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THE MELANOSOMAL/LYSOSOMAL PROTEIN OA1 HAS PROPERTIES OF A G PROTEIN-COUPLED RECEPTOR

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SUMMARY

OA1, the protein product of the ocular albinism type 1 gene, is a pigment cell-specific integral membrane glycoprotein, localized to melanosomes and lysosomes and possibly implicated in melanosome biogenesis. Although its function remains unknown, we previously showed that OA1 shares structural similarities with G protein-coupled receptors (GPCRs). To ascertain the molecular function of OA1 and in particular its nature as a GPCR, we adopted a heterologous expression strategy commonly exploited to demonstrate GPCR-mediated signaling in mammalian cells. Here we show that when expressed in COS7 cells OA1 displays a considerable and spontaneous capacity to activate heterotrimeric G proteins and the associated signaling cascade. In contrast, OA1 mutants carrying either a missense mutation or a small deletion in the third cytosolic loop lack this ability. Furthermore, OA1 is phosphorylated and interacts with arrestins, well-established multifunctional adaptors of conformationally active GPCRs. In fact, OA1 colocalizes and coprecipitates with arrestins, which downregulate the signaling of OA1 by specifically reducing its expression levels. These findings indicate that heterologously expressed OA1 exhibits two fundamental properties of GPCRs, being capable to activate heterotrimeric G proteins and to functionally associate with arrestins, and provide proof of principle that OA1 can actually function as a canonical GPCR in mammalian cells.

Keywords

Ocular albinism; Melanosome; Lysosome; GPCR; G protein; Arrestin

INTRODUCTION

Ocular albinism type 1, the most common form of ocular albinism, is an X-linked inherited disorder, characterized by severe reduction of visual acuity, nystagmus, strabismus, photophobia, iris translucency, hypopigmentation of the retina, foveal hypoplasia and misrouting of the optic tracts, resulting in loss of stereoscopic vision (King et al., 1995). Patients affected with the disorder also show the presence of melanosomal abnormalities, including giant melanosomes (macromelanosomes), in the pigment cells of the skin and eyes (melanocytes and retinal pigment epithelium, RPE), suggesting an underlying defect in melanosome biogenesis (Garner and Jay, 1980; O'Donnell et al., 1976). The protein product of the gene responsible for this disorder, named OA1 (also identified as GPR143 in GenBank),

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is a 404 amino acids integral membrane glycoprotein, exclusively expressed by melanocytes and RPE cells and whose function remains unknown (Schiaffino et al., 1996).

Previous morphological and biochemical studies performed by us and others showed that the endogenous OA1 in melanocytes is exclusively detectable on intracellular membranes, in particular on melanosomes and late-endosome/lysosomes, consistent with a role in the biogenesis of these organelles (Samaraweera et al., 2001; Schiaffino et al., 1996; Schiaffino et al., 1999). More recently, we identified structural similarities between OA1 and members of the G protein-coupled receptor (GPCR) superfamily (Schiaffino et al., 1999). In fact, OA1 displays seven predicted transmembrane domains and sequence homologies with members of families A, B, and E of GPCRs, including the presence of residues highly conserved in most receptors, some of which are the site of albinism-causing mutations (d'Addio et al., 2000; Schiaffino et al., 1999). However, since the overall amino acid identity between OA1 and any know receptor is lower than 10%, the protein could not be assigned to any GPCR subfamily and even its nature as a GPCR remains to be established.

In addition, the exclusive intracellular localization of OA1 and its accumulation into specialized organelles like the melanosomes are unprecedented among GPCRs, which are typically located at the plasma membrane. Nevertheless, since heterotrimeric G proteins are widely distributed in endomembranes, it has long been suggested that GPCR-mediated signal transduction systems might also operate at internal locations in mammalian cells (Nurnberg and Ahnert-Hilger, 1996). Thus, OA1 might possibly represent the first example of an exclusively intracellular receptor, functioning as sensor for an intra-lumenal ligand and triggering a signal transduction cascade across the organelle membrane (Schiaffino and Tacchetti, 2005). Consistent with this idea, OA1 was found to colocalize and coprecipitate with heterotrimeric G proteins in normal human melanocytes (Schiaffino et al., 1999) and to interact with a yeast/mammalian G protein chimera in a yeast-based signaling assay (Staleva and Orlow, 2005). Unfortunately, no demonstration has been provided so far that OA1 could actually activate heterotrimeric G proteins and thus function as a bona fide GPCR.

Since the ligand and downstream effectors of OA1 are unknown (hence not allowing direct investigation of the endogenous pathway), here we took advantage of a heterologous expression strategy commonly adopted to study GPCR-mediated signaling in mammalian cells. Our results indicate that, as expected for a member of the GPCR superfamily, OA1 is able to activate heterotrimeric G proteins and is recognized by the specific GPCR-adaptors arrestins.

RESULTS

OA1 is detectable at the plasma membrane when expressed at high levels in COS7 cells

The most sensitive and standardized approaches to measure the signaling capacity of GPCRs exploit second messenger accumulation assays, which are based on classical GPCR-mediated signal transduction pathways taking place at the plasma membrane. Consequently, these assays require the cell surface localization of the receptors and their local interaction with G proteins and downstream effectors, such as adenylyl cyclase (AC) or phospholipase Cβ (PLCβ). Conversely, in melanocytes the endogenous OA1 is localized to the membrane of melanosomes and late-endosome/lysosomes, being excluded from the plasma membrane (Samaraweera et al., 2001; Schiaffino et al., 1996; Schiaffino et al., 1999). Even when expressed at moderate levels in nonmelanocytic cells, OA1 is delivered to the lysosomes (d'Addio et al., 2000; Schiaffino et al., 1999; Shen et al., 2001), behaving as other typical melanosomal proteins (Vijayasaradhi, 2000). To overcome this problem and exploit second messenger accumulation assays to study OA1 signaling, we expressed the protein in COS7 cells for 48 hours using FuGENE as transfection reagent. As shown in Figure 1 by immunofluorescence analysis, the

considerable protein production obtained in these conditions resulted in the appearance of a small amount of OA1 at the plasma membrane.

OA1 is able to activate heterotrimeric G proteins and the downstream cascade

GPCRs are characterized by the ability to couple with and activate heterotrimeric G proteins in response to agonist binding. Nevertheless, many GPCRs physiologically display some degree of agonist-independent or constitutive activity and this ability can be enhanced by expressing the receptors with their partner G proteins in heterologous systems (Milligan, 2003; Seifert and Wenzel-Seifert, 2003). Previous co-immunoprecipitation and pull-down studies suggested that OA1 might be able to couple with Gi and possibly Go and Gq proteins (Schiaffino et al., 1999). However, the canonical activity of the i/o class of heterotrimeric G proteins, i. e. lowering of cAMP levels, is not easily measurable. In contrast, Gq proteins activate PLCβ and lead to intracellular accumulation of inositol phosphates (IPs, including inositol 1,4,5-tris-phosphate and its di- and mono-phosphate degradation products), which can be conveniently evaluated by a highly sensitive assay.

