Selective Interactions between Helix VIII of the Human \(\mu\)-Opioid Receptors and the C Terminus of Periplakin Disrupt G Protein Activation*

Analysis of interactions between the C-terminal tail of the MOP-1 and MOP-1A variants of the human \(\mu\)-opioid receptor with proteins derived from a human brain cDNA library resulted in identification of the actin and intermediate filament-binding protein periplakin. Mapping of this interaction indicated that the predicted fourth intracellular loop/helix VIII of the receptor interacts with the C-terminal rod and linker region of periplakin. Periplakin is widely expressed in the central nervous system of both man and rat and demonstrated an overlapping but not identical distribution with \(\mu\)-opioid (MOP) receptors. Co-expression of periplakin with MOP-1 or a MOP-1-eYFP fusion construct in HEK293 cells did not interfere with agonist-mediated internalization of the receptor. When co-expressed with a MOP-1-G\(_{\alpha}\) fusion protein periplakin significantly reduced the capacity of the agonist to stimulate binding of \(^{35}\)S-GTP\(_{\gamma}\)S to the receptor-associated G protein. By contrast, periplakin did not interfere with agonist-stimulation of \(^{35}\)S-GTP\(_{\gamma}\)S binding to either an \(\alpha_{2A}\)-adrenoreceptor-G\(_{\alpha}\)\(_{i}\) fusion protein or a \(\beta_{2}\)-adrenoreceptor-G\(_{\alpha}\) fusion protein, indicating its selectivity of function. This represents the first example of an opioid receptor-interacting protein that functions to disrupt agonist-mediated G protein activation.

The basic module of G protein-mediated signal transduction has long been considered to be a G protein-coupled receptor (GPCR), a G protein, and an effector. However, in recent times a wide range of proteins has been identified that interacts with either GPCRs (1–3) or G proteins (4–6), and these can modulate signal transduction efficiency, cellular localization, or the regulation of these polypeptides. Although certain protein-protein interactions can be anticipated based on the presence of well characterized protein interaction motifs in the primary sequence of the GPCR (1), many of the reported interactions do not involve previously characterized motifs. A widely used strategy to identify such interactions is the yeast two hybrid system (7).

The core opioid GPCR family comprises the MOP, KOP, and DOP receptors (8–9). These have been studied extensively in the search for non-addictive analgesics and particular interest centers on the MOP receptor because it mediates most of the actions of morphine and other clinically relevant analgesic agents as well as drugs of abuse such as heroin. Mice in which the genes for the various opioid receptors have been knocked out have contributed significantly to understanding (10). A number of MOP receptor subtypes have been defined pharmacologically (11–12), and it is possible that these represent hetero-dimers containing both the MOP and either DOP, KOP (12–15), or other related GPCRs (16). In rodents a number of distinct MOP splice variants have been described (17–19) that vary in distribution (20). In man, two variants, MOP-1 and MOP-1A, were described initially (21), but further potential variation has recently been indicated (22).

Recent studies have demonstrated a number of opioid receptor-interacting proteins. These include the ezrin-radixin-moesin binding phosphoprotein-50/Na\(^+\)/H\(^+\) exchanger regulatory protein that has been shown to interact with KOP and prevent agonist-induced down-regulation of the receptor by enhancing its recycling rate (23) and a GPCR-associated sorting protein shown to interact with DOP and alter the recycling characteristics of this receptor (24). Further studies have recently indicated an interaction between the rat MOP and phospholipase D2 (25). This also appears to be involved in the regulation of agonist-induced internalization of the receptor (25). These interactions either did not produce significant effects on G protein activation by the receptors or this issue was not examined.

