Dissociation of affinity and efficacy in KOR-3 chimeras

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Abstract KOR-3 chimeras were constructed in which the first coding exon of KOR-3 was exchanged for the corresponding first coding exon of either MOR-1 (MOR-1/KOR-3) or DOR-1 (DOR-1/KOR-3). All three clones were expressed in CHO cells and characterized with regards to their binding profiles for orphanin FQ/nociceptin (OFQ/N) and a variety of opioids as well as their functional activities in cyclase studies. ^{125}I[Tyr^{14}]OFQ/N labels both KOR-3 (K_D 37 pM) and the MOR-1/KOR-3 chimera (K_D 39 pM) equally well. Although its affinity for the DOR-1/KOR-3 chimera is quite good (K_D 135 pM), it is slightly lower than the other two. Competition studies confirm the high affinity of OFQ/N for all three clones. However, several competitors clearly distinguish the chimeras from KOR-3. OFQ/N(1–11) competes KOR-3 (K_i 55 nM) over 6-fold more potently than either of the chimeras (K_i values > 350 nM). Conversely, the modest affinity of naloxone benzoylhydrazone for KOR-3 (310 nM) is greatly increased in both the MOR-1/KOR-3 (K_i 69 nM) and DOR-1/KOR-3 (K_i 74 nM) chimeras. The remainder of the opioids tested have no appreciable affinity against any of the clones. Functionally, OFQ/N inhibits forskolin-stimulated cAMP accumulation in both the KOR-3 and the MOR-1/KOR-3 chimera by almost 40%, with IC_{50} values in the low nanomolar range. Little activity is seen against the DOR-1/KOR-3 chimera. Naloxone benzoylhydrazone inhibits cAMP accumulation in the KOR-3 and the DOR-1/KOR-3 chimera. Although naloxone benzoylhydrazone has higher affinity for the MOR-1/KOR-3 chimera in binding studies than KOR-3 itself, it is inactive in cyclase studies using the MOR-1/KOR-3 chimera, implying that the replacement of the first coding exon increases affinity while decreasing intrinsic activity.

Key words: Opioid receptor; Orphanin FQ; Nociceptin; KOR-3; OLR1

1. Introduction

A novel peptide, termed orphanin FQ [1] or nociceptin [2] (OFQ/N), recently has been identified which has very high affinity for a recently cloned member of the opioid receptor family [3–11]. We have cloned and characterized both the mouse homolog of this receptor, KOR-3 [12,13], and its gene [14]. Opioids demonstrate low affinity for this receptor and inhibit adenylyl cyclase activity only at high concentrations [8,12,13]. However, other approaches have implied a close association between the receptor encoded by KOR-3 and the κ_3 receptor found in brain. In addition to the recognition of the expressed KOR-3 clone by a monoclonal antibody generated against the κ_3 receptor [15], antisense studies raise the possibility that the κ_3 receptors may represent a splice variant of KOR-3 [12,13]. In these studies, six antisense oligodeoxynucleotide probes targeting the second and third coding exons of KOR-3 blocked κ_3 analgesia. However, an additional five probes based upon the first coding exon were inactive, raising the possibility that the κ_3 receptor might have an alternatively spliced first coding exon. To determine whether changes in the first coding exon of KOR-3 might influence the binding and functional characteristics of the receptor, we generated chimeras by exchanging the first coding exon of KOR-3 with the corresponding first coding exon from a traditional mu (MOR-1) or delta (DOR-1) opioid receptor and examined receptor binding and function.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from Promega (Madison, WI, USA). DNA sequencing kits were from Perkin-Elmer (Foster City, CA, USA) and US Biochemicals (Cleveland, OH, USA). OFQ(1–17), [Tyr^{14}]OFQ(1–17), OFQ(1–7), and OFQ(1–11) were synthesized by the Core Protein Facility and purified by HPLC. Morphine sulfate, DPDPE, and U50,488 were obtained from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). Naloxone benzoylhydrazone (NalBzoH) was synthesized as described previously [16]. Oligodeoxynucleotides were synthesized by Integrated DNA Technologis, Inc. (Coralville, IO, USA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

