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COMMENTARY

DELTA OPIOID RECEPTOR RADIOLIGANDS

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Receptor characterization using radioligands

Prior to the development of radioligand binding methods, receptors could only be characterized through biological assays where a behavioral or tissue response was related to a particular receptor. A number of different bioassays were developed for the characterization of opioid compounds including isolated tissue preparations such as the guinea pig ileum and mouse vas deferens and *in vivo* assays of analgesia [1, 2]. Effects produced by opioid agonists in these assays are indirect to the extent that they occur only after the activation of a polysynaptic pathway. The agonist potency measured by these assays may be described as a function of receptor binding affinity and the efficacy of the agonist. Measurements of drug potency are subject to at least three independent variables: the affinity of the drug, its efficacy, and the density of the target receptor in the tissue preparation [3]. This complicates comparisons between different drugs acting at the same receptor and for different receptors being acted on by the same drug.

Radioligand binding studies provide a simplified measure of drug–receptor interaction that is only dependent on receptor binding affinity. Receptors can be characterized in terms of their binding affinity for different ligands independent of the efficacy of the ligand or the receptor density in the tissue preparation. To be useful, however, it is necessary to know the identity of the receptor being labeled by the radioligand which is dependent on the selectivity of the radioligand for that receptor.

The binding selectivity of a ligand is defined in terms of its relative affinity between two or more receptors that must be labeled by selective radioligands [4]. These measurements are made by radioligand binding inhibition studies where the ability of the ligand to inhibit binding of a radioligand over a range of ligand concentrations is determined. The measurement is valid to the extent that the radioligand specifically labels the intended receptor and the inhibition is competitive. A competitive interaction between ligand and radioligand requires that the ligand prevents binding of the radioligand through the occupation of the radioligand's binding site. Noncompetitive inhibitory interactions include allosteric mechanisms where the ligand binds to a distant, but physically coupled, site recognized by the radioligand in a way that reduces the binding affinity of the radioligand. In this way the allosteric site modulates the binding affinity of the radioligand binding site.

Apart from ordinary errors of measurement, the precision of binding selectivity determinations is affected by the conditions used. The inhibition constant (IC_{50}) determined for a ligand is dependent on the radioligand concentration used for the experiment through the simplified Cheng–Prusoff relationship [5]:

$$IC_{50} = K_i\left(1 + \sqrt{\frac{[L]}{K_d}}\right).$$

In this formula K_i is the equilibrium inhibition constant, $[L]$ the concentration of the radioligand, and K_d the equilibrium dissociation constant of the radioligand. From this relationship it can be seen that the IC_{50} value measured will increase as the concentration of the radioligand becomes large relative to its binding affinity at the receptor. This error can be avoided by using K_i instead of IC_{50} values for the determination of selectivity ratios but this relationship is only valid if the K_d value of the radioligand under the experimental conditions used for the inhibition study is known.

The precision of a binding selectivity measurement is also dependent on the conditions used for the binding experiments in another way. It is possible, even likely, that the different receptors selected for the studies will have different optimal binding conditions. This introduces a bias toward greater apparent selectivity if the experimental conditions used favor higher binding affinity to one receptor over the other.

The selectivity of a ligand can only be defined for the receptors chosen by the investigator. Typically, the choice of receptors is based on structural considerations of the ligand (e.g. an opioid derivative will be tested for binding to opioid, but not muscarinic, receptors). This can occasionally lead to surprises. Examples include the observations that certain cholecystokinin analogs have unusually high binding affinity at δ opioid receptors [6], while some somatostatin analogs have high affinity at μ opioid receptors [7].

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Opioid receptors

Opioid drugs are, and will continue to be, essential therapeutic agents [8]. They provide the ultimate treatment for pain, but their use is complicated by many non-therapeutic actions. The most notable include respiratory depression, sedation, and gastrointestinal dysfunction. (The gastrointestinal effects of opiates, such as constipation, can be considered therapeutic in the case of diarrhea, however.) Chronic use of opiates can also result in addiction and physical dependence though this is rare when they are used for therapeutic reasons. The non-analgesic effects of opiate drugs are a strong incentive to develop new opioids less capable of producing them. The identification of multiple opioid receptor types and the production of compounds selective for them may provide a means to safe analgesics [9].