By analysis of proteins identified to interact with the C-terminal tail of the human MOP-1 and MOP-1A isoforms herein we demonstrate the interactions of these receptors with periplakin (PPL). Periplakin does not significantly alter agonist-induced internalization of MOP-1, but by interacting with the postulated helix VIII of the receptor that likely runs parallel to the plasma membrane (26–29) it interferes with agonist-mediated activation of G protein. This region of rhodopsin has been demonstrated to play an integral role in G protein activation (30). This interaction is selective because the presence of periplakin did not interfere with the ability of agonists

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**To whom correspondence should be addressed: Davidson Bldg., University of Glasgow, Glasgow G12 8QQ, Scotland, UK. Tel.: 44-141-330-5557; Fax: 44-141-330-4620; E-mail: g.milligan@bio.gla.ac.uk.

† The abbreviations used are: GPCR, G protein-coupled receptor; DOP, \(\delta\)-opioid receptor; KOP, \(\kappa\)-opioid receptor; MOP, \(\mu\)-opioid receptor; PPL, periplakin; PPLC, C terminus of PPL; GTP\(_{\gamma}\)S, guanosine 5\(^\prime\)-3\(^\prime\)-(thio)triphosphate; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
at the α2A-adrenoceptor or the β2-adrenoceptor to activate their cognate G proteins.

EXPERIMENTAL PROCEDURES

[5HT]Dipropionophosphate and [5HBS-7994-197] were from Amebios Biosciences. [5H]Hydroxypropylnornorphine and [5S]GTP·S were from PerkinElmer Life Sciences. Reagents for cell culture were from Invitrogen. Pertussis toxin and general reagents were from Sigma. The C terminus of periplakin (PPLC) in PGEX-4T-1 and HA-tagged full-length periplakin (HA-PPL) in pCII-neo vector were gifts of Dr. S. Aho (Thomas Jefferson University, Philadelphia, PA).

Generation of DNA Constructs, Yeast Two-hybrid Analysis, TAQMAN Analysis, and Immuno blotting Studies—PPLC was inserted into pQE30 (Qiagen). Fusion proteins between MOP-1 and Cys351-Ile-Gi1α (31), the α2-adrenoceptor and Cys351-Ile-Gi1α, and the β2-adrenoceptor and Gi1α (33) have been described previously. Yeast two-hybrid analysis was conducted as described previously (34). RNA purification and TAQMAN reverse transcription-PCR analysis of human perfused as described previously (35). Immuno blotting studies were performed on homogenates of dissected rat brain regions or on pre-prepared gel-ready Medley samples of human brain obtained from BD Biosciences.

Cell Culture and Transient Transfection—HEK293 cells were maintained in DMEM containing 10% newborn calf serum and 2 m M glutamine. The day before transfection, cells were seeded either in 10-cm dishes or on coverslips in 6-well plates at 50–70% confluency. Transfection was performed using LipofectAMINE reagent (Invitrogen). 48 h later cells in the dishes were washed twice with ice-cold PBS in situ, harvested in 5 ml of PBS, and pelleted by centrifugation at 1,600 rpm at 4 °C. These pellets were kept at −80 °C until membrane preparation. For coverslips in 6-well plates, 24 h after transfection they were fixed or subjected to immunostaining as follows.

Fluorescence Staining and Confocal Microscopy—Cells on coverslips were fixed in 1% paraformaldehyde in PBS containing 5% sucrose for 10 min at room temperature. Cells were then permeabilized for 10 min in TM buffer (0.15% Triton X-100 and 3% nonfat milk in PBS). Coverslips were incubated for 1 h at room temperature with mouse anti-HA antibody (2.5 μg/ml Roche Applied Science), washed in TM buffer and PBS, and then incubated for a further 1 h with Alexa conjugated anti-mouse 594. After washing, coverslips were mounted onto glass slides and examined using a laser-scanning Zeiss LSM510 confocal microscope.

Preparation of Membranes—Cell pellets were resuspended in TE buffer (10 mM Tris HCl/0.1 mM EDTA, pH 7.5) and homogenized with 30–50 strokes of a Teflon-on-glass homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1,000 rpm for 10 min. The supernatant was then centrifuged at 50,000 rpm for 30 min. The pellets were resuspended in TE buffer at −80 °C until use.

