

## Desensitization and Internalization of Metabotropic Glutamate Receptor 1a Following Activation of Heterologous G<sub>q/11</sub>-Coupled Receptors<sup>†</sup>

Stuart J. Mundell,<sup>‡</sup> Giordano Pula,<sup>‡</sup> R. A. Jeffrey McIlhinney,<sup>§</sup> Peter J. Roberts,<sup>‡</sup> and Eamonn Kelly<sup>\*:‡</sup>

*Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K., and Medical Research Council Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, U.K.*

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**ABSTRACT:** In this study we characterized the heterologous desensitization and internalization of the metabotropic glutamate receptor 1 (mGluR1) splice variants mGluR1a and mGluR1b following activation of endogenous G<sub>q/11</sub>-coupled receptors in HEK293 cells. Agonist activation of M1 muscarinic acetylcholine or P2Y1 purinergic receptors triggered the PKC- and CaMKII-dependent internalization of mGluR1a. In co-immunoprecipitation studies, both glutamate and carbachol increased the association of GRK2 with mGluR1a. Co-addition of the protein kinase C (PKC) inhibitor GF109203X and the Ca<sup>2+</sup> calmodulin-dependent kinase II (CaMKII) inhibitor KN-93 blocked the ability of glutamate and carbachol to increase the association of GRK2 with mGluR1a. Glutamate also increased the association of GRK2 with mGluR1b, whereas carbachol did not. However, unlike mGluR1a, glutamate-stimulated association of GRK2 with mGluR1b was not reduced by PKC/CaMKII inhibition. Pretreatment of cells expressing mGluR1a or mGluR1b with carbachol rapidly desensitized subsequent glutamate-stimulated inositol phosphate accumulation. The carbachol-induced heterologous desensitization and internalization of mGluR1a was blocked by LY367385, an mGluR1a antagonist with inverse agonist activity. Furthermore, LY367385 blocked the ability of carbachol to increase the association of GRK2 with mGluR1a. On the other hand, LY367385 had no effect on the carbachol-induced desensitization and internalization of the nonconstitutively active mGluR1b splice variant. These results demonstrate that the internalization of mGluR1a, triggered homologically by glutamate or heterologously by carbachol, is PKC/CaMKII-, GRK2-, arrestin-, and clathrin-dependent and that PKC/CaMKII activation appears to be necessary for GRK2 to associate with mGluR1a. Furthermore, the heterologous desensitization of mGluR1a is dependent upon the splice variant being in an active conformation.

Metabotropic glutamate receptors (mGluRs),<sup>1</sup> members of the G protein-coupled receptor (GPCR) superfamily, are activated by the excitatory amino acid glutamate and play an essential role in regulating neural development and synaptic plasticity events (for reviews see refs 1 and 2). Members of the mGluR family bear little sequence or structural homology to most other GPCRs (except other

family C receptors such as Ca<sup>2+</sup>-sensing and GABA<sub>B</sub> receptors) apart from the retention of a seven transmembrane domain topology. On the basis of their pharmacology, sequence homology, and signal transduction mechanisms, mGluRs have been classified into three groups, with the group I mGluRs, mGluR1 and mGluR5, being coupled to G<sub>q/11</sub> and phospholipase C. Five splice variants of mGluR1 have been described thus far, all of which differ in the length of their COOH-terminal tail (2, 3). The functional significance of the different splice variants has not yet been fully explored. The intracellular COOH-terminal tail of mGluRs might play a role in the subcellular targeting of the receptor (4), and elements of the COOH terminus close to the inner surface of the plasma membrane are important for G protein coupling (2).

The desensitization of GPCRs is a crucial physiological mechanism of adaption to the continuous or repeated presence of stimuli (reviewed in refs 5 and 6). Mechanisms underlying desensitization are complex and can involve phosphorylation of the receptor, uncoupling from G proteins, internalization, and ultimately intracellular downregulation. Phosphorylation and subsequent desensitization of GPCRs can occur by two distinct mechanisms. Independent of the activation status of the GPCR, second messenger-regulated protein kinases (e.g., PKC) can phosphorylate GPCRs. In

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\* To whom correspondence should be addressed. Telephone: +44 117 95 46 406. Fax: +44 117 92 50 168. E-mail: E.Kelly@bristol.ac.uk.

<sup>‡</sup> University of Bristol.

<sup>§</sup> Medical Research Council Anatomical Neuropharmacology Unit.

<sup>1</sup> Abbreviations: mGluR1a, metabotropic glutamate receptor 1a; mGluR1b, metabotropic glutamate receptor 1b; mGluR1c, metabotropic glutamate receptor 1c; DM-II, deletion mutant II; arr-2, arrestin-2; arrestin-2-GFP, arrestin-2-green fluorescent protein; PKC, protein kinase C; CaMKII, Ca<sup>2+</sup>-calmodulin-dependent protein kinase II; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; DNM-GRK2, dominant negative mutant G protein-coupled receptor kinase 2; GRK2-GFP, G protein-coupled receptor kinase 2-green fluorescent protein; HEK293, human embryonic kidney 293 cells; HA, hemagglutinin; PBS, phosphate-buffered saline; AP-2, adaptor protein 2; PKC, protein kinase C; IP, inositol phosphate; GF109203X, 2-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide; KN-93, 2-[[*N*-(2-hydroxyethyl)-*N*-(4-methoxybenzenesulfonyl)amino]-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; LY367385, (+)-2-methyl-4-carboxyphenylglycine; CPCOEt, cyclopropan[b]chromen-1a-carboxylic acid ethyl ester.

contrast, the family of G protein-coupled receptor kinases (GRKs) phosphorylates only agonist-activated GPCRs, this latter process often being referred to as homologous desensitization. Receptor phosphorylation can in turn increase the affinity of the receptor for arrestins. Arrestin binding to receptors will not only occlude receptor–G protein coupling but in many cases arrestins act as adaptors for receptor internalization via their interaction with components of the trafficking machinery (6). From early endosomes, receptors may then either be dephosphorylated and returned to the cell surface for another round of activation or, alternatively, enter an intracellular degradative pathway (5). At present, there is limited knowledge of how mGluR signaling is switched off, despite the fact that this is a critical step in vivo for receptor functions such as the regulation of glutamate release (7). Recent studies have examined the desensitization and internalization of group I mGluRs. The desensitization of mGluR1a, which has a long intracellular COOH-terminal tail, appears to be both GRK- and PKC-dependent (8–10). Group I mGluRs are also known to internalize upon agonist addition, and in recent studies we have demonstrated the arrestin- and dynamin-dependent internalization of mGluR1a and mGluR1b in response to glutamate (10, 11). Furthermore, we have recently shown that the glutamate-stimulated GRK- and arrestin-dependent regulation of mGluR1a depends on the proximal COOH-terminal tail (12).

In addition to homologous desensitization, mGluR1a function can also be regulated by glutamate-independent heterologous mechanisms. Recently, we demonstrated that activation of  $G_{q/11}$ -coupled M1 muscarinic acetylcholine receptors induces a PKC- and  $Ca^{2+}$  calmodulin-dependent kinase II (CaMKII) dependent internalization of mGluR1 splice variants (10, 13, 14). In the present study we have further investigated the molecular mechanisms underlying this heterologous effect and also whether muscarinic receptor activation regulates the functional coupling of mGluR1a and mGluR1b. We find that activation of  $G_{q/11}$ -coupled receptors triggers heterologous desensitization of both mGluR1a and mGluR1b responsiveness in a PKC/CaMKII-dependent manner. Furthermore, the heterologous desensitization of mGluR1a responsiveness depends on a constitutively active conformation of this splice variant, since treatment with an inverse agonist at mGluR1a blocks the carbachol-induced desensitization and internalization of mGluR1a.

## EXPERIMENTAL PROCEDURES

**Materials.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and Lipofectamine 2000 transfection reagent were obtained from Life Technologies. The mouse monoclonal anti-HA antibody (3F10) was from Roche, the anti-HA monoclonal antibody (HA-11) from Molecular Probes, and the arrestin-2 polyclonal antibody (R-19) from Santa Cruz. The monoclonal antibody against GRK2 was generously donated by Professor J. L. Benovic, Thomas Jefferson University, Philadelphia, PA, while the DNM-Eps15 and control Eps15 constructs were kindly supplied by Dr. A. Benmerah, Institut Cochin, INSERM U567, University of Paris. *myo*-[ $^3H$ ]inositol (37 MBq  $mL^{-1}$ ) was purchased from Amersham. All other reagents were from Sigma.

**Construction of mGluR1 Constructs.** The construction of mGluR1a and mGluR1b with a HA-epitope tag (TRMYPY-

DVPDYA) located between amino acids 57 and 58 of the  $NH_2$  terminus and subcloned into pcDNA3 has been described previously (10, 11). The construction of the COOH-terminal tail truncation mutant of HA-tagged mGluR1a/mGluR1b (Arg<sup>868</sup> stop; DM-II) has also been previously described (12).

