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REVIEW

Therapeutic potential for novel drugs targeting the type 1 cholecystokinin receptor

Erin E Cawston and Laurence J Miller

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ, USA

Cholecystokinin (CCK) is a physiologically important gastrointestinal and neuronal peptide hormone, with roles in stimulating gallbladder contraction, pancreatic secretion, gastrointestinal motility and satiety. CCK exerts its effects via interactions with two structurally related class I guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs), the CCK_1 receptor and the CCK_2 receptor. Here, we focus on the CCK_1 receptor, with particular relevance to the broad spectrum of signalling initiated by activation with the natural full agonist peptide ligand, CCK. Distinct ligand-binding pockets have been defined for the natural peptide ligand and for some non-peptidyl small molecule ligands. While many CCK_1 receptor ligands have been developed and have had their pharmacology well described, their clinical potential has not yet been fully explored. The case is built for the potential importance of developing more selective partial agonists and allosteric modulators of this receptor that could have important roles in the treatment of common clinical syndromes.
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Abbreviations: AA, arachidonic acid; Ac, acetylated; cADPr, cyclic ADP-ribose; CCK, cholecystokinin; CCK_1 receptor, type 1 cholecystokinin receptor; CCK_2 receptor, cholecystokinin type 2 receptor; EGFR, epidermal growth factor receptor; eIF4E-BP, eukaryotic initiation factor 4E-binding protein; ERK, extracellular-regulated kinase; GPCR, guanine nucleotide-binding protein-coupled receptor; Grb2, growth factor receptor-bound protein 2; IBS, irritable bowel syndrome; IP_3, inositol 3, 4, 5-triphosphate; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; mTOR, mammalian Target of Rapamycin; NAADP, nicotinic acid adenine dinucleotide phosphate; NO, nitric oxide; $p90^{RSK}$, 90-kDa ribosomal S6 protein kinase; PI3K, class I phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; SAR, drug structure-activity relationship; Shc, Src homology/collagen related; SOS, Drosophila homolog, Son of Sevenless

The main purpose of this report is to explore the therapeutic potential of highly selective partial agonists and allosteric modulators of the type 1 cholecystokinin (CCK) receptor (CCK_1 receptor). To more fully appreciate this selective role, the normal physiology and pathophysiology of this receptor and of its natural, full agonist ligand, CCK, must first be reviewed.

Correspondence: Laurence J Miller, Mayo Clinic, 13400 East Shea Boulevard, Scottsdale, AZ 85259, USA. E-mail: miller@mayo.edu
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Cholecystokinin is one of the earliest gastrointestinal hormones to be discovered, identified more than 90 years ago based on its ability to stimulate gallbladder contraction (Ivy and Oldberg, 1928). It was soon recognized to be identical to the factor responsible for stimulating pancreatic exocrine secretion (Harper and Raper, 1943). This hormone has subsequently been shown to have effects on enteric smooth muscle and on nerves at various locations in the peripheral and central nervous system (Rehfeld, 2004). One of its most important neural effects is post-cibal satiety (Kissileff *et al.*, 1981; Smith and Gibbs, 1985; Muurahainen *et al.*, 1988;

Beglinger *et al.*, 2001), a critically important role that could provide the basis of a highly useful treatment for obesity. In addition to its roles in stimulating smooth muscle cell contraction and exocrine cell secretion, CCK stimulates cell growth, energy production, gene expression and protein synthesis (Williams, 2001), processes that have substantial potential implications for agonist drug development.

It is well recognized that mammals synthesize CCK peptides in the I-cells of the small intestine, as well as the central nervous system (Rehfeld, 1978; Miller *et al.*, 1984). CCK is found as variable length linear peptides, including 58, 39, 33 and eight residues, all of which share the pharmacophoric domain for the CCK_1 receptor, representing the carboxyl-terminal heptapeptide-amide (Rehfeld *et al.*, 2001). CCK peptides also share their carboxyl-terminal tetrapeptide-amide with all molecular forms of gastrin, with this region providing the pharmacophoric domain for the type 2 CCK receptor (CCK_2 receptor) (Tracy and Gregory, 1964; Mutt and Jorpes, 1968). Critical post-translational modifications found on biologically active CCK peptides include sulfation of the tyrosine that is present seven residues from the carboxyl terminus of CCK, as well as amidation of the carboxyl-terminal phenylalanine residue (Solomon *et al.*, 1984; Rehfeld, 1998).

CCK_1 receptor

Cholecystokinin exerts its physiological functions through the activation of two structurally related class I guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) identified as the CCK_1 receptor (also known as the Type A CCK receptor) and the CCK_2 receptor (also known as the Type B CCK receptor) (consistent with IUPHAR and BJP GPCR Nomenclature). These receptors are approximately 67% conserved and are in close approximation on the phylogenetic tree of GPCRs, within the class I grouping (Kolakowski, 1994). This review will focus only on the CCK_1 receptor.

Localization and function of CCK_1 receptor

The activation of the CCK_1 receptor by CCK has been shown to be responsible for a broad variety of important physiological functions, including the stimulation of gallbladder contraction and pancreatic exocrine secretion, delay of gastric emptying, relaxation of the sphincter of Oddi, inhibition of gastric acid secretion and induction of post-cibal satiety (Solomon *et al.*, 1984; Anagnostides *et al.*, 1985; Kerstens *et al.*, 1985; Schmitz *et al.*, 2001). The activation of the CCK_1 receptor by CCK also stimulates trophic and proliferative effects in some target cells (Matozaki and Williams, 1989; Matozaki *et al.*, 1990; Moralejo *et al.*, 1998; Moralejo *et al.*, 2001).

The CCK_1 receptor mRNA and protein have been localized in the human gastric mucosa within D cells, where CCK can stimulate somatostatin secretion, thereby affecting gastric acid secretion (Schmitz *et al.*, 2001). The CCK_1 receptor is also located in the muscularis propria of human gastric antrum, fundus and pylorus (Reubi *et al.*, 1997), where activation by CCK has been shown to modulate contractile ability and thus affect gastric emptying (Fried *et al.*, 1991; Scarpignato *et al.*, 1996; Zerbib *et al.*, 1998).