Ligand Binding Assays—The level of expression of MOP-1 and the MOP-1-Cys341-Ile-Gi1α fusion protein was determined by the binding of [3H]Dipropionophosphate (2 nM) in TEM buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA, 12.5 mM MgCl2). Nonspecific binding was defined with 50 μM naloxone. Samples were incubated at 25 °C for 1 h and stopped by adding 5 ml of cold TE buffer followed by immediate filtration through GF/C filters and washing. Binding assays were to measure levels of expression of the α2-adrenoceptor-Cys351-Ile-Gi1α and the β2-adrenoceptor-Gi1α fusion proteins have been described previously (32–33).

Receptor Internalization Assay by Biotin Labeling of MOP-1—24 h after transfection with or without HA-PPL cells were transferred into 6-well plates and cultured for further 24 h. Cells were incubated with the MOP-selective agonist DAMGO (10 μM) for varying times and washed immediately 2× with PBS and 2× with PBS-CM (PBS containing 1 mM MgCl2, 0.1 mM CaCl2). Biotin labeling was performed in a dark room under dim light. Cells were treated with ice-cold 10 mM sodium periodate in PBS-CM and then with ice-cold 1 mM biotin-LC-hydrazide in acetate buffer (0.1 mM sodium acetate, 1 mM MgCl2, 0.1 mM CaCl2) for 10 min on ice. Unreacted biotinylated cells were lysed in radioimmunoprecipitation assay buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5% ethylene glycol, and a mixture of protease inhibitors). Solubilized proteins were collected by a 15-min centrifugation at 13,000 rpm at 4 °C. Equal amounts of protein from each sample were used for immunoprecipitation using an antibody against the C terminus of human MOP-1 (anti-MT43). Immuno complexes were immobilized by protein A-Sepharose 4B (Sigma), resolved in 4–12% Tris-Bis NuPAGE (Invitrogen), and transferred to nitrocellulose. Biotin-labeled MOP-1 was detected by horseradish peroxidase-conjugated streptavidin and visualized by ECL.

Purification of His-tagged Proteins—Plasmids bearing the desired His fusion inserts were transformed into competent Escherichia coli XL1-Blue. From an overnight culture, 1 ml was transferred to 10 ml of LB media containing 100 μg/ml ampicillin, and cells were allowed to grow at 37 °C until the culture reached an A600 of 0.4–0.6. 1 ml isopropyl-β-D-thiogalactopyranoside was added for 4 h before harvesting by centrifugation at 8,000 rpm for 15 min at 4 °C. The pellet was resuspended in 10 ml of lysis buffer, and His-tagged proteins were purified according to the manufacturer (Qiagen). Purification of proteins were dialized against at least three changes of PBS containing 5% glycerol at 4 °C over a period of 2 days before storage at −80 °C.

Purification of GST Fusion Proteins and GST Pull-down Assays—Bacterial cultures as above were harvested at 8,000 rpm for 15 min. The cell pellets were then lysed in 10 ml of BugBuster containing 10 μl of benzonase (Novagen) and a protease inhibitor mixture, incubated for 1 h at room temperature with rotation, and cleared by centrifugation at 16,000 rpm for 30 min at 4 °C after 2 × 1 min of sonication. The supernatants were then either kept at −80 °C until used for GST pull-down assays or directly purified using glutathione-Sepharose 4B beads (Amersham Biosciences).

GST pull-down assays, 1–5 μl of the soluble lysates were incubated with 100 μl of 50% (w/v) slurry of glutathione-Sepharose beads for 2 h at 4 °C. After a brief centrifugation (1,000 rpm, 2 min), the beads were washed 3× with PBS containing 1% Triton X-100 and resuspended in 1 ml of PBS/Triton X-100 containing 50 μg of required His fusions. The mix was then incubated for an additional 2 h before collection of the beads. These were washed 5× with PBS/Triton X-100, washed again with PBS, and then eluted in 50 μl of 10 mM glutathione in a Tris-HCl buffer, pH 8.0. The eluates were resolved, and the His-tagged protein was detected by immunoblotting.

