Conserved polar residues in the transmembrane domain of the human tachykinin NK₂ receptor: functional roles and structural implications

Dan DONNELLY^{*1,2}, Stuart MAUDSLEY^{*1,3}, J. Paul GENT^{*}, Rachel N. MOSER^{†4}, Craig R. HURRELL^{†5} and John B. C. FINDLAY[†]

^{*}School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, U.K., and [†]School of Biochemistry and Molecular Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, U.K.

We have studied the effects of agonist and antagonist binding, agonist-induced activation and agonist-induced desensitization of the human tachykinin NK₂ receptor mutated at polar residues Asn-51 [in transmembrane helix 1 (TM1)], Asp-79 (TM2) and Asn-303 (TM7), which are highly conserved in the transmembrane domain in the rhodopsin family of G-protein-coupled receptors. Wild-type and mutant receptors were expressed in both COS-1 cells and *Xenopus* oocytes. The results show that the N51D mutation results in a receptor which, in contrast with the wild-type receptor, is desensitized by the application of a concentration of 1 μ M of the partial agonist GR64349, indicating that the mutant is more sensitive to agonist activation than is the wild-type receptor. In addition, we show that, whereas the D79E mutant displayed activation properties similar to those of the wild-type receptor, the D79N and D79A mutants displayed a

severely impaired ability to activate the calcium-dependent chloride current. This suggests that it is the negative charge at Asn-79, rather than the ability of this residue to hydrogen-bond, that is critical for the activity of the receptor. Interestingly, the placement of a negative charge at position 303 could compensate for the removal of the negative charge at position 79, since the double mutant D79N/N303D displayed activation properties similar to those of the wild-type receptor. This suggests that these two residues are functionally coupled, and may even be in close proximity in the three-dimensional structure of the human tachykinin NK₂ receptor. A three-dimensional model of the receptor displaying this putative interaction is presented.

Key words: G-protein-coupled receptor, mutation, oocytes, three-dimensional model.

INTRODUCTION

The G-protein-coupled receptors (GPCRs) form a large superfamily of integral membrane proteins which mediate a multitude of signalling events throughout the body. The rhodopsin-like GPCRs are characterized by a number of highly conserved residues present in their seven-helix transmembrane domain, which include several polar residues (Asn, Asp and Arg) on TM1–TM3 and TM7 (where TM1 denotes transmembrane helix 1 etc.) that are proposed to form a ‘polar pocket’. These residues have been the subject of several studies which suggest that they play a critical role in mediating the extracellular signal through the bilayer to the G-protein on the intracellular side of the membrane (reviewed in [1]). A close proximity of the conserved Asp in TM2 and the conserved Asn in TM7 was proposed following a reciprocal mutation study in the gonadotropin-releasing hormone receptor (in which the Asp and Asn are naturally reversed), and further substantiated by the same groups in the 5-hydroxytryptamine (serotonin) 5HT_{2A} receptor [2,3]. However, since it is difficult to reconcile ‘two-dimensional’ projection models of GPCRs with these data (e.g. [4–6]), we set out both to verify this interaction in a separate receptor type and to interpret these data in the light of the latest structural information available for GPCRs. The role of these two conserved polar residues was also compared with that of the conserved Asn residue in TM1.

The human tachykinin NK₂ receptor (NK₂R) is a member of the rhodopsin family of GPCRs. The three mammalian tachykinin receptor subtypes each apparently show a preference for a

