Contribution of the second transmembrane helix of the secretin receptor to the positioning of secretin

Emmanuel Di Paolo[a,b], Philippe De Neef[a], Nicole Moguilevsky[b], Han Petry[b], Alex Bollen[b], Magali Waelbroeck[a], Patrick Robberecht[a,*]

[a] *Department of Biochemistry and Nutrition, Faculty of Medicine, Nivelles, Université Libre de Bruxelles, Building G/E, CP 611, 808 Route de Lennik, B-1070 Brussels, Belgium*
[b] *Applied Genetics, Faculty of Sciences, Nivelles, Université Libre de Bruxelles, Brussels, Belgium*

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Abstract The secretin amino-terminal residues are essential for high affinity binding to its cognate receptor and for its biological activity. Mutation of the [Asp^3] residue of secretin to [Asn^3] decreased the ligand's affinity for the rat wild-type receptor 100–300-fold. Receptor mutations in the transmembrane 2 domain and the beginning of the first extracellular loop allowed the identification of three residues involved in recognition of the [Asp^3] residue: D174, K173 and R166. Mutation of K173 and D174 not only reduced the secretin and [Asn^3]secretin affinities, but also changed the receptor's selectivity as judged by a decreased secretin and [Asn^3]secretin potency ratio. The most striking effect was observed when R166 was mutated to Q, D or L. This led to receptors with a very low affinity for secretin but an up to 10-fold higher affinity than the wild-type receptor for [Asn^3]secretin. This suggested that R166, highly conserved in that subgroup of receptor, is a major determinant for the recognition of the [Asp^3] of the ligand.

Key words: Secretin receptor; Mutagenesis; Secretin analogue

1. Introduction

The secretin receptor [1] belongs to a subfamily within the G protein-coupled receptors that includes the PACAP I [2], the VIP_1/PACAP [3], the Vip_2/PACAP [4], the glucagon [5], the glucagon-like peptide I [6], the growth hormone-releasing hormone [7], the gastric inhibitory peptide [8], the parathyroid [9], the calcitonin [10], and the corticotrophin-releasing factor [11] receptors. There is a high overall sequence homology in the transmembrane domains of these receptors, and a general model – different from that of the rhodopsin-like receptor family – for the positioning of these helices has been recently proposed [12].

The amino-terminal extracellular receptor sequence is rather long (120 amino acid residues) and is involved in selective ligand recognition [13,14]. In the case of the secretin receptor, this domain recognizes the central [15] and carboxy-terminal parts [16] of the ligand.

The amino-terminus of secretin, but also of VIP, PACAP and GLP-1, is absolutely required for a high affinity interaction with the cognate receptor as well as for a full expression of the biological activity [17–20]. Furthermore, at least for secretin, VIP and PACAP, the presence of the lateral chain of the aspartate in position 3 is necessary for high affinity interactions with the receptors. Indeed, replacement of [Asp^3] by [Glu^3] or [Asn^3] in secretin, VIP and PACAP markedly decreases the ligands' affinities for their cognate receptors. It is thus of interest to identify the receptor's amino acid residues that interact with this key residue.

We previously showed that one of the secretin amino-terminal moiety anchoring points was a lysine residue, [Lys^{173}], located at the end of the second transmembrane helix or at the beginning of the first exoloop of the receptor [21]. In the present work, we investigated the role of amino acids located in the vicinity of [Lys^{173}] (Fig. 1) and found that a highly conserved arginine residue [Arg^{166}], located within the second transmembrane helix, is crucial for the recognition of the [Asp^3] residue of secretin.

2. Materials and methods

The study was performed on the rat secretin receptor.

The products used for recombinant DNA expression and cell cultures have been listed in previous papers [17,21,22]. The mutant receptors N170→A, D174→V, D174→N, K173→I and K173→Q were prepared by replacement of a *Bss*HII-*Bsr*GI fragment with a synthetic duplex constructed from four oligonucleotides encoding the sequence of the mutant secretin receptor. The duplexes were ligated with a *Bsr*GI-*Nhe*I fragment and *Bss*HII-*Nhe*I pUC19/secretin receptor vector. The mutant receptors R166→L, R166→D and R166→Q were constructed as follows: a polymerase chain reaction fragment was prepared using a primer that encoded the sequence for the mutation and for the *Msl*I restriction site, and a second primer that encoded the sequence for the *Bbs*I restriction site. This PCR fragment was inserted into the TOPO vector (TOPO cloning kit, Invitrogen) and sequenced. Then, the *Msl*I-*Bbs*I fragment was obtained and ligated with the *Hin*dIII-*Msl*I secretin receptor fragment and *Hin*dIII-*Bbs*I pUC19/secretin receptor vector.