Multiple opioid receptors. Efforts to identify safer opioid drugs in the middle of this century led to the synthesis of many new opiate analogs and non-opiate compounds with opiate-like activity. Differences in the actions of these drugs suggested the possibility of multiple opiate receptors. This hypothesis was confirmed by the studies of Martin and colleagues [10, 11] that characterized three distinct sites of opiate drug action. Named for their prototype agonists, they were defined as the μ (morphine), κ (ketocyclazine), and σ (SKF-1044) receptors. While subsequent studies demonstrated the non-opioid nature of the σ receptor [12], the μ and κ receptors remain important opioid receptor types. The observation that these receptors mediated different non-analgesic effects opened the possibility that some opiate side-effects might be avoided by more selective drugs acting on different opioid receptor populations.

Isolation of endogenous opioid peptides. The existence of opioid receptors implied the presence of endogenous opioid compounds. This was demonstrated by the discovery of the pentapeptides leucine- and methionine-enkephalin [13]. Their discovery resulted in the almost immediate recognition of a third opioid peptide, β-endorphin, which was identified as part of the β-lipotropin sequence [14]. β-Endorphin contains the [Leu]-enkephalin sequence as its five N-terminal amino acids. Somewhat later another group of peptides containing the amino acid sequence of enkephalins, the dynorphins, was identified [15].

While the three classes of endogenous opioid peptides, enkephalins, endorphins, and dynorphins, are all related through the presence of N-terminal enkephalin sequences, they are the products of distinct precursor proteins and have different distributions in the CNS.

Identification of the delta opioid receptor. The δ opioid receptor was identified by virtue of the relatively high potency of enkephalins and β-endorphin in the mouse vas deferens compared to morphine [16]. The greater potency of these peptides in the mouse vas deferens contrasted to their lower potency relative to morphine in the guinea pig ileum. This difference in the rank order of potencies between mouse vas deferens and guinea pig ileum suggested the presence of a different receptor in the vas deferens. It is now known that the mouse vas deferens has all three of the major opioid receptor types (μ, δ, and κ) whereas the guinea pig ileum lacks functional δ opioid receptors. Selective δ opioid agonists are always more potent in the mouse vas deferens compared to the guinea pig ileum for this reason.

Radioligand binding to opioid receptors. Goldstein and colleagues [17] proposed that radiolabeled drugs could be used to investigate their binding to opioid receptors and suggested methods to distinguish receptor bound radioligand from that bound to other elements of tissue. Their efforts to demonstrate such receptor binding were unsuccessful due to the low specific activity of the radioligands available, but subsequent efforts by the laboratories of Snyder [18], Simon [19], and Terenius [20] using high specific activity radioligands were successful.

Enkephalin analogs

Enkephalins are rapidly degraded *in vivo* and by *in vitro* tissue preparations. This accounts for the apparent low potency of enkephalins observed in the absence of enzyme inhibitors [21, 22]. Efforts to produce more stable opioid receptor ligands with improved selectivity for opioid receptor types has led to the development of many new peptide analogs [23].

Early studies showed that the presence of the D-isomer of alanine in the 2-position of enkephalin greatly reduces enzymatic cleavage of the N-terminal tyrosine [24, 25]. This substitution greatly enhances the potency of the peptide by preventing its degradation. One of the first enkephalin analogs to exploit this characteristic, [D-Ala^2,D-Leu^5]enkephalin (DADLE)*, is substantially more potent than the endogenous opioid peptides by virtue of its greater metabolic stability [24, 25]. DADLE also has greater pharmacological selectivity for the δ opioid receptor relative to [Leu]enkephalin. While [^{3}H]DADLE was used as a radioligand for labeling δ opioid receptors for many years, it is now recognized as having very limited receptor binding selectivity.

Delta receptor selective linear peptides. Greater δ opioid receptor selectivity relative to DADLE was found for the enkephalin analogs [D-Ser^2,Leu^5,Thr^6]-enkephalin (DSLET) [26] and [D-Thr^2,Leu^5,Thr^6]-enkephalin (DTLET) [27]. Like DADLE, these analogs have good pharmacological selectivity between μ and δ opioid receptors as determined by their potency ratios in the mouse vas deferens and guinea pig ileum bioassays. However, like DADLE they show much more limited binding selectivity between these opioid receptors [28]. Substantially better δ opioid receptor binding selectivity is observed for the newer ligands [D-Ser^2(*O-tert*-

