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REVIEW

Lifting the lid on GPCRs: the role of extracellular loops

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GPCRs exhibit a common architecture of seven transmembrane helices (TMs) linked by intracellular loops and extracellular loops (ECLs). Given their peripheral location to the site of G-protein interaction, it might be assumed that ECL segments merely link the important TMs within the helical bundle of the receptor. However, compelling evidence has emerged in recent years revealing a critical role for ECLs in many fundamental aspects of GPCR function, which supported by recent GPCR crystal structures has provided mechanistic insights. This review will present current understanding of the key roles of ECLs in ligand binding, activation and regulation of both family A and family B GPCRs.

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Abbreviations
$A_{2A}R$, A_{2A} adenosine receptor; AT_1R, angiotensin II type 1 receptor; β_1AR, β_1-adrenergic receptor; β_2AR, β_2-adrenergic receptor; C5aR, complement factor 5a receptor; CAM, constitutively activating mutation; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CRF, corticotropin-releasing factor; D3R (D2R), D3 (D2) dopamine receptor; GLP-1, glucagon-like peptide-1; ECL, extracellular loop; H_1R, histamine H_1 receptor; HIV-1, human immunodeficiency virus type 1; M2R (M4R), M2 (M4) muscarinic acetylcholine receptor; NDI, nephrogenic diabetes insipidus; PACAP, pituitary adenylyl cyclase-activating peptide; PTH, parathyroid hormone; TM, transmembrane helix; $V_{1a}R$, V_{1a} vasopressin receptor; V_2R, V_2 vasopressin receptor

Introduction

GPCRs form the largest class of membrane proteins in the human genome, with >800 unique receptors. They are central to cell signalling and are of great commercial value to the pharmaceutical industry worldwide, with ~50% of clinically marketed drugs and ~25% of top-selling drugs targeting this receptor family (Lagerström and Schiöth, 2008). GPCRs are activated by a wide variety of agonists which differ with respect to chemical class, physical properties and size – from photons and small biogenic amines to peptides and large glycoproteins (Hill, 2006).

Historically, it was envisaged that binding any agonist induced the 'on' conformation that activated a single G-protein type to initiate an intracellular signal. It is now recognized that GPCR signalling is much more complex than this. Individual GPCRs can activate multiple types of G-protein, not just one type, and signalling can be G-protein independent, such as β-arrestin-dependent GPCR activation of MAPK (Azzi *et al.*, 2003). Furthermore, there is compelling evidence to indicate that the classification of an individual ligand can be dictated by the signalling system being observed. For example, the peptide ligand SP-G is an antagonist for V_{1a} vasopressin receptor ($V_{1a}R$) inositol phosphate signalling, but is an agonist for $V_{1a}R$-stimulated MAPK (MacKinnon *et al.*, 2009.). Likewise, the β_2-adrenergic receptor (β_2AR) ligand propanolol is an inverse agonist at Gs-mediated signalling but stimulates the β-arrestin/MAPK pathway (Baker *et al.*, 2003). Moreover, recent crystal structures have revealed that full agonists establish a different hydrogen bonding pattern within the β_1AR binding site compared with partial agonists (Warne *et al.*, 2011).

Consequently, instead of just one active conformation (R*), there is a wide spectrum of distinct active receptor conformations with different efficacies for different signalling systems. In addition, the signalling generated by a ligand binding to the natural ligand (orthosteric) binding site can be modulated by the receptor binding additional ligands to allosteric binding sites (Schwartz and Holst, 2007; Valant *et al.*, 2009).

Despite this diversity and complexity, GPCRs exhibit a remarkably conserved protein architecture comprising a bundle of seven transmembrane helices (TMs) linked by alternating extracellular loops (ECLs) and intracellular loops. Analysis of GPCR sequences has revealed that they can be subdivided into families. Three of these families are of particular interest; the rhodopsin/β-adrenergic receptor family (family A), the secretin receptor family (family B) and the metabotropic glutamate receptor family (family C). The largest of these by far is family A, followed by family B.

The binding pocket for small ligands such as biogenic amines and non-peptide analogues of peptide hormones is buried deep within the TM bundle and the chromophore 11-*cis*-retinal occupies a similar position within rhodopsin. Furthermore, G-proteins, kinases, arrestins and scaffolding proteins interact with the intracellular face of the receptor, not the extracellular side. Consequently, it may appear that the ECLs are somewhat remote from the 'action'. The extracellular face of GPCRs comprises the N-terminus plus three ECLs. These loops are highly diverse with respect to both sequence and length, even when comparing subtypes of the same receptor family. This lack of sequence conservation may perhaps be interpreted as indicating a lack of functional significance. However, this is certainly not the case, and in this review we will highlight important functional roles for ECLs of both family A and family B GPCRs. For reasons of space, it has not been possible to consider the ECLs of GPCRs belonging to other families. Moreover, most of the information regarding ECL function addresses family A and family B GPCRs.

Experimental approaches to the study of ECLs

The ECLs are intrinsically flexible and this makes their study problematical. Nevertheless, there are a number of complementary approaches that can be used to obtain useful information.

A few family A GPCR crystal structures have been solved recently, providing atomic-level detail of ECL architecture [Palczewski *et al.*, 2000; Li *et al.*, 2004; Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008; Chien *et al.*, 2010; Wu *et al.*, 2010; Shimamura *et al.*, 2011 (reviewed in Peeters *et al.*, 2011a)]. Such crystal structures provide a 'snapshot' of the architecture of the ECLs but cannot address their dynamics. In contrast, NMR spectroscopy can provide high-resolution structural data plus insights into the conformational dynamics of the protein (Tikhonova and Costanzi, 2009). Unfortunately, an entire GPCR is too large for current technology. Consequently, solution NMR has focused on a single isolated domain (e.g. the N-terminus of a family B GPCR), or a peptide mimetic that is assumed to resemble the ECL within the complete receptor. Typically, to achieve a native-like loop conformer, the ends of the peptide mimetic are constrained (e.g. Déméné *et al.*, 2003).

When there is a lack of structural information, homology modelling of GPCRs is often used to interpret experimental data. However, loop prediction is difficult due to the loops' inherent flexibility and low sequence conservation. A recent model of the secretin receptor shows a total of 10 different plausible arrangements of the ECLs (Dong *et al.*, 2010a). A plethora of computational loop prediction methods has been published which broadly fall into knowledge-based searches and *ab initio* methods (Soto *et al.*, 2008). Efforts have also been made to incorporate flexibility to mimic the effects of 'induced-fit' (Sherman *et al.*, 2006; Michino *et al.*, 2009). The availability of crystallographic data on ECL conformations is leading to improved loop prediction programmes (Goldfeld *et al.*, 2011).

To constrain molecular models, empirical data addressing the structure of ECLs are needed. Given the technical problems associated with crystallography and NMR cited previously, indirect methods for probing structure–activity relationships remain very valuable. Site-directed mutagenesis and chimeric proteins have been used extensively. Specifically, alanine scanning has been successfully used to identify 'hot spots' in GPCR loops (e.g. Conner *et al.*, 2007). The substituted cysteine accessibility method (SCAM) is a technique whereby native residues are individually substituted by cysteine and reactivity to sulfhydryl-modifying reagents (e.g. biocytin maleimide or derivatives of methanethiosulfonate) used to determine exposure to the aqueous environment and hence provide structural insight (Shi and Javitch, 2004). Photoaffinity cross-linking studies typically incorporate a photoactivatable moiety (e.g. p-benzoylphenylalanine) within a peptide ligand to determine receptor contacts. This method has been applied to various GPCRs including the angiotensin II type 1 receptor (Fillion *et al.*, 2010) as well as numerous family B GPCRs (Assil-Kishawi and Abou-Samra, 2002; Dong *et al.*, 2004). More recently, a disulfide-trapping approach has been used to map the interaction sites between parathyroid hormone (PTH) and its receptor (PTHR1), negating the need for incorporating bulky moieties into the ligand (Monaghan *et al.*, 2008).

