Novel Receptor Partners and Function of Receptor Activity-modifying Proteins*

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The receptor activity-modifying proteins (RAMPs) comprise a family of three accessory proteins that heterodimerize with the calcitonin receptor-like receptor (CL receptor) or with the calcitonin receptor (CTR) to generate different receptor phenotypes. However, RAMPs are more widely distributed across cell and tissue types than the CTR and CL receptor, suggesting additional roles for RAMPs in cellular processes. We have investigated the potential for RAMP interaction with a number of Class II G protein-coupled receptors (GPCRs) in addition to the CL receptor and the CTR. Using immunofluorescence confocal microscopy, we demonstrate, for the first time, that RAMPs interact with at least four additional receptors, the VPAC1 vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor with all three RAMPs; the glucagon and PTH1 parathyroid hormone receptors with RAMP2; and the PTH2 receptor with RAMP3. Unlike the interaction of RAMPs with the CL receptor or the CTR, VPAC1R-RAMP complexes do not show altered phenotypic behavior compared with the VPAC1R alone, as determined using radioligand binding in COS-7 cells. However, the VPAC1R-RAMP2 heterodimer displays a significant enhancement of agonist-mediated phosphoinositide hydrolysis with no change in cAMP stimulation compared with the VPAC1R alone. Our findings identify a new functional consequence of RAMP-receptor interaction, suggesting that RAMPs play a more general role in modulating cell signaling through other GPCRs than is currently appreciated.

The discovery of receptor activity-modifying proteins (RAMPs) has led to a re-evaluation of what defines G protein-coupled receptor (GPCR) phenotypic behavior toward agonists and/or G proteins (1). The RAMP family comprises three accessory proteins (designated RAMP1, RAMP2, and RAMP3) that were originally identified during attempts to clone the receptor for calcitonin gene-related peptide (CGRP). Phenotypic receptor behavior corresponding to that of the native CGRP receptor could be demonstrated only in recombinant expression systems when another seven-transmembrane receptor, the calcitonin receptor-like receptor (CL receptor) was co-expressed with RAMP1 (1). Additional studies extended these observations to identify a general role of RAMPs in modifying the expression and the pharmacology of receptors related to the calcitonin family of peptides (1–5).

To date, studies of RAMP-GPCR interactions have focused predominantly on the receptors for calcitonin and its related peptides (i.e. CGRP, adrenomedullin, and amylin). However, these receptors belong to the Class II family of GPCRs, members of which share a number of structural features and are all activated by peptide ligands (6). Given these similarities, it is possible that other Class II GPCRs may also interact with RAMP proteins to yield novel receptor phenotypes or receptors with altered pharmacological profiles. Importantly, RAMPs display a ubiquitous tissue distribution (1, 7, 8), and cellular background can have a significant impact on the effects of RAMP (5, 8). Therefore, it is conceivable that RAMPs play a more generalized role in cellular signaling mediated by Class II GPCRs than is currently appreciated.

EXPERIMENTAL PROCEDURES

Materials—All peptides were from Auspep (Parkville, Victoria, Australia) except for human GHRH, human secretin, and helodermin, which were from Bachem (Bubendorf, Switzerland). 125I-VIP was prepared using IODO-BEADS (Fierce), with the mono-iodinated peptide purified by reverse-phase high pressure liquid chromatography. All other chemicals were reagent grade or better and were purchased from Sigma (St Louis). Plasmid DNA for the hGHRH receptor was a gift from Dr. Bruce Gaylinn (9). Plasmid DNA for the hPTH1 receptor was a gift from Dr. Michael Chorev (10). Plasmid DNA for the hPTH2 receptor was a gift from Dr. Ted Usdin (11). Plasmid DNA for wild-type hRAMP1, c-Myc RAMP1, and hCL receptor was a gift from Dr. Steve Foord (1). Plasmid DNA for c-Myc RAMP2 and 3 was prepared as described previously (12). Plasmid DNA for the hVPAC1, hVPAC2, and hVPAC3 receptors was prepared as described previously (13, 14). Plasmid DNA for the human glucagon, GLP-1, and GLP-2 receptors was provided by Dr. Anette Sams Nielsen (Novo Nordisk, Copenhagen, Denmark).

