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The Biochemical Characterization of the Native Pancreatic Cholecystokinin Receptor Using Affinity Labeling Approaches

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Affinity labeling has been a powerful tool for the biochemical characterization of sparse molecules which bind to a ligand probe in a specific, high-affinity manner. The rat pancreatic acinar cell receptor for cholecystokinin (CCK), the major physiologic hormonal stimulant of pancreatic exocrine secretion, has been the target of such investigation. Of interest, affinity-labeling studies have identified two distinct plasma membrane glycoproteins as candidates to represent this receptor. The initial candidate, which was identified using ^{125}I-Bolton Hunter-labeled CCK-33 as probe, migrates on a SDS-polyacrylamide gel as a broad band in the M_r = 80,000 range. Subsequently, using shorter probes in which the site of covalent attachment was closer to the receptor-binding domain of the probe, a band of M_r = 85,000–95,000 was specifically labeled. Deglycosylation and protease-peptide mapping demonstrated that these bands represent distinct molecules. Using "intrinsic" probes of the receptor, in which a photolabile residue was sited within the pharmacophoric domain of the ligand, attention was focused on the latter candidate as representing the binding protein. Insight into the relationship between these proteins as they reside in the plasma membrane was contributed by labeling with a "topographical mapping" probe, which incorporates a flexible spacer of variable length between a CCK-like ligand and a photolabile residue. This procedure confirmed that these two minor membrane proteins are spatially associated with each other. In addition to the identification of proteins which represent the hormone-binding protein and proteins associated with it, affinity-labeling approaches can provide structural information about post-translational modifications of these proteins, which clearly complement and extend the primary amino acid sequence data now made available by receptor cDNA cloning. There are data to support the glycosylation and phosphorylation of the rat pancreatic CCK receptor. In addition, it is possible to use photoaffinity labeling with "intrinsic" probes to label directly domains and residues in the receptor which are critical to agonist binding.

Cholecystokinin (CCK) is the major physiologic hormonal stimulant of pancreatic exocrine secretion. Its effects are initiated by a high-affinity, specific binding interaction with a plasmalemmal receptor for this hormone on the pancreatic acinar cell. Although CCK was one of the earliest recognized gastrointestinal hormones, discovered over 60 years ago [1], the characterization of receptors for this hormone is a very recent phenomenon. The reason for this delay relates to the extremely small number of these receptors on target cells, and the methodological difficulties in characterizing these.

As is the case for many hormone receptors, the earliest characterization of the

Abbreviations: CCK: cholecystokinin PEG: poly(ethyleneglycol)

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CCK receptor was functional, using classical pharmacological techniques of description of the biological response to structural analogs of the native hormone. These studies clearly localized the biologically active domain of cholecystokinin peptides to their carboxyl-terminal heptapeptide-amide [2]. This domain is shared by all molecular forms of this hormone and is the domain which defines the CCK/gastrin family of hormones. Gastrin shares the identical carboxyl-terminal pentapeptide amide with CCK and indeed can interact with CCK receptors as a weak agonist [3].

Currently, receptors for this family of hormones can be classified into two main groups [4]: *(i)* the type A, or "alimentary" type of receptor, present on the pancreatic acinar cell, the gallbladder muscularis smooth muscle, pyloric smooth muscle, and a few select brain nuclei; and *(ii)* the type B/gastrin receptor, which is the predominant type in the "brain" and on the gastric parietal cell. The type A receptor, which is the focus of this review, is the most selective receptor in the CCK-gastrin family, requiring the entire carboxyl-terminal heptapeptide, including a sulfated tyrosine for high-affinity binding. The type B and gastrin receptors require only the carboxyl-terminal tetrapeptide for high-affinity binding. There has been considerable discussion as to whether the type B and gastrin receptors represent the same or different receptors [5]. Subtle affinity and potency differences have been reported for different structural analogs of CCK and gastrin at these two receptors; however, these observed differences may represent only differences in species and tissues, or differences in laboratory technique. Although a variety of receptor antagonists can clearly distinguish between the type A and type B/gastrin receptors [6], none reported to date can distinguish between the type B receptor and the gastrin receptor.

DESIGN OF CCK RECEPTOR PROBES

The chemical structure of the carboxyl-terminal domain of CCK has been responsible for some of the difficulty in the direct characterization of this receptor. This region includes a sulfated tyrosyl residue which is critical for high-affinity binding, and which is labile in acidic conditions. The carboxyl-terminal phenylalanine-amide is also important for high-affinity binding and is base-labile. Present in this region of the peptide are two methionines, which are sensitive to oxidation, markedly interfering with binding and biological activity. These structural features of CCK made the development of a high specific radioactivity probe based on the structure of the native hormonal ligand a challenge.

