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Agonist-Stimulated and Tonic Internalization of Metabotropic Glutamate Receptor 1a in Human Embryonic Kidney 293 Cells: Agonist-Stimulated Endocytosis Is β-Arrestin1 Isoform-Specific

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ABSTRACT

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors (GPCRs) that contribute to the regulation of integrative brain functions such as cognition, motor control, and neural development. Metabotropic glutamate receptors are members of a unique class of GPCRs (class III) that include the calcium sensing and γ-aminobutyric acid type B receptors. Although mGluRs bear little sequence homology to well-characterized members of the GPCR superfamily, both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs) contribute to mGluR desensitization. Therefore, in the present study, we examined whether β-arrestins, regulators of GPCR desensitization and endocytosis, are required for mGluR1a desensitization and internalization in human embryonic kidney (HEK) 293 cells. Unlike what has been reported for other GPCRs, we find that in response to agonist stimulation, mGluR1a internalization is selectively mediated by β-arrestin1 in HEK 293 cells. However, even though β-arrestin1 binds directly to the carboxyl-terminal tail of mGluR1a and redistributes with mGluR1a to endosomes, neither β-arrestin1 nor β-arrestin2 seems to contribute to mGluR1a desensitization in HEK 293 cells. We also observed extensive tonic mGluR1a internalization via clathrin-coated vesicles in the absence of agonist. The tonic internalization of mGluR1a is insensitive to antagonist treatment, dominant-negative mutants of GRK2, β-arrestin1, and dynamin as well as treatments that disrupt caveolae, but is blocked by hypertonic sucrose and concanavalin A treatment. Internalized mGluR1a is colocalized with clathrin, transferrin receptor, β_2-adrenergic receptor, and Rab5 GTPase in endocytic vesicles. Therefore, although mGluR1a internalizes with β-arrestin in response to agonist, the agonist-independent internalization of mGluR1a involves the β-arrestin-independent targeting of mGluR1a to clathrin-coated vesicles.

Metabotropic glutamate receptors (mGluRs) are members of the G protein-coupled receptor (GPCR) superfamily that are activated by the excitatory amino acid glutamate (Nakanishi, 1994). Glutamate is the major excitatory neurotransmitter in the central nervous system and is responsible for regulating integrative brain functions such as cognition, motor control, and neuronal development (Nakanishi, 1994). In addition to activating mGluRs, glutamate activates ionotropic glutamate receptors that are cation-specific ion channels (Nakanishi, 1994). Whereas ionotropic glutamate receptors mediate fast excitatory glutamate responses, mGluRs mediate slower glutamate responses by regulating the activity of intracellular second messenger cascades (Nakanishi, 1994). Consequently, mGluR activation is translated into long-lasting changes in synaptic activity (Aiba et al., 1994; Ichise et al., 2000).

The mGluR family of GPCRs consists of eight receptor subtypes that are subclassified into three groups on the basis of sequence homology, pharmacology, and G protein coupling specificity (Nakanishi, 1994). Group 1 mGluRs (mGluR1 and mGluR5) are coupled via Gq to the stimulation of phospholipase Cβ, leading to increases in intracellular inositol 1,4,5-triphosphate formation, the release of calcium from intracellular stores, and the activation of protein kinase C (Nakanishi, 1994). Group 2 (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR6, mGluR7 and mGluR8) are each negatively coupled to adenylyl cyclase (Nakanishi, 1994).

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ABBREVIATIONS: mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; MEM, minimal essential medium; 4C3HPG, 4-carboxy-3-hydroxy-phenylglycine; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HBSS, HEPES-buffered saline solution; IP, inositol phosphate; TBS, Tris-buffered saline; GFP, green fluorescent protein; β_2AR, β_2-adrenergic receptor.

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Members of the mGluR family bear no sequence or structural homology to other GPCRs (except calcium-sensing and $GABA_B$ receptors) other than the retention of a seven transmembrane topology characteristic of GPCRs. However, the molecular mechanisms contributing to the desensitization of "classical" GPCRs are conserved for this unique class of GPCR (Gereau and Heineman 1998; Dale et al., 2000; Sallese et al., 2000). In particular, group 1 mGluRs are phosphorylated by both second messenger-dependent protein kinases (Gereau and Heinemann, 1998) and G protein-coupled receptor kinases (GRKs) (Dale et al., 2000; Sallese et al., 2000).

GPCR desensitization by both second messenger-dependent protein kinases and GRKs represents an important mechanism by which receptor activity is attenuated (Ferguson, 2001). GRK-mediated phosphorylation promotes the binding of β-arrestin proteins that function to both uncouple GPCRs from their cognate heterotrimeric G proteins (Lohse et al., 1990; Attramadal et al., 1992) and target GPCRs to clathrin-coated pits for internalization (Ferguson et al., 1996; Zhang et al., 1996). The overexpression of either β-arrestin1 or β-arrestin2 has been demonstrated to augment the desensitization and internalization of several GPCRs (Pippig et al., 1993; Zhang et al., 1998; Oakley et al., 1999). Although both β-arrestin isoforms interact with the β2-adaptin subunit of the AP2 adaptor complex to target GPCRs for endocytosis via clathrin-coated vesicles (Laporte et al., 2000), β-arrestin2 has emerged as the principal GPCR endocytic adaptor protein (Oakley et al., 2000; Kohout et al., 2001). In addition, although β-arrestins regulate the internalization of many GPCRs, there is evidence supporting the existence of a β-arrestin-insensitive GPCR endocytic mechanism (reviewed by Ferguson, 2001).

Recently, we reported that GRK2 and GRK5 contribute to the desensitization of both agonist-stimulated and intrinsic mGluR1a activity in human embryonic kidney (HEK) 293 cells (Dale et al., 2000) suggesting that the mechanisms contributing to the regulation of GPCR desensitization are conserved across all classes of GPCRs. Therefore, in the present study, we have tested the hypothesis that GRK-mediated phosphorylation contributes to the β-arrestin–dependent desensitization and internalization of mGluR1a. Unexpectedly, we find that in HEK 293 cells, the agonist-stimulated internalization of mGluR1a is mediated solely by β-arrestin1 and that, unlike all other GPCRs, which have been observed to internalize in a β-arrestin–dependent manner, β-arrestin2 does not contribute to the internalization of mGluR1a. Furthermore, β-arrestin binding to mGluR1a does not seem to contribute to mGluR1a desensitization. This observation suggests that GRK-mediated phosphorylation may be sufficient to mediate the full desensitization of this class of GPCR in the absence of other mGluR interacting proteins, such as homer. In addition to agonist-stimulated endocytosis, mGluR1a also internalized in an agonist-independent manner that is insensitive to both β-arrestin- and dynamin-dominant negative mutants but is mediated by clathrin-coated vesicles. Taken together, these data indicate that, although mGluR1a internalizes with β-arrestin in response to agonist, the agonist-independent internalization of mGluR1a involves the β-arrestin-independent targeting of mGluR1a to clathrin-coated vesicles.

Experimental Procedures

Materials. Human embryonic kidney (HEK) 293 cells and COS-7 cells were from American Type Culture Collection (Manassas, VA). Fetal bovine serum was from Hyclone Laboratories Inc (Logan, UT). Gentamicin, minimal essential medium (MEM), and Trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA). Quisqualate and 4C3HPG were obtained from Tocris Cookson Inc (St. Louis, MO). The anti-Flag monoclonal and polyclonal antibodies were purchased from Research Diagnostics (Flanders, NJ). myo-[^{3}H]Inositol was acquired from PerkinElmer Life Sciences Products (Boston, MA) The Dowex 1-X8 (formate form) resin with 200–400 mesh was purchased from Bio-Rad (Hercules, CA). Protein G-Sepharose beads, glutathione Sepharose beads, anti-rabbit-HRP antibody, and enhanced chemiluminescence Western blotting detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). Rhodamine-conjugated transferrin, Rhodamine Red- and FITC goat anti-mouse secondary antibody were purchased from both Sigma (St. Louis, MO) and Molecular Probes (Eugene, OR). All other biochemical reagents were purchased from Sigma, Fisher Scientific (Pittsburgh, PA), and VWR (West Chester, PA).

cDNA Constructs. For the expression of the mGluR1a carboxyl-terminal tail as a GST fusion protein, the mGluR1a tail (amino acid residues 841-1199) was cloned into pEBG3. Briefly, the carboxyl-terminal tail was amplified by polymerase chain reaction using as forward primer 5′-CATCGGATCCAAACCTGAGAGGAACGTC-CGCAGTG and the SP6 promoter primer as reverse primer, using pcDNA3.1 mGluR1a as the template cDNA. The polymerase chain reaction product was digested with *Bam*HI and *Xba*I and subcloned in frame with GST into the pEBG3 mammalian expression vector digested with the same enzymes. The β-arrestin1 mutant, 185 to 418, was also constructed by polymerase chain reaction. 5′ Oligonucleotide primers introduced an amino-terminal *Eco*RI restriction site, minimal Kozak sequence, initiation ATG at the appropriate site of β-arrestin1, and 3′ oligonucleotide primers introduced a carboxyl-terminal *Xho*I restriction site, stop codon, Flag-epitope tag sequence (DYKDDDDK) at the C terminus of each β-arrestin1 mutant. The sequence integrity was confirmed by DNA sequencing. β-Arrestin1-GFP and β_2AR-GFP constructs were the gift of Dr. Marc G. Caron (Duke University Medical Center, Durham, NC).