2.2. Constructing and expressing the chimeras

The Chimeras were constructed by using an overlapping PCR strategy. The first coding exon 1 of mouse MOR-1 was amplified in PCR using a sense primer A (5′-TGGGTACCGGGCCCCCCC-3′) including a *Kpn*I site, located in the vector upstream of the 5′ MOR-1 clone and an antisense primer which contained 22 bases corresponding to the 3′-end of the MOR-1 exon 1 and 13 bases corresponding to the 5′-end of the KOR-3 exon 2 (5′-CATCTTGGTGTGCCTTACAATCACATACATGACCA-3′). The template was the mouse MOR-1 construct [17]. The amplified fragment contained all the coding exon 1 of the MOR-1 tailing a 13 bp of KOR-3 exon 2. The second and third coding exons were separately amplified using a sense primer which contained 11 bases of the 3′-end of the MOR-1 exon 1 and 20 bases of the 5′-end of the KOR-3 exon 2 (5′-GTGATTGTAAGGCACACCAAGATGAAGACTG-3′) and an antisense B corresponding to the nucleotide sequence of the KOR-3 clone [12,13] at position 1420–1438 tailing a *Xho*I site. The template was the mouse KOR-3 clone. The PCR fragment included the complete exons 2 and 3 of KOR-3 capping a 11 bp of the MOR-1 exon 1. The chimera containing the MOR-1 exon 1 and KOR-3 exon 2 and 3 (MOR-1/KOR-3) (Fig. 1) was produced through PCR by using the mixture of the above two PCR fragments as templates since the two fragments had 24 bp overlapping that was dictated by the above primers. The sense primer A and the antisense B were also included in the reaction. The expected PCR product was purified, digested with the *Kpn*I and *Xho*I and subcloned into the *Kpn*I and *Xho*I sites of a pcDNA3 (Clontech) expression vector. The clones were sequenced using either Sequenase version 2 (USB) or a Amplicycle sequencing kit (Perkin-Elmer) to confirm the structure and to ensure the absence of any mutations induced by the PCR. The chimera containing the DOR-1 exon 1 and KOR-3 exons 2 and 3 (DOR1/KOR3) (Fig. 1) was generated

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using the same strategy. The sense primer A and an antisense primer (5′-CTTGGTGTGCCGGACGATGCCAAACATGA-3′) were used to amplify the DOR-1 exon 1, and the antisense B and a sense primer (5′-GGCATCGTCCGGCACACCAAGATGAAGACTG-3′) to amplify the KOR-3 exon 2 and 3. Both constructs were translated into proteins with the predicted molecular weight in a in vitro transcription coupled in vitro translation system (Novagen) (data not shown).

The resulting MOR1/KOR3 and DOR1/KOR3 constructs and the KOR-3 clone in pcDNA3 were transfected into CHO cells using lipofectAMINE reagent (Gibco BRL) and single stable clones selected in the presence of G418.

2.3. Characterization of the chimeras and the KOR-3 constructs

[Tyr^{14}]OFQ/N was iodinated with Na^{125}I using chloramine-T, as previously described [18]. The mixture was applied to a C18 Sep-Pak cartridge (Waters, Milford, MA) and the monoiodinated peptide separated with a step gradient. The radiochemical purity (>95%) of the iodinated peptide was confirmed by reverse-phase high-performance liquid chromatography (rpHPLC). There was no detectable di-iodo compound in the purified material. [^{125}I][Tyr^{14}]OFQ/N binding was conducted in KPO_4 buffer (10 mM, pH 7.0) containing $MgCl_2$ (5 mM), bacitracin (0.25 mg/ml) and bovine serum albumin (BSA, 0.1%) with washed membranes from the indicated clones. Samples were incubated for 60 min at 25°C. After incubation, the membranes were pelleted in a microcentrifuge and counted in a Packard Cobra II Automated Gamma counter. Non-specific binding was determined in the presence of OFQ/N (1 μM). Specific binding was defined as the difference in binding between total and non-specific binding. Saturation studies were subjected to non-linear regression analysis to determine K_D values. Competition studies yielded IC_{50} values which were converted to K_i values as described in the literature [19,20].

Adenylyl cyclase activity was assessed by measuring cAMP levels, as previously reported [21,22]. Briefly, inhibition of forskolin-stimulated cAMP accumulation was assessed in membranes (0.6–0.8 mg protein) after a 10 min incubation at 37°C in the presence of (0.5 μM), the phosphodiesterase inhibitor RO-20 and various concentrations of agonists. The assay was stopped by boiling the samples for 5 min, after which they were centrifuged at $1000\times g$ for 10 min and the supernatant assayed for cAMP levels. The amount of cAMP present was calculated from a standard curve determined with unlabeled cAMP and the inhibition determined.