* Abbreviations: DADLE, [D-Ala^2,D-Leu^5]enkephalin; DSLET, [D-Ser^2,Leu^5,Thr^6]enkephalin; DTLET, [D-Thr^2,Leu^5,Thr^6]enkephalin; DSTBULET, [D-Ser^2(*O-tert*-butyl),Leu^5,Thr^6]enkephalin; BUBU, [D-Ser^2(*O-tert*-butyl),Leu^5,Thr^6(*O-tert*-butyl)]enkephalin; DPDPE, [D-Pen^2, D-Pen^5]enkephalin; and *p*-Cl-DPDPE, [D-Pen^2,4'-Cl-Phe^4,D-Pen^5]enkephalin.

butyl),Leu^5,Thr^6]enkephalin (DSTBULET) and [D-Ser^2(*O*-*tert*-butyl), Leu^5,Thr^6(*O*-*tert*-butyl)]-enkephalin (BUBU).

DSTBULET is an analogue of DSLET, differing by the presence of an *O*-*tert*-butyl substitution on the serine hydroxyl side chain group [29, 30]. This modification produces a small enhancement of DSTBULET binding affinity for δ opioid receptors (less than 2-fold relative to DSLET) but substantially reduces its binding affinity to μ opioid receptors (about 10-fold relative to DSLET) when measured using rat brain membranes. The binding affinity of DSTBULET is 130-fold higher at δ relative to μ opioid receptors in contrast to about 10-fold for DSLET. [^{3}H]DSTBULET rapidly binds to sites in rat brain with an association rate constant (k_{+1}) of $1.04 \times 10^8\,M^{-1}\,min^{-1}$ at 35°. The dissociation rate constant (k_{-1}) measured under the same conditions is $3.94 \times 10^{-2}\,min^{-1}$. The K_d value obtained from the kinetic studies ($k_{-1}/k_{+1} = 379$ pM) is nearly 6-fold less than what was measured by equilibrium binding ($K_d = 2.3$ nM).

The further modification of DSTBULET though the addition of a second *O*-*tert*-butyl group on the threonine side chain hydroxyl group to produce BUBU provides a marginal 2-fold increase in δ opioid receptor binding afffinity and δ to μ selectivity [29, 30].

Structural comparisons between heterocyclic opiate drugs and the enkephalins suggest that the tyrosyl ring of enkephalins is analogous to the phenolic hydroxyl group of morphine while the α-amino group of the N-terminal tyrosine may be analogous to the secondary nitrogen. These analogies provided a rationale for the preparation of an enkephalin antagonist by *N,N*-bis diallyl substitutions of the tyrosine α-amino group of a [Leu]enkephalin analog where the glycine-phenylalanine peptide bond was replaced by a methylene thio linkage. The resulting [Leu]enkephalin analog (ICI 154,129) is a low potency antagonist at the δ opioid receptor [31]. Improved selectivity and potency are observed for the related [Leu]enkephalin analog, ICI 174,864 where the two glycines are replaced by two α-amino isobuytyric acid substitutions [32]. Neither ICI 154,129 nor ICI 174,864 has sufficient affinity for δ opioid receptors to permit their use as radioligands though they are important ligands for the characterization of agonists in δ receptor bioassays.

Conformationally constrained enkephalin analogs

Conformational studies of enkephalins. It is generally assumed that receptors bind ligands that are in a specific conformational state. The characterization of the receptor-bound conformational state would be helpful for the rational design of selective ligands since chemical modifications that force a ligand to assume that state would facilitate its binding to the receptor while hindering its binding to other sites. It is difficult to obtain such information for the enkephalins, however.

Enkephalins, like other linear peptides comprised of naturally occurring amino acids, are capable of assuming a large number of different conformations in solution. Both solution and solid phase studies suggest that enkephalins can adopt a folded structure where the C-terminal amino acid approaches the N-terminal tyrosine [33]. From this information it was hypothesized that analogs of the enkephalins forced to assume a folded conformation through the introduction of an intermolecular bridge might be better ligands for opioid receptors. This hypothesis was tested by the synthesis of cyclic enkephalin analogs.

Cyclic enkephalin analogs. The first systematic effort to examine the effect of an intermolecular bridge on enkephalin interactions with opioid receptors was made by Schiller and colleagues [34, 35]. The major finding of these studies was that enkephalin analogs constrained in this way retained activity at μ and δ opioid receptors with a small degree of pharmacological selectivity for the μ receptor.

