0026-895X/90/020159-05$02.00/0

MOLECULAR PHARMACOLOGY, 38:159-163

ACCELERATED COMMUNICATION

Biochemical Characterization of the Cholecystokinin Receptor on CHP212 Human Neuroblastoma Cells

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Received January 19, 1990; Accepted May 5, 1990

SUMMARY

Cholecystokinin (CCK) receptors reside on a large number of cell types along the digestive tract and in the nervous system. A human neuroblastoma cell line (CHP212) has recently been described to express a type A receptor, with structural specificity similar to that on pancreatic acinar cells and gall bladder smooth muscle cells but different from the predominant type of binding site found in brain (type B). In this work, we have performed photoaffinity labeling and protease peptide mapping of the CHP212 receptor and have compared it to other type A CCK receptors. ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})-CCK-26-33], a probe that possesses a photolabile residue at position 33 within the theoretical receptor-binding domain of this hormone, specifically labeled a M_r = 80,000-90,000 glycoprotein on this cell line, while labeling larger proteins (M_r = 85,000-95,000) on rat pancreas and human gall bladder. Deglycosylation with endo-β-*N*-acetylglucosaminidase F yielded bands of M_r = 43,000 from CHP212 and gall bladder and M_r = 42,000 from pancreas. Peptide mapping of the deglycosylated bands using *Staphylococcus aureus* V8 protease demonstrated identical patterns in CHP212 and gall bladder and a similar but different pattern in pancreas. Thus, although possessing heterogeneity in their carbohydrate domains, CCK receptors on human neuroblastoma cells (CHP212) and human gall bladder smooth muscle cells have highly similar or identical protein cores. The core protein on another type A CCK receptor, from rat pancreas, appears to differ from these, likely representing molecular heterogeneity between species.

CCK is a peptide hormone synthesized and secreted from cells along the intestine and in the brain (1). Targets for this hormone include the pancreas, gall bladder, enteric smooth muscle, and various neuronal cells (2). Radioligand binding studies using these tissues have demonstrated that at least two patterns of structural specificity exist (3). Using the nomenclature of Moran *et al.* (4), the type A binding site is found on pancreas, gall bladder, and specific areas of the brain, whereas the type B binding site is the predominant brain site (4, 5).

To date, extensive biochemical characterization has been performed on the type A CCK receptors on pancreas (6, 7) and gall bladder (8, 9), but only two reports of such characterization exist for receptors that may be neuronal (10, 11). Both reports focused on type B receptors, one from brain and the other from retina. There are no existing biochemical data on type A neuronal receptors or their comparison with type A CCK receptors on peripheral tissues. The recent report of the presence of type A CCK receptors on the human neuroblastoma cell line CHP212 (12) has made it possible to perform biochemical characterization of such a receptor.

A series of molecular probes exist for the biochemical characterization of CCK receptors (1, 7, 13, 14). These include probes that are cross-linked through the N-terminus of CCK-33, a site far removed from the receptor-binding domain (1), as well as those that can be cross-linked to the CCK receptor through domains adjacent to (7) and even within (13, 14) the theoretical receptor-binding region of this hormone. Of interest, a single protein (M_r = 85,000-95,000) has been labeled by all the probes that span the receptor-binding domain, whereas the CCK-33-based probe has consistently affinity labeled a different glycoprotein (M_r = 80,000). Indeed, both of these proteins can be affinity labeled under certain conditions using the CCK-33-based probe (15). Consistent with these data, it has been postulated that the M_r = 85,000-95,000 protein represents the hormone-binding subunit, whereas the M_r = 80,000 protein represents an associated subunit (13, 14).

For this study, we have chosen a probe with a photolabile residue in the position of Phe^{33}, within the receptor-binding

This work was supported by grants from the National Institutes of Health (DK32878 and DK34988) (L.J.M.), the Chadwick Foundation (L.J.M.), and Deutsche Forschungsgemeinschaft (KL 607/1-1) (U.G.K.).

ABBREVIATIONS: CCK, cholecystokinin; endo F, endo-β-*N*-acetylglucosaminidase F; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate.

domain, ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33] (14). This has proven to be an efficient and highly specific probe for labeling the CCK receptors on pancreas and gall bladder (8, 14). Therefore, we have used it to characterize the CCK receptors present on the CHP212 cells and to compare these with CCK receptors present on peripheral gastrointestinal tissues. The structures of these receptors have been further defined using enzymatic deglycosylation and protease peptide mapping.

