Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors

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Class B G-protein-coupled receptors are a small family of 15 peptide-binding receptors. This family includes at least six biologically attractive therapeutic targets for both peptide ligands (osteoporosis and Type II diabetes) and nonpeptide ligands (anxiety, depression and migraine). A general mechanism of peptide binding has emerged for this receptor family, termed the two-domain model. In this mechanism, the C-terminal ligand region binds the extracellular N-terminal domain of the receptor. This interaction acts as an affinity trap, promoting interaction of the N-terminal ligand region with the juxtamembrane domain of the receptor. Peptide binding to the juxtamembrane domain activates the receptor and stimulates intracellular signaling. Nonpeptide ligands bind the juxtamembrane or N-terminal domain and, in most cases, allosterically modulate peptide-ligand binding. Here, these mechanisms of peptide and nonpeptide ligand binding are reviewed, then applied in a discussion of the future strategies of drug development for Class B G-protein-coupled receptors.

The Class B family of G-protein-coupled receptors (GPCRs) (also referred to as the secretin receptor family) comprises 15 peptide-binding receptors in humans, with no apparent orphan receptors [1,2] (Table 1). These receptors comprise an extracellular N-terminal domain (of ~100–160 residues) and a juxtamembrane domain of seven membrane-spanning α-helices. Class B GPCRs share little apparent sequence homology with Class A receptors (rhodopsin-like) or Class C receptors (e.g. $GABA_B$) [1,2]. Class B receptors are activated by endogenous peptide ligands of intermediate size (typically ~30–40 amino acid residues). These peptides include hormones, neuropeptides and autocrine factors that mediate diverse physiological functions (Table 1). The peptide-binding Class B receptors share low sequence homology with two other groups of receptors that have been suggested to belong to the Class B family: (i) the frizzled/smoothened receptors, which bind proteins of 350–360 amino acids (Wnts); and (ii) large N-terminal receptors, which bear single or repeating extracellular consensus sequences within their N-termini [2]. These two receptor groups are not considered in this review.

Several Class B GPCR–ligand systems are biologically attractive for treatment of disease (see Table 2 for a detailed list). For example, calcitonin and parathyroid hormone (PTH) regulate bone turnover and calcium homeostasis [3,4], and both of these peptides are effective treatments for osteoporosis [3,5] (Table 2). Two peptides that modulate insulin release and glucose homeostasis are potential novel therapies for Type II diabetes [glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) [6]; see Table 2]. Corticotropin-releasing factor (CRF) is a principle mediator of the body's response to stress [7], and antagonism of central CRF_1 receptors has been rationalized as a potential next-generation treatment for anxiety and depression

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TABLE 1

Classification of human Class B G-protein-coupled receptors and their peptide ligands[a]

Receptor	Peptide ligand(s)	Principal biological actions	Refs
CRF_1	CRF	ACTH release, central stress responses	[7]
	UCN1	Central stress responses	[7]
CRF_2	UCN1	Central stress responses	[7]
	UCN2	Cardiac contractility	[7]
	UCN3	Hearing	[7]
GHRH	GHRH	Release of growth hormone	[22,78]
GIP	GIP	Insulin secretion	[22,78]
Glucagon	Glucagon	Regulation of blood glucose	[22,78]
GLP-1	GLP-1	Insulin and glucagon secretion	[22,78]
GLP-2	GLP-2	Gut mucosal growth	[22,78]
PTH1	PTH	Ca^{2+} homeostasis	[4]
	PTHrP	Developmental regulator	[79]
PTH2	TIP39	Hypothalamic secretion, nociception	[80]
Secretin	Secretin	Pancreatic secretion	[22,78]
$VPAC_1$	VIP	Vasodilation, neuroendocrine functions	[22,81]
	PACAP	Neurotransmission, neuroendocrine functions	[22,81]
$VPAC_2$	VIP	Vasodilation, neuroendocrine functions	[22,81]
	PACAP	Neurotransmission, neuroendocrine functions	[22,81]
PAC_1	PACAP	Neurotransmission, neuroendocrine functions	[22,81]
Calcitonin	Calcitonin	Ca^{2+} homeostasis	[3]
Calcitonin; RAMP1	CGRP	Vasodilation	[20,23]
	Amylin	Reduces feeding	[20,23]
Calcitonin; RAMP3	Amylin	Reduces feeding	[20,23]
CL; RAMP1	CGRP	Vasodilation	[20,23]
CL; RAMP2	Adrenomedullin	Vasodilation	[20,23]
CL; RAMP3	Adrenomedullin	Vasodilation	[20,23]
	CGRP	Vasodilation	[20,23]

[a]Abbreviations: ACTH, adrenocorticotropin hormone; CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; CRF, corticotropin-releasing factor; GHRH, growth hormone-releasing hormone; GIP, glucose-dependent insulinotropic peptide; GLP, glucagon-like peptide; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; TIP39, tuberoinfundibular peptide of 39 residues; PAC, pituitary adenylate cyclase; PACAP, pituitary adenylate cyclase-activating polypeptide; RAMP, receptor activity-modifying proteins; VIP, vasoactive intestinal peptide; UCN, urocortin.

[8]. Nonpeptide antagonists of the CRF_1 receptor have reached early stages of clinical development [9] (Table 2). These results and physiological findings indicate that Class B GPCRs are highly attractive therapeutic targets from a biological perspective. However, the receptors are frequently considered difficult targets for drug development. The ligands that are in clinical use or at a late stage of development are all peptides, and drug-like nonpeptide ligands have only been identified for a small number of receptors (Table 2).

The mechanisms of ligand interaction with Class B GPCRs have been studied in detail. A general mechanism of peptide binding has emerged, and the mechanisms by which nonpeptide ligands bind and function are beginning to be elucidated. The purpose of this review is to present an overview of these mechanisms, and to consider how these mechanisms can be used to aid the future development of therapeutic ligands targeting Class B GPCRs.