Cell Culture and Transfection. HEK 293 cells were maintained in MEM and COS-7 cells in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 100 μg/ml gentamicin at 37°C in a humidified atmosphere containing 5% CO_2. The cells used in each of the experiments were transfected using a modified calcium phosphate method as described previously (Zhang et al., 1996). After transfection (~18 h), the cells were incubated with fresh medium and allowed to recover 8 h and then reseeded and grown an additional 18 h before any experimentation.

Agonist-Dependent Internalization Assays. Receptor sequestration was assessed by flow cytometry as described previously (Zhang et al., 1996; Anborgh et al., 2000). In brief, sequestration was defined as the fraction of total cell surface receptors lost from the cell surface and therefore inaccessible to antibodies from outside the cell following agonist treatment for 30 min. For these assays, the cells were exposed to saturating agonist concentrations before antibody staining. Under these conditions, receptors were able to undergo multiple rounds of internalization and recycling and receptor internalization represented the loss of cell surface receptor at steady state. Antibody staining was performed as follows. Flag-tagged mGluR1a were labeled on ice with an anti-Flag antibody (1:500) for 45 min. The cells were washed with cold PBS and subsequently labeled with a goat anti-mouse IgG antibody conjugated to FITC (1:500) for 45 min on ice. The cells were harvested and cell surface immunofluorescence was assessed by flow cytometry.

Agonist-Independent Internalization Assays. The agonist-independent internalization of cell surface receptors was measured by prelabeling cell surface epitope-tagged receptors with primary

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mouse anti-epitope tag antibody (1:500 dilution) on ice for 45 min and then warming cells to 37°C in the absence of agonist for the times indicated in the figure legends. Cells were then transferred back to ice and labeled with the secondary FITC-conjugated anti-mouse IgG antibody (1:500 dilution) for 45 min. Under these conditions, receptors were able to undergo only a single round of internalization, and measures of internalization do not reflect either receptor recycling or the loss of cell surface receptor at steady state. Receptor internalization was defined as the fraction of total cell receptors lost from the cell surface and thus not available to secondary antibodies outside the cell.

Inositol Phosphate Formation. Transiently transfected HEK 293 cells were seeded into 24-well dishes. The cellular inositol lipids were radiolabeled by incubating the cells overnight with 1 μCi/ml myo-[^{3}H]inositol in inositol-free Dulbecco's modified Eagle's medium. Unincorporated myo-[^{3}H]inositol was removed by washing the cells with HBSS (116 mM NaCl, 20 mM HEPES, 11 mM glucose, 5 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, pH 7.4). The cells were preincubated for 1 h in HBSS 37°C and then preincubated in the same buffer containing 10 mM LiCl for an additional 10 min at 37°C. The cells were then incubated in either the absence of presence of quisqualate for 30 min at 37°C. The reaction was stopped on ice by adding perchloric acid and then neutralized with 0.72 M KOH/0.6 M $KHCO_3$. The total [^{3}H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 μl of the cell lysate. Total inositol phosphate (IP) was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) anion exchange resin with 200–400 mesh. [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Beckman LS 6500 scintillation system (Beckman Coulter, Fullerton, CA).

***β*-Arrestin Coimmunoprecipitation.** Transiently transfected HEK 293 cells were incubated for 1 h at 37°C in HBSS. The cells were solubilized in lysis buffer containing protease inhibitors (25 mM HEPES, pH 7.5, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). The protein content of the lysates was determined using the Bio-Rad D_c Protein Assay Kit. Flag-mGluR1a was immunoprecipitated with a monoclonal anti-Flag M2 antibody using Protein G Sepharose beads from cell lysates containing 500 μg of protein. GST fusion proteins were precipitated using glutathione Sepharose beads. The coprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by electroblotting onto nitrocellulose membranes. The membranes were blocked with 10% milk in TBS-Tween (150 mM NaCl, 10 mM Tris · HCl, pH 7.0, 0.05% Nonidet P-40, and 0.05% Tween 20) and then incubated with polyclonal antibody raised against the amino terminus of β-arrestin1 diluted 1:5000 in TBS-Tween containing 3% skimmed milk. The membranes were rinsed with TBS-Tween and then incubated with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:2500 in wash buffer containing 3% milk. The membranes were rinsed with TBS-Tween, incubated with enhanced chemiluminescence Western Blotting detection reagents, and then exposed to X-Omat Blue XB-1 film (Eastman Kodak, Rochester, NY).

Confocal Microscopy. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning confocal microscope using a Zeiss 63×, 1.3 numerical aperture, oil immersion lens. HEK 293 cells expressing mGluR1a with or without β_2AR, Rab5a, or Rab5a-Q79L were plated on 35-mm glass-bottomed culture dishes and were kept warm at 37°C in serum-free MEM on a heated microscope stage as described previously (Anborgh et al., 2000; Seachrist et al., 2000). Flag-mGluR1a staining of HEK 293 cells grown on coverslips and fixed with 4% paraformaldehyde in HBSS with 0.2% Triton X-100 for 20 min was performed using the anti-Flag monoclonal antibodies in conjunction with a Rhodamine Red- or FITC-conjugated goat anti-mouse secondary antibody. Clathrin staining was performed using the monoclonal antibody X22. Transferrin receptor staining was performed by incubating cell cultures on ice for 45 min with 15 μg/ml Texas Red-conjugated transferrin and then warming the cells at 37°C for 10 min. Colocalization studies were performed using dual excitation (488, 543 nm) and emission (515–540 nm, GFP and FITC; 590–610 nm, Rhodamine) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.

Data Analysis. The mean and the standard error of the mean are expressed for values obtained from the number of separate experiments indicated. Dose response data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was determined by analysis of variance and corrected for multiple comparisons.

Results

***β*-Arrestin-Dependent mGluR1a Internalization.** Because mGluR1a is phosphorylated by GRKs and many of the GPCRs tested thus far internalize in a GRK-and/or β-arrestin–dependent manner (reviewed by Ferguson 2001), we examined whether β-arrestins are required for agonist-stimulated mGluR1a internalization. In HEK 293 cells transfected with Flag-mGluR1a alone, 100 μM quisqualate stimulation for 30 min resulted in a loss of only 11 ± 2% of cell surface receptor fluorescence (Fig. 1). The coexpression of either GRK2, GRK5, β-arrestin1, or β-arrestin2 individually had no significant effect on the agonist-stimulated internalization of Flag-mGluR1a (Fig. 1). Because both GRK2 and GRK5 contribute to the phosphorylation and desensitization of mGluR1a in HEK 293 cells, we tested the effect of coexpressing GRK2 and GRK5 with β-arrestin1 and β-arrestin2 on mGluR1a endocytosis. When β-arrestin1 was coexpressed with either GRK2 or GRK5, the maximal extent of agonist-stimulated Flag-mGluR1a internalization was doubled, with 20 ± 5% and 23 ± 5% loss of cell surface receptors, respectively (Fig. 1). However, even though β-arrestin2 is considered to be the more effective GPCR endocytic adaptor protein (reviewed by Ferguson, 2001), the coexpression of GRK2 and