corresponding endogenous tachykinin peptide: NK₁R for substance P, NK₂R for neurokinin A (NKA) and NK₃R for neurokinin B (but for additional complexity, see [7]). The tachykinin NK₂R, like the other tachykinin receptor subtypes, displays an ability to stimulate intracellular signalling pathways involving phospholipase C, leading to phosphoinositide breakdown and elevation of intracellular calcium [8,9]. In the experiments reported here, the signalling behaviour and desensitization capability of NK₂R were studied using the *Xenopus laevis* oocyte heterologous expression system. In *Xenopus* oocytes, activation of the receptor and the resulting elevation of intracellular Ca²⁺ levels leads to the activation of an endogenous Ca²⁺-dependent chloride current [10–13] which can be readily measured using the two-electrode voltage-clamp technique [14,15]. Using this system, we have previously analysed in detail the effects of altering receptor expression levels, agonist concentrations and agonist efficacy upon agonist-induced desensitization using both the full agonist NKA and the lower-efficacy agonist GR64349 [16]. We demonstrated that, in this system, the threshold to agonist-induced desensitization is a sensitive measure of the extent of the signalling activity of the receptor, and we have gone on to exploit this property in this present work.

MATERIALS AND METHODS

Materials

NKA was obtained from Calbiochem. SR48968 was kindly donated by Sanofi Recherche. ¹²⁵I-NKA and [³H]SR48968 were

Abbreviations used: GPCR, G-protein-coupled receptor; NKA, neurokinin A; NK₂R, NK₂ receptor; TM1 (etc.), transmembrane helix 1 (etc.).

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail d.donnelly@leeds.ac.uk).

³ Present address: Duke University Medical Center, P.O. Box 3821, Room 468, CARL Building, Research Drive, Durham, NC 27710, U.S.A.

⁴ Present address: Celltech PLC, 216 Bath Road, Slough SL1 4EN, U.K.

⁵ Present address: Genosys Biotechnologies (Europe) Ltd., London Road, Pampisford, Cambs. CB2 4EF, U.K.

obtained from Dupont-NEN. Collagenase 1A and other basic chemical reagents were obtained from Sigma.

Vector constructs and RNA synthesis

A 1260 bp *Bam*HI/*Nde*I-restricted fragment containing the human NK₂R cDNA was introduced into the polylinker region of pEF-BOS using *Bst*XI adaptors [17]. Site-directed mutagenesis was carried out using this construct and confirmed by DNA sequencing as described previously in [17]. This pEF-BOS construct was used for the transfection of COS-1 cells and for the injection of oocytes with wild-type and mutant cDNAs. Mutant cDNAs were later subcloned, downstream of the T7 polymerase initiation site, into the pcDNA3 transcription vector (Invitrogen) to allow *in vitro* transcription of receptor cRNA primed with cap dinucleotide m⁷G(5')ppp(5')G using a MEGAscript RNA synthesis kit (Ambion).

COS-1 cell expression and ligand analysis

Wild-type and mutated cDNAs were introduced into COS-1 cells by lipofection using DOTAP[®] (Boehringer Mannheim). At 72 h post-transfection, inhibition of binding of 0.1 nM ¹²⁵I-NKA or 4 nM [³H]SR48968 was carried out using homologous competition binding assays [17]. Assays were performed at least three times in triplicate, and the data were analysed using GraphPAD Prism v2.0.

Electrophysiological recording

The preparation of *Xenopus* oocytes, their injection with receptor cRNA or cDNA and the subsequent electrophysiological recordings were all carried out exactly as described in [16]. Standard two-electrode voltage-clamp recordings were made from oocytes at 24 and 72 h post-injection of cRNA and cDNA respectively. Agonist-induced desensitization was assessed by measuring the ratio (R_2/R_1) of the amplitude of the first agonist-induced response (R_1) to that resulting from a second application 10 min after the first (R_2), with constant washing with buffer between agonist applications.

Molecular modelling

The three-dimensional C α -model of Baldwin et al. [18] was used as a template on which a model of the tachykinin NK₂R was built. Whereas the residues on TM1 and TM4 in this model were simply substituted with the corresponding NK₂R sequence (followed by the automatic building of the backbone and side chains using the tools within the molecular modelling software SYBYL v6.3), the remaining helices were positioned differently, although the helix-helix angles and spacing were maintained. TM7 was initially built as an ideal α -helix using SYBYL. The distortion predicted from Fourier-transform analysis at the bilayer interface on the extracellular end of this helix [5,19] was constructed interactively and the transmembrane region was then positioned on top of the equivalent helix in the rhodopsin template, in order that its predicted internal face was directed towards the centre of the helix bundle [5]. Unger et al. [20] observed a distortion in a similar location in the electron-density map of frog rhodopsin, and hence in our model the path of the helix on the N-terminal side of the distortion reflects this. The remaining helices (TM2, TM3, TM5 and TM6) were positioned relative to each other in order to minimize the distance between the C α atoms of the residues identified experimentally to be in