3. Results

First, we examined ^{125}I[Tyr^{14}]OFQ/N binding in CHO cells stably transfected with KOR-3 or the two chimeras (Fig. 1). All three cell lines bind ^{125}I[Tyr^{14}]OFQ/N quite potently. Saturation studies reveal that ^{125}I[Tyr^{14}]OFQ/N labels the KOR-3 cells with an affinity (K_D 37 pM) (Table 1) which is similar to that reported previously with an homologous clone [1]. The MOR-1/KOR-3 chimera shows no change in its affinity for ^{125}I[Tyr^{14}]OFQ/N, while its affinity in the DOR-1/KOR-3 chimera is lowered slightly.

Competition studies confirm the unique selectivities of the KOR-3 clone and the chimeras (Table 2). Both OFQ/N and [Tyr^{14}]OFQ/N potently compete binding in all three clones, although its affinity for the DOR-1/KOR-3 chimera is slightly lower, as anticipated based upon the saturation studies. OFQ/N(1–7) and OFQ/N(1–11) represent potential peptides which could be generated from OFQ/N, a possibility supported by their pharmacological activity in mice [23,24]. Although OFQ/N(1–11) labels the native KOR-3 receptor reasonably well (K_i 55 nM), its affinity for the chimeras is lowered over 6-fold ($p<0.03$). Conversely, the modest affinity of the κ_3 ligand naloxone benzoylhydrazone (NalBzoH) is increased over 4-fold in the chimeras ($p<0.04$). Thus, the relative affinity of OFQ/N(1–11) compared to NalBzoH shifts over 25-fold from KOR-3 to the chimeras. Traditional opiates and OFQ/N(1–7) all display no appreciable affinity for any of the three receptors.

Table 1
^{125}I[Tyr^{14}]OFQ/N binding sites in CHO cells transfected with the KOR-3 and chimeric receptor clones

Clone	K_D (pM)	B_{max} (fmol/mg protein)
KOR-3	36.7 ± 1.6	17.3
MOR-1/KOR-3	39.4 ± 1.4	7.1
DOR-1/KOR-3	135 ± 5.7	3.8

Saturation studies were performed with ^{125}I[Tyr^{14}]OFQ/N on CHO cells transfected with the indicated clone. Binding was best fitted by a one-site model. Values are the means ± S.E.M. of three independent determinations.

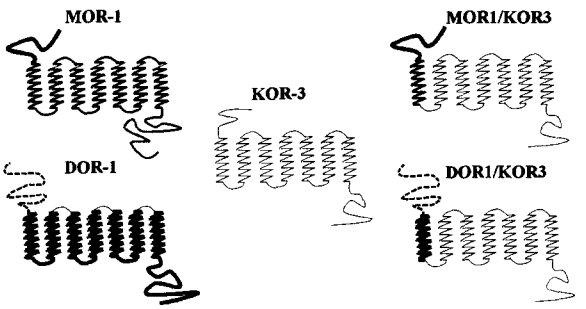

Fig. 1. Schematic of KOR-3 and the chimeras. Schematic representation of KOR-3 (light lines) and the chimeras. The exchanged first exons from MOR-1 and DOR-1 are denoted by a heavy line.

We also examined the ability of OFQ/N to inhibit forskolin-stimulated cAMP accumulation in the three cell lines. OFQ/N inhibits cAMP accumulation in the KOR-3 cells by nearly 40%, with an IC_{50} less than 1 nM (Fig. 2), results similar to those previously reported with homologous clones [1,2]. Although OFQ/N inhibits cAMP accumulation to the same degree in the MOR-1/KOR-3 chimera, higher doses are needed to achieve these effects. The effects of OFQ/N in the DOR-1/KOR-3 chimera are limited at concentrations under 100 nM and are not significantly different from values in non-transfected CHO cells.

NalBzoH inhibits cAMP accumulation in the KOR-3 cells, consistent with prior observations [12,13]. NalBzoH also inhibits cAMP accumulation in the DOR-1/KOR-3 chimera to the same degree and slightly more potently than in the KOR-3 cells, consistent with its 4-fold greater binding affinity (Fig. 3). Despite its increased affinity for the MOR-1/KOR-3 chimera in binding assays, NalBzoH is inactive at all concentrations tested in the cyclase assays and antagonizes OFQ/N.