Family A GPCRs: ECL structural aspects

The structure of ECL2

Although GPCRs share a conserved overall protein fold comprising 7TMs, the limited number of GPCR crystal structures solved to date do not possess a single ECL2 structure common to them all (Figure 1). In family A GPCRs, ECL2 is usually the longest ECL, although this is not always the case. For example, the ECL2 of melanocortin receptors is very short indeed, containing just a few residues (Holst and Schwartz, 2003). In rhodopsin, ECL2 comprises two β-sheets (β3-β4) which form a twisted β-hairpin that plunges down into the TM bundle (Figure 1). This β-hairpin interacts with another β-hairpin (β1-β2) in the structured N-terminal region to form an effective 'lid', or 'plug' (not shown in Figure 1 for clarity),

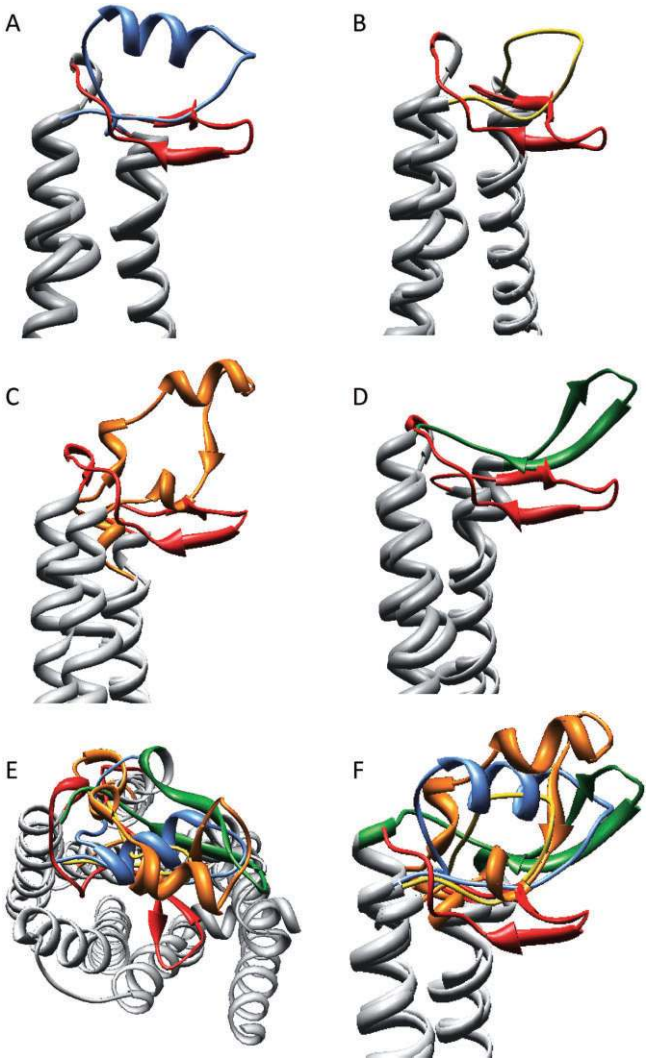

Figure 1

ECL2 conformational diversity in Family A GPCRs. In Panels A–D, the ECL2 of the diffusible-ligand GPCR is compared with the ECL2 of rhodopsin (red; PDB accession 3CAP): A, β_2AR (blue; PDB accession 2RH1); B, D3R (yellow; PDB accession 3PBL); C, A_{2A}R (orange; PDB accession 2YDO); D, CXCR4 (green; PDB accession 3OE0). An overlay of ECL2 of all five GPCRs viewed from above (Panel E) or from within the plane of the membrane (Panel F). Only the TM bundle of rhodopsin is shown in Panels E and F for clarity.

isolating the binding crevice from solvent and thereby preventing inappropriate hydrolysis of the Schiff base of the covalently bound chromophore 11-*cis*-retinal (Palczewski *et al.*, 2000; Li *et al.*, 2004).

In contrast, the ECL2 of the β_2AR possess a radically different structure comprising a short α-helix that is stabilized by an intra-helical disulfide bond (Figure 1A) and unlike rhodopsin, the β_2AR N-terminus is disordered (Cherezov *et al.*, 2007). The additional intra-helical disulfide bond is absolutely required for stabilizing the short ECL2 helix in the β_2AR, as its disruption by mutagenesis decreased ligand affinity 1000-fold (Fraser, 1989). The α-helix feature of ECL2 is also present in the β_1AR (Warne *et al.*, 2008). This marked difference in ECL2 conformation between rhodopsin and βARs seems to correlate with differences in their ligand binding modes. It is thought that the tight 'lid' conformation of ECL2 in rhodopsin reflects the fact that 11-*cis*-retinal is already covalently bound in the inactive rhodopsin. In contrast, the ECL2 segment of the βARs is held away from the binding cavity, thereby facilitating diffusible ligand access from the extracellular medium and also egress from the binding pocket. However, the highly structured ECL2 conformation of the βARs is not a universal motif for GPCRs with diffusible ligands. The β_1AR, β_2AR and the dopamine D3 receptor (D3R) are all catecholamine receptors and consequently bind agonists of very similar structure. Nevertheless, the ECL2 α-helix is not conserved in the D3R (Figure 1B) and this loop is also shorter in the D3R than in the βARs (Chien *et al.*, 2010). Consequently, the ECL2 helical secondary structure is more a feature of βARs than a feature of GPCRs with diffusible ligand, or even of catecholamine receptors in general. The absence in αARs of the important stabilizing intra-helical disulfide bond in ECL2 of βARs is consistent with this and furthermore, SCAM studies indicate that ECL2 of the D2R has a similar fold to rhodopsin (Shi and Javitch, 2004).

The ECL2 of the adenosine A_{2A} receptor (A_{2A}R) possesses three disulfide bonds with ECL1/top of TM3. The crystal structures from the Stevens laboratory indicated that ECL2 forms a spatially constrained random coil, albeit with a very short helix that contributes to the ligand binding pocket (Jaakola *et al.*, 2008; Xu *et al.*, 2011). However, these structures lacked a nine-residue segment of ECL2 due to insufficient electron density to allow modelling (Gln148–Ser156, Jaakola *et al.*, 2008; Pro149-Gln157, Xu *et al.*, 2011). The complete ECL2 was revealed to be highly structured in a subsequent structure from the Tate laboratory (Figure 1C). Despite the presence of an α-helix, the ECL2 of A_{2A}R is structurally different to the ECL2 of βARs (Figure 1E,F). The ECL2 of the only peptide-GPCR crystallized to date, the CXCR4 chemokine receptor (Wu *et al.*, 2010), adopts a β-hairpin conformation rather than an α-helix as in βARs and A_{2A}R. This, however, projects away from the binding cavity and therefore is a very different ECL2 feature to the β-hairpin 'lid' of rhodopsin previously cited (Figure 1D). The overall structure of the histamine H_1 receptor (H_1R) is closer to the βARs and D3R than to rhodopsin, A_{2A}R or the CXCR4 chemokine receptor (Shimamura *et al.*, 2011). Unfortunately, the conformation of ECL2 of the H_1R is unknown currently as the segment of ECL2 preceding the conserved disulfide bond was not resolved sufficiently to define the structure.

In addition to the crystallographic information already cited, NMR studies using synthetic ECL2 peptides have suggested that ECL2 is structured in other GPCRs. For example, it has been proposed that there is a helix preceding the conserved Cys in the κ-opioid receptor (Zhang *et al.*, 2002) and central to ECL2 in the neurokinin-1 receptor (Pellegrini *et al.*, 2001; Lequin *et al.*, 2002), whereas it was reported that ECL2 of the thromboxane A_2 receptor contains two β-turns extending away from the TM bundle (Ruan *et al.*, 2001). Overall, it is apparent that GPCRs exhibit a range of different ECL2 conformations; in the case of rhodopsin ECL2 (together with the N-terminus) forms a lid over the binding pocket protecting the pre-bound ligand, but for

'diffusible ligand-GPCRs' ECL2 lies more peripheral to the binding crevice entrance, thereby facilitating ligand access and egress (Figure 1E,F).