close proximity (by the introduction of zinc-binding sites in the closely related tachykinin NK₁R [21,22]) and to conform with the Fourier-transform predictions in [5]. In order to accurately reflect the observed distortion in the rhodopsin template at the extracellular/C-terminal end of TM6, this helix was built directly from the C α co-ordinates of the template, although the alignment was altered so that our model was moved by four residues (approximately one helical turn) towards the cytoplasmic side of the bilayer. The remaining transmembrane helices (TM2, TM3 and TM5) were built as ideal α -helices in SYBYL and positioned on top of the equivalent helices in the rhodopsin model template, in order to optimize the constraints outlined above. The model was energy-minimized in SYBYL using 2000 steps of conjugated gradient optimization with the Tripos forcefield in order to remove side-chain clashes.

RESULTS AND DISCUSSION

It might be expected that highly conserved residues, such as Asn-51, Asp-79 and Asn-303, are likely to be involved in functions common to all of the rhodopsin-like receptors, i.e. the maintenance of the structural integrity of the seven-helix bundle, the mediation of the conformational changes involved in receptor activation, or the interaction with intracellular G-proteins. However, this last role seems unlikely for these particular residues, since they appear to reside within the bilayer-protected region of the receptor. In order to investigate the role of these conserved residues in receptor structure and function, ten site-directed mutant receptors were constructed, verified by DNA sequencing and analysed for binding and signalling activities. Three highly conserved residues were targeted: Asn-51 was mutated to Ser, His and Asp; Asp-79 was mutated to Asn, Ala and Glu; and Asn-303 was mutated to Asp, Ala and Ser. In addition, a double mutant D79N/N303D was constructed.

Asn-51 mutants

The receptors resulting from point mutations at the Asn-51 locus could not be detected using either the ¹²⁵I-NKA or the [³H]SR48968 homologous competition binding assays after transfection of COS-1 cells. However, these mutant receptors surprisingly produced responses with wild-type-like amplitudes and desensitization properties when expressed in oocytes injected with cDNA, showing that they are correctly folded and translocated to the plasma membrane (Table 1). Further analysis of the Asn-51 receptor mutants is shown in Table 2. Using oocytes injected with cDNA, the Asn-51 mutants produced a similar degree of NKA-induced chloride-current activation as the wild-type receptor. Despite the inability of these mutants to detect [³H]SR48968 antagonist binding, the NKA-induced responses were, unexpectedly, almost completely abolished in the presence of 10 nM SR48968 (approx. $2 \times K_d$ of the wild-type NK₂R). Hence our functional data demonstrate that the Asn-51 mutations do not significantly affect the ligand-binding properties of the receptor, despite the inability to directly measure the affinities at the mutant receptors. One possible explanation for this, albeit indirect, is that the expression levels of the Asn-51 mutants may be reduced below the point at which they can be detected by radioligand binding, but not below the point at which they can be detected by the highly sensitive electrophysiological assay.

A major problem with using oocytes injected into the nucleus with cDNA is that the resultant high expression levels can mask subtle differences between the intrinsic activities of different agonists or between the activities of receptor variants. For example, we have shown previously that the partial agonist

Table 1 Estimated K_d and B_{max} values, chloride-current amplitudes and response ratios for wild-type and mutant NK₂Rs

WT, wild type NK₂R; mutant NK₂Rs are indicated using the one-letter amino acid code. ND, no binding detected. K_d and B_{max} values were estimated from homologous competition binding assays. Chloride-current amplitudes were determined following application of 100 nM NKA to oocytes that had been injected with 10 ng of cDNA. R_2/R_1 ratio refers to the ratio of the current amplitude resulting from a second application of 100 nM NKA to that resulting from a first application 10 min previously. All values are means $\pm$ S.E.M.; numbers in parentheses indicate the numbers of experiments carried out.

