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Distinct Mechanisms for Activation of the Opioid Receptor-Like 1 and κ-Opioid Receptors by Nociceptin and Dynorphin A

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ABSTRACT

To understand how two structurally analogous ligand-receptor systems, the nociceptin/opioid receptor-like 1 (ORL1) and dynorphin A/κ-opioid receptor 1 (KOR1) systems, achieve selectivity, receptor chimeras were generated and analyzed. Replacing discrete domains located between the N-terminus and top of the third transmembrane helix of the KOR1 by the homologous domains of the ORL1 receptor yields hybrid receptors, which, in comparison with the parent KOR1, display up to 300-fold increased affinity but low sensitivity toward nociceptin, and unaltered (high) affinity and sensitivity toward dynorphin A. These substitutions contribute elements for binding of nociceptin but do not suppress determinants necessary for binding and potency of dynorphin A. More importantly, further replacement in these chimeras of the second extracellular loop with that of the ORL1 receptor fully restores responsiveness to nociceptin without impairing responsiveness to dynorphin A. A bifunctional hybrid receptor has thus been identified that binds and responds to both nociceptin and dynorphin A as efficiently as the ORL1 receptor does to nociceptin and the KOR1 to dynorphin A. Together, these results suggest that distinct peptide activation mechanisms operate in the two receptor systems. In particular, the second extracellular receptor loop appears to be an absolute requirement for activation of the ORL1 receptor by nociceptin, but not for activation of the KOR1 by dynorphin A.

The heptadecapeptide nociceptin (Meunier et al., 1995), also known as orphanin FQ (Reinscheid et al., 1995), was recently identified as the endogenous ligand of the orphan opioid receptor-like 1 (ORL1) receptor, a G protein-coupled receptor that shares high sequence identity with opioid receptors (Mollereau et al., 1994). Nociceptin itself resembles opioid peptides, particularly dynorphin A, which is also a heptadecapeptide and the presumed endogenous ligand of the κ-opioid receptor 1 (KOR1). However, the structural similarity of both peptides and receptors is not entirely reflected pharmacologically. In common with opioids, nociceptin induces spinal analgesia (Xu et al., 1996), impairs locomotion (Devine et al., 1996a), suppresses spatial learning (Sandin et al., 1997), and stimulates food intake (Pomonis et al., 1996). Unlike opioids, however, nociceptin is motivationally neutral (Devine et al., 1996b), and most remarkably, is endowed with supraspinal pronociceptive/antiopioid properties (Meunier et al., 1995; Reinscheid et al., 1995; Mogil et al., 1996a,b). Nociceptin is also active at the periphery to relax smooth muscle (Champion and Kadowitz, 1997) and suppress natriuresis and stimulate diuresis (Kapusta et al., 1997). The broad pharmacological spectrum of nociceptin implies a variety of potential therapeutic applications.

The mechanism by which nociceptin binds and activates the ORL1 receptor is important for the identification of novel lead compounds, most desirably nonpeptidic ligands. Based upon the structural analogy of nociceptin and dynorphin A and the homology of the ORL1 and KOR1 receptors, it was originally argued that the two neuropeptides must have very similar functional architectures, and hence employ equivalent receptor pathways for signal transduction (Meunier et al., 1995). However, studies of the structure-activity relationships of nociceptin using truncated peptides (Dooley and Houghten, 1996; Reinscheid et al., 1996; Butour et al., 1997) and/or hybrids of nociceptin and dynorphin A (Lapalu et al., 1997; Reinscheid et al., 1998), together with the identification in a combinatorial library of highly potent, basic hexapeptide agonists of the ORL1 receptor (Dooley et al., 1997), have provided evidence that the biologically active ("message") domains of nociceptin and dynorphin A are lo-

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ABBREVIATIONS: ORL1, opioid receptor-like 1; KOR1, κ-opioid receptor 1; CHO, Chinese hamster ovary.

cated in different regions. Likewise, the ORL1 and KOR1 receptors do not seem to bind nociceptin and dynorphin A, respectively, at a topologically equivalent site, because we recently identified a chimeric receptor, *O*-K (ORL1[1–133]-KOR1 [142–380], human receptor numbering, hereafter designated O[Nt-e1]-K), which, unlike the two parent receptors, binds both nociceptin and dynorphin A with high affinity (Lapalu et al., 1998). This chimeric receptor was however not a genuinely bifunctional receptor, because although it was as responsive (in terms of ability of the peptide to inhibit adenylyl cyclase) to dynorphin A as the KOR1 receptor, it was much less so (~200-fold) to nociceptin in comparison with the ORL1 receptor. In the present study, we have sought, again using the same hybrid receptor approach, to delineate more precisely the domains of the ORL1 receptor that are required for high affinity binding of nociceptin, and to identify those that would confer the O[Nt-e1]-K hybrid receptor full responsiveness to nociceptin. Most significantly, we found that replacing the second extracellular (e2) loop of the KOR1 with that of the ORL1 receptor in the O[Nt-e1]-K chimera yields a hybrid receptor, O[Nt-e1,e2]-K, that is fully responsive to dynorphin A and nociceptin, and hence truly bifunctional. We conclude that the e2 loop of the ORL1 receptor is essential for activation of the receptor by nociceptin, whereas in marked contrast, the e2 loop of the KOR1 is not an absolute requirement for activation by dynorphin A. In other words, notwithstanding the inherent structural similarities of the ligand-receptor systems, the two neuropeptides appear to use distinct receptor activation pathways for signal transduction.