One of the best described physiological roles of CCK activation of the CCK_1 receptor is stimulation of pancreatic enzyme secretion. In the exocrine pancreas, the CCK_1 receptor has been shown to be present with distinct cellular distributions in rodents, guinea-pigs and humans (Christophe *et al.*, 1978; Deschodt-Lanckman *et al.*, 1978; Jensen *et al.*, 1980; Yu *et al.*, 1987). The majority of the literature pertaining to the functional role of the CCK_1 receptor in the pancreas has been examined using the rodent pancreatic acinar cell model. Yet, until recently, the expression of the CCK_1 receptor in human pancreas has been difficult to demonstrate, possibly reflecting very low levels of this receptor in this species (Ji *et al.*, 2001). Galindo *et al.* (2005) were finally able to apply qRT-PCR to multiple human specimens to clearly demonstrate expression of the CCK_1 receptor mRNA in human pancreas, but this did not establish cell of origin. Human pancreatic acinar cell expression of this receptor was finally supported by Murphy *et al.* (2008) when they demonstrated direct activation of isolated human pancreatic acinar cells at physiologic concentrations of CCK, with this hormone eliciting oscillatory increases in cytosolic calcium activation and stimulation of enzyme secretion. Substantial evidence also supports the presence of this receptor on intrapancreatic neurons and on abdominal branches of the vagus nerve in several species (Owyang and Logsdon, 2004). Indeed, CCK_1 receptors on vagal afferent fibres have been shown *in vivo* to mediate pancreatic enzyme secretion (Li *et al.*, 1997).

The CCK_1 receptor has also been detected in the endocrine pancreas in human insulin- and glucagon-secreting cells (Morisset *et al.*, 2003), and CCK has been shown to stimulate the release of insulin from the pancreas (Karlsson and Ahren, 1989; 1991). In the gallbladder, CCK_1 receptor expression on smooth muscle cells has been shown in humans and is responsible for stimulation of gallbladder contraction (Tokunaga *et al.*, 1993).

It is well established that the CCK_1 receptor is present on distinct neuronal nuclei in the central nervous system, while the predominant CCK receptor present in the brain is the CCK_2 receptor (Innis and Snyder, 1980; Moran *et al.*, 1987; Wank, 1995). Vagal afferent nerve expression of CCK_1 receptors has not only been shown to mediate pancreatic acid secretion, but has also been shown to be important in the response to gastric load (Schwartz *et al.*, 1994) and eliciting satiety signals (Weatherford *et al.*, 1993).

Pathophysiological role of CCK_1 receptor

The CCK_1 receptor has been implicated in common and important pathological disorders such as irritable bowel syndrome (IBS) that is characterized by abdominal pain and disturbed bowel habits (Zhang *et al.*, 2008). Sjolund *et al.* (1996) have shown that CCK release may contribute to the intestinal dysmotility in IBS patients, and recently Zhang *et al.* (2008) have shown an increase in plasma CCK levels in IBS patients.

The CCK_1 receptor has also been implicated in the production of cholesterol gallstones. Cholesterol gallstone disease

has been associated with reduced CCK binding to the CCK_1 receptor and with impaired gallbladder smooth muscle contraction in response to this hormone, while patients with pigment gallstones have been reported to have normal CCK binding and responsiveness. The impaired responses to CCK have been demonstrated to be reversed by removal of excess membrane cholesterol (Xiao *et al.*, 1999).

One of the most interesting and relevant functions of the CCK_1 receptor stimulation by CCK is related to its role in modulating food consumption, which may provide the basis of a highly useful treatment for obesity. One of the first descriptions of the role of CCK as a food inhibitor was by Gibbs *et al.* in 1973, when they showed that intraperitoneal administration of CCK suppressed intake of both solid and liquid in a dose-dependent manner in rats (Gibbs *et al.*, 1973). Since that initial observation, much research has been performed on the role CCK plays as a satiety factor. By definition, administration of a satiety factor at the start of a meal results in a smaller-than-normal meal being consumed. Satiety signals result from exogenously administered or endogenously produced mediators that activate specific receptors and result in cessation of eating. It is advantageous when this occurs early in the course of a meal. Studies in human have shown that intravenous infusion of CCK-8 and CCK-33 increase the perception of fullness, decrease hunger and reduce energy intake (Kissileff *et al.*, 1981; Muurahainen *et al.*, 1988; Lieverse *et al.*, 1994; MacIntosh *et al.*, 2001).

CCK_1 receptor signalling

In order to discuss the potential importance of developing more selective partial agonists and allosteric modulators of the CCK_1 receptor, we must first understand the broad spectrum of signalling initiated by activation with the natural full agonist peptide ligand, CCK. Here, we provide careful reference to signalling pathways that may have potential implications for agonist drug development, with these pathways depicted graphically in Figure 1.

CCK_1 receptor G_q signalling pathways

Over the years, it has become clear that multiple affinity states of the CCK_1 receptor can be observed that are most clearly related to its status of coupling with its heterotrimeric G protein (Jensen *et al.*, 1980; Sankaran *et al.*, 1980). There is general agreement that the agonist-occupied CCK_1 receptor that is coupled with G_q represents a high affinity state of this receptor. However, as noted below, multiple G proteins are capable of coupling with this receptor, yet the specific functional effects on ligand binding of each are not clear. Similarly, it is well established that the cellular environment in which this receptor resides can affect its specific binding characteristics. This might reflect differences in the lipid composition of the plasma membrane, as well as differences in the relative stoichiometry of receptor and individual G protein expression and in the cellular concentrations of guanine nucleotides. Ultimately, it will be key to determine the molecular and structural basis for observed affinity states and for the initiation of particular signalling pathways. Unfortunately, at the present time, these levels of knowledge and understanding are absent.

The signalling pathways involved in the spectrum of action of CCK at the CCK_1 receptor have become an area of great interest. Stimulation of this receptor by CCK is known to result in the proximal initiation of several different vectorial signalling pathways. These include coupling with G_q and G_s, as well as the possibility of activating non-G protein-mediated signalling pathways. The predominant pathway studied for CCK activation of the CCK_1 receptor includes the G_q family of G proteins, G_q, G_{11} and G_{14} (Xu *et al.*, 1998; Yule *et al.*, 1999). Stimulation of the CCK_1 receptor by an agonist such as natural CCK results in a conformational change in this receptor, including its third intracellular loop where G proteins often couple. This exposes a region of the receptor that binds G_q and results in the exchange of GTP for bound GDP at the G protein, with the dissociation of the GTP-bound α-subunit from its β and γ subunits. The GTP-bound α-subunit then activates phospholipase C (PLC). PLC is known to cleave phosphatidylinositol 4, 5-bisphosphate (PIP_2) to form inositol 3, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) (Trimble *et al.*, 1987; Matozaki and Williams, 1989). IP_3 moves to the endoplasmic reticulum where it stimulates the release of calcium stores (Muallem *et al.*, 1985). The diacylglycerol activated by PLC then activates various protein kinase C (PKC) isoforms.