Structure of Class B GPCRs and their peptide ligands

Class B GPCRs comprise a moderately sized, extracellular N-terminal domain of ~100–160 amino acid residues (here termed the N-domain), connected to a juxtamembrane domain (J-domain) of seven membrane-spanning α-helices with intervening loops and a C-terminal tail (exemplified by the PTH1 receptor in Figure 1). The receptors are glycosylated, with consensus sequences for asparagine-linked glycosylation located within the N-domain and, in some instances, extracellular loops. Intracellular loops interact with G-proteins to stimulate intracellular signaling, predominantly through G_s-coupled pathways and generally, to a lesser extent, through G_q and G_i. However, the relative strength of these signaling pathways can be regulated by accessory proteins [10].

The structure of the N-domain has been determined by nuclear magnetic resonance (NMR) spectroscopy for the $CRF_{2(b)}$ receptor [11] (Figure 2a). A central core contains a salt bridge sandwiched between aromatic side chains, surrounded by conserved residues. Two anti-parallel β-sheets are interconnected by the core [11]. The tertiary structure is stabilized by three disulfide bonds between six conserved cysteine residues, in an arrangement that has also been demonstrated for PTH1 (Figure 1, [12]), CRF_1 [13], and GLP-1 [14] receptors. The structure of the J-domain is largely unknown. This region displays no significant overall sequence homology with rhodopsin, for which the crystal structure has been determined [1]. Limited mutagenesis [15] and zinc-bridging studies [16] suggest that certain inter-helix interactions are similar to rhodopsin, for example, a putative interaction between transmembrane (TM) helix two and seven of the PTH1 receptor [15] (Figure 1). The arrangement of the TM regions has been investigated using computer modeling [1], and homology models based on the rhodopsin crystal structure have been developed (e.g. Figure 2b for the PTH1 receptor [17,18]), but the predictive utility of these models remains to be determined.

Some Class B GPCRs are highly unusual in that their ligand-binding properties are profoundly affected by accessory proteins [19]. The calcitonin receptor and calcitonin receptor-like receptor (CL receptor) interact non-covalently with receptor activity-modifying proteins (RAMPs), changing the responsiveness of these receptors to peptides of the calcitonin family (Table 1). The mechanism of the RAMP effect has been reviewed elsewhere [20]. The effect on ligand binding involves close contact between the RAMP and the GPCR, suggesting a direct participation

TABLE 2

Drug development status of peptide and nonpeptide ligands targeting Class B G-protein-coupled receptors[a]

Receptor target	Indication	Agonist or antagonist	Name	Peptide	Company	Status	Refs
Peptide ligands							
Calcitonin	Osteoporosis	Agonist	Miacalcin	Salmon calcitonin	Novartis	On market	[3]
	Paget's disease	Agonist	Cibacalcin	Human calcitonin	Novartis	On market	
	Hypercalcemia	Agonist	Calcimar	Salmon calcitonin	Rhone-Poulenc Rorer and Aventis	On market	
PTH	Osteoporosis	Agonist	Forteo	PTH(1–34)	Lilly	On market	[5,82]
			PREOS	PTH(1–84)	NPS	Phase III	
GLP-1	Type II diabetes	Agonist	Exenatide	Exendin-4	Amylin and Lilly	Phase III	[6,83]
			Liraglutide	GLP-1 analogue	Novo Nordisk	Phase III	
			CJC-1131	GLP-1 analogue	ConjuChem	Phase I and II	
Secretin	Autism	Agonist	RG1068	Porcine secretin	RepliGen	Unknown	[84]
Nonpeptide ligands							
CGRP	Migraine	Antagonist	BIBN4096BS	NA	Boehringer Ingelheim	Phase II	[71]
CRF_1	Depression, anxiety, IBS	Antagonist	Numerous	NA	Numerous	Phase I/II	[9,62]
Glucagon	Type II diabetes	Antagonist	Numerous	NA	Numerous	Unknown	[74,85]

[a]Abbreviations: CGRP, calcitonin gene-related peptide; CRF, corticotropin-releasing factor; GLP, glucagon-like peptide; IBS, irritable bowel syndrome; NA, not applicable; PTH, parathyroid hormone.

of the RAMP in ligand binding and/or an indirect conformational effect on the receptor [20]. RAMPs interact with other Class B GPCRs in transfected cells, but have little overall effect on ligand pharmacology for these receptors when compared with the RAMP effects on the CL receptor [21].

Peptide ligand structure has been extensively investigated. At the primary structure level, peptide ligands within certain families share similarities of amino acid sequence, for example, peptides of the secretin, glucagon, growth hormone-releasing hormone, GIP, GLP-1 and GLP-2 family [22]. Members of the calcitonin family are unusual in that the N-terminal region contains a six- or seven-residue ring formed by an intramolecular disulfide bond [23]. The secondary structure of peptide ligands has been evaluated in aqueous solution and under secondary-structure-inducing conditions. The peptides are generally disordered in solution, with local and transient formation of α-helical structure, often amphipathic, which can be stabilized under secondary-structure-inducing conditions [24,25]. Turn structures and bends have also been identified generally in the mid-region of the peptide [25]. In crystal structures, PTH and glucagon are almost exclusively α-helical [26,27]. These crystal structures support the propensity of the peptides to form α-helical structures, but their physiological significance is difficult to interpret because of the limited solvent exposure and extensive protein–protein interactions within the crystals. Much less is known about the receptor-bound ligand structure. These structures have been inferred from structure-activity relationships (SAR), modeling and, in one case, directly determined by NMR. The C-terminal portion of the ligand is probably α-helical when bound to the receptor, for most if not all ligands (e.g. CRF [28,29] and PTH [30]). The bound structure of the N-terminal portion probably differs between ligands – extensive SAR of PTH strongly implies a bound α-helical structure [18,31], whereas NMR spectroscopy of pituitary adenylate cyclase-activating polypeptide (PACAP) bound to purified PAC_1 receptors revealed a β-coil formed from two consecutive β-turns, preceded by a N-terminal tail [32].

Mechanisms of peptide interaction with Class B GPCRs

A general mechanism of peptide ligand interaction with Class B GPCRs has emerged, termed the 'two-domain' model (Figures 1–3). In this low-resolution model, the C-terminal portion of the peptide binds the N-domain of the receptor, often conferring high affinity. The N-terminal ligand region binds the J-domain, an interaction that activates the receptor and so stimulates intracellular signaling. The two-domain model provides a useful conceptual and analytical framework to evaluate ligand-binding properties. The following are reviewed: (i) structural models of ligand–receptor interaction; (ii) binding energy (micro-affinity) of the two interactions; and (iii) their role in receptor activation. It is important to stress that the two-domain model is a low-resolution model – at higher resolution, multiple receptor–ligand contacts are present within each of the two broadly defined interactions (Figures 1,2) and these contacts differ between receptors.