Materials and Methods

Construction of Hybrid Receptor cDNAs. The hybrid receptor cDNAs were constructed in pRC/CMV (Invitrogen, San Diego, CA) or pEFIN (Euroscreen, Brussels, Belgium) eucaryotic expression vectors at existing or created restriction sites in the human ORL1 and KOR1 (a gift of Dr. B. Kieffer, Illkirch, France) receptor cDNAs (see Fig. 1). When unique sites were present in both cDNAs (for example MseI and *Afl*III), digestion and fragment exchange were carried out directly. Otherwise, a *Bst*EII restriction site was introduced in the Bluescript SK$^+$ KOR1 construct by mutagenesis with mutated oligonucleotides using the ExSite PCR mutagenesis kit (Stratagene, La Jolla, CA) and Vent polymerase (New England Biolabs, Beverly, MA). Exchange of the e2 loop of the KOR1 for that of the ORL1 receptor was accomplished by ligating a synthetic 79-mer double-stranded oligonucleotide encoding the ORL1-(192–215) amino acid sequence into the *Ban*I and *Bgl*II sites of KOR receptor cDNA. To facilitate screening of the clones, a silent mutation creating a *BsmI* site was also introduced in the oligomer. Sequences of recombinant cDNAs were checked before transfection. To simplify, the chimeric receptor nomenclature emphasized, in brackets, those KOR1 (K) domains (Nt: N-terminal, tm: transmembrane, e: extracellular) that were exchanged for the corresponding ORL1 receptor (O) domains, and vice versa. For instance, O[Nt]-K denotes a KOR1 in which the N-terminal domain has been replaced by that of the ORL1 receptor. Eleven hybrid receptors were thus engineered, as depicted in Fig. 1: O[Nt]-K and reciprocal K[Nt]-O O[tmI-2]-K, O[e1]-K, O[Nt, e1]-K, O[Nt-tm1]-K, O[Nt-e1]-K, O[Nt, e1,e2]-K, O[e1,e2]-K, O[tm1-e1,e2]-K, and O[Nt-e1,e2]-K.

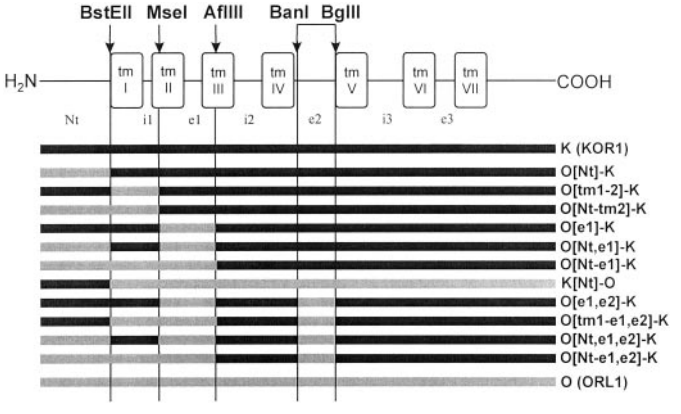


Fig. 1. Construction, structure, and nomenclature of KOR1-ORL1 hybrid receptors. cDNA restriction sites used for constructions are shown with respect to encoded receptor sequence. Nt, N-terminal domain; tm, transmembrane domain; i, cytoplasmic loop; e, extracellular loop. Gray bars, ORL1 receptor sequences; black bars, KOR1 receptor sequences.

Expression in Cell Lines. Chinese hamster ovary (CHO)-K1 cells were transfected with recombinant vectors by the calcium phosphate precipitation method (Chen and Okayama, 1987), and grown in Ham's F-12 medium (Gibco BRL, Gaithersburg, MD) containing G418 (400 μg/ml; Gibco BRL) for selection, as previously described (Mollereau et al., 1994). Screening of the clones was based on the ability of nociceptin or dynorphin A to inhibit forskolin-induced accumulation of cAMP. For membrane preparations, cells were harvested, frozen at −70°C for a minimum of 1 h, and homogeneized in 50 mM Tris-HCl, pH 7.4, in a Potter Elvehjem tissue grinder. The nuclear pellet was discarded by centrifugation at 1,000*g* and the membrane fraction was collected upon centrifugation at 100,000*g*.

Binding Experiments. [^{3}H]Nociceptin (23 Ci/mmol), custom-labeled by Amersham (Little Chalfont, UK) and [^{3}H]diprenorphine (30–60 Ci/mmol; Amersham) were used. Binding experiments were performed at 25°C in polypropylene tubes. Membranes (5–30 μg) were incubated with tritiated ligand (1 nM) and the various unlabeled ligands for 1 h in 0.5 ml 50 mM Tris, pH 7.4, supplemented with proteinase-free bovine serum albumin (final concentration, 0.1 mg/ml) in the case of [^{3}H]nociceptin to avoid tube wall adsorption of the radioligand. Nonspecific binding was determined in the presence of 10^{-6} M nociceptin or diprenorphine. Bound radioligand was collected by filtration on polyethyleneimine-treated glass fiber filters (GF/B, Whatman Inc., Clifton, NJ), and radioactivity counts made in a Kontron model MR300 liquid scintillation counter (Zurich, Switzerland)

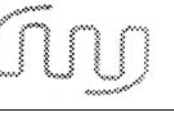
Intracellular cAMP Assay. Sterile hemolysis tubes were seeded with 2×10^5 recombinant CHO cells in culture medium and incubated for approximately 16 h at 37°C. The culture medium was removed and 200 μl fresh medium containing 0.1 μM adenine and 0.6 μCi [^{3}H]adenine (24 Ci/mmol; Amersham) was added. After 1 h at 37°C, the cells were rinsed with 400 μl of HEPES-buffered Krebs-Ringer saline (KRH: 124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, 8 mM glucose, and 0.5 mg/ml BSA, pH 7.4), and 180 μl of fresh KRH was added to each tube. Intracellular accumulation of cAMP was initiated by the addition of 20 μl of KRH containing 100 μM forskolin (Sigma Chemical Co., St. Louis, MO), 1 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM Ro20–1724 (BIOMOL Res. Labs., Plymouth Meeting, PA) and the ligand(s) to be tested at the desired concentration. After exactly 10 min at 37°C, the reaction was stopped by addition of 20 μl HCl 2.2 N and rapid mixing (Vortex). The [^{3}H]cAMP content of each tube was determined by selective batch elution on acidic alumina columns, essentially as described by Alvarez and Daniels (1992).