Family A GPCR ECLs – the role of disulfide bonds and other constraints

Despite the high degree of structural diversity with respect to ECL2 in family A GPCRs previously discussed, there is one feature that is conserved in the vast majority of GPCRs, both family A and family B *viz.* a disulfide bond between ECL2 and the top of TM3 ($Cys^{3.25}$). This effectively tethers ECL2 to the helical bundle and provides a conformational constraint. Some GPCRs have additional disulfide bonds between ECLs or linking ECLs to the N-terminus, for example between N-terminus-ECL2 (gonadotropin releasing hormone receptor; Cook and Eidne, 1997; Millar *et al.*, 2004), N-terminus-ECL3/TM7 junction (CXCR4; Wu *et al.*, 2010), the N-terminus-ECL3 ($AT_{1a}R$; Ohyama *et al.*, 1995) and between ECL2-ECL1 ($A_{2A}R$; Jaakola *et al.*, 2008). The $A_{2A}R$ also possesses an intra-loop disulfide bond within ECL3, in common with melanocortin receptors (Tarnow *et al.*, 2003; Yang *et al.*, 2007) and the human H_1R (Shimamura *et al.*, 2011). In each case, these 'additional' disulfide bonds will constrain the ECLs and also sculpt the topography of the approach to the binding crevice. Increasing extracellular constraint by engineering an 'additional' disulfide bond between the N-terminus and ECL3 (with the mutation N2C/D282C) generated a thermally stable rhodopsin (Xie *et al.*, 2003) that facilitated subsequent crystallisation from detergent solutions (Standfuss *et al.*, 2007; 2011). Reducing the flexibility of the M2 muscarinic acetylcholine receptor (M2R) ECL2 by engineering a disulfide bond between ECL2-TM7, effectively 'closed the lid' over the binding crevice and impaired binding of both orthosteric and allosteric ligands (Avlani *et al.*, 2007).

Removing a constraint can also have effects of course. Disruption of the conserved TM3-ECL2 disulfide is detrimental to function for many family A GPCRs (Davidson *et al.*, 1994; Noda *et al.*, 1994; Cook and Eidne, 1997; Zeng *et al.*, 1999; Elling *et al.*, 2000; Conner *et al.*, 2007). For other disulfide bonds, the observed effects can be more receptor specific. For example, breaking the N-terminus-ECL3 disulfide bond decreased agonist affinity in the CXCR4 (Zhou and Tai, 2000) but engendered constitutive activity on the angiotensin II type 1 receptor (AT_1R; Correa *et al.*, 2006). Likewise, breaking the N-terminus-ECL2 disulfide in GPR39 increased agonist (Zn^{2+}) potency (Storjohann *et al.*, 2008). A recent study on the N-terminus-ECL3 interface of the complement factor 5a receptor and CXCR4 concluded that this domain interface may actually act as a 'micro-switch' to regulate activation in some GPCRs (Rana and Baranski, 2010). Given all the evidence that significant changes in receptor functionality can be induced by perturbing the disulfide bond constraints of GPCRs, it is not surprising that naturally occurring cysteinyl mutations provide the molecular basis for some diseases. Cysteinyl mutations of the V_2 vasopressin receptor, rhodopsin and the MC_4 melanocortin receptor underlie cases of nephrogenic diabetes insipidus (NDI), retinitis pigmentosa and obesity respectively (Tarnow *et al.*, 2003; Bichet, 2009; Rakoczy *et al.*, 2011).

Disulfide bonds are not the only form of constraint in ECLs; ionic interactions also play a role. For example, the stability of dark-adapted rhodopsin is mediated in part by the ionic pair Arg^{177}/Asp^{190} within ECL2 and naturally occurring mutations of Asp^{190} result in unstable rhodopsin giving rise to the disease retinitis pigmentosa (Janz *et al.*, 2003). In addition, NMR data indicate that activation of the β_2AR weakens a salt-bridge present in the inactive receptor between ECL2 and ECL3/TM7 (Bokoch *et al.*, 2010). For GPCRs in general, it is true to say that disulfide and ionic bonds within ECLs provide important constraints although their precise location and function can be receptor specific.

Family A GPCR ECLs – the role of glycosylation

The extracellular face of GPCRs is usually N-glycosylated, with at least one glycosylation site (N-X-S/T) on the N-terminus, often more (Wheatley and Hawtin, 1999). There are exceptions of course; for example, the α_{2b}AR lacks any putative glycosylation sites and the $A_{2A}R$ lacks N-terminal glycosylation sites but has a glycosylated ECL2. In fact, N-glycosylation of ECL2 is quite common in GPCRs. Sequence analysis of 613 family A GPCRs revealed that 32% contain at least one consensus N-glycosylation site in ECL2. The large majority of these sites (85%) are located between the top of TM4 and the conserved Cys in ECL2, that is, not deeply buried. Such a location would be expected to accommodate the oligosaccharide chain within the GPCR tertiary fold. Although it is possible for loops other than ECL2 to be glycosylated, there is a minimum distance between the acceptor site Asn and the membrane for the oligosaccharyltransferase to operate (Nilsson and von Heijne, 1993). The ramification of this is that short loops are not glycosylated, even if they contain a consensus N-glycosylation site. The fact that ECL2 is often the longest loop in GPCRs predisposes it as a locus for N-glycosylation.

Glycosylation of ECL2 may serve to stabilize the conformation of the loop, or may orientate the loop away from the binding pocket entrance to enable unhindered ligand access. It is known that glycosylation can stabilize extracellular segments of GPCRs. Ablation of one of the two N-terminal glycosylation sites in rhodopsin (Asn15Ser) is a cause of the progressive retinopathy in humans autosomal dominant retinitis pigmentosa (Kranich *et al.*, 1993), probably due to destabilization of this region in the mutant rhodopsin (Standfuss *et al.*, 2007). Technical limitations have prevented crystal structures providing mechanistic insight into the roles of GPCR glycosylation. Only a short stretch of oligosaccharide chain was defined by density in rhodopsin crystal structures (Palczewski *et al.*, 2000; Li *et al.*, 2004) due to the inherent structural heterogeneity of the carbohydrate chains. Consequently, in order to decrease heterogeneity, the oligosaccharide chains on 'diffusible-ligand GPCRs' were absent prior to crystallization, due to either glycosylation site deletion (Warne *et al.*, 2008) or enzymatic deglycosylation (Jaakola *et al.*, 2008). Nevertheless, there is a compelling body of evidence indicating that in general terms, GPCR glycosylation has a role in trafficking to the cell membrane and/or receptor stability (Davidson *et al.*, 1996; Wheatley and Hawtin, 1999; Hawtin *et al.*, 2001).

Family A GPCRs: ECL functional aspects

Activation of GPCRs

In the absence of agonist, the ground state conformation of the receptor (R) is maintained by a network of intramolecular contacts that restrict conformational change. On binding agonist, these restraining interactions are broken and new interactions are established that facilitate the formation of the active receptor conformation (R*). The agonist-driven transition from R to R* is not an on/off switch but a multistep process that proceeds through a series of conformational intermediates in which contacts between the receptor and agonist change. A picture is now emerging of the molecular changes underlying GPCR activation, in which an agonist-induced re-arrangement of the TM helices involving TM5 leads to a rotation and outward movement of the cytoplasmic end of TM6. This opens a binding crevice for the C-terminal α5 helix of the Gα subunit and G-protein activation (reviewed in Oldham and Hamm, 2008 and Hofmann *et al.*, 2009; Choe *et al.*, 2011; Standfuss *et al.*, 2011; Rasmussen *et al.*, 2011b). A recent crystal structure of a modified β_2AR stabilized in an active conformation by a bound camelid antibody fragment (termed a nanobody) indicated that activation of a diffusible ligand GPCR involves similar TM re-arrangements as the somewhat 'atypical' rhodopsin, although specific details obviously vary (Rasmussen *et al.*, 2011a).

