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Functional Inactivation of the Nociceptin Receptor by Alanine Substitution of Glutamine 286 at the C Terminus of Transmembrane Segment VI: Evidence from a Site-Directed Mutagenesis Study of the ORL1 Receptor Transmembrane-Binding Domain

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ABSTRACT

A site-directed mutagenesis approach has been used to gain insight into the molecular events whereby the heptadecapeptide nociceptin binds and activates the opioid receptor-like 1 (ORL1) receptor, a G protein-coupled receptor. Alanine mutation, in the human ORL1 receptor, of transmembrane amino acid residues that are conserved in opioid receptors, Asp^{130} and Tyr^{131} in transmembrane segment (TM) III, Phe^{220} and Phe^{224} in TM V, and Trp^{276} in TM VI, yields mutant receptors with reduced affinity, and proportionally decreased reactivity, toward nociceptin. Least to most deleterious in this respect are Ala substitutions of $Phe^{220} \sim W276A < Tyr^{131} \ll Phe^{224} \leq Asp^{130}$. The dramatic effects of the D130A mutation on nociceptin binding and activity are not reversed in the D130N mutant, whereas those of the Y131A mutation are totally suppressed in Y131F. This suggests that a negative charge at position 130, and a phenyl ring at position 131 in TM III, are critical for occupancy and/or activation of the receptor by nociceptin. Alanine replacement of glutamine 286, located at the C terminus of TM VI, yields a mutant receptor that binds nociceptin with nearly the same affinity as does the wild-type receptor (K_d values of 0.13 and 0.22 nM, respectively) but, unlike the latter, is unable to mediate nociceptin inhibition of forskolin-induced cAMP synthesis in recombinant Chinese hamster ovary cells ($ED_{50} > 10{,}000$ nM compared with 0.8 nM at the wild-type receptor). In all respects, this mutant receptor appears to be functionally inactive, indicating that residue Gln^{286} may play a pivotal role in ORL1 receptor-mediated transduction of the nociceptin signal.

Nociceptin (noc) (Meunier et al., 1995), also known as orphanin FQ (Reinscheid et al., 1995), a neuropeptide with multiple functions (reviewed by Henderson and McKnight, 1997; Meunier, 1997; Darland et al., 1998), is the natural agonist of the orphan opioid receptor-like 1 (ORL1) receptor, a G protein-coupled receptor (GPCR) (Mollereau et al., 1994). The ORL1 receptor shares high sequence similarity with opioid receptors. Likewise, noc (FGGFTGARKSARKLANQ) resembles in several respects dynorphin A (dyn), also a heptadecapeptide (YGGFLRRIRPKLKWDNQ), and the presumed natural agonist of the κ-opioid receptor. However, in spite of receptor homology, and common chemical features between ligands, noc and dyn appear to bind and stimulate their cognate receptors by distinct mechanisms. Studies of the structure-activity relationships of noc, based on the properties of truncated (Dooley and Houghten, 1996; Reinscheid et al., 1996; Shimohigashi et al., 1996; Butour et al., 1997; Guerrini et al., 1997), "mutant" (Dooley and Houghten, 1996; Reinscheid et al., 1996; Guerrini et al., 1997; Calo' et al., 1998), or hybrid noc/dyn and dyn/noc (Lapalu et al., 1997; Reinscheid et al., 1998) peptides, have now established noc[1–13]-amide as the smallest noc fragment displaying the affinity, biological potency, and ORL1 versus κ-opioid receptor selectivity of noc. Moreover, positively charged hexapeptides of general sequence Ac-RYY(R/K)(W/I)(R/K)-NH_2 have been shown to be potent ORL1 receptor agonists (Dooley et al., 1997). Taken together, these data suggest that noc activ-

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ABBREVIATIONS: noc, nociceptin; CHO, chinese hamster ovary; dyn, dynorphin A; e2 and e3, second and third extracellular loop; GPCR, G protein-coupled receptor; ORL1, opioid receptor-like 1; hORL1, human ORL1; HP5, hexa- or Houghten peptide 5, Ac-Arg-Tyr-Tyr-Lys-Trp-Lys-NH_2; mDOR1, mouse δ-opioid receptor; nor-BNI, nor-binaltorphimine; TM, transmembrane segment; wt, wild type.

ity may primarily reside in its positively charged R^8KSARK domain that would therefore fulfil a "message" function (Schwyzer, 1977). This is in marked contrast to dyn, whose capacity to induce a biological response is contained within the N-terminal Y^1GGF sequence (Chavkin and Goldstein, 1981; Mansour et al., 1995). The notion that noc and dyn have distinct functional architectures has gained further support from the finding that the two neuropeptides use alternative mechanisms for receptor activation. In particular, the second exofacial (e2) loop of the receptor appears to be required for stimulation of the ORL1 receptor by noc, but not for activation of the κ-opioid receptor by dyn (Lapalu et al., 1998; Mollereau et al., 1999). Recently, a molecular model of the ORL1 receptor complex with noc has been built (Topham et al., 1998), showing the N-terminal F^1GGF tetrapeptide of noc to bind in a highly conserved transmembrane region, comprising elements from helices 3, 5, 6, and 7, that is the topological equivalent of the presumed opioid binding pocket in the μ-, δ-, and κ-opioid receptors, and the basic R^8KSARK segment to establish multiple interactions with the acidic e2 loop. Topham et al. (1998) have proposed that the binding energy contributed by noc[1–4] may be partially uncoupled from activation mediated by the e2 loop, hence that interactions of the N-terminal tetrapeptide in the vestigial opioid pocket serve a primarily locatory function, consistent with the extremely low biological potency of most opiates at the ORL1 receptor. Thus, the TM binding cavity may serve distinct, and even inverse, functions in the ORL1 and opioid receptors, providing a differential array of binding and recognition elements for the necessary but biologically insufficient noc F^1GGF "address" sequence in the former and for effective transduction of the Y^1GGF "message" sequence in the latter (Mollereau et al., 1999). Within this conceptual framework, the vestigial opioid binding pocket of the ORL1 receptor would appear better suited as a template for antagonist rather than agonist design (Mollereau et al., 1999).