Therefore, to amplify the potential agonist-independent activity of OA1 and translate it into intracellular accumulation of IPs, we co-expressed the putative receptor either with wild-type Gαq, or with other chimeric or promiscuous Gα subunits able to stimulate PLCβ. In particular, we utilized the chimeric G proteins Gαqo5, Gαqi5 and Gαqz5, which are identical to Gαq except that the 5 C-terminal residues are replaced with those of Gαo, Gαi and Gαz, respectively. Since the last 5 residues of Gα subunits have been shown to determine the receptor-G protein coupling specificity (Liu et al., 1995), these chimeras are expected both to recognize Gi/o coupled receptors and to stimulate PLCβ. In addition, we took advantage of the hematopoietic cell-specific G protein Gα15, which shows functional promiscuity and is able to redirect or enhance the coupling of a variety of different receptors to PLCβ, thus representing a convenient tool to reveal the signaling of GPCRs with unknown downstream pathways (Amatruda et al., 1991; Offermanns and Simon, 1995). Each of these G proteins was expressed in COS7 cells in the absence or in the presence of OA1. PLCβ activation was estimated by measuring the increase of intracellular IPs during a 1 hour interval, a time frame ensuring a linear IP accumulation in the presence of the inositol monophosphatase-inhibitor Litium (Zhu et al., 1994). The results of this analysis are shown in Figure 2.

Western analysis determined that the expression levels of the different G proteins were comparable in the presence or in the absence of OA1 co-expression (Figure 2A), implying that any variation of PLCβ activity in the two conditions should be ascribed to a functional interaction between putative receptor and signal transducer. While cells expressing OA1 alone showed a barely visible increase of PLCβ activity compared to mock transfected cells, those expressing the G proteins showed a significant ability to accelerate the phosphoinositide (PI) turnover, particularly when they were co-expressed with OA1 (Figure 2B, compare black to empty bars for each G protein). Among the G proteins tested, Gα15 was capable of eliciting the greatest increase of PLCβ activity in co-transfection with OA1, not only relative to basal, but also in absolute values (Figure 2B). Indeed, when OA1 and Gα15 were co-expressed, the amount of IPs recovered was largely exceeding the sum of those obtained with OA1 and G protein alone, indicating the presence of a functionally productive coupling between putative receptor and signal transducer (Figure 2C).

The specificity of the OA1-Gα15 interaction was confirmed by comparing the G protein-activation ability of the wild-type OA1 with that of 1) a mutant carrying a single amino acid substitution, which determines ER retention of the protein and its inability to interact with Gi by co-immunoprecipitation (OA1 C116S) (d'Addio et al., 2000; Schiaffino et al., 1999) and 2) a mutant carrying a small deletion in the third cytosolic loop (deletion of 18 amino acids, from A221 to M238) (OA1 Δ18). This latter mutant displays a subcellular localization identical to

the wild-type (M.V.S., unpublished results), nevertheless it is expected to exhibit a defective ability to activate Gα15, since the third cytosolic loop is known to play a critical role in G protein-coupling in canonical GPCRs (Wess, 1998). As shown in Figure 3, none of the mutants was able to appreciably activate the PLCβ cascade, displaying IP production levels similar to those obtained with Gα15 alone.

Finally, we tested the Gα15-activation ability of OA1 by comparison with other well-recognized GPCRs, including the formyl peptide receptor (fPR), the V2 vasopressin receptor (V2R) and the β2 adrenergic receptor (β2AR), which were previously shown to couple with Gα15 (Offermanns and Simon, 1995). Equal amounts of plasmid DNA encoding for the different receptors were co-transfected with Gα15 and PLCβ activation was estimated by measuring the increase of intracellular IPs in stimulated and non-stimulated conditions. The results of this analysis are shown in Figure 4. In non-stimulated conditions, only fPR showed levels of IP production similar to OA1. By converse, V2R and β2AR were able to exceed the activity of OA1 only upon addition of their respective ligands. Overall, the ligand-activated canonical GPCRs fPR, V2R and β2AR displayed IP levels 5-12 times those obtained by Gα15 alone. In the same expression conditions, but presumably less represented at the cell surface and in the absence of any specific agonist supplement, OA1 showed the capacity of amplifying 2-3 times the activity of the G protein. Collectively, these results indicate that OA1 displays a substantial and spontaneous ability to activate heterotrimeric G proteins, possibly due to amplification of its agonist-independent activity in the recombinant expression system.

OA1 is phosphorylated and interacts with arrestins

In addition to trigger G protein coupling and downstream signaling, activation of GPCRs promotes processes resulting in the downregulation of receptors' activity, including phosphorylation and arrestin binding. As in most GPCRs, serine and threonine residues are well represented in the cytosolic domains of OA1, particularly in the C-terminal tail, where they account for 22% of total amino acids. A prediction for phosphorylated protein sequence motifs (http://scansite.mit.edu) (Obenauer et al., 2003) revealed three potential PKC phosphorylation sites, conserved in mouse, rat, chicken, zebrafish and fugu, within the second (S148, T153) and third (S222) intracellular loops; at least four conserved casein kinase phosphorylation sites within the OA1 C-terminus (S369, S375, S378, T379); and a potential Src kinase phosphorylation site within the third intracellular loop (Y231). To investigate whether OA1 is actually phosphorylated, we immunoprecipitated the protein form transiently transfected and [^{35}S]Met/Cys labelled COS7 cells and analyzed it by in gel fluorography to reveal phosphorylated proteins (Pro-Q Diamond kit, see Experimental Procedures). The total amount of immunoprecipitated OA1 was subsequently quantified by autoradiography (Figure 5).

As previously reported, OA1 was found to accumulate in COS7 cells mostly as a doublet, corresponding to the protein backbone and a partially glycosylated form (d'Addio et al., 2000). Since in COS7 cells OA1 can efficiently exit the ER even in the presence of glycosylation inhibitors (tunicamycin; d'Addio et al., 2000), we believe that in the overexpression condition utilized nonglycosylated and partially glycosylated OA1 do not necessarily correspond to newly synthesized proteins retained in the ER, but may traffic normally in the secretory pathway. Indeed, both protein species were found phosphorylated and could be effectively de-phosphorylated by treating the immunoprecipitate with λ-phosphatase, an enzyme capable of removing phosphoric groups from serines, threonines and tyrosines. Activation and phosphorylation of GPCRs are believed to precede arrestin binding, which in turn promotes receptor uncoupling from G proteins, internalization and degradation (Ferguson, 2001; Gurevich and Gurevich, 2004). Thus, the above findings prompted us to explore whether OA1 interacts with these well-known GPCR adaptors. COS7 cells have been

previously reported to express low levels of ubiquitous arrestin isoforms (arrestin 2 and arrestin 3) (Menard et al., 1997). Accordingly, the overexpression of arrestins has been widely utilized in this system to study their functional interaction with various GPCRs, including PLCβ-coupled receptors (Castro et al., 2002; Chen et al., 2004; Diviani et al., 1996).