**Cell Culture and Transfection.** HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units  $mL^{-1}$  penicillin G, and 100  $\mu g mL^{-1}$  streptomycin sulfate at 37 °C in a humidified atmosphere of 95% air and 5%  $CO_2$ . For transient transfections, HEK293 cells were grown in 60 or 100 mm dishes to 80–90% confluence and transfected with 0.5–10  $\mu g$  of DNA using Lipofectamine 2000 according to the manufacturer's instructions. Cells were incubated with the DNA/Lipofectamine mixture for 24 h, the media replaced, and the cells analyzed 48 h after transfection.

**Internalization of mGluR1a or mGluR1b Transiently Expressed in HEK293 Cells.** mGluR1a or mGluR1b cell surface loss was assessed by ELISA as described previously (11). Briefly, cells plated at a density of around  $6 \times 10^5$  cells/100 mm dish were transiently transfected with pcDNA3 containing mGluR1a or mGluR1b (5  $\mu g$ ) and in some cases with wild-type GRK2 (5  $\mu g$ ), dominant negative mutant GRK2 (DNM-GRK2, 5  $\mu g$ ), dominant negative mutant Eps15 (DNM-Eps15, E $\Delta$ 95–295, 5  $\mu g$ ), or control Eps15 construct (DIII $\Delta$ 2, 5  $\mu g$ ). Twenty-four hours posttransfection, cells were split into 24-well tissue culture dishes coated with 0.1  $mg mL^{-1}$  poly(L-lysine). Twenty-four hours later, cells were incubated with DMEM (without L-glutamine), to which glutamate (10  $\mu M$ ), carbachol (1  $mM$ ), ADP (10  $\mu M$ ), or isoprenaline (10  $\mu M$ ) was added for up to 60 min at 37 °C. In some experiments GF109203X (2  $\mu M$ )/KN-93 (10  $\mu M$ ), LY367385 (100  $\mu M$ ), CPCCOEt (100  $\mu M$ ), or filipin (5  $\mu g mL^{-1}$ ) was added for a period before and then during glutamate or carbachol addition. Reactions were stopped by removing the medium and fixing the cells with 3.7% formaldehyde in TBS (20  $mM$  Tris-HCl, pH 7.5, 150  $mM$  NaCl, and 20  $mM$   $CaCl_2$ ) for 5 min at room temperature. Cells were then washed three times with TBS, incubated for 45 min with TBS containing 1% BSA, and then incubated with a primary antibody (anti-HA monoclonal HA-11, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, reblocked with TBS/BSA for 15 min at room temperature, and then incubated with secondary antibody (goat anti-mouse antibody conjugated with alkaline phosphatase, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were then washed three times with TBS, and a colorimetric alkaline phosphatase substrate was added. When adequate color change was achieved, 100  $\mu L$  of sample was added to 100  $\mu L$  of 0.4  $M$  NaOH to terminate the reaction, and the samples were read at 405 nm using a microplate reader. Throughout, internalization of mGluR1a was compared against surface receptor expression at time zero. Results are expressed as percent loss of surface receptor, with the background signal from pcDNA3-transfected controls subtracted from all receptor-transfected values.

**Co-immunoprecipitation Experiments.** HEK293 cells were transiently transfected with 5  $\mu g$  of mGluR1a, mGluR1b, or DM-II, along with 2  $\mu g$  of either empty pcDNA3 or pcDNA3 containing arrestin-2-GFP, GRK2-GFP, or DNM-GRK2.

Following drug treatment, cells from 60 mm dishes were washed twice with ice-cold PBS and lysed in 500  $\mu$ L of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100 containing the protease inhibitors leupeptin at 20  $\mu$ g mL<sup>-1</sup>, aprotinin at 20  $\mu$ g mL<sup>-1</sup>, and phenylmethanesulfonyl fluoride at 20  $\mu$ g mL<sup>-1</sup>). The particulate fraction was removed by microcentrifugation at 14000 rpm, and 300  $\mu$ g of supernatant protein was incubated in the presence of anti-HA monoclonal antibody (3F10, 1:500) and 100  $\mu$ L of 20% slurry protein G Sepharose beads in immunoprecipitation buffer (300 mM NaCl, 2% deoxycholate, and 2% Triton X-100) at 4 °C for 12–18 h. The beads were subsequently washed three times with immunoprecipitation buffer and solubilized in 2  $\times$  SDS sample buffer and the immunoprecipitates subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. Arrestin-2 was detected by immunoblotting with a polyclonal arrestin-2 antibody (R-19) diluted 1:200, GRK2 by a monoclonal antibody that recognizes an epitope within residues 500–531 of the COOH terminus of bovine GRK2 diluted 1:100, and mGluR1a by a polyclonal anti-NH<sub>2</sub>-terminal mGluR1 antibody diluted 1:100 in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) wash buffer containing 5% skimmed milk. The nitrocellulose membranes were washed three times with TBS-T and then incubated with horseradish peroxidase-conjugated goat-anti rabbit or sheep-anti mouse-IgG antibody diluted 1:2000 in wash buffer containing 5% skimmed milk. The nitrocellulose membranes were then rinsed three times with TBS-T and incubated with ECL Western blotting detection reagents. The extent of co-immunoprecipitation was quantified by densitometric analysis of the resulting autoradiographs.

**Inositol Phosphate (IP) Determination.** This was undertaken as previously described (15). Briefly, cells plated at a density of around  $6 \times 10^5$  cells in each 60 mm dish were transiently transfected with 5  $\mu$ g of mGluR1a or mGluR1b. Twenty-four hours posttransfection, cells were split into 24-well tissue culture dishes coated with 0.1 mg mL<sup>-1</sup> poly(L-lysine). The following day cells were labeled for 18–24 h with *myo*-[<sup>3</sup>H]inositol (4  $\mu$ Ci mL<sup>-1</sup> of culture medium) in DMEM (high glucose, without inositol). After labeling, cells were washed once in PBS and incubated in prewarmed DMEM (without L-glutamine) containing various combinations of carbachol (1 mM; 0–60 min), GF109203X (2  $\mu$ M), KN-93 (10  $\mu$ M), LY367385 (100  $\mu$ M), and CPCCOEt (100  $\mu$ M). Cells were subsequently washed three times in ice-cold PBS and incubated in prewarmed media containing 20 mM LiCl, the muscarinic acetylcholine receptor antagonist atropine (100  $\mu$ M), and glutamate (10  $\mu$ M) for 0–30 min. Reactions were terminated by removing the stimulation media and adding 0.8 mL of 0.4 M perchloric acid. Samples were harvested in 1.5 mL Eppendorf tubes to which 0.4 mL of 0.72 M KOH and 0.6 M KHCO<sub>3</sub> were added. Tubes were vortexed and centrifuged for 5 min at 14000 rpm in a microcentrifuge. Inositol phosphates were separated on Dowex AG 1-X8 columns exactly as described previously (15). Total labeled inositol phosphates were determined by liquid scintillation counting.

**Experimental Design and Statistics.** Data were analyzed using a GraphPAD Prism (GraphPAD Software). Where appropriate, the statistical significance of differences between means was assessed by the Mann Whitney U-test.

