1. Introduction

1.1 Signal transduction

During the evolution of multicellular organisms, highly complex and integrative communication networks have evolved to coordinate growth, differentiation and metabolism of cells. Extracellular signalling molecules play a key role in these communication mechanisms and are produced by cells to signal to their neighbours or to cells further away. In higher animals these signal molecules include amino acids, small peptides, proteins, nucleotides, steroids, retinoids, fatty acid derivates, dissolved gasses such as nitric oxide and carbon monoxide. The majority of these signal molecules are secreted from the signalling cell into the extracellular space by exocytosis. It is essential that each cell contains an elaborate system of proteins in order to respond to these signals and react in a cell-specific way. Among these proteins are cell surface receptors, which bind signal molecules, thereby transducing extracellular signals to a variety of intracellular signalling proteins, such as kinases, GTP-binding proteins, and phosphatases that distribute signals to appropriate parts of the cells. At the end of each intracellular signalling cascade are effector proteins, which are altered when the signal transduction pathway is active and change the behaviour of the cell.

1.2 G protein-coupled receptors

Receptors are macromolecules that transmit extracellular regulatory signals to the cell interior, thus modulating cell activity and function. Three major types of plasma membrane receptors are known,

a) receptors that interact with G proteins (G protein-coupled receptors),

b) ligand-gated ion channels (e.g. nicotinic cholinergic receptor), and

c) receptors with enzymatic activity (e.g. insulin receptor).