Thus, we co-expressed OA1 and arrestin 2-GFP or arrestin 3-GFP and looked for their colocalization by confocal immunofluorescence analysis (Figure 6). Arrestins-GFP showed a diffuse cytosolic distribution and in several cells were found to colocalize with OA1 in intracellular vesicles, similarly to what observed with constitutively active and desensitized receptors (Barak et al., 2001;Innamorati et al., 1997;Pei et al., 1994). As expected, arrestins-GFP were also found to colocalize extensively on intracellular structures with activated V2R, while no colocalization was found between arrestins-GFP and rat LAMP1, which accumulates in late-endosomes and lysosomes as OA1 (Figure 6). Furthermore, to test whether OA1 and arrestins interact by co-immunoprecipitation, we expressed arrestin 2-FLAG or arrestin 3-FLAG in COS7 cells, either in the absence or in the presence of OA1. Protein extracts were immunoprecipitated with anti-OA1 antibodies and the presence of coprecipitated arrestins was evaluated by Western blotting using anti-FLAG antibodies. As shown in Figure 7, both arrestin 2 and to a minor extent arrestin 3 were found to coprecipitate with OA1, indicating the presence of an in vivo interaction between putative receptor and GPCR adaptors.

Finally, to search for the functional consequences of the OA1-arrestin interaction, we co-expressed OA1 and Gα15 in COS7 cells either in the absence or in the presence of arrestin 2 or arrestin 3. The impact of arrestin co-expression on OA1 was tested by Western analysis and PLCβ activity assays. As shown in Figure 8A, arrestin co-expression induces a considerable reduction in the yield of OA1, both when the transfected OA1:arrestin plasmid DNA ratio was 1:5 and 1:1 (Figure 8A, lanes with OA1 at 0.1 and 0.5μg/well, respectively). Considering a mean of 9 different experiments performed using a 1:1 plasmid DNA ratio, arrestin 2 and 3 reduced the amount of OA1 by 57% and 47%, respectively, as determined by densitometric analysis of unsaturated blot films. In contrast, arrestin co-expression elicited no significant effects on the expression levels of Gα15 (Figure 8A), unstimulated V2R or β2AR (not shown), or a control construct (LAMP/CT, Figure 8A), carrying the C-tail of OA1 fused at the C-terminus of rat LAMP1. This chimeric protein shows a lysosomal/plasma membrane distribution (M.V.S. and R.P., unpublished results) and contains a domain of OA1 that is typically involved in desensitization and downregulation of GPCRs, however its expression was not reduced by arrestins, further supporting the specificity of the effect observed on full-length OA1.

Consistent with these findings, the OA1-dependent PLCβ activity was strongly reduced by arrestin co-expression (by about 60%, Figure 8B), confirming the exclusive role of the putative receptor in the activation of Gα15 in this assay. The decrease of OA1 yield by arrestins does not depend on an artifactual interference with the exogenous expression system (such as competition between plasmid promoters), since both OA1 and negative control proteins were expressed by the same vector and comparable results were obtained when OA1 was expressed by a pRc/RSV or a pcDNA3.1 vector (carrying a RSV or CMV promoter, respectively; not shown). Instead, pulse-chase analyses revealed a significantly faster degradation of OA1 in the presence of co-expressed arrestin (Figure 8C). Together, these findings indicate that arrestins bind and accelerate the degradation of OA1, further supporting the concept that the putative receptor displays the conformation of an activated GPCR in transfected COS7 cells.

DISCUSSION

The GPCR superfamily represents the most large, ubiquitous and functionally diverse group of plasma membrane receptors (Pierce et al., 2002). Despite their primary sequences may be

quite different, all GPCRs share common structural and functional features, including a seven-transmembrane topology, the ability to activate heterotrimeric G proteins and the sensitivity to downregulatory mechanisms (Pierce et al., 2002). Although receptor activation is typically elicited in response to agonist binding, GPCRs are assumed to exist in an equilibrium between multiple active and inactive conformations, so that even in the absence of the ligand a certain proportion of receptors spontaneously resides in the activated state and is able to couple with heterotrimeric G proteins (Gudermann et al., 1996). Indeed, agonist-independent activity has been reported not only for a number of GPCR mutants, but to variable extent also for most wild-type receptors (Milligan, 2003; Seifert and Wenzel-Seifert, 2003). In the absence of a known ligand for OA1, we exploited these features to provide evidence that this protein can actually function as a canonical GPCR.

Assuming that spontaneous activation might also occur with OA1, we expressed the putative receptor in COS7 cells together with PLCβ-coupled G proteins and evaluated the intracellular accumulation of IPs. Among the G proteins tested, Gα15 displayed the most substantial ability to reveal the signaling of OA1. In fact, when co-expressed with Gα15, OA1 was able to induce a significant accumulation of IPs compared to G proteins alone, OA1 mutants, and even unstimulated or ligand-activated canonical GPCRs. The G protein-mediated PLCβ activity induced by OA1 and demonstrated in the present study represents the first functional evidence that OA1 can actually behave as a GPCR in vivo in mammalian cells. Our findings are in agreement with a recent study by Staleva and Orlow (2005), reporting that OA1 displays a behavior similar to that shown by other GPCRs in a yeast-based signaling assay. In fact, when co-expressed with a yeast/mammalian G protein chimera, OA1 is able to reduce the basal activity of a *Fus1-LacZ* reporter by a mechanism distinct from G protein activation and thought to involve the formation of a receptor/G protein complex (Dosil et al., 2000). Nevertheless, the ability of OA1 to promote G protein activation in this system remains to be established.

Ligand-activated GPCRs are subjected to downregulatory mechanisms that control their downstream signaling and avoid excessive and/or continuous receptor activation (Ferguson, 2001). These processes have been shown to operate also on constitutively active GPCRs, which can display phosphorylation and association with arrestins even in the absence of agonist (Barak et al., 2001; Innamorati et al., 1997; Pei et al., 1994). Consistent with the idea that at least part of the OA1 molecules in transfected COS7 cells displays the conformation typical of activated GPCRs, we found that, in addition to show G protein-activation abilities, OA1 is phosphorylated and interacts in vivo with arrestins, as demonstrated by colocalization and coprecipitation assays. Furthermore, arrestins appear to induce an accelerated degradation of OA1, leading to decreased levels of the protein in transfected cells.

Arrestins are critical players in a number of GPCR-associated processes, including receptor desensitization (G protein-uncoupling), internalization, degradation, and signaling (Ferguson, 2001; Lefkowitz and Whalen, 2004). Since arrestins are thought to mediate the degradation of ligand-activated GPCRs by promoting their internalization from the plasma membrane, it is unclear how they might exert a substantial effect on the downregulation of OA1, which is by most already localized to internal membranes. Nevertheless, these adaptors have been also found to directly induce GPCR degradation by a process distict from internalization, i. e. by promoting receptor ubiquitination through recruitment of E3 ligases and subsequent lysosomal and/or proteasomal-mediated destruction (Shenoy et al., 2001; Martin et al., 2003). It is possible that arrestins recognize a fraction of OA1 as conformationally active and mark it for degradation by a similar mechanism, regardless of the subcellular localization of the protein. Further studies will be required to determine the mechanisms regulating the turnover of OA1 and the precise role of arrestins in this process.

Collectively, our results indicate that heterologously expressed OA1 behaves as a GPCR with a substantial spontaneous activity. It is conceivable that the high expression levels obtained in COS7 cells increase the absolute number of receptors in the active conformation, resulting in detectable G protein coupling, phosphorylation and arrestin binding. Alternatively, the agonist-independent activity of OA1 might be enhanced in COS7 cells due to the lack of inhibitors normally existing in melanocytic cells, a phenomenon previously observed with other GPCRs (Milligan, 2003; Seifert and Wenzel-Seifert, 2003). For instance, glutamate receptors show constitutive activity in HEK-293 cells, but are controlled by Homer proteins in neurons (Ango et al., 2001). Melanocortin receptors display a constitutive activity that is regulated on the target tissues by molecules acting either as agonists or as inverse agonists and antagonists (Adan and Kas, 2003). Similarly, OA1 might behave as a spontaneously active receptor, whose signaling in the melanocyte environment is controlled by specific inhibitors or inverse agonists. Finally, we cannot exclude that OA1 is activated by a ligand present in the experimental system, either contained in the culture medium or directly produced by the cells. Although DMEM does not activate OA1 (data not shown), probably only the identification of the actual OA1 ligand will completely solve this issue.