If activation of GPCRs requires the cytoplasmic face of the TM bundle to open, what is happening at the extracellular side of the receptor? Distance constraints, derived from precisely engineered metal ion binding sites, established that the extracellular ends of TM3, TM6 and TM7 move towards each other during receptor activation (Holst *et al.*, 2000; Schwartz *et al.*, 2006). Likewise, agonist contact with Ser203$^{5.42}$ and Ser207$^{5.46}$ in the β_2AR-nanobody complex stabilizes a 2.1 Å inward movement of TM5 and smaller inward movements of TM6 and TM7. Agonist-induced contraction of the A_{2A}R binding pocket is very similar but involves hydrogen bonding between the agonists and residues at the top of TM7 (Ser$^{7.42}$ and His$^{7.43}$) rather than TM5 (Lebon *et al.*, 2011). Consequently, the overall structural changes associated with GPCR activation are similar in each case, despite receptor-specific differences in the detail (Lebon *et al.*, 2011). To summarize, activation of GPCRs is accompanied by a coordinated movement in which the extracellular segments of TM helices draw together concomitant with the intracellular segment of the same TM helices moving apart. This has been referred to as the 'global toggle switch model' of GPCR activation (Holst *et al.*, 2000; Schwartz *et al.*, 2006).

Family A GPCRs – the role of ECL2 in receptor activation

ECL2 is usually the longest loop in family A GPCRs and is also the most structured loop as revealed by recent crystal structures (Figure 1). Furthermore, there is an increasing body of data highlighting the importance of ECL2 to GPCR function. Consequently, due to limitations of space, greater emphasis will be given to ECL2 in this review than to the other loops and consequently ECL2 will be addressed first.

Important contributions to GPCR activation are not restricted to residues within the TM bundle. It is known that the ECLs are important for ligand binding (Howl and Wheatley, 1996; Millar *et al.*, 2004; Shi and Javitch, 2004). Random saturation mutagenesis of ECL2 of the chemoattractant C5a receptor identified multiple mutations conferring constitutive activity (i.e. signalling in the absence of agonist; Klco *et al.*, 2005). Likewise, mutating ECL2 of the thrombin receptor also resulted in constitutive activation (Nanevicz *et al.*, 1996), suggesting that ECL2 acts as a negative regulator or damper, restricting the R → R* transition (Massotte and Kieffer, 2005). Consistent with this idea, solid state NMR data indicate that ECL2 of rhodopsin locks the extracellular ends of TM5, TM6 and TM7 in the inactive conformation. On exposure to light, ECL2 moves away from the retinal binding site to allow the active conformation to be adopted and this movement is linked to rotation of TM5 (Ahuja *et al.*, 2009). The reciprocal interaction has also been reported, with mutation of Tyr$^{5.58}$ in TM5 causing structural changes in ECL2 (Goncalvesa *et al.*, 2010). So there is an emerging picture of a regulatory function for ECL2 in GPCR activation. Such a role would certainly explain why disrupting specific contacts between ECL2 and the TM bundle in some GPCRs can result in constitutive activation. For example, this was observed when two ECL2-TM contacts in the free fatty acid receptor 1 were disrupted by site-directed mutagenesis (Sum *et al.*, 2009) and the pathogenic mutants Ile568Val and Ile568Thr within ECL2 of the thyroid-stimulating hormone receptor generate constitutive activity by breaking an ECL2-TM6 interaction (Kleinau *et al.*, 2007). It is also interesting in this regard that auto-antibodies directed against ECL2, which probably perturb ECL2 structure and its associated interactions when they bind, can induce receptor signalling and are linked to human pathologies (Goin *et al.*, 1997; Zhou *et al.*, 2008).

It should not be construed, however, that mutagenesis of ECL2 uniformly results in receptor activation. Systematic individual mutation of the 30 residues comprising the entire ECL2 domain of the V_{1a}R did not identify any mutation affecting basal signalling but did identify six residues required for agonist binding and receptor activation (Conner *et al.*, 2007). Furthermore, random mutagenesis of the M3 muscarinic acetylcholine receptor identified several ECL2 residues important for stabilizing the *active* state of the receptor rather than stabilizing the *inactive* state of the receptor (Scarselli *et al.*, 2007). Likewise, the pathogenic mutation Ala204Glu in ECL2 of the ghrelin receptor abolishes the pre-existing high natural constitutive activity of this receptor while retaining responsiveness to ghrelin (Pantel *et al.*, 2006). Overall, there is now compelling evidence that ECL2 modulates GPCR signalling but precise molecular details are receptor specific.

Family A GPCRs – the role of ECL2 in ligand binding

As noted earlier, the conformation of ECL2 adopted by family A GPCRs is very diverse (Figure 1). Nevertheless, ECL2 contributes to the ligand binding pocket irrespective of these striking differences. This is apparent when receptors are compared using a standard nomenclature for ECL2 residues (Conner *et al.*, 2007) in which the conserved Cys is desig-

Table 1

ECL2 'hot-spot' residues of family A GPCRs interacting with bound ligand

Receptor	Ligand	ECL2 residues interacting with ligand							
		C – 3	C – 2	C – 1	C0	C + 1	C + 2	C + 3	C + 4
rhodopsin	11-*cis*-retinal			Ser186		Gly188	Ile189		Tyr191
β_1AR	cyanopindolol						Phe201		Thr203
β_2AR	carazolol						Phe193		
A_{2A}R	ZM241385						Phe168	Glu169	
D3R	eticlopride						Ile183		
CXCR4	IT1t	Arg183		Ile185	Cys186	Asp187			
CXCR4	CVX15					Asp187	Arg188		Tyr190

ECL2 residues forming interactions with the named ligand are cited by their residue number for each receptor listed (Palczewski *et al.*, 2000; Li *et al.*, 2004; Cherezov *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008; Chien *et al.*, 2010; Wu *et al.*, 2010). ECL2 residue nomenclature (C + 1, C + 2 *etc.*) uses a standard nomenclature in which the ECL2 Cys forming the well-conserved disulfide bond to TM3 is designated as the reference residue C0 (see text for details).

nated as the reference residue (Cys0), the residue following this Cys is C+1 and the residue preceding is C-1. A particular 'hot-spot' in ECL2 for ligand binding contacts is revealed for family A GPCRs in the area adjacent to the highly conserved Cys which forms a disulfide bond to the top of TM3, tethering the orientation of ECL2 at this locus (Table 1). This region can also have a role in binding allosteric modulators, as Phe186(C+1) of the M4 muscarinic acetylcholine receptor is possibly a direct contact site for the allosteric modulator LY2033298 (Nawaratne *et al.*, 2010). It is also noteworthy that many of the CXCR4 ECL2 residues that interact with ligands also decrease human immunodeficiency virus type 1 (HIV-1) infectivity when mutated (Brelot *et al.*, 2000; Tian *et al.*, 2005) and that CCR5 inhibitors of HIV-1 interaction bind to ECL2 residues (Maeda *et al.*, 2008). A key functional role for ECL2 in ligand binding correlates well with our own studies on the V_{1a}R which demonstrated that most ECL2 residues were not essential for receptor function but C+1 and C+4 were important for agonist binding and signalling (Conner *et al.*, 2007). Such a close physical relationship between ECL2 and the ligand binding pocket of GPCRs explains why mutating specific residues in ECL2 can alter subtype selectivity of ligands (Zhao *et al.*, 1996), convert an antagonist to an agonist (Ott *et al.*, 2002) and modulate agonist-induced receptor internalization (Li *et al.*, 2001). It is known that agonist binding can induce a conformational change in ECL2 (Banères *et al.*, 2005), with agonists inducing a different conformation to antagonists and the unoccupied receptor possessing a more exposed ECL2 than when the binding pocket was occupied (Unal *et al.*, 2010). This suggests that the binding of a diffusible ligand to a GPCR helps to close the ECL2 'lid' over the entrance to the binding crevice, thereby restraining the ligand within the pocket, but that the precise shape of this lid is dictated by the efficacy of the ligand.