In the rodent pancreatic acinar cell model, physiological concentrations of CCK binding to the high affinity state of this receptor have been shown to generate transient calcium oscillations (Matozaki *et al.*, 1990). In contrast, stimulation of the CCK_1 receptor with high concentrations of CCK that would be expected to saturate both high and low affinity states of the receptor has been shown to cause a rapid transient elevation of intracellular calcium that peaks within seconds, followed by a smaller sustained increase (plateau) in intracellular calcium without oscillations. IP_3 has been shown to be responsible for the calcium responses to stimulation of this receptor by high concentrations of CCK in rodent pancreatic acinar cells, yet low concentrations of CCK are reported to result in no measurable IP_3 response (Matozaki *et al.*, 1990). The latter may reflect the relative insensitivity of the assay.

It has been postulated that the initial release of calcium in response to physiological concentrations of CCK is from IP_3-sensitive stores (Wakui *et al.*, 1990). This subsequently may result in the calcium-induced release of calcium from other IP_3-insensitive stores (Wakui *et al.*, 1990). ADP-ribose (cADPr) may be involved in one of these IP_3-insensitive pathways, acting on ryanodine receptors (Galione *et al.*, 1991; Meszaros *et al.*, 1993; Lee *et al.*, 1997). Both IP_3- and cADPr-sensitive calcium release channels have been shown to be involved in CCK-stimulated calcium spiking, with blockade of cADPr resulting in enhancement of the IP_3-induced calcium spiking (Thorn *et al.*, 1994). Other work in pancreatic acinar cells has suggested that CCK could elicit calcium spiking via either the cADPr or IP_3 pathways, with the level of intracellular glucose determining which of the two pathways would be predominant (Cancela *et al.*, 1998).