Receptor	K_d for [³ H]SR48968 (nM)	$10^{-6} \times B_{max}$ (receptors/cell)	Current amplitude (nA)	R_2/R_1 ratio
WT	4.5 $\pm$ 0.9 (7)	0.45 $\pm$ 0.01	1799 $\pm$ 193 (27)	0.13 $\pm$ 0.04 (27)
N51D	ND	—	1822 $\pm$ 405 (4)	0.03 $\pm$ 0.02 (4)
N51H	ND	—	2484 $\pm$ 467 (4)	0.08 $\pm$ 0.04 (4)
N51S	ND	—	1339 $\pm$ 681 (5)	0.17 $\pm$ 0.16 (5)
D79A	8.7 $\pm$ 3.4 (6)	0.40 $\pm$ 0.02	1205 $\pm$ 134 (33)	0.90 $\pm$ 0.10 (33)
D79E	5.6 $\pm$ 0.9 (4)	0.34 $\pm$ 0.10	2354 $\pm$ 526 (12)	0.15 $\pm$ 0.06 (12)
D79N	7.6 $\pm$ 1.2 (6)	0.35 $\pm$ 0.01	1258 $\pm$ 412 (15)	0.05 $\pm$ 0.02 (15)
N303A	7.1 $\pm$ 1.9 (6)	0.41 $\pm$ 0.02	1406 $\pm$ 702 (7)	0.07 $\pm$ 0.03 (7)
N303D	5.4 $\pm$ 1.7 (5)	0.28 $\pm$ 0.01	2732 $\pm$ 960 (4)	0.07 $\pm$ 0.04 (4)
N303S	5.1 $\pm$ 1.1 (7)	0.31 $\pm$ 0.08	1482 $\pm$ 378 (10)	0.05 $\pm$ 0.02 (10)

Table 2 Agonist-induced activation of wild-type and TM1 mutant receptors

Oocytes that had been injected with the amounts of cDNA or cRNA indicated were then treated with NKA or GR64349. ND, no current detected. All values are means $\pm$ S.E.M.; numbers in parentheses represent numbers of experiments. Significant differences compared with wild-type (WT): * P < 0.05; ** P < 0.01 (Student's t -test).

Receptor	1 ng of cDNA; 100 nM NKA		25 ng of cRNA; 1 μ M NKA		25 ng of cRNA; 1 μ M GR64349	
	Amplitude (nA)	Inhibition by 10 nM SR48968 (%)	Amplitude (nA)	R_2/R_1 ratio	Amplitude (nA)	R_2/R_1 ratio
WT	943 $\pm$ 133 (9)	80.3 $\pm$ 1.5 (3)	975 $\pm$ 150 (4)	0.07 $\pm$ 0.01	880 $\pm$ 130 (6)	0.93 $\pm$ 0.14
N51D	803 $\pm$ 149 (35)	93.1 $\pm$ 1.0 (6)	137 $\pm$ 16 (14)*	0.01 $\pm$ 0.00	128 $\pm$ 88 (5)**	0.12 $\pm$ 0.07**
N51H	979 $\pm$ 115 (11)	85.2 $\pm$ 0.6 (4)	160 $\pm$ 42 (10)**	0.46 $\pm$ 0.09*	141 $\pm$ 66 (4)*	0.91 $\pm$ 0.2
N51S	742 $\pm$ 189 (11)	95.8 $\pm$ 0.7 (6)	ND	ND	ND	ND

Figure 1 Typical examples of agonist-induced activation and desensitization of wild-type, N51H and N51D receptors expressed in oocytes

Each box shows the response obtained following two applications of agonist, to the same oocyte, separated by 10 min of washing. In oocytes injected with 25 ng of cRNA, the wild-type (WT) receptor was highly desensitized by the application of 1 μ M NKA (centre, top), but not by 1 μ M GR64349 (centre, bottom). In contrast, the N51H mutant was not desensitized by either agonist (left), while the N51D mutant was desensitized by both agonists (right). See Table 2 for full data.