In conclusion, this study demonstrates that OA1 shares two fundamental properties with canonical GPCRs, i.e. it is capable to functionally associate with both heterotrimeric G proteins and arrestins, which represent the two most typical interactors of this family of receptors. In addition, we showed that in the experimental conditions utilized a substantial fraction of OA1 exhibits an active conformation, which will be possibly modulated by agonists, as well as inverse agonists, in future screenings for ligands. These studies were entirely conducted in a mammalian expression system distinct from pigment cells and therefore could not address the specifics of the signaling pathway mediated by OA1 in physiological conditions, including activation mechanism and downstream effectors. Nevertheless, they provide proof of principle that OA1 can act as a GPCR in mammalian cells, corroborating the idea that in its natural setting the protein might function by transducing signals at the melanosomal/lysosomal membranes to direct proper organelle biogenesis.

METHODS

Reagents, cell culture and expression vectors

All chemical reagents were purchased from Sigma-Aldrich, Inc., unless otherwise stated. COS7 cells were cultured as described (d'Addio et al., 2000). Expression vectors for the wild-type OA1 and the C116S mutant in pRc/RSV were described previously (d'Addio et al., 2000). The rat LAMP1 cDNA and LAMP/CT construct were cloned into the pCR3 vector (Invitrogen; M.V.S. and R.P., unpublished results). The pcDNA1-GαqWT-HA, pcDNA1-Gαqo5-HA, pcDNA1-Gαqi5-HA, and pcDNA1-Gαqz5-HA plasmids were kindly provided by Dr. Bruce Conklin (Gladstone Institutes, University of California, San Francisco, CA). The pCIS-Gα15 and pcDNA3-Gα15-EE plasmids were kindly provided by Dr. Stefan Offermanns (Universitat Heidelberg, Germany) and by the Guthrie cDNA Resource Center (www.cdna.org), respectively. Expression vectors for wild-type and GPF-tagged arrestins were generous gifts of Dr. Antonio De Blasi (Istituto Neurologico Mediterraneo Neuromed, Italy). Vectors for FLAG-tagged arrestins were kindly provided by Dr. Robert J. Lefkowitz (Duke University, Durham, NC). The pcDNA3-fPR (fPR-98) plasmid was a generous gift of Dr. Silvano Sozzani (Istituto Mario Negri, Milan, Italy). The pcDNA3-V2R and pcDNA3-β2AR plasmids were previously described (Bowen-Pidgeon et al., 2001). The pCDM8-αL and pCDM8-β2 (encoding the αL and β2 subunits of the heterodimeric lymphocyte function-associated antigen-1, LFA1) plasmids and anti-LFA1 (anti-β2) mouse mAbs TS1.18 and TS1.22 (Denti et al., 2004) were kindly provided by Dr. Ruggero Pardi. In all transfections the total amount of DNA was kept constant by using a pcDNA3/βgal vector.

Phospholipase Cβ (PLCβ) activity assay

Accumulation of inositol phosphates (IPs) was measured by a modification of the method by Hung et al. (Hung et al., 1992; Zhu et al., 1994). COS7 cells were grown in 12-well tissue culture plates and transfected by FuGENE 6 (Roche Diagnostics, Inc.) according to the manufacturer instructions. Unless otherwise stated, in double-transfection experiments, in each well 0.5 μg of plasmid DNA encoding for OA1 or other GPCRs were co-transfected with 0.1 μg of plasmid DNA for G proteins. In triple-transfection experiments, in each well 0.5 μg of plasmid DNA for OA1 or other GPCRs were co-transfected with 0.5 μg of plasmid DNA for arrestins and 0.1 μg of plasmid DNA for Gα15. 36 hours after transfection, each well was supplemented with 2 μCi/ml of myo-[^{3}H]inositol. Following overnight labeling, cells were rinsed three times at room temperature with 1 ml of washing buffer (DPBS supplemented with 5.5 mM glucose, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$). The cells were then incubated at 37°C for 30 minutes in 0.5 ml of the same buffer, before the addition of 5 μl of 1 M LiCl, to inhibit inositol monophosphatase. The incubation was continued for 1 hour at 37°C. In some experiments, agonists were added 10 minutes after LiCl. At the end of the incubation, the supernatant was removed and 0.75 ml of ice-cold 20 mM formic acid was added to each well to extract the IPs produced. IPs were separated from myo-inositol by a simplified Dowex chromatography procedure (Hung et al., 1992). Briefly, after 1 hour on ice, the 20 mM formic acid extracts were applied to Dowex columns (Bio-Rad AG 1-X8, 100-200 mesh, formate form, 0.6 cm diameter, 1.0 ml bed volume; Bio-Rad Laboratories, Inc.) that had been pretreated sequentially with 2 M ammonium formate/0.1 M formic acid, water, and 20 mM ammonium hydroxide adjusted to pH 9.0 with formic acid. Immediately after sample loading, 3 ml of 40 mM ammonium hydroxide, pH 9.0, were added to each column and the eluates (~3.75 ml) were collected in scintillation vials containing 10 ml of scintillation fluid (ULTIMA-FLO AF; Packard Instruments, Inc.). These first eluates were previously shown to recover the vast majority (98%) of myo-[^{3}H]inositol present in the samples (Zhu et al., 1994). The columns were then washed three times with 4 ml of 40 mM ammonium formate and IPs were eluted with 5 ml of 2 M ammonium formate/0.1 M formic acid into scintillation vials containing 15 ml of scintillation fluid. To normalize the accumulation of IPs for the total incorporated [^{3}H] inositol, the [^{3}H]IP counts (last eluate) were divided by the sum of the counts for myo[^{3}H] inositol (first eluate) plus [^{3}H]IPs and expressed in percentage.