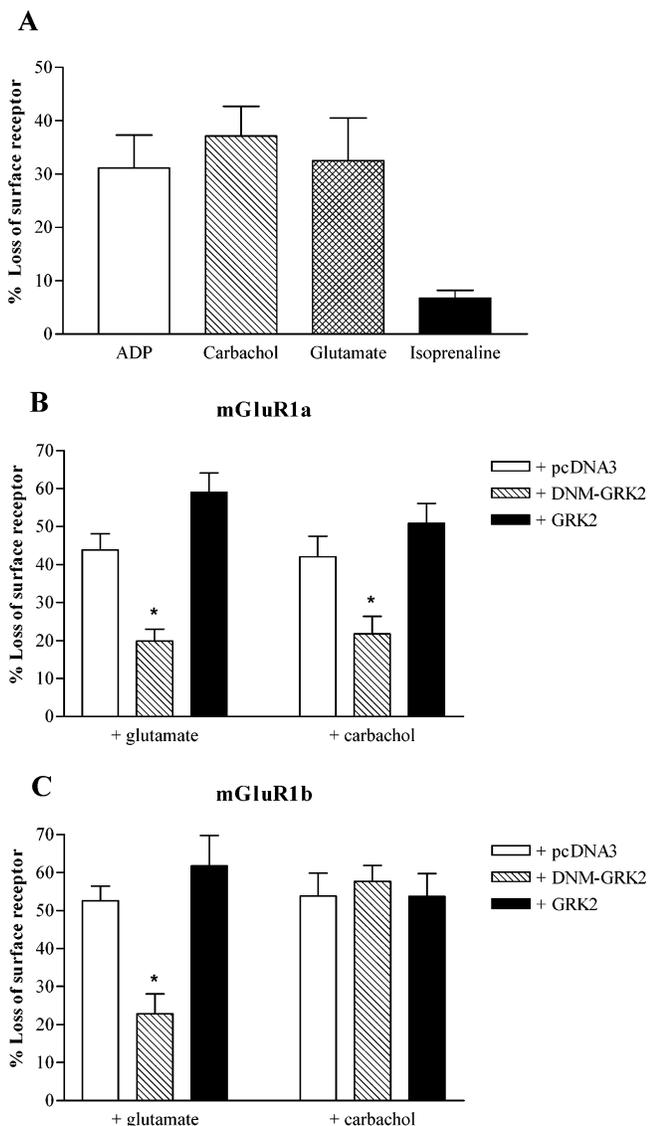
## RESULTS

### *Heterologous Internalization of mGluR1a and mGluR1b.*

To determine whether endogenous GPCRs in HEK293 cells other than the M1 muscarinic acetylcholine receptor trigger mGluR1a internalization, cells were treated with ADP, which activates endogenous G<sub>q/11</sub>-coupled P2Y1 purinergic receptors in these cells (15). The addition of ADP (10  $\mu$ M; 30 min) induced mGluR1a internalization to levels comparable with those observed with carbachol (1 mM; 30 min) and glutamate (10  $\mu$ M; 30 min) (Figure 1A). In contrast, activation of endogenous G<sub>s</sub>-coupled  $\beta_2$ -adrenoceptors with isoprenaline (10  $\mu$ M; 30 min) failed to promote mGluR1a receptor internalization. Therefore, only the activation of G<sub>q/11</sub>-coupled GPCRs is sufficient to promote mGluR1a internalization.

A number of recent studies have shown that GRKs are directly involved in the phosphorylation, desensitization, and internalization of mGluR1a (8, 9). We therefore investigated the role of GRK2 in the glutamate- and carbachol-induced internalization of mGluR1a and mGluR1b. Coexpression of receptor and DNМ-GRK2 (GRK2-Lys<sup>220</sup>Arg) attenuated glutamate-induced internalization of mGluR1a and mGluR1b (Figure 1B,C), in agreement with our previous studies (12). However, while DNМ-GRK2 also inhibited carbachol-induced internalization of mGluR1a, it had no effect on carbachol-induced internalization of mGluR1b. On the other hand, overexpression of wild-type GRK2 either had no effect or produced a modest increase in mGluR1a and mGluR1b internalization due to glutamate or carbachol (Figure 1B,C).

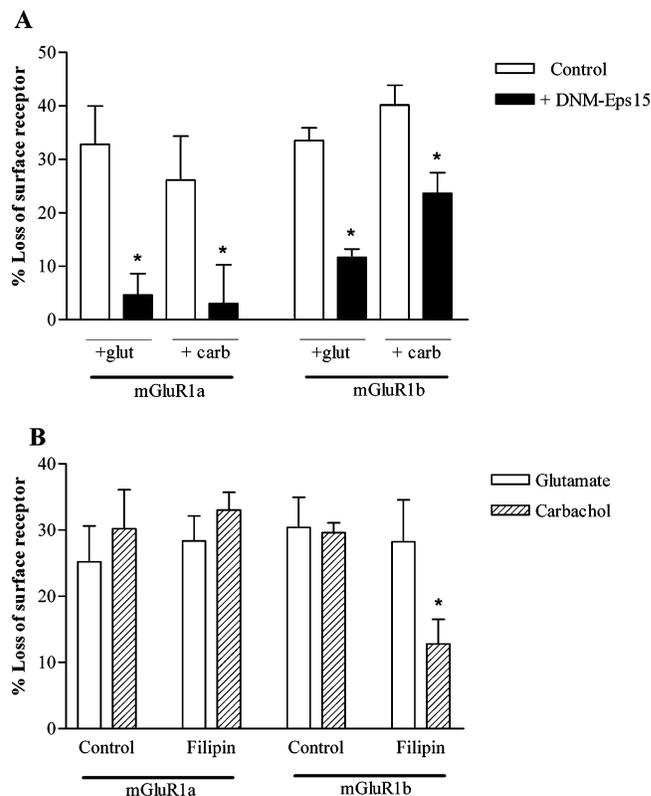
We have previously shown that carbachol induces PKC- and CaMKII-dependent internalization of mGluR1a and mGluR1b and that this confers differential arrestin dependence, with carbachol-induced internalization of mGluR1a being arrestin-dependent and that of mGluR1b being largely arrestin-independent (10). To determine the involvement of clathrin-coated pits in carbachol-induced internalization of these splice variants, cells were cotransfected with receptor and DNМ-Eps15 (E $\Delta$ 95–295), which disrupts the formation of clathrin-coated pits (16, 17). In cells expressing DNМ-Eps15, glutamate-induced internalization of mGluR1a and mGluR1b was markedly inhibited, as was carbachol-induced internalization of mGluR1a (Figure 2A). However, DNМ-Eps15 only partially inhibited carbachol-induced internalization of mGluR1b. Expression of a control Eps15 construct (DIII $\Delta$ 2), which does not contain the AP-2 binding site and so is unable to inhibit clathrin-mediated internalization (17, 18), did not affect glutamate-induced mGluR1a internalization ( $n =$  two independent experiments; data not shown). Together, these results demonstrate that, in response to carbachol, mGluR1b internalizes by both clathrin-dependent and -independent mechanisms. An alternative internalization pathway for GPCRs involves caveolae or lipid rafts (19, 20). To investigate this, glutamate- and carbachol-induced internalization was assessed in mGluR1a- and mGluR1b-expressing cells in the presence or absence of filipin (Figure 2B), which is reported to disrupt caveolae and block internalization by this mechanism (19, 20). The concentration of filipin that we used (5  $\mu$ g mL<sup>-1</sup>) has been employed in receptor trafficking studies both in HEK293 cells (21) and in Chinese hamster ovary cells (19) and has not been reported to overtly affect cell integrity. Whereas filipin had no effect on agonist-



**FIGURE 1:** Activation of heterologous  $G_{q/11}$ -coupled receptors triggers internalization of mGluR1a: GRK2 dependency of internalization. HEK293 cells were transiently transfected with pcDNA3-mGluR1a (A), pcDNA3-mGluR1a  $\pm$  pcDNA3, pcDNA3-DNM-GRK2, or pcDNA3-GRK2 (B), or pcDNA3-mGluR1b  $\pm$  pcDNA3, pcDNA3-DNM-GRK2, or pcDNA3-GRK2 (C) and used in experiments 2 days later. (A) Cells were challenged for 30 min with the P2Y1 purinergic receptor agonist ADP (10  $\mu$ M), the M1 muscarinic acetylcholine receptor agonist carbachol (1 mM), glutamate (10  $\mu$ M), or the  $\beta_2$ -adrenoceptor agonist isoprenaline (10  $\mu$ M), and surface receptor loss was assessed by ELISA. The data are the mean  $\pm$  SE from four independent experiments. (B, C) Cells were challenged for 30 min with carbachol (1 mM) or glutamate (10  $\mu$ M), and surface receptor loss was assessed by ELISA. The data are the mean  $\pm$  SE from six independent experiments. \* $p$  < 0.05 compared to the respective plasmid vector only transfected controls (Mann Whitney U-test).

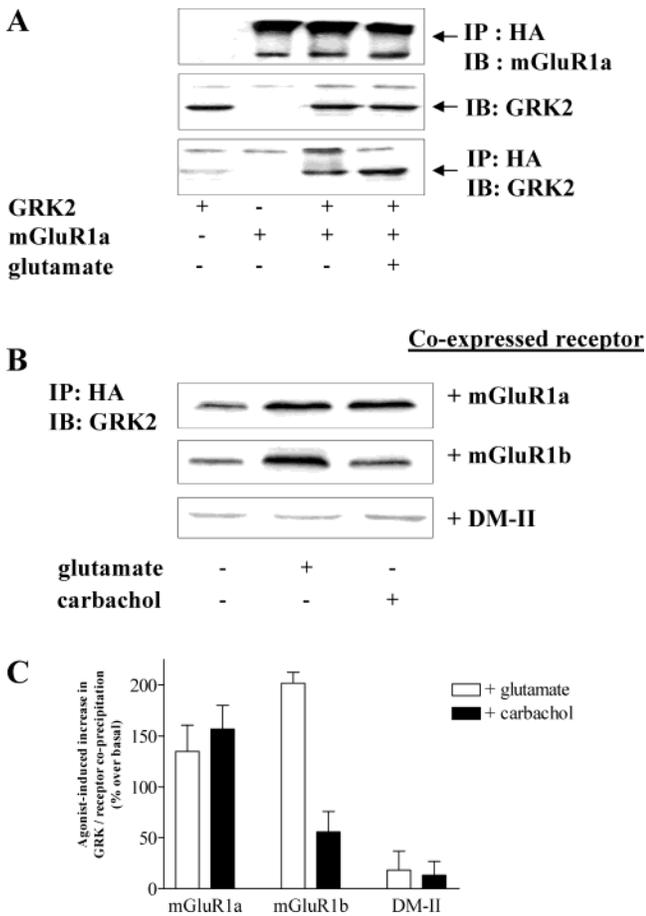
induced internalization of mGluR1a, it significantly inhibited carbachol-induced internalization of mGluR1b (Figure 2B). Taken together, these data strongly suggest that, in response to carbachol, mGluR1b internalizes by both clathrin- and caveolae/lipid raft-dependent mechanisms.