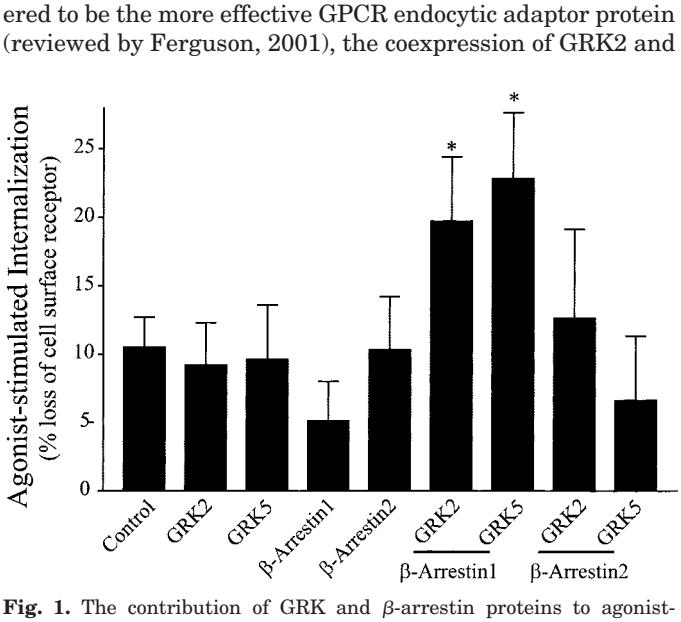

Fig. 1. The contribution of GRK and β-arrestin proteins to agonist-stimulated mGluR1a internalization. Effect of GRK2, GRK5, β-arrestin1 and β-arrestin2 expressed alone, or in combination, on the internalization of Flag-mGluR1a in response to a 30-min stimulation with 100 μM quisqualate. HEK 293 cells were transfected transiently with plasmid cDNAs encoding Flag epitope-tagged mGluR1a (5 μg) with either empty plasmid cDNA or 10 μg total of plasmid cDNAs encoding GRK2, GRK5, β-arrestin1, and β-arrestin2. Data represent the mean ± S.D. of eight independent experiments. *, $P < 0.05$ versus control mGluR1a.

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GRK5 with β-arrestin2 did not increase Flag-mGluR1a internalization in HEK 293 cells (Fig. 1). The coexpression of GRK4 and GRK6 in our hands did not contribute to Flag-mGluR1a desensitization (Dale et al., 2000). Expression of GRK4 and GRK6 in either the absence or the presence of β-arrestin1 and β-arrestin2 did not increase agonist-stimulated mGluR1a internalization (data not shown).

β-Arrestin1 Specifically Associates with mGluR1a. To further address the possibility that mGluR1a internalization in HEK 293 cells is β-arrestin1-specific, we tested whether green fluorescent protein (GFP) tagged β-arrestin1 and/or β-arrestin2 constructs redistribute in response to mGluR1a activation. In the absence of agonist (control), both β-arrestin1-GFP and β-arrestin2-GFP were diffusely localized throughout the cytoplasm of HEK 293 cells (Fig. 2, A and B). In response to Flag-mGluR1a activation with 100 μM quisqualate, only β-arrestin1-GFP redistributed over time to intracellular vesicular structures (Fig. 2, A and B). However, the lack of β-arrestin2-GFP translocation upon activation of mGluR1a was mGluR1a-specific, because β-arrestin2-GFP translocation was observed after the subsequent activation of β_2-adrenergic receptor (β_2AR) coexpressed in the same cells (Fig. 2B). Moreover, wild-type β-arrestin1 could be coimmunoprecipitated with Flag-mGluR1a from HEK 293 cells (Fig. 3A, Top Panel). Coimmunoprecipitation of β-arrestin2 with mGluR1a was not observed (Fig. 3A, Bottom Panel). The association of β-arrestin1 with mGluR1a was increased following receptor activation with agonist (Fig. 3B). Both wild-type β-arrestin1 and β-arrestin1-GFP could be specifically precipitated using a GST-mGluR1a-carboxyl-terminal fusion protein (Fig. 3C, Lower Panel) indicating that the site of β-arrestin1 interaction is localized to the mGluR1a carboxyl-terminal tail.

Recently, Bhatnagar et al. (2001) demonstrated that in response to 5-hydroxytryptamine 2A receptor activation, β-arrestin proteins redistributed to a vesicular compartment that is distinct from the compartment to which the receptor was internalized. Therefore, we examined whether β-arrestin1-GFP and Flag-mGluR1a redistributed to the same intracellular compartment in response to quisqualate treatment. At 4°C, Flag-mGluR1a labeling was restricted to the cell surface and β-arrestin1-GFP fluorescence was evenly distributed throughout the cytoplasm of the cell (Fig. 4A). Similar to a previous report, some nuclear β-arrestin1-GFP fluorescence was observed (Oakley et al., 2000). When cells were warmed to 37°C, substantial Flag-mGluR1a immunofluorescence was found in intracellular vesicles (Fig. 4B), indicating that mGluR1a internalization occurs in the absence of agonist stimulation. However, β-arrestin1-GFP was not observed in the Flag-mGluR1a positive intracellular vesicles (Fig. 4B). Consistent with the hypothesis that β-arrestin1 may contribute to the regulation of agonist-stimulated internalization of mGluR1a, we found that both Rhodamine-labeled Flag-mGluR1a and β-arrestin1-GFP were localized to the same intracellular vesicular compartment after the exposure of cells to 100 μM quisqualate for 45 min at 37°C (Fig. 4C). Consequently, we conclude that only β-arrestin1 contributes to the agonist-stimulated endocytosis of mGluR1a and redistributes to an intracellular vesicular compartment with the receptor via direct association with the mGluR1a carboxyl-terminal. To our knowledge, these results provide the first example of a GPCR that internalizes solely in a β-arrestin1-dependent manner in HEK 293 cells.

β-Arrestins and mGluR1a Desensitization. β-Arrestins were originally characterized as proteins required as

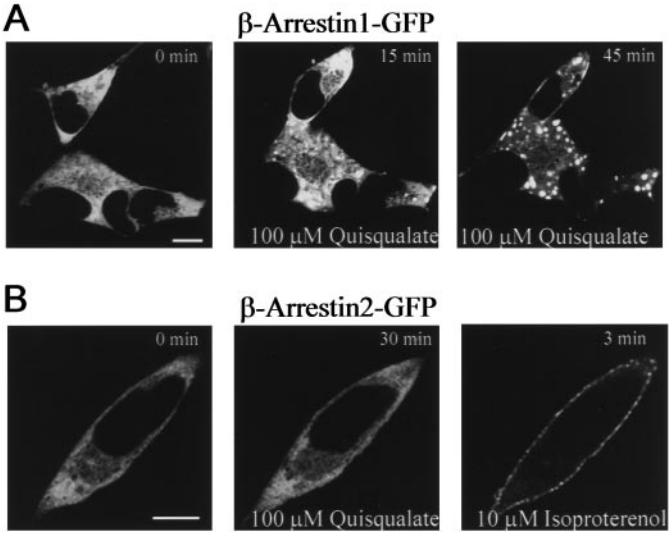

Fig. 2. β-Arrestin 1-specific vesicular redistribution in response to mGluR1a activation. Shown are representative laser scanning confocal images of β-arrestin1-GFP (A) and β-arrestin2-GFP (B) distribution in Flag-mGluR1a expressing HEK 293 cells before and after exposure of the same cells to 100 μM quisqualate. The Flag-mGluR1a cells in B also express 12CA5-β_2AR and were exposed to 10 μM isoproterenol for 3 min after a 30-min exposure of the same cells to 100 μM quisqualate. Shown is a representative image from four independent experiments.