In the present study, a site-directed mutagenesis approach has been used to better delineate the role of individual amino acid residues in the transmembrane noc-binding domain of the human ORL1 (hORL1) receptor. Alanine mutation of conserved residues in ORL1 and opioid receptors results in a loss of affinity, and a commensurate loss of biological reactivity, toward noc, consistent with the provision of recognition elements to noc "address" domain. Most critical in this respect were residues Asp^{130} in transmembrane segment (TM) III, and Phe^{224} in TM V. We also report that alanine mutation of glutamine 286, located near the C terminus of TM VI, at the extracellular margins of the modeled F^1GGF binding site (Topham et al., 1998), yields a mutant receptor whose binding characteristics are undistinguishable from those of the wild-type (wt) receptor, but which is unresponsive to noc and other ORL1 receptor agonists. The mutant ORL1[Q286A] receptor is thus functionally inactive. Therefore, residue Gln^{286} appears to play an important role in ORL1 receptor-mediated transduction of the noc signal.

Materials and Methods

Construction of Mutant Receptor cDNAs. Point mutations were introduced in the Bluescript SK^+/hORL1 construct with mutated oligonucleotides (Genosys Biotech., Pampisford, U.K.) using the ExSite PCR mutagenesis kit (Stratagene, La Jolla, CA) and Vent polymerase (New England Biolabs, Beverly, MA). To facilitate screening of the clones, an additional silent mutation was routinely incorporated into the oligonucleotides. The mutated cDNAs were custom-sequenced (Genome Express, Paris, France) and subcloned in the *Bste*II and *Xba*I sites of the eukaryotic expression vector pEFIN (Euroscreen, Brussels, Belgium).

Expression in Cell Lines. Chinese hamster ovary (CHO)-K1 cells were transfected with recombinant vectors using calcium phosphate precipitation (Chen and Okayama, 1987) or polycation-dimethyl sulfoxide (Kawai and Nishizawa, 1984) and grown in Ham's F-12 medium (Life Technology, Gaithersburg, MD) containing G418 (Life Technologies; 400 μg/ml) for selection, as described previously (Mollereau et al., 1994). Clones in which noc was able to cause maximum inhibition of forskolin-induced accumulation of cAMP (see below) were considered to express a functional receptor.

Membrane Preparation. Recombinant cells were harvested, frozen at −70°C for at least 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 collected on centrifugation at 100,000*g*.

Binding Studies. Custom-labeled [^{3}H]noc (23 Ci/mmol; Amersham, Little Chalfont, U.K.) was used. Saturation binding and competition experiments were performed at 25°C in polypropylene tubes. Membranes (5–30 μg) were incubated for 1 h with tritiated ligand alone at variable concentrations (for saturation), or with tritiated ligand at fixed concentration (1 nM) and variable concentration of unlabeled ligand (for competition), in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, supplemented with proteinase-free BSA (to 0.1 mg/ml; Sigma, St Louis, MO) to avoid tube wall adsorption of the radioligand. Nonspecific binding was determined in the presence of 1 μM unlabeled noc. Bound radioligand was collected by filtration on polyethyleneimine-treated glass fiber filters (GF/B; Whatman, Clifton, NJ), and radioactivity counts were made in a Packard model 2100TR liquid scintillation analyzer (Packard, Meriden, CT)

Intracellular cAMP Assay. Sterile hemolysis tubes were seeded with 2×10^5 recombinant CHO cells in culture medium and incubated for ~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 added to each tube. Intracellular accumulation of cAMP was initiated by the addition of 20 μl of KRH containing 100 μM forskolin (Sigma), 1 mM 3-isobutyl-1-methylxanthine (Sigma), 1 mM Ro20–1724 (Biomol Research, Plymouth Meeting, PA) and the ligand(s) to be tested at the desired concentration. The reaction was stopped after exactly 10 min at 37°C, by the 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).

Analysis of Data. Experimental data were fitted to a sigmoidal dose-response curve with variable slope parameter 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. $[I]_{50}$ values (the concentration of inhibitor that halves specific binding of radioligand) were converted to K_I values using the Cheng and Prussof (1973) equation: $K_I = [I]_{50}/[1 + ([L]/K_d)]$, where [L] and K_d are the concentration and dissociation constant of radioligand, respectively.