**Agonist-Induced Association of GRK2 and Arrestin-2 with mGluR1a and mGluR1b.** Using mGluR1a transiently expressed in HEK293 cells, we have found it difficult to detect significant levels of agonist-induced mGluR1a phosphorylation. As an alternative approach, we attempted to dem-



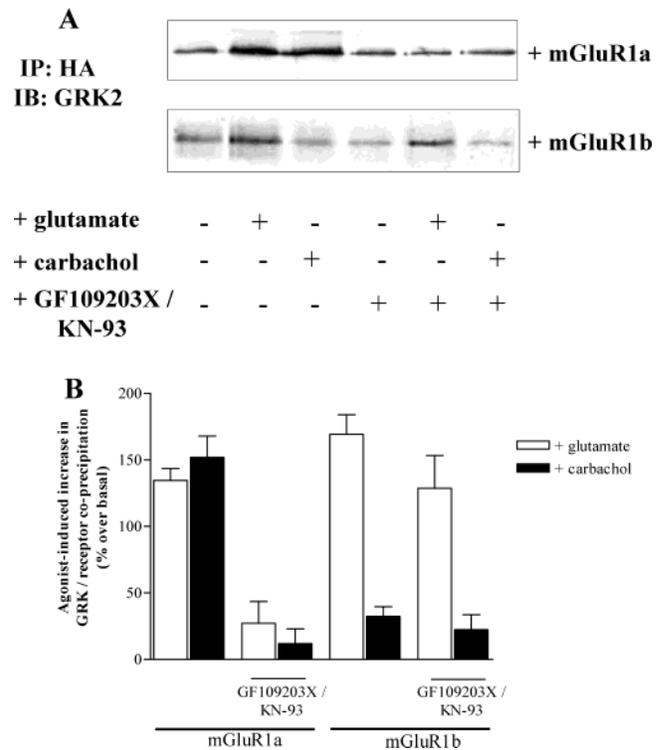
**FIGURE 2:** Effect of inhibitors of clathrin- or caveolae/lipid raft-dependent function on agonist-induced internalization of mGluR1a and mGluR1b. (A) HEK293 cells were transiently transfected with pcDNA3-mGluR1a or pcDNA3-mGluR1b  $\pm$  EGFP-C2 or EGFP-C2-E $\Delta$ 95-295 and used in experiments 2 days later. Cells were challenged for 30 min with glutamate (10  $\mu$ M) or carbachol (1 mM), and surface receptor loss was assessed by ELISA. The data are the mean  $\pm$  SE from four independent experiments. \* $p$  < 0.05 compared to the respective plasmid vector only transfected controls (Mann Whitney U-test). (B) Cells were transiently transfected with pcDNA3-mGluR1a or pcDNA3-mGluR1b and 2 days later challenged for 30 min with glutamate (10  $\mu$ M) or carbachol (1 mM) in the presence of filipin (5  $\mu$ g mL $^{-1}$ ; added for 15 min before and then during agonist treatment) or vehicle control, and surface receptor loss was assessed by ELISA. The data are the mean  $\pm$  SE from four independent experiments. \* $p$  < 0.05 compared to internalization in the absence of filipin treatment (Mann Whitney U-test).

onstrate that GRK2 and the mGluR1 splice variants interact, by undertaking a series of co-immunoprecipitation experiments (Figure 3). Using the HA epitope of the mGluR1a receptor for immunoprecipitation, we initially demonstrated that wild-type GRK2 co-immunoprecipitates with mGluR1a when the two constructs are coexpressed in HEK293 cells (Figure 3A). Treatment of cells with glutamate or carbachol increased the association between GRK2 and mGluR1a (Figure 3B,C). On the other hand, whereas glutamate treatment increased the association of GRK2 with mGluR1b, carbachol produced relatively little GRK2/mGluR1b association (Figure 3B,C). To further determine which regions of mGluR1 interact with GRK2, we utilized a COOH-terminal tail deletion mutant of mGluR1a/mGluR1b: DM-II (Arg<sup>868</sup> stop). This deletion mutant, which in response to glutamate undergoes limited internalization which is GRK2-independent and also displays little if any carbachol-induced internalization (12), did not associate with GRK2 following treatment with either glutamate or carbachol (Figure 3B,C). To investigate the role of PKC/CaMKII activity in the



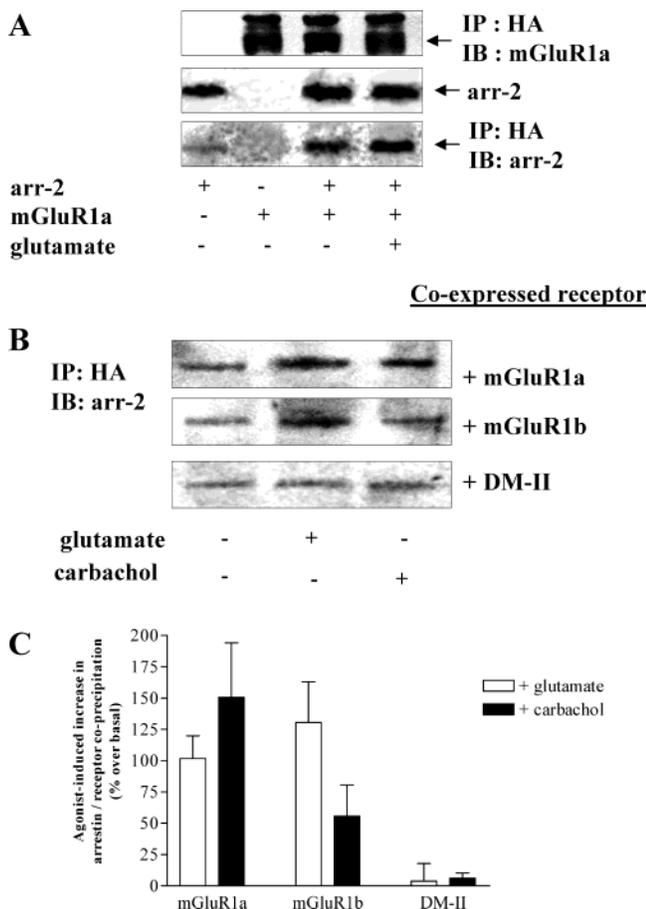
**FIGURE 3:** Glutamate and carbachol increase the association of GRK2 with mGluR1a. HEK293 cells were transiently transfected with pcDNA3-mGluR1a, pcDNA3-mGluR1b, or pcDNA3-DM-II, along with either empty pcDNA3 or pcDNA3-GRK2-GFP, and used 2 days later. (A) Co-immunoprecipitation of GRK2 with mGluR1a. In the top panel mGluR1a was immunoprecipitated from cell lysates using an anti-HA antibody (3F10) and subsequently identified by Western blotting with an anti-mGluR1 antibody. Note the presence of mGluR1a in monomeric and dimeric forms and that overexpression of GRK2 does not affect mGluR1a expression. The middle panel shows the presence of GRK2 in lysates of GRK2-transfected cells. Note that endogenous GRK2 could be detected in cell lysates following longer film exposure (data not shown). The lower panel shows that GRK2 is co-immunoprecipitated in the presence of mGluR1a and that the co-immunoprecipitation is increased following addition of glutamate (10  $\mu$ M; 10 min). (B) Agonist-stimulated co-immunoprecipitation of GRK2 with mGluR1 constructs following 10 min exposure to either glutamate (10  $\mu$ M) or carbachol (1 mM). (C) Densitometric analysis of GRK2/receptor construct co-immunoprecipitation. In these experiments the amount of GRK2 co-immunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of receptor immunoblot). The data are the mean  $\pm$  SE of three independent experiments.

carbachol-stimulated association of GRK2 and mGluR1 splice variants, cells were treated with the selective PKC inhibitor GF109203X (2  $\mu$ M) in combination with the CaMKII inhibitor KN-93 (10  $\mu$ M) prior to and during agonist stimulation (Figure 4). Pretreatment with these protein kinase inhibitors blocked the carbachol-stimulated association of GRK2 with mGluR1a, but unexpectedly, inhibition of PKC and CaMKII also blocked the glutamate-induced association of GRK2 with mGluR1a. On the other hand, the glutamate-induced association of GRK2 with mGluR1b was not reduced by inhibition of PKC and CaMKII.