Parallel studies by Hruby and associates focused on the introduction of additional conformational constraint to cyclic enkephalins. This was achieved by the use of penicillamine substitutions in the 2- and 5-positions of the enkephalin sequence [36, 37]. Penicillamine (Pen) is related to cystine but differs by the presence of two methyl groups on the β-carbon. These methyl groups reduce rotation around the dithio bridge formed with another sulfhydryl amino acid by steric hindrance. The incorporation of two Pen substitutions at the 2- and 5-positions produced the first highly selective δ opioid receptor agonist, [D-Pen^2,D-Pen^5]enkephalin (DPDPE). This cyclic enkephalin is over 3000-fold more potent at δ relative to μ opioid receptors and generally shows over 600-fold greater δ to μ receptor binding affinity.

[^{3}H]DPDPE has long served as the most selective radioligand available for the characterization of δ opioid receptors. While it has high affinity ($K_d = 1$–5 nM) for δ receptors of NG 108-15 cells and those of rat and guinea pig brain, its low association rate constant requires extended incubation times (>4 hr for concentrations below 1.0 nM) to achieve equilibrium [38, 39]. [^{3}H]DPDPE shows similar binding properties to intact NG 108-15 cells under physiological conditions (binding in cell culture medium at 37°) as are observed for cell homogenates in 50 mM Tris buffer [40].

An important advance in the understanding of the difference between the ligand requirements of the μ and δ opioid receptors resulted from studies designed to explore the effects of electronegative group substitutions in the phenylalanine ring of DPDPE [41]. Nitro group and halogen atom substitutions in the 4′-position of the Phe^4 ring gave small but significant improvements in binding affinity at the δ opioid receptor, but produced dramatic reductions in binding affinity at the μ receptor. One of these ligands, [D-Pen^2,4′-Cl-Phe^4,D-Pen^5]enkephalin (*p*-Cl-DPDPE), was selected for radiolabeling.

p-Cl-DPDPE was tritium-labeled (sp. act. ≈ 40 Ci/mmol) and binding measurements were made with crude rat brain membranes less cerebellum in Tris buffer [42]. Saturation binding data analyzed by nonlinear regression methods for one- and two-site models consistently showed binding to a single site with a mean K_d of 330 pM and a receptor density of 87 fmol/mg protein at 25°. As expected, elevation

of the assay temperature to 37° decreased the binding affinity (K_d = 603 pM). The receptor density value was also reduced (B_{max} = 69 fmol/mg protein) which is interpreted as being a result of receptor, rather than radioligand, degradation. [^{3}H]*p*-Cl-DPDPE is an unusually stable peptide and is recovered intact even after systemic administration in rats [43].

In contrast to the single site binding observed by saturation binding studies, [^{3}H]*p*-Cl-DPDPE consistently shows biphasic receptor association. Dissociation is monophasic so two K_d values can be calculated. Approximately 90% of the binding at a [^{3}H]*p*-Cl-DPDPE concentration of 750 pM was associated with a site having a K_d value of 201 pM while about 10% of the binding had a K_d value of 8.4 pM. The high-affinity site detected by the kinetic studies could not have been observed over the concentration range used for the saturation studies (50–5000 pM). The higher affinity seen for the second site in the kinetic studies relative to the lower affinity measured by saturation studies is most likely due to the effect of receptor concentration on saturation binding measurements. Observed binding affinity in saturation studies is inversely proportional to receptor concentration with high affinity being measured at lower receptor concentrations [44, 45]. This effect cancels out when the same tissue preparation is used for both association and dissociation kinetic studies. The two sites observed by the kinetic studies are thought to represent different affinity states for the same receptor rather than independent receptor sites. This conclusion is consistent with the agonist properties of the radioligand and has also been observed for the μ opioid receptor.

[^{3}H]*p*-Cl-DPDPE is beginning to replace [^{3}H]-DPDPE as the radioligand of choice for the radioligand characterization of δ opioid receptors. Compared to DPDPE, *p*-Cl-DPDPE is 5-fold more selective for δ relative to μ opioid receptors and is more selective than the linear enkephalin analogs described above but it is less selective than the recently described amphibian δ opioid ligands (see below). [^{3}H]*p*-Cl-DPDPE has more than twice the affinity of [^{3}H]DPDPE and has somewhat lower nonspecific binding.