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1 The abbreviations used are: RAMP, receptor activity-modifying protein; GPCR, G protein-coupled receptor; CGRP, calcitonin gene-related peptide; CL receptor, calcitonin receptor-like receptor; CTR, calcitonin receptor; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; VPAC1R, VIP and PACAP 1 receptor; PTH, parathyroid hormone; PTH1R, parathyroid hormone 1 receptor; PTH2R, parathyroid hormone 2 receptor; PI, phosphoinositide; h, human; GHRH, growth hormone-releasing hormone; HA, hemagglutinin; GHRHR, growth hormone-releasing hormone receptor; GLP, glucagon-like peptide.
Preparation of Epitope-tagged RAMPs—Double (RAMP2 or triple (RAMP3) hemagglutinin (HA) tags were inserted in the RAMP N terminus, downstream of the signal peptide (between amino acids 55 and 56 of RAMP2 and amino acids 33 and 34 of RAMP3), by overlap extension PCR and cloned directionally into the HindIII and XhoI restrictions sites of pcDNA3.1/Zeo (Invitrogen). For incorporation of the V5 tag at the C terminus of RAMPs, coding regions were generated by PCR using the High Fidelity Taq polymerase (Roche Molecular Biochemicals) and ligated in-frame into pcDNA3-V5His6 (Invitrogen) using TA-topoisomerase. All constructs were confirmed by sequencing.

Cell Culture and Transfection—HEK-293 and COS-7 cells were maintained as previously described (3). HEK-293 cells were grown to 60% confluence in 6-well plates or 4-well chamber slides and were transfected with the indicated plasmid using LipofectAMINE 2000 (Invitrogen). COS-7 cells were grown in 175-cm² flasks (membrane preparations) or in 24-well plates (whole cell binding and signaling assays) to 90% confluence and transfected using LipofectAMINE (3).

Immunofluorescence Microscopy—Immunofluorescence experiments were carried out 24 h post-transfection. HEK-293 or COS-7 cells, transiently transfected with 150 ng of epitope-tagged RAMP cDNA with/without 100 ng cDNA of an individual Class II GPCR (see "Results"), were fixed with 3.4% paraformaldehyde in phosphate-buffered saline with subsequent detection of the subcellular distribution of the RAMPs using anti-c-Myc (9E10, gift from Dr. Jorg Heierhorst, SVIMR, Fitzroy, Australia), anti-HA (12CA5, Roche Molecular Biochemicals) or anti-V5 antibodies (Invitrogen) performed as described previously (3).

Western Blot Analysis—N-terminally c-Myc-tagged RAMP1 or C-terminally V5-tagged RAMP1 was transfected into 6-well plates as described above with or without increasing amounts of CL receptor or VPAC1 receptor. Whole cell lysates were separated on 12% SDS-PAGE, transferred to nitrocellulose, and probed using either anti-c-Myc antibody or VPAC1 receptor. The c-Myc tag or the artificial signal sequence of the RAMPs. Using these constructs, a much lower level of cell surface RAMP2 or -3 expression was seen, and indeed the total level of RAMP expressed was also lower than that seen with the c-Myc-tagged RAMPs, particularly for RAMP2. Given the divergence in subcellular distribution with the different tags, we also looked at the localization of RAMP3 incorporating a V5 tag at the C terminus. Like the HA-tagged RAMP3, this construct was principally retained inside the cell in the absence of co-transfection of an interacting receptor, suggesting that either the c-Myc tag or the artificial signal sequence of the c-Myc-tagged construct contributed to the high levels of receptor-independent cell surface expression of RAMPs 2 and 3. It has been suggested that the presence of either the c-Myc or HA tags can differentially affect protein transit and terminal glycosylation in the Golgi (19), which would be consistent with our findings. We have principally used the HA-tagged RAMPs 2 and 3 in our primary assay for RAMP-receptor interaction.

Co-transfection of the GHRH (Fig. 1, D–F), VPAC2, GLP-1, or GLP-2 (not shown) receptors with each of the RAMPs failed to alter the level of cell surface-expressed RAMP1, -2, or -3. In contrast, co-transfection of the CL receptor (Fig. 1, G–I), CTR (not shown), or VPAC1 receptor (Fig. 1, J–L) led to a significant relocation of each of the RAMPs to the cell surface. Furthermore, co-expression of the PTH2 receptor with RAMP3 (Fig. 1O), but not the other RAMPs (not shown), led to increased cell surface expression of RAMP3, whereas expression of the PTH1 (Fig. 1N) or glucagon (Fig. 1M) receptors with RAMP2, but not other RAMPs (not shown), led to translocation of RAMP2 to the cell surface as well as an increase in total RAMP2 levels. This latter property is likely to be a result of stabilization of the RAMP2 protein when complexed with its receptor partner and is also seen with the classic RAMP partners CTR and CL receptor (not shown). The specificity of RAMP-receptor interactions was also maintained when the c-Myc-tagged RAMPs were used, although the capacity to observe modulation of RAMP distribution required titration of the level of RAMP co-transfected, because of the high level of receptor-independent cell surface expression (not shown). Our findings with the VPAC1, glucagon, PTH1, and PTH2 receptors are the first to demonstrate an interaction between these receptors and RAMPs. Previous study of "unglycosylated" murine RAMPs and PTH1 and glucagon receptors in oocytes have failed to reveal the association between these receptors and RAMP2 (18). Although this discrepancy in results may represent a species difference in RAMP-receptor interaction or an effect of removal of the consensus glycosylation sites, we believe it is more likely a consequence of the highly divergent cellular back-
grounds. We have shown previously that interaction of the CTR with RAMPs, particularly RAMP2, is sensitive to the cellular background in which it is expressed, indicating that other cellular components, such as G proteins, are likely to contribute to RAMP-receptor interactions (5). The specific pairing of the PTH1, PTH2, and glucagon receptors with only one of the RAMPs highlights the specificity of RAMP-receptor interactions and the diversity of the impact that RAMPs may have on receptor function.