Radioiodination is the technique of choice to radiolabel a peptide ligand in order to achieve the high specific radioactivity which is necessary to characterize the small number of receptors present on native targets of this hormone (on the order of 5,000 molecules per cell) [7]. Native CCK cannot be radioiodinated oxidatively due to the absence of an unsubstituted tyrosine and to the presence of the critical methionine residues. The other major method for radioiodination utilizes acylation with a radioiodinated residue, such as the Bolton-Hunter reagent [8]. Bolton-Hunter labeling is applicable to CCK since there is an alpha amino group present and available for acylation, with structure-activity data supporting the possibility of derivatization of that residue with multiple different chemical moieties [9].

Of interest, the first high specific radioactivity analog of CCK to be produced was CCK-33, radioiodinated using the Bolton-Hunter reagent [10]. While this analog was initially used as a radioligand in radioimmunoassay, it is also useful as a receptor

ligand. Indeed, it was subsequently used in both direct radioligand binding studies and in affinity-labeling studies [7]. Subsequently, methodology for the acylation of CCK-8 by Bolton-Hunter reagent was also reported [9]. Bolton-Hunter-labeled CCK-8 has proven to be quite useful in CCK receptor-binding studies; however, the absence of an available nucleophilic group in that molecule makes it unacceptable for affinity labeling of the receptor.

A major advance occurred when it was appreciated that the two methionines present within the carboxyl-terminal heptapeptide of CCK could be replaced with threonines and/or norleucine, without interfering with receptor binding [11]. These substitutions made the peptide stable to oxidative conditions, and potentially amenable to oxidative radioiodination. Oxidative radioiodination is much preferable to the acylation type of radiolabeling using Bolton-Hunter reagent, since it is less expensive, more rapid, and provides a greater yield of product. The latter, in particular, is important for a technique such as affinity labeling, which utilizes a very large amount of radiolabeled probe.

A CCK analog in which the methionines were replaced with oxidation-resistant residues was, therefore, further modified to incorporate a tyrosine on its amino terminus [12]. Oxidative radioiodination of this peptide was successful, providing a radiolabeled probe which bound to the receptor with appropriate affinity and specificity [12]. That probe, however, as originally designed, was degraded quickly when incubated with a protease-rich tissue such as pancreas. To overcome this problem, a probe was designed in which unnecessary basic residues were eliminated, and which incorporated a D-tyrosine at its amino terminus [13]. This process provided an aminopeptidase-resistant probe which could be oxidatively radioiodinated to high specific radioactivity while maintaining high-affinity binding to the CCK receptor [13]. Also, unlike Bolton-Hunter-labeled CCK-8, this probe had an available alpha amino group for affinity labeling, using bifunctional cross-linking. This probe has become the template for a series of additional probes of the CCK receptor which our laboratory has developed.

AFFINITY LABELING OF THE CCK RECEPTOR

Affinity labeling has been a powerful method for the biochemical characterization of sparse hormone receptors [14]. These molecules, although relatively few in number, bind to ligand probes with high affinity and specificity. By incorporating a high specific radioactivity radiolabel into the probe, this technique provides an opportunity to radiolabel the receptor in a specific manner. In an affinity-labeling experiment, the probe is allowed to bind to the receptor under optimal conditions, and unbound probe is washed away. Then, using either bifunctional chemical cross-linking reagents or a photolabile residue which may actually be incorporated into the probe, covalent attachment is attempted. In the case of the use of bifunctional cross-linking reagents, it is critical that an appropriately reactive group be present and available in the radioligand probe. Even when that is the case, the efficiency of this type of cross-linking is typically very low, since the spatial approximation and geometry of that residue and an appropriate target residue on the receptor are critical [15]. Using too high a concentration of cross-linker may also result in the serial attachment of several adjacent proteins, matting them together to provide a misleading apparent size of the receptor complex. Photoaffinity labeling provides the theoretical advantage of being more reactive, and therefore less selective, than the

bifunctional chemical cross-linking reagents. In addition, by incorporating such a reagent into the probe, only a single bond can be formed, and the possibility of the serial cross-linking of several adjacent proteins is eliminated.

All of these approaches have been used in the characterization of the pancreatic CCK receptor. The initial report of specific affinity labeling using a CCK probe utilized Bolton-Hunter-labeled CCK-33 and a series of bifunctional chemical and photochemical cross-linkers [7]. In that work, the major membrane protein which was consistently labeled migrated on a SDS-polyacrylamide gel as a broad band around M_r = 80,000. Of interest is the fact that a number of other proteins were also labeled using this approach, but their labeling was not consistent from one cross-linker to another. A protein of approximately M_r = 40,000 was present, linked via disulfide bond to this M_r = 80,000 protein for part of the time. The observations of these proteins in affinity labeling, using CCK-33-based probes, was subsequently confirmed by other groups [16]. All of the traditional controls were satisfied by these studies: namely, the labeling was inhibited in a concentration-dependent manner by peptides structurally related to CCK, the labeling was dependent on the presence of cross-linker, and, in photoaffinity labeling, it was photolysis-dependent.