A second halogenated analog of DPDPE, [D-Pen^2, 4′-^{125}I-Phe^4,D-Pen^5]enkephalin ([^{125}I]DPDPE), has also proved to be an exceptional radioligand. The recognition that iodine substitution into the Phe^4 aromatic ring of DPDPE produced a more selective δ opioid receptor ligand with enhanced affinity led to efforts to incorporate ^{125}I into this position. The procedure of Escher [46], based on the classic Sandmeyer approach for the replacement of an aromatic diazonium group with a halogen atom, was modified to produce [^{125}I]DPDPE [47]. Described briefly, the precursor ([D-Pen^2,4′-NH$_2$-Phe^4,D-Pen^5] enkephalin) is diazotized with nitrous acid and then reacted with sodium ^{125}I in the presence of cuprous cyanide. The monoiodinated product with a specific activity of 2200 Ci/mmol is easily separated from the parent peptide by reverse phase high performance liquid chromatography.

[^{125}I]DPDPE has two advantages over tritium-labeled δ opioid radioligands. First, the high specific activity facilitates measurements of binding to tissues with low (<20 fmol/mg protein) δ opioid receptor densities. Second, [^{125}I]DPDPE is a better radioligand for receptor autoradiography studies since the high energy γ radiation does not show different quenching resulting from variations in tissue composition compared to the lower energy β particle emitted by tritium.

The δ opioid receptor binding properties of [^{125}I]DPDPE were characterized using rat brain membranes in 50 mM Tris buffer containing 5 mM $MgCl_2$ [48]. [^{125}I]DPDPE binds to a single class of sites in whole rat brain membranes with a dissociation constant of 420 pM. This affinity is essentially the same as is observed for [^{3}H]*p*-Cl-DPDPE (330 pM) and is significantly higher than that of [^{3}H]DPDPE (1.0 to 5.4 nm for rat). The receptor density measured for whole rat brain membranes (including cerebellum) is 36 fmol/mg protein which is about half that observed for [^{3}H]*p*-Cl-DPDPE or [^{3}H]-DPDPE using membranes prepared from rat brains without the cerebellum. The reason for this difference remains unclear.

The fast receptor binding kinetics of [^{125}I]DPDPE provides another important advantage of this radioligand over the non-iodinated DPDPE analogs. A 1.0 nM concentration of [^{125}I]DPDPE reaches steady-state binding in less than 1 hr at 25° while similar concentrations of [^{3}H]*p*-Cl-DPDPE (0.75 nM) and [^{3}H]DPDPE (1.0 nM) at this temperature require 4.5 and 8 hr, respectively [39, 42]. The extremely slow binding kinetics of [^{3}H]DPDPE are seldom recognized by investigators using this radioligand and probably account for the wide range of binding affinities described for it. Both the association and dissociation binding processes of [^{125}I]DPDPE are monophasic with k_{+1} and k_{-1} rate constants of $5.8 \times 10^7\ M^{-1}\ min^{-1}$ and $0.9 \times 10^{-2}\ min^{-1}$, respectively. The K_d value calculated from these rate constants is 160 pM or about 2-fold less than the K_d obtained by equilibrium binding.

As expected, [^{125}I]DPDPE is an excellent radioligand for δ opioid receptor autoradiography studies. Receptor labeling in the granular cell layer of the cerebellum is clearly evident in rat brain tissue sections treated with 1.0 nM [^{125}I]DPDPE and exposed for only 48 hr. [^{125}I]DPDPE has also been used to measure the δ opioid receptor density of mouse vas deferens tissue (unpublished). The δ receptor density measured for this tissue is 5.3 fmol/mg protein.