The most conclusive early demonstration of the binding orientation was a study of PTH–calcitonin ligand and receptor chimeras [33]. A peptide comprising the C-terminal portion of PTH and the N-terminal portion of calcitonin bound with high affinity to a receptor comprising the N-domain of the PTH1 receptor and J-domain of the calcitonin receptor. The same result was obtained with reciprocal chimeras. A similar experimental approach

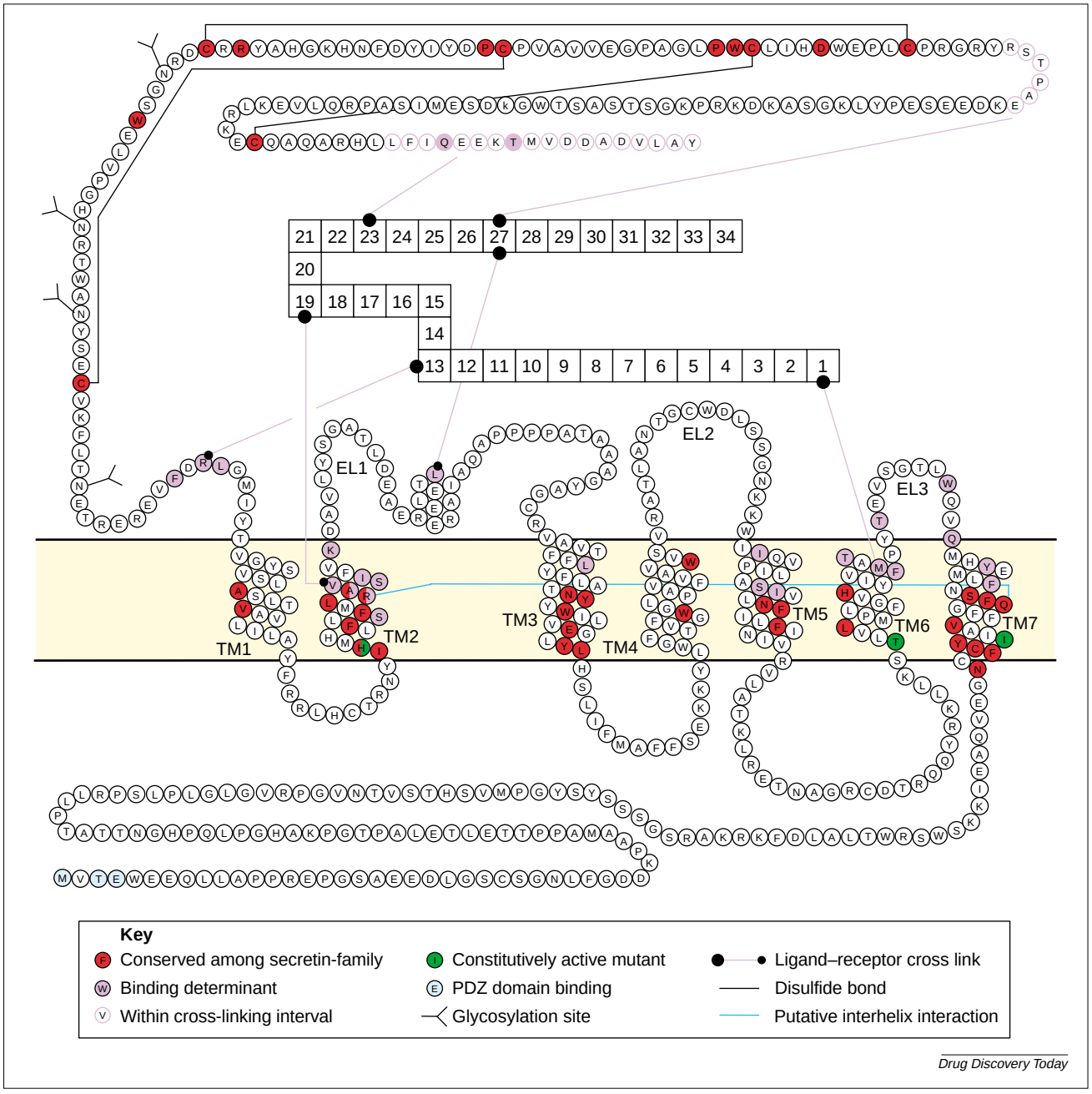

FIGURE 1

Topography of the human parathyroid hormone 1 receptor. The locations of TM α-helices in the receptor sequence are currently uncertain [1]. An α-helix is represented in EL1, based on a NMR study of a synthetic EL1 loop peptide [86]. The extracellular N-domain contains six conserved cysteines arranged in three disulfide bonds [12], and four consensus sequences for N-linked glycosylation [87]. A signal sequence of 22 amino acids has been excluded [12]. The peptide (PTH or PTHrP) is represented in the center of the figure with residues boxed and numbered in ascending order from the N-terminus. Receptor–ligand cross-linking coordinates are from studies using photoactivated amino-acid side-chains incorporated into PTH and PTHrP analogues [17,47,48,60,88,89]. Note that position 27 cross-links EL1 when the photoactive group is attached distally to the peptide backbone [47], whereas position 27 cross-links to the N-domain when the photoactive group is more backbone-proximal [48]. Residues shaded purple have been identified as determinants of peptide ligand binding by mutagenesis or cross-linking (reviewed in Refs [88,90,91]). Residues in red are conserved between Class B GPCRs [1]. Mutation of the conserved histidine at the intracellular face of TM2 can result in constitutive receptor activation of the PTH1 [55], glucagon [56], secretin [58] and $VPAC_1$ [59] receptors, but not the GIP receptor [57]. The conserved arginine in TM2 and glutamine in TM7 have been proposed to interact [15]. C-terminal residues are determinants of PDZ-domain binding; the PTH1 receptor interacts with the PDZ domain of NHERFs, altering the intracellular signaling pathways of the activated receptor [10]. Abbreviations: EL, extracellular loop; GIP, glucose-dependent insulinotropic peptide; GPCR, G-protein-coupled receptor; NHERF, Na^+/H^+ exchanger regulatory factor; PDZ , PSD-95/discs-large/ZO-1; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; TM, transmembrane.

