Replacement of Gln^{280} by His in TM6 of the human ORL1 receptor increases affinity but reduces intrinsic activity of opioids

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Abstract **The ORL1 (Opioid Receptor-Like) receptor is the G protein-coupled receptor whose amino acid sequence is closest to those of opioid receptors. Residues that are conserved in ORL1 and the three types of opioid receptor, but also a residue, His in the sixth putative transmembrane (TM6) helix, which is present in all opioid receptor types but absent in ORL1, appear to play a key role in receptor recognition and/or activation. Here we have sought to create an opioid binding pocket in the non-opioid ORL1 receptor by replacing residue Gln^{280} in its TM6 by the corresponding His residue of opioid receptors. The mutation affects neither the affinity of nociceptin – the natural ORL1 agonist – for the receptor, nor the potency of nociceptin to inhibit adenylyl cyclase via ORL1. In contrast, we find that a few opioid ligands, the agonists lofentanil, etorphine and dynorphin A, and especially the antagonists diprenorphine and nor-BNI, bind the mutant Q280H receptor with substantially (5- to >100-fold) higher apparent affinity than they do the wild-type receptor. Moreover, lofentanil and etorphine no longer act as pure agonists, as they do at the native ORL1 receptor, but are endowed with clear antagonist properties at the mutant receptor. The mutation Q280H, which increases affinity while decreasing intrinsic activity of opioids at ORL1, emphasizes the importance of the His residue for opioid recognition and activation.**

Key words: Opioid receptor; Nociceptin/orphanin FQ; Site-directed mutagenesis; Agonist/antagonist binding; Adenylate cyclase inhibition

1. Introduction

The ORL1 (Opioid Receptor-Like) receptor [1–8] is the G protein-coupled receptor whose amino acid sequence is closest to those of the μ, δ and κ types of opioid receptor. In particular, ORL1 and opioid receptors display extensive amino acid identity in their putative transmembrane (TM) helices (70–80% in TMs 2, 3 and 7). The ORL1 receptor endogenous ligand was recently identified as nociceptin [9] or orphanin FQ [10], a heptadecapeptide structurally related, however distantly, to the opioid peptide dynorphin A. In recombinant CHO cells expressing it, the ORL1 receptor mediates inhibition of adenylyl cyclase not only by nociceptin (which is not an opioid peptide) but also by etorphine [1] and other potent opiates, such as lofentanil (Butour et al., manuscript in preparation). Although the doses of opiate which maximally inhibit the cyclase via ORL1 are substantially higher than those required to inhibit the enzyme via opioid receptors, the ORL1 receptor does recognize and respond to opiates, an indication that the ORL1 receptor contains a functional binding pocket whose structure resembles the opioid binding pocket of opioid receptors. In practice then, it should be possible to improve the opioid binding abilities of the ORL1 receptor, i.e. create an opioid binding pocket in ORL1, by operating only minor changes in the amino acid sequence of the receptor.

There is abundant circumstantial evidence in favor of an opiate binding pocket located within the bundle of transmembrane helices and common to the three types of opioid receptor: (i) the putative membrane-spanning domains in μ-, δ- and κ-opioid receptors display very high sequence homologies (≥80% amino acid identity in TMs 2, 3 and 7), (ii) all endogenous opioid peptides contain the same Tyr-Gly-Gly-Phe opioid message sequence, and (iii) there are numerous rigid opiates (the so-called 'universal' opiates) that bind the three types of opioid receptor as well as receptor chimeras thereof with comparable high affinities, for instance the two tetrahydro-endoethano-thebaine derivatives etorphine and diprenorphine, and benzomorphans such as ethylketocyclazocine and bremazocine. Site-directed mutagenesis and 3-D models of opioid receptors have begun to identify conserved amino acid residues that may play a key role in opiate recognition and/or receptor activation. These include not only residues that are conserved in ORL1 and the three types of opioid receptor [11–14,18], but also and most interestingly, a residue which is present in all opioid receptor types but absent in ORL1, namely His in the sixth putative transmembrane helix (TM6). Then, substituting for His the corresponding amino acid residue (Gln^{280}) in ORL1 should endow the mutant ORL1 receptor (Q280H) with increased binding affinity for opiates. Here, we have generated this mutant ORL1 receptor, have it stably expressed in CHO cells, and compared its binding and functional coupling properties with those of the native ORL1 receptor. We find that opioid ligands bind the mutant Q280H receptor with substantially higher apparent affinity than they do the wild-type receptor. Yet unexpectedly, these opioids no longer act as pure agonists, as they do at the native receptor, but are endowed with clear antagonist properties at the mutant receptor.