Naltrindole: A delta opioid receptor antagonist

The heterocyclic δ opioid receptor antagonist 17-cyclopropylmethyl-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7,2′,3′-indolomorphinan ("naltrindole") developed by Portoghese and colleagues [49] is an example of the use of the "message-address" concept of ligand design [50]. It contains an oxymorphone nucleus (representing the antagonist "message") with an element thought to be functionally related to the Phe^4 aromatic ring of enkephalin (representing the binding site "address"). Regardless of the validity of this design hypothesis, naltrindole acts as a selective and highly effective δ opioid receptor

Table 1. [^{3}H]Naltrindole binding constants for selected tissue preparations

Tissue	K_d (pM)	B_{max} (fmol/mg protein)
Rat brain	37 ± 3	63 ± 2
Mouse brain	51 ± 9	64 ± 2
Mouse vas deferens	32 ± 17	5.0 ± 2.0

[^{3}H]Naltrindole binding was measured by filtration methods using membranes suspended in 50 mM Tris buffer (pH 7.4) at 25°. $MgCl_2$ (5.0 mM) was present for the mouse brain and vas deferens tissues. Nonspecific binding was defined as radioligand bound in the presence of 1.0 μM [4′-Cl-Phe^4]DPDPE. Tissue protein values were determined using the protein assay of Lowry *et al.* [52]. Values are the means (± SEM) of three determinations.

antagonist. Naltrindole has much higher affinity for δ opioid receptors compared to the linear peptide antagonists (see above) which allows its use as a radioligand.

Tritium-labeled naltrindole was prepared from the 5′-bromo-naltrindole precursor by catalytic exchange to a specific activity of 20.5 Ci/mmol by Tóth and associates at the Isotope laboratory of the Biological Research Center at Szeged, Hungary [51]. The receptor binding properties of this radioligand were characterized using rat brain membranes in 50 mM Tris buffer [51]. [^{3}H]Naltrindole bound to a single site in whole rat brain membranes (including cerebellum) with a K_d value of 37 pM and a receptor density of 63 fmol/mg protein when *p*-Cl-DPDPE was used to define nonspecific binding. The site labeled by [^{3}H]naltrindole at a concentration of 100 pM shows high affinity for selective δ opioid ligands including [D-Ala^2,Glu^4]deltorphin (see below) and *p*-Cl-DPDPE and low affinity for μ and κ opioid receptor ligands consistent with the expected properties of a δ opioid receptor.

More recently, the receptor binding properties of [^{3}H]naltrindole were also characterized in mouse brain and mouse vas deferens. The results of these studies in comparison with those obtained from rat brain membranes are given in Table 1. The binding affinity of [^{3}H]naltrindole is probably not significantly different for the three tissues though clearly the B_{max} value for the mouse vas deferens tissue is different from either of the brain membrane preparations. The B_{max} value for the mouse vas deferens (5.0 fmol/mg protein) is consistent with that measured using [^{125}I]DPDPE (5.3 fmol/mg protein, see above). [^{3}H]Naltrindole binding for all three tissues showed no significant improvement when fitted to a two-site model showing that a single class of binding sites was labeled.

[^{3}H]Naltrindole is probably the highest affinity radioligand with selectivity for the δ opioid receptor. The antagonist properties of this ligand are suggested by its displacement from multiple sites when its binding is inhibited with agonists. The potent agonists [D-Ala^2,Glu^4]deltorphin and *p*-Cl-DPDPE inhibit [^{3}H]naltrindole binding with low Hill slope values consistent with the presence of at least two sites showing different affinities for the agonists. The most likely explanation for these observations is that the two sites represent different affinity states of the same δ opioid receptor. [^{3}H]Naltrindole binds to these affinity states with equal affinity while the agonists have different binding affinity for the states. The presence of agonist affinity states is seen for opioid and non-opioid receptors. Antagonists appear to bind with equal affinity to such conformational forms of these receptors. The observation that naloxone inhibits [^{3}H]naltrindole binding from a single site is consistent with this hypothesis. The observation of agonist affinity states by radioligand binding represents a facet of receptor binding studies that is influenced by ligand efficacy.

The δ opioid receptor, like other opioid receptor types, is probably coupled to a guanylyl nucleotide binding protein (G-protein) effector mechanism [53]. Agonist binding affinity to G-protein coupled receptors is influenced by the presence or absence of bound G-protein. G-proteins can be induced to dissociate from the cytoplasmic portion of the receptor by the addition of guanosine 5′-triphosphate (GTP) or more stable analogs like guanylyl-imidodiphosphate (Gpp(NH)p). Sodium ions are also required for this dissociation. The dissociation of the receptor–G-protein complex produces a receptor conformational state with reduced binding affinity for agonists that can be measured by radioliagand binding studies.