demonstrated that the N-domain of glucagon and GLP-1 receptors determined the selectivity of the C-terminal portion of glucagon and GLP-1, respectively [34,35]. Studies of receptor chimeras formed from a diverse range of receptor pairs are consistent with the same binding orientation [36–38]. The two-domain model is also consistent with

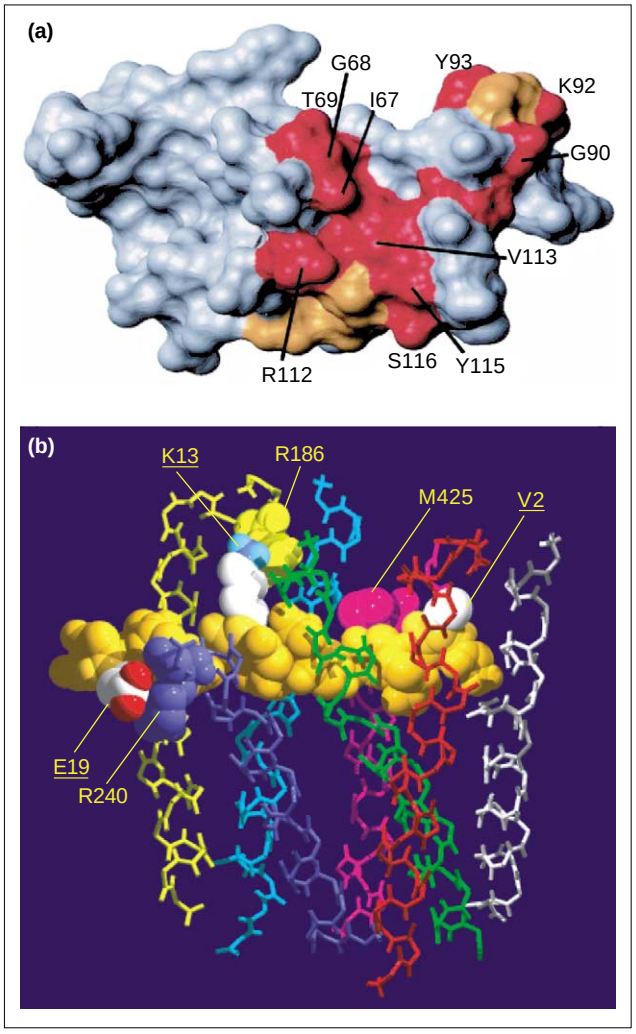

FIGURE 2

Ligand interaction sites on N- and J-domains. (a) Surface representation of the NMR structure of the mouse $CRF_{2(b)}$ receptor. Peptide ligand-interacting residues were determined using NMR chemical shift perturbation in the N-domain structure on binding astressin, a peptide antagonist (red, large perturbation >0.2ppm; orange, ~0.1–0.2ppm). Most of these residues are highly conserved between CRF receptor subtypes and isoforms, suggesting a conserved peptide-binding site among these receptors. Note the binding region for peptide ligand is a rather flat surface. Figure 2a is reproduced, with permission, from Ref. [11]. **(b)** Molecular model of PTH(1-21) bound to the J-domain of the human PTH1 receptor. PTH(1-21) is shown in α-helical conformation, bound to the extracellular portion of the J-domain (modeled on the crystal structure of rhodopsin). Ligand was docked to the receptor as described in Ref. [17]. The complex was then subjected to iterations of energy minimization without constraints until a steady-state structure was reached. (A more detailed model is presented in Ref. [18].) The atoms of PTH(1-21), in space-filled format, are colored orange, except for the side-chains of Val2, Lys13 and Glu19, which are colored by atom type (light blue, nitrogen; red, oxygen; white, carbon). The seven TM regions, in wire-frame format, are indicated as follows: TM1, yellow; TM2, dark blue; TM3, green; TM4, red; TM5, white; TM6, pink; TM7, light blue. The side-chain atoms of Met425, Arg186 and Arg240 in the receptor are shown in space-filled format and in the color of their respective TM domain (dark blue, Arg240; purple, Met425; yellow, Arg186). Figure 2b is reproduced, with permission, from Ref. [17]. Abbreviations: CRF, corticotropin-releasing factor; PTH, parathyroid hormone; TM, transmembrane.

studies of peptide and receptor fragments. N-terminal fragments of PTH interact with the isolated J-domain of the PTH1 receptor [31], and C-terminal fragments of PTH, exendin-4 and CRF bind the N-domain of PTH1 [39], GLP-1 [40] and CRF [41] receptors, respectively. Photochemical cross-linking studies are also generally consistent with the model. In these experiments, a photoreactive side chain is incorporated within the ligand and its site of covalent attachment to the receptor is mapped following exposure to photoactivating conditions. With some exceptions, photoactivated side chains in the C-terminal peptide portion bind residues in the N-domain of the receptor, and side chains in the N-terminal portion of the peptide bind residues in the J-domain [17,42–44] (see Figure 1 for the PTH1 receptor).

Structural models of receptor–ligand interaction have been obtained based on cross-linking coordinates, mutagenesis and NMR chemical-shift perturbation data. The structural basis of ligand–N-domain interaction was recently evaluated for the $CRF_{2(b)}$ receptor [11] (Figure 2a). The C-terminal ligand region probably binds as an α-helix to a broad surface on the N-domain with specific interactions, potentially electrostatic and hydrophobic, between side chains of ligand and receptor. The structural basis of J-domain binding has been explored extensively for the PTH1 receptor (Figure 1 and 2b). J-domain residues involved in binding ligand have been identified principally toward the extracellular face of the PTH1 receptor (Figure 1), but these residues are distributed diffusely throughout the sequence. (This diffuse distribution of binding determinants has been observed for many other Class B GPCRs, e.g. VPAC receptors [38].) Mutation and cross-linking data for the PTH1 receptor are consistent with a recent molecular model [17,18] (Figure 2b). In this model, a N-terminal α-helix of PTH (1–21) lies across almost the entire extracellular face of the J-domain of the PTH1 receptor. J-domain interactions of other ligand–receptor pairs are probably different, given the considerable diversity of primary and secondary structure within the N-terminal region of the peptide ligands for the receptor family. For example, the N-terminal region of PACAP contains a β-coil when bound to the J-domain of the PAC_1 receptor [32].

