Structures That Delineate Orphanin FQ and Dynorphin A Pharmacological Selectivities*

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Strict pharmacological selectivity in families of structurally related ligands and receptors may result from a key process in evolution aiming at increasing diversity in neurotransmission. An intriguing example of such exclusive specificity can be found in the newly discovered orphanin FQ (OFQ) system when it is compared with the opioid system. Both OFQ and its receptor share a high degree of sequence similarity to the opioid peptides and their corresponding receptors, respectively. However, OFQ does not activate opioid receptors, nor do the opioid peptides elicit biological activity at the OFQ receptor. We have therefore investigated the basis for the inherent selectivity of the primary structures of OFQ and dynorphin A, its closest counterpart. A series of truncated and/or chimeric peptides led to the conclusion that both peptides contain domains which establish their pharmacological selectivity. In the OFQ molecule we could delineate a domain that prevents its ability to activate the κ-opioid receptor by apparently repelling its binding. In both peptides the selectivity-generating domains are composed of single residues in key positions together with short stretches of amino acids which do not overlap. To prove this concept, we designed a universal agonist and found it active at both the OFQ receptor and the κ-opioid receptor. Our observations suggest that a coordinated mechanism of evolution has separated the orphanin FQ system from the opioid system.

Orphanin FQ (OFQ),1 also called nociceptin, is an endogenous ligand for an opioid-like G protein-coupled receptor (1, 2). This heptadecapeptide shows striking structural homology to the opioid peptides, especially dynorphin A (DynA). However, OFQ does not activate opioid receptors, nor do the opioid peptides elicit any biological effect at the orphanin FQ receptor (OFQR). Despite the high degree of homology between the two ligand/receptor systems, they seem to exert opposite physiological functions. The opioids are known to produce analgesia, whereas OFQ is able to reverse opioid-mediated stress-induced analgesia as well as morphine-induced analgesia (3). Thus, OFQ seems to function as an anti-opioid peptide in certain physiological paradigms. These observations prompted us to investigate potential structural determinants in OFQ and DynA which could ensure their pharmacological selectivity.

In a structure-activity relationship study we have recently determined the individual amino acid residues as well as some overall structural requirements responsible for the biological activity of OFQ at its receptor (4). This study indicated that OFQ exhibits a structure strikingly different from that of the opioid peptides regarding receptor binding and activation. For example, the amino-terminal Phe can be replaced by Tyr without any loss of binding affinity and biological activity. In addition, although the amino-terminal half of OFQ appeared to be more important for receptor binding than the carboxyl-terminal part, the entire OFQ molecule was required for receptor activation. This is in sharp contrast to the opioid peptides whose binding to their receptors obey the “message-address” concept in which the first part of the peptide is sufficient to activate the receptors, whereas the second part, the address, differentiates the receptor sites (5). OFQ interaction with its receptor on the other hand fits the more general model of effector and cooperative domains, where the effector part confers binding to a complementary site on the receptor but is often not sufficient to exert biological activity on its own. In turn, the cooperative domain alone is not able to induce activation of the receptor but may control specificity. The latter model implies that both effector and cooperative domains are necessary for full agonism (15, 16).

Thus far only selectivity-enhancing properties of the address domains in opioid peptides have been described, because all naturally occurring opioid peptides are promiscuous ligands at all three opioid receptors, albeit with different selectivity. This observation is surprising, because no conserved structural motif can be found in the address domains of the opioid peptides. Therefore, one cannot explain on a structural level why a peptide like Tyr¹-OFQ does not activate the opioid receptors. An intriguing discovery from the structure-activity studies on OFQ is that the OFQR does not discriminate against a Tyr residue in position 1 of the peptide. This raised the question as to why the opioid peptide DynA, which contains the same amino-terminal sequence as Tyr¹-OFQ and is of the same length, cannot activate the OFQR and whether structures can be identified within DynA which regulate such activity. Conversely, Tyr¹-OFQ was not able to activate opiate receptors although its amino-terminal tetrapeptide contains the Tyr-Gly-Gly-Phe motif which is believed to be the minimal structure required for opioid peptide activity. The aim of this study was therefore to determine the structural basis for this exclusive selectivity.