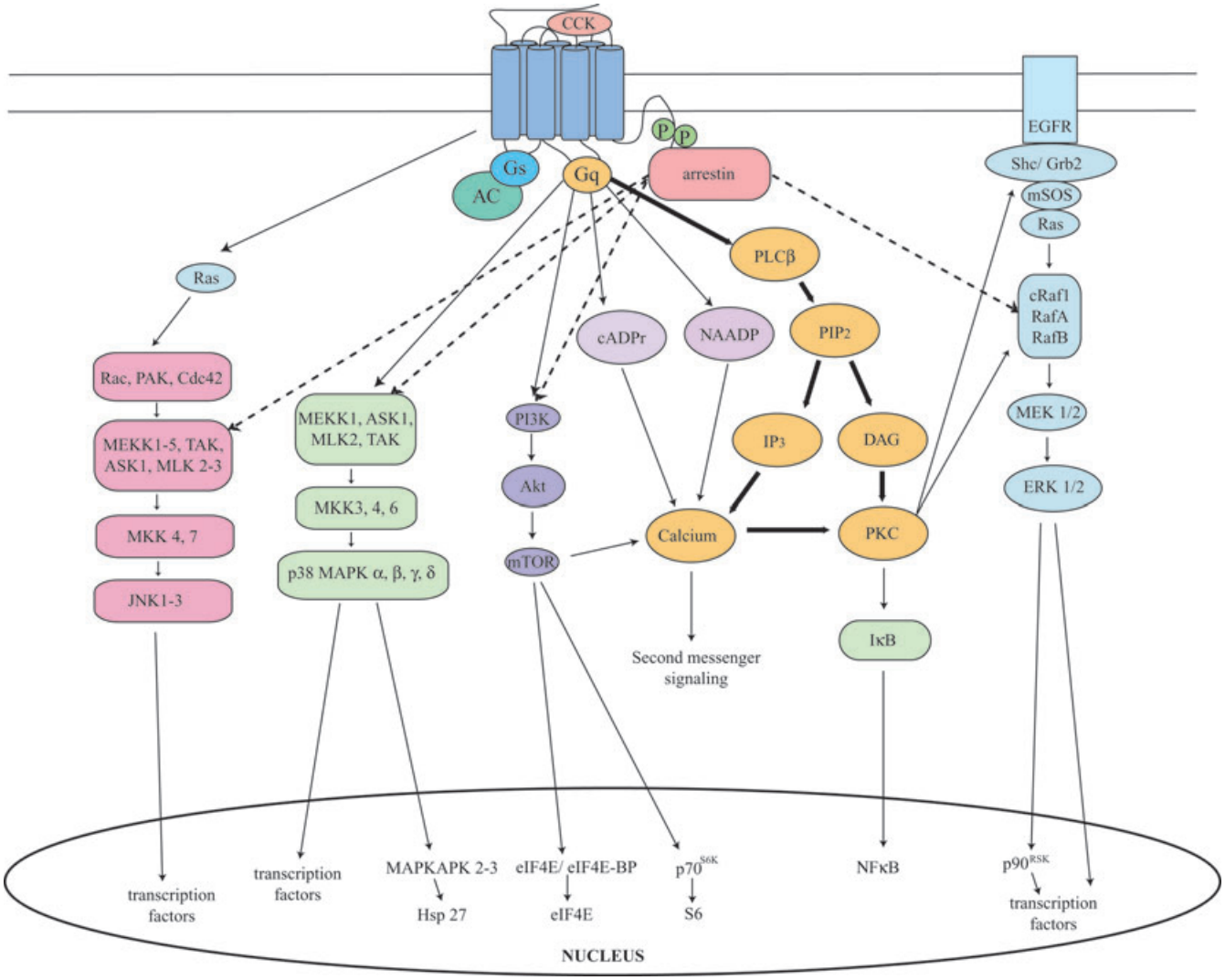

Figure 1 Schematic depiction of the signalling pathways stimulated by cholecystokinin (CCK) activation of the CCK_1 receptor. CCK_1 receptor activation by CCK is known to result in activation of G_q-initiated pathways with stimulation of classical second messengers (bold line) such as phospholipase C (PLC)/phosphatidylinositol 4,5-bisphosphate (PIP_2)/diacylglycerol (DAG) and protein kinase C (PKC)/inositol 3, 4, 5-triphosphate (IP_3) resulting in the release of intracellular calcium. Additionally, nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPr) pathways have also been implicated in the release of calcium after ligand stimulation of the receptor. A number of other signalling pathways have been shown to be activated by ligand stimulation of the CCK_1 receptor. These include the class I phosphatidylinositol 3-kinase (PI3K) pathway involving Akt and stimulation of mammalian Target of Rapamycin (mTOR) that results in downstream activation of transcriptions factors, the NF-κB pathway as well as the G_s-initiated adenylate cyclase (AC) pathway. Three mitogen-activated protein kinase (MAPK) pathways have been shown to be stimulated by CCK_1 receptor activation, p38-MAPK, extracellular-regulated kinase (ERK) and c-Jun amino-terminal kinase (JNK). The activation of the CCK_1 receptor has also been shown to stimulate the phosphorylation of epidermal growth factor receptor (EGFR), resulting in activation of Ras that in turn has been shown to be important in the activation of the JNK and ERK pathways. While no direct evidence has shown CCK activation of arrestin family proteins, these are known to have various signalling roles in many GPCRs and bind and potentially regulate several subsets of proteins from various signalling pathways (dashed lines).

Along with the cADPr and IP_3 pathways, the NAADP (nicotinic acid adenine dinucleotide phosphate) pathway has been implicated in the release of calcium from intracellular stores. NAADP is a metabolite of $NADP^+$ that is also synthesized by ADPr cyclase and releases calcium from stores that are physically separate from IP_3 and cADPr (Lee and Aarhus, 1995; Lee *et al.*, 1997). Yamasaki *et al.* (2005) produced some of the first evidence linking CCK_1 receptor stimulation to NAADP production, as well as demonstrating a difference in time course between NAADP and cADPr responses to physiological concentrations of CCK. They also postulated that physiological concentrations of CCK could initially enhance NAADP production and stimulate a localized increase in calcium release that would then activate cADPr-sensitized receptors. This, in turn, would allow the maintenance of calcium spiking for the duration of agonist stimulation of pancreatic acinar cells.

Mitogen-activated protein kinases (MAPKs) are serine-threonine-directed kinases that are activated by a variety of stimuli including various hormones. MAPKs have been shown to regulate a number of important cellular processes such as gene transcription, protein translation, metabolism and

cytoskeletal function. Three MAPK pathways, representing the ERK (extracellular-regulated kinase), JNK (c-Jun amino-terminal kinase) and p38 MAPK pathways, have been described in pancreatic acinar cells in response to CCK stimulation of the CCK_1 receptor. ERK1 and ERK2 are activated by mitogens in all cells and serve an important role in cell growth and mitogenic proliferation, as well as being implicated in the enhancement of cell cycle entry (Robinson and Cobb, 1997). Isolated rat pancreatic cells have been used to identify increases in ERK1 and ERK2 activities after stimulation with CCK, implicating both the PKC and tyrosine kinase pathways (Duan and Williams, 1994). CCK stimulation has also been shown to activate Ras, which then can activate Raf family members (RafA, RafB and cRaf1) (Dabrowski *et al.*, 1997). This group went on to show that CCK stimulation of the CCK_1 receptor activated MEK1/2 (MAP kinase or ERK kinase) that then could activate ERK1/2. CCK stimulation of pancreatic acinar cells has also been shown to result in the formation of a complex of Shc (Src homology/collagen related)-Grb2 (growth factor receptor-bound protein 2)-SOS (Drosophila homolog, Son of Sevenless) using a PKC-dependent mechanism. This group proposed that this may provide the link between G_q-coupled CCK receptor stimulation and Ras (Dabrowski *et al.*, 1996b). Using the pancreatic acinar carcinoma cell line AR42J, it was shown that CCK activation can cause tyrosine phosphorylation of EGFR (epidermal growth factor receptor), resulting in the subsequent formation of the Shc-Grb2-SOS complex (Piiper *et al.*, 2003). Additionally, it has been shown that CCK-stimulated activation of these signalling events can be abolished by transfection with a dominant negative Ras construct (Nicke *et al.*, 1999). Downstream of ERK1/2, the 90-kDa ribosomal S6 protein kinase ($p90^{RSK}$) is activated in a time- and dose-dependent manner (Bragado *et al.*, 1997b). ERKs and $p90^{RSK}$ have also been shown to activate transcription factors in other cell systems, but little is known about the specific transcription factors activated by CCK stimulation of the CCK_1 receptor.

Stress and CCK can activate the p38 MAPK pathway in pancreatic acinar cells, which can lead to the activation of MAPKAP kinase-2, resulting in an increase in Hsp27 phosphorylation (Schafer *et al.*, 1998). It has been shown that p38 MAPK activation is maximal 5 to 10 min after stimulation, which is similar to that seen with ERK1/2 stimulation. Additionally, it has been found that the CCK concentration needed to activate p38 MAPK is approximately in the range of 300 pM to 1 nM, similar to that needed for ERK1/2 activation and closer to the physiological range than that needed to obtain JNK stimulation. Higher concentrations of CCK could lead to a decrease in p38 MAPK activity. Other experiments showed that CCK stimulation of the CCK_1 receptor resulted in activation of the p38 MAPK pathway via G_q-coupled signalling (Schafer *et al.*, 1998). Other than Hsp27, the specific transcription factors that are activated by CCK-induced p38 MAPK activation are not known.

Another MAPK pathway that has been shown to be activated by CCK is that of JNK. It was identified and named after its ability to phosphorylate the amino terminus of the c-Jun transcription factor (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kallunki *et al.*, 1994). JNK has been implicated in mitogenic signalling and activation of JNK has been shown to induce apoptosis (Xia *et al.*, 1995; Ip and Davis, 1998). Early work in isolated pancreatic acinar cells stimulated with CCK described activation of two forms of JNK first described as protein 46 (p46) and protein 55 (p55) based on their apparent molecular weights, with the maximal activation produced after 30 min (Dabrowski *et al.*, 1996a). This work also demonstrated that the CCK concentration needed to stimulate JNK was ten times higher than that of ERK1/2 (Dabrowski *et al.*, 1996a). This group noted that in their cell model JNK was shown to be independent of PKC, calcium and cAMP, and was dependent on Ras (Dabrowski *et al.*, 1996a). While little is known about the role the JNK pathway plays in physiologic CCK_1 receptor activation, it has been suggested that JNK activation by high concentrations of CCK may induce pancreatitis (Dabrowski *et al.*, 1996a).

