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Effects of pH and temperature on photoaffinity labeling of Family B G protein-coupled receptors

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Abstract

The efficiency of covalent labeling of a receptor by a photolabile analogue of its natural ligand is dependent on the spatial approximation of the probe and its target. Systematic application of intrinsic photoaffinity labeling to the secretin receptor, a prototypic Family B G protein-coupled receptor, demonstrated reduced efficiency of labeling for amino-terminal and mid-region sites of labeling relative to carboxyl-terminal sites. Reduction of pH from 7.4 to 5.5 and reduction of temperature from 25 °C to 4 °C improved the efficiency of covalent labeling of the receptor with these probes. This correlated with sites of labeling at the interface between the receptor amino terminus and the receptor core, a region containing histidine residues that have their ionization affected in this pH range. Application to the calcitonin receptor, another Family B G protein-coupled receptor, yielded analogous results. These results support the consistent mode of docking peptide ligands to this group of receptors.

Keywords

G protein-coupled receptors; secretin receptor; calcitonin receptor; photoaffinity labeling

1. Introduction

Photoaffinity labeling can be utilized for the identification of molecules that interact, and can even be used for the identification of domains and residues at the interface of such interactions. When this technique is applied to ligand-receptor systems, it can provide a powerful tool for the elucidation of the molecular basis of ligand docking [1]. However, there are substantial limitations in the application of photoaffinity labeling to this type of molecular interaction, with effective covalent labeling dependent on spatial approximation of the probe residue with the target. Bulky photolabile residues that are often aromatic may not be well accommodated in receptor ligands in positions of critical residue-residue interactions. In other positions that are better accommodated, the photolabile moiety may not be held in adequate apposition to the target sequence to establish a covalent bond.

Much has been recently learned about the structure of the disulfide-bonded amino-terminal domains of Family B guanine nucleotide-binding protein (G protein)-coupled receptors, with

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a series of NMR and crystal structures of these domains [2–8]. Several of these structures also include ligands, suggesting the molecular basis of ligand docking to this important domain. This involves the carboxyl-terminal region of natural peptide ligands. However, there continues to be substantial uncertainty regarding the positioning of the receptor amino terminus with the helical bundle domain, and uncertainty in the siting of the amino-terminal regions of the ligands for these receptors.

In the current work, we explored the ability of modification of temperature and pH to enhance the efficiency of covalent labeling of the secretin receptor through different positions spread throughout the pharmacophore. The pattern that emerged was also tested by application to another Family B G protein-coupled receptor, the calcitonin receptor. For both of these, amino-terminal probes had substantially improved labeling efficiency at pH 5.5 relative to pH 7.4, and at 4 °C relative to 25 °C (room temperature). Of note, carboxyl-terminal probes were much less affected by these variables. This likely reflects the very distinct environments of the different ends of the peptides when docked at this family of receptors. We attempt to put these observations into perspective, based on current understanding of this important group of receptors.

2. Materials and methods

2.1 Materials

Amino acids for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY). *N*-chlorobenzene-sulfonamide (Iodo-beads) was from Pierce Chemical Co (Rockford, IL). Ham's F-12 and Dulbecco's Modified Eagle medium, and soybean trypsin inhibitor were from Invitrogen (Carlsbad, CA). Fetal Clone II culture medium supplement was from Hyclone laboratories (Logan, UT). All other reagents were analytical grade.

2.2. Cell and receptor preparations

Cell lines expressing the rat secretin receptor (CHO-SecR) [9] and the human calcitonin receptors for the current study, isoform II receptor (HEK293-CT_(a)) [10], were used as sources of Cells were cultured at 37 °C in a 5% environment on Falcon tissue culture plasticware in CO₂ Ham's F-12 medium (for CHO-SecR) or Dulbecco's Modified Eagle medium (for HEK293-CT_(a)) supplemented with 5% Fetal Clone II. Cells were passaged approximately twice a week. Enriched plasma membranes from these cell lines were prepared as previously described and stored in Krebs-Ringer-HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM 1.2 mM 2 mM soybean trypsin inhibitor, 1 mM KH₂PO₄, MgSO₄, CaCl₂, 0.01% phenylmethylsulfonyl fluoride) [11]

2.3. Peptides

Photolabile secretin probes used in this study (Fig 1) were previously synthesized: [Bpa⁻¹,Tyr¹⁰]rat secretin (Bpa⁻¹ Sec) [12], [Bpa⁶,Tyr¹⁰]rat secretin (Bpa⁶ Sec) [13], [Tyr¹⁰, (BzBz)Lys¹²]rat secretin ((BzBz)Lys¹² Sec) [14], [Tyr¹⁰,Bpa¹³]rat secretin (Bpa¹³ Sec) [15], [Tyr¹⁰, (BzBz)Lys¹⁸]rat secretin ((BzBz)Lys¹⁸ Sec) [16], [Tyr¹⁰,Bpa²²]rat secretin (Bpa²² Sec) [17], and [Tyr¹⁰,Bpa²⁶]rat secretin (Bpa²⁶ Sec) [18]. In addition to the incorporated photolabile moiety, either Bpa or (BzBz)Lys, all the secretin peptides contained a tyrosine residue in position 10 for radioiodination.