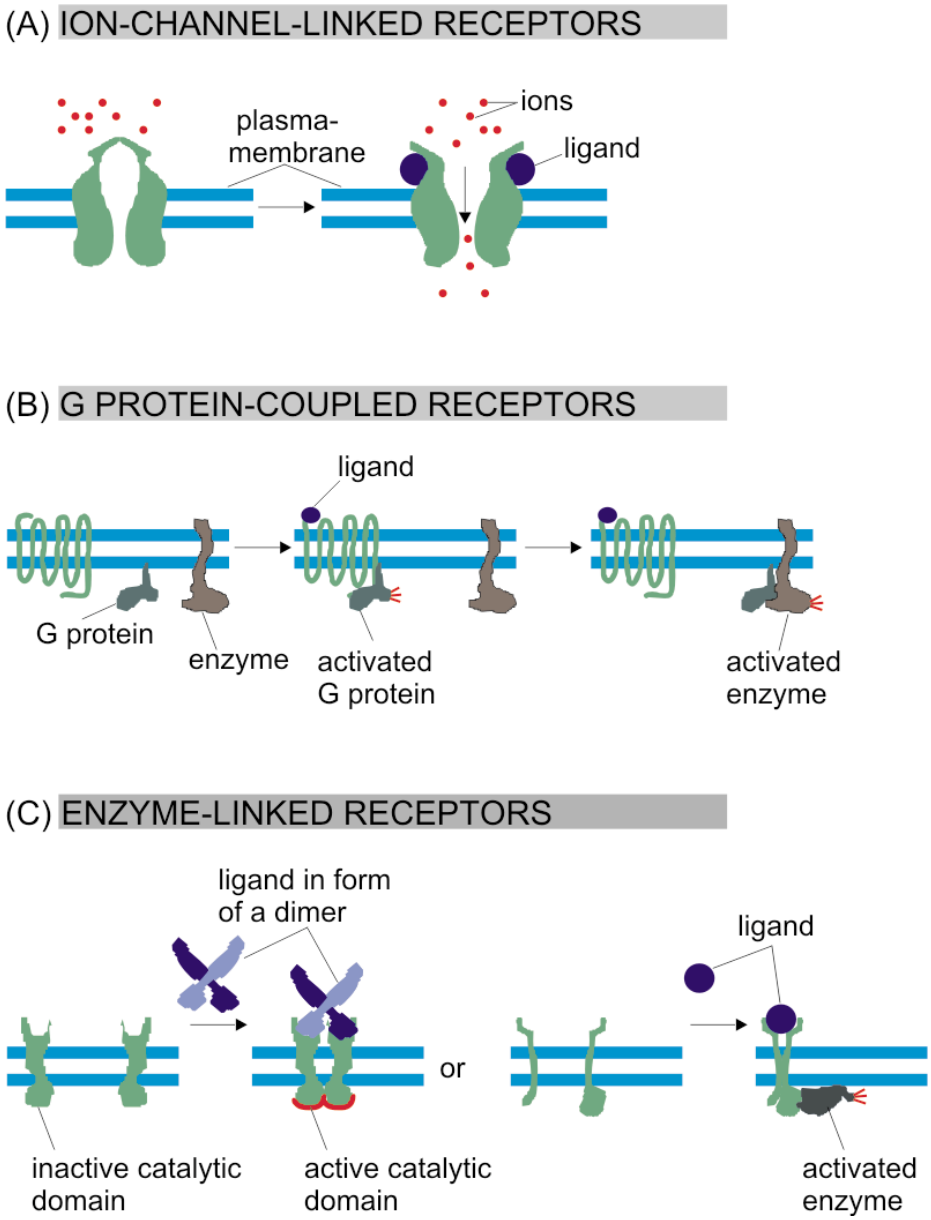

Fig.1. **Three classes of cell-surface receptors.** Scheme showing the three main classes of cell surface receptors. In all cases the binding of the ligand induces a conformational change leading to the activation of the receptor. In A) ligand binding causes opening of an ion channel. In B) the activated receptor recruits a G protein which itself activates a membrane linked enzyme. In C) the activated receptor induces the intrinsic enzyme activity or leads to the activation of an associated enzyme (modified after Alberts et al., 2002).

G protein-coupled receptors (GPCRs) form the largest class of cell-surface receptors. They are widely expressed among the different organisms and are certainly one of the oldest devices devoted to signal transduction. They are present in plants (Plakidou-Dymock et al. 1998), yeast (Dohlman et al. 1991), the slime mold (*Dictyostelium discoideum*) (Devreotes 1994), as well as in protozoa and the earliest diploblastic metazoa (Vernier et al. 1995; New et al. 1998). In vertebrates for example, this family contains 1000-2000 members (Bockaert et al. 1999). Sequence analysis suggests that the human genome contains about 600-700 distinct GPCR genes (Venter et al. 2001).

Classification of GPCRs. All GPCRs have a common central core domain consisting of seven transmembrane helices, connected *via* three intracellular and three extracellular loops (Baldwin 1993). According to sequence data GPCRs are divided into three main receptor families, which share no sequence similarity. This might indicate that they are phylogenetically unrelated and that the similarity of their transmembrane structure be only to fulfil common functional requirements (George et al. 2002).

Family A or the rhodopsin-like family. It is the largest family, which consists of receptors for odorants, small molecules like catecholamines and amines, some peptides and glycoprotein hormones. Family A receptors are further divided into three subfamilies. Subfamily 1a includes receptors for small ligands like the rhodopsin and the β–adrenergic receptors. The binding site is localised within the seven transmembrane domains. Subfamily 1b consists of receptors for peptides where binding sites are localised within the N-terminus, the extracellular loops and the superior parts of the transmembrane domains. Subfamily 1c includes GPCRs for glycoprotein hormones. They have a large extracellular domain and a binding site, which is mostly extracellular. Furthermore family A receptors are characterised by several highly conserved amino acids and a disulphide bridge which connects the first and second extracellular loops, which could have a role in stabilising the conformation of the seven transmembrane helices of GPCRs (Bockaert and Pin 1999). The majority of the receptors have one or multiple palmitoylated cysteins in the

carboxy-terminal tail. The determination of the crystal structure of rhodopsin indicated that the transmembrane domains of family A receptors are 'tilted' and 'kinked' as shown in figure 3 (Palczewski et al. 2000).

Family B. The receptors are characterised by a long amino terminus that contains several cysteins. Presumably these cysteins form a network of disulphide bridges. The general structure of the family B receptors display similarities to some family A receptors, in particular to members of the subfamily 1c. However they do not share any sequence homology. Family B receptors also contain a disulphide bridge that connects the first and second extracellular loop, but do not display a palmitoylation site in the C terminus and the conserved prolines differ from those of the family A receptors. The DRY (aspartic acid, arginine, tyrosine) motif, which is adjacent to the third transmembrane domain in family A receptors is also absent in family B receptors. How the transmembrane domains are orientated is relatively unknown. When taken into consideration that the amino acid sequence is quite divergent, it is likely that the orientation is very dissimilar from that of rhodopsin.

Family C. Family C receptors include the Ca^{2+}-sensing, the γ-aminobutyric acid ($GABA_B$) and the metabotropic glutamate receptors. The amino terminus and the carboxyl tail in this receptor family are long. Ligand binding occurs within the N terminus. According to its structure this N terminus is described as being like a 'Venus fly trap'. Two cysteins form a putative disulphide bridge between the first and second extracelluar loop. Apart from this similarity to family A and family B receptors, family C receptors do not have any of the key features that characterise the other families. Unique among the family C receptors is the third intracellular loop, which is short and highly conserved. The structure of the amino terminus, similar to family B receptors, is well characterised, but little is known about the orientation of the transmembrane domains.