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Abbreviations: ORL, opioid receptor-like; TM, transmembrane segment; G-protein, guanine nucleotide-binding regulatory proteins; DAGO, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; DTLET, [D-Thr^2,D-Leu^5]enkephalin; U-50488, trans,3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclo-hexyl]-benzeneacetamide

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2. Materials and methods

2.1. Construction of the ORL(Q280H) mutant

Replacement of glutamine-280 by histidine in the TM6 of ORL1 was achieved by site-directed mutagenesis, according to Kunkel et al. [15], of human ORL1 cDNA fragment *Hin*dIII-*Pst*I, cloned in plasmid Bluescript SK$^+$ (Stratagene). Uracylated single-strand DNA was generated in *E. coli* bacterial strain BW313 with helper phage M13KO7 (Invitrogen), and annealed with the 5'-phosphorylated oligonucleotide 5'-CTGCTGGACGCCTGTGCACGTCTTCGTGCTGGCC-3' carrying the mutation as well as a silent one, to generate an *Apa*LI restriction site in hORL1 cDNA. The double-stranded DNA was synthesized and ligated with Sequenase (Pharmacia) and T4 DNA ligase (Boehringer Mannheim). Selective amplification of the mutated DNA was achieved upon transformation in *E. coli* bacterial strain *mutL*, and the appropriate clones were selected on the basis of *Apa*LI restriction. The mutated cDNA was subcloned in eukaryotic expression vector pRc/CMV (Invitrogen) at *Hin*dIII and *Xba*I sites, after sequence verification.

2.2. Expression in cell lines

CHO-K1 cells were transfected with pRc/CMV/ORL(Q280H) by the calcium phosphate precipitation method [16] and selected as resistant to G418 (Gibco BRL; 400 μg/ml), as described earlier [1]. Screening of the clones was based on the ability of nociceptin to inhibit forskolin-induced accumulation of cAMP. For membrane preparations, cells were harvested, frozen at −70°C for at least 60 min, and homogenized in 50 mM Tris-HCl, pH 7.4 in a Potter Elvehjem tissue grinder. The nuclear pellet was discarded by centrifugation at $1000\times g$ and the membrane fraction was collected upon centrifugation at $100\,000\times g$.

2.3. Binding experiments

[^{3}H]Nociceptin (23 Ci/mmol), custom-labelled by Amersham, was used. Binding experiments were performed at 25°C in polypropylene tubes. Membranes (30–50 μg) were incubated with the tritiated ligand and various unlabelled ligands, for 60 min, in 0.5 ml Tris 50 mM, pH 7.4 supplemented with protease-free BSA (0.1 mg/ml final) to avoid adsorption of the radioligand on the walls of the tubes. Non-specific binding was determined in the presence of 10^{-6} M nociceptin. Bound radioactivity was collected by filtration on polyethyleneimine-treated GF/B filters (Whatman), and counted in a Kontron model MR300 liquid scintillation counter.

2.4. Assay for intracellular cAMP

Sterile hemolysis tubes were seeded with 2×10^5 recombinant CHO cells in culture medium and incubated for about 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, 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 addition of 20 μl of KRH containing 100 μM forskolin (Sigma), 1 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM Ro20-1724 (Biomol Res.) 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), and 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 [17].

2.5. Data analysis

Analysis of the data was performed with the InPlot ver 4.03 software from GraphPad Inc. (San Diego, CA, USA).