4. Discussion

Chimera studies must be interpreted cautiously due to the extensive conformational changes which can result from the exchange of such a large region. Nevertheless, the effects of substituting exon 1 from a traditional opioid receptor with that in KOR-3 yields interesting results. This study confirms the high affinity of OFQ/N for the mouse homologue of the orphan receptor, KOR-3, along with its expected selectivity profile for opioids and OFQ/N. Both chimeras maintain high affinity for OFQ/N with only a small decrease in affinity in the DOR-1/KOR-3 chimera compared to KOR-3. Prior work from our laboratory has revealed a complex behavioral pharmacology for OFQ/N and its fragments [23,24]. In these stud-

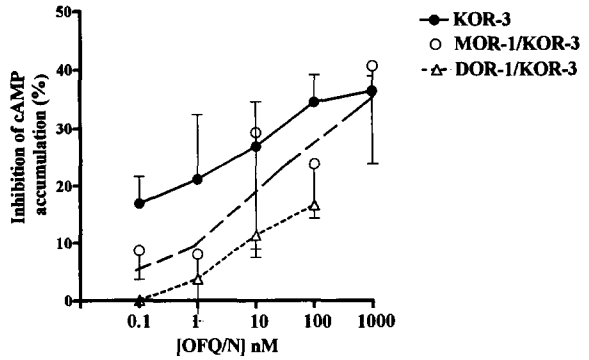

Fig. 2. Effects of OFQ/N on cAMP accumulation in transfected CHO cells. The ability of OFQ/N at the stated concentrations to inhibit forskolin-stimulated cAMP accumulation was determined in CHO cells transfected with KOR-3, MOR-1/KOR-3, or DOR-1/KOR-3, as described in Section 2.

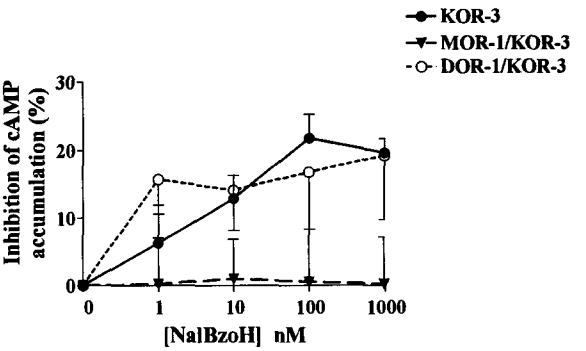

Fig. 3. Effects of NalBzoH on cAMP accumulation in transfected CHO cells. The ability of NalBzoH at the stated concentrations to inhibit forskolin-stimulated cAMP accumulation was determined in CHO cells transfected with KOR-3, MOR-1/KOR-3 or DOR-1/KOR-3, as described in Section 2.

ies, OFQ/N(1–11) produces a potent, naloxone-reversible analgesia implying that it, too, may be physiologically relevant. OFQ/N(1–11) shows reasonable affinity for KOR-3, but its affinity is lowered 6–10-fold in the chimeras. Thus, it is interesting that the binding affinity of OFQ/N(1–11) can be dissociated from OFQ/N.

Despite the close relationship between KOR-3 and the κ_3 receptor, they are likely distinct receptors, possibly splice variants [12,13]. Their relationship is further supported by the NalBzoH studies. NalBzoH has only a modest binding affinity against KOR-3. Replacing the first coding exon enhances this affinity 4-fold in both chimera receptors. These studies indicate that the structural requirements for OFQ/N binding also can be differentiated from those of NalBzoH. Whereas the affinity of OFQ/N is independent of which first exon is present, the affinity of NalBzoH is significantly improved with an exon from a traditional opioid receptor. This also contrasts with the opposite change in affinity for OFQ/N(1–11) seen in the same chimeras.

The cyclase results discriminate between binding affinity and functional activity. OFQ/N potently inhibits forskolin-stimulated cAMP accumulation with an IC_{50} of approx. 1 nM. OFQ/N also inhibits stimulated cAMP accumulation in the MOR-1/KOR-3 chimera, but is less effective in the DOR-1/KOR-3 cells, perhaps related in part to its lower affinity for this receptor. The greatest functional effect is seen with NalBzoH. Substituting a first coding exon from either MOR-1 or DOR-1 significantly enhances the binding affinity of NalBzoH to both chimeras by 4-fold. Yet, NalBzoH potently inhibits cAMP accumulation in the DOR-1/KOR-3 chimera and is inactive against the MOR-1/KOR-3 chimera. This loss of intrinsic activity is interesting. NalBzoH is an effective κ_3 agonist in cyclase assays [21,22], but it is an antagonist at mu receptors [25]. Substituting the first coding exon of MOR-1 eliminates the efficacy of NalBzoH without affecting its affinity. Thus, these chimeras illustrate the ability to change the affinity of OFQ/N(1–11) and NalBzoH in opposite directions and demonstrate that changes in affinity can be dissociated from intrinsic activity.