Photolabile calcitonin probes used in this study (Fig 1) were [Bpa⁸,Arg¹⁸]human calcitonin (Bpa⁸ CT) [19], [Ile⁸,Bpa¹⁶,Arg¹⁸]calcitonin (Bpa¹⁶ CT) [10], and [Ile⁸,Arg¹⁸,Bpa²⁶]calcitonin (Bpa²⁶ CT) [10]. In addition to the photolabile Bpa residue, they all incorporated an arginine in position 18 to facilitate the specific digestion of the labeled receptor by endoproteinase Lys-C without the cleavage of the probes themselves. The methionine in

position 8 of the Bpa¹⁶ and Bpa²⁶ calcitonin probes was replaced by an isoleucine to eliminate a site for potential oxidative damage during radiolabeling. All the three calcitonin probes contained a naturally-occurring tyrosine residue in position 12 as the site for radioiodination.

All above peptides were synthesized using manual solid-phase techniques and were purified to homogeneity by reversed-phase HPLC [20], with the chemical identities of the products established by mass spectrometry. They were radioiodinated using Na¹²⁵I and a 15 s exposure to the solid phase oxidant, *N*-chlorobenzene-sulfonamide (Iodo-beads). The products were purified to homogeneity by reversed-phase HPLC to yield specific radioactivities of 2,000 Ci/mmol [20].

2.4. Photoaffinity labeling

Covalent photoaffinity labeling of the secretin and calcitonin receptors was performed using 50 µg of enriched receptor-bearing plasma membranes from CHO-SecR or HEK293-CT_(a) cells incubated with 0.1 nM radiolabeled photolabile secretin or calcitonin (see above) in the dark in KRH medium under varied pH (5.5, 6.5 and 7.4) for 1 h at 4 °C or RT. The KRH medium at pH 5.5 contained 25 mM MES. The reaction was then photolyzed for 30 min at 4 °C in a Rayonet photochemical reactor (Southern New England Ultraviolet Company, Hamden, CT) equipped with 3500-Å lamps. Membranes were then washed, solubilized in Laemmli SDS sample buffer, and resolved by 10% SDS-polyacrylamide gels [21]. Labeled proteins were visualized by autoradiography and quantified by densitometry using the ImageJ program from the National Institutes of Health.

3. Results

3.1. Efficiencies of covalent labeling of the secretin receptor by a battery of photolabile probes

The efficiencies of covalent labeling of the secretin receptor under standard conditions (pH 7.4, room temperature, incubation time 60 min) varied relative to the position of the photolabile site of covalent attachment (Fig 2). Each of these probes incorporated a benzophenone moiety for labeling into an analogue of the natural peptide ligand, secretin. The efficiencies were observed to decrease as the position of attachment moved from the carboxyl-terminal end of the probe toward its amino-terminal end.

3.2. Effect of pH and temperature on the photoaffinity labeling of the secretin receptor by a battery of probes

Shown in Figure 3 are representative autoradiographs of the covalent labeling of the secretin receptor at pH 7.4, 6.5, and 5.5 at both room temperature and 4 °C using probes with photolabile sites distributed from the amino terminus to the carboxyl terminus. Also shown is a graph plotting the ratio of covalent labeling of the receptor at pH 5.5 to that at pH 7.4, with positions of the photolabile residue illustrated along the X axis. These data demonstrate that decreasing the pH from 7.4 to 5.5 increases the efficiency of covalent labeling of the secretin receptor most dramatically for amino-terminal probes, somewhat for mid-region probes, and not at all (or decreases the labeling) for carboxyl-terminal probes. Similarly, decreasing the temperature from room temperature to 4 °C had a pronounced effect on covalent labeling of amino-terminal probes, less pronounced effect on mid-region probes, and minimal (or inhibitory) effect on carboxyl-terminal probes.

For the secretin probes with Bpa moiety in positions -1 and 6, the specific receptor residue that was labeled at pH 5.5 was fully characterized (data not shown) and found to be identical to the residue that was labeled with the same probe at pH 7.4 (receptor residue Tyr³³³ for Bpa⁻¹ probe

and receptor residue Val⁴ for Bpa⁶ probe [12,13]). This is consistent with the docking of the probes in their natural poses and positions.