Receptor activation. The binding of a ligand induces a change in receptor conformation, which is responsible for receptor activation. When activated

GPCRs recruit heterotrimeric G proteins, which are composed of three protein subunits- α, β, and γ, and catalyse the GDP-GTP exchange on the α subunit of the G protein. This exchange causes the dissociation of the trimeric complex in an α–subunit and a βγ–complex, both of which can regulate target proteins in the plasma membrane, i.e. controlling the activity of enzymes, ion channels and the transport of vesicles.

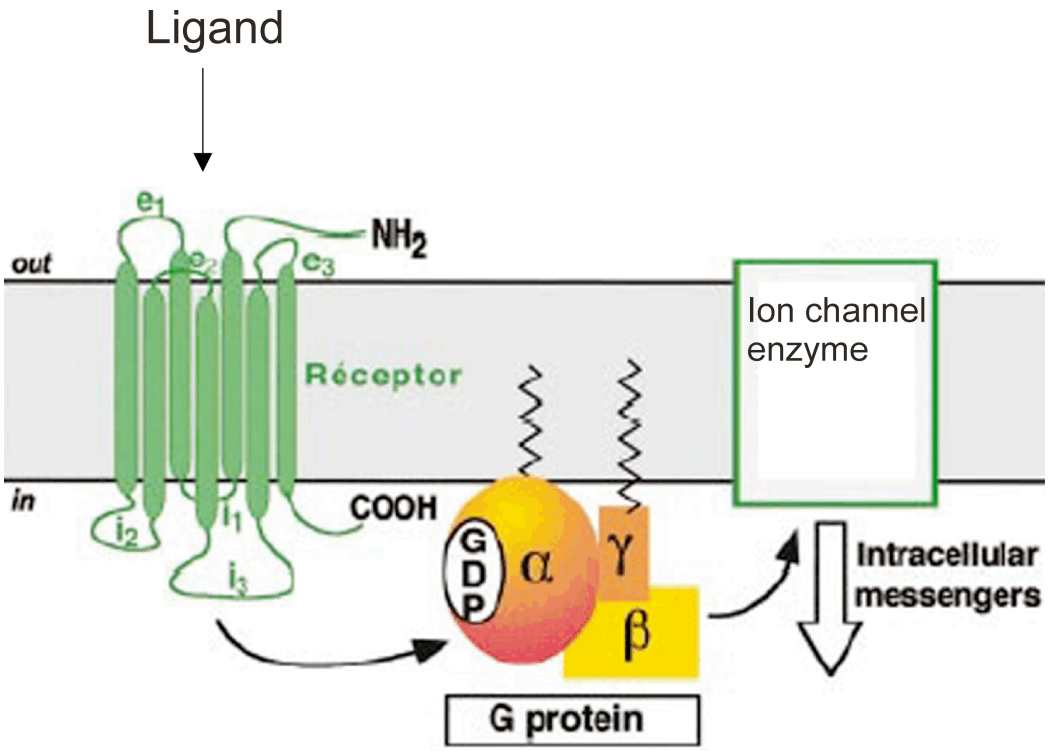

Fig. 2. **Illustration of a G protein-coupled receptor.** G protein-coupled receptors display a central core domain consisting of seven transmembrane helices (TM-I to TM-VII). The transmembrane helices are connected *via* three intracellular (i1, i2, i3) and three extracellular (e1, e2, e3) loops. GPCRs are activated through an enormous spectra of ligands, ranging from light, Ca^{2+}, odorants, pheromones, small molecules like amino acids, amines, peptides and nucleoside to proteins like chemokines and interleukins. The stimulated receptor recruits G proteins, which dissociate in a α- and βγ- subunit, thereby activating effectors like enzymes and ion channels (modified after Bockaert and Pin, 1999).

In family A GPCRs for example an Asp residue in the transmembrane helix 2 and, a tripeptide (DRY or ERW) between transmembrane helix 3 and the intracellular loop 2, play an important role in activation of the receptor (Oliveira et al. 1994). Conformational changes within the transmembrane domains generally affect the conformation of the intracellular loops 2 and 3, which are one of the key sites for G protein recognition and activation (Oliveira et al. 1994). It is believed that the C-terminal end of the G protein α-subunit binds to this pocket formed by the intracellular loops. There are several mechanisms how the natural ligand induce this change in conformation of the receptor, which leads to the activation of the receptor. For example small ligands like catecholamines activate GPCRs of the family A (subfamily 1a) by binding in a cavity formed by TM-III to TM-VI. In the subfamily 1b activated by short peptides, the binding of the ligand to the extracellular loops and the N terminus of the GPCR induce the change in conformation. When large proteins activate the GPCR like in the case of the subfamily 1c, the large N-terminal domain recognizes the protein and mediates activation of the transmembrane domains *via* its interaction with e1 and e3. In family B receptors, which are activated by large peptides such as glucagon or secretin, the long N-terminal part of the receptor plays an important role in the binding of the ligand. The family C GPCRs are characterised by a very large extracellular domain. The ligand-binding domain is localised in the N terminus and functions like a Venus flytrap, closing upon the binding of the ligand resulting in a conformational change within the transmembrane domains.