The mutant cDNAs were then introduced into pRc/RSV mammalian expression vector. The recombinant genes were identified by restriction endonuclease mapping and by sequencing. The plasmids were transfected in Chinese hamster ovary (CHO) cells and the cells expressing the protein were selected first by their resistance to geneticin, then for their capacity to increase adenylate cyclase activity in response to secretin or secretin analogues. Cell cultures, membrane preparation and adenylate cyclase activity determinations have been previously detailed [17].

Secretin – with an aspartate in position 3 – and [Asn^3]secretin were synthesized by solid phase methodology and their conformity and purity evaluated by capillary electrophoresis, amino acid sequencing and electrospray mass spectrometry.

3. Results and discussion

The importance of the carboxylic function of [Asp^3] in the

*Corresponding author. Fax: (32) (2) 555.62.30.
E-mail: probbe@ulb.ac.be

Abbreviations: Sn, secretin; Sn-R, secretin receptor; VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase activating polypeptide; GLP-1, glucagon-like peptide 1

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secretin molecule for its receptor recognition was deduced from the observation that [Asn^3]secretin was 100-fold less potent than secretin for secretin receptor occupancy and adenylate cyclase activation (Fig. 2, Table 1, [17]). We thus hypothesized that receptor mutants that resulted in the expression of a protein with a reduced capacity to discriminate secretin from [Asn^3]secretin would give indications on the residues involved directly or indirectly with the carboxylic function recognition. We anticipated that the relevant mutant receptors should display a decreased affinity for secretin – a rather unfavorable condition for binding studies – and therefore evaluated the receptors' properties by their capacity to stimulate adenylate cyclase activity.

We previously demonstrated [17] that the secretin K_{act} value is equivalent to its binding K_d value only at low receptor expression levels: if the receptor concentration is too high, the agonists' apparent affinity can be significantly overestimated in functional studies due to the presence of spare receptors. We were careful to chose a wild-type receptor clone expressing a low receptor density ($EC_{50} = K_d$). For each mutant receptor we studied at least four different clones and detailed those with the highest EC_{50} value and the lowest maximal stimulatory effect in the presence of secretin, assuming that these clones were expressing the lowest receptor density and thus had EC_{50} values as close as possible to the receptor occupancy K_d values. We nevertheless cannot exclude the hypothesis that some of the clones expressing mutant receptors expressed spare receptors, and that the deleterious effect of these mutations on secretin recognition was underestimated. We therefore chose to compare the ratio of the secretin/[Asn^3]secretin EC_{50} values to evaluate the effect of the receptor mutations in the recognition of the aspartate at position 3 of secretin: spare receptors do not affect the EC_{50} value ratio of full agonists.

We first confirmed that the K173→I mutated secretin receptor [21] had a reduced affinity for secretin and a reduced capacity to discriminate secretin from [Asn^3]secretin and that similar results were obtained for the K173→Q mutation

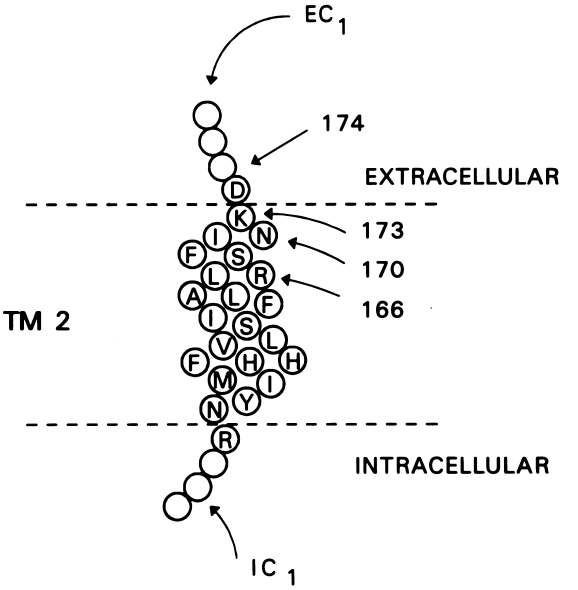

Fig. 1. Schematic representation of a possible alignment [24] of the TM 2 of the rat secretin receptor. The arrows indicate the amino acid residues that were mutated.

(Fig. 3, Table 1). We then mutated the polar amino acid residues that were assumed to be located in the second transmembrane domain on the same face of the helix [12,23,24] and Fig. 1), namely N170 and R166, as well as D174, which is adjacent to K173 in the first extracellular loop.