Data Analysis. Data were fitted to a sigmoidal dose-response curve with variable slope using the Prism program (GraphPad Software, San Diego, CA). Fitting of equilibrium binding inhibition data always yielded slope factors near unity, indicative of a homogenous population of binding sites in the membrane preparations examined. IC_{50} values were thus converted to K_i values using the Cheng and Prussof (1973) relationship: $K_i = IC_{50} \times (1 + [L]/K_d)^{-1}$, where [L] and K_d are the concentration and dissociation constant of the radioligand, respectively.

Results

Eleven chimeras and the parent ORL1 and KOR1 receptors were compared for affinity and biological potency of nociceptin and dynorphin A, the respective endogenous ligands of the ORL1 and KOR1 receptors, and [Tyr^1]nociceptin, a nociceptin analog containing the Tyr-Gly-Gly-Phe N-terminal opioid "message" sequence of dynorphin A. [^{3}H]Nociceptin and [^{3}H]diprenorphine were used to probe the ORL1 and KOR1 receptors, respectively, and the most appropriate radioligand to probe individual chimeras. The chimeric receptors, expressed at densities in the range 1 to 10 pmol/mg protein, all exhibited high affinity for either [^{3}H]nociceptin (K_ds in the range of 0.1–0.2 nM) or [^{3}H]diprenorphine (K_ds in the range of 0.1–0.8 nM), thus allowing their pharmacological characterization and indicating overall preservation of receptor structural integrity. Biological potency was estimated by the ability of the peptide to inhibit forskolin-induced accumulation of cAMP in intact transformed CHO cells expressing the receptor. Table 1 shows that nociceptin is a high-affinity, potent agonist of the ORL1 but not of the KOR1 receptor (K_i: 0.1 versus 107 nM; ED_{50}: 0.8 versus >10,000 nM). Conversely, dynorphin A is a high-affinity, potent agonist of the KOR1 but not of the ORL1 receptor (K_i: 0.1 versus 99 nM; ED_{50}: 0.4 versus >10,000 nM). [Tyr^1]Nociceptin dis-

TABLE 1
Affinity (K_i, nM) and potency (ED_{50}, nM) of nociceptin, [Tyr^1]nociceptin, and dynorphin A at KOR1 receptor chimeras containing e2 loop of KOR1 receptor

		Nociceptin		[Tyr^1]Nociceptin		Dynorphin A	
		K_i	ED_{50}	K_i	ED_{50}	K_i	ED_{50}
KOR1 (K)		107 ± 12 (4)	>10000	3.4 ± 0.2 (4)	865 ± 3 (3)	0.10 ± 0.01 (3)	0.4 ± 0.1 (4)
O[Nt]-K		73 ± 17 (4)	>10000	7.2 ± 1.6 (4)	258 ± 70 (4)	0.20 ± 0.06 (3)	0.9 ± 0.1 (3)
O[tm1-2]-K		126 ± 20 (3)	>10000	6.6 ± 1.2 (3)	543 ± 15 (2)	0.06 ± 0.00 (4)	1.0 ± 0.5 (2)
O[Nt-tm2]-K		27 ± 1 (3)	>10000	1.7 ± 0.4 (3)	285 ± 45 (2)	0.07 ± 0.01 (3)	1.1 ± 0.2 (3)
O[e1]-K		23 ± 6 (4)	>1000	0.12 ± 0.01 (3)	3.3 ± 0.3 (3)	0.24 ± 0.04 (4)	0.3 ± 0.1 (3)
O[Nt, e1]-K		13 ± 4 (4)	>1000	0.07 ± 0.01 (3)	12 ± 2 (3)	0.23 ± 0.01 (3)	0.8 ± 0.5 (3)
O[Nt-e1]-K		0.49 ± 0.02 (3) *0.30 ± 0.01 (3)	115 ± 6 (3)	0.29 ± 0.01 (2)	0.6 ± 0.1 (3)	0.06 ± 0.01 (3) *0.22 ± 0.01 (3)	0.13 ± 0.02 (3)
K[Nt]-O		*0.11 ± 0.03 (3)	11 ± 2 (4)	*0.06 ± 0.01 (3)	21 ± 9 (4)	*125 ± 52 (3)	>10000
ORL1 (O)		*0.11 ± 0.01 (4)	0.84 ± 0.02 (4)	*0.48 ± 0.05 (3)	1.2 ± 0.1 (3)	*99 ± 12 (3)	>10000

K_i values were calculated from IC_{50} values determined from competition curves using [^{3}H]diprenorphine or [^{3}H]nociceptin (values denoted by an asterisk), according to Cheng and Prussof (1973) equation. ED_{50} values represent concentration of unlabeled ligand that half-maximally inhibits forskolin-induced accumulation of cAMP in recombinant CHO cells expressing wild-type or hybrid receptors. Each value is mean ± S.E.M. Number of separate estimates is indicated in parentheses.

played mixed properties: it binds the ORL1 and KOR1 receptors with high affinity (K_i: 0.5 and 3.4 nM, respectively) but is considerably (~1000-fold) more potent at the ORL1 than at the KOR1 receptor (K_i: 0.5 versus 0.1 nM; ED_{50}: 1.2 versus 900 nM).

