A Putative Selectivity Filter in the G-protein-coupled Receptors for Parathyroid Hormone and Secretin*

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Paul R. Turner, Tom Bambino, and Robert A. Nissenson†

From the Endocrine Unit, Veterans Affairs Medical Center and the Departments of Medicine and Physiology, University of California, San Francisco, California 94121

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‡Research Career Scientist of the Department of Veterans' Affairs.

†To whom all correspondence should be addressed: Endocrine Unit, VAMC (111N), 4150 Clement St., San Francisco CA 94121. Tel.: 415-750-2089; Fax: 415-750-6929.

The superfamily of G-protein-coupled receptors (GPCRs)1 initiates the biological effects of a remarkably diverse array of agonists, ranging from ions to glycoproteins (1). Each GPCR has encoded within it the structural information required for appropriate ligand affinity and selectivity. Although all GPCRs share the common topologic feature of seven transmembrane domains (TMs), subfamilies of GPCRs differ markedly in the strategies used to maintain ligand affinity and selectivity. At one extreme, determinants of affinity and selectivity may reside exclusively in the TMs (e.g. opsin and adrenergic receptor subfamilies) (2, 3). At the other extreme, agonist affinity and selectivity may be largely a function of residues in the receptors’ N-terminal extracellular domains (e.g. luteinizing hormone/thyrotropin receptor subfamily) (4).

The present studies were designed to identify determinants of ligand specificity in a recently recognized subfamily of GPCRs that includes receptors for the peptide hormones secretin, parathyroid hormone (PTH)/PTH-related protein (PTHrP), gastric inhibitory polypeptide, calcitonin, and several others (5). Receptors in this subfamily resemble the larger superfamily members in displaying an apparent 7-TM topology but do not share significant sequence homology with them. Agonist binding to these receptors leads to activation of adenyl cyclase (via Gα) and, in several cases, phospholipase C (via Gβγ or a related G-protein). Although receptors in this subfamily display 30–65% sequence homology with one another, their cognate peptide ligands are structurally diverse. Thus, these receptors are ideally suited for studies of receptor specificity using molecular chimeras. We chose to initiate such studies with the receptors for PTH/PTHrP and secretin, since their ligands are highly divergent and accordingly do not cross-react with each other’s receptor.

Previous studies have suggested an important role for the relatively large N-terminal extracellular domain of PTH/PTHrP and secretin receptors in ligand binding affinity and specificity (6–9). However, the results of the present study point to a critical role of specific residues in the second TM domain of these receptors in maintaining ligand selectivity.

EXPERIMENTAL PROCEDURES

Reagents—A cDNA encoding the WT opossum PTH/PTHrP receptor in the expression vector pCMV (10) was kindly supplied by Drs. H. J. Jüppner and G. Segre (Massachusetts General Hospital, Boston, MA). A cDNA encoding the rat secretin receptor in the expression vector pCDM8 (11) was a generous gift of Dr. S. Nagata (Osaka Bioscience Institute, Osaka, Japan). Synthetic peptides bPTH(1–34), PTHrP(1–34), porcine secretin, porcine gastric inhibitory polypeptide, and salmon calcitonin were obtained from Bachem. Synthetic oligonucleotides were designed so as to both introduce the mutation of choice and to either add or delete a restriction endonuclease site to facilitate the mapping of mutated clones. Dideoxy sequencing (12) was routinely performed to confirm that the expected mutations were successfully introduced.

Preparation of Constructs Encoding N-terminal Chimeric PTH/Secretin Receptors—Site-directed mutagenesis was used to introduce unique Acc sites into the corresponding positions encoding amino acids at the extracellular end of the first TM of the PTH/PTHrP and secretin receptors. This did not alter the amino acid sequence of the secretin receptor and produced a single conservative change (I187M) in the PTH/PTHrP receptor. The latter mutation produced no detectable effect on the functional activity of the PTH/PTHrP receptor. The presence of an Acc site in the polylinker region of pBluescript allowed us to excise the cDNA encoding the N-terminal domain of each receptor and to subclone into the plasmid encoding the “body” of the heterologous receptor. Restriction mapping was carried out to identify clones with the proper orientation of the insert.

Transfection—cDNAs encoding WT and mutated forms of the PTH/PTHrP and secretin receptors were subcloned into the mammalian expression vector pcDNAIamp (Invitrogen). Column-purified plasmids were transfected into COS-7 cells at a concentration of 5 μg/ml, using the chloroquine DEAE method (13). Cells were sub cultured 24 h in 9205
Determinants of Specificity in PTH and Secretin Receptors

Molecular chimeras of PTH/PTHrP and secretin receptors transiently expressed in COS-7 cells were utilized to assess the contribution of the N-terminal extracellular domains in determining the specificity of ligand binding and activation (cAMP production). As expected, the Wt PTH/PTHrP receptor did not display specific binding of 125I-secretin or respond to secretin with an increase in cAMP accumulation; likewise, the Wt secretin receptor did not bind 125I-PTHrP-(1–34) or 125I-secretin plus unlabeled peptides, where appropriate. Cells were washed twice with ice-cold phosphate-buffered saline and extracted with 0.8 N NaOH, and cell-associated 125I was counted. For cAMP assays, COS-7 cells were incubated for 10 min at room temperature with serum-free Dulbecco’s modified Eagle’s medium containing 1 mM Hepes, 0.1% bovine serum albumin, 1 mM isobutylmethylxanthine, and 100 nM bPTH-(1–34) or secretin. Cells were washed twice with ice-cold phosphate-buffered saline, and cellular cAMP was extracted with 95% ethanol and measured by radioimmunoassay.