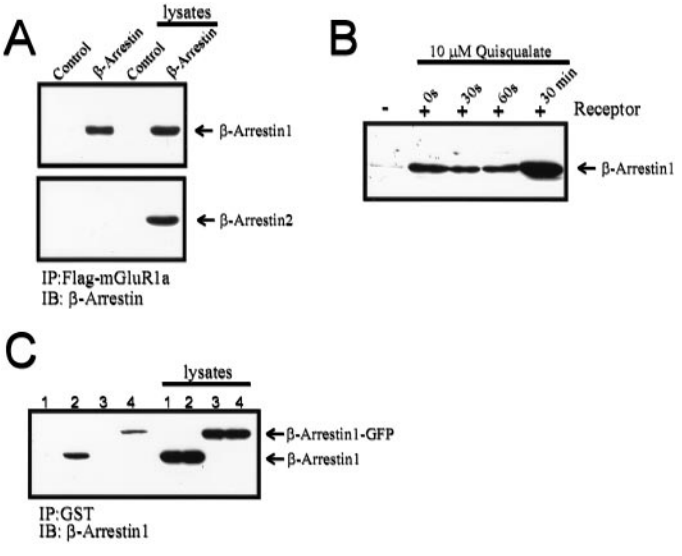

Fig. 3. β-Arrestin1-selective interaction with mGluR1a. HEK 293 cells were transfected transiently with plasmid cDNAs encoding Flag epitope-tagged mGluR1a (10 μg) and with either empty plasmid cDNA or plasmid cDNAs encoding either β-arrestin1-GFP (5 μg) or β-arrestin2-GFP (5 μg). A, coimmunoprecipitation of β-arrestin1 (top) and β-arrestin2 (middle) with Flag-mGluR1a from HEK 293 cells. B, the agonist-stimulated coimmunoprecipitation of β-arrestin1 with mGluR1a in HEK 293 cells in response to 0, 20 s, 60 s, and 30 min of exposure to 100 μM quisqualate. C, the precipitation of both wild type and GFP-tagged β-arrestin1 using a GST-mGluR1a-carboxyl-terminal fusion protein expressed in COS-7 cells. COS-7 cells were transfected transiently with plasmid cDNAs encoding cDNAs encoding GST (5 μg) (lane 1 and 3) or GST-mGluR1a-carboxyl-terminal fusion protein (5 μg) (lanes 2 and 4) along with plasmid cDNAs encoding either β-arrestin1 (5 μg) (lanes 1 and 2) or β-arrestin1-GFP (5 μg) (lanes 3 and 4). All data shown are representative of three to five independent experiments.

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cofactors for GRK-mediated desensitization of GPCRs (Benovic et al., 1987; Lohse et al., 1990). Therefore, we tested whether the overexpression of either β-arrestin1 or β-arrestin2 with and without GRK2 altered mGluR1a desensitization in HEK 293 cells. Similar to our previous observations (Dale et al., 2000), the maximal extent of mGluR1a-stimulated IP formation in response to increasing concentrations of quisqualate was reduced by 30% after the coexpression of GRK2 with mGluR1a in HEK 293 cells (Fig. 5, A and B). However, the dose-response for quisqualate-stimulated IP formation was not altered by the overexpression of either β-arrestin1 or β-arrestin2 (Fig. 5, A and B). Similarly, GRK2-mediated mGluR1a desensitization was not significantly increased by the coexpression of either β-arrestin1 or β-arrestin2 with the kinase (Fig. 5, A and B). Because GRK2 overexpression substantially reduced basal mGluR1a activity (Dale et al., 2000), we assessed whether β-arrestins expressed either alone or together with GRK2 reduced basal mGluR1a activity. Whereas GRK2 overexpression reduced basal mGluR1a-stimulated IP formation under all conditions tested, coexpression of β-arrestins had no effect on basal mGluR1a activity in either the presence or absence of GRK2 (Fig. 5C). Taken together, these data suggest that, unlike what is observed for many other GPCRs, GRK-mediated mGluR1a desensitization in HEK 293 cells does not require β-arrestin proteins as cofactors.

Agonist-Independent mGluR1a Internalization. Previous reports indicated that mGluRs internalize in the absence of agonist-stimulation (Doherty et al., 1999) and that in neurons, substantial group 1 mGluR immunoreactivity was localized to the intracellular compartment of postsynaptic specializations (Hanson and Smith, 1999). Consistent with

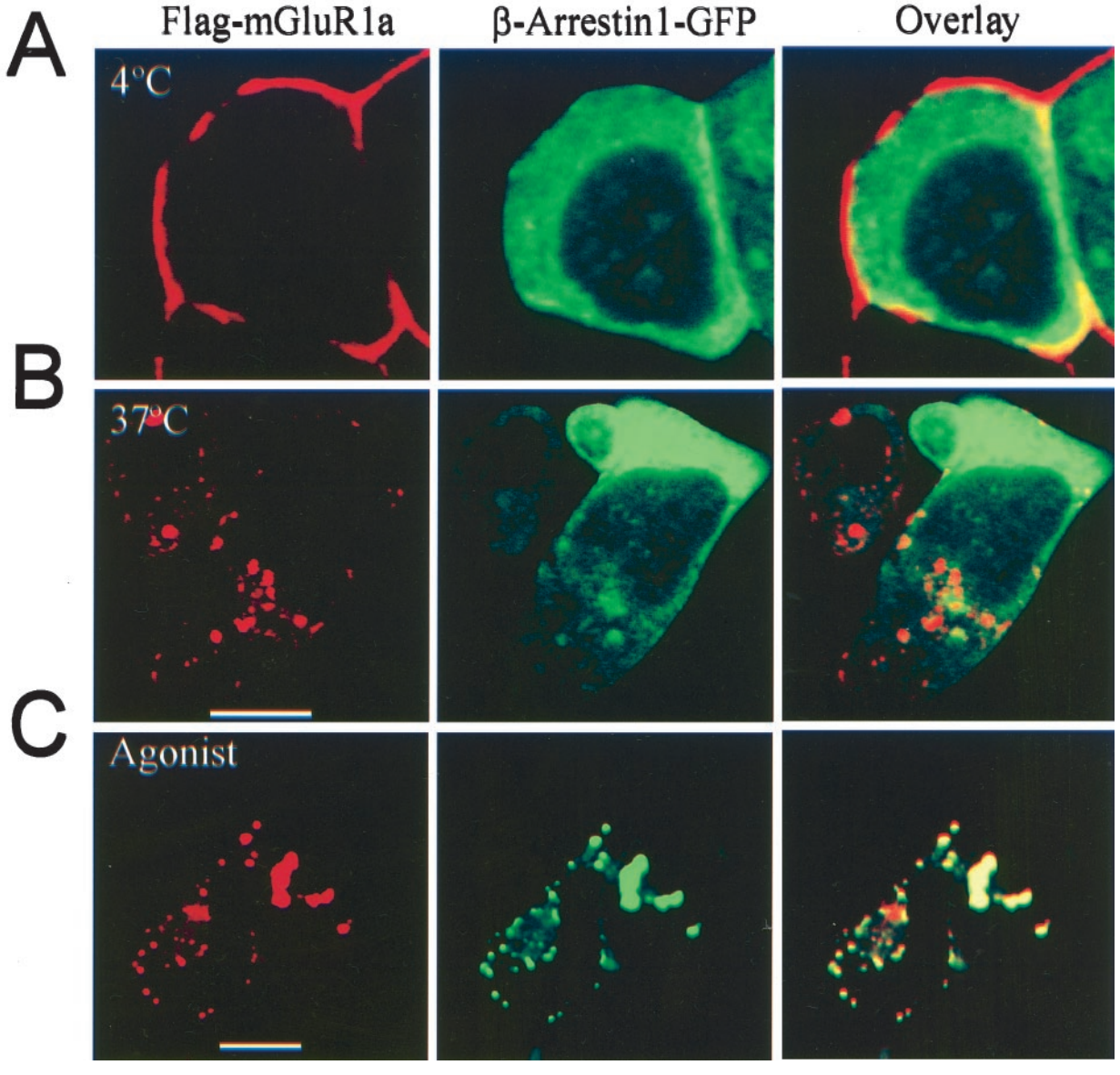

Fig. 4. Colocalization of Flag-mGluR1a and β-arrestin1-GFP. Representative laser scanning confocal micrographs demonstrating the distribution and colocalization (yellow) of monoclonal anti-Flag antibody labeled Flag-mGluR1a (red) and β-arrestin1-GFP (green) at 4°C (A) warmed to 37°C for 45 (B) and after 45-min stimulation with 100 μM quisqualate at 37°C (C). HEK 293 cells were fixed in PBS containing 3.6% paraformaldehyde and 0.2% Triton X-100. Cells were transfected with plasmid cDNAs encoding Flag-mGluR1a (10 μg) and β-arrestin1-GFP (5 μg). Data shown are representative of three experiments. Bar represents 10 μm.

