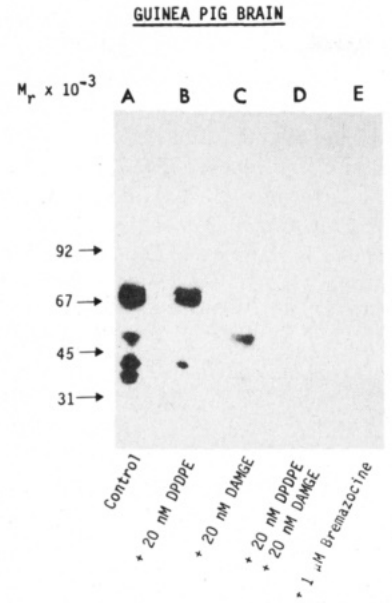

FIGURE 3: Affinity cross-linking of guinea pig whole brain membranes: (lane A) control, no competing ligand added; (lane B) +20 nM DPDPE; (lane C) +20 nM DAMGE; (lane D) +20 nM DPDPE and 20 nM DAMGE; (lane E) +1 μM bremazocine.

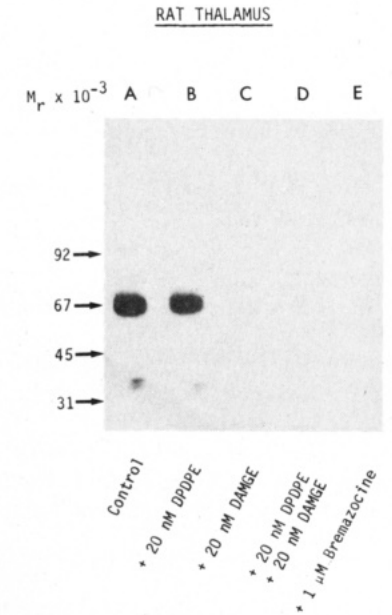

FIGURE 4: Affinity cross-linking of rat thalamus membranes: (lane A) control, no competing ligand added; (lane B) +20 nM DPDPE; (lane C) +20 nM DAMGE; (lane D) +20 nM DPDPE and 20 nM DAMGE; (lane E) +1 μM bremazocine.

peptides was ~1:0.3 for bovine caudate and ~1:0.6 for bovine frontal cortex. That all four peptides are constituents of opioid receptors was indicated by the finding that the labeling of these bands was completely abolished when the binding of ^{125}I-β-end$_H$ was carried out in the presence of 1 μM bremazocine, an opiate alkaloid unrelated in structure to β-end$_H$ (Figures 1–4, lanes E). Moreover, the labeling of all four peptides was pharmacologically relevant since it was decreased or prevented when binding was conducted in the presence of Na^+ (100 mM) or GTP (50 μM) added separately or together (data not shown).

When ^{125}I-β-end$_H$ binding was conducted in the presence of the δ-selective ligand DPDPE, autoradiographic analysis revealed a significant decrease in (cortex—Figure 1, lane B) or complete abolition of (caudate—Figure 2, lane B; guinea pig—Figure 3, lane B) the labeling of the 53-kDa peptide. The labeling of the 65-kDa peptide in all tissues was unchanged or only slightly reduced by DPDPE treatment. In marked contrast, the effect of the μ-selective ligand DAMGE was to decrease the labeling of the 65-kDa peptide in all tissues (Figures 1–4, lanes C) while exhibiting minimal, or no, effect on the labeling of the 53-kDa peptide. It should be noted that the concentration of DAMGE and DPDPE used in the above experiments was based on competition studies against [^{3}H]-bremazocine binding which indicated that 20 nM was a reasonable concentration to inhibit binding at μ sites by DAMGE or at δ sites by DPDPE with relatively little cross-reactivity at the nonpreferred site (data not shown). In the presence of both DAMGE and DPDPE, the labeling of the 65- and 53-kDa peptides is further reduced or completely absent (Figures 1–4, lanes D). The labeling of the lower molecular weight peptides of 41 and 38 kDa, while clearly opioid receptor related, was not selectively depressed by either DAMGE or DPDPE but tended to be depressed by either the μ or the δ ligand.