The global spatial arrangement between N- and J-domains is presently unclear. On the one hand, the N- and J-domain peptide interactions have been shown to be functionally independent for some receptors (e.g. the CRF_1 receptor [45,46]). On the other hand, results of some photochemical cross-linking experiments suggest that the N- and J-domain regions might be close to each other. A cross-link has been identified between the unusually large first extracellular loop of the PTH1 receptor and residue 27 of PTH when the photoactive group is attached distally from the peptide backbone (Figure 1) [47]. A more backbone-proximal photoactive substituent at position 27 cross-links to the N-domain (Figure 1) [48]. These findings suggest the first extracellular loop of the PTH1 receptor might be located close to the N-domain, at least when ligand is bound to receptor. A close spatial arrangement

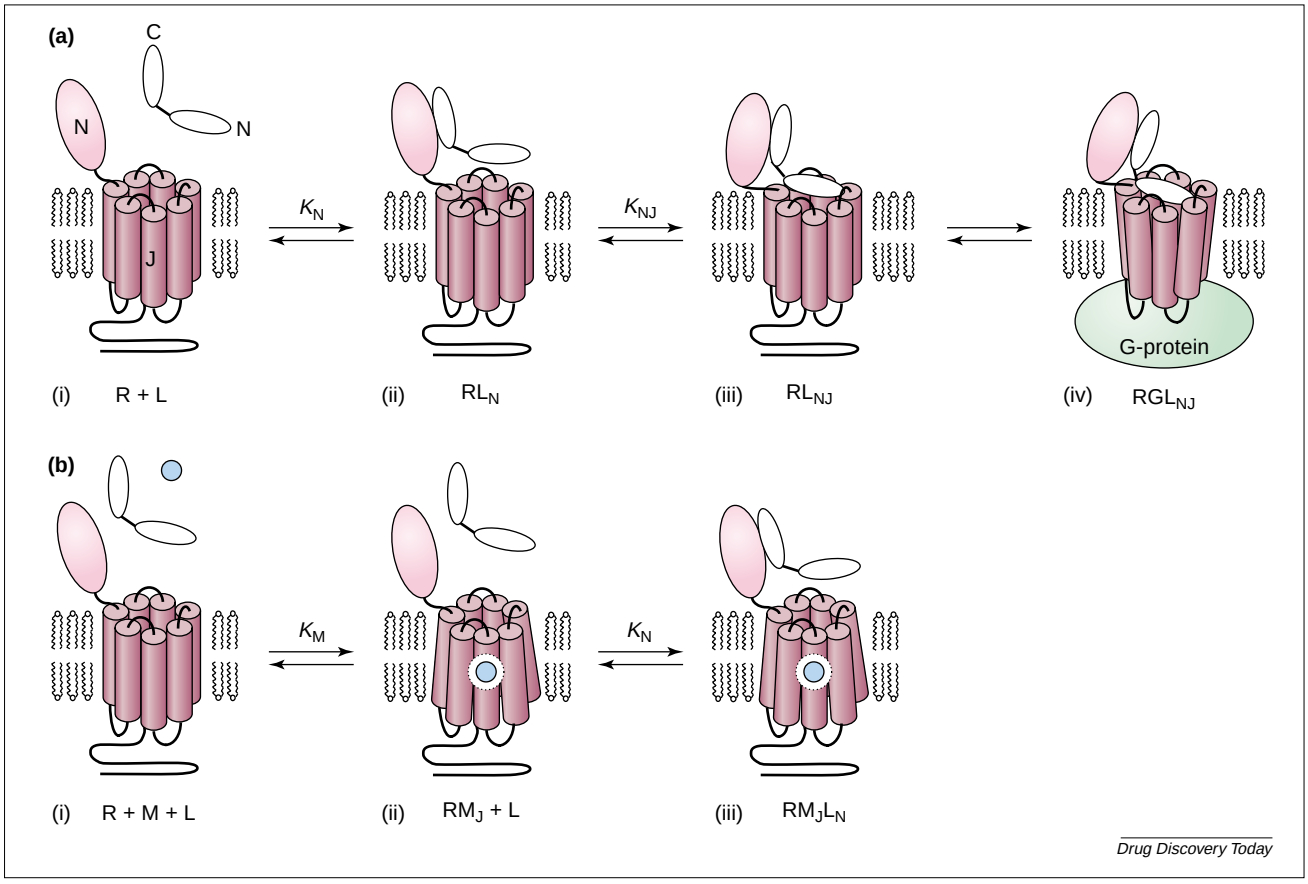

FIGURE 3

Binding models of peptide and nonpeptide interaction with Class B G-protein-coupled receptors. (a) General peptide binding model for Class B GPCRs. (i) The C-terminal region of the peptide L binds the N-domain of the PTH1 receptor forming RL_N (ii). This interaction enormously increases the local concentration of the N-terminal peptide region in the vicinity of the J-domain of the receptor, allowing their weak interaction to occur, resulting in formation of the bi-tethered RL_{NJ} from RL_N (iii). J-domain interaction of peptide increases receptor interaction with G-protein (iv), and vice versa. This enhanced interaction probably results from conformational change within the J-domain that stabilizes peptide interaction with this region. A more global conformational change has been suggested (the 'closed' conformation [61]), but this putative change is not illustrated because its structural basis is unknown. **(b)** Nonpeptide binding and antagonism model for the CRF_1 receptor (described in more detail in Ref. [45]). (i) Nonpeptide antagonist M (solid blue circle) binds within the J-domain, forming RM_J (ii). This interaction causes a change within the J-domain that prevents peptide binding to the J-domain (probably a conformational change of the receptor although this remains unproven) (ii). By blocking peptide–J-domain interaction, the nonpeptide antagonist blocks peptide-stimulated receptor signaling because peptide–J-domain interaction is required for G-protein activation. Nonpeptide antagonist binding to the J-domain does not affect peptide binding to the N-domain, hence the RM_JL_N ternary complex forms (iii). Abbreviations: CRF, corticotropin-releasing factor; GPCR, G-protein-coupled receptor; J, J-domain; L, ligand; M, nonpeptide antagonist; N, N-domain; R, receptor.

of N- and J-domains might also explain photochemical cross-linking points for secretin, in which multiple residues in the N-terminal ligand region bind the N-domain of the receptor [49].