The most striking pharmacological consequence of RAMP association with receptors for the calcitonin family of peptides is the generation of unique receptor phenotypes that can readily be detected pharmacologically in both radioligand binding and cell signaling studies (1, 3, 5). Because we established a significant interaction between the VPAC1 receptor and each of the RAMPs, we chose to investigate the pharmacological consequences of this association in greater detail to determine whether the VPAC1 receptor phenotype was also altered. For the sake of comparison with our functional experiments (see below), binding assays were conducted on both membrane homogenates and whole cells. Saturation binding experiments using $^{125}$I-VIP as the radioligand revealed no significant effect of co-expression of RAMPs on the maximal density of cell surface VPAC1 receptor binding sites when compared with those determined in the absence of RAMP (not shown). Table I shows the results of subsequent competition binding assays between $^{125}$I-VIP and a variety of low and high affinity VPAC1 receptor ligands. Unlike their behavior with either the CTR or CL receptor, the association of RAMPs with the VPAC1 receptor did not cause a significant alteration in the binding properties of the receptor in either whole cells or membranes (Table I).

The VPAC1 receptor is known to couple promiscuously to different classes of G proteins, leading to the intracellular stimulation of cAMP accumulation and PI hydrolysis (20). We chose to quantify the generation of these second messengers in response to VPAC1 receptor agonists in the absence or presence of co-transfection of RAMPs (Fig. 2, A and B, left panels) and compared the responses of these agonists with agonists acting via the CTR (Fig. 2, D and E, right panels). PI and cAMP accumulation in response to the indicated agonist ligands were determined in parallel in the same group of cells 48 h post-transfection. Bars represent the mean ± S.E. of 5–6 experiments conducted in triplicate; *, p < 0.05. C, concentration-response curves to VIP signaling via the VPAC1 receptor in the absence (open symbols) or presence (solid symbols) of RAMP2 in COS-7 cells. Each point represents the mean ± S.E. of six experiments conducted in triplicate; **, p < 0.01. All other details are as described for A and B. h, human; s, salmon, r, rat.
Novel RAMP-Receptor Interactions

Ligand potency estimates (negative logarithm of the concentration yielding 50% displacement of specific radioligand binding \((pIC_{50})\) for the VPAC1 receptor expressed in COS-7 cells were determined via \(^{125}\)I-VIP competition binding in the absence or presence of co-expression of each RAMP. Values represent mean \pm S.E. of 3–4 experiments performed in triplicate. PHI, peptide histidine isoleucine.

| Agonist | VIP | PACAP-27 | PACAP-38 | Helodermin | PHI-27 |
|---------|-----|----------|----------|------------|--------|
| Membranes\(^b\) |     |          |          |            |        |
| Vector  | 8.93 ± 0.05 | 8.94 ± 0.04 | 8.67 ± 0.06 | 6.28 ± 0.05 | nd     |
| RAMP1   | 8.98 ± 0.04 | 8.98 ± 0.04 | 8.59 ± 0.08 | 6.26 ± 0.04 | nd     |
| RAMP2   | 9.08 ± 0.05 | 9.04 ± 0.04 | 8.74 ± 0.09 | 6.32 ± 0.04 | nd     |
| RAMP3   | 8.92 ± 0.03 | 8.90 ± 0.06 | 8.55 ± 0.07 | 6.29 ± 0.05 | nd     |
| Whole cells\(^a\) |     |          |          |            |        |
| Vector  | 7.47 ± 0.18 | 7.84 ± 0.14 | 7.88 ± 0.14 | <6         | <6     |
| RAMP1   | 7.82 ± 0.11 | 8.12 ± 0.15 | 8.17 ± 0.12 | <6         | <6     |
| RAMP2   | 7.53 ± 0.19 | 8.12 ± 0.19 | 7.99 ± 0.14 | <6         | <6     |
| RAMP3   | 7.62 ± 0.14 | 8.04 ± 0.20 | 7.89 ± 0.09 | <6         | <6     |

\(^a\) Additional competition experiments using secretin or GHRH (100 nm, membranes; 1 \(\mu\)m, whole cells) as competitor had no effect on specific \(^{125}\)I-VIP binding.