**FIGURE 4:** Glutamate- and carbachol-stimulated association of GRK2 with mGluR1a is blocked by inhibition of PKC and CaMKII. HEK293 cells were transiently transfected with pcDNA3-mGluR1a or pcDNA3-mGluR1b, along with either empty pcDNA3 or pcDNA3-GRK2-GFP, and used 2 days later. (A) Cells were incubated in the presence or absence of GF109203X (2  $\mu$ M) and KN-93 (10  $\mu$ M) or vehicle alone for 30 min. Following this, co-immunoprecipitation of GRK2 and receptor constructs was assessed following 10 min exposure to either glutamate (10  $\mu$ M) or carbachol (1 mM) in the continued presence or absence of GF109203X/KN-93. (B) Densitometric analysis of GRK2/receptor construct co-immunoprecipitation. In these experiments the amount of GRK2 co-immunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of the receptor immunoblot). The data are the mean  $\pm$  SE of three independent experiments.

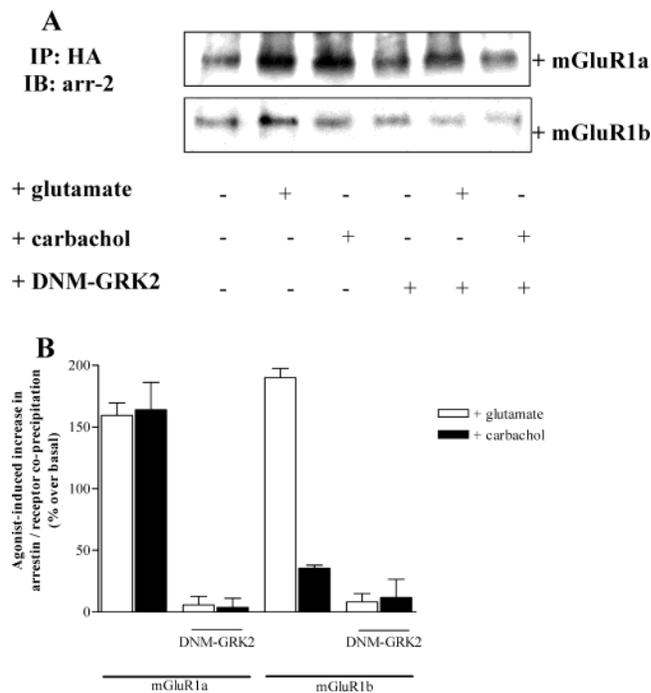
Since arrestin is thought to interact with GRK-phosphorylated receptors (5), we also used the co-immunoprecipitation protocol to assess the association of arrestin-2 with the mGluR1 splice variants. Again, using the HA epitope of the mGluR1a for immunoprecipitation, we initially demonstrated that arrestin-2 co-immunoprecipitates with mGluR1a (Figure 5A), in agreement with a previous study (22). Both glutamate and carbachol treatment increased the association of arrestin-2 with mGluR1a (Figure 5B,C). Also, in agreement with our previous studies (10) showing that glutamate- but not carbachol-induced internalization of mGluR1b is strongly arrestin-dependent, glutamate treatment markedly increased the association of arrestin-2 with mGluR1b, while carbachol promoted relatively little co-immunoprecipitation of arrestin-2 (Figure 5B,C). With the deletion mutant DM-II, neither glutamate nor carbachol treatment increased the association of arrestin-2 with the construct (Figure 5B,C). Furthermore, coexpression of DNM-GRK2, which has previously been shown to co-immunoprecipitate with mGluR1a (8), completely blocked the glutamate- and carbachol-induced association of arrestin-2 with mGluR1a and the glutamate-induced association of arrestin-2 with mGluR1b (Figure 6). Together, these results demonstrate that the kinase activity



**FIGURE 5:** Glutamate and carbachol increase the association of arrestin-2 with mGluR1a. HEK293 cells were transiently transfected with pcDNA3-mGluR1a, pcDNA3-mGluR1b, or pcDNA3-DM-II, along with either empty pcDNA3 or pcDNA3-arrestin-2-GFP (5  $\mu$ g), and used 2 days later. (A) Co-immunoprecipitation of arrestin-2 with mGluR1a. In the top panel mGluR1a was immunoprecipitated from cell lysates using an anti-HA antibody (3F10) and subsequently identified via Western blotting with an anti-mGluR1 antibody. The middle panel shows the presence of arrestin-2 in lysates of arrestin-2-transfected cells. Note that endogenous arrestin-2 could be detected in cell lysates following longer film exposure (data not shown). The lower panel shows that arrestin-2 is co-immunoprecipitated in the presence of mGluR1a and that the co-immunoprecipitation is increased following addition of glutamate (10  $\mu$ M; 10 min). (B) Agonist-stimulated co-immunoprecipitation of arrestin-2 with receptor constructs following 10 min exposure to either glutamate (10  $\mu$ M) or carbachol (1 mM). (C) Densitometric analysis of arrestin-2/receptor construct co-immunoprecipitation. In these experiments the amount of arrestin-2 co-immunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of the receptor immunoblot). The data are the mean  $\pm$  SE of three independent experiments.

of GRK2 is essential for arrestin-2 association with mGluR1a and mGluR1b. Also, the glutamate-induced association of both GRK2 and arrestin-2 with mGluR1a and mGluR1b requires the COOH-terminal tails of the splice variants.

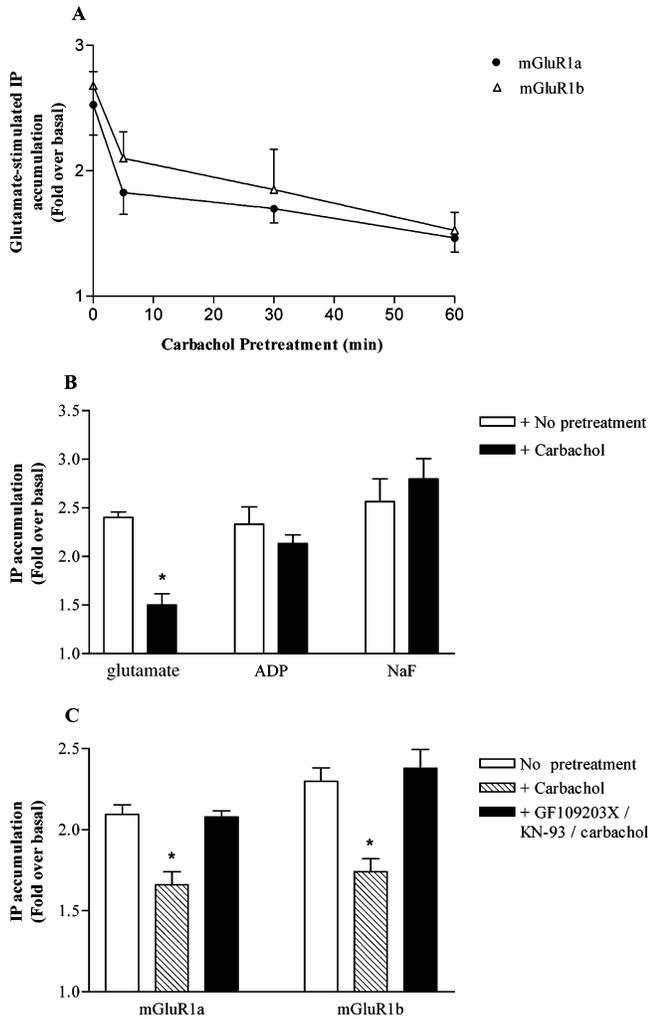
**Heterologous Desensitization of mGluR1a and mGluR1b Coupling.** Since M1 muscarinic receptor activation promoted mGluR1 splice variant internalization, we investigated whether this cross-talk could also modulate mGluR coupling. Carbachol pretreatment (1 mM; 0–60 min) markedly inhibited subsequent glutamate-stimulated mGluR1a and mGluR1b coupling to inositol phosphate (IP) accumulation (Figure 7A). This desensitization was receptor-specific since carbachol pretreatment (1 mM; 10 min) did not affect the



**FIGURE 6:** Agonist-induced association of arrestin-2 with mGluR1a and mGluR1b is dependent upon the kinase activity of GRK2. HEK293 cells were transiently transfected with pcDNA3-mGluR1a or pcDNA3-mGluR1b, along with pcDNA3-arrestin-2-GFP (5  $\mu$ g) and either empty pcDNA3 or pcDNA3-DNM-GRK2 (5  $\mu$ g), and used 2 days later. (A) Agonist-stimulated co-immunoprecipitation of arrestin-2 with mGluR1a (upper panel) and mGluR1b (lower panel) in the absence or presence of coexpressed DNM-GRK2. The co-immunoprecipitation of arrestin-2 with receptor constructs was assessed following 10 min exposure to either glutamate (10  $\mu$ M) or carbachol (1 mM). (B) Densitometric analysis of arrestin-2/receptor construct co-immunoprecipitation. In these experiments the amount of arrestin-2 co-immunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of the receptor immunoblot). The data are the mean  $\pm$  SE of three independent experiments.