GR64968 resembles the full agonist NKA in this cDNA-injected oocyte system [16]. However, at lower expression levels (produced by the injection of specific quantities cRNA into the cytoplasm),

the differences between the agonist efficacies are apparent. For example, using oocytes injected with 25 ng of cRNA, the wild-type receptor is typically resistant to GR64349-induced desensi-

Figure 2 Homologous competition binding curves using 0.1 nM ^{125}I -NKA as the radioligand

The data show that the substitution of Asn-303 with Ala, Asp or Ser, or the substitution of Asp-79 with Glu or Asn, had little effect upon the receptor's affinity for NKA. The IC_{50} for the wild-type NK_2R (wt) was 1.1 ± 0.4 nM. The mutant IC_{50} values were not significantly different (all fell within a factor of two), with mutant/wild-type ratios of: N303A, 1.45 ± 0.39 ; N303D, 1.08 ± 0.11 ; N303S, 0.63 ± 0.15 ; D79N, 1.42 ± 0.38 ; D79E, 0.80 ± 0.20 . No binding could be detected for N51S and N51H. Low levels of binding were occasionally detected for N51D and D79A, but reliable IC_{50} values could not be calculated from such data.

tization, whereas it is almost completely desensitized by NKA ([16,23]; Table 2). Since it was surprising that mutation of the highly conserved Asn-51 residue had such a minimal effect in cDNA-injected oocytes, we postulated that the apparent similarity between the agonist-induced activation and desensitization of the mutant and wild-type receptors may be a result of a masking of differences in receptor activity by overexpression. Therefore we went on to use cRNA-injected oocytes to analyse the mutant receptors further.

In oocytes injected with 25 ng of cRNA, the N51D and N51H mutants displayed significantly lower NKA-induced current amplitudes compared with the wild-type receptor, while current activation was undetectable for the N51S mutant (Table 2). The inability to detect N51S-mediated activity, even at saturating levels of NKA, was surprising, since this mutant receptor was clearly functional in cDNA-injected oocytes (Table 1) and hence was correctly folded in the plasma membrane. A possible explanation is that the decrease in expression levels resulting from the use of cRNA injection [16] may have resulted in an inability to achieve the threshold of activated receptors required for pathway stimulation, even at saturating agonist concentrations. Indeed, the reduced expression levels also affected the N51D and N51H mutants, albeit to a lesser extent, since the NKA-induced current amplitudes for the cRNA-injected oocytes were reduced by more than 10-fold compared with the cDNA-injected cells. In addition, the N51H mutant, expressed in oocytes injected with 25 ng of cRNA, displayed a significantly reduced ($P < 0.05$) NKA-induced desensitization compared with the wild-type receptor (Table 2; Figure 1). Although this may be the result of the decreased activity of the receptor, such an effect may also be a direct result of reduced expression levels compared with the wild-type receptor (as we have shown previously [16]). On the other hand, despite its likely lowered expression levels, the N51D mutant receptor was significantly desensitized ($P < 0.01$) by the partial agonist GR64349 (Table 2; Figure 1), and hence the

N51D mutation has apparently increased the activity of the receptor. Scheer et al. [24] have shown previously that the mutation of the equivalent conserved Asn residue to Ala in the $\alpha_{1\text{B}}$ -adrenergic receptor results in an increase in the activity of the receptor to the extent that it shows constitutive activity.