[5S]GTP·S Binding Assays—24 h after transfection with MOP-1-Cys351-Ile-Gi1α, plus or minus HA-PPL cells were treated with 25 ng/ml pertussis toxin for 16 h before harvest. [5S]GTP·S binding experiments were initiated by the addition of membranes containing 25 fmol of MOP-1-Cys351-Ile-Gi1α, measured by the binding of [3H]Dipropionophosphate, to an assay buffer (20 mM HEPES, pH 7.4, 5 mM MgCl2, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% (w/v) bovine serum albumin,10 μM guanosine 5′-diphosphate, 50 nCi of [5S]GTP·S containing the indicated concentrations of DAMGO. Nonspecific binding was determined in the presence of 10 μM GTP·S. Reactions were incubated for 15 min at 30 °C and terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM MgCl2, and 100 mM NaCl. The samples were centrifuged (16,000g, 15 min, 4 °C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P-40) plus 0.2% SDS. Protein samples were preclarified by centrifugation (30,000g, 10 min) and immuneprecipitated with an antisera that identifies the C-terminal decapeptide of Gi1α. Finally, the immunocoupled complexes were washed twice with solubilization buffer, and bound [5S]GTP·S was measured by liquid scintillation spectrometry. Equivalent experiments were performed after transfection of fusion proteins between the α2-adrenoceptor and Cys351-Ile-Gi1α (32) or between the β2-adrenoceptor and Gi1α (33) in the presence or absence of HA-PPL. End of assay immunocapture of the β2-adrenoceptor-Gi1α fusion protein utilized an antisera that identifies the C-terminal decapeptide of Gi1α (33).

RESULTS

Protein Interaction Studies—Bio-informatic analysis of expressed sequence tags suggests only two forms of MOP are expressed in man. These differ only in the extreme C-terminal tail, resulting in MOP-1A being eight amino acids shorter than MOP-1. Based on the presence of a potential, weak coiled-coil domain in the C terminus of human MOP-1 we sought protein-interacting partners for the C-terminal tails of MOP-1 and MOP-1A. The C-terminal 75 amino acids of MOP-1 and 67 amino acids of MOP-1A were employed as bait for yeast two-hybrid screens using a human brain-derived cDNA library. Of 147 hits for the MOP-1 tail from 1.2 × 107-transformed cDNAs and 58 hits for the MOP-1A tail from 6 × 107-transformed cDNAs, multiple clones corresponded to PPL (36–38) (Fig. 1a).
The interaction of PPL with both MOP-1 and MOP-1A eliminated the possibility that binding was to the extreme C terminus of MOP-1. The interaction with the C-terminal tails of the MOP-1 and MOP-1A receptors required the extreme C-terminal linker domain of PPL and/or part of the C-terminal region of its rod domain as all the characterized hits from the yeast two-hybrid assays contained these regions. A series of fragments based on the C-terminal 75 amino acids of MOP-1 delimited the site of interaction. Cys\(^{348}\) and Cys\(^{353}\), that may be sites for \textit{in vivo} post-translational acylation, are boxed. FL, full-length.

Fig. 1. The C-terminal region of periplakin interacts with the C-terminal tail of MOP receptor isoforms. Yeast two-hybrid analysis is shown. Interactions between PPL and the C-terminal tail of both MOP-1 and MOP-1A were detected by yeast two hybrid analyses of cDNAs transformed from a human brain cDNA library. a, the domain structure of PPL. All the characterized yeast two-hybrid hits with the C-terminal region of MOP-1 and MOP-1A contained the denoted (YTH) C-terminal segment of PPL. The domain nomenclature is defined in Green et al. (47). a.a., amino acids. b, a series of fragments based on the C-terminal 75 amino acids of MOP-1 delimited the site of interaction. Cys\(^{348}\) and Cys\(^{353}\), that may be sites for \textit{in vivo} post-translational acylation, are boxed. FL, full-length.