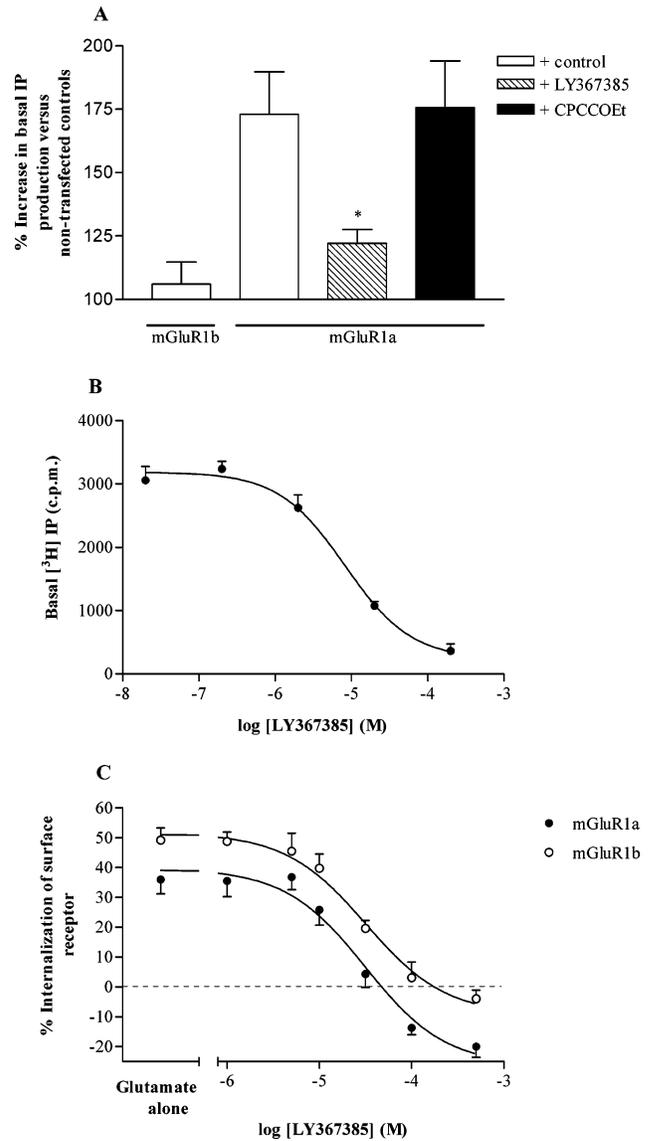
subsequent ability of ADP (10  $\mu$ M; 30 min) or NaF (10 mM; 30 min) to stimulate IP accumulation in the cells (Figure 7B). Since carbachol-stimulated internalization of mGluR1a and mGluR1b is inhibited by combined blockade of PKC and CaMKII activity (10), we determined whether inhibition of these kinases would also reverse the heterologous desensitization of the mGluR1 splice variants. Indeed, inclusion of a combination of GF109203X and KN-93 completely blocked the ability of carbachol pretreatment to desensitize subsequent glutamate-stimulated mGluR1a and mGluR1b coupling to IP accumulation (Figure 7C).

**Effect of an mGluR1a Inverse Agonist on the Desensitization and Internalization of mGluR1a and mGluR1b.** The mGluR1a splice variant is known to display a high degree of constitutive, agonist-independent activity, whereas the short splice variant mGluR1b does not (1). When expressed in HEK293 cells, mGluR1a increases basal IP accumulation relative to nontransfected or mGluR1b-transfected cells (Figure 8A). To determine whether a low concentration of endogenous glutamate present in the culture media was activating the mGluR1a receptor, we tested the effects of the mGluR1 antagonists LY367385 (100  $\mu$ M) and CPCCOEt (100  $\mu$ M) on basal IP accumulation in mGluR1a-transfected cells (Figure 8A). Whereas the noncompetitive antagonist CPCCOEt did not affect basal IP production, the competitive



**FIGURE 7:** M1 muscarinic receptor activation triggers heterologous desensitization of mGluR1a- and mGluR1b-stimulated inositol phosphate (IP) accumulation. HEK293 cells were transiently transfected with either pcDNA3-mGluR1a or pcDNA3-mGluR1b 2 days before assessment of agonist-induced IP accumulation. (A) Cells were pretreated with the M1 muscarinic acetylcholine receptor agonist carbachol (1 mM; 0–60 min) in medium without LiCl. Cells were subsequently washed, and IP accumulation was assessed following addition of glutamate (10  $\mu$ M; 30 min) in the presence of the muscarinic receptor antagonist atropine (1 mM) in medium containing 20 mM LiCl. Carbachol pretreatment did not affect subsequent basal IP accumulation (basal accumulation in mGluR1a-expressing cells in the absence and presence of carbachol pretreatment was  $0.59 \pm 0.14$  and  $0.62 \pm 0.10$  expressed as total [ $^3$ H]IP production/total [ $^3$ H]inositol incorporation). (B) Cells transiently transfected with mGluR1a were preincubated in the presence or absence of carbachol (1 mM; 10 min) and then washed, and IP accumulation was measured following addition of glutamate (10  $\mu$ M; 30 min), ADP (10  $\mu$ M; 30 min), or NaF (10 mM; 30 min). \* $p < 0.05$  compared to the respective non-carbachol pretreated control (Mann Whitney U-test). (C) Cells transiently transfected with mGluR1a or mGluR1b were treated in the presence or absence of GF109203X (2  $\mu$ M) and KN93 (10  $\mu$ M) for 15 min before and then during carbachol pretreatment (10  $\mu$ M; 30 min). Cells were subsequently washed, and IP accumulation was measured after the addition of glutamate (10  $\mu$ M; 30 min) in the presence of the muscarinic receptor antagonist atropine (1 mM). \* $p < 0.05$  compared to the respective non-carbachol pretreated control (Mann Whitney U-test). In each case the data are the mean  $\pm$  SE of four independent experiments.

antagonist LY367385 reduced basal IP production to levels close to those seen in mGluR1b-transfected cells. The lack of effect of CPCCOEt indicates that glutamate that may be



**FIGURE 8:** The constitutive activity of mGluR1a is inhibited by LY367385. (A) Cells transfected with mGluR1a were pretreated with vehicle, LY367385 (100  $\mu$ M), or CPCCOEt (100  $\mu$ M) for 60 min. Basal inositol phosphate (IP) levels were subsequently measured. The lack of constitutive activity in mGluR1b-expressing cells is also shown. The data are the mean  $\pm$  SE of four independent experiments. \* $p < 0.05$  compared to the respective vehicle treated control in mGluR1a expressing cells (Mann Whitney U-test). (B) Concentration-dependent inhibition of basal inositol phosphate formation by LY367385. The drug was added to mGluR1a-expressing cells at varying concentrations for 30 min before basal IP levels were measured. The data shown are the mean  $\pm$  SE of triplicate measurements in a single experiment, the same result being obtained in one further experiment. The  $IC_{50}$  value for LY367385 in the experiment shown is 8  $\mu$ M. (C) Concentration-dependent inhibition of glutamate-induced internalization of mGluR1a and mGluR1b by LY367385. Varying concentrations of LY367385 were added to the cells for 10 min before and then during 30 min treatment with 30  $\mu$ M glutamate, and the internalization of receptor was determined by ELISA as described in Experimental Procedures. The data shown are the mean  $\pm$  SE of quadruplicate measurements in a single experiment, the same result being obtained in one further experiment. The  $IC_{50}$  values for LY367385 in this experiment were 23 and 27  $\mu$ M for mGluR1a and mGluR1b, respectively. Note that higher concentrations of LY367385 actually increase cell surface expression of mGluR1a.