Asp-79 and Asn-303 mutants

In our experiments, all receptors resulting from single mutations at either Asp-79 or Asn-303 displayed both expression levels and [^3H]SR48968 antagonist-binding affinities similar to those of the wild-type receptor (Table 1). Hence, since antagonist binding was maintained, it seems likely that the overall structure of the protein had not been significantly altered by the mutations. The D79N and D79E mutants, as well as the three Asn-303 mutants, also displayed wild-type-like binding affinities for ^{125}I -NKA (Figure 2). In contrast, the D79A mutant receptor could not be easily detected in ^{125}I -NKA homologous binding assays, despite its wild-type-like expression levels and antagonist-binding properties, suggesting that the mutation had affected the affinity of the receptor for agonist. Huang et al. [25] have shown previously that the D79A mutation in NK_2R expressed in COS cells results in a 35-fold decrease in affinity for NKA (measured using a heterologous competition binding assay with [^3H]SR48968 as the radioligand), while the affinity for SR48969 was unaltered. Since it is unlikely that this residue is involved in direct interaction with the ligand, the decrease in agonist affinity may be the result of the reduced ability of the receptor to achieve its active form, i.e. the form that has highest agonist affinity [26]. Early studies on the equivalent Asp $\rightarrow$ Ala mutant in the β_2 -adrenergic receptor also showed a decrease in agonist binding affinity, with no significant affect on antagonist binding [27].

Surprisingly, in oocytes expressing receptors from cells injected with 10 ng of cDNA, all of the Asp-79 and Asn-303 mutant receptors displayed an agonist-induced current amplitude similar to that of the wild-type receptor upon the application of 100 nM NKA (Table 1). The ability of all the mutant receptors to undergo agonist-induced desensitization also resembled that of the wild-type receptor, with the exception of the D79A mutant, which was almost completely resistant to 100 nM-NKA-induced desensitization ($R_2/R_1 = 0.90 \pm 0.10$; $n = 33$; Table 1). However, this mutant was fully desensitized upon the application of 1 μM NKA, despite a similar current amplitude (amplitude = 1434 ± 542 nA; $R_2/R_1 = 0.08 \pm 0.02$; $n = 8$). Hence it would appear that the Asn-303 and Asp-79 mutations do not prevent the receptors from folding, binding agonist and activating their second-messenger system. Huang et al. [25] did not detect receptor activation for the D79A mutant expressed in COS cells using a total inositol phosphate assay; this discrepancy is probably due to the difference in the sensitivities of the two methods used. The requirement for a higher agonist concentration to produce wild-type-like desensitization for the D79A mutant probably reflects the reduced agonist affinity of this mutated receptor (discussed above).

Although all of the Asp-79 and Asn-303 mutants displayed wild-type-like current amplitudes and desensitization properties in oocytes injected with 10 ng of cDNA, the analysis of cRNA-injected oocytes revealed differences in both of these characteristics. The D79A and D79N mutant receptors displayed a marked decrease in the current amplitude induced by 100 nM NKA. In addition, it was noted that such agonist-induced responses failed to induce a significant degree of desensitization. On the other hand, 100 nM NKA produced desensitization and current amplitudes at the D79E mutant receptor that were both

Figure 3 Agonist-induced calcium-dependent chloride-channel activation in oocytes expressing wild-type and Asp-79 mutant receptors

The bar chart represents the full data, while the boxes display typical recordings from two applications of agonist separated by 10 min. Although the D79E mutant resembled the wild-type (WT) receptor, the activation of the D79A and D79N mutants resulted in a greater-than-10-fold lower maximal current amplitude. These data are derived from oocytes injected with 25 ng of cRNA and challenged with 100 nM NKA. * Significantly different from WT at $P < 0.05$ (Student's *t*-test).