Mutation of D174→N markedly reduced the receptor's affinity for both secretin and [Asn^3]secretin, and also the ratio between the K_{act} values of secretin and [Asn^3]secretin (Fig. 3 and Table 1). Mutation of D174→A could not be investigated as receptor expression could not be detected among 48 clones transfected with the construction and resistant to geneticin (data not shown). D174 is a highly conserved residue in this family of receptors and was also found to be essential

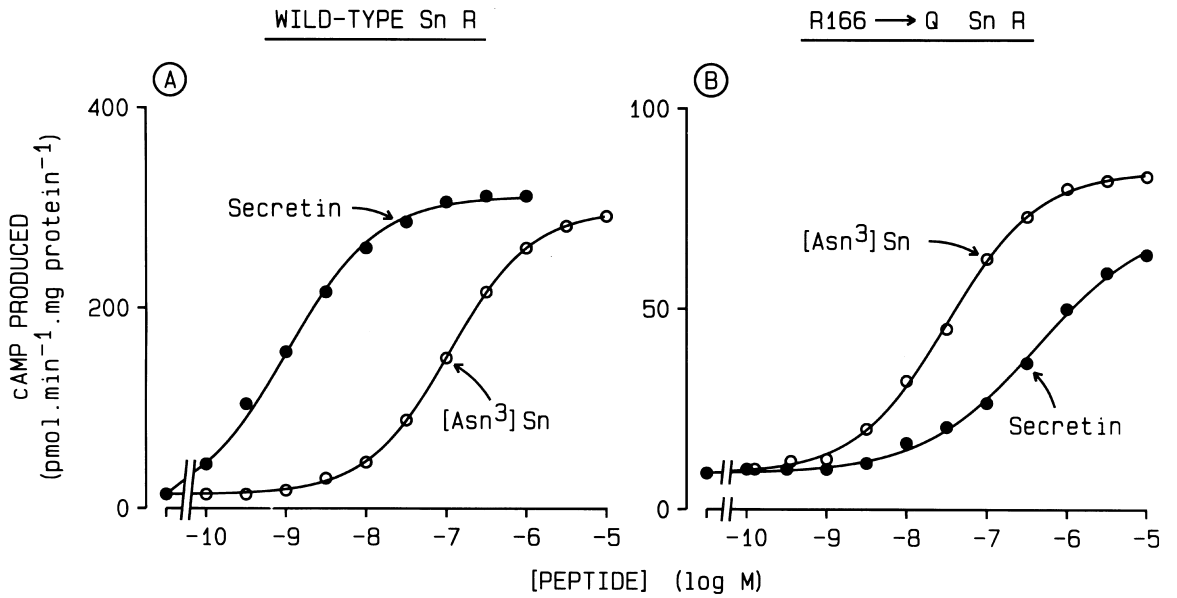

Fig. 2. Dose-response curves of adenylate cyclase activation in the presence of secretin (●) or [Asn^3]secretin (○) on membranes from CHO cells expressing the rat secretin receptor wild-type (left panel) or mutated R166→Q (right panel). The results are the mean of three determinations.