3.3. Effect of pH and temperature on the photoaffinity labeling of the calcitonin receptor by a battery of probes

Shown in Figure 4 are representative autoradiographs of the covalent labeling of the calcitonin receptor at pH 7.4, 6.5, and 5.5 at both room temperature and 4 °C using probes with photolabile sites within the amino-terminal half (Bpa⁸ CT) [19], mid-region (Bpa¹⁶ CT) [10] and carboxyl-terminal half (Bpa²⁶ CT) [10] of the 32-residue calcitonin peptide. Also shown is a graph plotting the ratio of covalent labeling of the receptor at pH 5.5 to that at pH 7.4, with positions of the photolabile residue illustrated along the X axis. Similar to the results with secretin and the secretin receptor, calcitonin labeling of its receptor was most enhanced by low pH and low temperature for the amino-terminal probe.

4. Discussion

Intrinsic photoaffinity labeling often utilizes a radiolabeled probe that incorporates a photolabile moiety within the pharmacophore of the ligand to identify spatially approximated receptor regions or even specific receptor residues that are adjacent to the photolabile moiety within the docked ligand. This approach has been particularly useful for the study of Family B G protein-coupled receptors, where the natural ligands are moderately large peptides with extended pharmacophoric domains. The prototypic secretin receptor has been extensively studied using this technique to establish spatial approximation constraints potentially useful in molecular modeling [12–18,22–25]. We have previously used secretin probes incorporating a photolabile benzophenone, Bpa or (BzBz)Lys, in various positions throughout the length of the 27-residue peptide [12–18,22–25]. These spatial constraints have been key in building a working model of the ligand-bound secretin receptor complex [24].

However, during these studies it became clear that the labeling efficiency of some photolabile probes, particularly the amino-terminal and mid-region probes, was quite low when the incubation was routinely performed at pH 7.4 at room temperature [12–15,24]. This is also the case for photoaffinity labeling of the calcitonin receptor, another Family B G protein-coupled receptor [10,19]. In this work, we have attempted to optimize the conditions for photoaffinity labeling the secretin receptor by varying pH and temperature conditions. We found that the labeling efficiency of the amino-terminal and mid-region probes increased by as much as 18-fold at pH 5.5 and 4 °C and these conditions had little effect on the labeling with carboxyl-terminal probes. Similar observations were made for calcitonin receptor labeling.

These observations are particularly interesting in light of recent data suggesting that the carboxyl-terminal regions of Family B receptor ligands are present in helical conformation [26] and appear to reside within a cleft between two other helical regions of the amino terminus of their receptors [3]. Such helix-helix interactions are typically quite stable and may not be dramatically affected by these manipulations. The decrease in pH from 7.4 to 5.5 would only be expected to affect the ionization status of histidine residues having a pKa of approximately 6.0. There are no histidine residues predicted to reside within the predicted cleft in the secretin receptor.

In contrast, the amino-terminal region of secretin [12,24] and other Family B G protein-coupled receptor ligands [19,27], is believed to interact with the helical bundle and loop regions of its receptor. The contrast in environments of the different regions of secretin was also supported by fluorescence probe studies in which the microenvironments of residues in different regions of secretin were directly probed [28]. In those studies, fluorophores within the amino terminus were most exposed to the aqueous solvent and most easily quenched by hydrophilic potassium

iodide, while fluorophores within the carboxyl-terminal region were more protected and less easily quenched. Consistent with this, the amino-terminal probes were most affected by changes in pH and in temperature in the current studies. It is noteworthy that there are seven histidines in various transmembrane segments of the helical bundle and one histidine in the first extracellular loop region of the secretin receptor. These histidines could affect the conformation of the loop and helical bundle domains that have been postulated to be important for interaction with the amino-terminal region of secretin [12,19,27].

Family B G protein-coupled receptors contain several important potential drug targets. A detailed understanding of their structures, mechanisms of ligand binding, and mechanisms of activation should facilitate the development of these drugs. The structures and activity profiles of the natural peptide ligands for these receptors have been quite consistent, as have the regions of their receptors where they are believed to interact [29]. Similarly, the receptor structures and functional themes have been highly conserved. The effects we have observed in the current work for the effects of pH and temperature on two typical members of this family further support this as a consistent theme. It is noteworthy that some receptors in this family, such as the calcitonin gene-related peptide receptor and the calcitonin receptor-like receptor, may follow a distinct theme, since their ligand binding has been demonstrated to be modified by interaction with receptor activity-modifying proteins (RAMPs).