Protein phosphorylation assay

COS7 cells were grown in 100 mm tissue culture dishes and transfected by a modification of the DEAE-dextran method by Luthman and Magnusson (1983). The next day, cells were labeled overnight with 100 μCi of Pro-mix L-[^{35}S] in vitro cell labeling mix/plate (Amersham-Pharmacia Biotech, Inc.). Cells were then chilled, washed twice with PBS and solubilized for 1 hour at 4°C in 300 μl of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS), supplemented with protease inhibitors (200 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml pepstatin, 200 μg/ml aprotinin, 200 μg/ml leupeptin) and 1% (v/v) phosphatase inhibitor cocktail I (Sigma-Aldrich, Inc.). After 1 hour at 4°C, samples were centrifuged and cell extracts were clarified by the addition of 50 μl of a 50% slurry of prewashed protein G-Sepharose 4B (Amersham-Pharmacia Biotech, Inc.) for 30 minutes and subsequent centrifugation. The clarified extracts were incubated 4-6 hours at 4°C with anti-OA1 polyclonal antibodies at 9 μg/ml (Schiaffino et al., 1996). The antigen-antibody complexes were separated by incubation with prewashed protein G-Sepharose 4B overnight at 4°C. The beads were centrifuged and washed 5 times with 800 μl of RIPA. Proteins were eluted from the beads for 20 minutes at room temperature with 80 μl of Laemmli buffer containing 10% β-mercaptoethanol and 12% SDS. The samples were separated by SDS-PAGE as described (Schiaffino et al., 1996) and phosphorylated proteins were visualized using a fluorescent phosphoprotein gel stain, according to the manufacturer instructions (Pro-Q Diamond phosphoprotein gel stain; Molecular Probes, Inc.). Positive bands were visualized

utilizing a Typhoon (Molecular Dynamics) to scan either directly the wet gels, or a LE storage phosphor screen (Molecular Dynamics) previously exposed to the dried gels. Dephosphorylation of OA1 was achieved utilizing bacteriophage λ-phosphatase. After immunoprecipitation, the beads were directly resuspended in 20 μl of phosphatase buffer (50 mM HEPES, 5 mM dithiothreitol, 0.1 mM Na_2EDTA, 0.01% Brij 35, pH 7.5, supplemented with 2 mM $MnCl_2$). 400 units of λ-phosphatase were added and overnight incubation was carried out at 30°C. At the end of the treatment the protein was eluted from the beads in Laemmli buffer as described above.

Co-immunoprecipitation and western immunoblotting

COS7 cells were grown in 100 mm tissue culture dishes and transfected by a modification of the DEAE-dextran method by Luthman and Magnusson (1983), using 3 μg of plasmid DNA encoding for OA1 and/or FLAG-tagged arrestins. 48 hours post-transfection, cells were lysed in 1 ml of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, supplemented with protease inhibitors (200 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml pepstatin, 200 μg/ml aprotinin, 200 μg/ml leupeptin) and 1% (v/v) phosphatase inhibitor cocktail I (Sigma-Aldrich, Inc.). After 1 hour at 4°C, the particulate was removed from the samples by centrifugation. Immunoprecipitation was performed for 16 hours at 4°C using anti-OA1 polyclonal antibodies (Schiaffino et al., 1996), previously crosslinked to CNBr-activated Sepharose 4B beads (Amersham-Pharmacia Biotech, Inc.). Immune complexes were washed three times with 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% (v/v) Nonidet P-40 and eluted from the antibody with 40 μl of 0.1 M glycine pH 2.5 for 3 minutes. After 5 minutes of centrifugation, the supernatants were recovered and returned to neutral pH by addition of 2 μl of 1 M Tris-HCl pH 9.0. Samples were separated by SDS-PAGE and analyzed by immunoblotting as described (Schiaffino et al., 1996). Primary antibodies were as follows: anti-OA1 rabbit polyclonal (Schiaffino et al., 1996) at 2 μg/ml, anti-FLAG-M2 mouse mAb (Sigma-Aldrich, Inc.) at 1:400, anti-calnexin rabbit polyclonal (Sigma-Aldrich, Inc.) at 1:5000, anti-EE mouse mAb (Covance) at 1:1000 and anti-HA mouse mAb (12CA5 protein A-purified) at 1:1000. Secondary antibodies were anti-mouse and anti-rabbit horseradish peroxidase-conjugated (Amersham-Pharmacia Biotech, Inc.). Visualization of antibody binding was carried out with Enhanced ChemiLuminescence Plus (Amersham-Pharmacia Biotech, Inc.) according to the manufacturer instructions.

Pulse-chase assay

18 hours after transfection, cells in 12 wells plates were starved for 1 hour in methionine/cysteine-free DMEM supplemented with 1% dialyzed fetal bovine serum, followed by 30 minutes pulse in the same medium containing 40 μCi of Pro-mix L-[^{35}S] (Amersham-Pharmacia Biotech, Inc.). Cells were then rinsed three times in PBS and chased for 2 and 6 hours in DMEM 10% FCS. At the end of the chase period, OA1 was extracted in RIPA buffer and immunoprecipitated as described above. The bands were visualized utilizing a Typhoon (Molecular Dynamics) to scan a LE storage phosphor screen (Molecular Dynamics) previously exposed to the dried gels.

Immunofluorescence microscopy

Transiently transfected COS7 cells were grown on glass coverslips. 48 hours after transfection cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% saponin in PBS and stained with anti-OA1 (Schiaffino et al., 1996), anti-HA (mAb 12CA5, protein A-purified) or anti-rat LAMP1 (mAb LY1C6, Stressgen Biotechnologies) antibodies, followed by Cy3-conjugated donkey anti-rabbit/mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories). The plasma membrane was decorated by staining the V2R tagged with the extracellular epitope HA. Briefly, before fixation and permeabilization, transfected cells were

incubated 1 hour at 4°C with 12CA5 antibodies directly coupled to Alexa 488 (Molecular Probes, Inc.) at 0.5 μg/ml in PBS, supplemented with 10% fetal bovine serum. Images were collected using a Zeiss Axiophot epifluorescence microscope or a Leica TCS SP2 laser scanning microscopy system. The final composite images were created using Adobe Photoshop 7.0 (Adobe Systems, Inc.).

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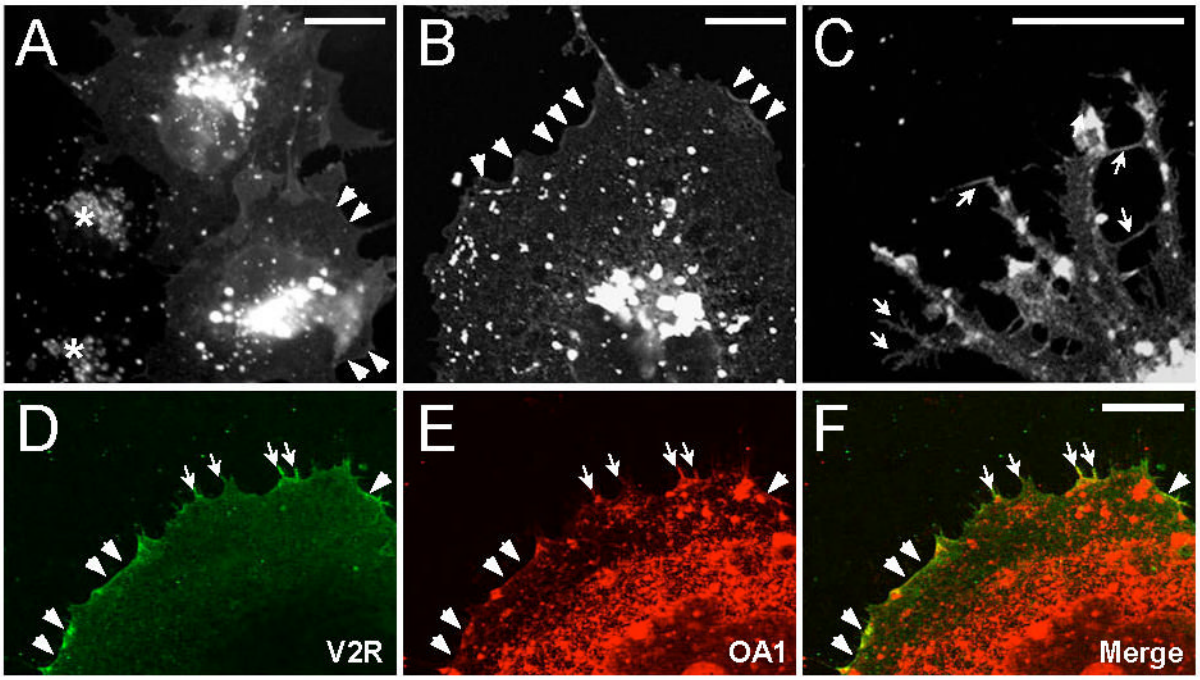

Figure 1.