Figure 4 Agonist-induced calcium-dependent chloride-channel activation in oocytes expressing wild-type and Asn-303 mutant receptors

The bar chart represents the full data, while the boxes display typical recordings from two applications of agonist separated by 10 min. Although the N303D mutant resembled the wild-type (WT) receptor, the N303A and N303S mutants showed greater than 10-fold decreases in the maximal current amplitude. These data are derived from oocytes injected with 25 ng of cRNA and challenged with 100 nM NKA. *Significantly different from WT at $P < 0.05$ (Student's *t*-test).

similar to those of the wild-type receptor (Figure 3). The wild-type-like characteristics of the D79E mutant were not surprising, since the closely related tachykinin NK₁R has Glu at the equivalent site. The results show that a negative charge at this position is critical for receptor signalling activity, but not for high-affinity agonist or antagonist binding, and hence not for correct receptor expression and folding. Our data agree with a previous study with the 5HT_{2A} receptor, which showed that the substitution of the TM2 Asp residue by either Asn or Ala resulted in uncoupling of the receptor from its second-messenger system, while having minimal effects upon ligand-binding affinities [3]. Chung et al. [28], however, reported a decrease in both

Figure 5 Agonist-induced calcium-dependent chloride-channel activation and desensitization in oocytes expressing wild-type, Asp-79, Asn-303 and D79N/N303D mutant receptors

The bar charts represent the full data, while the boxes display typical recordings from two applications of agonist separated by 10 min. Although the removal of the negative charge at Asp-79 severely impaired the agonist-induced activation of the receptor, the additional placement of a negative charge at Asn-303 restored the signalling capability. In addition, the ability of the double-mutant receptor to undergo agonist-induced desensitization was restored.

the agonist affinity and second-messenger turnover due to the Asp → Asn mutation in the β -adrenergic receptor.

The properties of the three Asn-303 mutant receptors also displayed differences when cRNA-injected oocytes were analysed (Figure 4). Whereas N303D displayed wild-type-like activation and desensitization properties, the N303A and N303S mutants displayed marked decreases in agonist-induced current amplitudes and the ability to be desensitized by NKA. Hence the carbonyl group at this position may be the moiety that is essential for full receptor activity. Previous studies involving mutation of this TM7 Asn residue in both the β_2 -adrenergic and the 5HT_{2A} receptors have also shown that its substitution by Ala severely impairs the ability of the receptor to activate the second-messenger system, while the more conservative substitution by Asp has little significant effect [3,29]. In the case of the β_2 -adrenergic receptor, the Asn → Ala mutation also impairs high-affinity binding, which is not observed in either the 5HT_{2A} receptor or the NK₂R. Hunyady et al. [30] have also shown that the Asn → Ala mutation in the AT₁ angiotensin receptor results in impaired G-protein coupling.

Summary of results from single mutants

The agonist- and antagonist-binding assays, coupled with the electrophysiological data from the cDNA-injected oocytes, suggest that all nine single-mutant receptors are expressed, maintain antagonist- and agonist-binding affinities and are therefore structurally intact. Hence these conserved residues are not essential for maintaining the structural integrity of GPCRs. However, it can be inferred, although not directly confirmed, that the Asn-51 mutants are expressed at a lower level than the wild-type receptor, and that the N51D mutant displays an elevated sensitivity towards agonist-induced desensitization. In addition, the D79A mutant has reduced agonist affinity, while the negative charge at Asp-79 and the carbonyl group at Asn-303 are required for correct agonist-induced transmembrane signalling. Since these conserved polar residues appear not to be

Figure 6 Schematic diagrams of the transmembrane domain of the human tachykinin NK₂R

(a) A two-dimensional projection model of a typical rhodopsin-like GPCR showing how Asp-79 (D₇₉) cannot easily interact with Asn-303 (N₃₀₃), since the necessity to place the position equivalent to the retinal-binding lysine of rhodopsin ('K') on the inside face of the helix results in Asn-303 facing TM6. (b) If the relative tilt between TM2 and TM7 is considered, it is possible that Asp-79 can interact with Asn-303 below the cross-over point of the helices, while the 'K' position interacts with TM2 above the cross-over.

essential for agonist or antagonist affinity, showing that the mutant receptors are structurally intact, these residues clearly play a critical role in the ability of the receptor to adopt the conformation required for G-protein activation.