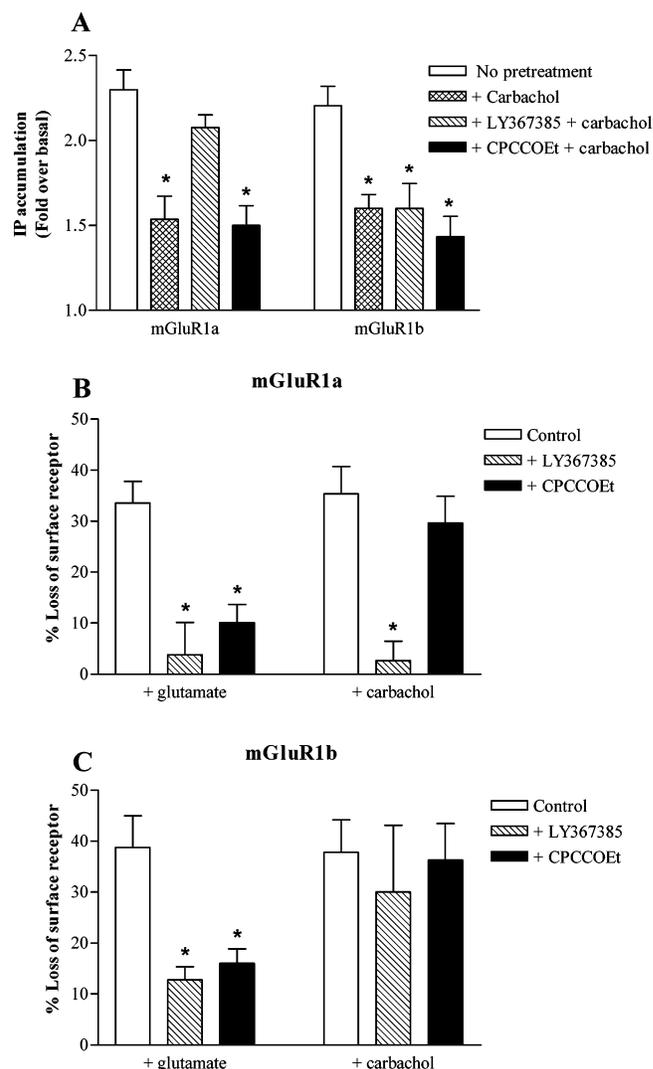
present in the culture medium does not contribute to the activation of mGluR1a. In further experiments, LY367385

inhibited basal IP accumulation in mGluR1a-expressing cells with an  $IC_{50}$  of 8  $\mu$ M (Figure 8B), while LY367385 inhibited the glutamate-induced internalization of mGluR1a and mGluR1b with  $IC_{50}$  values of 23 and 27  $\mu$ M, respectively (Figure 8C). These values are consistent with those previously reported (23, 24) for the competitive interaction of LY367385 with mGluR1a. Interestingly, pretreatment with LY367385 actually increased the cell surface expression of mGluR1a but not mGluR1b (Figure 8C). Taken together, these results indicate that LY367385 exhibits significant inverse agonist activity.

Given this property of LY367385, we were interested to see if it would modify carbachol-induced desensitization of the mGluR1 splice variants. Indeed, co-addition of LY367385 with carbachol reversed the latter's ability to desensitize mGluR1a coupling to IP accumulation (Figure 9A). On the other hand, LY367385 had no effect on the carbachol-induced desensitization of mGluR1b. Furthermore, the mGluR1 antagonist CPCCOEt, which did not display inverse agonist activity, had no effect on carbachol's ability to desensitize either mGluR1a or mGluR1b responsiveness (Figure 9A). In internalization experiments, both LY367385 and CPCCOEt blocked glutamate-induced internalization of mGluR1a and mGluR1b, as expected (Figure 9B,C). However, whereas LY367385 blocked carbachol-induced internalization of mGluR1a, it had no effect on carbachol-induced internalization of mGluR1b. Also, carbachol-induced internalization of both splice variants was unaffected by CPCCOEt (Figure 9B,C). Finally, we assessed the effect of the inverse agonist on the ability of GRK2 to associate with mGluR1a (Figure 10). As expected, treatment of cells with LY367385 blocked the glutamate-stimulated association of GRK2 with mGluR1a and mGluR1b. Furthermore, the inverse agonist blocked the carbachol-stimulated association of GRK2 with mGluR1a. In a similar way, LY367385 blocked the ability of carbachol to increase association of arrestin-2 with mGluR1a, whereas CPCCOEt did not (two independent experiments; data not shown). Together, these data suggest that mGluR1a must be in an active conformation before it can undergo carbachol-induced desensitization and internalization and that LY367385 blocks these effects probably by inhibiting the ability of carbachol to increase association of GRK2 and arrestin-2 with mGluR1a.

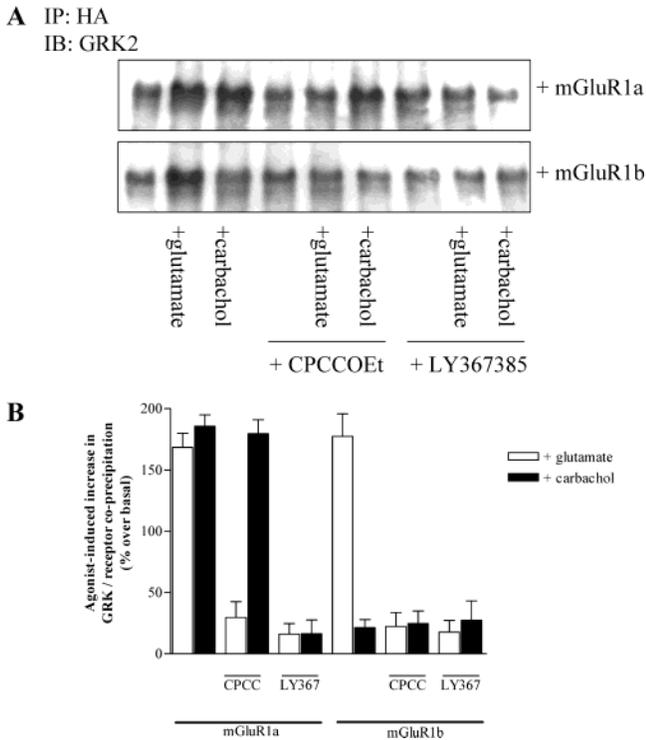
## DISCUSSION

We have previously shown that muscarinic acetylcholine receptor activation using carbachol triggers the internalization of both mGluR1a and mGluR1b in a PKC/CaMKII-dependent manner (10). We now report that carbachol-induced internalization of mGluR1a and mGluR1b ultimately proceeds by different mechanisms. When cells were cotransfected with a DNM-GRK2 construct, the glutamate-induced internalization of mGluR1a and mGluR1b was inhibited, as was the carbachol-induced internalization of mGluR1a. However, DNM-GRK2 had no effect on carbachol-induced internalization of mGluR1b. Since we have previously shown that carbachol induces little or no arrestin translocation in mGluR1b-expressing cells (10), then carbachol-induced internalization of mGluR1b, unlike that for mGluR1a, proceeds largely in the absence of GRK2 and arrestins. Furthermore, inhibition of clathrin-dependent internalization with a DNM-Eps15 construct (16, 17) only



**FIGURE 9:** The inverse agonist LY367385 blocks carbachol-induced heterologous desensitization and internalization of mGluR1a. HEK293 cells were transiently transfected with either pcDNA3-mGluR1a or pcDNA3-mGluR1b. (A) Cells were treated with LY367385 (100  $\mu$ M), CPCCOEt (100  $\mu$ M), or vehicle for 15 min before and then during carbachol pretreatment (10  $\mu$ M; 30 min). Cells were subsequently washed, and IP accumulation was measured after the addition of glutamate (10  $\mu$ M; 30 min) in the presence of the muscarinic receptor antagonist atropine (1 mM). The data are the mean  $\pm$  SE from four independent experiments. \* $p$  < 0.05 compared to the respective non-carbachol pretreated controls (Mann Whitney U-test). Cells transiently transfected with mGluR1a (B) or mGluR1b (C) were treated with LY367385 (100  $\mu$ M), CPCCOEt (100  $\mu$ M), or vehicle for 15 min before and then during subsequent challenge with glutamate (10  $\mu$ M; 30 min) or carbachol (1 mM; 30 min), and surface receptor loss was assessed by ELISA. The data are the mean  $\pm$  SE from six independent experiments. \* $p$  < 0.05 compared to the vehicle pretreated control (Mann Whitney U-test).

weakly inhibited the carbachol-induced internalization of mGluR1b, whereas both the glutamate- and carbachol-induced internalization of mGluR1a was strongly inhibited. This is in agreement with a previous study where natively expressed mGluR1a in C6 glioma cells were shown to internalize in a clathrin-dependent manner (25). Presumably, removal of the long COOH terminus of mGluR1a uncovers an alternative internalization pathway for mGluR1b in response to muscarinic acetylcholine receptor activation. Interestingly, we found that filipin, which disrupts caveolae/