Yeast two-hybrid

|     | FL | Y1 | Y2 | Y3 | Y4 | Y5 | Y6 | Y7 | N1 | N2 |
|-----|----|----|----|----|----|----|----|----|----|----|
| pACT2 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Periplakin | +++ | +++ | +++ | +++ | +++ | +++ | +  | -  | +++ | -  |

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To confirm the results of the yeast two-hybrid analyses, a GST fusion protein containing the C-terminal 80 amino acids of MOP-1 was generated and linked to glutathione-Sepharose 4B beads. This was able to capture a His-tagged form of the C-terminal 208 amino acids of PPL (PPLC) (Fig. 2a). When GST fusion proteins of both the C-terminal 43 amino acids and the remaining 37 amino acids of the 80 amino acid MOP-1 fragment were generated, interaction with PPLC was preserved only for the membrane proximal 37-amino acid section (Fig. 2a). Elimination of 10 or 20 amino acids from the N terminus of the 80-amino acid fragment did not prevent interactions with PPLC (Fig. 2a). However, removal of a further 10 amino acids abolished the interaction (Fig. 2a). The combination of these data (Fig. 2b) delimited the site of interaction of the C-terminal 208 amino acids of PPL with the region between amino acids 341–351 (LDENFKRCFRE) of MOP-1. Based on the structure of bovine rhodopsin (26) and comparisons of other related GPCRs (29), this sequence is likely to represent helix VIII of the MOP-1 and MOP-1A receptors. Further GST fusion proteins, including a 23-amino acid segment corresponding to the third intracellular loop of the human MOP receptors, failed to indicate interactions between PPLC and other linear, intracellular regions of the MOPs (data not shown). Importantly for subsequent studies, the addition of enhanced green fluorescent protein to the C-terminal end of the MOP-1 tail did not prevent interaction between the C-terminal tail of the receptor and PPLC (Fig. 2c). Reciprocal experiments demonstrated that a GST fusion protein containing the C-terminal 208 amino acids
of PPL (amino acids 1548–1756) was able to capture a His-tagged form of the MOP-1 C-terminal tail (Fig. 2d). However, we were unable to define the region of PPLC responsible for this interaction in detail as two similar-sized sections derived from this C-terminal region (periplakin 1548–1666 and periplakin1667–1756) were both unable to capture the His-tagged form of the MOP-1 C-terminal tail (data not shown).

Expression and Distribution of Periplakin—Low levels of MOP-1 transcripts were detected in a number of distinct regions of human brain using TAQMAN quantitative reverse transcription-PCR (Fig. 3a). Although PPL has been most actively studied as a 195-kDa protein of the keratinocyte cytoskeleton and desmosomes (36–37), its transcript was also highly and widely expressed in the central nervous system. High levels of mRNA were detected in regions of human brain including the frontal and temporal...
lobes, amygdala, thalamus, hippocampus, and cerebellum (Fig. 3b). Immunoblotting studies with antibodies directed toward the C-terminal region of PPL identified a polypeptide of some 195 kDa in lysates of all available regions of human (Fig. 3c) and rat (Fig. 3d) brain. Immunodetected levels of PPL were relatively similar in individual, gross regions of human brain and were especially high in the pituitary and olfactory bulb from rat. Antibodies directed toward the N- (Fig. 3e) or C-terminal (data not shown) of PPL also easily detected a single 195-kDa protein in human keratinocytes. However, PPL expression in the neuron-derived cell lines, NG108–15 and SHSY-5Y, was below immunodetectable levels (Fig. 3e). Anti-PPL antibodies were also unable to detect protein expression in HEK293 cells (Fig. 3e).