Results

Several entirely conserved ORL1 receptor residues, lining the structural equivalent of the opioid binding pocket in opioid receptors (Paterlini et al., 1997; Pogozheva et al., 1998;

Bikker et al., 1998; Topham et al., 1998), Asp^{130} and Tyr^{131}, located in TM III, Phe^{220} and Phe^{224} in TM V, and Trp^{276} in TM VI, were selected for mutagenesis. Gln^{286}, adjacent to the pocket at C terminus of TM VI, was also chosen (Fig. 1). This position is occupied by different amino acid residues in the ORL1, μ-, δ-, and κ-opioid receptors. The wt and mutant receptors were probed in terms of affinity and biological reactivity toward the known ORL1 receptor agonists, noc (Meunier et al., 1995), [Tyr^1]noc (Reinscheid et al., 1996), Ac-Arg-Tyr-Tyr-Lys-Trp-Lys-NH_2 (HP5; Dooley et al., 1997), and lofentanil (Butour et al., 1997). Specific [^{3}H]noc binding (B_{max} in the range 1 to 5 pmol/mg protein) was readily detected in membrane preparations from transformed CHO cells expressing the wt and mutant ORL1 receptors, mutants D130A, D130N, and F224A excepted. However, the latter were able to mediate inhibition of forskolin-induced accumulation of cAMP in recombinant CHO cells (Fig. 2), indicating that the receptor was expressed and functional.

Figure 3A shows that noc, [Tyr^1]noc, HP5, and lofentanil potently inhibited binding of [^{3}H]noc in a crude membrane fraction from recombinant CHO cells expressing the wt ORL1 receptor. The calculated K_i values were 0.13, 0.26, 1.2, and 24 nM, respectively. The four ligands also potently and maximally inhibited forskolin-induced accumulation of cAMP in intact recombinant CHO[$ORL1^+$] cells (Fig. 3B), thus behaving as "pure" ORL1 receptor agonists in this test. ED_{50} values were estimated to be 0.8, 1.1, 0.7, and 7.2 nM for noc, [Tyr^1]noc, HP5, and lofentanil, respectively.

Mutations of Asp^{130} in TM III. Replacement of Asp^{130} by an alanine residue in the hORL1 receptor resulted in a mutant ORL1[D130A] receptor, at which no specific binding of [^{3}H]noc, used at concentrations of up to 5 nM, could be demonstrated. However, the mutant receptor was still able to mediate inhibition of forskolin-induced accumulation of cAMP in recombinant CHO cells, yet only in the presence of elevated concentrations of agonist (Table 1). Similar results were obtained with the mutant ORL1[D130N] receptor. Mutation of Asp^{130} to glutamate did not improve noc affinity and potency relative to D130A (data not shown). The noc derivative *N*-acetyl-noc[1–13]-amide, which has an uncharged N terminus, retained moderate affinity and potency at the wt receptor, but was totally inactive toward the mutant D130A and D130N receptors (Table 1). These results suggest that the steric and electronic demands of a putative direct interaction between Asp^{130} and the N terminus of noc are either quite strict or that Asp^{130} assumes another role in receptor activation (see *Discussion*).

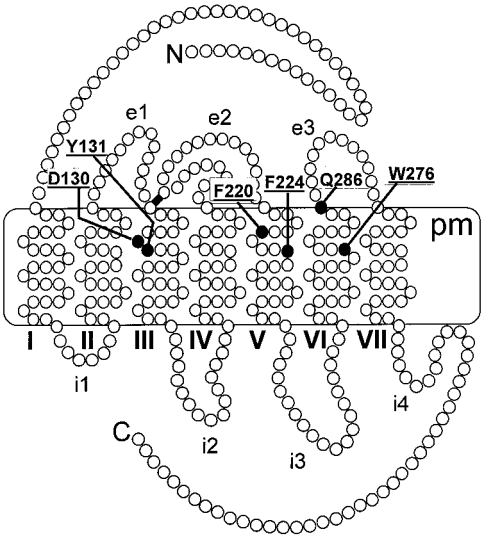

Fig. 1. Schematic representation of hORL1 receptor indicating (●) amino acid residues mutated in present study. These are also identified using one letter code followed by the receptor residue number. Conserved amino acid residues in the ORL1, μ-, δ-, and κ-opioid receptors are underlined. N, N terminus; C, C terminus; pm, plasma membrane (box); e, extracellular loop; i, intracellular loop. Transmembrane segments are labeled with Roman numerals. The filled rectangle represents the putative disulphide bridge between cysteine residues 123 and 200.

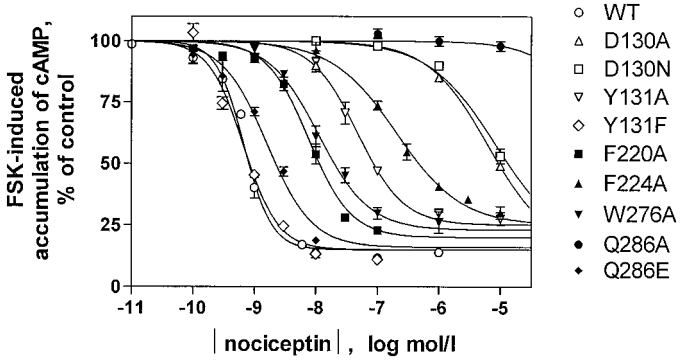

Fig. 2. Inhibition by noc of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing the wt and mutant ORL1 receptors. Control refers to accumulated cAMP in the absence of noc. Vertical capped bars represent S.E.M. from triplicate measurements.