The class I phosphatidylinositol 3-kinases (PI3K) signalling pathway has been implicated in many different aspects of cell biology including vesicle trafficking, cell growth, DNA synthesis, as well as regulation of apoptosis and cytoskeletal changes (Vanhaesebroeck *et al.*, 2001). The molecular mechanism for PI3K activation by CCK is not well understood. However, it is able to phosphorylate and to activate Akt, which is known to have several targets including mammalian Target of Rapamycin (mTOR) (Vanhaesebroeck *et al.*, 2001). In acinar cells, mTOR activation has been related to the translational control of protein synthesis. Specifically, ribosomal protein S6 has been shown to be phosphorylated by $p70^{S6K}$ in response to CCK stimulation of pancreatic cells (Bragado *et al.*, 1997a). Another target for mTOR that has been described in pancreatic acinar cells stimulated by CCK is the binding protein for eukaryotic initiation factor 4E (eIF4E-BP). In isolated acinar cells and *in vivo*, CCK has been shown to stimulate eIF4E-BP phosphorylation, resulting in the release of eIF4E and the formation of an eIF4E-eIF4G complex (Bragado *et al.*, 1998).

The rat pancreatic acinar cell model has implicated the PI3K pathway in some potentially pathologic responses of pancreatic acinar cells, such as activation of the NF-κB transcription factor and intracellular activation of trypsinogen (Gukovsky *et al.*, 2004). These authors suggested that the PI3K pathway may play a role in acute pancreatitis induced by stimulation with supramaximal concentrations of CCK (Gukovsky *et al.*, 2004).

Recently, further investigation of the PI3K-Akt-mTOR pathway by Berna *et al.* (2009) identified some important distinctions between the Akt responses with high concentrations of CCK compared with that of low concentrations of this hormone. This group demonstrated that physiological concentrations of CCK could stimulate a 'biphasic' Akt response, with an increase in p85 phosphorylation and Akt activation by a Src-dependent mechanism. In contrast, high concentrations of CCK, possibly acting through a low affinity state of the CCK_1 receptor, were shown to be dependent on PLC and independent of Src or ERK (Berna *et al.*, 2009). In addition to this, it was found that high concentrations of CCK lead to a decrease in p85 tyrosine phosphorylation, PI3K activity, Akt activation and translocation to the membrane (Berna *et al.*, 2009).

An additional signalling pathway of interest includes activation of transcription factor NF-κB. This has been implicated

in inflammatory and cell death pathways (Gukovsky *et al.*, 1998; Steinle *et al.*, 1999). Supramaximal concentrations of cerulein have been shown to activate NF-κB in the isolated pancreas, and *in vivo* studies of experimental pancreatitis showed that NF-κB activation is one of the earliest events in pancreatic inflammation (Grady *et al.*, 1997; Gukovsky *et al.*, 1998). The hypothesis put forward by Gukovsky *et al.* (1998) was that NF-κB activation resulted in upregulation of cytokines associated with pancreatitis that mediate acinar cell death and inflammation (Gukovsky *et al.*, 1998). In rat pancreatic acinar cells, the translocation of PKC isoforms δ and ε is necessary for CCK-8-induced NF-κB activation (Satoh *et al.*, 2004). Activation has been shown to be regulated through calcium signalling (Gukovsky *et al.*, 2004) and to involve both phosphoinositol (PI)-specific PLC and phosphocholine (PC)-specific PLC (Satoh *et al.*, 2004).

Additional G protein-coupled pathways

The CCK_1 receptor has also been shown to be capable of coupling to G_S, with stimulation by high concentrations of CCK leading to increases in cAMP (Sjodin and Gardner, 1977; Yule *et al.*, 1993). Wu *et al.* (1997) have shown that CCK_1 receptors on HEK-293 cells are coupled to adenlyl cyclase (AC) exclusively through G_S. Furthermore, the specific residue, Asn82 in the first intracellular loop of the CCK_1 receptor, has been shown to be necessary for the cAMP responses observed after agonist stimulation (Wu *et al.*, 1999).

Another G protein that can be activated by CCK stimulation of this receptor is G_{13}. In NIH3T3 cells, stimulation of the CCK_1 receptor by CCK has been shown to activate G_{13} and to stimulate RhoA, resulting in the induction of actin stress fibres (Le Page *et al.*, 2003).

Other signalling pathways

Additional signalling molecules that have been shown to be stimulated after activation of the CCK_1 receptor include protein kinase D1 (PKD1), p125 focal adhesion kinase (125^{FAK}) and Src. PKD1 is a serine/threonine kinase (Valverde *et al.*, 1994) that has been shown to be activated by PKC family members (Waldron *et al.*, 2001). In rat pancreatic acini, the most relevant PKC isoform for this response was believed to represent PKC-δ (Berna *et al.*, 2007b). Activation of PKD1 may be of substantial importance, as it has been implicated in cell proliferation and invasion (Bowden *et al.*, 1999; Zhukova *et al.*, 2001).

The focal adhesion tyrosine kinase (FAK) $p125^{FAK}$ and the closely related proline-rich kinase 2 (PYK2) are cytoplasmic protein kinases that have been implicated in important processes such as cellular growth, motility, adhesion and changes to the cellular cytoskeletal system (Schaller, 2001). Pace *et al.* (2003) have shown that activation of the CCK_1 receptor results in rapid tyrosine phosphorylation of $p125^{FAK}$ and PYK2 in rat pancreatic acini. An additional protein implicated in the FAK signalling pathway is Lyn, a member of the Src family of kinases (SFK). Activation of the CCK_1 receptor by CCK has been shown to activate Lyn, resulting in association with PKC-δ, SHC, $p125^{FAK}$ and PYK2 in pancreatic acinar cells (Pace *et al.*, 2006).

Non-G protein-coupled signalling pathways

In addition to the classical G protein-mediated signalling pathways, it is now accepted that many GPCRs may also function through an additional pathway that involves arrestin family proteins. Studies with rhodopsin and the β-adrenergic receptor first described the role of arrestins in GPCR desensitization (Kohout and Lefkowitz, 2003). This mechanism involves the phosphorylation of agonist-stimulated GPCRs by one or more of seven G protein-coupled receptor kinases (GRK) that leads to the recruitment of one of four arrestin family members that impairs signalling by uncoupling of the receptor from its respective G protein (Freedman and Lefkowitz, 1996; Lefkowitz and Shenoy, 2005; Tobin *et al.*, 2008). It is now recognized that arrestin may also be involved in signal transduction, thought to be produced by the conformational change elicited when arrestin binds to GPCRs. Arrestin family members have been shown to bind to a number of signalling proteins and have been implicated in signalling via various pathways such as JNK, ERK and p38 MAPK (Shenoy and Lefkowitz, 2003). While no direct studies of this phenomenon have been performed with the CCK_1 receptor, the CCK_2 receptor has been shown to recruit and bind arrestin after agonist stimulation (Barak *et al.*, 2003), making it likely that arrestin plays a similar role in CCK_1 receptor activation.