Multiple ORL1 Receptor Domains Are Required for Nociceptin Binding. Based on our previous identification of an ORL1-KOR1 receptor chimera (O[Nt-e1]-K; see *Introduction*) that showed high affinity for both nociceptin and dynorphin A (Lapalu et al., 1998), we first sought to structurally delineate more precisely which ORL1 receptor domains in the O[Nt-e1]-K chimera are responsible for binding of nociceptin. Table 1 shows that substitution of the ORL1 receptor N-terminal domain (O[Nt]-K chimera) or the tm1, i1 and tm2 domains (O[tm1–2]-K chimera) in the KOR1 receptor had limited effect on nociceptin affinity. However, when these substitutions were combined, as in the O[Nt-tm2]-K hybrid receptor, nociceptin affinity was increased by approximately 4-fold (K_i: 27 versus 107 nM). A similarly improved (4-fold) affinity was recorded in the O[e1]-K chimera, generated by substitution in the KOR1 receptor of the exofacial loop 1 by that of the ORL1 receptor. An approximate 8-fold increase in nociceptin affinity was also observed for the O[Nt, e1] chimera, a combination of O[Nt]- and O[e1]-K. None of these hybrid receptors could be activated by nociceptin (ED_{50} >10 μM). The highest affinity for nociceptin (K_i = 0.3–0.5 nM, close to its affinity for the ORL1 receptor) and measurable potency (ED_{50} = 115 nM, however >100-fold lower than that for the ORL1 receptor) was observed in the O[Nt-e1]-K hybrid receptor. Thus, although individual domains located in the N-terminal third of the ORL1 receptor each contributed affinity (but not potency), they needed be combined (as in O[Nt-e1]-K) to restore "wild-type" binding ability (but not biological activity) to nociceptin. Interestingly, substitutions in the KOR1 receptor that were ineffective in improving binding and/or potency of nociceptin, drastically increased affinity and, above all, activity of [Tyr^1]nociceptin. This was particularly true for the O[e1]-, O[Nt, e1]-, and O[Nt-e1]-K hybrid receptors whose affinity for and response to the hybrid peptide were 10- to 50-fold and 70- to 1500-fold higher, respectively, compared with the KOR1 receptor. Most significantly, the O[Nt-e1]-K hybrid and ORL1 receptor had comparable affinities for [Tyr^1]nociceptin (K_i: 0.3 versus 0.5 nM) and responded equally well to the peptide (ED_{50}: 0.6 versus 1.2 nM), although (see above) the chimera was much less responsive to nociceptin than the ORL1 receptor. In marked contrast, none of these substitutions affected the affinity (K_is in the range of 0.06–0.24 versus 0.1 nM) or biological potency (ED_{50}s in the range of 0.13–1.10 versus 0.41 nM) of dynorphin A. Finally, replacing the ORL1 receptor N-terminal domain by that of the KOR1 receptor, resulted in a hybrid receptor (K[Nt]-O), which, in comparison with the ORL1 receptor, displayed a significantly reduced sensitivity to nociceptin (ED_{50}: 11.2 versus 0.84 nM) and [Tyr^1]nociceptin (ED_{50}: 20.6 versus 1.2 nM). This substitution did not result in any change in nociceptin affinity, nor did it improve dynorphin A binding or biological activity, compared with the ORL1 receptor.

e2 Loop of ORL1 Receptor Is Required for Nociceptin Activity. To identify ORL1 receptor domains conferring high affinity and biological potency to nociceptin, a second series of chimeric receptors was constructed. These hybrids, still largely based on the KOR1 receptor, all additionally comprised the second exofacial loop of the ORL1 receptor. Table 2 shows that these new chimeras, designated O[e1,e2]-, O[tm1-e1,e2]-, O[Nt, e1,e2]-, and O[Nt-e1,e2]-K were considerably more sensitive (>200- to >20,000-fold) to nociceptin than was the parent KOR1 receptor. Indeed, the O[Nt-e1,e2]-K chimera was slightly more sensitive to nociceptin than the ORL1 receptor (ED_{50}: 0.54 versus 0.84 nM). The presence of the ORL1 receptor e2 loop also improved binding of nociceptin, as evidenced in the O[e1,e2]- and O[Nt, e1,e2]-K chimeras whose affinity for the peptide was nearly 20- to 30-fold higher than that of the O[e1]- and O[Nt, e1]-K chimeras. However, introduction of the ORL1 receptor e2 loop led to a moderate increase in potency of [Tyr^1]nociceptin, accompanied only by a marginal improvement in binding affinity, as is apparent from a comparison of the O[Nt-e1]- and O[Nt-e1,e2]-K hybrid receptors (K_i: 0.29 versus 0.17 nM; ED_{50}: 0.59 versus 0.09 nM). Again, although these chimeras contained the ORL1 receptor e2 loop, no impairment of affinity and sensitivity toward dynorphin A was observed, in comparison with the KOR1 receptor (K_is in the range of 0.1–0.7 nM versus 0.1 nM; EC_{50}s in the range of 0.4–0.8 nM versus 0.4 nM). Thus, the O[Nt-e1,e2]-K chimera corresponding to the KOR1 receptor whose N-terminal, tm1, i1, tm2, and e2 domains were replaced by the homologous domains of the ORL1 receptor (Fig. 2A) behaved as a genuinely bifunctional receptor: it displayed an affinity and reactivity toward nociceptin comparable with those of the ORL1 receptor (K_is: 0.5–0.6 versus 0.1 nM; ED_{50}s: 0.5 versus 0.8 nM) and, it displayed an affinity and reactivity toward dynorphin A comparable with those of the KOR1 receptor (K_is: 0.5–0.7 versus 0.1 nM; ED_{50}s: 0.4 versus 0.4 nM) (Fig. 2B). Here again, it was apparent that no one individual substitution was sufficient to restore "wild-type" affinity and/or potency to nociceptin. Several ORL1 domains, particularly the extracellular elements (Nt, e1, and e2), appeared to act synergistically to allow for peptide recognition and activation.

Discussion

The aim of this study was to understand how two structurally homologous ligand-receptor systems, the nociceptin/ORL1 and dynorphin A/KOR1 receptor systems, achieve selectivity. Toward this end, we used receptor chimeras consisting of the KOR1 receptor in which selected domains were exchanged for the corresponding domains of the ORL1 receptor. We find that replacing discrete domains in the N-terminal third (between the N-terminus and top of the third transmembrane helix) of the KOR1 receptor by homologous domains of the ORL1 receptor yields chimeric receptors (O[Nt-tm1]-, O[e1]-, O[Nt, e1]-, and O[Nt-e1]-K) which, in comparison with the parent KOR1 receptor, display substantially increased (up to 300-fold) affinity for but not sensitivity to nociceptin. The chimeras however retain "wild-type" affinity for and sensitivity to dynorphin A. Thus, these substitutions each contribute determinants for high-affinity binding but not biological potency of nociceptin, while not suppressing important determinants for high-affinity binding and potency of dynorphin A. These results provide firm evidence that the two peptides have distinct receptor requirements for coupling binding with activation.