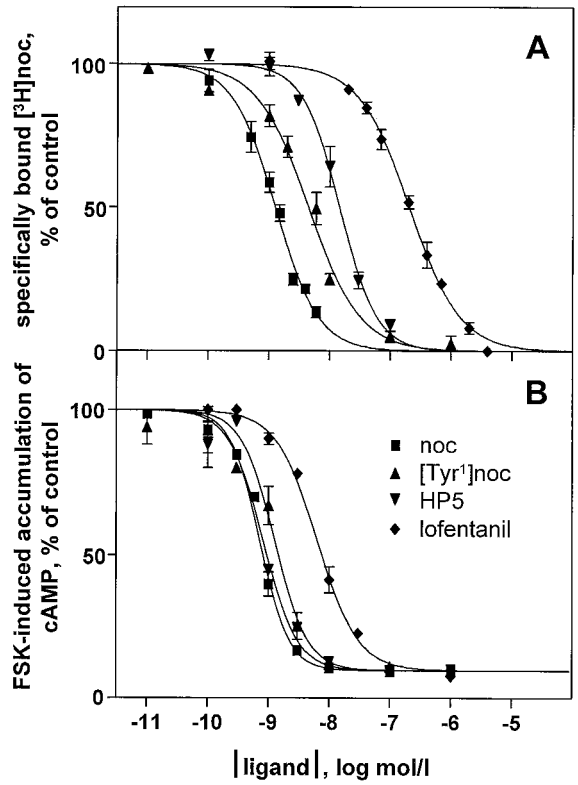

Fig. 3. Potency of various agonists to bind (A) and activate (B) the wt ORL1 receptor. A, Inhibition of specific [^{3}H]noc (1 nM) binding in a crude membrane preparation from recombinant CHO[$ORL1^+$] cells. B, Inhibition of forskolin (FSK)-induced accumulation of cAMP in intact CHO[$ORL1^+$] cells. In A and B, control refers to bound [^{3}H]noc and accumulated cAMP, respectively, in the absence of any (unlabeled) ligand. Vertical capped bars represent S.E.M. from triplicate measurements.

Mutations of Tyr^{131} in TM III (see Table 1). Substituting Tyr^{131} for an alanine residue adversely affected affinity and potency of all the ligands. However, the relative magnitude of these effects was ligand dependent. Noc and [Tyr^1]noc showed substantially reduced affinity (~30-fold), and a commensurate 50-fold loss in biological potency, toward the ORL1[Y131A] receptor. The mutation had little impact on affinity of HP5 and lofentanil, but decreased biological activity of the two ligands 50- and >140-fold, respectively. Interestingly, replacing Tyr^{131} by phenylalanine rather than alanine resulted in a mutant receptor with minimally impaired pharmacological properties. Compared with the wt receptor, the ORL1[Y131F] receptor showed only a 2-fold reduced affinity for, and essentially unaltered response to, the peptide agonists noc, [Tyr^1]noc, and HP5. Lofentanil displayed only moderately diminished binding (6-fold) and potency (15-fold) at this mutant receptor.

Mutations of Phe^{220} and Phe^{224} in TM V (see Table 1). The F220A mutation resulted in a <2-fold decreased affinity, and a more pronounced, although moderate reduction in biological potency (5- to 15-fold), for the four agonists. The F224A mutation was considerably more deleterious because no specific binding of [^{3}H]noc to membrane preparations from recombinant CHO cells could be demonstrated. However, noc and [Tyr^1]noc were still able to produce a maximum biological response in these cells, albeit with a nearly 200-fold decreased potency.

Mutation of Trp^{276} in TM VI (see Table 1). Alanine replacement of Trp^{276} differentially affected the pharmacological properties of the four ligands tested. Noc and [Tyr^1]noc showed diminished binding (2- to 4-fold reduced affinity), and biological potency (10- to 30-fold), but not efficacy. These changes are quantitatively very similar to those seen with the F220A mutant receptor. In contrast, the W276A mutant receptor could not be fully activated by HP5 and lofentanil. These ligands, which retained nearly wild-type affinity for the ORL1[W276A] mutant receptor, only produced ~25 and ~65%, respectively, of the maximum attainable inhibition level in the cAMP synthesis assay (data not shown). Both ligands, especially HP5, thus behave as low-efficacy agonists of the ORL1[W276A] mutant receptor. Indeed, HP5, in addition to exerting its own partial agonist action, was also able to prevent activation of the mutant ORL1[W276A] receptor by noc (Fig. 4A), thereby exhibiting antagonist properties. A Schild plot of the data (Fig. 4B) was linear (r = 0.99; slope = 1.25), and yielded a pA_2 value of 8.1, in close agreement with the logarithmically transformed dissociation constant for HP5 (pK_i = 8.4).

Mutations of Gln^{286} in TM VI/e3. The Q286A mutant ORL1 receptor possessed unique characteristics. As shown in Fig. 5A, ORL1[Q286A] bound [^{3}H]noc with a high affinity (K_d = 0.13 nM), identical with that of the wt receptor. However, the mutant receptor was totally unresponsive to noc (ED_{50} > 10 μM), being unable to mediate inhibition of forskolin-induced accumulation of cAMP in recombinant CHO cells (Fig. 5B). The retention of high affinity and sensitivity loss in ORL1[Q286A] generalized to the other ORL1 receptor agonists [Tyr^1]noc, HP5, and lofentanil (Table 2). Thus, in every respect, this mutant ORL1 receptor is functionally inactive, compared with the wt receptor. Residue Gln^{286}, which is unique to the ORL1 receptor, appears to selectively stabilize the active agonist-bound receptor form, making little or no contribution to noc, [Tyr^1]noc, or HP5 binding per se. However, the mutation did increase the affinity for lofentanil, dyn, and etorphine by an order of magnitude (see Table 2). The affinity for nor-binaltorphimine (nor-BNI), a κ-selective opioid receptor antagonist, remained unchanged. The deleterious effect of mutating Gln^{286} to alanine could be almost completely reversed in the Q286E mutant ORL1 receptor, which displayed nearly wt affinity and response toward noc, [Tyr^1]noc, and lofentanil. However, the HP5 peptide was ~13-fold less potent at the ORL1[Q286E] mutant compared with the wt receptor.