Although Tyr¹-OFQ is an artificial molecule that has so far
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not been identified in nature, this is not a purely academic question, because at the genetic level, just a single nucleotide exchange could create a mutation leading to the generation of Tyr\(^1\)-OFQ (5). As outlined before, the OFQR would not select against such a mutant ligand.

To attempt answering these questions we designed a number of chimeric OFQ/DynA peptides and investigated their biological activities at both the OFQ and the opioid receptors. Our results indicate that the primary structures of both OFQ and DynA contain domains which ensure proper selectivity of each ligand molecule for only its cognate receptor. Interestingly, derivatives of Tyr\(^1\)-OFQ truncated at the carboxyl terminus showed considerable activity toward the opioid receptors, whereas the intact Tyr\(^1\)-OFQ has none. This indicates that the carboxyl-terminal half of OFQ may serve as a domain which excludes it from binding to opioid receptors. From these studies we were able to predict the structure of a universal agonist which activates both the \(\kappa\)-opioid receptor (KOR) and the OFQR. Finally, our studies may provide new insights into the evolutionary events which could have separated the opioid from the OFQ system.

MATERIALS AND METHODS

Peptides and Chemicals—OFQ and all chimeric peptides were synthesized by Research Genetics (Huntsville, AL). Opioid peptides were from Bachem (Bubendorf, Switzerland) and non-peptidergic opioid ligands were from RBI (Natick, MA). cAMP assay reagents and \([\text{H}]\)OFQ were obtained from Amersham Corp. \([\text{H}]\)Naloxone was purchased from NEN Life Science Products. Forskolin was from Calbiochem, and tissue culture media were from Life Technologies, Inc. All other chemicals were of analytical grade and obtained from Merck or Sigma.

Transfected Cell Lines and Culture Conditions—Cloning of the rat OFQR as well as transfection of CHO cells and the characterization of clones expressing the receptor have been described previously (1, 4). The cDNAs of the rat \(\delta\), \(\kappa\), and \(\mu\)-opioid receptors were cloned into the eukaryotic expression vector pRcRSV (Invitrogen) and transfected into CHO cells. Stably expressing clones (kindly provided by D. Grandy, Vollum Institute, Portland, OR) were selected with G418 (Life Technologies, Inc.) and screened by binding and functional assays. All cells were cultured at 5% CO\(_2\) in modified Eagle's medium containing 5% fetal calf serum and 500 \(\mu\)g/ml G418.

Measurement of Adenylyl Cyclase Activity in Receptor Transfected Cells—Adenylyl cyclase assays were carried out as described previously (1). All experiments were repeated at least three times in triplicate. Dose-response curves were fitted and calculated with Kaleidagraph.

Receptor Binding and Competition Experiments—OFQ binding assays were done as described before using \([\text{H}]\)OFQ as a radioligand (7). \(\kappa\)-Opioid binding was determined on membranes prepared from CHO cells expressing the rat \(\kappa\)-opioid receptor with \([\text{H}]\)naloxone as a radioligand. Incubation of membranes (40 \(\mug\) of protein) with \([\text{H}]\)naloxone was carried out in the dark at room temperature for 60 min in a total volume of 500 ml of binding buffer (50 mM Tris-HCl, pH 7.8, 1 mM EGTA, 5 mM MgCl\(_2\), 0.1% bovine serum albumin). For competition binding experiments 1 \(\mu\)M \([\text{H}]\)naloxone was added together with the indicated concentrations of unlabeled peptides. Non-specific binding was determined in the presence of 2 \(\muM\) cold naloxone. Bound and free ligand were separated by rapid vacuum filtration through Unifilter GF/C glass fiber filters using a Unifilter 96 harvester (Canberra Packard). GF/C filters had been pretreated with 0.3% polyethylenimine containing 0.1% bovine serum albumin for 1 h at room temperature. Filters were washed six times with 1 ml of ice-cold 50 mM Tris-HCl, pH 7.5. After washing, 60 \(\mul\) of scintillation fluid (Micro Scint, Canberra Packard) was added and the plates were counted in a \(\beta\) counter. All experiments were done at least three times in triplicate. Binding data were analyzed by determination of IC\(_{50}\) values using nonlinear curve fitting in Kaleidagraph, and \(K\) values were obtained according to the method of Cheng and Prusoff (8).