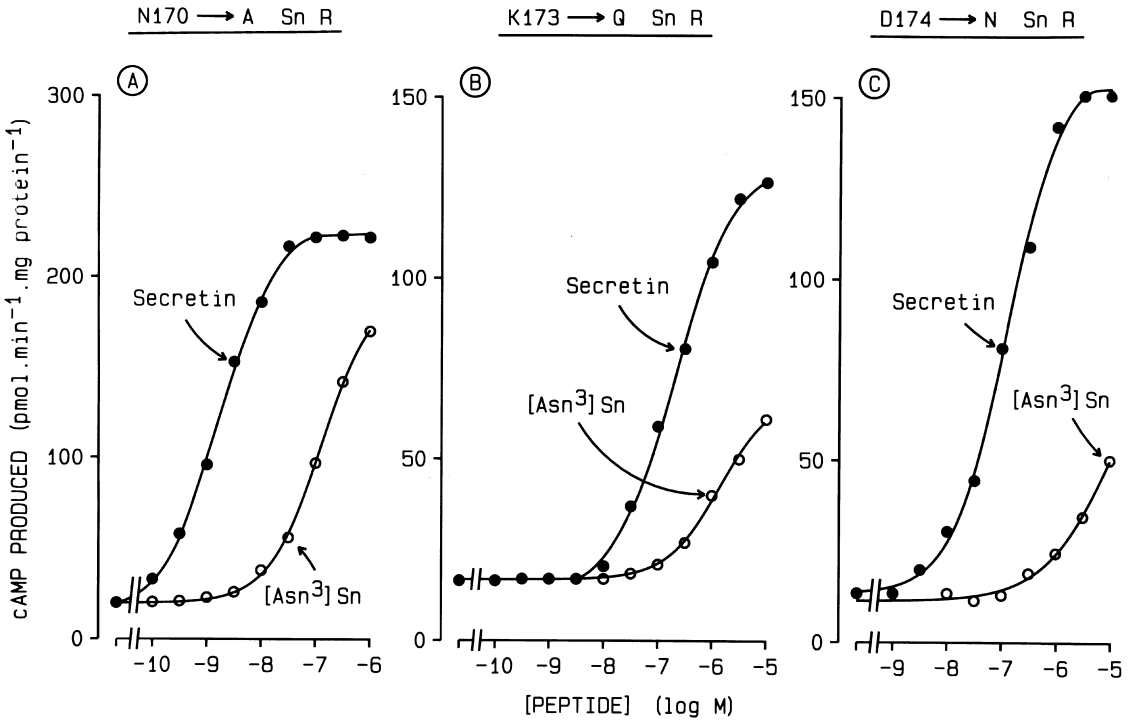

Fig. 3. Dose-response curves of adenylate cyclase activation in the presence of secretin (●) or [Asn^3]secretin (○) on membranes from CHO cells expressing three mutated secretin receptors: the N170→A (left panel), K173→Q (middle panel) and D174→N (right panel) secretin receptors.

for high affinity VIP binding on the VIP$_1$/PACAP receptor [25]. This residue might be more important for the receptor structure than for a precise contact between the ligand and the receptor.

Mutation of N170→A affected neither the receptor affinity nor its capacity to discriminate secretin from [Asn^3]secretin (Fig. 3 and Table 1). Similar results (not shown) were obtained with the N170→V mutant receptor.

The most spectacular change in receptor selectivity was observed for the mutation of residue R166 to Q, L or D: secretin was 500–1000-fold less potent on the mutated than on the wild-type receptor (Fig. 2 and Table 1), whereas [Asn^3]secretin's potency was unchanged or increased 10-fold. Thus, the suppression of the positive charge in position 166 was highly unfavorable for secretin recognition but not for [Asn^3]secretin recognition. We in fact observed lower EC$_{50}$ values when investigating the effect of [Asn^3]secretin on the R166→L and R166→Q mutants. These results might be explained by two hypotheses: (1) spare receptors might be present in the clones studied (see above); (2) it is also possible that [Asn^3]secretin (and secretin) recognizes slightly different binding sites in the wild-type and mutant receptors. The difference must, however, be subtle since both mutant receptors were capable of activating the adenylate cyclase in response to agonists: we know that the proper recognition of the amino acids at positions 1–4 of secretin by the receptor is essential for receptor activation [17].

Arginine^{166} is an excellent candidate for a precise contact point between the ligand and the receptors: (a) this charged residue is conserved in the whole family of receptors except in the calcitonin receptor; (b) receptor modeling suggests it to be rather accessible as its side chain is facing an internal pocket limited by TM 2, 3, 4, 5, 6 and 7, TM 1 being more largely exposed to the lipid bilayer [12].

Table 1
EC$_{50}$ values for wild-type and mutant secretin receptors

Secretin receptor	EC$_{50}$(nM)		
	Secretin	[Asn^3]secretin	N^3/D^3
Wild-type	2	200	100
K173→I	16	200	13
K173→Q	200	900	5
D174→N	125	3800	30
N170→A	2	200	100
R166→Q	1000	30	0.03
R166→L[a]	400	50	0.125
R166→D[a]	1500	300	0.2

EC$_{50}$ values were obtained from dose-response curves of adenylate cyclase activation and established using the Ligand program. Each value was the mean of three determinations. The S.E.M. values were below 0.1 concentration logarithm units. N^3/D^3 indicates the ratio between the [Asn^3]secretin and secretin EC$_{50}$ values.
[a]Indicates that the maximal effect of secretin was lower than that of [Asn^3]secretin.

As the residue is buried relatively deeply in the transmembrane domain the present results suggest that the peptide ligand, like the small aminergic ligands, may enter into the receptor to change receptor conformation. The precise localization of secretin on its receptor is far from completely established. Considering the large similarities between the secretin, VIP and PACAP receptors, converging studies of different research groups have identified critical regions for peptide binding. The N-terminal domain and particularly residues 1–10 [26], the C-terminus of the first extracellular loop [21] and four residues in the N-terminal half of the second extracellular loop are implicated in ligand recognition and discrimination [27]. The present data demonstrate that the carboxy-terminal moiety of TM 2 is a major binding site for the amino-terminal sequence of secretin. In view of the conservation of this receptor region in the secretin receptor family, the homologous amino acids probably have the same importance for other ligand-receptor interactions.

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