TM2/TM7 double mutant

The decreases in both current amplitude and agonist-induced desensitization resulting from the replacement of Asp-79 by Asn could be reversed by the additional substitution of Asn-303 with Asp (Figure 5). The functional coupling of these two residues has been demonstrated previously in the mouse gonadotropin-releasing hormone receptor [2] although, surprisingly, no regeneration of binding or signalling activity for the equivalent mutations in the rat gonadotropin-releasing hormone receptor was observed [31]. Further evidence of this functional coupling has been observed in the 5HT_{2A} receptor [3], and now in the human NK₂R in the present work. Indeed, the correlation between the phenotypes of the equivalent mutant NK₂ and 5HT_{1A} receptors is remarkable, considering their diverse nature. The simplest explanation for such functional coupling is a direct interaction between the side chains of the two residues. Alter-

natively, the functional coupling could be mediated through water molecules, a sodium ion or another side chain, such as that of the highly conserved Arg residue which forms part of the Asp-Arg-Tyr ('DRY') motif at the cytoplasmic end of TM3 [1].

A direct interaction between Asp-79 and Asn-303 appeared to be unlikely in our previous two-dimensional 'projection' models, and also in the three-dimensional models of rhodopsin-like receptors [4,5,19,32]. Analysis of transmembrane helices often involves the analysis of helical wheels and the assumption that neighbouring helices are more or less parallel. Using such assumptions, it would appear that Asp-79 and Asn-303 cannot directly interact, since the necessity to place the position equivalent to the retinal-binding lysine of rhodopsin ('K' in Figure 6) on the internal face of TM7 results in Asn-303 pointing towards TM6 and away from TM2 (Figure 6a). Confidence in the placement of the retinal-binding lysine at this location is increased when the data suggesting the close proximity of this site in rhodopsin to both the Glu-113 counter-ion on TM3 and Gly-90 on TM2 are considered [33]. At first sight, then, it appears that the proposed Asp-Asn interaction cannot be satisfied without distorting either TM2 and/or TM7. However, if the relative tilts of the helices are taken into account, it can be seen that the

Figure 7 Extracellular (a) and in-membrane (b) views of the three-dimensional model of the human tachykinin NK₂R

The NK₂R is represented as a solid ribbon, with Asn-51, Asp-79 and Asn-303 shown in ball-and-stick representation. For clarity, only TM2 and TM7 are shown in (b).

internal face of TM2 can interact with one face of TM7 in the cytoplasmic half of the receptor, while interacting with the opposite face in the extracellular half (Figure 6b). This makes it possible to allow an interaction between Asp-79 and Asn-303 without either distorting the helices or compromising the internal/TM2-facing location of the retinal-binding position on TM7 of rhodopsin.

In the light of the latest direct structural data from frog rhodopsin, which show that several of the helices are indeed highly tilted [20], an updated generic molecular model of the rhodopsin-like receptors was built in order to investigate the possibility of a direct interaction between Asp-79 and Asn-303. The helix-helix angles and distances were derived directly from the experimentally based C α -model of Baldwin et al. [18], although the precise placement of the residues within the helices generally differed. The analysis of the orientation of the transmembrane helices using the Fourier-transform-based algorithms encoded in the computer program PERSCAN v7.0 [5,34] was used to aid the placement of the helices, while maximal consideration was given to the satisfying the experimentally determined distance constraints derived from the introduction of zinc-binding sites into the related tachykinin NK₁R [21,22]. The final model was also compatible with other experimentally estimated distance constraints derived from mutagenesis and ligand-binding analyses [33,35,36]. Figure 7 shows two views of the final three-dimensional model which highlight the close proximity of Asp-79 and Asn-303 (C α -C α distance = 8.1 Å; closest atom-atom distance = 3.8 Å) and show how a direct interaction is possible within the context of this receptor model (the co-ordinates of this and related models can be found on the EMBL server at <http://swift.embl-heidelberg.de/7tm/models/>). These data show the limitations of 'two-dimensional' models of transmembrane proteins, which assume neighbouring helices to be approximately parallel, and highlight the need for firm experimental data: both direct, such as the electron-density map of frog rhodopsin, and indirect, such as the data from the mutagenesis experiments described here.

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