RESULTS

Inactivity of OFQ at the Opioid Receptors—As shown in Fig. 1, OFQ is not able to inhibit forskolin-stimulated adenylyl cyclase activity in CHO cells stably transfected with the three different opioid receptors. On the other hand, these three re-

FIG. 1. Biological activity of OFQ and some typical opioid ligands at the \(\delta\), \(\kappa\), and \(\mu\)-opioid receptors. A, activity at the \(\delta\)-opioid receptor (DOR) of OFQ (\(\bullet\)), methadone (\(\bigcirc\)), and deltorphin I (\(\oplus\)). B, activity at the \(\kappa\)-opioid receptor (KOR) of OFQ (\(\bullet\)), U50,488 (\(\bigcirc\)), DynA (\(\oplus\)), and DynB (\(\bigodot\)). C, activity at the \(\mu\)-opioid receptor (MOR) of OFQ (\(\bullet\)), methadone (\(\bigcirc\)), and dermorphin (\(\oplus\)). Ligand activity was measured by their ability to inhibit forskolin-stimulated cAMP accumulation in receptor-transfected CHO cells. Data were normalized to cAMP levels in forskolin-stimulated cells being 100%. Values are given as means ± S.E. and were derived from at least three independent experiments.

ceptors responded well to challenge with both peptidergic and non-peptidergic opioids, showing their proper functional expression. The EC\(_{50}\) values for inhibition of forskolin-stimulated cAMP accumulation by the opioid compounds at the respective receptors are summarized in Table I.

Activity of Tyr\(^1\)-OFQ at the Opioid Receptors—As reported previously, the Phe residue in position 1 of OFQ can be substituted by Tyr without loss of biological activity or binding affinity at the OFQR (4). Tyr\(^1\)-OFQ contains the amino-terminal tetrapeptide Tyr-Gly-Gly-Phe, thus rendering this part of the molecule identical to the opioid motif contained within all mammalian opioid peptides (for sequences of peptides, see Table I). However, as depicted in Fig. 2, Tyr\(^1\)-OFQ cannot activate the opioid receptors using physiologically relevant concentrations. Only at the KOR was a weak agonistic behavior observed above concentrations of 1 \(\muM\). Surprisingly, Tyr\(^1\)-OFQ inhibited
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| OFQ and DynA-derived peptides | OFQR | DOR | KOR | MOR |
|-------------------------------|------|-----|-----|-----|
| OFQ                           |      |     |     |     |
| Tyr1-OFQ                      | YGGFTGARKSARKLANQ | 1.68 ± 1.03 | inact. | inact. | inact. |
| Tyr1-OFQ 1–9                  | YGGFTGARKSARKLANQ | 1.03 ± 0.67 | >10^4 | >10^4 | >10^4 |
| Tyr1-OFQ 1–7                  | YGGFTGARK | inact. | >10^4 | 6.89 ± 1.71 | >10^4 |
| OD Y1–17                      | YGGFTGARKPKLKWDNQ | inact. | 138.2 ± 50.1 | >10^4 | >10^4 |
| OD R8                         | YGGFTGARKSARKLANQ | inact. | 400 ± 192.5 | 82.56 ± 14.86 | >10^4 |
| Dyn R8                        | YGGFLRRRPKLKWDNQ | 86.67 ± 26.36 | ND | 659 ± 191 | ND |
| Dyn R6–9                      | YGGFLRRRPKLKWDNQ | 61.12 ± 38.29 | ND | 10.99 ± 2.93 | ND |
| Dynorphin A                   | YGGFLRRRPKLKWDNQ | inact. | 0.11 ± 0.02 | ND | ND |
| Opioid agonists               |      |     |     |     |
| Dynorphin B                   | YGGFLRRQFKVVT | inact. | ND | 3.48 ± 1.98 | ND |
| Deltorphin I                  | YaFDVVG-NH2 | inact. | 0.18 ± 0.03 | ND | ND |
| Dermorphin                    | YaFORYS-NH2 | inact. | ND | ND | 0.41 ± 0.23 |
| (+) Methadone                 | U 50,488 | inact. | 7.78 ± 1.62 | ND | 1.59 ± 0.99 |
|                               |     |     |     |     |