Discussion

The present study has used site-directed mutagenesis to experimentally probe the presumed transmembrane binding subsite for noc in the ORL1 receptor. Molecular modeling of the complex of receptor with noc (Topham et al., 1998) shows this subsite to be the structural equivalent of the opiate binding pocket of opioid receptors, and to host the noc N-terminal Phe^1-Gly-Gly-Phe sequence, that closely resembles the N-terminal Tyr^1-Gly-Gly-Phe sequence of many opioid peptides. Indeed, the existence of a vestigial opioid binding pocket within the ORL1 receptor receives strong support from the finding that relatively few point mutations in this region suffice to confer the hORL1 receptor with improved

TABLE 1

Affinity (K_i, nM), potency (ED_{50}, nM), and efficacy (% maximum attainable inhibition of forskolin-induced cAMP synthesis) of noc, [Tyr^1]noc, HP5, lofentanil, and *N*-acetyl-noc[1-13]-amide at wt and mutant ORL1 receptors expressed in CHO cells

Mutations are designated using the one letter amino acid code and the ORL1 receptor numbering system. K_i values were calculated from the concentration of unlabelled drug that halves specific binding of [^{3}H]noc (1 nM) in a crude membrane preparation from transformed CHO cells, according to Cheng and Prussof (1973). ED_{50} values represent the concentration of unlabelled ligand that half-maximally inhibits forskolin-induced accumulation of cAMP in intact recombinant CHO cells. Each value is mean ± S.E. from at least three separate estimates. F_{mut}, Mutation factor, i.e. ratio K_i (mutant)/K_i (wt). NB, No demonstrable specific binding of [^{3}H]noc, used at concentrations of up to 5 nM. ND, Not determined.

Ligand	ORL1 Receptor											
	wt			D130A			D130N			Y131A		
	K_i	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max
Noc	0.13 ± 0.03	0.8 ± 0.05	88	NB (ND)	~6,000	84	NB (ND)	~7,000	86	3.3 ± 1 (25)	39 ± 3	74
[Tyr^1]noc	0.26 ± 0.02	1.1 ± 0.04	85	ND (ND)	>10,000	ND	ND (ND)	>10,000	ND	8.5 ± 0.5 (33)	54 ± 9	74
HP5	1.2 ± 0.06	0.7 ± 0.08	87	ND (ND)	>10,000	ND	ND (ND)	~3,000	86	2.9 ± 0.1 (2.4)	36 ± 5	74
Lofentanil	24 ± 2.5	7.2 ± 1.3	85	ND (ND)	~5,000	84	ND (ND)	~5,000	86	36 ± 3 (1.5)	>10,000	ND
N-Acetyl-noc[1-13]-amide	22 ± 2	37 ± 2	88	ND	>10,000	ND	ND	>10,000	ND	ND (ND)	ND	ND

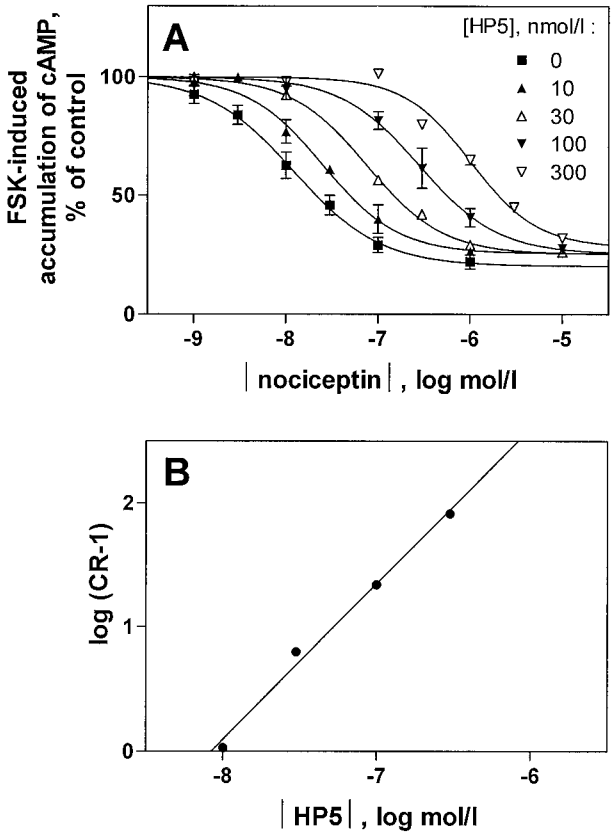

Fig. 4. A, Inhibition by noc of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing mutant ORL1[W276A] receptor in the absence or presence of increasing concentrations of HP5. Control refers to accumulated cAMP in the presence of HP5 alone at the indicated concentration. Vertical capped bars represent S.E.M. from triplicate measurements. B, Schild transform of the data. CR, Concentration ratio.