Periplakin Does Not Interfere with Internalization of MOP-1—
Transient expression of full-length MOP-1-eYFP in HEK293 cells resulted in a predominantly plasma membrane-delineated distribution when examined by confocal microscopy (Fig. 4a). Exposure of these cells to the highly MOP selective enkephalin analogue DAMGO (10 μM) resulted in rapid redistribution of the eYFP signal to punctate intracellular vesicles that are likely to represent recycling endosomes (Fig. 4a). HA-tagged PPL was distributed more widely in cells but was excluded from the nucleus and showed a distinct corona of staining close to the plasma membrane (Fig. 4b). With co-expression, there was a clear overlap of the signals corresponding to the two polypeptides at the cell surface (Fig. 4c). The presence of HA-PPL did not prevent DAMGO mediated internalization of MOP-1-eYFP in HEK293 cells (Fig. 4c), but there was no evidence that DAMGO treatment altered the cellular distribution of HA-PPL. Indeed, the signals corresponding to the two polypeptides separated during exposure to the agonist (Fig. 4c). Unlike the receptor, HA-PPL did not move into an endocytic compartment after agonist treatment as there was no overlap of the signals in the MOP-1-eYFP-positive intracellular vesicles (Fig. 4c). The internalization of MOP-1 in intact HEK293 cells in response to DAMGO was also assessed by the removal from the cell surface of receptors available to be biotinylated (Fig. 5a). Biotinylated MOP-1 migrated in SDS-PAGE predominantly as an ~80-kDa species. Higher molecular mass species may represent dimeric and aggregated forms of the receptor. Co-expression with HA-PPL did not prevent the agonist-induced removal of MOP-1 from the cell surface (Fig. 5). Over short time periods, removal of MOP-1 from the cell surface did not reflect a down-regulation of the total cellular levels of the receptor (Fig. 5b).

Periplakin Interferes Selectively with MOP Activation of G Protein—Given that helix VIII of the rhodopsin-family receptors is believed to be a key functional contact site for the N-terminus of G protein α subunits, we explored whether PPL would interfere with G protein activation by MOP-1. After transient expression in HEK293 cells of a fusion protein in which the N terminus of a pertussis toxin-resistant (Cys351-Ile) variant of Gi1 subunits, we explored subunits, we explored interactions between helix VIII of the rhodopsin-family receptors and PPLC, this does not appear to be the only region of PPL that contributes to its effect on agonist-activation of G protein. An HA-tagged version of PPL lacking the C-terminal 208 amino acids was constructed and expressed (Fig. 7a). This was also able to inhibit DAMGO-stimulated binding of[^35S]GTPγS to the MOP-1-Gi1α fusion protein (Fig. 7b).

The effects of PPL were selective. Yeast two-hybrid assays using the C-terminal tails of the α2δ-adrenoceptor, the 5-hydroxytryptamine 5-HT1A receptor, or the β2-adrenoceptor did not identify PPL as a potential interacting protein (data not shown). The ability of adrenaline to stimulate the binding of[^35S]GTPγS to an α2δ-adrenoceptor-Gi1α fusion protein (Fig. 8) or of isoprenaline to stimulate binding of[^35S]GTPγS to a
DISCUSSION

Opioid receptors signal predominantly via members of the Gi family of heterotrimeric G proteins (8). They also internalize and recycle to the cell surface after challenge with efficacious peptide and alkaloid ligands as part of the processes of receptor desensitization and resensitization. Recently, interactions of each of the DOP (24), the KOP (23), and the MOP receptors (25) with proteins that alter their intracellular sorting and recycling rates have been reported. By using the C-terminal tails of the human MOP-1 and MOP-1A receptors as bait in yeast two-hybrid screens with proteins generated from a human brain cDNA library we identified an interaction with PPL. Such interactions were confirmed in a range of pull-down studies. PPL is a member of the plakin family of cytolinker proteins (36). It has been most fully studied in keratinocytes and produces the scaffold on which the cornified envelope is formed (37). It is well suited to such a role as it is a large, 195-kDa, multi-domain protein known to interact with actin and intermediate filament proteins (40–42). This role reflects specific contributions from the N-terminal domain and the ability of the rod segment of the polypeptide to allow both homodimerization and heterodimerization with other related plakins such as envoplakin. The C terminus appears to play roles in interactions with intermediate filaments and has recently been shown to be the region involved in interaction with protein kinase B (43). However, although detailed studies on the function of PPL have been largely restricted to skin, early cloning and mapping studies indicated it to be expressed in the brain (38). Quantitative reverse transcription-PCR confirmed this and in combination with direct immunoblotting studies demonstrated that significant levels of PPL mRNA and protein expression could be detected in a wide range of human and rat brain regions, including those that express the MOP receptors.