\(^b\) Differences in \(pIC_{50}\) values for whole cells and membranes likely reflect the presence of excess guanine nucleotide in whole cells.

CTR (Fig. 2, A and B, right panels). As shown in Fig. 2A, the co-expression of the VPAC1 receptor and RAMP2 led to a significant enhancement of the PI response to the VPAC1 receptor agonists. Although there was a slight enhancement in the PI response when the receptor was co-expressed with RAMP3, it was not statistically significant. In contrast, the cAMP response mediated by the VPAC1 receptor was not significantly affected by co-expression of any of the RAMPs (Fig. 2B). When similar experiments were performed with the CTR and its agonists, no selective augmentation of the PI response by any of the RAMPs was noted (Fig. 2A). In subsequent experiments, we established complete concentration-response curves to VIP for both PI and cAMP accumulation. Fig. 2C shows that the potency of VIP to mediate PI hydrolysis remained unaltered, whereas the maximal response was significantly enhanced in the presence of RAMP2. When VIP-mediated cAMP accumulation was investigated, there was no significant effect of RAMP2-VPAC1 receptor co-expression on either the maximal agonist response or agonist potency. The lack of change in agonist potency is consistent with our competition binding data in which no change in ligand affinity was observed. To ensure that the increased PI response seen with RAMP2 was not due to increased cell surface receptor expression, additional experiments were performed in which N-terminally FLAG-tagged VPAC1 receptors were co-transfected with each of the RAMPs and both \(^{125}\)I-VIP and \(^{125}\)I-IgG binding (to detect anti-FLAG antibody as an independent measure of cell surface-expressed receptor) were assessed in parallel. No significant difference was observed in either \(^{125}\)I-VIP binding or anti-FLAG antibody binding, confirming that co-expression with RAMPs did not modify the levels of cell surface-expressed receptor. Taken together, these findings indicate that the association of RAMP2 and the VPAC1 receptor differentially affects the coupling efficiency of the receptor through different signal transduction pathways. Importantly, the effect of RAMPs on intracellular signal strength is receptor specific, with no parallel modulation of CTR-mediated PI hydrolysis found. Thus, we have identified a novel behavior of RAMPs that is distinct from their effect on expression of receptor phenotype.

The finding that PI signal strength is specifically augmented by RAMP2 is highly relevant because the classic coupling pathway associated with VPAC1 receptor activation in a number of cell and tissue types has always been the stimulation of cAMP accumulation via coupling to G\(_s\) proteins; receptor-mediated PI hydrolysis is either weakly stimulated or not observable (20). Our finding of a selective enhancement of the VPAC1 receptor PI response in the presence of RAMP2 is one possible mechanism that would explain some of the contradictory results observed in other studies of VPAC1 receptor signaling. Specifically, the endogenous RAMP complement may exert a hitherto unappreciated impact on the signaling efficiency of the VPAC1 receptor.

Important questions remain regarding the functional roles of the VPAC1-RAMP1, VPAC1-RAMP3, PTH1-RAMP2, glucagon receptor-RAMP2, and PTH2-RAMP3 complexes that we have identified. In the case of the VPAC1-RAMP1 heterodimers, despite very potent translocation of RAMP1 to the cell surface and reduction of RAMP1 homodimerization (not shown), we were unable to observe any obvious effects on receptor binding or classic signal pathways such as cAMP or PI accumulation. However, this does not mean that the complexes are pharmacologically indistinguishable from the VPAC1 receptor monomer. For example, the VPAC1-RAMP1 complexes may display altered trafficking properties, altered protein–protein interactions, or differential signaling through other intracellular pathways. Indeed, it is now clear that ligand efficacy is manifested in a variety of both classic and nonclassic modes (21), and thus RAMP-receptor complexes may impact upon these modes to differing extents.

In conclusion, we have provided evidence for the direct interaction of RAMPs with four new receptor partners, thus illustrating for the first time that RAMP-receptor complexing extends to receptors beyond those activated by the calcitonin family of peptides. The ability of RAMP2 to augment VPAC1 receptor signaling with no concurrent alteration in CAMP identifies a new functional consequence of RAMP-receptor interactions and suggests that RAMPs may play a more general role in modulating cell signaling through other GPCRs than is currently appreciated.

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