The binding energy of each of the N- and J-domain interactions (micro-affinity) has been estimated for PTH1, PTH2, CRF_1, CRF_2 and GLP-1 receptors [40,41,45,50,51] (Figure 3a). Ligand interaction with the N-domain, forming RL_N, is defined by the micro-affinity constant K_N (Figure 3a). Overall, these measurements indicate moderate to high micro-affinity interaction with the N-domain (~1–100 nM). Interestingly, different ligands can bind with distinct affinities to the same N-domain, suggesting different molecular interactions [40,41]. For example, exendin-4 binds the GLP-1 receptor N-domain with an affinity of 13 nM, whereas GLP-1 affinity is much lower (800 nM). This difference is explained by the nine-residue C-terminal extension of exendin-4, which forms an additional N-domain-binding determinant [40,51]. Native peptide interaction with the isolated J-domain is extremely weak (~10 μM for PTH1 [31] and CRF_1 [45] receptors, 100,000-fold weaker than the affinity for the whole receptor). These findings suggest a sequential mechanism based on mass action (Figure 3a, discussed in detail in Ref. [45]), in which the peptide first binds the N-domain of the receptor. This interaction provides an affinity 'trap', which enormously increases the local concentration of peptide in the vicinity of the J-domain. This concentrating effect enables significant ligand interaction with the J-domain to occur at physiological levels of ligand (Figure 3a). The formation of the bi-tethered complex (RL_{NJ}) from RL_N can be described by an isomerization constant, K_{NJ} (Figure 3a). The value of

K_{NJ} is low (<1) in the absence of G-protein coupling for PTH1 and CRF_1 receptors, suggesting little formation of RL_{NJ} at the uncoupled receptor [45,50].

The J-domain mediates G-protein activation. J-domain fragments of PTH and CRF receptors are stimulated by peptide ligand to a similar maximal extent (E_{max}) as full-length receptors, albeit with dramatically reduced potency (increased EC_{50}) resulting from the loss of N-domain interaction [31,45]. PTH(1–14) and GIP(1–14) fragments stimulate signaling to a similar maximal extent as the full-length peptides [31,52]. Tethering N-terminal CRF or PTH sequences to J-domain fragments results in receptor activation [53,54]. Finally, constitutively activating mutations have been identified within the J-domain of the PTH1 (Figure 1), glucagon, GIP, secretin and $VPAC_1$ receptors [55–59]. These observations indicate that the J-domain alone is necessary and sufficient for G-protein activation by receptor.

G-protein binding to the receptor increases the affinity of agonist ligands for the J-domain of PTH1 and CRF_1 receptors [45,50]. In quantitative models, this effect is described by an increase of K_{NJ} – i.e. increased formation of RL_{NJ} from RL_N (tenfold and 1000-fold increase for PTH1 [50] and CRF_1 [45] receptors, respectively). The change of affinity is presumably a manifestation of conformational change within the receptor (forming the 'active' state). Conformational change within the J-domain is suggested by photochemical cross-linking experiments, in which a specific ligand residue can cross-link to different J-domain regions depending on the signaling efficacy of the ligand [60]. G-protein binding might also promote a more global conformational change within the receptor. Ligand-binding experiments for the PTH1 receptor suggest a more 'closed' conformation of the receptor in complex with G-protein. At the uncoupled PTH receptor, a PTH(1–14) analogue and PTH(3–34) appear to bind simultaneously to the receptor, whereas at the receptor–G-protein complex the ligands effectively compete each other for receptor binding [61]. Cross-linking evidence for close proximity between N- and J-domains might be a manifestation of this putative 'closed' receptor conformation. Comparison of ligand cross-linking at G-protein-coupled and -uncoupled receptor states could further test this hypothesis.

Mechanisms of nonpeptide ligand binding to Class B GPCRs

Nonpeptide antagonists have been identified and developed for CRF_1, CGRP, glucagon and GLP-1 receptors (Figure 4). Molecular details of their receptor interaction are not well characterized at present. There is more known about the functional mechanism by which the antagonists block peptide-stimulated signaling, particularly for the CRF_1 receptor. Nonpeptide antagonists of CRF_1, glucagon and GLP-1 receptors probably act allosterically, that is they probably bind sites at least partially distinct from the peptide ligand-binding regions.

Nonpeptide antagonists for the CRF_1 receptor comprise a central core, a 'top' side chain and a 'bottom' aromatic ring [62] (Figure 4a). The central core features an essential hydrogen bond acceptor, separated from the bottom aromatic ring by a one-atom linker (Topology I, e.g. SSR125543A) or by a two-atom linker (Topology II, e.g. DMP-696) (Figure 4a) [62]. Nonpeptide antagonists bind the J-domain of the CRF_1 receptor predominantly, if not exclusively. The ligands bind a J-domain fragment with the same affinity as the whole receptor [45], and two mutations have been identified within the J-domain that affect nonpeptide antagonist affinity (H199V in TM2 and M276I in TM5) [63]. The binding site for nonpeptide ligands appears distinct from that of peptide ligands because H199V and M276I mutations do not affect peptide ligand interaction [63]. In addition, quantitative analysis of the ligand-binding data is consistent with nonpeptide antagonist allosterically modulating peptide ligand binding and vice versa [64,65]. The allosteric effect is dependent on the receptor conformational state [64]. At the receptor uncoupled from G-protein (R state), the negative cooperativity between nonpeptide and peptide binding is weak (e.g. NBI 35965 binding reduces peptide agonist affinity by only fourfold to sevenfold). By contrast, at the receptor–G-protein complex (RG state), the negatively cooperativity between nonpeptide and peptide ligand binding is strong (NBI 35965 reduces peptide agonist affinity by >180-fold). This strong allosteric inhibition at the RG state explains the antagonist action of the ligands because RG formation is a prerequisite for peptide-stimulated signaling. These and other observations are consistent with a functional model, as shown in Figure 3b and discussed in detail in Ref. [45]. In this model for the CRF_1 receptor, nonpeptide antagonist binds the J-domain, producing a change that blocks peptide binding to its site(s) on the J-domain (Figure 3b). Because the peptide can no longer bind the J-domain, it can no longer activate the CRF_1 receptor. It is likely that nonpeptide binding to the J-domain does not appreciably affect peptide binding to the N-domain (Figure 3b). It is possible that a similar mechanism underlies glucagon receptor antagonism by L-168,049 (Figure 4b) because the compound binds the J-domain of this receptor and allosterically modulates glucagon binding [66].