GPCR interacting proteins. GPCRs don't function exclusively through the activation of heterotrimeric G proteins, but also interact with a plethora of multidomain scaffolding proteins and accessory/chaperone molecules. Arrestins for example binds specifically to GPCRs, which were phosphorylated by G protein-coupled receptor kinases (GRKs). This interaction plays a role in the homologous desensitization of the receptors by disturbing their coupling to G proteins.

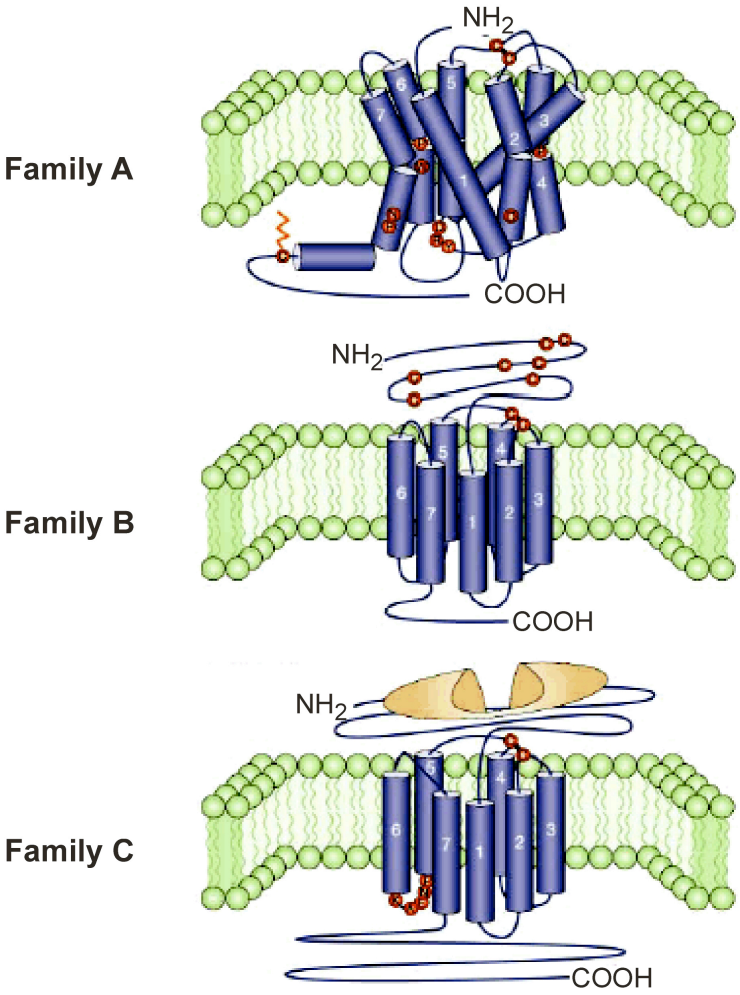

Fig. 3**. Classification of G protein-coupled receptors.** G protein-coupled receptors are phylogenetically divided into six families. This figure shows the main structural aspects of the three biggest families. Receptors of the family A form by far the largest subgroup. They are characterised by a set of highly conserved amino acids (some are indicated as red dots) and a disulphide bridge, connecting the first and second extracellular loop. Most of the family A receptors have a palmitoylated cysteine in the carboxy-terminal tail. Family B receptors are characterised by a relatively long amino terminus that contains several cysteins. In their general structure they display great similarities to family A receptors, although there is no sequence homology. The first and second extracellular loop is as well connected via a disulphide bridge, whereas there is no palmitoylation within the carboxy-terminal tail. Family C receptors have a long amino- and carboxy-terminal tail. The ligand binding domain is located within the amino terminus. According to its structure it is often compared to a "venus flytrap". A unique characteristic is that the third intracellular loop is short and highly conserved (modified after George et al., 2002).