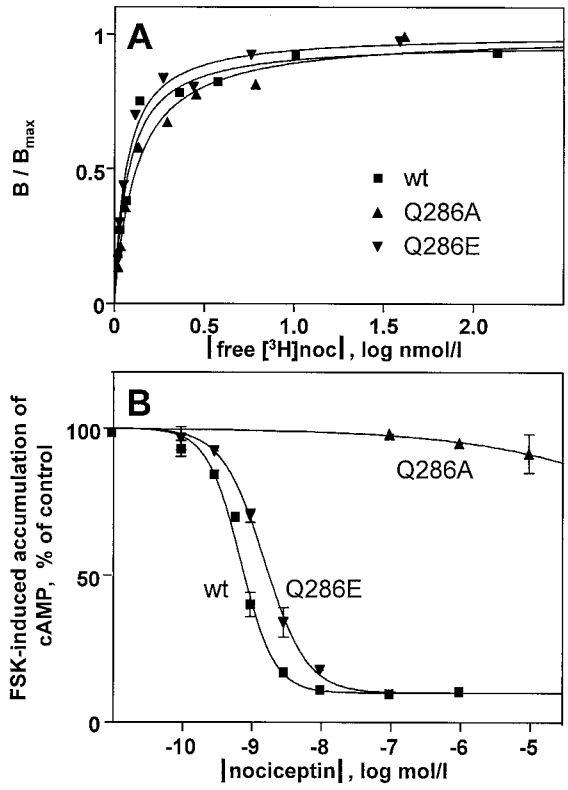

Fig. 5. Ability of wt and mutant Q286A and Q286E receptors to bind (A) and be activated by (B) noc. A, Equilibrium saturation binding of [^{3}H]noc in a crude membrane preparation from recombinant CHO cells expressing the wt, Q286A, or Q286E receptor. Data have been normalized to the same B_{max} value. In this representative experiment, the calculated K_d values (nM) were 0.07 (wt), 0.12 ([Q286A], and 0.10 ([Q286E]. B, Inhibition of forskolin (FSK)-induced accumulation of cAMP in intact recombinant CHO cells expressing wt, Q286A, or Q286E receptor. ED_{50} values were: 0.8, >10,000, and 2.0 nM for noc to cause half-maximal inhibition via the wt, [Q286A], and [Q286E] ORL1 receptors, respectively. Control refers to forskolin (FSK)-induced accumulation of cAMP in the absence of noc.

opioid binding properties, whereas noc binding and activity are unaffected (Mollereau et al., 1996; Meng et al., 1996; Meng et al., 1998).

Alanine mutation of five conserved transmembrane residues in this binding pocket, Asp^{130} and Tyr^{131} in TM III, Phe^{220} and Phe^{224} in TM V, and Trp^{276} in TM VI, had functional consequences that varied both quantitatively and qualitatively, dependent on location of the mutation and receptor agonist tested. However, these mutations did affect the properties of noc and its closest structural analog, [Tyr^1]noc, identically, indicative of a common mode of interaction with the ORL1 receptor. Affinity and biological potency were both diminished, consistent with either uniform losses in binding energy contributions in the active and resting receptor states or pure binding effects. This supports the hypothesis that the ORL1 receptor equivalent of an opioid binding pocket assumes a mainly locatory function and that binding energy gains from the interaction of the N-terminal Phe^1-Gly-Gly-Phe noc sequence can only be fully realized through the recruitment of interactions elsewhere with the receptor, in particular with e2 (Mollereau et al., 1999).

The most deleterious of the five alanine substitutions in the series, with respect to noc and [Tyr^1]noc affinity and potency, was the ORL1[D130A] (TM III) mutation, indicating that Asp^{130} plays a major role in ligand recognition. Because no recovery of receptor function was observed when Asp^{130} was exchanged for an asparagine residue, it can be reasonably argued that a negative charge at this locus is required for efficient binding, and hence activity of noc and [Tyr^1]noc. The aspartate residue equivalent to Asp^{130} in the ORL1 receptor is conserved in the opioid and other GPCR families,

TABLE 1 (*Continued*)

ORL1 Receptor											
Y131F			F220A			F224A			W276A		
K_i (F_{mut})	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max	K_i (F_{mut})	ED_{50}	% max
0.29 ± 0.04 (2.2)	0.7 ± 0.1	86	0.22 ± 0.09 (1.7)	7 ± 1	76	NB (ND)	160 ± 12	73	0.47 ± 0.14 (3.6)	11 ± 2	76
0.47 ± 0.03 (1.8)	0.9 ± 0.2	86	0.44 ± 0.14 (1.7)	15 ± 2	78	ND (ND)	178 ± 15	73	0.59 ± 0.07 (2.3)	15 ± 1	76
1.8 ± 0.2 (1.5)	1.6 ± 0.2	86	1.3 ± 0.3 (1.1)	3.9 ± 0.3	76	ND (ND)	>10,000	ND	4.2 ± 0.7 (3.5)	ND	20
142 ± 15 (5.9)	106 ± 5	84	39 ± 5 (1.6)	118 ± 10	73	ND (ND)	>10,000	ND	47 ± 3 (2)	199 ± 9	50

TABLE 2
Affinity (K_i), potency (ED_{50}), and efficacy (% maximum) of various ligands at wt and mutant ORL1[Q286A] and [Q286E] receptors, expressed in CHO cells. F_{mut}, Mutation factor. See legend to Table 1 for details.