Experimental Procedures

Materials. Synthetic CCK-8 was from Peninsula Laboratories (Belmont, CA). The CCK analogue D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33] was synthesized, iodinated oxidatively, and purified by high pressure liquid chromatography to yield a specific radioactivity of 2000 Ci/mmol, as we have described (14). The CCK antagonist L-364,718 was the kind gift of Dr. Roger Freidinger from Merck Sharp and Dohme Research Laboratories.

Staphylococcus V8 protease was from ICN (Lisle, IL). Endo F was prepared from *Flavobacterium meningosepticum*, as described by Elder and Alexander (16).

Cell culture. The human neuroblastoma cell line CHP212 was the kind gift of Dr. Joan Beidler (Memorial Sloan-Kettering Cancer Center). The cells were grown at 37° under 5% carbon dioxide in a 1:1 mixture of Eagle's minimum essential medium and Ham's F-12 medium with 10% heat-inactivated fetal calf serum, nonessential amino acids, 300 μg/ml L-glutamine, 100 IU of penicillin, and 100 μg/ml streptomycin. For binding and affinity labeling studies, cells were lifted mechanically without the use of proteases.

Plasma membrane preparations. For membrane preparation, the CHP212 cells in logarithmic phase were harvested from culture flasks using rubber policemen and were pelleted by centrifugation at 750 × g for 5 min. The cells were homogenized using a motor-driven Teflon-glass homogenizer (six strokes) at 4° in 10 volumes of iced buffer containing 16 mM HEPES (pH 7.4), 0.22 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, and 3 μM phosphoramidon. Unbroken cells and debris were sedimented by centrifugation at 800 × g for 10 min. The supernatant was saved while the pellet was rehomogenized in approximately 10 volumes of buffer and centrifuged again. Both supernatants were then pooled and the sucrose concentration was raised to 40% by the addition of 60% sucrose. This was then overlayered with 20% sucrose and centrifuged in a Beckman 70TI rotor at 54,000 rpm for 60 min. Plasma membranes were collected at the interface between the two sucrose layers, diluted in aqueous buffer, and sedimented by centrifugation at 200,000 × g for 60 min. The resulting pellets were resuspended in 50 mM MES buffer, pH 6.0, containing 130 mM NaCl, 7.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, and were stored at −80°.

Enriched plasma membranes from the tissues to be used for comparison were prepared as we have described (6, 9). For the rat pancreatic preparation, 125–150-g Harlan Sprague-Dawley rats were used. Human gall bladder muscularis plasma membranes were prepared from tissue obtained from patients who underwent clinically indicated cholecystectomies for chronic calculous cholecystitis. This tissue was obtained under guidelines approved by the Mayo Clinic Institutional Review Board and Pathology Tissue Use Committee. We have previously demonstrated that the CCK receptor on this type of tissue cannot be distinguished from that on healthy gall bladders removed incidentally during unrelated surgery (8).

Photoaffinity labeling studies. Membranes from CHP212 cells, rat pancreas, or human gall bladder muscularis were incubated with 500 pM ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33], in the presence of specified concentrations of agonist (CCK-8) or antagonist (L-364,718), for 60 min at 25° in 0.5 ml of MES buffer. The membranes were washed to remove unbound radioligand, resuspended in fresh buffer, and photolyzed by exposure to a 200-W medium pressure Hanovia lamp through a Pyrex filter for 30 min at 4°. Photolysis conditions were previously established and validated so that cross-linking occurred only via the nitrophenylalanyl residue (14). The membrane proteins were then solubilized, separated on 10% SDS-polyacrylamide gels (15.5 × 1.5 mm) with 5% stacking gels (1.5 × 1.5 mm) using the protocol of Laemmli (17), and visualized by autoradiography. Molecular weight values for labeled proteins were determined by interpolation on a plot of log M_r versus mobilities of standard proteins [myosin (M_r = 200,000), β-galactosidase (M_r = 116,000), phosphorylase B (M_r = 92,500), bovine serum albumin (M_r = 66,000), ovalbumin (M_r = 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r = 36,000), carbonic anhydrase (M_r = 29,000), trypsinogen (M_r = 24,000), and soybean trypsin inhibitor (M_r = 20,100)].