Inhibition of forskolin-stimulated cAMP, EC_{50} ±SEM at the

Amino Acid Residues Critical for Biological Activity of OFQ—
Previous structure-activity relationship studies have identified amino acid residues 1–5 together with Arg^2 in the OFQ molecule as the most critical for biological activity (4). In addition, it was shown that the entire OFQ peptide structure was necessary for activation of the OFQR. Because DynA shows the highest degree of homology to OFQ and is of identical length, we chose this peptide as a model molecule for investigating selectivity domains which could preclude activation of the OFQR. First, we introduced an arginine residue at position 8 of DynA, creating Dyn R6–9 (Table I). This molecule proved to be a very potent k agonist, inhibiting forskolin-stimulated cAMP accumulation with an EC_{50} of 0.11 ± 0.02 nM in CHO cells expressing the KOR (Fig. 4). It also displaced [3H]naloxone binding at the KOR with a K_i of 0.2 ± 0.19 nM. However, Dyn R6–9 did not display biological activity at the OFQR, although a moderate binding affinity with a K_i of 25.3 ± 21.7 nM was detected (Table II). The next peptide tested was Dyn R8, in which the sequence of amino acids 8 and 9 of DynA were inverted (Table I). This change resulted in a reduction of potency at the KOR but it also produced a partial agonist at the OFQR (Fig. 4). Dyn R8 could activate both the KOR and the OFQR with EC_{50} values of 10.99 ± 2.93 nM and 61.1 ± 38.3 nM, respectively. The binding potency at the KOR was high, showing a K_i of 0.09 ± 0.08 nM, whereas Dyn R8 displaced [3H]naloxone only moderately at the OFQR (K_i = 34.9 ± 23.8 nM; Table II). However, it appears that Dyn R8 either has an imperfect cooperative binding domain or contains inhibitory structures preventing full agonism at the OFQR.

Design of a Universal Agonist for the OFQ and k-Opioid Receptor—The existence of domains which generate selectivity in peptides of related structure can be tested by designing an agonist which would act at both related receptors. Owing to the fact that the OFQR appears to require a 17-amino-acid peptide for biological activity, we aimed at a heptadecapeptide structure combining all the information about selectivity domains in OFQ as well as DynA. Our studies on Tyr1-OFQ 1–9 and OD Y1–17 had shown that amino acids 1–9 of Tyr1-OFQ are permissive with regard to opioid receptor activity. We therefore used this part of the molecule as an amino-terminal building block. This was combined with a modified carboxyl-terminal half of DynA containing two substitutions: 1) the proline residue in position 10 of DynA was replaced with alanine, as proline is known to induce profound changes in the secondary
structure of peptide chains; 2) the negative charge of the aspartate at position 15 in DynA was eliminated by replacement with alanine. The resulting chimeric peptide (OD R8; Table I) proved to be a full agonist at both the OFQR and the KOR with EC50 values of 86.7 ± 26.4 nM and 659 ± 191 nM, respectively (Fig. 4). OD R8 displaced [3H]naloxone at the KOR with a Ki of 4.37 ± 4.89 nM. A lower affinity value was observed at the OFQR, where OD R8 displaced [3H]OFQ with a Ki of 45.7 ± 32.5 nM (Table II). Although OD R8 is not a highly potent molecule, by being a full agonist at both receptors it verifies our concept of selectivity-generating domains within the two parent peptides. These domains in both OFQ and DynA might be responsible for the observed lack of receptor cross-activation.