Peptidic analogues

Two particularly useful peptide analogues of CCK have been identified as JMV180 and OPE (Galas *et al.*, 1988; Gaisano *et al.*, 1989). The JMV180 analogue corresponds to the carboxyl-terminal heptapeptide of CCK in which the carboxyl-terminal amidated phenylalanine residue has been replaced by a phenylethyl ester moiety and the amino terminus is blocked with a t-BOC moiety (t-BOC-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-O-phenylethyl ester) (Galas *et al.*, 1988). The OPE analogue ($_D$Tyr-Gly-Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-O-phenylethyl ester) differs in its amino terminus, where a Gly spacer and a $_D$Tyr site for direct radioiodination have been provided (Gaisano *et al.*, 1989; Gaisano and Miller, 1992).

The OPE and JMV180 peptide analogues of CCK have been shown to bind to the CCK_1 receptor in manner similar to CCK, yet they elicit only a subset of the biological responses as those stimulated by the natural hormone (Gaisano *et al.*, 1989; Sato *et al.*, 1989; Gaisano and Miller, 1992). Hence, they represent partial agonists. The biological responses stimulated by these agents correlate with the responses attributed to the high affinity state of the receptor, while they have been described as low affinity CCK_1 receptor antagonists, even reversing CCK-induced supramaximal inhibition of enzyme secretion (Gaisano *et al.*, 1989). JMV180 and OPE are fully efficacious stimulants of amylase secretion and do not show supramaximal inhibition with high concentrations as is

typical of CCK (Gaisano *et al.*, 1989; Sato *et al.*, 1989; Gaisano and Miller, 1992). These analogues stimulate intracellular calcium oscillations with spiking similar to that stimulated by low concentrations of CCK (pM) (Galas *et al.*, 1988; Gaisano and Miller, 1992). Both JMV180 and OPE do not stimulate measureable IP_3 responses, similar to that observed with low concentrations of CCK. At supramaximal concentrations of these agents, a low level of IP_3 response has been observed, but it is significantly lower than that observed after stimulation with equivalent concentrations of CCK (Gaisano *et al.*, 1994). Additional pathways have been investigated for JMV180 and OPE, such as pancreatic growth and the phospholipase A_2 signalling pathways. JMV180 has been shown to stimulate pancreatic growth, but its effect was shown to be 1000 times less potent than CCK (Rivard *et al.*, 1994). The intracellular calcium response elicited by JMV180 stimulation of the CCK_1 receptor was investigated further, and it was found that the calcium oscillations could be inhibited by a phospholipase A_2 (PLA_2) inhibitor, and a 2.5-fold increase in the intracellular levels of arachidonic acid (AA) was obtained in a biphasic manner (Tsunoda and Owyang, 1995). The authors of this study concluded that the high affinity CCK_1 receptor is coupled to the PLA_2 pathway to produce AA that mediates calcium oscillations and monophasic amylase secretion in rat pancreatic acinar cells. The authors did note that the intracellular calcium pool that is sensitive to the PLA_2 pathway is the same as or similar to the IP_3-sensitive pool (Tsunoda and Owyang, 1995).

CCK_1 receptor peptide analogues of CCK

This review will highlight some of the CCK_1 receptor agonists identified that have shown the most promise as anti-obesity therapeutic agents; however, this study will only briefly explore these compounds, as excellent detailed reviews have been published describing the chemistry of development of specific CCK_1 receptor agonists (Szewczyk and Laudeman, 2003; Berna *et al.*, 2007a; Garcia-Lopez *et al.*, 2007).

Tilley *et al.* published several studies in which they modified Ac-CCK-7 to change its functional profile (Tilley *et al.*, 1991; 1992b,c; Danho *et al.*, 1992). The modifications that produced functional changes were the replacement of the dipeptide Met28 and Gly29 with analogues incorporating 3-aminobenzoic acid and (1S)-trans-2-aminocyclopentanecarboxylic acid. These were high affinity, potent anorectic agents (Tilley *et al.*, 1992b). Modification of Asp32 with (R)-5,5-dimethylthiazolidine-4-carboxylic acid produced a peptide that exhibited high affinity, very good CCK_1 receptor/CCK_2 receptor selectivity and more effective inhibition of food intake than that of Ac-CCK-7 (Tilley *et al.*, 1992a).

A CCK-7 heptapeptide analogue, A71378 (des-NH_2-Tyr(SO_3H)-Nle-Gly-Trp-Nle-(NMe)Asp-Phe-NH_2), was described in the literature in 1990 and was found to be 700-fold more selective for pancreatic CCK_1 receptor and a full agonist for stimulation of intracellular calcium (Lin *et al.*, 1990). Another class of potent and selective CCK_1 receptor agonists characterized was based on the replacement of Met31 in CCK-4 tetrapeptide with a substituted lysine. The two most potent compounds identified were A71623 and A70874, both without the tyrosine residue thought to be required for potent CCK_1 receptor activity in larger peptides (Liddle, 1989). A71623 (Boc-Trp-Lys(o-tolylaminocarbonyl)-Asp-MePhe-NH_2) and A70874 (Boc-Trp-Lys(p-hydroxycinnamoyl)-Asp-(NMe)Phe-NH_2) are 1200- to 140-fold selective for pancreatic CCK_1 receptors over brain CCK_2 receptors respectively. Functionally, A71623 is a fully efficacious agonist in producing PI hydrolysis, similar to CCK-8, and shows supramaximal inhibition of amylase secretion. In contrast, A70874 is only partially efficacious in stimulating PI hydrolysis and does not show supramaximal inhibition of amylase secretion, as well as reversing the inhibition produced by supramaximal concentrations of CCK-8. The amylase secretion profile of A70874 is very similar to JMV180 and OPE, except A70874 possesses greater efficacy to stimulate amylase secretion (Lin *et al.*, 1991).