RESULTS AND DISCUSSION

AssaysofReceptorFunction—LigandbindingandcAMPassayswerecarriedout,withslightmodificationsofpublishedmethods(14,15).For binding studies, transfected COS-7 cells were incubated in serum-free Dulbecco’s modified Eagle’s medium containing 20 mM Hepes and 0.1% bovine serum albumin for 1 h at 37 °C, followed by a 1-h incubation at room temperature with the same medium containing approximately 50,000 cpm 125I-PTHrP-(1–34) or 125I-secretin plus unlabeled peptides, where appropriate. Cells were washed twice with ice-cold phosphate-buffered saline and extracted with 0.8 N NaOH, and cell-associated 125I was counted. For cAMP assays, COS-7 cells were incubated for 10 min at room temperature with serum-free Dulbecco’s modified Eagle’s medium containing 20 mM Hepes, 0.1% bovine serum albumin, 1 mM isobutylmethylxanthine, and 100 nM bPTH-(1–34) or secretin. Cells were washed twice with ice-cold phosphate-buffered saline, and cellular cAMP was extracted with 95% ethanol and measured by radioimmunoassay.

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Recently, we have shown that an arginine residue that is conserved in the putative second membrane-spanning region (TMII) of the PTH/PTHrP and secretin receptors is crucial for full agonist binding affinity and for consequent receptor activation (16). To determine whether residues in TMII that differ between PTH/PTHrP and secretin receptors contribute to ligand specificity, we evaluated the functional properties of chimeric receptors in which the TMII domains were exchanged. A secretin receptor bearing TMII of the PTH/PTHrP receptor (Sec(PTHII)R) resembled the Wt secretin receptor in its affinity for secretin; remarkably, this chimeric receptor (unlike the Wt secretin receptor) also displayed specific binding of 125I-PTHrP-(1–34) (Fig. 2A). Corresponding results were obtained with the reciprocal chimeric PTH/PTHrP receptor (PTH(SeII)R), which bound PTH with an affinity similar to that of the Wt PTH/PTHrP receptor but also displayed specific binding of 125I-secretin (Fig. 2B). The affinities of these chimeric receptors for...
the heterologous ligands were reduced about 40–60-fold compared with the Wt receptors, consistent with a contribution of regions outside of TMII to optimal binding affinity.

These results demonstrate that the TMII PTH/secretin receptor chimeras are able to interact both with secretin and with PTH/PTHrP. Secretin does not share any amino acid sequence homology with either PTH or PTHrP, and it was thus of interest to determine whether the chimeras contained distinct binding sites for these ligands. Strikingly, PTH and secretin were roughly equipotent in the competitive inhibition of radioligand binding (Fig. 2C), indicating that the two ligands interact with similar affinities to a common binding site in the chimeric receptors.

The altered specificity of the chimeric receptors also extended to ligand-dependent signaling. Thus, Sec(PTHII)R signaled in response to secretin in a similar fashion to the Wt secretin receptor but also signaled in response to PTH; and PTH(SecII)R displayed a Wt signaling response to PTH but also signaled (albeit weakly) in response to secretin (Fig. 2D).

Two other peptides whose receptors are members of the PTH/secretin receptor subfamily, gastric inhibitory polypeptide and calcitonin, failed to raise cAMP levels or to bind to COS-7 cells expressing the chimeric receptors (not shown).

Inspection of the sequences of TMII of the PTH/PTHrP and secretin receptors reveal that they are identical save for three positions; Ile-186, Leu-190, and Asn-192 in the secretin receptor correspond to Met-228, Val-232, and Ile-234 in the PTH/PTHrP receptor (Fig. 3). Asn-192 (secretin receptor)/Ile-234 (PTH/PTHrP receptor) is predicted to face the TM cavity, lining the polar face of TMII, immediately adjacent to a serine residue and one helical turn on the extracellular side of an arginine residue, shown to be essential for optimal binding affinity and efficient signaling to adenylyl cyclase (16). Mutation of N192I in the secretin receptor reproduced the specificity change for secretin binding was 2.6 ± 0.22% of added tracer (n = 9). D, signaling (cAMP) responses of COS-7 cells expressing WtPTHR, WtSecR, PTH(SecII)R, and Sec(PTHII)R. Left panel, response to secretin; right panel, response to bPTH-(1–34).
ligand binding (Fig. 4A) and cAMP response (Fig. 4B) seen with the TMII chimera bearing mutations at all three positions, whereas (I186M, L190V)-secretin receptors failed to respond to PTH (not shown). Conversely, (I234N)-PTH/PTHrP receptors displayed a WT response to PTH and also responded to secretin (Fig. 4). The (N192I)-secretin receptor and (I234N)-PTH/PTHrP receptor exhibited dose-response curves for ligand binding and cAMP production that were indistinguishable from those of Sec(PTHII)R and PTH(PTHrP)R, respectively (not shown). Thus, in the WT secretin and PTH/PTHrP receptors, Asn-192 and Ile-234 may confer specificity by preventing the non-cognate ligands from making contact with key TM residues. This restriction function is partially abrogated in the (N192I)-secretin and (I234N)-PTH/PTHrP receptors, allowing either ligand to contact the activation site and thus to trigger signaling.

The receptors for PTH/PTHrP and secretin are members of a subfamily of G-protein-coupled receptors, which display sequence homologies not evident in other superfamily members (5). They presumably arose by divergence from a common ancestral receptor and have retained very similar modes of signal transduction but have evolved responsiveness to diverse peptide ligands. The present results provide a new perspective on the course of these studies. We also thank H. J. Jüppner and S. Nagata for providing the PTH/PTHrP and secretin receptor cDNAs, respectively.

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