**DISCUSSION**

In the present study we have sought to investigate the structural basis of pharmacological selectivity in two closely related neuropeptides, the recently discovered orphanin FQ and dynorphin A. Our results indicate the presence of specific domains in both peptides that prevent cross-activation of inappropriate receptors. To support this concept, we designed a universal agonist in which the restrictive structures have been eliminated and showed that this peptide is active at both the OFQR and the KOR.

Within the primary structure of OFQ the amino-terminal Phe is the first determinant which excludes it from being an opioid agonist, as all the opioid receptors stringently require a Tyr in that position (9, 10). However, the present study shows that OFQ also contains a domain located between amino acids

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**FIG. 2.** Activity of OFQ-derived peptides at the δ-, κ-, and μ-opioid receptors. A, activity of Tyr1-OFQ (○), OD Y1–17 (●), and Tyr1-OFQ 1–7 (▲) at the δ-opioid receptor (DOR). B, activity of Tyr1-OFQ (○), OD Y1–17 (●), and Tyr1-OFQ 1–9 (▲) at the κ-opioid receptor (KOR). C, activity of Tyr1-OFQ (○) and OD Y1–17 (●) at the μ-opioid receptor (MOR). Ligand activity was measured by their ability to inhibit forskolin-stimulated cAMP accumulation in receptor-transfected CHO cells. Data were normalized to cAMP levels in forskolin-stimulated cells being 100%. Values are given as means ± S.E. and were derived from at least three independent experiments.

**FIG. 3.** Interaction of Tyr1-OFQ with the κ-opioid receptor. A, binding of Tyr1-OFQ to the κ-opioid receptor. Membranes prepared from CHO cells expressing KOR were incubated with 1 nM [3H]naloxone and increasing concentrations of Tyr1-OFQ. The average percent specific binding (± S.E.) of three independent experiments is shown. B, inability of Tyr1-OFQ to interfere with κ-opioid receptor function determined as the inhibition of forskolin-stimulated cAMP accumulation in the presence of the indicated concentrations of DynA (●) or U50,488 (▲). Preincubation with 100 nM Tyr1-OFQ does not change the response to DynA (○) or U50,488 (△). C, Tyr1-OFQ (○) alone shows only weak activity at the κ-opioid receptor at high concentrations. Increasing amounts of Tyr1-OFQ are unable to alter the response elicited by 0.1 (●) or 1 (▲) nM DynA, respectively. The effect of DynA (●) is shown for comparison. Incubations were done in triplicate and repeated three times. Results of representative experiments are shown in B and C. All data were normalized to cAMP levels in forskolin-stimulated cells being 100%.
Table II

| Peptide   | Radioligand displacement ($K_i$ ± S.E.) at the |
|-----------|-----------------------------------------------|
|           | OFQR                                          |
|           | KOR                                          |
| Tyr1-OFQ  | 0.9 ± 0.72 (n = 4)                            | 3.35 ± 2.41 (n = 3) |
| Dyn R8    | 34.9 ± 23.8 (n = 4)                           | 0.09 ± 0.08 (n = 3) |
| Dyn R6–9 | 23.5 ± 21.7 (n = 3)                           | 0.2 ± 0.19 (n = 3)  |
| OD R8     | 45.7 ± 32.5 (n = 4)                           | 4.37 ± 4.89 (n = 3) |

**FIG. 5. Location of functional domains in OFQ and DynA.** The length and positions of the minimally active or minimally binding structures (4, 5) were included to demonstrate the mostly non-overlapping arrangement of positive and negative regulatory domains. Amino acid residues (besides the amino-terminal tetrapeptide) which are most critical for biological activity are marked by arrows (4, 5).

Although our studies have focused mainly on DynA and the $\kappa$-opioid receptor as the closest relatives to OFQ and its receptors, it is likely that similar results would be obtained with the other opioid peptides and their receptors. For example, the size of the enkephalins or $\beta$-endorphin together with the absence of an arginine residue in position 8 should be sufficient to exclude them from activating the OFQR. Conversely, the fact that Tyr1-OFQ is unable to activate the DOR or MOR suggests that a similar mechanism of structural restriction excludes Tyr1-OFQ from these receptors, as shown for KOR. However, the individual amino acids responsible for the selectivity between OFQR and DOR or MOR might be different from the ones identified as preventing KOR activation, because Tyr1-OFQ 1–9 is unable to activate DOR or MOR. Further mutation experiments will be necessary to delineate these domains in other opioid peptides and those in Tyr1-OFQ which exclude it to interact with DOR and MOR.