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References

[1] Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J. and Civelli, O. (1995) Science 270, 792–794.
[2] Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazargull, H., Vassart, G., Parmentier, M. and Costentin, J. (1995) Nature 377, 532–535.
[3] Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C.A. and Yu, L. (1994) FEBS Lett. 347, 279–283.
[4] Keith, D., Jr., Maung, T., Anton, B. and Evans, C. (1994) Regul. Pept. 54, 143–144.

Table 2
Competition of ^{125}I[Tyr^{14}]OFQ/N binding in transfected CHO cells

Ligand	K_i values (nM)		
	KOR-3	Chimera	
		MOR-1/KOR-3	DOR-1/KOR-3
OFQ/N	0.088 ± 0.007	0.110 ± 0.05	0.17 ± 0.034
[Tyr^{14}]OFQ/N	0.071 ± 0.019	0.073 ± 0.019	0.14 ± 0.06
OFQ/N(1–7)	> 1000	> 1000	> 1000
OFQ/N(1–11)	55 ± 22	350 ± 130	520 ± 140
Naloxone benzoylhydrazone	310 ± 75	69 ± 12	74 ± 16
Morphine	> 1000	> 1000	> 1000
U50,488H	> 1000	> 1000	> 1000
DPDPE	> 1000	> 1000	> 1000
Diprenorphine	> 1000	> 1000	> 1000

[5] Uhl, G.R., Childers, S. and Pasternak, G.W. (1994) Trends Neurosci. 17, 89–93.
[6] Wick, M.J., Minnerath, S.R., Lin, X., Elde, R., Law, P.-Y. and Loh, H.H. (1994) Mol. Brain Res. 27, 37–44.
[7] Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M. and Uhl, G.R. (1994) FEBS Lett. 348, 75–79.
[8] Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G. and Meunier, J.C. (1994) FEBS Lett. 341, 33–38.
[9] Lachowicz, J.E., Shen, Y., Monsma, F.J., Jr. and Sibley, D.R. (1995) J. Neurochem. 64, 34–40.
[10] Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M. and Grandy, D.K. (1994) FEBS Lett. 347, 284–288.
[11] Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T. and Siguimoti, T. (1994) FEBS Lett. 343, 42–46.
[12] Pan, Y.X., Cheng, J., Xu, J. and Pasternak, G.W. (1994) Regul. Pept. 54, 217–218.
[13] Pan, Y.-X., Cheng, J., Xu, J., Rossi, G.C., Jacobson, E., Ryan-Moro, J., Brooks, A.I., Dean, G.E., Standifer, K.M. and Pasternak, G.W. (1995) Mol. Pharmacol. 47, 1180–1188.
[14] Pan, Y.X., Xu, J. and Pasternak, G.W. (1996) Gene 171, 255–260.
[15] Brooks, A.I., Standifer, K.M., Rossi, G.C., Mathis, J.P. and Pasternak, G.W. (1996) Synapse 22, 247–252.
[16] Luke, M.C., Hahn, E.F., Price, M. and Pasternak, G.W. (1988) Life Sci. 43, 1249–1256.
[17] Rossi, G.C., Pan, Y.-X., Brown, G.P. and Pasternak, G.W. (1995) FEBS Lett. 369, 192–196.
[18] Mathis, J.P., Ryan-Moro, J., Chang, A., Hom, J.S.H., Scheinberg, D. and Pasternak, G.W. (1996) submitted.
[19] Cheng, Y.-C. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099–3108.
[20] Chou, T.-C. (1974) Mol. Pharmacol. 10, 235–247.
[21] Standifer, K.M., Cheng, J., Brooks, A.I., Honrado, C.P., Su, W., Visconti, L.M., Biedler, J.L. and Pasternak, G.W. (1994) J. Pharmacol. Exp. Ther. 270, 1246–1255.
[22] Cheng, J., Standifer, K.M., Tublin, P.R., Su, W. and Pasternak, G.W. (1995) J. Neurochem. 65, 170–175.
[23] Rossi, G., Leventhal, L., Boland, E. and Pasternak, G.W. (1996) submitted.
[24] Rossi, G.C., Leventhal, L. and Pasternak, G.W. (1996) Eur. J. Pharmacol., in press,
[25] Gistrak, M.A., Paul, D., Hahn, E.F. and Pasternak, G.W. (1990) J. Pharmacol. Exp. Ther. 251, 469–476.