The IC_{50} values for agonist inhibitors of [^{3}H]-natrindole binding to mouse brain δ opioid receptors were increased substantially by treatment with 50 μM Gpp(NH)p and 100 mM sodium chloride.* Both [4′-Cl-Phe^4]DPDPE and [D-Ala^2,Glu^4]deltorphin IC_{50} values were increased from 30- to 35-fold by this treatment relative to untreated control tissue. In contrast, the IC_{50} value of naltrindole was unchanged. Even larger (≈100-vold) reductions in agonist binding affinities were observed for [^{3}H]naltrindole binding studies using membranes prepared from mouse vas deferens.*

Amphibian peptides

Isolation of amphibian delta receptor peptides. The recent identification of several naturally occurring opioid peptides containing D-amino acids is an important development for opioid pharmacology. The structural dissimilarity to previously identified endogenous opioid peptides and remarkable selectivity of these peptides for either μ or δ opioid receptor types (Table 2) offer new information on the ligand requirements of these receptors. This is in sharp contrast to the other naturally occurring opioid peptides including the enkephalins, endorphin, and dynorphin that have very limited selectivity between the three major (i.e. μ, δ, and κ) opioid receptor types [4].

Three different D-amino acid containing peptides selective for the δ opioid receptor have been isolated from amphibian skin. Unfortunately the names used to identify these peptides can lead to confusion and in one case several different names have been used

* L. Fang *et al.*, manuscript in preparation. Cited with permission.

Table 2. IC_{50} values for deltorphin inhibition of [^{3}H][4′-Cl-Phe^4]DPDPE and [^{3}H]-CTOP binding

Deltorphin analog	IC_{50} (nM)		N
	δ	μ	
[D-Met^2]Deltorphin	0.473 ± 0.111	1,950 ± 156	5
[D-Ala^2,Asp^4]Deltorphin	0.597 ± 0.304	2,140 ± 693	3
[D-Ala^2,Glu^4]Deltorphin	0.732 ± 0.334	16,800 ± 4,310	3

Ten duplicate concentrations of each deltorphin were used to inhibit the binding of 0.75 nM [^{3}H][4′-Cl-Phe^4]DPDPE (δ) or 0.5 nM [^{3}H]CTOP (μ) binding to rat brain membranes. The data represent the arithmetic means ± the standard error for the number of determinations (N) shown and were obtained by nonlinear regression analysis. All binding data were best fit by a one-site model with Hill coefficient values not significantly different from unity.

for the same peptide. This peptide, Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, was originally named deltorphin [54] but in subsequent papers has been referred to as dermorphin gene-associated peptide ([D-Met^2]DGAP) [55] and dermenkephalin (DREK) [56]. During this same period a pair of peptides were identified in extracts of *Phyllomedusa bicolor* skin, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 and Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2, named deltorphin I and deltorphin II, respectively [57]. To avoid confusion here we will refer to Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 as [D-Met^2]deltorphin, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 as [D-Ala^2,Asp^4]-deltorphin, and Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 as [D-Ala^2,Glu^4]deltorphin.

Deltorphin radioligands. The naturally occurring deltorphins are remarkably selective for δ opioid receptors considering the low selectivity seen for the other endogenous opioid peptides. It is not surprising for this reason that efforts were made to produce radiolabeled forms of these peptides.

[D-Ala^2,Asp^4]Deltorphin was radioiodinated in the N-terminal tyrosine by oxidative methods using Iodogen in the presence of 1.0 mCi sodium ^{125}I [58]. Binding affinity (K_d) measured using mouse brain membranes (less cerebellum) by kinetic and equilibrium methods was reported as 1.1 and 0.48 nM, respectively. The receptor density measured for this tissue was 89 fmol/mg protein.

Iodination of the N-terminal tyrosine does not appear to have a strong adverse effect on δ receptor binding affinity since these values are similar to those of native [D-Ala^2,Asp^4]deltorphin (0.20 nM as reported by Dupin *et al.* [58]). Binding affinity to μ opioid receptors (as labeled by 0.6 nM tyrosine-iodinated [^{125}I]Tyr-D-Ala-Gly-(NMe)Phe-Met (O)-ol; FK-33-824) is reduced by less than 2-fold so that the overall selectivity of iodinated [D-Ala^2,Asp^4]-deltorphin (μ:δ) is reduced by about 2-fold from 2890 to 1390.