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these reports, we observe a loss of cell surface Flag-mGluRs at 37°C by confocal microscopy in the absence of agonist stimulation (Fig. 4B). Accordingly, we tested whether the relatively low levels of agonist-stimulated mGluR1a internalization observed in the present study may be masked by a rapid agonist-independent turnover of cell surface receptor. To assess the agonist-independent loss of cell surface Flag-mGluR1a, cell surface Flag epitope-tagged mGluR1a were antibody labeled on ice and warmed to 37°C for the times indicated in the figures. Then the loss of cell surface immunofluorescence was assessed by flow cytometry. The time course for agonist-independent Flag-mGluR1a internalization was rapid ($t_{1/2}$ = 3.3 ± 0.3 min) and extensive (V_{max} = 80 ± 3.6%) but could not be blocked by pretreatment with the group 1 mGluR antagonist 4C3HPG (Fig. 6A). This observation indicates that mGluR internalization was not mediated by glutamate excreted by the cells into the culture medium. The treatment of antibody-prelabeled Flag-mGluR1a with agonist also did not significantly increase the loss of Flag-mGluR1a from the cell surface (Fig. 6A). Agonist-independent mGluR1a internalization was reduced after the treatment of cells with either hypertonic sucrose or concanavalin A but was unchanged after expression of the dominant-negative dynamin I-K44A mutant (Fig. 6B). The treatment of HEK 293 cells with agents (50 μg/ml nystatin or 1 μM PMA) that block caveolae-mediated endocytosis (Anderson et al., 1996) had no effect on agonist-independent mGluR1a internalization (Fig. 6C). Furthermore, internalization was unaltered in cells coexpressing either wild-type GRK2 or dominant-negative GRK2-K220R and GRK2-CT constructs, which reduce mGluR1a phosphorylation (Dale et al., 2000) (Fig. 6D). Finally, the overexpression of either wild type β-arrestin1 or a dominant-negative β-arrestin1 185-418 mutant that blocks β-arrestin-mediated GPCR internalization had no significant effect on either the rate or maximal extent of agonist-independent mGluR1a internalization (Fig. 6E). The reduction of agonist-independent Flag-mGluR1a internalization after treatment with hypertonic sucrose did not alter Flag-mGluR1a stimulated IP formation in response to increasing concentrations of quisqualate (Fig. 6F). Furthermore, the expression of a β-arrestin1 (185-418) dominant-negative inhibitor of GPCR internalization had no effect on Flag-mGluR1a signaling (Fig. 6F). Taken together, these data suggest that agonist-independent mGluR1a internalization is not mediated by caveolae but may involve a clathrin-coated vesicle-mediated pathway that is GRK-, β-arrestin- and dynamin-insensitive.

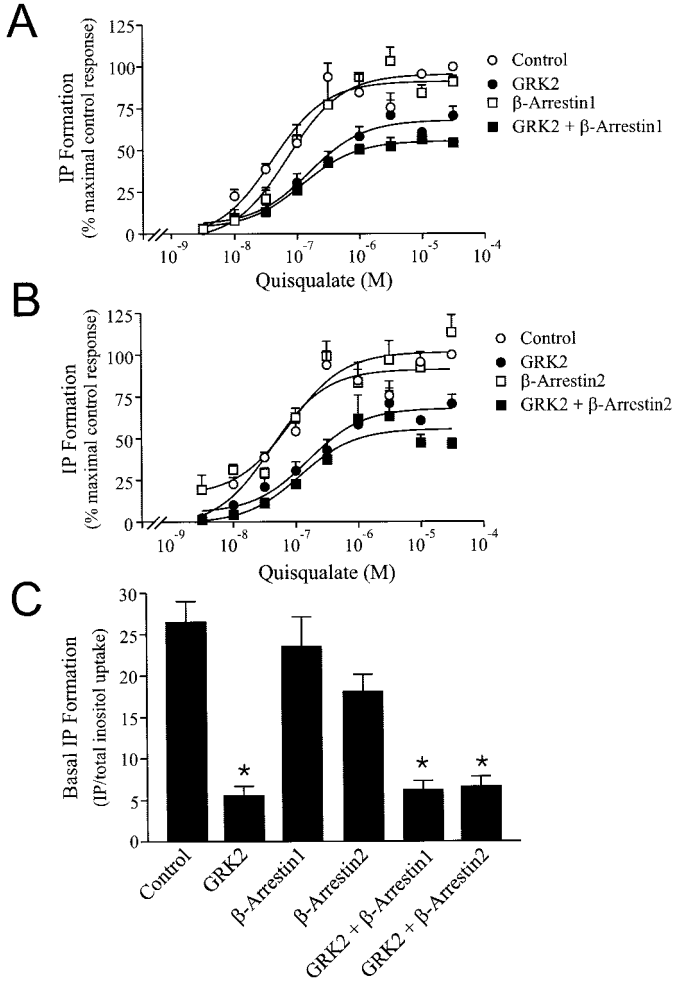

Fig. 5. Effect of β-arrestin expression on Flag-mGluR1a-stimulated IP formation. The effect of coexpressing GRK2 and β-arrestin1 (A) or β-arrestin2 (B) either alone or together on mGluR1a-stimulated IP formation in response to increasing concentrations of quisqualate for 30 min at 37°C. C, effect of coexpressing GRK2, β-arrestin1, and β-arrestin2 either individually or in combination on basal IP formation in cells expressing Flag-mGluR1a. Cells were labeled with 1 μCi/ml [^{3}H]inositol overnight, and mGluR1a basal activity was measured as the fraction of the incorporated [^{3}H]inositol converted to [^{3}H]inositol phosphates in the absence of agonist stimulation. HEK 293 cells were transfected with 10 μg of plasmid cDNA encoding Flag-mGluR1a with and without 10 μg each of plasmid cDNAs encoding either GRK2, β-arrestin1, or β-arrestin2. The data represent the mean ± S.E.M. for four experiments. *, $P < 0.05$ versus control (mGluR1a alone).

Agonist-Independent mGluR1a Internalization Is Mediated by Clathrin-Coated Vesicles. Because constitutive agonist-independent mGluR internalization is inhibited by hypertonic sucrose, which is known to impair clathrin-mediated endocytosis (Heuser and Anderson, 1989), we examined whether internalized mGluR colocalizes with clathrin and/or transferrin receptor. In cells fixed and stained for clathrin, colocalization of clathrin and Flag-mGluR1a immunofluorescence is observed in vesicular structures and at the cell surface (Fig. 7A). Consistent with the idea that agonist-independent mGluR1a internalization is mediated by a clathrin-dependent mechanism, Flag-mGluR1a is also colocalized with transferrin receptor in endocytic vesicles (Fig. 7B). It has been suggested that biochemically distinct endocytic vesicles may exist (Cao et al., 1998). Therefore, we examined whether Flag-mGluR1a that is internalized in the absence of agonist is internalized in the same endocytic vesicles that mediate the agonist- and β-arrestin-dependent internalization of the β_2AR. In the absence of the β_2AR agonist isoproterenol at 4°C, Flag-mGluR1a and β_2AR-GFP are both localized at the cell surface (Fig. 7C). Upon warming of cells to 37°C and the stimulation of β_2AR-GFP with 10 μM isoproterenol for 5 min, both the Flag-mGluR1a and β_2AR colocalized in the same endocytic vesicles (Fig. 7D).

Flag-mGluR1a internalized in the absence of agonist is also found to colocalize with the early endosomal marker protein GFP-Rab5a (Fig. 8A). Coexpression of a constitutively active GFP-Rab5a-Q79L mutant promotes the localization of Flag-mGluR1a immunostaining to enlarged endosomes, where it is colocalized with GFP-Rab5a-Q79L

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(Fig. 8B). However, unlike what was observed for the β_2AR (Seachrist et al., 2000), Flag-mGluR1a internalization is not blocked by the coexpression of a dominant-negative Rab5a-S34N mutant (data not shown). These data provide clear evidence that, although agonist-independent mGluR1a endocytosis is insensitive to dominant-negative GRK, β-arrestin, and dynamin mutants, it is apparently mediated by a clathrin-dependent endocytic pathway required for the β-arrestin-dependent internalization of other GPCRs.