3. Results

Saturation binding studies of the mutant ORL(Q280H) receptor in membranes from recombinant CHO cells indicated that [^{3}H]nociceptin specifically labeled a homogenous population of sites (B_{max} in the range 0.8–2 pmol/mg protein, depending on the preparation) with nearly the same high affinity ($K_D = 0.065 \pm 0.004$ nM, $n = 3$) as it does the wild-type receptor (Fig. 1 and Table 1). In addition, the Tyr^1 analogue of nociceptin was found to be equally potent in competing with equilibrium binding of [^{3}H]nociceptin at the native ($K_I = 0.26$ nM) and mutant ($K_I = 0.38$ nM) receptors (Fig. 2 and Table 1). Taken together, these data suggest that residue Q280 of the ORL1 receptor is not a key component of the nociceptin binding pocket. The ability of various opioid ligands to bind the mutated Q280H ORL1 receptor was then examined. The results are shown in Fig. 2 and Table 1. The mutation did not seem to improve binding of the μ-, δ- and κ-selective opioid agonists DAGO, DTLET and U50488, respectively. At concentrations up to 10 μM, these were inefficacious in competing with binding of [^{3}H]nociceptin. In contrast, the mutation resulted in a substantial (5- to >100-fold) increase of the receptor's apparent affinity for other opioid ligands, including etorphine and diprenorphine, lofentanil, the κ-selective agonist dynorphin A and the κ-selective antagonist norbinaltorphimine (nor-BNI). Most pronounced was the effect of the mutation on binding of the antagonists nor-BNI and diprenorphine, whose apparent affinities were augmented more than 100-fold. In other words, the Q280H mutation definitely endowed the ORL1 receptor with improved opioid receptor binding characteristics and, in this sense, could be considered a gain-of-function mutation.

The ability of the mutant receptor to mediate inhibition of forskolin-induced accumulation of cAMP in recombinant

Table 1
Comparison of the binding properties and cyclase inhibition potencies of diverse ligands, in CHO cells stably expressing the wild-type or mutant ORL(Q280H) receptor

	Binding (K_i, nM)		cAMP (ED_{50}, nM)	
	Wild-type	ORL(Q280H)	Wild-type	ORL(Q280H)
Nociceptin	0.13 ± 0.01 (n = 3)	0.38 ± 0.02 (n = 4)	0.84 ± 0.02 (n = 4)	3.06[a] ± 0.12 (n = 8)
[Tyr^1]-nociceptin	0.26 ± 0.01 (n = 2)	0.37 ± 0.05 (n = 2)	1.19 ± 0.09 (n = 3)	2.44[a] ± 0.1 (n = 3)
Lofentanil	24.2 ± 0.26 (n = 3)	3.1 ± 0.07 (n = 2)	6.67 ± 0.23 (n = 2)	44[b] ± 3.77 (n = 6)
Etorphine	530 ± 40 (n = 3)	35 ± 2.4 (n = 4)	400 ± 48 (n = 3)	560[c] ± 182 (n = 3)
Dynorphin A	111 ± 9 (n = 3)	21 ± 3.6 (n = 3)	>10000	551[c] ± 66 (n = 3)
Nor-BNI	>10000	74 ± 10 (n = 3)	>10000[e]	>10000[d]
Diprenorphine	>10000	137 ± 17 (n = 3)	>10000[e]	>10000[d]
DAGO	>10000	>10000	>10000[e]	>10000[e]
DTLET	>10000	>10000	>10000[e]	>10000[e]
U-50488	>10000	>10000	>10000[e]	>10000[e]

Values are means ± S.E.M.
[a] >75% maximal inhibition, [b]60% maximal inhibition, [c]20–30% maximal inhibition, [d]antagonist, [e]inactive.

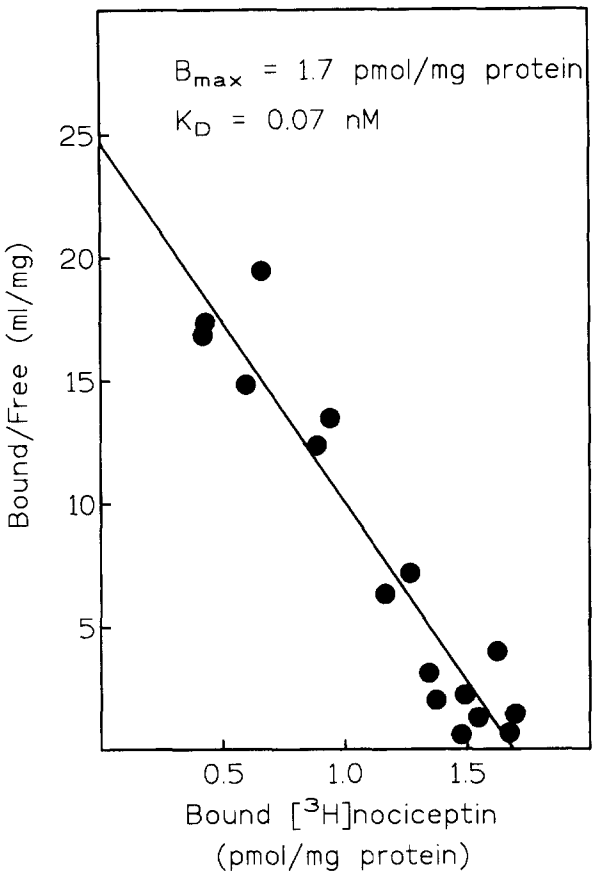

Fig. 1. Scatchard analysis of [^{3}H]nociceptin binding to membranes of CHO cells stably expressing the mutant receptor ORL(Q280H).