OA1 is detectable at the cell surface in COS7 cells. Confocal immunofluorescence analysis of COS7 cells transiently transfected with expression vectors for OA1 (A-C) or for OA1 and HA-tagged V2 vasopressin receptor (V2R) (D-F). After 48 hours, cells were fixed, permeabilized and decorated with anti-OA1 antibodies (A-C) or were labeled with anti-HA antibodies prior to fixation, permeabilization and incubation with anti-OA1 antibodies (D-F). OA1 is detectable at the plasma membrane, visible as staining of cell margins (arrowheads) and/or membrane spikes (arrows), and colocalizes with the cell surface labeled V2R (D-F). Cells that express low levels of OA1 do not show staining of cell margins (A, asterisks). Bars, 10 μm.

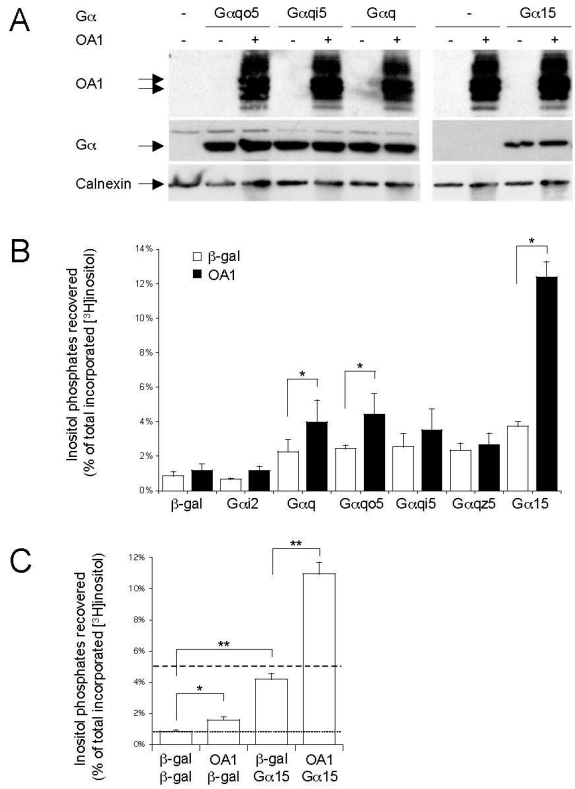

Figure 2.

OA1 shows G protein-activation abilities. (A) Western blot analysis of COS7 cells transfected with expression vectors for OA1 and/or various G proteins. Identical aliquots of cell lysates (20 μg) were separated by SDS-PAGE and analyzed by immunoblotting using anti-OA1 antibodies (in COS7 cells, OA1 accumulates mostly as an incompletely glycosylated doublet, indicated by the arrows; when blots are overexposed, as they are here, the small amount of fully glycosylated protein becomes visible as a higher molecular weight smear; d'Addio et al., 2000), anti-HA antibodies (to detect HA-tagged Gαqo5, Gαqi5 and Gαq), anti-EE antibodies (to detect EE-tagged Gα15) and anti-calnexin antibodies (for normalization). G proteins are expressed at comparable levels either in the presence or in the absence of OA1 co-expression. (B) PLCβ activity assay performed in COS7 cells transfected with expression vectors for OA1 and/or G proteins. PLCβactivation was estimated by measuring the accumulation of intracellular IPs. Significant IP production over the basal obtained with G proteins alone (empty bars) is observed when OA1 is co-transfected with Gαq, Gαqo5 and Gα15 (filled bars). As expected, no effect was obtained with Gαi2, suggesting that PLCβ activation in these assays does not depend on generalized Gβγ signaling. Results represent the mean ± S.D. of the data pooled from 2-4 independent experiments performed in triplicate. *, $P < 0.001$ (unpaired t test). (C) PLCβ activity assay performed in COS7 cells transfected with expression vectors for OA1 and Gα15, either alone or together. The dotted line represents the background obtained in mock-transfected cells. The dashed line represents the sum of the values obtained with OA1 and G protein alone (over the background), which should represent the maximum result expected in cotransfected cells in the absence of any synergistic effect on PLCβ activity. Instead, in cells expressing both OA1 and Gα15 the dashed line is largely overcome, implying effective receptor-G protein coupling. Results represent the mean ± S.E.M. of the data from eight independent experiments performed in triplicate. *, $P < 0.003$; **, $P < 0.000001$ (unpaired t test).

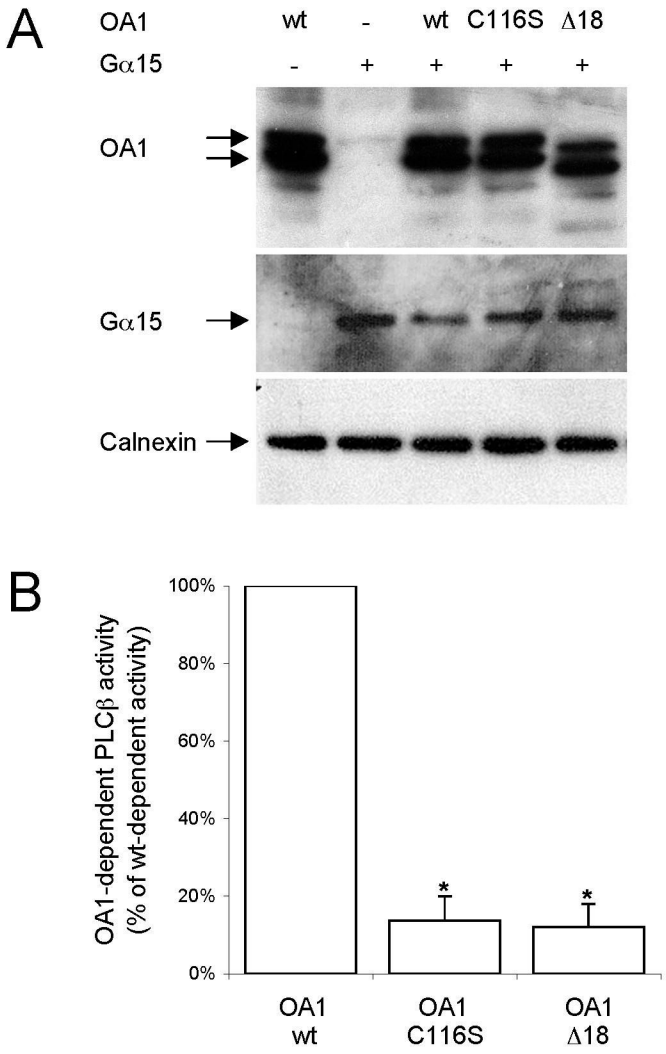

Figure 3.