Discussion

In the present study, we investigated the mechanisms involved in mGluR1a internalization. Our results indicate that GRK2 and GRK5 selectively promote β-arrestin1-specific mGluR1a internalization in HEK 293 cells. To our knowledge, mGluR1a represents the first GPCR to internalize in a β-arrestin-dependent manner that does not utilize β-arrestin2 as an endocytic adaptor protein. Furthermore, mGluR1a does not appear to require β-arrestin proteins as cofactors for desensitization in response to GRK-mediated

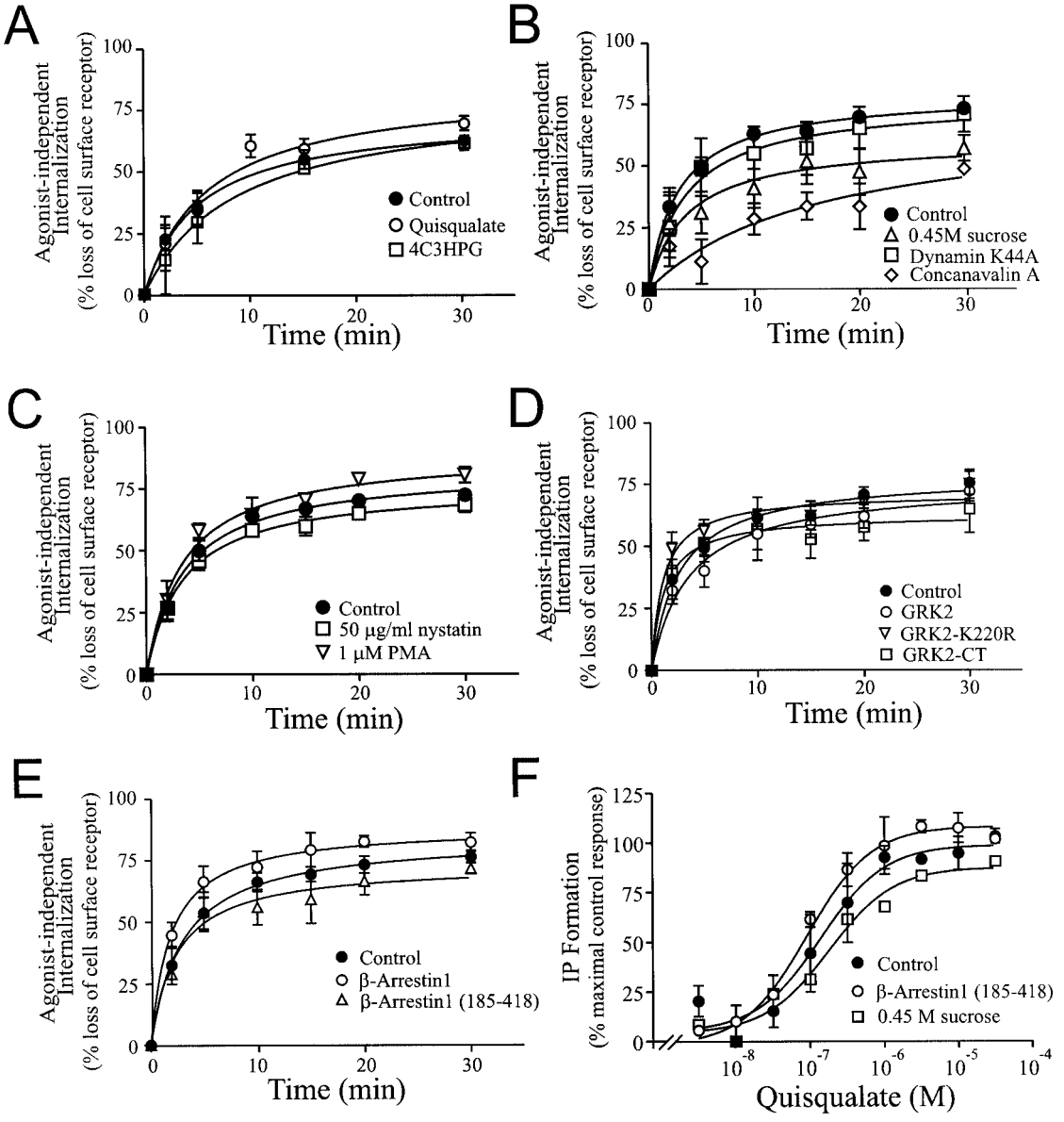

Fig. 6. Effect of antagonist and inhibitors of clathrin- and caveolin-mediated endocytosis on tonic mGluR1a internalization. A, time course for the agonist-independent Flag-mGluR1a internalization in the absence (control) and presence of either the mGluR1 agonist quisqualate (100 μM) or antagonist 4C3HPG (100 μM). Cells were pretreated for 1 h in the presence of the antagonist before assessing the loss of cell surface Flag-mGluR1a. B, effect of dynamin I-K44A coexpression on the pretreatment of HEK 293 cells with 0.45 M sucrose and 30 μg/ml of concanavalin A for 30 min on the agonist-independent internalization of Flag-mGluR1a. C, effect of 50 μg/ml nystatin (3 h pretreatment) and 1 μM PMA (30-min pretreatment) on the agonist-independent internalization of Flag-mGluR1a. D, the effect of GRK2, GRK2-K220R, and GRK-CT expression on agonist-independent internalization of Flag-mGluR1a. E, the effect of β-arrestin1 and β-arrestin1 (185-418) dominant-negative mutant on the agonist-independent internalization of Flag-mGluR1a. F, the effect of either expressing β-arrestin1 (185-418) or the 20-min treatment with 0.45 M sucrose on mGluR1a-stimulated IP formation in response to increasing concentrations of quisqualate for 30 min at 37°C. HEK 293 cells were transfected with 5 μg of plasmid cDNA encoding the Flag-mGluR1a along with 10 μg of plasmid cDNAs encoding dynamin I-K44A, GRK2, GRK2-K220R, GRK-CT, β-arrestin1, and β-arrestin1 (185-418). The data represent the mean ± S.E.M. for 3–8 independent experiments.

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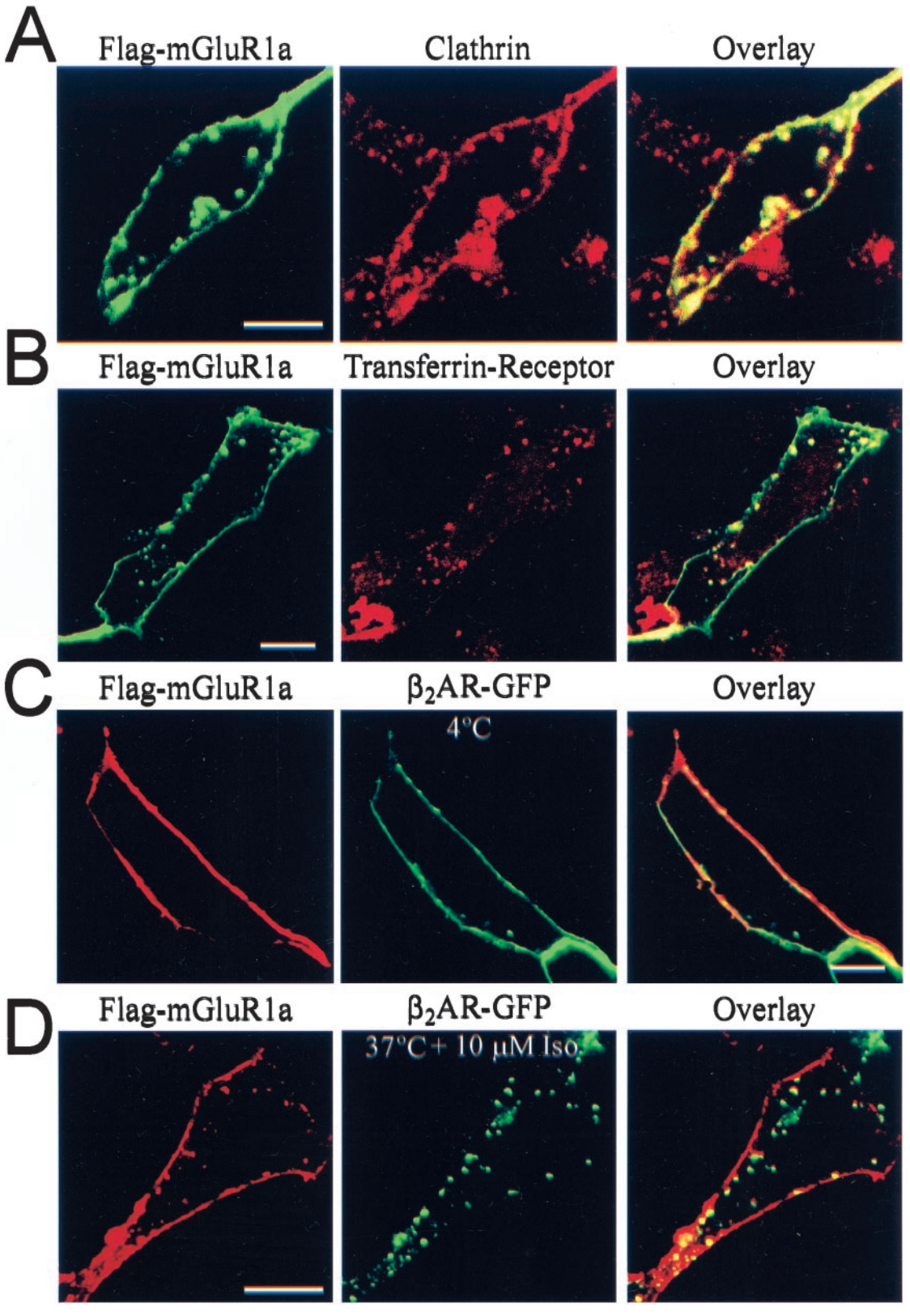