The most important finding is that the e2 loop appears to be a major determinant of nociceptin activity at the ORL1 receptor but not of dynorphin A activity at the KOR1 recep-

tor. In particular, substitution of the e2 loop in the O[Nt, e1]- and O[Nt-e1]-K chimera with that from the ORL1 receptor results in a dramatic increase in nociceptin potency. This substitution, however, does not affect the potency of dynorphin A. The requirement of the e2 loop for activation of the ORL1 receptor constitutes a further example of the involvement of extracellular loops in activation of G protein-coupled receptors, including the bradykinin B2 (Alla et al., 1996), chemokine CCR5 (Samson et al., 1997), and acetylcholine M2 (Elies et al., 1998) receptors. More surprising, however, is the finding that a pair of structurally similar ligands, nociceptin and dynorphin A, appear to bind and activate two structurally homologous receptors, the ORL1 and KOR1 receptors, by distinct mechanisms.

The different structural requirements for nociceptin and dynorphin A may be inferred from the present data and what is already known of the functional architectures of the two peptides and of opioid receptors. Dynorphin A has been shown to comprise two distinct domains, a N-terminal opioid "message" domain, Y^1GGF, responsible for biological activity, and a positively charged, "address" domain, $L^5RRIRPKLK$, responsible for enhanced potency (Chavkin and Goldstein, 1981). Dynorphin A is thought to bind the KOR1 receptor at two subsites: the hydrophobic opiate binding pocket proper, located within the bundle of transmembrane helices (Befort et al., 1996), which accommodates the "message" domain, and the acidic e2 loop that binds the "address" domain of the peptide. There is, however, some controversy as to the type and specificity of interactions between the "address" and e2. Replacing as many as the 11 C-terminal amino acid residues of dynorphin A by those of nociceptin yields a hybrid peptide that is as potent as dynorphin A at the KOR1 receptor (Lapalu et al., 1997), indicating that a specific dynorphin A sequence following the Y^1GGFLR sequence is not mandatory for biological activity, as previously emphasized by Mansour et al. (1995). Likewise, although several reports have shown the e2 loop of the KOR1 receptor to contribute elevated affinity and activity of dynorphin A (Wang et al., 1994; Xue et al., 1994), our data suggest that this contribution is of minimal importance, because exchanging this loop for that of the ORL1 receptor, as in the O[e1,e2]-, O[tm1-e1,e2]-, and O[Nt, e1,e2]-K chimeras, affects neither the affinity nor the biological activity of the peptide. Paterlini et al. (1997) recently modeled binding of dynorphin A-(1–10) to the KOR1 receptor, showing in particular that recognition of the peptide may occur, in part, through classical hydrophobic amphipatic helix-amphipatic helix interactions, rather than ion-pairing, between residues 4 to 10 of dynorphin and the receptor e2 loop. The KOR1 receptor extracellular domain (Nt domain, and e1, e2, and e3 loops) may in fact contribute a selective rather than instructive function in terms of ligand recognition, allowing access of κ-selective but not of μ- and δ-selective ligands to the opiate binding pocket, as proposed by Metzger and Ferguson (1995). This hypothesis can explain why the O[Nt-e1]-K chimera, which contains the Nt and e1 domains of the ORL1 receptor and the e2 and e3 loops of the KOR1 receptor, behaves as a "universal" opioid receptor, binding and responding to opioid ligands that are normally

TABLE 2
Affinity (K_i) and potency (ED_{50}) of nociceptin, [Tyr^1]nociceptin, and dynorphin A at KOR1 receptor chimeras containing e2 loop of ORL1 receptor

		Nociceptin		[Tyr^1]Nociceptin		Dynorphin A	
		K_i	ED_{50}	K_i	ED_{50}	K_i	ED_{50}
KOR1		107 ± 12 (4)	>10000	3.40 ± 0.15 (4)	865 ± 3 (3)	0.10 ± 0.01 (3)	0.41 ± 0.07 (4)
O[e1, e2]-K		0.71 ± 0.05 (2) *0.53 ± 0.05 (2)	46 ± 2 (3)	0.14 ± 0.02 (2) *0.10 ± 0.07 (2)	2.8 ± 0.8 (4)	0.13 ± 0.01 (2) *0.07 ± 0.01 (2)	0.63 ± 0.09 (4)
O[tm1-e1, e2]-K		0.92 ± 0.06 (2)	17.5 ± 0.9 (3)	0.11 ± 0.02 (3)	1.9 ± 0.2 (3)	0.11 ± 0.02 (3)	0.83 ± 0.25 (3)
O[Nt, e1, e2]-K		0.51 ± 0.12 (3)	5.7 ± 0.6 (3)	0.08 ± 0.01 (3)	0.6 ± 0.1 (3)	0.12 ± 0.01 (3)	0.69 ± 0.08 (3)
O[Nt-e1, e2]-K		0.58 ± 0.13 (3) *0.50 ± 0.04 (3)	0.54 ± 0.10 (3)	0.17 ± 0.01 (3) *0.09 ± 0.01 (3)	0.09 ± 0.01 (3)	0.72 ± 0.07 (3) *0.48 ± 0.06 (3)	0.41 ± 0.02 (3)
ORL1		*0.11 ± 0.01 (4)	0.84 ± 0.02 (4)	*0.48 ± 0.05 (3)	1.2 ± 0.1 (3)	*99 ± 12 (3)	>10000

See legend to Table 1 for details.

rejected by the parent ORL1 and KOR1 receptors (Lapalu et al., 1998). It may also explain why [Tyr^1]nociceptin, unlike nociceptin, displays nearly "wild-type" (ORL1) affinity and potency at the O[Nt-e1]-K hybrid receptor, which contains the KOR1 instead of the ORL1 receptor e2 loop. Because [Tyr^1]nociceptin contains the YGGF "message", it may then bind and activate by interacting with the opiate binding pocket present in the chimera, thus behaving more like an opioid than an analog of nociceptin. This is consistent with the presence of a histidine residue in TM6 both in the chimera (280), and the μ- (299), δ- (278), and κ- (291) opioid receptor types with which the phenolic hydroxyl of Tyr^1 may interact (Pogozheva et al., 1998). The equivalent residue in the ORL1 receptor is a glutamine whose replacement by His has been previously shown to confer the ORL1 receptor with improved opioid binding characteristics (Mollereau et al., 1996a).