Family A GPCRs – a role for ECLs in allosteric ligand function

In addition to being regulated by ligands binding to the 'classical' or orthosteric binding site, GPCR activity can also be modulated by ligands binding to topographically distinct allosteric sites (reviewed in May *et al.*, 2007). Some allosteric sites have been mapped to ECLs, for example, the EDGE+Y motif in ECL2 of the M2R (Gnagey *et al.*, 1999). It can be envisaged that receptor activity could be modulated by the allosteric ligand imposing conformational changes/constraints onto key ECL segments to influence the affinity at the orthosteric site and downstream intracellular signalling. It has been known for many years that novel pharmacology can be generated by fusing two ligands to generate a chimeric ligand and this applies to both homo- and hetero-chimeric ligands (Howl *et al.*, 1997). A variation on this approach is currently being explored in which allosteric and orthosteric ligands are linked to form a 'bitopic ligand', which can potentially interact with both binding sites simultaneously to engender improved subtype selectivity and novel pharmacological agents (reviewed in Valant *et al.*, 2009). Furthermore, given the near identity of orthosteric sites in related GPCR subtypes, this bitopic ligand approach can exploit interaction of the allosteric moiety with the divergent ECLs to bestow subtype specificity on the ligand.

Family A GPCRs – the roles of ECL1 and ECL3

From the evidence presented in this review, it can be seen that the importance of ECL2 is well established but this is also true for the other loops comprising the extracellular face of GPCRs. It is known that GPCR activation involves movement between TM3 and TM6 (Hofmann *et al.*, 2009). ECL1 tethers TM2 to TM3 and ECL3 links the extracellular ends of TM6 and TM7. This means that both loops are ideally positioned to bind ligand, influence the orientation of TMs and modulate receptor activation. This is highlighted by the GPCR-dysfunction diseases NDI and retinitis pigmentosa, which can be caused by specific mutations in all three ECLs of the V_2 vasopressin receptor (Bichet, 2009) and throughout the extracellular face of rhodopsin (Rakoczy *et al.*, 2011) respectively. The co-receptor activity of CXCR4 is markedly impaired by mutations not just in ECL2 but also in ECL3 or the

N-terminus (Brelot *et al.*, 2000). Likewise, constitutively activating mutations (CAMs) of the thyroid stimulating hormone receptor have been reported for all three ECLs (Duprez *et al.*, 1998), and agonist-mediated receptor activation of GPCRs can include an essential role for residues in ECL1 (Hawtin *et al.*, 2006; Peeters *et al.*, 2011b) and ECL3 (Lawson and Wheatley, 2004).

Family A GPCRs – ECLs and ligand specificity

The ligand binding cavity of the D3R and β_2AR is very similar, which is not surprising given the high structural homology between their natural agonists, dopamine and noradrenaline respectively. Such a similarity in the architecture of the ligand binding site is even more pronounced when comparing receptor subtypes, such as D2R and D3R or β_1AR and β_2AR. However, receptor-specific ligand binding is not dictated simply by the residues lining the binding pocket. The pharmacological profile exhibited by a receptor will also be dependent on residues peripheral to the binding site, including those in ECLs, and these are usually not well conserved even between closely related receptor subtypes.

Extracellular residues may influence ligand specificity in several ways which include:

(i) *Differences in the electrostatic characteristics of the extracellular face may alter ligand–receptor interaction.* When a water-soluble ligand approaches a receptor from the extracellular medium, subtle differences in the ECL charge characteristics of closely related receptors could influence specific ligand–receptor interactions. Sequence differences in the ECLs of D2R and D3R result in marked differences in the localized electrostatic surfaces presented by these two receptor subtypes, which have been proposed to contribute to the subtype selectivity exhibited by some ligands (Chien *et al.*, 2010).

(ii) *Residues located on the approach route taken by the ligand from the extracellular milieu to the binding crevice may regulate ligand access.* For example, residue 3.26 adjacent to the conserved disulfide bond tethering ECL2 is positively charged in ~85% of peptide-GPCRs but is negatively charged in ~70% of amine-GPCRs. Molecular modelling indicated that the nature of the charge at this locus affects the membrane lipid packing and solvent access into the TM bundle, with a negative charge at 3.26 resulting in increased solvent access (Hawtin *et al.*, 2006). Consequently, the conservation of contrasting charges in peptide-GPCRs (positively charged residue) versus amine-GPCRs (negatively charged residue) may reflect the different binding modes of the ligands, with biogenic amines accessing a binding site buried within the TM bundle whereas peptides bind to ECLs in addition to TMs (Hawtin *et al.*, 2006).

(iii) *Differences in the physical characteristics of ECLs can change the geometry of the TM bundle.* The ECLs physically connect individual TM helices within the conserved GPCR architecture; consequently, changes in the loop characteristics can influence the precise packing of individual TM helices within the bundle. For example, the segment of ECL2 between the conserved Cys (which contributes to the disulfide bond linking ECL2 with Cys$^{3.25}$ at the top of TM3) and the top of TM5 is one residue shorter in the D3R than the corresponding segment of ECL2 in the β_2AR. This reduction in length effectively draws the connected TM helices closer together, such that the distance between the extracellular ends of TM3-TM5 is ~3.5 Å shorter in the D3R compared with the β_2AR (Chien *et al.*, 2010).

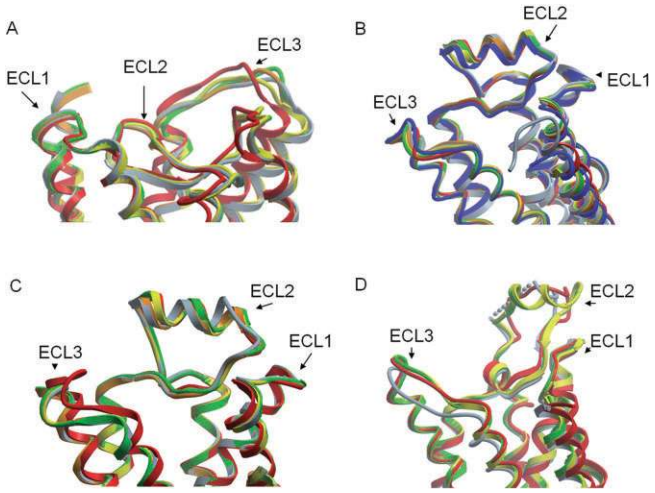

Figure 2

ECL conformations in different receptor activation states. Panel (A) Rhodopsin: red – inactive rhodopsin (PDB accession 1U19), grey – metarhodopsin II (PDB accession 3P0X); green – constitutively active rhodopsin (PDB accession 2X72); yellow – opsin (PDB accession 3CAP); orange – opsin-GαCT (PDB accession 3DQB). In each case, the N-terminus has been omitted for greater clarity. Panel (B) β_2AR: red – carazolol (partial inverse agonist) bound (PDB accession 2RH1B2); orange – alprenolol (antagonist) bound (PDB accession 3NYA); yellow – compound 2 (inverse agonist) bound (PDB accession 3NY9); green – ICI 118551 (inverse agonist) bound (PDB accession 3NY8); blue – FAUC50 (irreversible agonist) bound (PDB accession 3PDS); grey – BI-167107 (full agonist) bound, nanobody stabilized (active conformation) (PDB accession 3P0G). Panel (C) β_1AR: grey – dobutamine (partial agonist) bound (PDB accession 2YO1); orange – carmeterol (full agonist) bound (PDB accession 2YO2); yellow -isoprenaline (full agonist) bound (PDB accession 2Y03); green – salbutumol (partial agonist) bound (PDB accession 2Y04); red – cyanopindolol (antagonist) bound (PDB accession 2VT4). Panel (D) A_{2A}R: red – antagonist ZM241385 bound (PDB accession 3EML); grey – agonist UK-423097 bound (PDB accession 3QAK); yellow – agonist NECA bound (PDB accession 2YDV); green – agonist adenosine bound (PDB accession 2YDO).

Family A GPCRs – activation-associated changes to ECLs revealed by crystal structures

The recent availability of high resolution crystal structures of four family A GPCRs in both active and inactive conformations has enabled the conformational changes in ECLs associated with the transition from R to R* states to be observed directly (Figure 2).