Further modifications to CCK-8 resulted in FPL14294 (also known as ARL142941 and ARR142941) (Hpa(SO3H)-Met-Gly-Trp-Met-Asp-(Me)Phe-NH2) (Simmons *et al.*, 1994) and then the improved analogue ARR15849 (also known as ARL15849) (4-hydroxyphenylacetyl(SO_3H)-Nle-Gly-Trp-Nle-(Me)Asp-Phe-NH_2) (Pierson *et al.*, 1997). ARR15849 was synthesized by moving the N-methyl group from Phe to Asp, which resulted in a decreased affinity to the CCK_2 receptor, without affecting its affinity for the CCK_1 receptor, giving a 6600-fold selectivity for the CCK_1 receptor (Pierson *et al.*, 1997). ARR15849 was shown to inhibit food intake with nanomolar potency following intraperitoneal administration in rat and intranasal administration in beagle dogs (Simmons *et al.*, 1994; Pierson *et al.*, 1997), supporting a CCK receptor-mediated effect.

Non-peptidyl CCK_1 receptor agonists

Peptide ligands have limited therapeutic utility due particularly to limited bioavailability and substantial metabolism. For this reason, there is much more enthusiasm for the development of non-peptidyl ligands that can be administered orally. One such approach was the characterization of the non-peptidyl tetra-substituted tryptophan derivative, PD149164, that was shown to have full agonist activity at the CCK_1 receptor, but lower potency than natural CCK (Hughes *et al.*, 1996). This research group took the approach of designing a non-peptidyl agonist, PD149164, by appending a key feature of the selective peptide agonist A71623, described above. PD149164 was shown to have a higher affinity for the CCK_2 receptor than for the CCK_1 receptor, but the enantiomer, PD151932, was shown to have a much higher binding affinity for the CCK_1 receptor; however, it was found to represent an antagonist. Functional studies of the PD149164 agonist showed that it elicited a maximal amylase response both *in vitro* and *in vivo*, and produced intracellular calcium oscillations similar to CCK-8 (Hughes *et al.*, 1996). The same group made further modifications to PD149164 to improve its affinity and potency at the CCK_1 receptor and the resulting compound was called PD170292 (Bernad *et al.*, 2000). The pharmacological profile was noted by the authors to be similar to that of JMV180 (Galas *et al.*, 1988).

Thiozole derivatives: (SR146131)

The biological activity *in vitro* of the thiazole derivative, SR14613, was described by Bignon *et al.* in 1999 and it was shown to be a potent full agonist for intracellular calcium responses and IP_1 formation at the CCK_1 receptor, but only a partial agonist for MAPK activation and early gene expression (Bignon *et al.*, 1999b). The *in vivo* effects of SR14613 investigated by the same group showed it was able to inhibit gastric emptying and reduction in gallbladder volume in mice after administration of low oral doses (Bignon *et al.*, 1999a). SR14613 was also shown to reduce food intake in rodents as well as in one primate species (Bignon *et al.*, 1999a).

1,5-benzodiazepine derivatives

In 1996, Aquino *et al.* explored a company registry for novel leads towards the development of non-peptidyl CCK_1 receptor agonists. Compounds were selected based on structural features of CCK_1 receptor agonist peptides that had been described, in particular the features present in tetrapeptide, A71623, and then were evaluated for contractibility in isolated guinea pig gallbladder. The group identified a series of 1,5-benzodiazepines as potential CCK_1 receptor agonist candidates (Aquino *et al.*, 1996). The efficacy of the compounds in this series was shown to be modulated by variation of substituents on the N1-anilinoacetamide moiety. Further studies of the series of 1,5-benzodiazepine compounds were shown to have decreased affinity for the CCK_1 receptor compared with CCK-8, but some showed equipotent anorectic effects in rats following intraperitoneal administration (Aquino *et al.*, 1996). Since the first reports of the 1,5-benzodiazepine series exhibiting CCK_1 receptor agonist effects, the GSK compound GI181771 progressed to phase II clinical trials for the treatment of obesity (Szewczyk and Laudeman, 2003). Two clinical trials in humans have been reported investigating the role of GI18177X as a novel oral CCK_1 receptor agonist (Castillo *et al.*, 2004; Jordan *et al.*, 2008). The first study carried out by Castillo *et al.* in 2004 looked in particular at the effects of GI18177X on gastric emptying of solids, gastric accommodation and postprandial symptoms. The study looked at the effects of a four dose regime and a placebo on gastric functions and postprandial symptoms in a group of 61 healthy men and women (for more details of the study parameters refer to Castillo *et al.*, 2004). The results showed GI18177X treatment resulted in delays in gastric emptying of solids, increased fasting gastric volumes and an acceptable safety profile. In 2008, Jordan *et al.* extended the clinical studies by investigating whether GI18177X could induce weight loss in obese patients. Unfortunately, GI18177X did not reduce the body weight of obese patients and had no effects on waist circumference or cardiometabolic risk markers. The authors noted that mild tolerability-limiting gastrointestinal events, including vomiting, were observed and a small number of study subjects showed treatment-related events of cholecystitis and gallstone-related pancreatitis (Jordan *et al.*, 2008).

While the clinical studies of the 1,5-benzodiazepine, GI18177X, have showed varied results, this class of compound has lead to interesting developments in relation to drug discovery for a CCK_1 receptor agonist. Our group identified the binding sites for active enantiomers in racemic mixtures of structurally related benzophenone derivatives of 1,5-benzodiazepine for both agonist and antagonists to the CCK_1 receptor (Hadac *et al.*, 2006). We have also carefully examined the molecular basis of binding of other benzodiazepine ligands for this receptor using both receptor mutagenesis and classical analysis of allosterism (Gao *et al.*, 2008). This work has clearly established the allosteric nature of the binding of such ligands within the helical bundle, in a site totally distinct from the natural peptide-binding site that resides within the extracellular loop and tail regions at the surface of the lipid bilayer.

The therapeutic potential of drugs that act on CCK_1 receptor

To this point, we have described some of the important roles of the CCK_1 receptor, both physiologically and pathophysiologically, as well as some of the key signalling pathways initiated after stimulation with the natural ligand, CCK. We have also described some of the most promising CCK_1 receptor agonists developed to date and the findings of the clinical trials with these candidate agonists. It is clear from reviewing the literature that there is a need for the development of a more selective and safer CCK_1 receptor agonist that could have key roles in the treatment of common clinical syndromes, in particular obesity. There are three potential concepts that could be explored to increase specificity in relation to the activation of the CCK_1 receptor by an agonist. These concepts are allosteric modulation, partial agonism and biased agonism.