The binding and biological properties of truncated (Dooley and Houghten, 1996; Reinscheid et al., 1996; Butour et al., 1997) and hybrid (Lapalu et al., 1997; Reinscheid et al., 1998) peptides indicate that nociceptin, unlike dynorphin A, owes its biological activity to its positively charged domain (G^{6}ARKSARK) rather than its N-terminal hydrophobic tetrapeptide. This hypothesis has received considerable support from the identification, in a combinatorial library, of highly basic hexapeptides (Ac-RYY(R, K)(W, I)(R, K)-NH_2,, which bind and activate the ORL1 receptor at nanomolar or lower concentrations (Dooley et al., 1997). It is therefore tempting to suggest that nociceptin stabilizes an active conformation of the ORL1 receptor primarily through specific interactions between the positively charged peptide core and the acidic e2 loop of the receptor. On the other hand, dynorphin A might stabilize an active conformation of the KOR1 receptor primarily through interactions of the hydrophobic N-terminal tetrapeptide and the transmembrane opiate binding pocket. The N-terminal FGGF sequence of nociceptin, which is also important for biological activity (Dooley and Houghten, 1996; Reinscheid et al., 1996; Butour et al., 1997), and the opioid "message" YGGF sequence of dynorphin A are clearly very similar, and in all likelihood, the ORL1 receptor contains a binding pocket that resembles the opioid binding pocket of opioid receptors, as evidenced by the findings that the ORL1 receptor binds and responds to certain opiates, in particular lofentanil, to inhibit adenylyl cyclase (Butour et al., 1997), and that very few point mutations suffice to confer the ORL1 receptor with improved opioid binding properties (Meng et al., 1996; Mollereau et al., 1996a). The N-terminal tetrapeptide of nociceptin might therefore occupy, in the ORL1 receptor, an equivalent position to that of the opioid "message" in the KOR1 receptor. We have recently modeled the binding of nociceptin-(1–13)-NH_2, the shortest fully active analog of nociceptin (Guerrini et al., 1997), to the ORL1 receptor (Topham et al., 1998). The model shows that the N-terminal FGGF tetrapeptide binds in a hydrophobic region that is highly conserved across the ORL1 and opioid receptors, the equivalent of the opiate binding pocket, and that the basic