Discussion

These findings lend strong support to our previous results which suggested that μ and δ opioid binding sites contain major subunits that differ in molecular size. The assignment of the 65-kDa peptide to μ binding sites is based on (1) its appearance in all tissues that contain μ sites and its absence in a tissue that does not (NG 108-15 cells) (Howard et al., 1985) and (2) the selective displacement of radioactivity from this peptide by DAMGE, a highly selective μ ligand, and not by the highly selective δ ligand, DPDPE. The assignment of the 53-kDa peptide to δ sites similarly rests on (1) the appearance of this peptide in all tissues that are known to contain δ sites but its absence in rat thalamus, which is virtually devoid of δ sites, and (2) the selective displacement of radioactivity from this peptide by DPDPE and not by DAMGE. The ratio of radioactivity incorporated into these peptides varies from tissue to tissue in the direction of the variation in μ:δ ratios previously ascertained by radioreceptor assays of the membranes of the tissues. Quantitatively, the magnitude of the observed shift in radiolabel into the 53-kDa peptide in comparing, for example, bovine caudate (high in μ) to bovine frontal cortex (high in δ) is not as great as expected. This is most likely due to a finding we previously reported (Howard et al., 1985), namely, that the 53-kDa peptide is cross-linked to ^{125}I-β-end$_H$ with significantly lower efficiency than the 65-kDa peptide. Two smaller molecular weight peptides are also observed, a 38-kDa species seen to some extent in every brain tissue studied and a 41-kDa species, observed only in bovine caudate and guinea pig whole brain. These peptides are clearly opioid receptor constituents since, like the other peptides, their labeling is completely prevented by an excess of various opiates. These peptides, however, do not display μ or δ specificity since their labeling is reduced by the presence of either DAMGE or DPDPE. There are at least four possible explanations for these observations, namely, that these peptides (1) may be generated by proteolysis of the 65- and 53-kDa peptides during solubilization giving rise to fragments of 41 and 38 kDa, (2) could represent binding site subunits common to μ and δ receptors, (3) could be the binding site subunits of other receptors that have been postulated to have affinity for both μ and δ ligands, such as μ_1 (Nishimura et al., 1984) or ε (Chang et al., 1984) receptors, or (4) could represent cross-linking of ^{125}I-β-end$_H$ to proteins in very close proximity to the binding site, for example, coupled proteins such as the inhibitory guanyl nucleotide binding protein (N_i), whose subunit molecular mass

is in the range of 35–45 kDa.

One further observation requires discussion. It appears that when DPDPE and DAMGE are added together, the disappearance of the labeled peptides is more pronounced than expected from the individual effects of these ligands. While this phenomenom is not understood, it could be interpreted as providing evidence for an allosteric relationship between μ and δ receptors, as previously suggested by others (Rothman et al., 1982).

In work reported elsewhere (Gioannini et al., 1985), our laboratory has purified to apparent homogeneity an active opioid binding protein of molecular mass ~65 kDa from bovine caudate by ligand affinity and lectin chromatography. This preparation binds opiates with high affinity and stereospecificity in a saturable manner and is ~70 000-fold enriched as compared to crude soluble or membrane-bound receptors. The finding that the purified protein of 65 kDa can be specifically cross-linked to ^{125}I-β-end$_H$ lends strong support to the idea that the purified protein is identical with the polypeptide of 65 kDa visualized by affinity cross-linking in crude membrane homogenates, as reported here.

The observed difference in molecular size for μ and δ binding site subunits may reflect a difference in primary structure or the degree and type of glycosylation of these proteins (Gioannini et al., 1982). Because of the broad, diffuse nature of these bands, which may be attributed to their glycoprotein nature, peptide heterogeneity, and the use of the radioisotope ^{125}I, exact molecular sizes are difficult to determine. The report that subunits of μ and δ receptors seemed to have the same molecular size (Newman & Barnard, 1984) is very likely due to variations in sample preparation and the manner in which SDS–PAGE was conducted in the two laboratories. However, when μ and δ sites are covalently labeled in the same tissue and analyzed under identical conditions, there is no doubt about their difference in size.

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Registry No. DAMGE, 78123-71-4; DPDPE, 88373-73-3.

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