Researchers have long been interested in cell surface receptors such as GPCRs as targets for drug development. Until recently, the focus of drug design for GPCRs has been on the development of agonists acting at the orthosteric site of a specific GPCR, but many of these have been shown to have a high incidence of tolerance and dependence. Therefore, the concept of an allosteric modulator that has been defined by the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification in 2003 (Neubig *et al.*, 2003) as 'a ligand that increases or decreases the action of an agonist or antagonist by combining with a distinct site on the receptor macromolecule' has become an area of great interest in the pharmaceutical industry with regard to GPCRs.

The development of allosteric modulators to GPCRs is of benefit as such agents may not exhibit excessive activation of a specific GPCR. As stimulation of the receptor with an allosteric modulator would be dependent on the presence of both the natural agonist ligand and the allosteric modulator simultaneously, the enhanced effect would only occur during the brief post-cibal periods when the peptide hormone, having a very short half-life, would be in the circulation. Thus, an allosteric modulator theoretically might decrease the risk of desensitization and tolerance that could be observed with a ligand acting at the orthosteric site. Secretion of the endogenous hormone CCK that occurs after ingestion of food has a

major role in the digestion of food, as well as in stimulating the feeling of satiety, therefore an allosteric modulator that might enhance the effects of CCK in patients in whom it does not have strong effects, could return towards a more physiologic state. Additionally, the enhancement elicited by the allosteric modulator would be dependent on the number of receptors occupied by both the allosteric ligand and the endogenous hormone, perhaps limiting the possibility of overdose compared with an orthosteric drug (Christopoulos, 2002).

One of the more important issues that need to be raised in relation to drug design of a CCK_1 receptor agonist is its selectivity between CCK_1 and CCK_2 receptors. A desirable drug needs to have minimal or no biological activity at the CCK_2 receptor, as this receptor may mediate panic attacks and has other CNS actions (Dauge and Lena, 1998) that may be undesirable. Having a therapeutic drug that has either no stimulation of the CCK_2 receptor or is a CCK_2 receptor antagonist would be important, due to the chronic nature of obesity and due to the need for such a drug to be administered over a long term (Szewczyk and Laudeman, 2003). Another theoretical benefit of an allosteric ligand is that allosteric sites are generally less conserved than orthosteric sites in a particular receptor family, thus potentially enabling even greater specificity (Birdsall and Lazareno, 2005). Additionally, targeting of an allosteric modulator may be more effective than the orthosteric site as topographically it may be difficult for a small molecule to gain access (Jensen and Spalding, 2004). Of interest is that recently the benzodiazepine antagonist devazepide has been shown to bind to an allosteric site within the helical bundle region of the CCK_1 receptor (Hadac *et al.*, 2006; Gao *et al.*, 2008), thus identifying a potential site for further development of an allosteric modulator that might act as an enhancer of CCK stimulation of the CCK_1 receptor and might increase specificity.

One of the first descriptions of 'partial agonism' was made by Ariens and de Groot in 1954, and since then a partial agonist has been defined by the IUPHAR Compendium of Receptor Characterization and Classification (1998) as an agonist that 'cannot elicit as large an effect . . . as can a full agonist acting through the same receptors'. Partial agonists have been shown to have several benefits, one of these being the reduced propensity to elicit side effects or to desensitize their target receptors (Clark *et al.*, 1999). This adds to their therapeutic potential. Additionally, partial agonist ligands acting at the orthosteric site of a receptor can also reduce the effects of the natural full agonist by competing for its binding (Ohlsen and Pilowsky, 2005). In regard to the CCK_1 receptor, the partial agonists OPE and JMV180 have been shown to stimulate a subset of the biological actions of CCK. OPE and JMV180 are partial agonists to the CCK_1 receptor and do not exhibit supramaximal stimulation of amylase secretion (Gaisano *et al.*, 1989; Sato *et al.*, 1989; Gaisano and Miller, 1992), and they stimulate calcium oscillations and IP_3 levels that are similar to those seen with low concentrations of the natural agonist, CCK (Galas *et al.*, 1988; Gaisano and Miller, 1992; Gaisano *et al.*, 1994). It is, therefore, conceivable that an allosteric partial agonist exhibiting similar functional characteristics to those of OPE and JMV180 could be of substantial therapeutic benefit.

Until recently, it has been thought that an agonist was a ligand that would bind to its receptor in an inactive state and would induce a conformational change promoting the active state of the receptor, thus initiating second messenger signalling that might ultimately be limited by desensitization. It was also thought that the binding of an agonist stimulated all receptor functions to an approximately equal extent due to the conformational change from an inactive to the active state (Benovic *et al.*, 1988). Therefore, in previous years, the determination of a ligand's efficacy was generally determined by its ability to stimulate one second messenger pathway. Over the past decade, however, evidence has been produced showing that the original explanation of agonist stimulation is more complex than had been believed. The terms 'biased agonists' or 'functionally selective agonists' were first described in relation to the ability of a GPCR to selectively interact with various G protein signalling pathways (Kenakin, 1995; Lawler *et al.*, 1999). These terms have now been extended to encompass other signalling pathways such as the G protein-independent, arrestin-mediated signalling which has been extensively described in relation in relation to GPCRs (Lefkowitz, 2004; Terrillon and Bouvier, 2004; Lefkowitz and Shenoy, 2005; Luttrell, 2005; Lefkowitz *et al.*, 2006). The concept of biased agonism may be extrapolated to the development of a CCK_1 receptor agonist that is biased towards the signalling pathways responsible for satiety, but that does not simulate pathways implicated in trophic effects on the pancreas that also may induce pancreatitis and other side effects that have hindered the development of a therapeutically useful CCK_1 receptor agonist. In relation to CCK_1 receptor, biased agonist pathways that may be selected for are those of the G_q second messenger pathway shown to be responsible for the secretion of amylase, in particular having a bias towards agonists that do not exhibit supramaximal stimulation of amylase and the signalling pathways that are associated with it.

In summary, there have been substantial increases in our knowledge of the CCK_1 receptor, its physiology and its potential role in the treatment of obesity. Additionally, several of the potentially important signalling pathways that are activated in response to both physiological and pathological levels of CCK have been described. To date, several peptide and non-peptidyl CCK_1 receptor agonists have been developed with only a few progressing to clinical trials, and these having had properties that precluded their further development. Therefore, future development of useful CCK_1 receptor-active drugs may include allosteric modulators, biased agonists and partial agonists.

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Conflict of interest

No specific conflicts recognized.

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