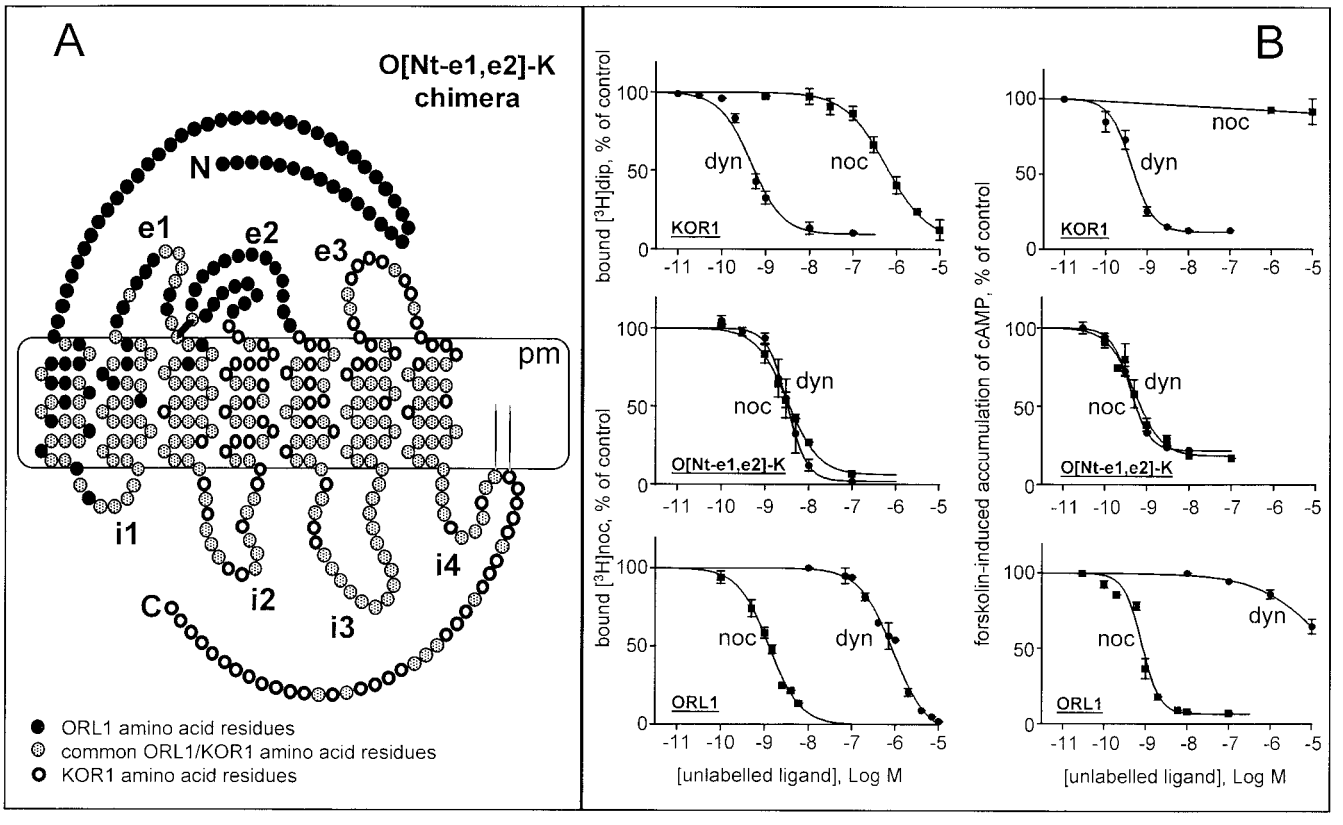


Fig. 2. Schematic representation (A) and functional properties (B) of O[Nt-e1, e2]-K hybrid receptor compared with those of parent ORL1 and KOR1 receptors. A, the ORL1 receptor-specific, KOR1 receptor-specific, and common amino acid residues are highlighted. Note that common amino acid residues are particularly abundant in the transmembrane and cytoplasmic domains. B (left), inhibition by nociceptin (noc) and dynorphin A (dyn) of [^{3}H]diprenorphine binding to KOR1 receptor and of [^{3}H]nociceptin binding to the O[Nt-e1,e2] hybrid and ORL1 receptors. B (right), inhibition by nociceptin and dynorphin A of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing the KOR1, O[Nt-e1,e2] hybrid or ORL1 receptor. Controls refer to specifically bound radioligand and accumulation of cAMP induced by forskolin in the absence of peptide, respectively.

R^8KSARK "core" of the peptide and the acidic e2 loop of the receptor establishes multiple intermolecular ion pairs.

Taken together, these data suggest that distinct peptide-receptor interactions are required for activation of the ORL1 and KOR1 receptors by nociceptin and dynorphin, respectively. Nociceptin would activate the ORL1 receptor primarily through interactions of its positively charged core with the receptor negatively charged e2 loop, whereas dynorphin would activate the KOR1 receptor mainly via interactions of its N-terminal hydrophobic domain (YGGF, the opioid "message" present in many opioid peptides) with the receptor hydrophobic opioid binding pocket, which is probably located within the transmembrane helix bundle. This may have important practical implications in terms of design of new ORL1 receptor ligands. In light of our findings, design of ORL1 receptor agonists should be based on the structure of the positively charged sequence of nociceptin, as opposed to the N-terminal domain, which itself may represent an interesting lead structure for the design of ORL1 receptor antagonists. More fundamentally, this also raises the question about how the two modes of receptor activation have diverged in the course of evolution, assuming that both the nociceptin and dynorphin A precursor genes and the ORL1 and KOR1 receptor genes derive from common ancestor genes (Mollereau et al., 1996b, Nothacker et al., 1996). According to Reinscheid et al. (1998), complete separation of the nociceptin (orphanin FQ) and opioid systems may have occurred by coordinated incorporation (in peptide and receptor) of structures that prevent activation of related but inappropriate binding sites, in very much the same way as Metzger and Ferguson (1995) had previously explained segregation between μ-, δ- and κ-opioid receptor systems. Implicit, however, in this hypothesis is the assumption that nociceptin and opioids use similar receptor pathway for signal transduction. This, as we have shown here, is unlikely. We therefore propose that the two peptides and the two receptors have diverged primarily by coordinated inversion of their "message" and "address" domains. How this may have occurred awaits the identification and pharmacological characterization of homologous systems in lower animal species.

The present study also identified a hybrid receptor, the O[Nt-e1,e2]-K chimera, which, unlike the parent ORL1 and KOR1 receptors, can be activated both by nociceptin and dynorphin A, and hence is genuinely bifunctional. This unique two-receptors-in-one chimera should be extremely useful in the identification and differentiation by site-directed mutagenesis of the amino acid residues that are important for the activity of nociceptin and dynorphin A.

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References

Alla SA, Quitterer U, Grigoriev S, Maidhof A, Haasemann M, Jarnagin K and Müller-Esterl W (1996) Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by anti-peptide antibodies. *J Biol Chem* **271:**1748–1755.

Alvarez R and Daniels DV (1992) A separation method for the assay of adenylylcyclase, intracellular cyclic AMP, and cyclic AMP phosphodiesterase using tritium-labeled substrates. *Anal Biochem* **203:**76–82.

Befort K, Tabbara L, Kling D, Maigret B and Kieffer B (1996) Role of aromatic transmembrane residues of the δ-opioid receptor in ligand recognition. *J Biol Chem* **271:**10161–10168.

Butour J-L, Moisand C, Mazarguil H, Mollereau C and Meunier J-C (1997) Recognition and activation of the opioid receptor-like ORL1 receptor by nociceptin, nociceptin analogs and opioids. *Eur J Pharmacol* **321:**97–103.

Champion HC and Kadowitz PJ (1997) Nociceptin, an endogenous ligand for the ORL1 receptor, has novel hypotensive activity in the rat. *Life Sci* **60:**PL241–245.

Chavkin C and Goldstein A (1981) Specific receptor for the opioid peptide dynorphin: Structure-activity relationships. *Proc Natl Acad Sci USA* **78:**6543–6547.

Chen C and Okayama H (1987) High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:**2745–2752.

Cheng Y-C and Prussof WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Devine DP, Reinscheid RK, Monsma FJ Jr, Civelli O and Akil H (1996b) The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion. *Brain Res* **727:**225–229.

Devine DP, Taylor L, Reinscheid RK, Monsma FJ Jr, Civelli O and Akil H (1996a) Rats rapidly develop tolerance to the locomotor-inhibiting effects of the novel neuropeptide orphanin FQ. *Neurochem Res* **21:**1387–1396.

Dooley CT and Houghten RA (1996) Orphanin FQ: Receptor binding and analog structure activity relationships in rat brain. *Life Sci* **59:**PL23–29.

Dooley CT, Spaeth CG, Bertzetei-Gurske IP, Craymer K, Adapa ID, Brandt SR, Houghten RA and Toll L (1997) Binding and in vitro activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL1. *J Pharmacol Exp Ther* **283:**735–741.