Rhodopsin. The inactive conformation of rhodopsin is stabilized by the covalently bound chromophore 11-*cis* retinal which acts as an inverse agonist (Palczewski *et al.*, 2000; Li

et al., 2004). Active state-like structures have been obtained at low pH using the apoprotein opsin, which lacks retinal, either alone (Park *et al.*, 2008) or complexed with a peptide corresponding to the C-terminal fragment of transducin Gα (GαCT; Scheerer *et al.*, 2008). Obviously, a more accurate determination of the active structure would also include the agonist all-*trans* retinal in the binding site of rhodopsin. Recently, two such crystal structures have been reported using different approaches. The structure of metarhodopsin II was obtained using crystals of opsin in an active conformation (with or without GαCT) after soaking with all-*trans* retinal, which formed a Schiff base bond between the retinal and Lys296$^{7.43}$ (Choe *et al.*, 2011). Employing a different strategy, a CAM (Glu113Gln$^{3.28}$) was introduced into a rhodopsin possessing an engineered stabilizing disulfide bond between the N-terminus and ECL3. The active state was generated prior to crystallization by illumination in the presence of GαCT (Standfuss *et al.*, 2011). This structure is an active conformation but is not identical to metarhodopsin II as the retinal is not covalently bound and there was an estimated 60:40 mix of all-*trans* retinal and a mixed population of conformers. Nevertheless, a consistent picture of rhodopsin activation emerges from these studies (Figure 2A). There is little change in ECL1 but there are movements of the backbone of ECL2 and ECL3 caused by movement in the positions of TM5 and TM6. In ECL2, the changes are mainly concentrated at the C-terminal end of the loop adjoining TM5. Moreover, there is a significant re-arrangement of the hydrogen bond network, which includes structural water molecules within rhodopsin, linking ECL2 to functionally important residues in the TM bundle. In addition, Glu181 on the β3 strand of ECL2 moves 2.1 Å closer to the Schiff base as Glu113$^{3.28}$ moves away. This is consistent with the change in the counter-ion for the protonated Schiff base during activation suggested by Fourier transform infrared studies (Lüdeke *et al.*, 2005). It is noteworthy that the activation-associated displacement of ECL2 detected by NMR (Ahuja *et al.*, 2009) referred to previously in this review, is not observed in the active rhodopsin crystal structures. This implies that the outward movement of ECL2 is a transitory conformational change on the activation pathway and that this displaced ECL2 orientation is not maintained in the activated state. This is consistent with the finding that ECL2 of the AT_1R is more exposed in the unoccupied receptor than when binding either antagonist or agonist (Unal *et al.*, 2010).

β_2AR. The first crystal structure reported for the inactive conformation of a GPCR with a diffusible ligand utilized a monoclonal Fab fragment to stabilize a carazolol-β_2AR complex (Rasmussen *et al.*, 2007). Unfortunately, the ECLs in these crystals were too disordered to provide structural information. Using an alternative approach however, in which ICL3 of the receptor was replaced by T4 lysozyme (T4L), has resulted in crystal structures for the β_2AR-T4L complexed with four different inverse agonists *viz.* carazolol, timolol, ICI 118551 and 'compound 2' (Cherezov *et al.*, 2007; Hanson *et al.*, 2008; Wacker *et al.*, 2010) and a neutral antagonist alprenolol (Wacker *et al.*, 2010). In addition, two different crystal structures of active β_2AR have been published; one using an irreversible agonist (FAUC50) disulfide- bonded to an [H93C]β_2AR-T4L mutant construct (Rosenbaum *et al.*, 2011) and a β_2AR-T4L:high affinity agonist (BI-167107) complex stabilized by a bound nanobody (Rasmussen *et al.*, 2011a). In both of these structures, the largest agonist-stabilized changes occur at the intracellular face with only small changes to the ECLs (Figure 2B). A crystal structure of the active state ternary complex comprising agonist:β_2AR:Gs heterotrimer has also been solved (Rasmussen *et al.*, 2011b), or more specifically a T4L-β_2AR fusion (with T4L fused to the N-terminus not replacing ICL3) occupied by the agonist BI-167107, in a complex with Gs stabilized by a nanobody (Nb35) binding to the interface between the Gsα and Gsβ subunits. The structure of the active receptor conformation in β_2AR-Gs is very similar indeed to that observed in β_2AR-Nb80 cited above, with specific differences largely confined to the intracellular end of TMs (notably TM5 and TM6) and not ECLs. Consequently, the ECLs in an active conformation of β_2AR possess a very similar structure to the ECLs in the inactive receptor conformation.

β_1AR. For the β_1AR, there are crystal structures for three antagonists (cyanopindolol, iodocyanopindolol and carazolol) (Warne *et al.*, 2008; Moukhametzianov *et al.*, 2011), two partial agonists (salbutamol and dobutamine) and two full agonists (carmoterol and isoprenaline) (Warne *et al.*, 2011). Occupancy by agonist (full or partial) results in subtle changes to the extracellular face, with ECL3 being displaced away from the binding pocket (Figure 2C).

A_2AR. There are crystal structures for the A_{2A}R-T4L bound to the antagonist ZM241385 (Jaakola *et al.*, 2008) and the agonist UK-432097 (Xu *et al.*, 2011). The principal change in the extracellular face of the A_{2A}R during activation is an outward movement of ECL3 by 4 Å away from the binding pocket to accommodate the docked agonist, which is bulkier than the antagonist (Figure 2D). In addition, there are rotamer conformation changes to individual residues, notably His264 (ECL3) swings by ~100° and the carboxyl of Glu169 (ECL2) moves by 4 Å. These rotomeric changes, however, are thought to be linked to the specific agonist bound rather than being linked to receptor activation *per se*. Recent crystal structures of A_{2A}R bound to the agonists adenosine and 5′-(N-ethyl carboxamido)adenosine (Lebon *et al.*, 2011) revealed that both agonists interact with ECL2 residues, forming π-stacking interactions with Phe168(C+2) and hydrogen bonding to Glu169(C+3). Interestingly, these are the same ECL2 residues that interact with the antagonist ZM241385 (Table 1; Jaakola *et al.*, 2008).

Caveat emptor. There are some caveats to the crystal structures that arise from the strategies which have been developed to overcome the inherent difficulties associated with obtaining diffracting crystals from such dynamic proteins as GPCRs. The various structures of β_2AR and two of the A_{2A}R structures (Jaakola *et al.*, 2008; Xu *et al.*, 2011) previously cited, exploited constructs in which ICL3 of the receptor, which connects TM5 to TM6, was replaced by T4L. Given the importance of TM5 and TM6 to the GPCR activation process, replacing ICL3 by T4L obviously has the potential to perturb the

conformational changes in the TM bundle and the ECLs associated with R → R* transitions. Furthermore, steric hindrance by the T4L fusion prevented direct G-protein activation by 'active' conformations of GPCR-T4L chimeric constructs. Nanobody binding and nanobody-induced high affinity agonist binding, however, were not prevented by the T4L domain. All of the β_1AR structures cited were obtained using a construct containing six point mutations which markedly increased the thermostability of the receptor in detergent solution, thereby facilitating crystallization, but which also shifted the R → R* equilibrium towards the inactive R state (Warne *et al.*, 2008) and considerably increased the activation energy barrier compared with wild-type β_1AR (Balaraman *et al.*, 2010). Likewise, the agonist-occupied A_{2A}R structures reported by Lebon and co-workers utilized an A_{2A}R construct possessing four thermostabilizing mutations (Lebon *et al.*, 2011). As a consequence, this thermostabilized receptor was very poorly activated by agonist, did not exhibit the increased agonist affinity associated with wild-type A_{2A}R : Gαs coupling and probably represented an intermediate conformational state on the R → R* transitional path (Lebon *et al.*, 2011). It is also noteworthy that ECL2 in A_{2A}R-T4L crystals (with antagonist or agonist bound) was relatively unstructured and included an ill-defined segment. In contrast, the agonist-occupied thermostabilized A_{2A}R was highly structured (Figure 1C). This could be a ramification of a very dynamic ECL2 which can move between ordered and disordered conformations (analogous to the presence/absence of a 2.5 turn α-helix in ICL2 of D3R crystal structures; Chien *et al.*, 2010), or it may represent a structural manifestation of the different receptor stabilization strategies employed for crystallization studies by the laboratories of Stevens (Jaakola *et al.*, 2008; Xu *et al.*, 2011) and Tate (Lebon *et al.*, 2011) respectively.