Deglycosylation and protease peptide mapping studies. Affinity labeled proteins that were visualized by autoradiography were cut from the frozen nonfixed gels. They were then electroeluted for 12 hr using the ISCO (Lincoln, NE) electrophoretic concentrator with cellulose dialysis membranes (molecular weight cutoff, 3500) and 0.01 M Tris-acetate buffer (pH 8.6) containing 0.1% Nonidet P-40 (18). Deglycosylation using endo F and peptide mapping using *Staphylococcus aureus* V8 protease were performed as we have described for the pancreatic and gall bladder CCK receptors previously (18–20). For deglycosylation, 3 units of endo F were added to the electroeluted proteins suspended in an equal volume of 0.1 M sodium phosphate, pH 6.1, 50 mM EDTA, 0.1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol. This reaction was permitted to proceed for 12 hr at 37° before SDS-polyacrylamide gel electrophoresis. For peptide mapping, 0.75 μg of *S. aureus* V8 protease was used to digest the sample for 1 hr at 37°. The buffer used contained 0.125 M Tris, pH 6.8, 0.3% SDS, 0.05% Nonidet P-40, 5% glycerol, and 0.5 mM EDTA. The resulting products were resolved on 15% polyacrylamide gels, as described above. Products of these reactions were visualized by autoradiography and interpolated among the standard proteins.

Results

Indeed, the photolabile CCK analogue ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33] bound to membranes prepared from the CHP212 cells in a saturable and specific manner. Binding was competed for in a concentration-dependent manner by CCK-8. Nonspecific binding, as assessed in the presence of 0.5 μM CCK-8, represented less than 8% of total binding.

Upon photolysis and separation by SDS-polyacrylamide gel electrophoresis, a protein of apparent M_r = 80,000–90,000 was specifically labeled and visualized by autoradiography. The migration of this band was not altered in the presence or absence of dithiothreitol, suggesting that the labeled protein did not represent a disulfide-linked complex or part of a larger such complex (Fig. 1). CCK-8 inhibited the labeling of the M_r = 80,000–90,000 band in a concentration-dependent manner, with 50% of labeling inhibited in the presence of 0.2 nM CCK-8 (Fig. 1). Labeling of this protein was also inhibited in a concentration-dependent manner by the CCK antagonist L-364,718 (Fig. 1).

The apparent size of the protein labeled on CHP212 neuroblastoma cell membranes was different from that of the proteins that were specifically labeled by the same photoaffinity ligand on rat pancreatic membranes and human gall bladder muscularis membranes (both M_r = 85,000–90,000) (Fig. 2).

In order to further characterize these labeled proteins, we deglycosylated them and performed protease peptide mapping. Previous characterization of the pancreatic and gall bladder CCK receptors, using a battery of deglycosylating enzymes, chemical deglycosylation, and lectin affinity chromatography,

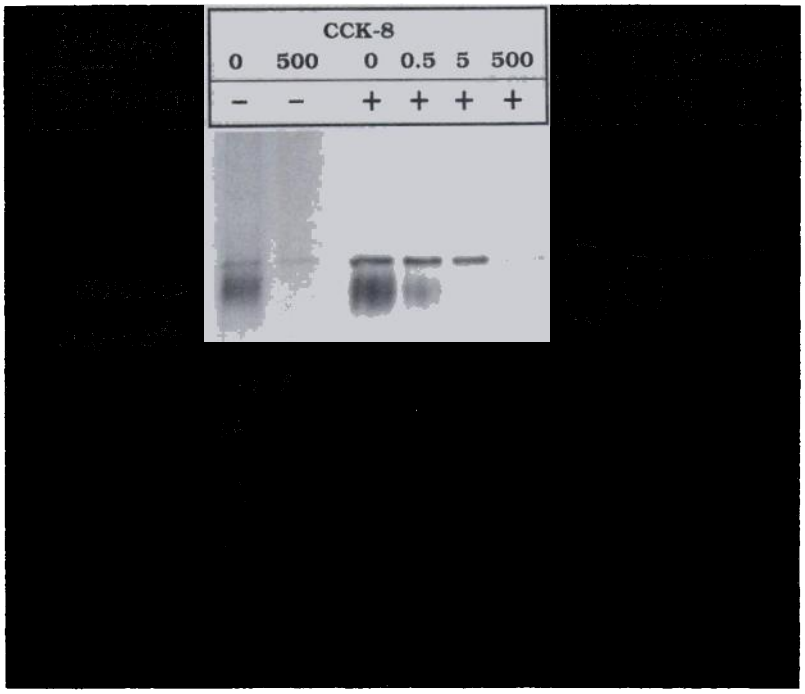

Fig. 1. Affinity labeling of CHP212 cell membranes using ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33]. Shown is a representative autoradiograph of a SDS-polyacrylamide gel used to separate the products of affinity labeling. The M_r = 80,000–90,000 band, which was specifically labeled, migrated similarly in the absence or presence of dithiothreitol (*DTT*). Labeling of this band was inhibited in a concentration-dependent manner by both CCK-8 and the antagonist L-364,718.