OA1 mutants lack G protein-activation abilities. Western blot analysis (A) and PLCβ activity assay (B) of COS7 cells co-transfected with expression vectors for Gα15 and either the wild-type OA1 or the OA1 C116S and OA1 Δ18 mutants. (A) Identical aliquots of cell lysates (20 μg) were separated by SDS-PAGE and analyzed by immunoblotting. The OA1 proteins are expressed at comparable levels. The OA1 Δ18 mutant displays a lower molecular weight due to the intramolecular deletion. (B) The ability of the OA1 mutants to couple with Gα15 and activate PLCβ was estimated by measuring the accumulation of intracellular IPs. The OA1-dependent PLCβ activity was calculated as the difference between the IP values obtained in OA1/Gα15 co-transfections and those obtained with Gα15 alone, and was expressed as percentage of the wild-type OA1 activity. Both mutants are basically silent. Results represent the mean ± S.E.M. of the data from four independent experiments performed in triplicate. *, $P < 0.001$ (compared to wild-type OA1) (paired t test).

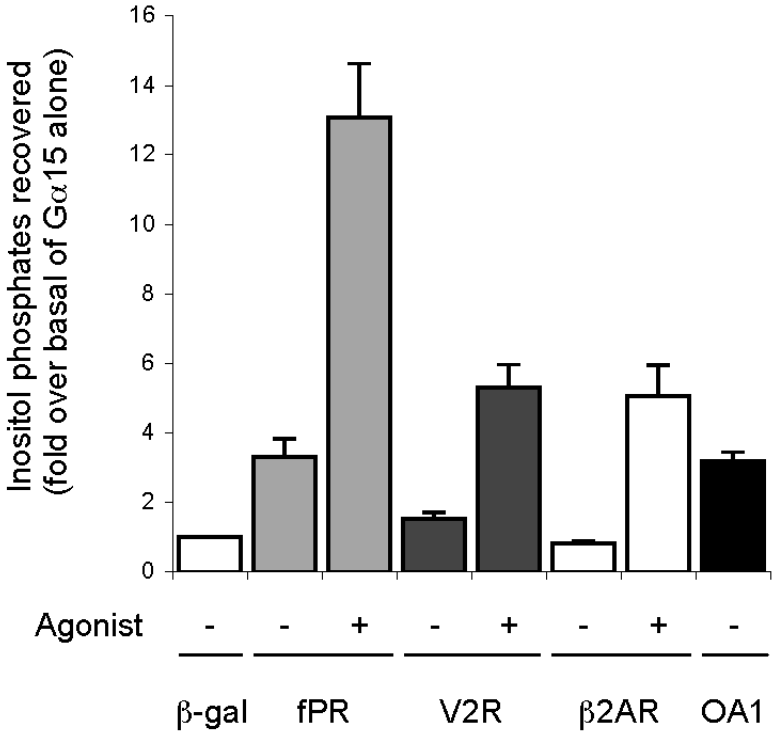

Figure 4.

OA1 activates G proteins similarly to well-established GPCRs. PLCβ activity assay performed in COS7 cells co-transfected with expression vectors for Gα15 and OA1, or Gα15 and well-established GPCR (fPR, V2R and β2AR). fPR demonstrated a level of activity similar to OA1 under non-stimulated conditions and a greater increase in the presence of the appropriate agonist (fMLP, 10 μM). By converse, V2R and β2AR showed activities exceeding that displayed by OA1 only upon ligand stimulation (AVP, 100 nM for V2R; isoproterenol, 100 nM for β2AR). Results represent the mean ± S.D. of the data from a representative experiment performed in triplicate.

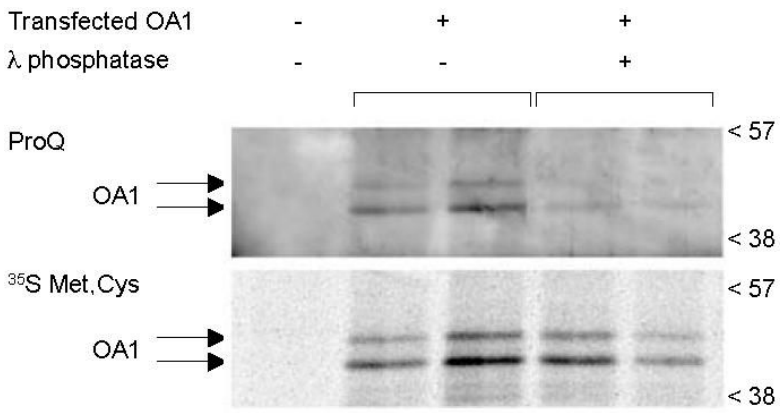

Figure 5.

OA1 is phosphorylated. OA1 was transiently expressed in COS7 cells and immunoprecipitated with anti-OA1 antibodies after overnight labeling with [^{35}S]Met/Cys. Immunoprecipitated proteins were or were not treated with λ-phosphatase before separation by SDS-PAGE. To visualize phosphoproteins, the gel was stained utilizing the Pro-Q Diamond solution and analyzed by fluorography (upper panel, see Experimental Procedures). To reveal all immunoprecipitated proteins, the gel was subsequently dried and analyzed by autoradiography (lower panel). Molecular weight markers (kD) are shown on the right.