Because the receptor-binding domain of CCK is known to be present in its carboxyl-terminal heptapeptide, far removed in primary sequence from the sites of potential cross-linking using CCK-33-based probes (epsilon amino groups on lysines in positions one and eleven), affinity labeling was then performed, using "shorter" probes in which the site of cross-linking was adjacent to this pharmacophoric domain [13,17]. For this procedure, the oxidatively labeled short CCK receptor probes discussed above were used. Though this work was originally performed as a control experiment, to confirm the identity of the M_r = 80,000 protein as the CCK-binding subunit of the receptor, it surprisingly identified a distinct protein of apparent M_r = 85,000–95,000 [17]. This finding proved true, using a series of short probes [18]. The distinct nature of these two proteins was further documented in studies in which both were deglycosylated to yield protein cores of different size [19] and in studies in which the protein cores were mapped, using specific proteases [20]. The M_r = 80,000 protein originally described appears to have a core protein of M_r = 65,000 [21], while the M_r = 85,000–95,000 protein, first identified using the shorter probes, appears to have a core protein of M_r = 42,000 [17].

While the two affinity-labeled proteins which were thought to be candidates to represent the pancreatic CCK receptor were clearly distinct, there was still a question as to which was the most likely candidate to represent the CCK-binding protein. Our bias was that the protein labeled when the site of cross-linking was closest to the receptor-binding domain was most likely to represent that protein. In order to test this hypothesis experimentally, new probes were developed, which incorporated photolabile residues for photoaffinity labeling within, or "intrinsic" to, the receptor-binding domain. Three such probes were developed, which utilized nitro- or azido-aromatic residues in positions of native aromatic residues within the pharmacophoric domain of CCK [22,23]. These probes included ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$, pN_3-Phe^{33})CCK-26-33], ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$,pNO_2-Phe^{33})CCK-26-33], and ^{125}I-D-Tyr-Gly-[($Nle^{28,31}$, 6-NO_2-Trp^{30})CCK-26-33]. The structures of two of these probes appear in Table 1. These were shown to be fully efficacious pancreatic secretagogues, which interacted specifically with the CCK receptor. The photolability of the modified aromatic residues was documented, and the ability to activate

TABLE 1
Some of the Substitutions in the Structure of Native CCK

	CCK Analogues
Native CCK-8	Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe(NH_2)
Oxidation resistant	-Nle- -Nle-
"Short"	[^{125}I]*Tyr*-*Gly*- -Nle- -Nle-
"Intrinsic"	[^{125}I]Tyr-Gly- -Nle- -Nle- *(NO_2)* -*Phe*(NH_2)
	[^{125}I]Tyr-Gly- -Nle- *(NO_2)* -*Trp*-Nle-
"Topographical mapping"	
NPA----PEG_n---[^{125}I]Tyr-Gly-	-Nle- -Nle-

These substitutions yield receptor probes which bind with appropriate specificity and high affinity, which can be radioiodinated to high specific radioactivity, and which are cross-linkable and/or photolabile (through residues shown in italics and underlined).

these specifically upon photolysis with a 300 nm lamp was established. All of these probes affinity-labeled the same M_r = 85,000–95,000 pancreatic plasma membrane glycoprotein with protein core of M_r = 42,000 [22,23] (Fig. 1). This result was interpreted as confirmation that this protein represented the hormone-binding subunit of the CCK receptor. It was postulated that the M_r = 80,000 protein originally identified was associated with this protein, since it was clear that it represented a minor membrane protein, which was specifically labeled by a number

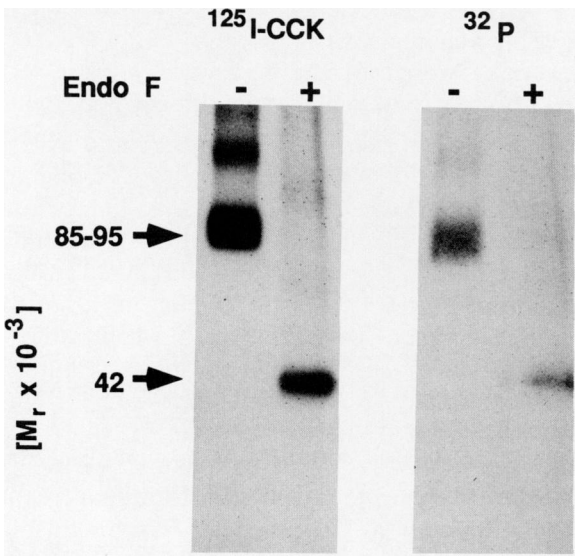

FIG. 1. Autoradiographs of typical SDS-polyacrylamide gels used to separate products of affinity labeling the rat pancreatic CCK receptor (*left*) or phosphorylating this molecule (*right*). Shown are lanes in which the mature (M_r = 85,000–95,000) and deglycosylated (M_r = 42,000) proteins are separated.

of different laboratories. That labeling could not be attributed solely to artifact or to the chance association of a prominent membrane protein.