CHO cells was then explored. Fig. 3 shows that nociceptin and [Tyr^1]nociceptin caused maximal (75–80%) inhibition (Fig. 3), however with slightly (2–3-fold) reduced apparent affinity compared to the wild-type receptor (Table 1). Surprisingly, lofentanil, etorphine and dynorphin A, which totally displaced binding of [^{3}H]nociceptin in competition experiments (Fig. 2), inhibited cAMP production only partially (Fig. 3), hence acted as partial agonists at the mutant receptor. The most pronounced effect was with etorphine which, although maximally (>90% inhibition) active via the wild-type ORL1 receptor, caused a maximum 20–30% inhibition of forskolin-induced accumulation of cAMP in cells expressing the mutant ORL(Q280H) receptor. Likewise, dynorphin A, despite a relatively high apparent affinity for the mutant receptor ($K_i \approx 20$ nM), was somewhat ineffective in inhibiting adenylate cyclase. In the presence of 10 μM etorphine or dynorphin A, the potency of nociceptin to inhibit residual (about 50% of maximally inhibitable) cyclase activity via the mutant receptor was decreased, as revealed by a shift towards the right of nociceptin's dose response curves (Fig. 4). This confirmed the notion that etorphine and dynorphin A acted as mixed agonists-antagonists at the mutant (Q280H) receptor.

4. Discussion

Replacement of residue Gln^{280} by His in the sixth putative membrane-spanning domain of ORL1 was achieved here to improve the receptor's affinity for opioid ligands, i.e. create an opioid binding pocket within the non-opioid ORL1 receptor. Mutation to Ala of the His residue, which is present at the equivalent position in the three opioid receptor types, dramatically reduces binding of the μ-selective agonist [^{3}H]DAGO and antagonist [^{3}H]naloxone to the μ-opioid receptor [12], and a 3-D model of the μ-opioid receptor complexed with lofentanil has proposed that this residue may be a constituent of the opioid binding pocket [18]. Indeed, the mutant ORL1(Q280H) receptor, although it retains the same high affinity as the native receptor for nociceptin, displayed significantly *increased* (5- to >100-fold) affinity for the non-selective opioid agonists (etorphine, lofentanil) and antagonist (diprenorphine) as well as for the κ-selective agonist dynorphin A and antagonist Nor-BNI. In these respects, the mutation appears to have shifted the pharmacological profile of the ORL1 receptor towards that of a κ-opioid receptor. However, U50488, a κ-selective compound of the arylacetamide series, failed to recognize the mutant receptor. This discrepancy could be explained by the fact that determinants specifically required for binding of U50488 [19] are lacking in the ORL1 receptor. Moreover, peptide and non-peptide ligands appear to bind opioid receptors differently [19,20], the exofacial loops filtering access of the former but not of the latter, to the opioid binding pocket. The fact that the mutant ORL1(Q280H) receptor failed to bind the μ- and δ-selective peptides, DAGO and DTLET respectively, could readily be explained by the extracellular loops of ORL1 preventing binding of receptor type-selective opioid ligands via an exclusion mechanism, as proposed by Metzger and Ferguson [21]. In contrast, dynorphin, taking advantage of its basic, 'address' core, could bypass the filter by interacting with the negatively charged second extracellular loop of ORL1, as it does at the κ-opioid receptor [19,20,22].

More recently, Uhl et al. [23] reported that the His residue in TM6 of the μ-opioid receptor could be replaced by Gln with minimal consequences on binding of μ-selective ligands, indicating that μ-selective drug recognition may simply de-