1.3 Endothelins and endothelin receptors

Endothelins (ET-1, ET-2, ET-3) are expressed from three different genes. They are important regulators of the vascular system (Yanagisawa et al. 1988; Inoue et al. 1989)and are acting *via* two GPCR receptors: the endothelin A (ET_A) and the endothelin B receptor (ET_B) (Arai et al. 1990; Sakurai et al. 1990). Both receptor subtypes share a high amino acid sequence identity, overall 59% exceeding to 75% at the cytoplasmic face and are encoded by two different genes located on chromosome 4 and 13 (Sakurai et al. 1992). The ET_B receptor binds the three isoforms of endothelin with similar affinity (Arai et al. 1990). The ET_A receptor is isopeptid-selective (ET-1>ET-2>>ET-3) (Ogawa et al. 1991). Despite the high sequence homology the receptor subtypes couple to different G proteins and differ in their ligand-induced internalisation and intracellular trafficking. The ET_A receptor stimulates G proteins of the $G_{q/11}$ and $G_{12/13}$ families. The ET_B receptor activates mainly G proteins of the G_i and $G_{q/11}$ families (Eguchi et al. 1993; Cramer et al. 2001). Upon ligand binding, both receptor subtypes rapidly desensitize by phosphorylation through the G protein-coupled receptor kinase type 2 (Freedman et al. 1997). While the ET_A receptor is internalized *via* caveolae and/or clathrin-coated pits and recycled back to the cell surface, the ET_B receptor is exclusively internalized *via* clathrin-dependent pathway and transported to late endosomes and lysosomes (Bremnes et al. 2000; Okamoto et al. 2000; Oksche et al. 2000) .

The ET_A receptor is mainly expressed in vascular smooth muscle cells. Its activation elicits a long-lasting contraction *via* an increase in intracellular Ca^{2+} and activation of Rho proteins (Seo et al. 1994; Seko et al. 2003). The ET_B receptor is predominantly expressed in endothelial cells and stimulates the release of NO and prostacyclin, thereby causing relaxation of vascular smooth muscle cells (Faro et al. 1998). In addition, ET_A and ET_B receptors are co-expressed in numerous cells, e.g. astrocytes, cardiomyocytes, epithelial cells of the choroid plexus and the anterior pituitary and certain vascular smooth muscle cells (Kitsukawa et al. 1994; Angelova et al. 1997; Harada et al. 2002). In

disease states such as atherosclerosis and hypercholesterolemia, vascular smooth muscle cells co-express ET_A and ET_B receptors (Iwasa et al. 1999). Since atypical ligand binding was observed for cells co-expressing ET_A and ET_B receptors, e.g. astrocytes, epithelial cells of the anterior pituitary or vascular smooth muscle cells, it was suggested that the two receptor subtypes form heterodimers (Kitsukawa et al. 1994; Ehrenreich 1999; Harada et al. 2002). For example, in epithelial cells of the anterior pituitary, ET_B receptor-selective ligands such as sarafotoxin 6c, ET-3 and IRL 1620 were competitors of ^{125}I-ET-1 binding only in the presence of the ET_A receptor-selective antagonist BQ123 (Harada et al. 2002). In astrocytes, ET_A and ET_B receptors cooperatively control ET-1 clearance, since only the combination of ET_A and ET_B receptor-selective antagonists, but not their individual application increased ET-1 in the extracellular fluid (Hasselblatt et al. 1998). Similarly, the gap junction permeability of astrocytes is cooperatively inhibited *via* ET_A and ET_B receptors: only the combined application of ET_A- and ET_B-selective antagonists blocks ET-1 action (Blomstrand et al. 1999). These experimental data fit well with the now widely accepted view that family A (rhodopsin-like) and family C ($GABA_B$-like) GPCR exist as homo- and/or heterodimers. The cooperatively of ET-1 action on both ET_A and ET_B receptors may be explained by the fact that ET-1 is a bivalent ligand, which could bind to the ET_A receptor *via* its cyclic N terminus and to the ET_B receptor *via* its extended C terminus. It was even speculated that the bivalent ligand ET-1 could mediate the formation of ET_A/ET_B heterodimers by bridging ET_A and ET_B receptors *via* its N- and C-terminal parts, respectively (Sakamoto et al. 1993; Harada et al. 2002).

1.4 The aim of this study

The endothelin A and endothelin B receptors and their ligands (ET-1, ET-2 and ET-3) are important regulators of the vascular system. Preliminary studies suggested a possible formation of heterodimers between the two receptor subtypes (Sakamoto et al. 1993; Himeno et al. 1998), however to date, no explicit experimental data have been provided to support this hypothesis. The aim of this study presented here, was to investigate homo- and heterodimerisation of endothelin receptor subtypes using immunoprecipitation and FRET studies.