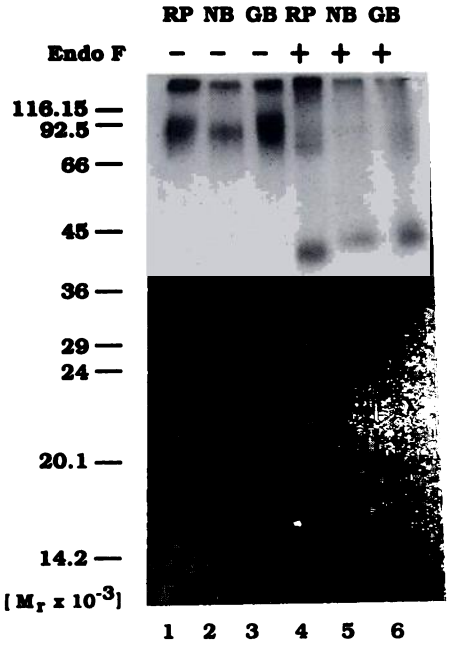

Fig. 2. Effect of endo F deglycosylation of membrane proteins from rat pancreas (*RP*), human neuroblastoma CHP212 cells (*NB*), and human gall bladder smooth muscle (*GB*), which were affinity labeled using ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33]. Shown is a representative autoradiograph of a SDS-polyacrylamide gel used to separate the products of affinity labeling in their native state (*lanes 1–3*) and after endo F treatment (*lanes 4–6*).

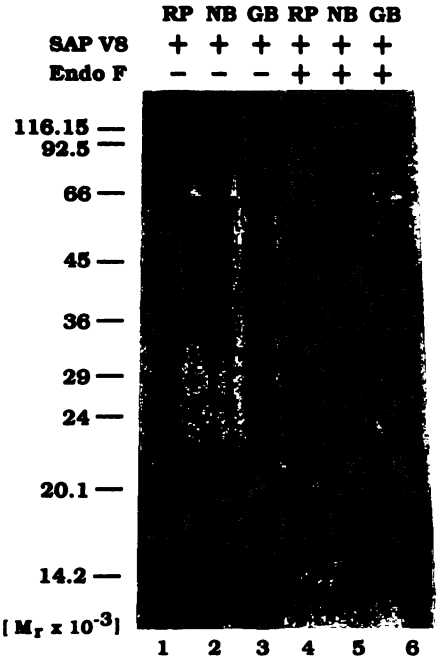

Fig. 3. Effect of *S. aureus* V8 protease (*SAP V8*) digestion of membrane proteins from rat pancreas (*RP*), human neuroblastoma CHP212 cells (*NB*), and human gall bladder smooth muscle (*GB*), which were affinity labeled using ^{125}I-D-Tyr-Gly-[(Nle^{28,31},pNO$_2$-Phe^{33})CCK-26–33]. Shown is a representative autoradiograph of a SDS-polyacrylamide gel used to separate the products of proteolytic cleavage of the products of affinity labeling in their native state (*lanes 1–3*) and after endo F deglycosylation (*lanes 4–6*).

has demonstrated that the enzyme endo F can fully deglycosylate these *N*-linked glycoproteins (19, 20). Endo F treatment of the M_r = 80,000–90,000 band from CHP212 cells yielded a labeled product of M_r = 43,000, identical to the product of digestion of the human gall bladder CCK receptor (Fig. 2). In contrast, the major product of endo F digestion of the rat pancreatic CCK receptor was smaller, migrating at M_r = 42,000 (Fig. 2).

When the native proteins were digested with *S. aureus* V8 protease, only incomplete cleavage of each was achieved, with a large proportion of radioactivity remigrating as starting material (Fig. 3). Even under these conditions, however, cleavage products that could be generated from the proteins labeled on CHP212 cells and human gall bladder muscularis were of

similar sizes (Fig. 3). In contrast, the *S. aureus* V8 fragments from the protein labeled on rat pancreas were different (Fig. 3).

As might be expected, the *S. aureus* V8 protease was much more active in cleaving the deglycosylated core proteins. When protease peptide mapping was performed after deglycosylation, again similar patterns of cleavage products were generated from the species labeled on CHP212 cells and human gall bladder muscularis plasma membranes (a major doublet at M_r = 17,000 and 18,000 and minor bands at M_r = 20,000, 22,000, and 35,000). In contrast, a similar but distinct map was seen when the deglycosylated rat pancreatic protein was cleaved with this enzyme.

Discussion

Biochemical characterization of receptors nicely complements their pharmacological characterization. The technique of photoaffinity labeling has proven to be an extremely powerful method that contributes to the biochemical characterization of molecules such as receptors, which are extremely sparse yet have a high affinity for potential probes (21). When this technique is used in conjunction with glycosidic and proteolytic enzymes, it can provide additional biochemical information about receptor molecules (18).