**FIGURE 10:** The inverse agonist LY367385 inhibits the carbachol-induced association of GRK2 and arrestin-2 with mGluR1a. HEK293 cells were transiently transfected with pcDNA3-mGluR1a or pcDNA3-mGluR1b, along with pcDNA3-GRK2-GFP, and used 2 days later. (A) Agonist-stimulated co-immunoprecipitation of GRK2 with mGluR1a (upper panel) and mGluR1b (lower panel) in the absence or presence of CPCCOEt or LY367385 as indicated. Cells were pretreated with CPCCOEt (100  $\mu$ M) or LY367385 (100  $\mu$ M) or vehicle alone for 15 min before and then during 10 min exposure to either glutamate (10  $\mu$ M) or carbachol (1 mM). Following these treatments, co-immunoprecipitation of GRK2 and receptor constructs was assessed. (B) Densitometric analysis of GRK2/receptor construct co-immunoprecipitation. In these experiments the amount of GRK2 co-immunoprecipitated is normalized against the amount of receptor coexpressed (as assessed by densitometric analysis of the receptor immunoblot). The data are the mean  $\pm$  SE of three independent experiments.

lipid rafts, selectively inhibited the carbachol-induced internalization of mGluR1b. Thus the mGluR1b exhibits a novel stimulus-dependent internalization profile (glutamate – clathrin; carbachol – clathrin and caveolae/lipid rafts). A previous study reported that neither mGluR1a nor mGluR1b associated significantly with caveolae/lipid rafts in brain (26), so it is possible that mGluR1b association with caveolae/lipid rafts is tissue-dependent or that mGluR1b only associates with caveolae/lipid rafts in response to activation of heterologous  $G_{q/11}$ -coupled receptors. Recently, in both COS7 cells and neurones, the constitutive internalization of mGluR5a was reported to be clathrin-independent (18) while the constitutive internalization of mGluR1a was reported to be arrestin-independent (22; but see ref 27). Therefore, group I mGluRs appear to internalize by multiple mechanisms depending upon the nature of the trigger for internalization as well as the particular splice variant studied. It will be important in future studies to identify the trafficking pathways of the mGluR1 splice variants in a neuronal environment.

To directly assess the interaction of GRK2 with mGluR1 splice variants, we utilized a co-immunoprecipitation approach in cells coexpressing receptor and wild-type GRK2

constructs. We observed significant immunoprecipitation of GRK2 with mGluR1a in the absence of agonist, as previously observed (8). This is unlikely to be related to constitutive activity of the receptor since it was also apparent with the mGluR1b splice variant, which is not constitutively active. Importantly, both glutamate and carbachol treatment increased the association of GRK2 with mGluR1a, indicating that this splice variant undergoes dynamic interaction with GRK2, even in the absence of glutamate occupation of the agonist binding site. On the other hand, only glutamate strongly increased the co-immunoprecipitation of GRK2 and mGluR1b, indicating that the carbachol-induced internalization of mGluR1b probably does not involve GRK2. Furthermore, using a COOH-terminal tail deletion mutant, we demonstrated that the glutamate-induced association of GRK2 with mGluR1a and mGluR1b, as well as the carbachol-induced association of GRK2 with mGluR1a, depends on the receptor tail as suggested by our previous internalization studies (12).

We have previously shown that inhibition of PKC/CaMKII activity blocks the carbachol-induced internalization of mGluR1a (10), and we now show that inhibition of PKC/CaMKII also blocks the carbachol-induced association of GRK2 with mGluR1a. Surprisingly, however, PKC/CaMKII inhibition also blocks the glutamate-stimulated association of GRK2 with mGluR1a. Previously, our results have suggested that GRK2 and PKC together mediate glutamate-induced mGluR1a internalization, as DNM-GRK2 expression plus PKC inhibition was required to effectively abolish internalization (12). We had assumed that these processes were independent and additive, but our present results suggest that the glutamate- or carbachol-induced association of GRK2 with mGluR1a is dependent upon prior PKC/CaMKII activation. Indeed, it seems possible that the glutamate- and carbachol-induced internalization of mGluR1a shares a common mechanism involving sequential activity of second messenger-dependent protein kinases and GRK2. PKC is known to directly phosphorylate and activate GRK2 (28), which could explain the increased association of GRK2 with mGluR1a. Recently, orphanin FQ-mediated desensitization of  $\mu$ -opioid receptors was shown to require PKC-dependent translocation of GRK2 and GRK3 to the cell membrane (29). However, in our study PKC/CaMKII inhibition did not reduce glutamate-stimulated GRK2/mGluR1b association, suggesting that a generalized increase in GRK2 activity after its phosphorylation by PKC may not be responsible. Alternatively, PKC phosphorylation of the mGluR1a COOH terminus or another intermediary protein could increase the affinity of GRK2 for its target sequence. Thus we may have uncovered a novel mechanism for GRK2 interaction with GPCRs, and it is interesting to note that olfactory GPCRs, which are also family C receptors, appear to require sequential interplay of second messenger-dependent protein kinases and GRKs in order to undergo desensitization (30).

We show here that carbachol treatment rapidly desensitizes mGluR1a and mGluR1b receptor responsiveness in a PKC/CaMKII-dependent manner. It is unlikely that mGluR1 internalization accounts for this, since the desensitization observed in this study occurs rapidly in comparison to internalization (10). The mGluR1 splice variants themselves appear to be the locus of this heterologous desensitization, since carbachol pretreatment did not affect the functional

response to another  $G_{q/11}$ -coupled receptor, the P2Y1 receptor, or direct activation of  $G_{q/11}$  with NaF. Cross-talk between other GPCRs and group I mGluRs in neurones remains to be examined in detail, but the desensitization of group I mGluR-mediated  $G_{q/11}$  signaling at hippocampal synapses is associated with a switch from facilitation to inhibition of excitatory synaptic transmission (31), which could be due to PKC-dependent mGluR desensitization (32).

In further experiments we examined the ability of mGluR1 antagonists to modify the carbachol-induced desensitization and internalization of the mGluR1 splice variants. Compared to the short splice variants, mGluR1a displays a high degree of constitutive, agonist-independent activity (33, 34), and in the present study basal IP levels were greater in mGluR1a-transfected HEK293 cells than in plasmid-transfected or mGluR1b-transfected cells. We identified the mGluR1 competitive antagonist LY367385 (23) as an inverse agonist, since it was able to inhibit basal IP accumulation in mGluR1a-expressing cells. On the other hand, the noncompetitive antagonist CPCCOEt did not display inverse agonism, in agreement with a previous study (35). According to accepted models, GRK2 preferentially targets GPCRs that are in an active, agonist-bound conformation ( $R^*$ ), as opposed to an agonist-unbound receptor conformation ( $R$ ) (36). Since GRK2 associates with mGluR1a following activation of M1 muscarinic receptors, we wondered whether the conformation of this GPCR plays a role in this association. To address this issue, we treated cells with LY367385 to promote formation of an inactive state of mGluR1a. Under these conditions, carbachol desensitization of mGluR1a responsiveness was blocked, indicating that an active conformation of the receptor is required for heterologous desensitization to occur. Interestingly, LY367385 did not affect heterologous desensitization of mGluR1b, showing that an inactive conformation of this splice variant, as indicated by the lack of constitutive activity of this receptor, remains susceptible to second messenger kinase regulation. The effects of LY367385 on carbachol-induced internalization mirrored those on desensitization; the inverse agonist blocked carbachol-induced internalization of mGluR1a but not mGluR1b, whereas CPCCOEt had no effect on carbachol-induced internalization of either splice variant. Thus the carbachol-induced desensitization and internalization of mGluR1b appear not to be dependent upon an active conformation of the receptor, unlike for mGluR1a. Furthermore, since GRK2 and arrestin-2 do not significantly associate with mGluR1b following carbachol treatment, then this splice variant appears to undergo heterologous desensitization by a GRK2/arrestin-independent yet PKC/CaMKII-dependent mechanism. Whether this reflects direct phosphorylation of mGluR1a and mGluR1b by these latter kinases remains to be determined. Finally, since LY367385 but not CPCCOEt blocked the ability of carbachol to increase the association of GRK2 and arrestin-2 with mGluR1a, then this seems the likely mechanism whereby the inverse agonist blocks the carbachol-induced desensitization and internalization of mGluR1a. Presumably, the inactive conformation of mGluR1a induced by LY367385 is unfavorable for GRK2 interaction; whether this is primarily because PKC/CaMKII regulation of mGluR1a is suppressed by the inverse agonist will be a focus of future experiments.

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