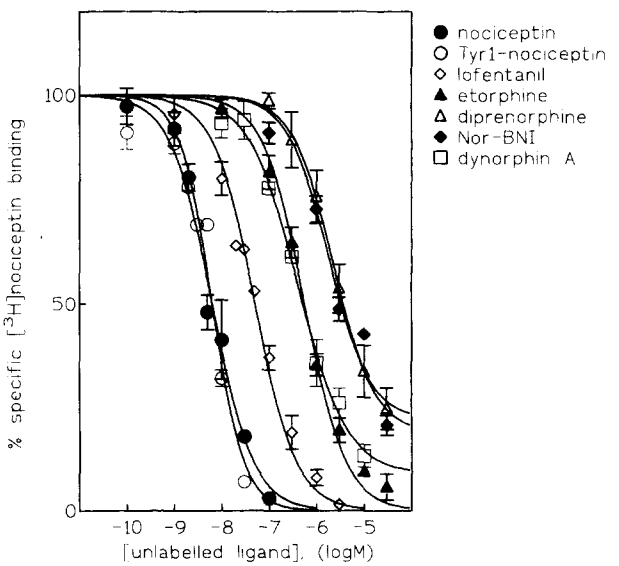

Fig. 2. Inhibition of [^{3}H]nociceptin (1 nM) binding by selected opioid and non-opioid ligands in membranes from CHO cells stably expressing the mutant receptor ORL(Q280H). Each point represents the mean ± S.E.M. from 2–6 determinations performed in duplicate.

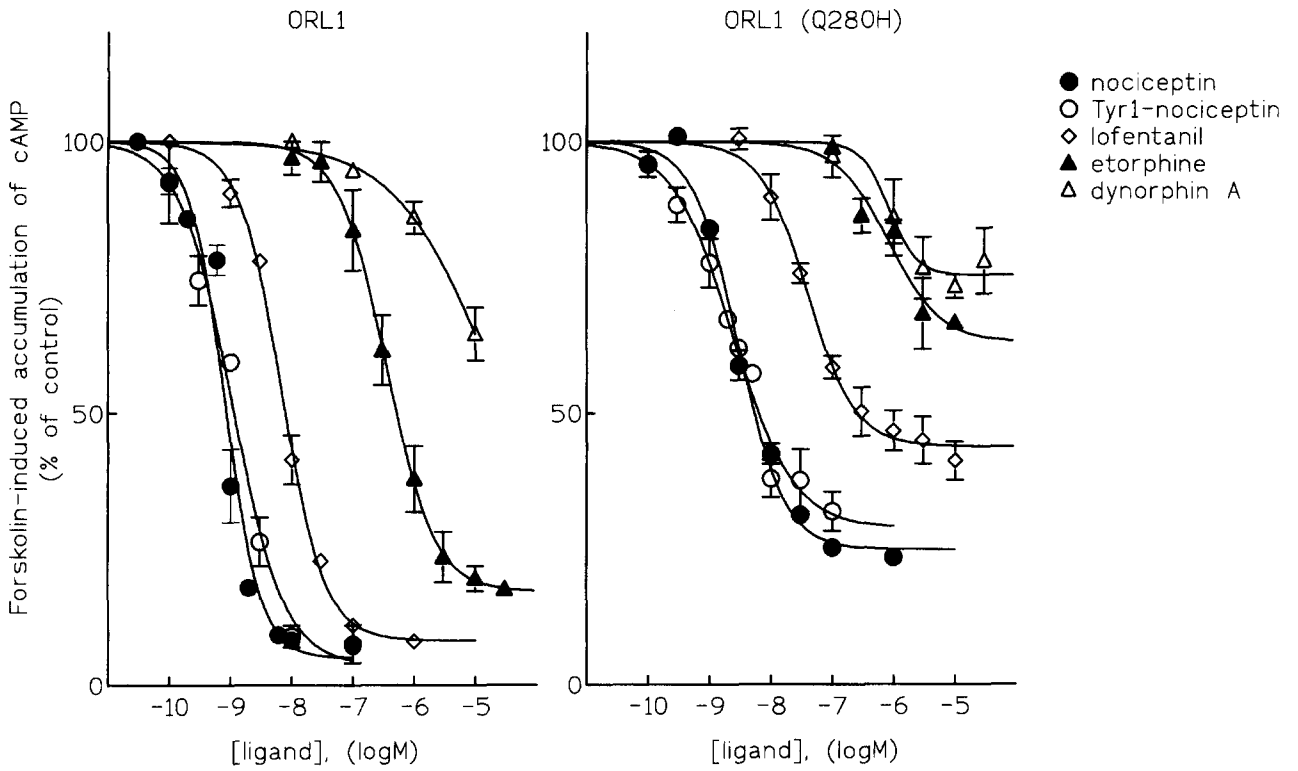

Fig. 3. Compared inhibition of forskolin-induced accumulation of cAMP by selected opioid and non-opioid ligands in intact CHO cells stably expressing the wild-type or mutant ORL(Q280H) receptor. Each point represents the mean ± S.E.M. from at least 3 experiments performed in triplicate.