To explore further the possible spatial association between the two affinity-labeled proteins, "topographical mapping" probes were designed and synthesized [24]. These probes were designed to separate the receptor-binding domain of the probe from a photolabile residue by a variable-length flexible spacer. For this spacer, we used poly(ethyleneglycol) (PEG), which is known to assume a random coil and not to interfere with the native conformation of associated peptides. The polymeric nature of this reagent made it easy to vary the length of the spacer in a systematic manner. It was postulated that the probes with the shortest spacers would label only the CCK-binding subunit, while associated subunits would be labeled when the spacer attained the appropriate length to reach it. Indeed, the shortest such probes labeled only the pancreatic plasma membrane glycoprotein with apparent M_r = 85,000–95,000, which has a protein core of M_r = 42,000. It was only when the probe incorporated the polymeric spacer with ten ethyleneglycol monomer units (approximately 16.2 Å) that the M_r = 80,000 protein began to be labeled [24].

These data are consistent with the M_r = 85,000–95,000 protein representing the CCK-binding subunit of the receptor, with an associated M_r = 80,000 protein. The CCK receptor is now known to represent a guanine nucleotide-binding protein (G protein)-associated receptor, with seven transmembrane segments predicted [25]. There is no current precedent for an additional protein to be associated with such a receptor protein, and it is clear from expression cloning that a single protein in this family is capable of binding ligand normally, associating with its guanine nucleotide-binding protein, and initiating intracellular activation cascades. If this association holds true, it may be possible that the associated protein could play a role in regulation of receptor function, such as modulation of the biological response or binding of the hormone. Studies of such a protein will be dependent on its further purification and characterization.

APPROACHES TO DEFINE POST-TRANSLATIONAL MODIFICATIONS AND RECEPTOR DOMAINS

In addition to the identification of the pancreatic CCK receptor and potentially associated proteins, methods of working with the mature, naturally expressed receptor such as affinity labeling have been able to demonstrate directly additional structural features of the receptor which cDNA cloning can only predict. Included among these features are the possibilities of the post-translational modifications of receptor glycosylation and receptor phosphorylation.

Glycosylation may be studied by removal of the carbohydrate from the mature receptor, using chemical or enzymatic methods. The enzymatic approach has the potential advantage of being specific for certain types of carbohydrate and certain linkages. By using limited amounts of enzyme and separating the products of receptor deglycosylation, it has also been possible to predict the number of carbohydrate chains associated with the receptor. Both the M_r = 85,000–95,000 and the M_r = 80,000 proteins have been shown to be complex, N-linked (via asparagine residues) sialoglycoproteins with three or more carbohydrate chains [19,21]. Neither glycoprotein has been demonstrated to have O-linked carbohydrate, although a small amount of this type cannot be fully excluded.

Receptor phosphorylation has been demonstrated by biosynthetic labeling of the

receptor in the intact cell after radiolabeling the ATP pool with ^{32}P [26] (Fig. 1). Using affinity adsorption methods developed and validated with affinity-labeled pancreatic CCK receptor, it was possible to demonstrate that the CCK receptor was phosphorylated in response to agonist stimulation; this process was appropriately blocked by CCK receptor antagonists. Also, increased receptor phosphorylation was observed after stimulation of the pancreatic acinar cells with heterologous agonists, which are known to act via intracellular cascades similar to those initiated by CCK [26]. Agents such as carbamylcholine and TPA have been shown to elicit heterologous desensitization of the CCK receptor, a well-described general effect of receptor phosphorylation. There are preliminary data to support the existence of at least two pancreatic enzymes which phosphorylate the CCK receptor *in vivo*. Investigation of the identity of these enzymes, their sites of action, their regulation, and the biological effects of their action are in progress.

Perhaps the most unique and important contribution to our understanding of receptor structure and function possible with affinity labeling is the use of this technique for the direct identification of the hormone-binding domain of a receptor. It is even theoretically possible to identify specific interacting residues within the receptor, using this approach. The development of "intrinsic" affinity-labeling probes, with a photolabile residue within the pharmacophoric domain of the ligand, as described above, is the first step toward this goal [22,23]. Using those probes and proteolytic cleavage of the labeled receptor, it has been possible to identify a general domain of the receptor on which to focus [20]. With the current availability of primary sequence information about receptors in this family, it should be possible to extend this "active site mapping" to a higher level of detail. This goal will also require the development of additional, more reactive "intrinsic" probes.

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