The domains that generate OFQ versus DynA selectivity are non-continuous and show virtually no overlap, indicating that a multistep mechanism of evolution, rather than a single point mutation, led to this separation. This hypothesis is supported by results from a recent study using site-directed mutagenesis showing that the OFQ receptor also contains structures which specifically exclude opioid peptides from binding (11). As few as four amino acid exchanges at remote sites of the protein are sufficient to endow the OFQR with high affinity opioid peptide binding while not affecting its affinity for OFQ itself. Thus, the wild type OFQR harbors opioid peptide-excluding structures which are clearly distinct from the binding sites for its endogenous ligand. The positions of the mutations are distributed over a larger area of the OFQR, indicating that, as proposed for the peptide ligands, a number of separate mutation events must have occurred on the receptor side. It appears that coordinate evolution in both the ligands and the receptors may have led to the complete pharmacological separation of the OFQR system from the opioid system and vice versa. This idea is also consistent with the finding that OFQ can act as an antiopioid peptide (3), as such an activity necessitates a mechanism to prevent any cross-activation of the two systems.

A similar theory has been proposed for the glycoprotein hormones lutropin and follitropin and their respective receptors. Based upon their similarity in sequence it is assumed that both the ligands and receptors might have originated from common ancestors, as in the case of the OFQ and opioid systems. However, lutropin and follitropin are clearly characterized by their distinct pharmacology, reflected by the exclusive
interaction with only one specific type of receptor. In an elegant study by Moyle et al. (12) it was demonstrated that distinct domains in both the glycoprotein hormones as well as their receptors restrict ligand-binding specificity. As noted for the OFQ and the opioid systems, the selectivity determinants in lutropin and follitropin and their receptors are composed of short stretches of amino acids which are non-overlapping. In the case of the glycoprotein hormone receptors this phenomenon could be explained by exon shuffling, because the individual selectivity domains of the lutropin and the follitropin receptor could each be matched to separate exons. However, a different genetic mechanism must be postulated for both the OFQR and the KOR, as the critical parts of the proteins are both contained within the third exon of the respective coding regions (13).

Finally, one may ask why such a stringent selectivity evolved. Restrictive control of ligand/receptor selectivity is a prerequisite in cases of anatomical separation between sites of release and sites of action. Such a situation obviously exists in the case of the glycoprotein hormones, where the humoral mode of ligand distribution provides an ubiquitous appearance of the ligand but simultaneously imposes a need for the development of selectivity. OFQ and the opioids, on the other hand, are believed to exert their activity at specific synaptic junctions as do other neuropeptides. Our observations of mutual exclusion in the two systems might therefore be interpreted in several ways. First, one may still consider that important functions of OFQ and the opioids might be found in the periphery which rely on humoral transport of the transmitters. Another possibility could be a colocalization of the receptors and/or the peptides at certain synapses, although recent anatomical studies seem to disprove this idea (14). But the broad distribution of the OFQR in the central nervous system indeed suggests that it might be found in close spatial proximity to opioid receptors. What this strict selectivity indicates is a need for OFQ and the opioid peptides to be clearly differentiated, which in turn suggests that OFQ may have additional functions unrelated to those it has in the modulation of nociception.

In summary, we have determined the structural basis for pharmacological selectivity between the OFQ and the k-opioid system which prevents cross-activation of their respective receptors. A coordinated evolution of the ligands and the receptors must have occurred to ensure proper separation of the physiological functions of the two systems. The incorporation of structures that prevent activation of related but inappropriate binding sites, rather than inventing two sets of totally new sites, might represent a general evolutionary strategy by which nature can gain variability in signal transduction. Knowledge of these specific motifs will also be helpful in the development of selective drugs to a single class of receptors.

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