Ligand	ORL1 Receptor									
	wt		Q286A				Q286E			
	K_i	ED_{50}	K_i	F_{mut}	ED_{50}	% max	K_i	F_{mut}	ED_{50}	% max
Noc	0.13 ± 0.03	0.8 ± 0.05	0.2 ± 0.1	1.5	>10,000	ND	0.26 ± 0.02	2.0	2.0 ± 0.3	84
[Tyr^1]Noc	0.26 ± 0.02	1.1 ± 0.04	0.25 ± 0.1	1.0	>10,000	ND	0.48 ± 0.1	1.8	1.7 ± 0.2	84
HP5	1.2 ± 0.06	0.7 ± 0.1	3.3 ± 0.3	2.8	>10,000	ND	2.0 ± 0.3	1.7	9.2 ± 2.6	86
Lofentanil	24 ± 2.5	7.2 ± 1.3	2.2 ± 0.3	0.09	>10,000	ND	14 ± 4	0.58	15 ± 2.3	86
Dyn	110	>10,000	16 ± 1.4	0.15	>10,000	ND	87 ± 3	0.8	>1,000	ND
Etorphine	530	460	39 ± 3	0.07	>10,000	ND	280 ± 20	0.52	ND	ND
nor-BNI	780	ND	750 ± 30	0.96	ND	ND	560 ± 40	0.71	ND	ND

including all cationic neurotransmitter receptors, where it is thought to participate in the binding of the ligand ammonium headgroup (reviewed by Trumpp-Kallmeyer et al., 1992, and Bikker et al., 1998). The aspartate residue equivalents in the opioid receptors have also been predicted to engage in ion-pair formation with the protonated N termini of opioid peptides (Surratt et al., 1994; Paterlini et al., 1997; Pogozheva et al., 1998), and, on the basis of a modeling study, we have proposed a similar electrostatic interaction between Asp^{130} and the N terminus of noc (Topham et al., 1998). In the case of the ORL1 receptor, the observation that the D130E mutant does not bind noc and is inactive (data not shown) suggests that were an intimate ion-pair to form between Asp^{130} and the positively charged N-terminal of noc, the presence of an extra carbon in the glutamate side chain is sufficient to disrupt the interaction. In contrast, there is no obvious steric impediment to hydrogen bond formation between the terminal NH function in the noc derivative *N*-acetyl-noc[1–13]-amide and the Asp^{130} carboxylate. Consistent with this, *N*-acetyl-noc[1–13]-amide remains able to stimulate the wt receptor, albeit with an increased ED_{50}. Removal of the hydrogen bond acceptor functionality in the D130A mutant leads to an inactive receptor (see Table 1). However, the isosteric D130N mutant, which possesses an uncharged bifunctional amide group, is unresponsive toward both noc and *N*-acetyl-noc[1–13]-amide (Table 1). Taken together, these results suggest that the presence of a negative charge at position 130 is a necessary but insufficient requirement for ORL1 receptor activation, and so the question of whether or not there is a direct interaction of Asp^{130} with the protonated N terminus of noc remains open.

In contrast to the results obtained here, Befort et al. (1996a) have provided evidence from a site-directed mutagenesis study that the aspartate residue in TM III of the δ-opioid receptor is not necessary for binding of peptide agonists, and can be replaced by an alanine without apparent effect. Although receptor activities were not reported, the authors conclude that the aspartate does not participate in direct Coulombic interactions. Thus although the exact roles and energetic contributions of the Asp residue in TM III may differ in the ORL1 receptor and the μ- (Surratt et al., 1994) and δ- (Befort et al., 1996a) opioid receptors, it is clear from our data that this residue is necessary for noc binding to the ORL1 receptor.

The present study has also identified transmembrane aromatic residues, Tyr^{131}, Phe^{220}, Phe^{224}, and Trp^{276}, as making important, although unequal contributions to binding and/or activation of the ORL1 receptor. They are conserved in the opiate binding pocket of μ-, δ-, and κ-opioid receptors (Paterlini et al., 1997; Pogozheva et al., 1998) and the analogous hydrophobic binding pocket of the ORL1 receptor where they interact with noc[1–4] (Topham et al., 1998). Most critical is Phe^{224} in TM V, whose replacement by alanine yields a practically inactive mutant receptor at which noc and [Tyr^1]noc showed ~200-fold reduced potency, and HP5 and lofentanil were inactive. Although less effective than mutation of Phe^{224}, alanine substitution of Tyr^{131}, Phe^{220}, and Trp^{276} caused a substantial reduction in affinity and/or potency of noc and [Tyr^1]noc. The finding that changing Tyr^{131} to phenylalanine acts to essentially restore receptor function, suggesting that a phenyl ring is required at this position for high noc affinity and potency. Modeling of the noc-ORL1 receptor complex suggests the possible formation of a hydrogen bond between the hydroxyl of Tyr^{131} and the peptide backbone carbonyl oxygen of Gly^3 (Topham et al., 1998). However, as is the case for other aromatic residues in the TM binding pocket, nonspecific interactions with the Phe^1 and Phe^4 side chains are also evident in the model, and favorable ligand desolvation effects are likely to contribute significantly to the free energy of binding. Alanine mutation of the equivalent aromatic residues in the mouse δ-opioid receptor (mDOR1), has been reported by Befort et al. (1996b). Their principal observations were that 1) mutation of residue Tyr^{129} (≡ Tyr^{131} in hORL1 receptor) markedly decreases affinity (receptor activity was not examined) for enkephalin and that this effect is partially reversed in the Y129F mutant receptor; 2) mutation of residues Phe^{222} (≡ Phe^{224}) and Trp^{274} (≡ Trp^{276}) produces mutant mDOR1 receptors with moderately decreased (5- to 10-fold) affinity for enkephalin; and 3) alanine mutation of mDOR1 residue Phe^{218} (≡ Phe^{220}) does not affect receptor affinity for enkephalin. With the notable exception of Phe^{222} (≡ Phe^{224}), alanine mutation of conserved transmembrane aromatic residues impairs the binding of the cognate ligands to the δ-opioid and ORL1 receptors in a similar way. Mutation of residue Phe^{224} is considerably more deleterious to the stability of the noc/ORL1 receptor complex than is Phe^{222} to the enkephalin/δ-opioid receptor system. This suggests that this conserved phenylalanine residue does not play exactly the same role in agonist binding and/or receptor activation. One interpretation is that the noc F^{1}GGF and enkephalin Y^{1}GGF tetrapeptides adopt alternative conformations when bound in their respective transmembrane hydrophobic pockets.