Fig. 7. Colocalization of Flag-mGluR1a with clathrin, transferrin receptors and β_2ARs. Representative laser scanning confocal micrographs demonstrating the distribution and colocalization (yellow) in HEK 293 cells of goat anti-Flag antibody labeled Flag-mGluR1a (green) and X-22 monoclonal antibody labeled clathrin (red) (A); Flag-mGluR1a (green) and transferrin receptor (red) (B); Flag-mGluR1a (red) and β_2AR-GFP (green) maintained on ice (C); or Flag-mGluR1a (red) and β_2AR-GFP (green) (D) warmed to 37°C for 5 min in the presence of 10 μM isoproterenol (Iso). Flag-mGluR1a were labeled at 4°C for 45 min with primary monoclonal anti-Flag antibody and 15 μg/ml Texas Red-conjugated transferrin prior to warming the cells to 37°C for 10 min. Cells were then fixed in PBS containing 3.6% paraformaldehyde with 0.2% Triton X-100 and receptors were stained with either FITC- or Rhodamine-conjugated goat anti-mouse secondary antibody. HEK 293 cells were transiently transfected with 10 μg of plasmid cDNA containing Flag-mGluR1a. The data in each panel are representative of at least three different experiments. Bars represent 10 μm.

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phosphorylation in HEK 293 cells. In addition to agonist-stimulated mGluR1a internalization, we also observe tonic mGluR1a internalization that is insensitive to either antagonist treatment or the expression of dominant-negative GRK, β-arrestin, and dynamin mutants. However, the agonist-independent internalization of mGluR1a is not only attenuated by hypertonic sucrose treatment, but the internalized receptor also colocalizes with proteins that are hallmarks of clathrin-mediated endocytosis: clathrin, transferrin receptors, and Rab5 GTPase. Consequently, our data provide: 1) an example of a GPCR that is internalized in a β-arrestin1 isoform-specific manner, 2) evidence that GRK phosphorylation in the absence of β-arrestin binding is sufficient to mediate mGluR1a desensitization in HEK 293 cells, and 3) support for the notion that multiple mechanisms may exist for the targeting of GPCRs for internalization via clathrin-coated vesicles and that these mechanisms may exhibit differential sensitivity to dominant-negative dynamin mutants.

On the basis of structural and sequence homology, the mammalian GPCR superfamily can be subdivided roughly into three subclasses: I) rhodopsin/β_2AR, II) secretin/glucagon, and III) mGluR families. Recently, Oakley et al. (2000) analyzed β-arrestin translocation responses to agonist activation of a series of class I GPCRs. These studies revealed two types of class I receptors: receptors that interact with β-arrestin2 with higher affinity than β-arrestin1 (e.g., β_2AR) and receptors that bind to β-arrestin1 and β-arrestin2 equally well (e.g., V2 vasopressin receptor) (Oakley et al., 2000). In the present study, we provide evidence of a β-arrestin1 selective GPCR interaction and propose that class III GPCRs, such as mGluR1a, may define a third class of GPCR/arrestin interactions that involve the selective and stable association with β-arrestin1. mGluR1a seems to be the first receptor to specifically associate with β-arrestin1 but not β-arrestin2, suggesting that the nature of the physical interaction between mGluR1a and β-arrestin1 must differ from group I GPCRs. In particular, chimeric β_2AR/β_3ARs have revealed that multiple intracellular loop domains, in addition to the carboxyl-terminal tail, are required for normal β_2AR endocytosis (Jockers et al., 1996). Thus, it is likely that multiple intracellular domains contribute to β-arrestin binding to class I GPCRs and that the carboxyl-terminal tail serves to regulate the avidity of β-arrestin binding. In contrast, it is likely that the selective interaction of β-arrestin1 with mGluR1a may not require multiple points of contact with the receptor. Consistent with this idea, β-arrestin1 binds to a GST-fusion protein that consists solely of the mGluR1a carboxyl-terminal tail, suggesting that other intracellular mGluR1a domains are not required for β-arrestin binding. Consequently, the binding of β-arrestins to mGluR1a may be more sensitive to subtle differences in β-arrestin protein structure.

β-Arrestin1 is a member of a family of intracellular proteins that comprises four members: visual arrestin, β-arrestin1, β-arrestin2, and cone arrestin (X-arrestin) (Ferguson 2001), originally identified as cofactors required for the inactivation of GRK-phosphorylated GPCRs (Pfister et al., 1985; Benovic et al., 1987). Agonist activation and GRK-mediated