Mapping of sites of interaction between PPL and the human β2-adrenoreceptor-Gα fusion protein (Fig. 8) was unaffected by the expression of PPL.

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Fig. 5. Periplakin does not prevent removal of MOP-1 from the cell surface.

a. HEK293 cells were transfected to express MOP-1 in the absence or presence of HA-PPL. Cell surface MOP-1 was monitored by biotinylation. Incubation of the cells with DAMGO for 0, 30, or 90 min resulted in extensive removal of MOP-1 from the cell surface in the absence or presence of PPL. b. At short time points this does not reflect down-regulation of PPL. HEK293 cells were transfected to express MOP-1 in the absence or presence of PPL. b, biotinylation experiments akin to those of panel a were conducted in the absence of DAMGO or after challenge for 20 min with 10 μM. Cell lysates were resolved by SDS-PAGE and immunoblotted to detect PPL (i) or MOP-1 (ii).

Fig. 6. Periplakin interferes with MOP-1 activation of Gα. A fusion protein between MOP-1 and a pertussis-toxin insensitive (Cys351-Ile) variant of Gα was expressed transiently in HEK293 cells in the absence (1) or presence (2) of HA-PPL. a, cell lysates were immunoblotted to detect HA-PPL. b, [3H]diprenorphine binding studies indicated lower levels of expression of MOP-1-Gα in the presence of PPL (2) than in its absence (1). c, MOP-1-Gα levels in membrane preparations from the cells were quantitated by the binding of [3H]diprenorphine and membrane amounts expressing 25 fmol of the fusion construct used in [35S]GTPγS binding studies in the absence (open bars) or presence (filled bars) of 10 μM DAMGO. At the termination of the assay samples were immunoprecipitated with an antiserum that identifies the C-terminal decapeptide of Gα and counted.
MOP isoforms indicated that it was a region within the last 208 amino acids of the C-terminal domain of PPL that interacted with the C-terminal tail region of MOP receptors. Fine mapping of the region of the MOP receptors responsible for this interaction defined a region of some 11 amino acids that are proximal to the plasma membrane. The vast majority of rhodopsin-like GPCRs contain one or more cysteine residues that can be post-translationally acylated within 10–15 amino acids of the end of transmembrane helix VII (44). Because direct studies on rhodopsin demonstrate that these acyl chains are able to insert into the plasma membrane to provide a point of anchorage (45), this region became known as the fourth intracellular loop. With crystallization it became apparent that this section forms an eighth helix that runs parallel to the plasma membrane (26). Structural similarity between the rhodopsin-like receptors suggests that this will be a common feature (29) and, thus, that the site of interaction of the C terminus of PPL with the MOP isoforms is at this helix. Models of the interaction of GPCRs with G proteins indicate a likely interaction between helix VIII and the N terminus of the G protein subunit (28). Furthermore, although a key site of interaction of GPCRs is provided by the extreme C terminus of the G protein α subunit, an important role for the N terminus of the G protein α subunit has long been appreciated. We thus considered that the presence of PPL might disrupt agonist activation of G protein rather than internalization of the MOP receptors, as regulation of internalization generally involves the distal elements of the C-terminal tail. Indeed, internalization of MOP-1 was not altered by the presence of PPL. To explore the capability of PPL to interfere with MOP-1 activation of G protein, we took advantage of a fusion strategy in which a pertussis toxin-resistant variant of the α2AR-α (α2AR-Gi1α) or the β2-adrenoreceptor- and Gi1α (β2AR-Gi1α) were expressed in HEK293 cells in the absence or presence of HA-PPL. Cell lysates were immunoblotted as in Fig. 6 to confirm expression of HA-PPL. [35S]GTP and [3H]dihydroalprenolol binding studies were performed on cell membranes to quantitate expression of the α2AR-adrenoreceptor- and β2-adrenoreceptor-containing fusion proteins, respectively. Membrane amounts expressing 20 fmol of the fusion constructs were used in [35S]GTPS binding studies in the absence of ligand (open bars) or in the presence of 10 μM adrenaline (α2AR-adrenoreceptor) or isoprenaline (β2-adrenoreceptor) (filled bars). At the termination of the assay samples were immunoprecipitated with antisera directed against the C-terminal decapeptide of the relevant G protein and counted.