[D-Ala^2,Glu^4]Deltorphin was also tritium-labeled to a specific activity of 20.6 Ci/mmol by catalytic dehalogenation of the precursor, [D-Ala^2,4′-I-Phe^3,Glu^4]deltorphin [59]. Saturation binding studies using rat brain membranes (less cerebellum) were consistent with binding to a single class of sites having a K_d value of 1.9 nM for the radioligand and a B_{max} value of about 90 fmol/mg protein.

The association rate constant reported for 35° (2×10^{-13} M^{-1} sec^{-1}) is incorrect.* The value should have been stated as 2×10^5 M^{-1} sec^{-1} or 1.2×10^7 M^{-1} min^{-1} which is a little slower than that measured for [^{125}I]DPDPE at 25° (see above). The dissociation rate constant for [^{3}H][D-Ala^2,Glu^4]-deltorphin is given as 3.3×10^{-4} sec^{-1} (2.0×10^{-2} min^{-1}).

Radioiodinated [D-Ala^2,Glu^4]deltorphin can also be prepared from [D-Ala^2,4′-NH_2-Phe^3,Glu^4]deltorphin by the same method used to produce [^{125}I]DPDPE from [4′-NH_2-Phe^3]DPDPE.† Unlike DPDPE which actually gains increased δ opioid receptor binding affinity and selectivity by iodination, [D-Ala^2,4′-I-Phe^3,Glu^4]deltorphin shows a significant loss of both. This is also seen for iodination of the tyrosine ring of [D-Ala^2,Asp^4]deltorphin by oxidative methods (see above). Given that the deltorphin peptides have better δ receptor binding affinity than DPDPE, it appears that the closer proximity of the tyrosine and phenylalanine aromatic rings of the deltorphins compared with DPDPE is more optimal for δ receptor binding and that halogenation of the DPDPE phenylalanine ring serves to overcome this deficiency. However, it is also possible that the aromatic ring structure of the deltorphins interacts differently with the ligand recognition site of the δ opioid receptor than does that of DPDPE. In this regard, it was shown that the Tyr-D-Met-Phe-His-NH_2 tetrapeptide obtained from the [D-Met^2]deltorphin sequence has about 160-fold greater affinity for the μ relative to the δ opioid receptor, suggesting that the C-terminal rather than the N-terminal portion of this deltorphin is responsible for its δ receptor selectivity [60]. This conclusion does not appear to hold for cyclic enkephalin analogs in that the addition of the Leu-Met-Asp-NH_2 C-terminal sequence of [D-Met^2]-deltorphin to DPDPE nearly eliminated δ opioid

* G. Toth, personal communication. Cited with permission.

† L. Fang *et al.*, manuscript in preparation (see above). Cited with permission.

receptor binding [61]. These observations suggest that despite the apparent similarity between the aromatic ring structures of the two classes of δ opioid receptor agonists (deltorphins and cyclic enkephalin analogs), they probably have different roles in ligand recognition site interaction.

Conclusions

The introduction of DPDPE allowed the characterization of δ opioid receptor-mediated analgesia at both spinal and then superspinal levels [62] which in turn led to greater interest in the development of more potent and selective ligands for this receptor. Just as [^{3}H]DPDPE came to replace less selective δ opioid receptor radioligands like [^{3}H]DADLE, it is likely that halogenated DPDPE analogues, deltorphins, and naltrindole will replace DPDPE. Each of these groups has specific advantages. The halogenated DPDPE analogues have good binding affinity and selectivity while being extremely stable. The deltorphin radioligands are even more selective, though less stable. Finally, naltrindole has the major advantage of being an antagonist which facilitates studies of G-protein coupling to the δ receptor. These ligands will further stimulate interest in the development of drugs for δ opioid receptors.

Recent studies showing that δ receptors have a reduced capacity to produce many of the non-therapeutic effects associated with conventional opiate drugs that act predominantly at μ opioid receptors such as respiratory depression [63–65] and gastrointestinal dysfunction [66] have increased this interest further.

The agonist ligands described here probably have limited clinical relevance due to their low penetration into the CNS after systemic administration [43]. However, their high affinity and selectivity for δ opioid receptors make them ideal for basic studies of these receptors. Three of the most exciting areas of this research are the investigation of δ receptor heterogeneity, the study of the proposed μ–δ receptor complex, and the identification of the δ opioid receptor gene. The new ligands discussed in this review will make an important contribution to these efforts.

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