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Abbreviations used in this paper

Bpa	<i>p</i> -benzoyl-L-phenylalanine
(BzBz)Lys	<i>p</i> -benzoylbenzoyl-L-lysine
CHO-SecR	rat secretin receptor-bearing Chinese hamster ovary cell line
CT	calcitonin
GPCR	G protein-coupled receptor
KRH	Krebs-Ringer/HEPES
HEK293-CT_(a)	human calcitonin receptor-bearing human embryonic kidney 293 cells
RT	room temperature
Sec	secretin

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Secretin (natural)

1 10 20
 H-S-D-G-T-F-T-S-E-L-S-R-L-Q-D-S-A-R-L-Q-R-L-L-Q-G-L-V-NH₂

Bpa⁴ Sec

125 I
 #-H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-S-A-R-L-Q-R-L-L-Q-G-L-V-NH₂

Bpa⁶ Sec

125 I
 H-S-D-G-T-#-T-S-E-Y-S-R-L-Q-D-S-A-R-L-Q-R-L-L-Q-G-L-V-NH₂

Lys(BzBz)¹² Sec

125 I
 H-S-D-G-T-F-T-S-E-Y-S-#-L-Q-D-S-A-R-L-Q-R-L-L-Q-G-L-V-NH₂

Bpa¹³ Sec

125 I
 H-S-D-G-T-F-T-S-E-Y-S-R-#-Q-D-S-A-R-L-Q-R-L-L-Q-G-L-V-NH₂

Lys(BzBz)¹⁸ Sec

125 I
 H-S-D-C-T-F-T-S-E-Y-S-R-L-Q-D-S-A-#-L-Q-R-L-L-Q-G-L-V-NH₂

Bpa²² Sec

125 I
 H-S-D-C-T-F-T-S-E-Y-S-R-L-Q-D-S-A-R-L-Q-R-#-L-Q-G-L-V-NH₂

Bpa²⁶ Sec

125 I
 H-S-D-G-T-F-T-S-E-Y-S-R-L-Q-D-S-A-R-L-Q-R-L-L-Q-G-#-V-NH₂

Calcitonin (natural)

1 10 20 30
 C-G-N-L-S-T-C-M-L-G-T-Y-T-Q-D-F-N-K-F-H-T-F-P-Q-T-A-I-G-V-G-A-P-NH₂

Bpa⁸ CT

125 I
 C-C-N-L-S-T-C-#-L-C-T-Y-T-Q-D-F-N-R-F-H-T-F-P-Q-T-A-I-C-V-C-A-P-NH₂

Bpa¹⁸ CT

125 I
 C-G-N-L-S-T-C-I-L-G-T-Y-T-Q-D-#-N-R-F-H-T-F-P-Q-T-A-I-G-V-G-A-P-NH₂

Bpa²⁶ CT

125 I
 C-G-N-L-S-T-C-T-L-G-T-Y-T-Q-D-F-N-R-F-H-T-F-P-Q-T-#-T-G-V-G-A-P-NH₂

Fig. 1.

Primary structures of photolabile secretin and calcitonin analogues used in this study. Shown are the amino acid sequences of natural secretin and calcitonin, as well as their photolabile analogues. Natural residues are illustrated in grey, while modified residues are illustrated in black. # indicates the positions of incorporation of photolabile Bpa or (BzBz)Lys moieties.

Fig. 2.

Efficiency of covalent labeling of the secretin receptor. Shown are relative efficiencies of covalent labeling of the secretin receptor using secretin analogue probes incorporating a photolabile benzophenone in positions spread throughout the pharmacophore (as noted). These were quantified by densitometric analysis of relevant bands, taking into consideration the exposure times and the specific radioactivity of the radioiodinated probes.

Fig. 3. Photoaffinity labeling of the secretin receptor. Shown are representative autoradiographs of the covalent labeling of the secretin receptor at pH 7.4, 6.5, and 5.5 at both room temperature and 4 °C using probes with photolabile sites distributed from the amino terminus to the carboxyl terminus. Also shown is a graph plotting the ratio of covalent labeling of the receptor at pH 5.5 to that at pH 7.4, with positions of the photolabile residue along the X axis. These data are representative of two to four experimental replicates of each condition.

Fig. 4. Photoaffinity labeling of the calcitonin receptor. Shown are representative autoradiographs of the covalent labeling of the calcitonin receptor at pH 7.4, 6.5, and 5.5 at both room temperature and 4 °C using probes with photolabile sites within the amino-terminal half (Bpa⁸ CT) [19], mid-region (Bpa¹⁶ CT) [10] and carboxyl-terminal half (Bpa²⁶ CT) [10] of the 32-residue calcitonin peptide. Also shown is a graph plotting the ratio of covalent labeling of the receptor at pH 5.5 to that at pH 7.4, with positions of the photolabile residue along the X axis. These data are representative of two to three experimental replicates of each condition.