Overall, the crystal structures suggest that there are likely to be some underlying themes to ECL conformational changes. ECL2 and ECL3 are contiguous with TM5 and TM6, respectively, the movements of which are central to GPCR activation. ECL configuration can be driven either by ligand binding or movement within the TM helical bundle and may be linked to activation of the receptor or be simply a local phenomenon, to accommodate the ligand. Where ECL movement is integral to receptor activation, then ligand binding, mutations in the ECLs or antibody binding that cause ECLs to adopt their R* conformations, are also likely to trigger receptor activation (and *vice versa* with respect to stabilizing inactive receptor conformations).

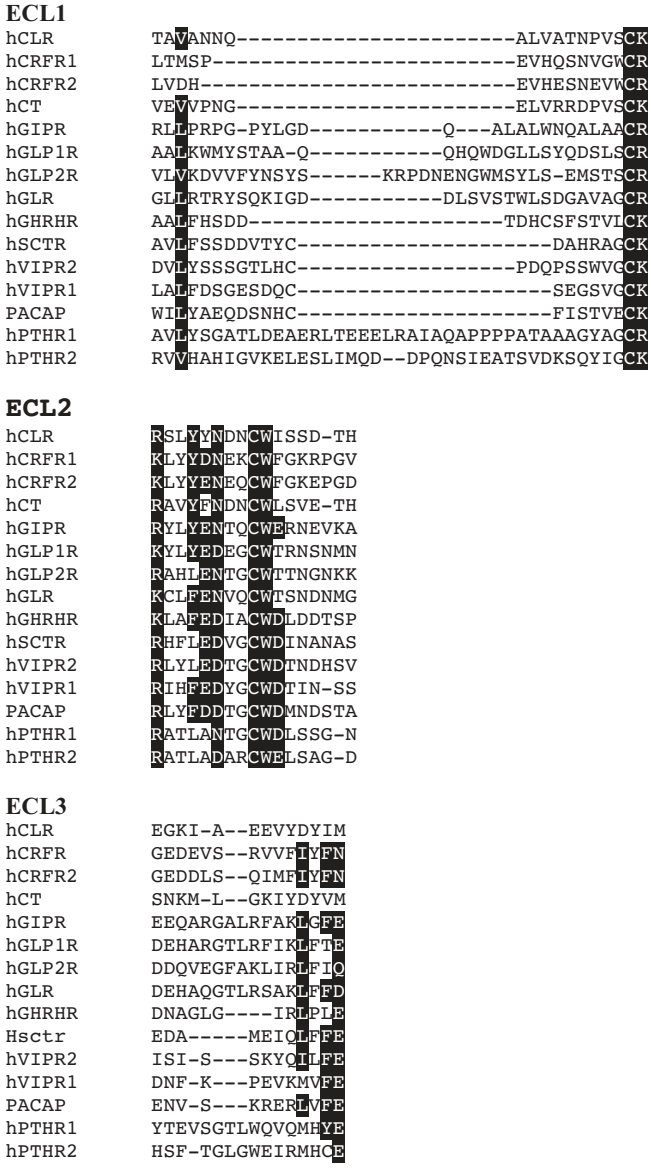

Figure 3

Alignment of the ECLs of Family B GPCRs. Human sequences of the entire receptors excluding the GLP2R were aligned with T-COFFEE Version_6.85 (http://www.tcoffee.org) after the putative signal peptides had been identified and manually removed. The GLP2R ECLs were subsequently aligned manually. Sequence conservation >50% is boxed in black.

Family B GPCRs: an introduction

Family B (secretin-like) GPCRs include receptors for medium-sized peptide hormones, typically 25–50 residues in length such, as secretin, calcitonin, glucagon, corticotrophin-releasing factor (CRF), PTH, amylin and the calcitonin gene-related peptide. One GPCR classification suggests that in humans there are 15 family B members that share between 30 and 50% sequence homology (Fredriksson and Schiöth, 2005). Family B GPCRs have some distinctive features, including a large N-terminal domain (~150–180 residues) possessing six cysteine residues that form three highly conserved disulfide bonds. The structure of this N-terminal domain complexed with ligand has been elucidated for several family B GPCRs, using solution NMR and X-ray crystallography, which allowed a common protein fold to be identified, referred to as 'the secretin family recognition fold' (Parthier *et al.*, 2009). The structures also reveal that the C-terminal and mid-region of the peptide ligands bind to this N-terminal extracellular domain. Consequently, the N-terminal region of the ligand is able to interact with the ECL and associated TM regions to induce receptor activation. This mode of ligand binding has aptly been referred to as the two-step model of activation (Hoare, 2005). Currently, there are no high-

resolution structural data describing the architecture of the TM bundle of family B GPCRs. Bioinformatic computational strategies have been employed but these often rely on a family A GPCR template. This is far from ideal, as family B GPCRs do not share the fingerprint motifs of family A GPCRs (such as 'DRY' and 'NPXXY') and do not possess the conserved reference residues used in the Ballesteros–Weinstein nomenclature (Ballesteros and Weinstein, 1995).

Family B ECLs

Identifying TM boundaries and loop prediction in GPCRs is problematical, although progress in predictive programmes is being made (Goldfeld *et al.*, 2011). Various alignment strategies have been put forward to model family B GPCRs (Donnelly, 1997; Frimurer and Bywater, 1999). Recently, Chugunov and co-workers have analysed the most prominent alignments and used an elaborate scoring procedure to evaluate TM packing properties and generated an alignment that scored higher than its predecessors (Chugunov *et al.*, 2010). In Figure 3, the loop boundaries proposed by Chugunov and co-workers have been followed to identify the likely sequences of the three ECLs in human family B GPCRs. Several trends are obvious from Figure 3, regardless of the precise location of the loop ends. ECL1 is the most variable in length, being particularly elongated in PTH receptors and possessing a 37-residue insert in one splice variant of the rat calcitonin receptor (Houssami *et al.*, 1995). The loop may terminate in a cysteine, analogous to the conserved Cys$^{3.25}$ in family A GPCRs, which would be expected to form a disulfide bond with the conserved cysteine in ECL2. There is a conserved basic amino acid immediately after the cysteine, which might interact with the negatively charged phosphate groups of membrane phospholipids, again analogous to family A peptide-GPCRs (Hawtin *et al.*, 2006).

PTH1R has the longest ECL1 within human family B GPCRs and this loop has been studied by NMR using a peptide mimetic. In the presence of detergent micelles, this forms an α-helical domain that may be critical for ligand binding (Piserchio *et al.*, 2000). This is an interesting finding, with the caveat that the structure of a single ECL isolated from other extracellular segments may differ from that in the intact receptor.

ECL2 shows the highest conservation among the ECLs. It probably begins with a highly conserved basic residue and then typically three hydrophobic followed by three to four hydrophilic residues, often acidic in nature (although this needs to be qualified as the precise starting point of the loop is difficult to determine). The mid-point of the loop has a highly conserved Cys-Trp (CW) motif, which in the majority of the human receptors is followed by an acidic residue (D/E). The rest of the loop is generally hydrophobic. In the CRF1 receptor, an alanine scan identified the equivalent of the CWD motif (CWF in this receptor) as essential for the binding of the agonist sauvagine (Gkountelias *et al.*, 2009), which is consistent with photoaffinity cross-linking studies (Assil-Kishawi and Abou-Samra, 2002). The equivalent Trp in the