Indeed, the CCK receptor has been extensively studied by affinity labeling and photoaffinity labeling, using a variety of probes that incorporate different sites of potential covalent attachment between probe and receptor (1, 7, 13, 14). Utilization of such a probe with its site of cross-linking within the theoretical receptor-binding domain of the ligand has proven to be efficient and highly specific (13, 14). CCK receptors best studied using these techniques include those on the pancreatic acinar cell and on the gall bladder muscularis smooth muscle cell (7–9, 20). Although CCK receptors have been well documented in the brain (10), only two reports of affinity labeling of CCK receptors that are likely neuronal currently exist (10, 11).

Additionally, it is now well established that at least two subtypes of CCK receptors exist in brain (4). The predominant type has structural specificity quite different from that of the pancreas and gall bladder and has been called a type B receptor (4). It is this receptor type that was probably studied in the previous work (10, 11). In addition, in specific locations in the brain, such as the area postrema and interpeduncular nucleus, binding sites with specificities similar to those of the gastrointestinal tissues exist and have been termed type A sites (5).

Indeed, pharmacological characterization of the receptor for CCK on the human neuroblastoma cell line CHP212 has demonstrated that it has structural selectivity typical of type A receptors (12), such as those found on the pancreatic acinar cell and the gall bladder muscularis smooth muscle cell (6, 9). This cell line clearly possesses a neuronal phenotype, with catecholaminergic and cholinergic synthetic enzymes and neuronal cell surface antigens (22–24). Being a neuroblastoma, it was likely derived from cells originating in the neural crest (25). Thus, the CHP212 cell may be a very useful source of a neuronal type A CCK receptor.

In the present study, we have confirmed the presence of a high affinity specific binding site on CHP212 cells for the peptide hormone CCK. Using techniques similar to those we have previously applied to the CCK receptors on the pancreatic acinar cell and the gall bladder muscularis smooth muscle cell (8, 9), we have demonstrated the specific labeling of a M_r = 80,000–90,000 glycoprotein on the CHP212 cells. This appears to represent a single molecule without associated covalently attached subunits and migrates similarly on a SDS-polyacrylamide gel under both reducing and nonreducing conditions. We have further demonstrated that it likely represents a type A CCK receptor, by competing for its labeling with the specific antagonist, L-364,718 (26).

The apparent size of this binding protein on such a gel suggests that it is different in its native form from that of type A CCK receptors on rat pancreatic membranes (M_r = 85,000–95,000) (7) and from that on human gall bladder muscularis membranes (M_r = 85,000–95,000) (8). Both of those receptors have been carefully studied using a series of glycosidic enzymes, chemical deglycosylation, and lectin affinity chromatography, to demonstrate that they represent *N*-linked complex glycoproteins (19, 20). Both can be completely deglycosylated using endo F (19, 20). In the present study, we treated the CHP212 binding site with endo F and demonstrated that its product of digestion was M_r = 43,000. That is the same apparent size as the protein core of the human gall bladder receptor (20). It is slightly larger than that of the rat pancreatic receptor (M_r = 42,000) (19). This suggests that the type A CCK receptors present on the human neuroblastoma cell line and on human gall bladder may have the same or closely related protein cores but are differentially glycosylated.

Of interest, there are also data supporting differential glycosylation of CCK receptors from the same tissue from two different species, human and bovine gall bladder smooth muscle (20). Both represent *N*-linked complex glycoproteins with differences in their carbohydrate moieties and with similar protein cores (20). These differences could not be explained simply by differences in sialylation, based on differences in the products of neuraminidase treatment (20). There is precedent for different tissues to glycosylate the same receptor differently (27) and to possess differences in sialyltransferase activity (28).

To gain further insight into the protein cores of the glycoproteins being compared, they were mapped using *S. aureus* V8 protease. After complete removal of all *N*-linked glycans, limited proteolysis generated peptide maps that were (essentially) identical for the human receptor proteins on CHP212 cells and gall bladder smooth muscle. The receptor on rat pancreas was slightly different but clearly related. Thus, there appears to be molecular heterogeneity in type A CCK receptors. This may well be explained by species differences. The peptide mapping further extends the suggestion that type A receptors on different tissues in the same species may have the same, or a closely related, protein core.

Acknowledgments

The authors would like to thank Stephen P. Powers, Ph.D., and Delia Pinon for providing synthetic peptides, Elizabeth Hadac for technical assistance, and Marilyn LeQve for assistance in preparation of the manuscript.

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