Different mechanisms have been proposed for nonpeptide antagonist action at CGRP and GLP-1 receptors. Binding determinants of BIBN4096BS (Figure 4c) are present within the extracellular N-terminal region of RAMP1, the accessory protein that is part of the CGRP receptor [67]. The GLP-1 receptor antagonist T-0632 (Figure 4d) binds the N-domain of the receptor and allosterically modulates GLP-1 binding [68]. These findings suggest nonpeptide ligands can target extracellular regions of the receptor. However, it should be pointed out that BIBN4096BS is a large nonpeptide ligand and that T-0632 binds the GLP-1 receptor with only moderate affinity (1.2 μM).

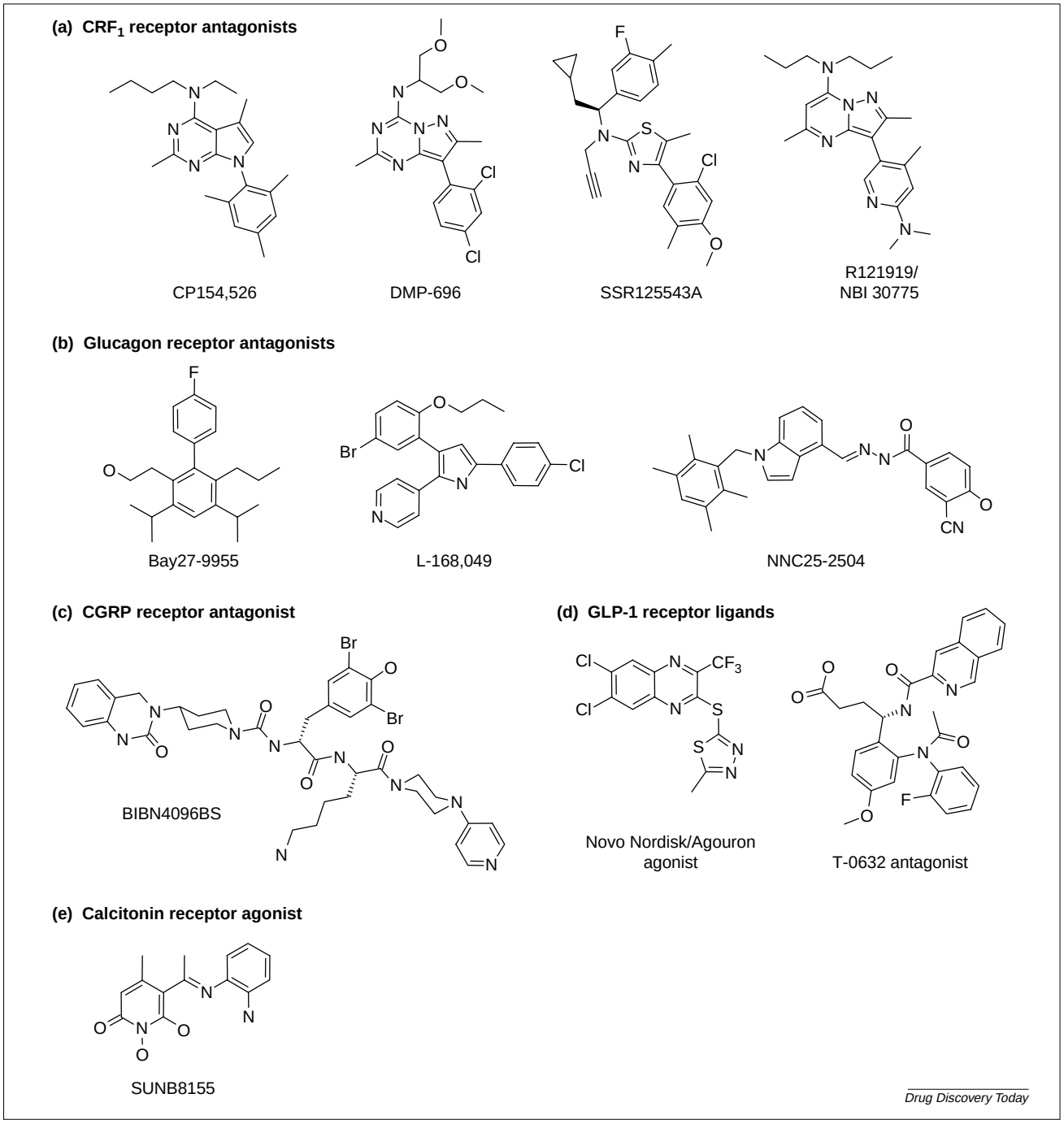

FIGURE 4

Chemical structure of nonpeptide ligands acting on Class B G-protein-coupled receptors. The representative compounds for each receptor are indicated. For comprehensive reviews of compounds for CRF_1, glucagon and CGRP receptors, see Refs [62], [74] and [92], respectively. Abbreviations: CGRP, calcitonin gene-related peptide; CRF, corticotropin-releasing factor; GLP, glucagon-like peptide.

Nonpeptide agonists have been identified for calcitonin [69] and GLP-1 [70] receptors. The calcitonin receptor agonist SUNB8155 (Figure 4e) stimulates cAMP accumulation with 20 μM potency and is selective for the calcitonin receptor over the PTH1 receptor [69]. GLP-1 receptor agonists have been described in patents. For example, a Novo Nordisk/Agouron compound (Figure 4d) was reported to bind with an affinity of 120 pM and is claimed to be an agonist (although no explicit functional data were provided) [70].