pend on the presence of an ε-nitrogen atom at this position. Likewise, the present study shows that the inverse substitution in ORL (Gln → His) induced only minor changes in nociceptin binding, while conferring greater affinity for opioid ligands. Taken together, these results act more in favor of the requirement of an ε-nitrogen at this position in the TM6 of opioid receptors and, perhaps, nociceptin receptors, rather than the His residue being one discriminative determinant between opioid receptors and ORL. This would explain why, even in the absence of histidine, opioid ligands such as etorphine and lofentanil could interact with ORL (Table 1).

The mutant ORL1(Q280H) receptor appeared to be fully functional, mediating robust (≥75%) inhibition of adenylyl cyclase by nociceptin and its Tyr^1 analogue. Surprisingly however, the opioid agonists, especially etorphine and lofentanil, which acted as low affinity, full agonists of the ORL1 receptor, behaved as higher affinity, partial agonists and even antagonists of the mutant receptor, as if the mutation had created an opioid antagonist binding pocket within the ORL1 receptor. This conclusion is sustained by the fact that the Gln → His mutation in ORL1 increases most the affinities of the antagonists diprenorphine and nor-BNI, and that these remain antagonists at the mutant receptor. By contrast, it has been observed that exchange of the histidine by alanine in TM6 of the μ-opioid receptor impaired opioid binding while enhancing intrinsic activity [12]. Taken together, these data suggest that the His residue in TM6 of opioid receptors is not only implicated in recognition of opioid ligands, particularly antagonists, but may also control coupling with G-proteins. Similar conclusions have been reached for other peptide hormone receptors in which His residues in TM5 or 6

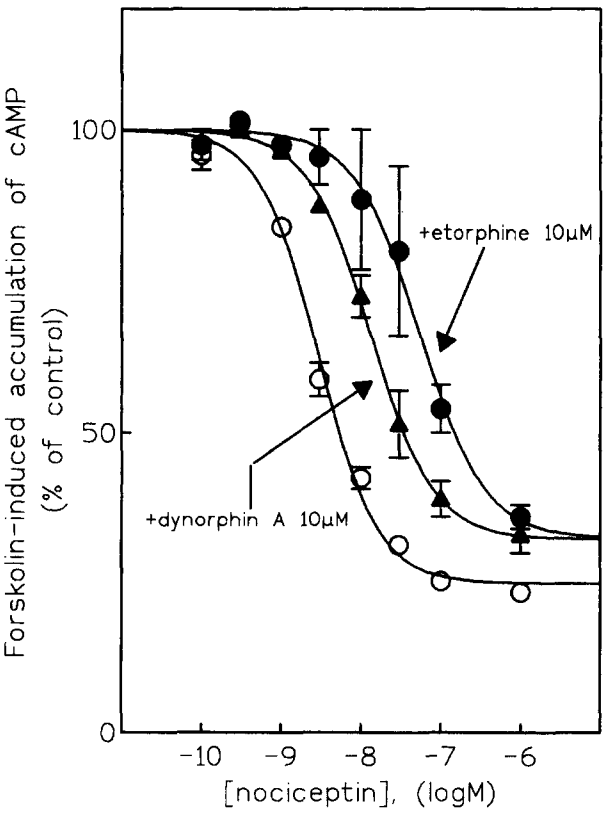

Fig. 4. Inhibition of the forskolin-stimulated cAMP content by nociceptin in the absence or presence of 10 μM dynorphin A or etorphine. The ED_{50} of nociceptin was increased from 3 nM to 13 nM and 56 nM in the presence of dynorphin A and etorphine respectively.

could be replaced by Gln without major effects, while other substitutions impaired more selectively antagonists binding [24–26].

In summary, the Q280H mutation of the ORL1 receptor, while not affecting the structure and functionality of the binding pocket for its natural peptide agonist, nociceptin, results in improved affinity for certain opioid ligands, especially antagonists. This mutation emphasizes a role of the His residue in TM6 of opioid receptors in maintaining an opioid receptor antagonist conformation. At present it is not known whether the corresponding residue (Gln) in TM6 of the ORL1 receptor plays a similar role. The answer to this question will await further mutagenesis studies on the ORL1 receptor and the development of other ligands, especially antagonists.

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