Elies R, Fu LXM, Eftekhari P, Wallukat G, Schulze W, Granier C, Hjalmarson A and Hoebeke J (1998) Immunochemical and functional characterization of an agonist-like monoclonal antibody against the M2 acetylcholine receptor. *Eur J Biochem* **251:**659–666.

Guerrini R, Calo' G, Rizzi A, Bianchi C, Lazarus LH, Salvadori S, Temussi PA and Regoli D (1997) Address and message sequences for the nociceptin receptor: A structure-activity study of nociceptin-(1–13)-peptide amide. *J Med Chem* **40:**1789–1793.

Kapusta DR, Sezen SF, Chang J-K, Lippton H and Kenigs VA (1997) Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ). *Life Sci* **60:**PL15–21.

Lapalu S, Moisand C, Butour J-L, Mollereau C and Meunier J-C (1998) Different domains of the ORL1 and κ-opioid receptors are involved in recognition of nociceptin and dynorphin A. *FEBS Lett* **427:**296–300.

Lapalu S, Moisand C, Mazarguil H, Cambois G, Mollereau C and Meunier J-C (1997) Comparison of the structure-activity relationships of nociceptin and dynorphin A using chimeric peptides. *FEBS Lett* **417:**333–336.

Mansour A, Hoversten MT, Taylor LP, Watson SJ and Akil H (1995) The cloned μ, δ and κ receptors and their endogenous ligands: Evidence for two opioid peptide recognition cores. *Brain Res* **700:**89–98.

Meng F, Taylor LP, Hoversten MT, Ueda Y, Ardati A, Reinscheid RK, Monsma FJ Jr, Watson SJ, Civelli O and Akil H (1996) Moving from the orphanin FQ receptor to an opioid receptor using four point mutations. *J Biol Chem* **271:**32016–32020.

Metzger TG and Ferguson DM (1995) On the role of extracellular loops of opioid receptors in conferring ligand specificity. *FEBS Lett* **375:**1–4.

Meunier J-C, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour J-L, Guillemot J-C, Ferrara P, Monsarrat B, Mazarguil H, Vassart G, Parmentier M and Costentin J (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor *Nature* **377:**532–535

Mogil JS, Grisel JE, Reinscheid RK, Civelli O, Belknap JK and Grandy DK (1996a) Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* **75:**333–337.

Mogil JS, Grisel JE, Zhangs G, Belknap JK and Grandy DK (1996b) Functional antagonism of mu-, delta- and kappa-opioid antinociception by orphanin FQ. *Neurosci Lett* **214:**131–134.

Mollereau C, Moisand C, Butour J-L, Parmentier M and Meunier J-C (1996a) Replacement of Gln280 by His in TM6 of the human ORL1 receptor increases affinity but reduces intrinsic activity of opioids. *FEBS Lett* **395:**17–21.

Mollereau C, Parmentier M, Mailleux P, Butour J-L, Moisand C, Chalon P, Caput D, Vassart G and Meunier J-C (1994) ORL1, a novel member of the opioid receptor family: Cloning, functional expression and localization. *FEBS Lett* **341:**33–38.

Mollereau C, Simons MJ, Soularue P, Liners F, Vassart G, Meunier J-Cl and Parmentier M (1996b) Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene. *Proc Natl Acad Sci USA* **93:**8666–8670.

Nothacker HP, Reinscheid RK, Mansour A, Henningsen RA, Ardati A, Monsma FJ Jr, Watson SJ and Civelli O (1996) Primary structure and tissue distribution of the orphanin FQ precursor. *Proc Natl Acad Sci USA* **93:**8677–8682.

Paterlini G, Portoghese PS and Ferguson DM (1997) Molecular simulation of dynorphin A-(1–10) binding to extracellular loop 2 of the κ-opioid receptor. *J Med Chem* **40:**3254–3262.

Pomonis JD, Billington CJ and Levine AS (1996) Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. *Neuroreport* **8:**369–371.

Pogozheva, ID, Lomize, AL and Mosberg, HI (1998) Opioid receptor three-dimensional structures from distance geometry calculations with hydrogen bonding constraints. *Biophys J* **75:**612–634.

Reinscheid RK, Ardati A, Monsma FJ Jr and Civelli O (1996) Structure-activity relationship studies on the novel neuropeptide orphanin FQ. *J Biol Chem* **271:**14163–14168.

Reinscheid RK, Higelin J, Henningsen RA, Monsma FJ Jr and Civelli O (1998) Structures that delineate orphanin FQ and dynorphin A pharmacological selectivities. *J Biol Chem* **273:**1490–1495.

Reinscheid RK, Nothacker HP, Bourson A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ and Civelli O (1995) Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* **270:**792–794.

Samson M, LaRosa G, Libert F, Paindavoine P, Detheux M, Vassart G and Parmen-

tier M (1997) The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J Biol Chem* **272:**24934–24941.
Sandin J, Georgieva J, Schött PA, Ogren SO and Terenius L (1997) Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. *Eur J Neurosci* **9:**194–197.
Topham CM, Moulédous L, Poda G, Maigret B and Meunier J-C (1998) Molecular modelling of the ORL1 receptor and its complex with nociceptin. *Protein Eng* **11:** 1163–1179.
Wang JB, Johnson PS, Wu JM, Wang WF and Uhl GR (1994) Human κ opiate receptor second extracellular loop elevates dynorphin's affinity for human μ/κ chimeras. *J Biol Chem* **269:**25966–25969.
Xu X-J, Hao J-X and Wiesenfeld-Hallin Z (1996) Nociceptin or antinociceptin: Potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. *Neuroreport* **7:**2092–2094.
Xue J-C, Chen C, Zhu J, Kunapuli S, DeRiel JK, Yu L and Liu-Chen L-Y (1994) Differential binding domains of peptide and non-peptide ligands in the cloned κ opioid receptor. *J Biol Chem* **269:**30195–30199.

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