Fig. 7. The effect of periplakin is not produced only by the C-terminal region. a, a form of HA-tagged PPL lacking the C-terminal 208 amino acids was generated. Full-length HA-PPL or Δ-208 HA-PPL was expressed in HEK293 cells along with the MOP-1-Gi1α fusion protein, and cell lysates were immunoblotted with an anti-HA antibody to detect expression. b, experiments akin to those of Fig. 6 were performed in membranes expressing MOP-1-Gi1α alone (open bars) or co-expressing MOP-1-Gi1α with either full-length (gray bars) or Δ-208 (black bars) HA-PPL. *, significantly different (p < 0.05) from MOP-1-Gi1α alone.

Fig. 8. The effects of periplakin are selective. Fusion proteins between the α2AR-adrenoreceptor and Cys351-Ile-Gi1α (α2AR-Gi1α) or the β2-adrenoreceptor and Gi1α (β2AR-Gi1α) were expressed in HEK293 cells in the absence or presence of HA-PPL. Cell lysates were immunoblotted as in Fig. 6 to confirm expression of HA-PPL. [3H]RS-79948 and [3H]dihydroalprenolol binding studies were performed on cell membranes to quantitate expression of the α2AR-adrenoreceptor- and β2-adrenoreceptor-containing fusion proteins, respectively. Membrane amounts expressing 20 fmol of the fusion constructs were used in [35S]GTPS binding studies in the absence of ligand (open bars) or in the presence of 10 μM adrenaline (α2AR-adrenoreceptor) or isoprenaline (β2-adrenoreceptor) (filled bars). At the termination of the assay samples were immunoprecipitated with antisera directed against the C-terminal decapeptide of the relevant G protein and counted.
proteins. When we generated and expressed a form of PPL lacking the C-terminal 208 amino acids, this was also able to interfere with agonist-stimulated binding of [35S]GTPγS to the MOP-1-Cys351-Ile-G1α fusion protein. Although yeast two-hybrid analyses are extremely useful in demonstrating interactions between linear peptide fragments from two proteins, they are not appropriate to examine complex interactions requiring sequences from more than one segment of a protein. We thus wished to confirm that the ability of PPL to interfere with agonist-stimulated binding of [35S]GTPγS to the MOP-1-Cys351-Ile-G1α fusion protein described that alters the effectiveness of G protein activation of the G proteins. Parallel yeast two-hybrid assays failed to demonstrate interactions of periplakin with the C-terminal tails of these GPCRs.

These results demonstrate interactions between helix VIII of the human MOP receptors and PPL and indicate that selective interactions between these polypeptides limit agonist activation of G protein. PPL is the first opioid receptor-interacting protein described that alters the effectiveness of G protein activation rather than the intracellular sorting and recycling of the receptor. The distribution of MOP and PPL in rat brain overlapped but was not identical. The current data suggest that MOP receptor signaling may be less effective in neurons that co-express the receptor and PPL than in those that do not. Future studies will test this hypothesis.

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