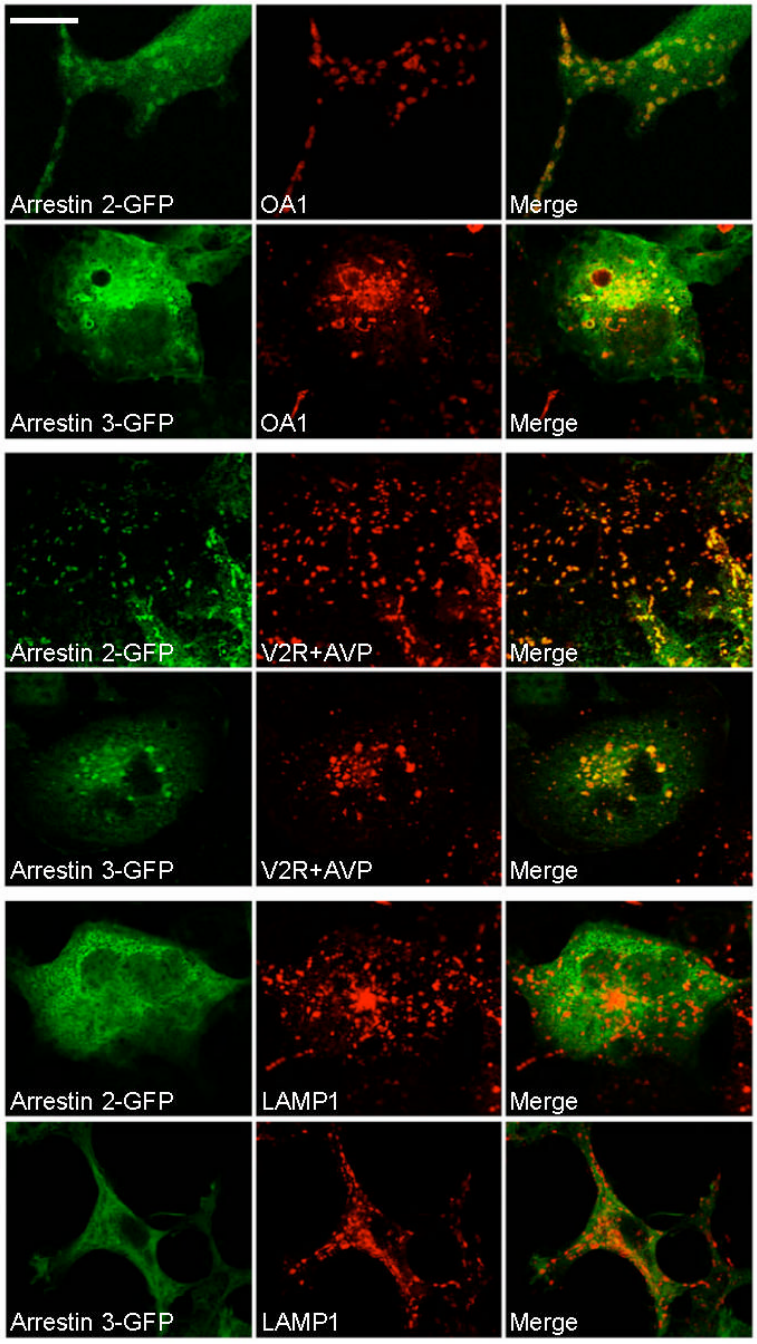

Figure 6.

OA1 colocalizes with arrestins. Confocal immunofluorescence analysis of COS7 cells transfected with expression vectors for OA1, V2R or rat LAMP1 together with vectors for arrestin 2-GFP or arrestin 3-GFP, as indicated. After 48 hours, cells were fixed and decorated with anti-OA1, anti-HA (for V2R) or anti-rat LAMP1 antibodies. In the merge, areas of colocalization can be observed in intracellular vesicles of OA1 and V2R transfected cells. Bar, 10 μm. Transfections were performed using a 1:1 plasmid DNA ratio for OA1 (or V2R or LAMP1) and arrestins.

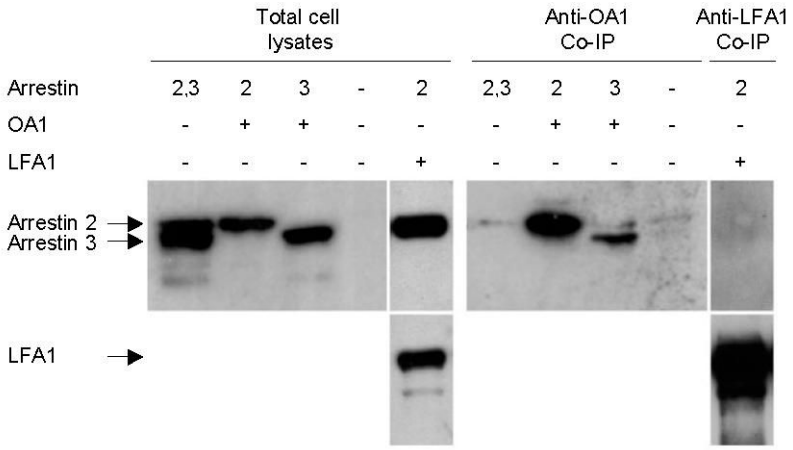

Figure 7.

OA1 coprecipitates with arrestins. Western blot analysis of whole cell lysates and anti-OA1 immunoprecipitates (anti-OA1 Co-IP) obtained from COS7 cells co-transfected with expression vectors for OA1 and arrestin 2-FLAG or arrestin 3-FLAG, as indicated. As negative control, COS7 cells were also co-transfected with expression vectors for the lymphocyte specific integrin LFA1 and arrestin 2-FLAG. An aliquot (20 μg) of the original lysate and the immunoprecipitated proteins were separated by SDS-PAGE and probed by Western blot utilizing anti-FLAG antibodies. Both FLAG-tagged arrestins were found in the anti-OA1 immunoprecipitates only in the presence of cotransfected OA1. Transfections were performed using a 1:1 plasmid DNA ratio for OA1 and arrestins.

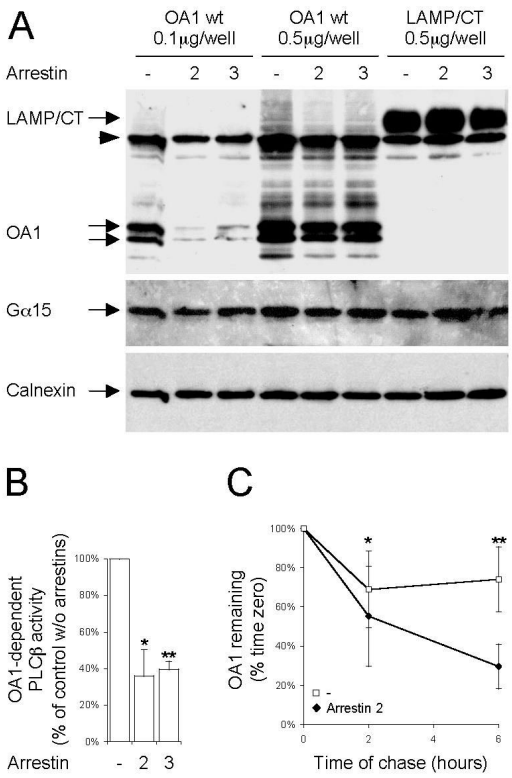

Figure 8.

OA1 is downregulated by arrestins. (A) An example of Western blot analysis of COS7 cells transfected with expression vectors for Gα15 and OA1 or a control protein (LAMP/CT, also recognized by anti-OA1 antibodies) in the absence or in the presence of co-transfected arrestins 2 or 3. Identical aliquots of cell lysates (20 μg) were separated by SDS-PAGE and analyzed by immunoblotting. Arrestin co-expression results in evident loss of OA1 (bands of OA1 at 0.5μg/well are partially saturated), but not of LAMP/CT or Gα15. The arrowhead indicates an endogenous protein cross-reacting with the antibody, which appears more intense in the lanes with OA1 due to the underlying OA1 smear. (B) PLCβ activity assay performed in COS7 cells transfected with expression vectors for Gα15 and OA1, in the absence or in the presence of co-transfected arrestins 2 or 3. The OA1-dependent PLCβ activity was calculated as in Figure 3B and was expressed as percentage of the OA1 activity observed in the absence of co-expressed arrestins. Results represent the mean ± S.E.M. of the data from four independent experiments performed in triplicate. *, $P < 0.02$ and **, $P < 0.001$ (compared to control) (paired t test). (C) Pulse-chase assay performed in COS7 cells transfected with expression vectors for Gα15 and OA1, in the absence or in the presence of co-transfected arrestin 2. Following metabolic labeling and chase, proteins were immunoprecipitated with anti-OA1 antibodies, separated by SDS-PAGE and analyzed by autoradiography. The density of OA1 bands at 2 or 6 hours of chase was expressed as percent of those at time 0 (immunoprecipitated immediately after metabolic labeling). The degradation rate of OA1 is considerably increased in the presence of co-transfected arrestin. Results represent the mean ± S.D. of the data from ten experiments. *, $P < 0.02$ and **, $P < 0.0001$ (paired t test). Transfections were performed in 12-well plates using the following amounts of plasmid DNAs: OA1, 0.1μg/well (A) or 0.5μg/well (A, B, C); Lamp1/CT, 0.5μg/well (A); arrestins, 0.5μg/well (A, B, C); Gα15, 0.1μg/well (A, B, C).