Future development of peptide and nonpeptide ligands targeting Class B GPCRs

At least six Class B GPCRs have been the focus of drug discovery programs (Table 2), but they have proven to be difficult targets. The ligands that have progressed into clinical use or late-stage trials are all peptides that are administered by injection (with the exception of calcitonin, which is available as a nasal spray). However, injection could affect patient compliance for diseases in which the drug is used preventatively in patients without debilitating

symptoms (e.g. osteoporosis). The furthest developed nonpeptide ligand (BIBN4096BS targeting the CGRP receptor) is a large molecule (M_r = 869.66 [67]) that is currently administered intravenously [71], which could limit its use in migraine treatment. There are numerous CRF_1 receptor nonpeptide antagonists that are orally available and can penetrate the brain [62], but it has proven difficult to combine reasonable pharmacokinetic properties with high binding affinity. The future development of peptide and nonpeptide ligands will be facilitated by knowledge of their receptor binding mechanisms. Here, the known mechanisms are used to suggest further avenues of drug development research on peptide and nonpeptide ligands.

Alternative routes of therapeutic peptide administration might offer greater compliance than injection. Nasal administration could represent an attractive option particularly as nasal delivery of calcitonin is known to be effective [3]. Nasal absorption is generally greater when using smaller peptides, so development of small bioactive peptide fragments might offer a strategy for nasal and other routes of administration. In this regard, a crucial observation is that the J-domain alone is sufficient for receptor activation, and therefore the entire peptide is not necessary for full efficacy. In principle, it might be possible to develop bioactive N-terminal fragments of the peptide ligand. An important issue with this approach is the low affinity of N-terminal peptide fragments. This difficulty can be overcome by introducing affinity-enhancing substitutions into the fragment. For example, combined substitutions in N-terminal PTH fragments increased affinity by five orders of magnitude [31]. Alternatively, screening could be used to identify short bioactive peptides. This approach has yielded short peptide agonists for the calcitonin receptor, based on screening of peptide ligands from synthetic libraries [72] and natural sources [73]. Although short peptides might be more readily absorbed, it will still be necessary to retain pharmacokinetic properties required for efficacy *in vivo*.

Nonpeptide antagonists could bind the N-domain or J-domain, blocking the principle binding interaction or activating interaction of the endogenous peptide, respectively. Developing competitive antagonists of the N-domain interaction could be difficult because the peptide-binding surface appears flat, an unattractive target for small ligands [11]. (It might be possible for a ligand to bind elsewhere on the N-domain and allosterically regulate peptide binding, as shown for the low-potency GLP-1 antagonist T-0632 [68].) The J-domain is probably the most attractive target for nonpeptide ligands. Most of the 'drug-like' nonpeptide ligands bind within the J-domain, especially CRF_1 antagonists and the glucagon antagonist L-168,049 [45,66]. It is possible that the J-domain contains pockets between TM helices that could be targeted. However, targeting the J-domain appears to be a challenge for Class B GPCRs compared with targeting most Class A GPCRs. The binding sites within the TM bundle in Class A GPCRs are highly amenable to medicinal chemistry; electrostatic, hydrogen bonding, π-stacking and hydrophobic interactions are frequently exploited. By contrast, ligand binding to the J-domain in the CRF_1 receptor, and potentially the glucagon receptor, appears to be driven largely by hydrophobic interactions, with few hydrogen bond interactions and no identified electrostatic interactions [62,74]. These properties suggest that the ligand-binding region(s) within the J-domain might be more hydrophobic than the corresponding domain of Class A GPCRs. As a result of these features, particularly the high lipophilicity, it has been difficult to develop compounds with high affinity and good pharmacokinetic properties, although advances are being made in the more recent medicinal chemistry efforts [62].

Orally available nonpeptide agonists of Class B GPCRs would offer several advantages over existing therapeutic peptide ligands, especially a nonpeptide PTH1 receptor agonist for osteoporosis. The J-domain is the most attractive target for nonpeptide agonists, given the importance of this domain for receptor activation. No high-potency drug-like nonpeptide agonists have been reported in literature. It is not yet clear whether a small nonpeptide agonist can activate Class B GPCRs. Taking into account the large number of contacts between peptide ligand and the J-domain (Figure 1 and 2b), it is possible that the energy barrier to the active receptor state is too high for a small nonpeptide ligand to overcome. However, the finding that single point mutations can result in constitutive receptor activity [55–59] might suggest that modifying a small number of constraining interactions is sufficient to activate the receptor.

Identifying nonpeptide ligands is the principle bottleneck in drug discovery for Class B GPCRs. HTS has apparently yielded few drug-like chemical starting-points to date. However, screens for these receptors might be particularly prone to false negatives because: (i) identifying ligands that bind the J-domain might be difficult in receptor-binding assays as a result of the large contribution of N-domain binding for many peptide radioligands; (ii) antagonist IC_{50} in functional assays is often more than tenfold higher than the binding affinity (K_i) for Class B GPCRs, thus decreasing the hit rate for low-affinity compounds; and (iii) many screens might not be optimally designed to detect allosterically acting compounds [75]. Screens can be designed to minimize these potential shortcomings, for example, the use of radioligands that bind to the J-domain extensively.

An alternative approach to identifying nonpeptide ligands is to mimic the structure of the binding determinants of the endogenous peptide ligand. Structural analyses of peptide ligands for Class A GPCRs have yielded nonpeptide mimics for these peptide-binding receptors, in particular, angiotensin-1, cholecystokinin, tachykinin and neuropeptide Y receptors [76]. Knowledge of peptide-ligand structure has also been useful in rationalizing and optimizing screening hits for Class A GPCRs, for example, melanocortin-4

receptor agonists [77]. For Class B GPCRs, it will probably be necessary to first determine the structure of the receptor-bound peptide; these ligands are largely unstructured in solution and the receptor-bound conformation is different from the structure in solution (at least for PACAP and PTH).

Conclusions

In summary, Class B GPCRs are involved in major biological and pathophysiological functions. These actions have stimulated drug discovery programs and a great deal of research into the mechanisms of receptor–ligand interaction. Peptide binding is described by the two-domain model, in which: (i) the C-terminal ligand portion binds the receptor N-domain, providing an affinity trap; and (ii) the N-terminal ligand portion binds the receptor J-domain, activating the receptor. Nonpeptide antagonists bind the N-domain or J-domain, respectively blocking the principle binding or activation interactions of the peptide ligand. Applying knowledge of these mechanisms prospectively should aid the identification, optimization and development of ligands targeting the receptors.

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