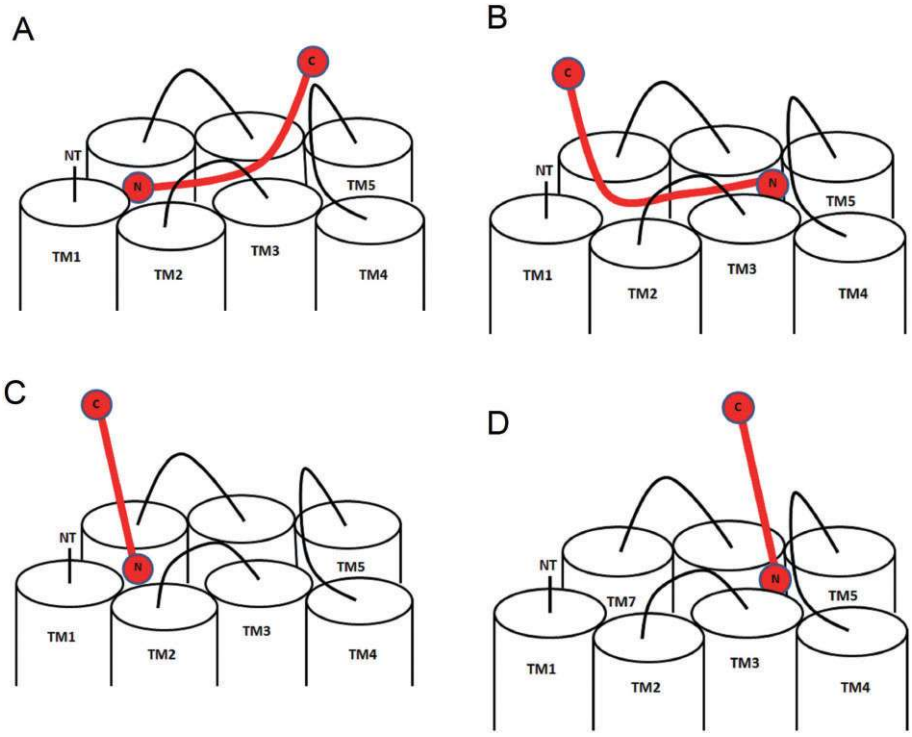

Figure 4

Modes of natural peptide agonist binding at the extracellular surface of a family B GPCR. Possible binding modes for peptide agonists interacting with ECLs and associated TM bundle of family B GPCRs. Panel (A) N-terminus parallel to the TM bundle, facing TM1-TM2-TM7; panel (B) N-terminus parallel to the TM bundle, facing TM3-TM4-TM5-TM6; panel (C) N-terminus perpendicular to the TM bundle, interacting with TM1-TM2-TM7; panel (D) N-terminus perpendicular to the TM bundle, interacting with TM3-TM4-TM5-TM6.

glucagon-like peptide-1 (GLP-1) receptor constitutes a photoaffinity contact for the ligand and deletion of this residue abolished high-affinity GLP-1 binding (Dong *et al.*, 2011). Consequently, this region of ECL2 may merit further investigation in other family B GPCRs, especially as the corresponding region adjacent to the conserved cysteine in ECL2 is critical for ligand binding in many family A GPCRs (Table 1; Conner *et al.*, 2007).

In general, ECL3 is predicted to be relatively short. There is some conservation of sequence around the likely ends of ECL3, but it is currently unclear if the residues involved are actually located within the loop itself. A recent model of the PTH1R, based on unpublished NMR data, suggested that there is a short section of α-helix in the middle of this loop (Thomas *et al.*, 2008).

A radical departure from conventional modes of agonist action was proposed by Dong and colleagues, who suggested a tripeptide motif (WDN) within the N-terminus of the receptor interacted with ECL3 and acted as an 'endogenous agonist' (Dong *et al.*, 2006). There are difficulties in applying this hypothesis to other family B GPCRs however; the Asn residue of the WDN motif in the VPAC1 receptor is N-glycosylated (Laburthe *et al.*, 2007), and $PTH_{1–14}$ activates both the intact and N-terminally truncated PTH1R (Luck *et al.*, 1999). Consequently, this model requires extensive revision (Dong *et al.*, 2010a,c). Nevertheless, cyclic WDN hexapeptides have biological activity on the secretin receptor (Dong *et al.*, 2010b), and it is noteworthy that another 'WD' located in ECL2 (part of the conserved CWD motif referred to previously; Figure 3) may be in a more optimal position to induce receptor activation, although this is speculative.

Family B GPCRs: role of ECLs in ligand binding and receptor activation

At present, it is unclear as to whether family B GPCRs share a global orthosteric binding site contributed by the TM bundle (Figure 4); depending on the receptor, models have been proposed in which the N-terminus of the ligand approaches the TM bundle from a perpendicular direction, or lies across it parallel to the plane of the membrane (Runge *et al.*, 2003; Monaghan *et al.*, 2008; Dong *et al.*, 2011; Miller *et al.*, 2011). The roles of individual ECLs will clearly differ depending on how individual peptide ligands interact with their cognate receptors. For receptor activation, it has been postulated that family B ligands contain a helical N-cap (Neumann *et al.*, 2008), based on NMR analysis of the PACAP1-21 bound to its receptor (Figure 5; Inooka *et al.*, 2001). It has been suggested that the helical N-caps give the peptide a specific constrained conformation that is needed to propagate receptor activation; residues at the bases of the ECLs are likely to play a role in this, although details remain elusive. This line of argument has been supported by experiments using disulfide constraints in the secretin peptide (Dong *et al.*, 2010c).

Extensive efforts have been used to identify the molecular determinants within the ECLs that make up the diffuse orthersteric binding site. Extensive photoaffinity cross-linking experiments have been conducted on secretin and its receptor (Dong *et al.*, 2008; 2010a) as well as GLP-1 and its receptor

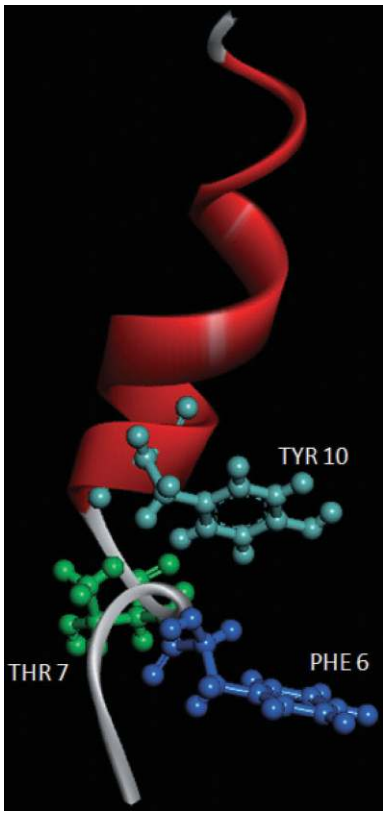

Figure 5

The N-cap motif for peptide agonists at family B GPCRs. The structure of the PACAP 1–21 bound to the PACAP receptor (Inooka *et al.*, 2001). Residues involved in the N-cap are indicated.

(Miller *et al.*, 2011). The authors used hypothetical molecular models to approximate the TM region and the orientation of the N-terminal extracellular domain relative to the TM region, then used experimentally derived distance constraints to dock the peptide ligand into its receptor. In addition, an elegant disulfide trapping approach has been used to map out the interaction sites between PTH and the PTHR1 (Monaghan *et al.*, 2008). It has been suggested that three distinct epitopes at the top of TM2, ECL2 and ECL3 in the glucagon receptor extracellular face govern ligand selectivity (Runge *et al.*, 2003).

Unlike family A GPCRs where major and minor binding crevices have been defined (Rosenkilde *et al.*, 2010), identification of family B binding crevices remain in its infancy. However, given the ability of family B GPCRs to activate multiple signalling cascades (Walker *et al.*, 2010) and the existence of ligand-biased agonism (Gesty-Palmer *et al.*, 2009), it is plausible that multiple ligand binding sites may govern key signalling switches within the receptor. Interactions between agonists and different parts of the ECLs would then be expected to influence these switches.

Conclusion

Overall, there is compelling evidence that although ECLs may appear peripheral within the GPCR architecture, they are

actually key elements modulating all aspects of receptor function and are not merely peptide linkers joining important TM segments (Lawson and Wheatley, 2004). Furthermore, given the structural diversity of the ECLs even between closely related receptor subtypes, exploitation of these regions may offer the greatest opportunities for developing receptor-specific drugs in the future.

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Conflict of interest

None.

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