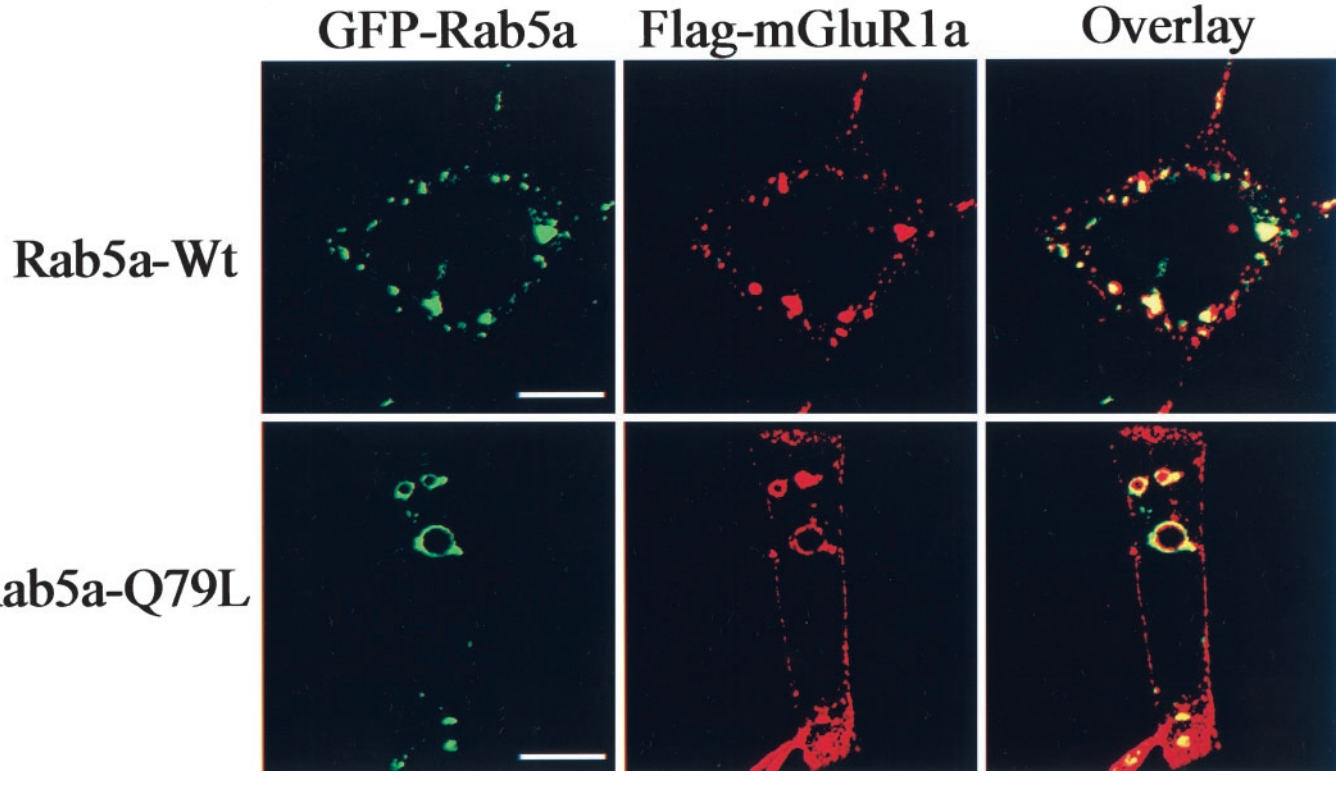

Fig. 8. Colocalization of Flag-mGluR1a with GFP-Rab5a and GFP-Rab5a-Q79L. Representative laser scanning confocal micrographs showing the distribution and colocalization (yellow) of Flag-mGluR1a (red) and either (A) GFP-Rab5a or (B) GFP-Rab5a-Q79L (green). mGluR1a expressing HEK 293 cells were labeled at 4°C for 45 min with primary monoclonal anti-Flag antibody, warmed to 37°C for 30 min prior to fixation with PBS containing 4% paraformaldehyde with 0.2% Triton X-100. HEK 293 cells were transiently transfected with 5 μg of plasmid cDNAs encoding the Flag-mGluR1a along with 5 μg of plasmid cDNA encoding either GFP-Rab5a or GFP-Rab5a-Q79L. Data are representative images of three to four different experiments. Bars represent 10 μm.

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phosphorylation promotes the association of arrestins with both class I and class II GPCRs resulting in the physical uncoupling of these GPCRs from heterotrimeric G proteins (Pippig et al., 1993; Shetzline et al., 1998; Zhang et al., 1998; Oakley et al., 1999). Unexpectedly, we find that β-arrestin overexpression does not increase mGluR1a desensitization in HEK 293 cells, even though β-arrestin1 binds to the carboxyl-terminal tail of mGluR1a and contributes to mGluR1a endocytosis. This is different from class I and II GPCRs where β-arrestin overexpression increases receptor desensitization in the presence and absence of GRKs (Pippig et al., 1993; Zhang et al., 1998; Shetzline et al., 1998; Oakley et al., 1999). These observations suggest that, although GRKs and arrestins retain the capacity to associate with all GPCR classes, the functional consequence of the interaction varies significantly from one receptor class to the next. For example, although β-arrestins serve as cofactors for the GRK-mediated desensitization of Class II GPCRs, they may not be required for the agonist-stimulated internalization of Class II receptors (Walker et al., 1999). In contrast, β-arrestin1 seems to contribute to agonist-stimulated mGluR1a endocytosis but is not absolutely required as a cofactor for GRK-mediated mGluR1a desensitization. Consequently, it will not be possible to predict a priori the relative roles of GRK and arrestin proteins in regulating the activity of distinct GPCR subtypes. Such differences in GPCR subtype regulation highlights the importance of studies examining the contribution of GRKs and arrestins in modulating the activity of different GPCRs.

The precise mechanism(s) contributing to the internalization of some GPCRs remains unclear. Considerable interest has arisen from the observation that the internalization of some GPCRs is relatively insensitive to β-arrestin and dynamin dominant-negative mutants (reviewed by Ferguson, 2001). However, varying results are obtained from one study to the next suggesting that multiple endocytic pathways may contribute to the internalization of the same GPCR (Zhang et al., 1996; Vogler et al., 1999, Werbonat et al., 2000; Gáborik et al., 2001). In the present study we observe tonic mGluR1a internalization that cannot be blocked by either antagonist treatment or the expression of GRK, β-arrestin, and dynamin dominant negative mutants. Furthermore, the treatment of cells with drugs that are proposed to block caveolae-mediated internalization (Anderson et al., 1996) has no effect on tonic mGluR1a internalization. However, agonist-independent mGluR1a internalization is impaired by hypertonic sucrose treatment and internalized mGluR1a is colocalized with clathrin, β_2AR, and transferrin receptors as well as the early endosomal marker Rab5. These proteins are hallmarks of clathrin-mediated endocytosis. Moreover, the inhibition of clathrin-mediated endocytosis does not influence mGluR1a responsiveness, suggesting that tonically internalized receptors may be either nonfunctional or normally replenished by recycled receptors. Taken together, these observations suggest that the tonic internalization of mGluR1a involves a clathrin-mediated endocytic recycling pathway that is relatively insensitive to dynamin-I-K44A and does not use β-arrestins as proximal GPCR adaptor proteins. Recently, it was proposed that β_2AR internalization may be mediated by a population of clathrin-coated vesicles that are functionally distinct from the vesicles mediating constitutive transferrin receptor endocytosis (Cao et al., 1998). However, our data do not support this contention and suggest that tonic mGluR1a internalization occurs via the same clathrin-coated, vesicle-mediated endocytic pathway that mediates both transferrin receptor endocytosis and the agonist- and β-arrestin-dependent internalization of the β_2AR.

The internalization of GPCRs in an agonist-independent manner has not been the subject of intense investigation because GPCR internalization has generally been considered an agonist-stimulated process (reviewed by Ferguson, 2001). However, several recent studies have demonstrated the agonist-independent internalization of protease activated receptors, angiotensin II type 1A receptor, cholecystokinin receptor, and thromboxane A2 receptor (Shapiro et al., 1996; Hein et al., 1997; Anborgh et al., 2000; Parent et al., 2001). Both agonist-independent β-arrestin binding and tyrosine-based endocytic motifs have been implicated in the tonic internalization of these receptors (Anborgh et al., 2000; Parent et al., 2001). In the case of mGluR1a, the mechanism(s) underlying the tonic mGluR1a internalization remains to be determined. However, it does not seem to involve either GRK-mediated phosphorylation or β-arrestin binding in the absence of agonist. Moreover, the agonist-dependent internalization of mGluR1a does not seem to be associated with constitutive receptor activity because the overexpression of GRK2, which effectively reduces basal mGluR1a activity, does not reduce its agonist-independent internalization. This suggests that an endocytic motif and/or a novel endocytic adaptor protein underlies tonic mGluR1a internalization. Although it is unclear why mGluRs are constitutively internalized, it is possible that neurons maintain an intracellular pool of mGluRs that are mobilized in response to neuronal activity. The mobilization of an intracellular pool of mGluRs may parallel the plasma membrane recruitment and insertion of ionotropic glutamate receptors during long-term potentiation (Lu et al., 2001). Alternatively, mGluRs expressed in the soma may be internalized to endosomes before being targeted to dendrites and nerve terminals similar to what is suggested for other synaptic proteins (Nakata et al., 1998).

In the present study, we have assessed the potential role of GRK and β-arrestin proteins in the regulation of mGluR1a signaling in HEK 293 cells without the contribution of other mGluR1a regulatory proteins. However, it is possible that the functional regulation of mGluR1a in HEK 293 cells may differ from that observed in primary neurons that express other mGluR regulatory proteins such as homer. However, mGluR1a expression is not limited to neurons; its expression exists in rat heart and testes (Gill et al., 1999; Storto et al., 2001) and it is likely that mGluR1a expression will be localized to additional tissues. Nonetheless, Sallese et al. (2000) have reported that GRK4 contributes to the desensitization and internalization of mGluR1a in both cerebellar Purkinje and HEK 293 cells. Although our data is in apparent disagreement with the data of Sallese et al. (2000), GRK4 regulation of mGluR1a signaling in Purkinje cells may involve the GRK4-dependent phosphorylation of cellular components that do not exist in the particular HEK 293 cell line used in the present study. Moreover, because mGluR expression in the brain is not limited to Purkinje cells, mGluR1a desensitization in other neuronal cell types is probably regulated by other GRK isoforms.

In summary, although many of the molecular mechanisms contributing to the regulation of GPCR activity are conserved

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across the GPCR superfamily, we report here that mGluR-specific differences in GRK and β-arrestin regulation exist. In particular, we show that β-arrestin1 specifically contributes to mGluR1a internalization but is not involved in the desensitization of mGluR1a-mediated responses in HEK 293 cells. These observations highlight how GPCR activation is translated into diverse receptor-specific patterns of GRK and β-arrestin interactions that arise as the consequence of distinct differences in GPCR structure/function. The observation that a β-arrestin–independent clathrin-vesicle–mediated pathway mediates tonic mGluR1a internalization also suggests that multiple mechanisms and/or endocytic adaptor proteins contribute to the internalization of individual GPCRs. The challenge in the future will be to identify these alternative mediators of GPCR endocytosis.

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