Two mutations, Y131A and W276A, differentially affect the properties of HP5 and lofentanil, compared with noc and [Tyr^1]noc. Mutation Y131A results in a decreased potency of

the four ligands, whereas leaving the binding of both HP5 and lofentanil essentially unchanged. This is perhaps not surprising in the case of HP5, because this peptide is expected to bind elsewhere in the receptor, most probably to e2 (Dooley et al., 1997). As for lofentanil, the disproportionate effect on activity compared with noc may be explained if the opiate employs a similar activation mechanism in the ORL1 receptor to that which pertains in opioid receptors. However, even more discriminatory is the W276A mutation. This yields a receptor at which HP5 exhibits antagonist, rather than agonist, properties. If, in view of its highly cationic nature, one accepts that HP5 does bind to the acidic e2 loop of the receptor, it can be surmised that Trp^{276} is involved in stabilizing a conformational change mediated at long range by interactions with e2, independent of whether the vestigial opioid ORL1 receptor binding pocket is occupied or not. In any event, these data indicate that residues Tyr^{131} and Trp^{276} play distinct roles in receptor complexes with noc and [Tyr^1]noc, HP5 and lofentanil, consistent with their lack of obvious structural similarity.

Finally, we have identified a mutant receptor, ORL1[Q286A], that binds strongly but does not respond to either noc, [Tyr^1]noc, HP5, or lofentanil. Thus, it may be described as functionally inactive or, in the context of allostery, desensitized (Monod et al., 1965). In the κ- and δ-opioid receptors, mutations of the residues equivalent to Gln^{286} in the hORL1 receptor, i.e., glutamate and tryptophan, respectively, have been shown to impair binding of κ-selective antagonists (Hjorth et al., 1995; Jones et al., 1998) and δ-selective agonists (Valiquette et al., 1996), respectively, but not of non-type-selective ligands. Although mutant receptor functionality was not examined in these studies, this residue position was concluded to be an important determinant of opioid receptor-type selectivity. Likewise, we observe that mutant ORL1[Q286A] receptor binds the opioids lofentanil, dyn, and etorphine with significantly higher affinity than does the wt receptor (see Table 2), suggesting that glutamine 286 may also play a role, albeit modest, in the ligand binding selectivity of the ORL1 receptor. Clearly, however, the major consequence of the Q286A mutation is functional inactivation of the receptor, indicating that residue Gln^{286} may play a pivotal role in receptor transduction of the nociceptin signal. Activation of GPCRs involves positive heterotropic, long-range interactions between the agonist- and G protein-binding sites, and there is a growing body of evidence that movements of transmembrane helix VI (F in rhodopsin) are critical in this process (Dunham and Farrens, 1999, and references therein). Within this structural context, Gln^{286} occupies a strategic position, at the C terminus of TM VI in the ORL1 receptor, where it might act as a switch in a noc-promoted, TM VI-mediated structural transition of the ORL1 receptor from resting to active state. Changing this residue for an alanine residue leaves the switch in the "off" position, resulting in functional inactivation. When Gln^{286} is replaced by a glutamate rather than an alanine residue, inactivation is no longer observed. These findings are consistent with the involvement of at least one hydrogen bond in selectively stabilizing the activated form of the wt receptor. The demonstration of partial or complete loss in receptor activity upon replacement of Gln^{286} by a shorter amide-bearing asparagine residue side chain would provide evidence in favor of hydrogen bond formation. Although modeling of the noc-ORL1 receptor complex (Topham et al., 1998) shows Gln^{286} to be close to the Thr^5 side chain of the peptide, intermolecular hydrogen bonding between Gln^{286} and the noc main chain, or intramolecular hydrogen bonding of Gln^{286} with another residue in the activated receptor, are equally feasible.

In conclusion, the present study has provided experimental evidence for the existence, in the ORL1 receptor, of a noc transmembrane binding pocket that is the topological equivalent of the opiate binding pocket in opioid receptors. Within this pocket are the conserved aspartate residue in TM III, and several conserved aromatic residues in TMs III, IV, and VI. However, not all of the conserved aromatic residues contribute in the same way to binding and/or activity of noc and opioids in the ORL1 and opioid receptor, respectively. Identification of the allosterically desensitized, functionally inactive mutant ORL1[Q286A] receptor raises the question whether alanine mutations at the equivalent residue position in the opioid receptors also lead to functional inactivation. A total loss in binding, or complete retention of biological activity, would provide firm evidence of different activation mechanisms operating in the two receptor systems (Mollereau et al., 1999). In the latter case, this would also delimit the extent of the agonist binding site in opioid receptors to the transmembrane region. With regard to the ORL1 receptor, the finding that Gln^{286} is able to stabilize an active receptor conformation at a position level with the membrane interface, but yet does not appear to contribute to cognate agonist intrinsic binding per se, lends further support to our belief (Mollereau et al., 1999) that the transmembrane region proper of an extended noc binding site is better suited as a template for antagonist as opposed to agonist design.

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